

**ISOLATION OF BACTERIAL STRAINS FOR IMPROVED
MAIZE PRODUCTION**

BY

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A dissertation submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE

(BIOLOGY)

**DEPARTMENT OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE, AGRICULTURE AND TECHNOLOGY
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November 2016

DECLARATION

I, the undersigned, declare that this thesis submitted to the North-West University for the degree of Master of Science in Biology in the Faculty of Science, Agriculture and Technology, School of Environmental and Health Sciences, and the work contained herein is my original work with the exception to the citations and that this work has not been submitted at any other University partially or entirely for the award of any degree.

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Signature:

Date:

Supervisor: Professor Olubukola Oluranti Babalola

Signature:

Date:

DEDICATION

This work is dedicated to my loving mother, Miss. Oluwafunmilayo A. Adeleke. You have been my support and my pillar all through these years. God bless you abundantly and you will reap the fruit of your labor in Jesus name. Amen.

ACKNOWLEDGEMENTS

My sincere appreciation goes to God almighty for being my help from the beginning to the end. Without You I would not have been able to do anything. Thank you Lord. I am forever loyal. I would like to express my sincere and deepest, gratitude and appreciation to my supervisor, Professor Olubukola Oluranti Babalola for giving me the opportunity to pursue my Masters degree under her supervision and for her encouragement, help and kind support. Her invaluable advice, suggestions, discussions and guidance were a real support to me. God bless you ma. You will never lack help.

I acknowledge with honor and gratitude the National Research Foundation, South Africa for a grant (Ref: UID81192 to Professor Olubukola Oluranti Babalola) and North-West University for offering me a bursary/scholarship award to pursue the MSc degree.

A special thanks to those who helped me with my techniques or/and part of my research at one stage or another, Dr Shivakumar Banakar, Mrs. Caroline Ajilogba, Dr Mobolaji Adegboye, Dr Rhoda Aremu, Mrs. Omotola Fashola, I can never thank you enough for all my troubles.

I cannot forget to appreciate my twin and sisters that really helped me out on the field at every stage of the work, Isaiah Simon, Oladejo Victoria, Giwa Fiyinfoluwa, Ayandokun Florence not forgetting Oche Mary, and Badmus Mojisola. God bless you all. You will never lack help. I appreciate my wonderful babies and sisters in the house, Victoria Ajilogba, Emmanuel Ajilogba, my sweet Demilade Ajilogba, my 'lerato' Damilola Ajilogba and my wonderful Adetomiwa Adelekan. I also thank my families back home for their support in words and prayers, Pastor Mark Ajilogba, Oluwadamilare Aluko, and Ayomide Olanrewaju. You are very special to me. God bless you.

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LIST OF MANUSCRIPTS SUBMITTED FOR PUBLICATIONS

Chapter 2: The Rhizosphere and Plant Health. *This chapter has been submitted in this format for publication in the Journal of Plant Growth Regulation*

Authors: Oluwaseyi Samuel Olanrewaju and Olubukola Oluranti Babalola

Candidate's Contributions: designed the study, managed the literature searches, and wrote the first draft of the manuscript.

Chapter 3: Functions and mechanisms of action of plant growth promoting rhizobacteria. *This chapter has been submitted in this format for publication in Microbiology Open.*

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Candidate's Contributions: designed the study, managed the literature searches, and wrote the first draft of the manuscript.

Chapter 4: Development of bacterial strain for improved maize crop production. *This chapter has been submitted in this format for publication in American and Environmental Microbiology journal.*

Authors: Oluwaseyi Samuel Olanrewaju and Olubukola Oluranti Babalola

Candidate's Contributions: designed the study, managed the literature searches, wrote the protocol, carry out the laboratory work, performed all the analyses, interpretation of results and wrote the first draft of the manuscript.

Chapter 5: Metabolite profiling of *Bacillus subtilis* rhizobacteria isolated from maize rhizosphere. *This chapter has been submitted in this format for publication in journal of natural products.*

Authors: Oluwaseyi Samuel Olanrewaju and Olubukola Oluranti Babalola

Candidate's Contributions: designed the study, managed the literature searches, wrote the protocol, carry out the laboratory work, performed all the analyses, interpretation of results and wrote the first draft of the manuscript.

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GENERAL ABSTRACT

BACKGROUND

The ever increasing world population has led to a continued demand for food. If the increase and sustenance of available food crops are not taken seriously, then there might be a crisis of food shortage to sustain all. Maize happens to be an important food crop, as a matter of fact, it is the third largest staple food crop in the world. In South Africa, it is the largest staple crop produced for human consumption. Its importance is not only for food purposes, but also for other vital uses which ease the existence of mankind. Maize is used in the pharmaceutical industry, it is a major ingredient in poultry feed, used in energy industries, paper manufacturing, and brewing industries. The major problems hindering maize production arises from the unavailability of fertile land for good cropping systems caused by land shortage from urbanization and by land pollution mainly through contamination. In order to combat these problems, farmers resort to using chemical fertilizers on the available land. Although this helps to improve the yield to some extent, it is derogatory in the sense that it causes loss of soil fertility on the long run as well as becomes a hazard and a threat to the population it is meant to help. In other words, the advantages of using chemical inputs can never be compared to the destructive activities it causes to the ecosystem and health. This entails the need for a more reliable and safe alternative which is found in nature itself in the form of plant growth promoting rhizobacteria (PGPR). These PGPRs have been used over time as bio-fertilizers. The aim of this study is to identify some potent PGPRs and their synergistic effect to produce a “super” effect on maize crop yield on the farm.

METHOD

This study was carried out using a randomized block design. Each treatment was used in triplicates with the control having no treatment. Treatments were used in single organisms, consortia of two organisms and three organisms. Length of leaves, roots, stem, plant heights,

numbers of leaves and weight of 100 seeds were taken at 4 and 8 weeks. The readings were compared to the control.

OUTPUT

In this study, 31 strains designated A1-A31 were isolated from the rhizospheric soil of maize plants grown in the North West University farm, Molelwane, South Africa. Morphological, biochemical and physiological characteristics of these isolates as well as their plant growth promoting abilities were carried out. 16S rDNA gene sequencing and the nucleotide sequence phylogenetic analysis were determined. 93.5% were able to produce ammonia and just 35.5% could produce indole (IAA). Based on these assays, 3 isolates that showed the most promising result as PGPR were finally selected from the 31 isolates. Three *Streptomyces* isolates designated NWU4, NWU14 and NWU198 which were also assayed with the 31 isolates that were also selected. All 6 isolates produced oxidase as well as catalase but not all could produce protease. Only A18 and NWU4 produced HCN.

Antifungal and antibacterial assays were carried out on the 6 selected isolates. All showed antagonistic activity against the fungal pathogen *Fusarium graminearum* except A18 that was not effective. A1 and NWU198 were the most active against this pathogen showing more than 70% activity while NWU14, NWU4 and A29 were all moderate in their activities showing 50% and the last two showed 40% activity. Based on these assays, the isolates were categorized in groups of two and three as well as single organisms and were used to inoculate maize seeds which were then planted on the farm inside the university campus.

Isolate A1 was screened for its metabolite production due to its high antifungal activity against the fungal pathogen. Metabolites such as phthalan, tropone, ethylbenzene etc were detected.

This study demonstrates the potential benefits of using microbial consortia in plant growth promotion as compared to single inoculant treatments. Significant increase was observed in all

the parameters compared to the control as well as between the consortia treatments. The study also demonstrates the screening of useful metabolites from one of the effective isolates. Lastly, the study showed that the use of microbial consortia can be of advantage in the eradication of low maize yield as well as serve as reliable alternatives to chemical fertilizers.

CONCLUSION

Eradication of chemical fertilizers is becoming more realistic as potent biofertilizers are being discovered daily. These studies show that combination of microorganisms as consortia organisms can enhance all round growth yield in maize. These can be used as efficient PGPR for maize production in field as it is friendly and safe to the environment as well as cost effective.

CHAPTER 1

1.1 General Introduction

Soil is the habitat to many microorganisms. These microbes can be harmful, i.e. causing diseases or they can be useful and advantageous to their hosts and their immediate environments. Examples of the microorganisms present in soil include fungi, bacteria, virus and protozoa. Some microbes are under the influence of plant roots as they are able to colonize the surroundings of the root (Kennedy, 2005). These rhizosphere competent bacteria are called rhizobacteria (Antoun and Kloepper, 2001). The soil in which the roots of the plants are directly in contact with is called rhizospheric soil. The word rhizosphere was first used by Hiltner in 1904. The most abundant microbes found in rhizosphere happen to be bacteria. Microorganisms interact with their environment either parasitically, saprophytically or in a mutualistic way (Qiang et al., 2012, Saharan and Nehra, 2011). Mutualistic beneficial interactions are the major characteristics of rhizobacteria as they exert a beneficial effect on the plants even in the presence of other organisms (Bakker et al., 2012, Kloepper, 2003). Rhizobacteria that effect positive impact on the growth of plant as well as crop yield are termed plant growth promoting rhizobacteria (PGPR). The commercial aspect of PGPR and their plant interactions is on a high (Podile and Kishore, 2006), which is great in the future sustenance of agriculture. Studies of these interactions have been examined in a variety of crops including wheat, peas, canola, soy, oat, radicchio, barley, potatoes, maize, lentils, tomatoes, and cucumber (Gray and Smith, 2005).

Actinomycetes are filamentous gram positive bacteria, known for their complex life cycles. They are good root colonizers and widely distributed in nature, and are present in aquatic and terrestrial environments. They belong to the phylum Actinobacteria (one of the largest taxonomic units that is currently recognized) (Ventura et al., 2007). In soil, they play a very

cogent part of biomaterials recycling through the catabolism of complex mixtures of organic molecules in fungi, plants, and animals that are already dead. Their importance is also exhibited in bioremediation and soil nutrient recycling as they are known to recycle the nutrients which are associated with polymers, such as chitin, keratin, and lignocelluloses (Stach and Bull, 2005). *Streptomyces*, which is arguably the most important actinomycetes, are widely known for their antibiotic production and also for degrading different macromolecules. They also synthesize different metabolites which help in carrying out the afore-mentioned tasks. They are filamentous gram positive actinomycetes. Their life cycle is complex in nature compared to other microbes including mycelial, spores and aerial hyphae formation. *Streptomyces* have been reported to be good plant growth promoters in some research studies as well as being excellent biocontrol agents. They are good colonizers of rhizospheres; therefore, this makes them viable plant growth promoters. Antagonistic activity of rhizobacteria can be through competition, production of antibiotics, or secretion of hydrolytic enzymes (Van Loon and Bakker, 2003), which makes them effective as antipathogens. The main bacteria known to exhibit this antipathogenic traits are of the genera *Pseudomonas*, *Bacillus*, and *Streptomyces* as it has been shown in numerous studies (Kloepper et al., 1988).

Maize (*Zea mays*) which belong to the family of grasses, (Fig. 1.2) is cultivated globally and it is partly the most important cereal crop all over the world (Glover and Mertz, 1987). Increasing demand for food and livestock feed causes maize to be introduced as an important crop in temperate and semi- arid regions. Maize is one of the most consumed staple crops all over the world which has many functions which include human consumption. Maize thrives well in a healthy and rich soil that consists of beneficial rhizosphere microorganisms. Maize is not only important for human consumption, it is also an important raw material in making animal feed. Maize is also a starting material for the production of many products like maltodextrins, corn starch, corn syrup, corn oil, with fermentation and distillation industries products not left out.

Its recent usage comes to its use as bio-fuel, all these have shown its excellent ability in food, feed and industrial utilization. Its importance as food cannot be overemphasized due to its wide applications in food industries; however, it should be noted that it is deficient in these essential amino acids; lysine and tryptophan (Galili and Amir, 2013, Nguyen et al., 2012).



Figure 1.1: Maize plants at vegetative and reproductive states

A and E: vegetative stage, C: tassel, B and D: maize cobs

The genetic variation in maize may be strongly the result of the environmental condition of the area. Maize is basically a warm climate crop, although it has been grown and thrived in a wide range of climatic conditions. No toxins or tangible amount of anti-nutritional factors have been reported in maize. It is nonpathogenic as it is not known to cause any disease in humans, animals or plants with the only exception being maize allergy that occurs after ingestion or inhalation of maize flour or pollen.

Day by day the world population is increasing and agricultural land is decreasing due to the concrete network of buildings. There is a need for sufficient food to be made available to fulfil the nutritional requirements of the growing human population. This requires large hectares of land for the production of plenty of food crops. Unfortunately, land is limited and it is a great challenge to solve this problem as crops need lots of nutrients to reach their maximum potential yield. Initially, soil fertility was increased by use of chemical fertilizer, but frequent use of

chemical fertilizer decreased crop yields and soil fertility. These also have adverse effects on the environment including soil and water deterioration and contamination. There are other problems associated with chemical fertilizer which include its increasing cost and the wide gap between supply and demand. That is the reason for the continuing search for another option to combat this problem. The use of PGPR to enhance plant growth has proven to be a reliable alternative. The right application of PGPR for a better crop yield is key to their success as good biofertilizer.

CHAPTER 2

The Rhizosphere and Plant Health

Abstract

The microbial community densely populating the rhizobiome is aided by the plant's root exudates and other factors. Working towards maintaining a good plant health is a very important factor for continuous living. Many factors are necessary to consider but only the aspect of the effect the rhizobiome has on the plant health will be discussed. As minute as rhizospheric microbes are, their existence is purely based on the plant host in their habitat. They practically depend on the plants for nutrients, and other necessary requirements. There is a strong relationship between the rhizobiome and plant hosts which can be beneficial or pathogenic depending on the microbes involved. This relationship, to a large extent, determines the fate of the host plant's survival. Modern molecular techniques have to a large extent been used to unravel the rhizobiome species composition, but the interplay between the rhizobiome root exudates and other factors in the maintenance of a healthy plant have really not been thoroughly looked into. To get the best out of the host plants, research should be directed towards understanding the rhizobiome diversity in the plant's rhizosphere, so that the best modulatory methods and techniques for the rhizobiome for improved plant health can be well understood.

Keywords: biofertilization, plant-microbe interaction, quorum sensing, rhizobiome, rhizodeposition

2.1 Introduction

There is a daily increase in demand for more crop yield as a result of the ever increasing world population. Soil can categorically be said to contain the largest population of microbial diversities ever known to man. The direct contact of plants with the soil brings them in contact

with a large species of diverse microbes both pathogenic and nonpathogenic. Plants and the rhizobiome interact for disease suppression and nutritional values (Lundberg et al., 2012, Chaparro et al., 2012, Lakshmanan et al., 2014, Lapsansky et al., 2016). The rhizobiome comprises of the rhizosphere and the organisms inhabiting the rhizospheric soil. The microbes function in pathogen resistance, growth hormone production, water retention and nutrient conversion to accessible forms for plants, while the plants in return exude carbon metabolites for the microbes (Bais et al., 2006, Berendsen et al., 2012).

The plant rhizobiome multifariousness is determined by the soil physical and chemical properties, genetics, and the type of the host plants (Schreiter et al., 2015, Peiffer et al., 2013, Li et al., 2014, Yuan et al., 2015). Plants and the rhizosphere microbes relationship have been comprehensively studied for pathogens and biocontrol activities with less knowledge and work on the plant growth supporting microbes which are also health supporting. This has necessitated the need to decipher the rhizosphere diversity for the exploitation of plant beneficial microbes in terms of growth supporting.

2.2 Soil and rhizospheric soil

2.2.1 The soil

Soil microbiome is one of the richest in microbes on earth (Bulgarelli et al., 2013). The impact of various types and constituents of the soil is evident on the rhizobiome due to the different bionomics obtained in the type of soils as shown in different plant species (Mendes et al., 2013). The complex nature of the soil, (which involves the chemical properties, constituents of the soil, different reactions going on in the soil, microbial interactions in the soil as well as plant-microbe interactions) and its environment is paramount in the effect it has on root exudate compositions, plant physiology, and invariably influencing the rhizobiome composition (Rohrbacher and St-Arnaud, 2016). The soil composition and property determines the type of plant that can exist, thereby influencing the microbiome of the soil through the type of exudates

that is released by the plant present. An important factor that has an effect on the soil microbiome is the pH as was demonstrated by Fierer and Jackson (2006) on soil samples collected from North and South America as the temperature was seen not to have any meaningful effect on bacterial variations. This was also confirmed by Lauber et al. (2009) as well as being documented in the work of Rousk et al. (2010). In as much as most rhizospheric bacteria are dependent on their mutualistic relationship with plants, the different array present in an environment have an important part to fulfil in that environment whether they are beneficial or not (Panizzon et al., 2016).

2.2.2 The rhizosphere

The rhizosphere is the area of soil around the root system. It is the soil attached to the roots and the immediate root environment (figure 2.1).

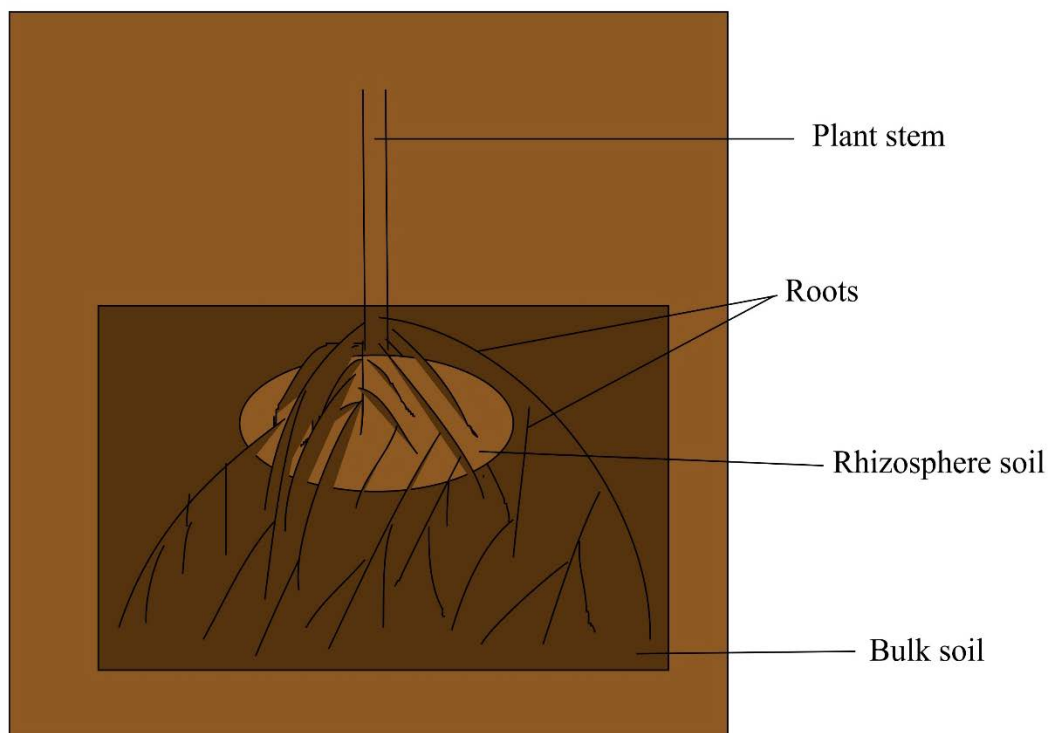


Figure 2.1: Rhizospheric soil and bulk soil

The rhizosphere constituent of a plant is determined by the synergistic relationship between the soil, the plant root, and the microbes present. It is influenced by the soil pH, type and complexity (Mendes et al., 2013), not forgetting plant roots through the production of root

exudates which can be amino acids, sugars, and different nutrients (Moe, 2013, Lakshmanan et al., 2014). Plant roots have a mutualistic effect on the rhizospheric soil and microbes, thus, the rhizobiome inadvertently acts as a second functional niche for plants (Lakshmanan et al., 2014, Spence et al., 2014, Pineda et al., 2015).

2.3 The rhizobiome, root exudates and plant health

2.3.1 The rhizobiome

Berendsen et al. (2012) referred to the rhizobiome as the plant's second genome. Rhizobiome functioning in plant growth has since been recognized, but the different interactions involved have been less studied due to the unavailability of required tools and techniques (Brink, 2016), although there have been achievements in the understanding of the root microbiome through different separation techniques. The surfacing of sequencing technologies has helped in the studies of microbiome as seen in the case of *Arabidopsis thaliana* (Lundberg et al., 2012, Brink, 2016, Hacquard, 2016). Sonication treatments and high throughput sequencing techniques have been established as major effective techniques in the targeting and studying of the rhizobiome (Lundberg et al., 2012). These techniques combined with other ones will prove to be helpful in rhizospheric microbial diversity studies as well as to elucidate the relationship between the rhizobiome and the plant, including how this relationship affects the plant health status.

The rhizobiome has been shown to consist of both beneficial and non-beneficial microbes. The composition, position and abundance of the rhizobiome will affect human health as well as plant growth. The plant genotype and type of soil largely determine the plant's rhizobiome (Berg and Smalla, 2009, Bakker et al., 2012, Mendes et al., 2013), making them to be specific in their soil microbiome which is evident in the stimulation of *Bacillus subtilis* by malic acid present in the rhizobiome (Mendes et al., 2013, Lakshmanan et al., 2014). Through the Janzen-Connell effect, the rhizobiome influences the plant community invariably leading to mutualistic coexistence of competitors in the same environment (Bever, 2003, Fitzsimons and

Miller, 2010, Liu et al., 2015). It can either be positive involving host symbiosis or negative involving pathogens and predators (Bever et al., 2012, Reinhart, 2012, Mack and Bever, 2014). Whether positively or negatively, the rhizobiome affects plant growth and stress tolerance and its importance are gaining more attention as shown in recent works (Reinhart, 2012, Mendes et al., 2013).

2.3.2 Root exudates and rhizobiome relationship

The rhizosphere is the soil around the roots of plants. It is affected by root exudates of plants as this and other rhizodeposition secreted by the plants determine the microbiota that will be present in the rhizosphere of the plant (Moe, 2013). These root exudates can also serve as signal molecules in microbe-microbe interactions, plant-microbe interactions, and plant-plant interactions among others.

Most rhizobiome organisms make use of the nutrients being exuded by the plants. It has been established that many rhizospheric microbes have pronounced effect on the growth and development of plants by making nutrients available, suppressing and controlling diseases, and increasing yield (Mendes et al., 2013, Raaijmakers, 2015). These microbes are commonly referred to as plant growth promoting rhizobacteria and they constitute the plant's secondary genome (Berendsen et al., 2012). There is a symbiotic relationship between the plants and the rhizosphere microbes as the plants feed the microbes with fixed carbon and other exudates, the microbes in turn help in aiding the growth of the plants (Berendsen et al., 2012, Minz et al., 2013, Mitter et al., 2013).

2.4 Rhizobiome and plant health

The rhizobiome is crucial to the growth, nutrition and health of plants. It includes the different diversities of genomes from eukaryotes, viruses and prokaryotes which are found in the plant ecosystem (Rout and Southworth, 2013, Lakshmanan et al., 2014). They all form different relationships with the plant host and other organisms. They form an ingrained interaction with

the plant as a result aiding in plant growth and affecting the health of the plant positively or negatively (Turner et al., 2013, Lapsansky et al., 2016). Positively they can improve seed germination, seedling vigor, plant growth, nutrition and plant development, while negatively they can cause diseases and stress competition for nutrients among others.

The rhizobiome acts as a highly developed secondary habitat of plants (Shi et al., 2011, Turner et al., 2013, Spence et al., 2014, Lareen et al., 2016). Rhizosphere microbes influence the diversity in plant habitat thereby increasing the richness of nutrients below ground (Lau and Lennon, 2011, Schnitzer et al., 2011, Wagg et al., 2011). According to Wagg et al. (2011), this richness in below-ground diversity can help in maintaining productivity under adverse conditions.

Plant rhizobiome can have either beneficial or nonbeneficial influence or both on plant health which can occur through beneficial microbes or pathogens. The positive effects can be carried out through the secretion of plant growth hormones, nutrient solubilization, pathogen antagonism and plant immune system induction (Vessey, 2003, Rudrappa et al., 2010, Verbon and Liberman, 2016). This establishment between the rhizobiome and plant is influenced by the mutualistic interaction between the host plant and surrounding soil. This fact has been established in so many works like that of Peiffer et al. (2013) and Chaparro et al. (2014).

As shown in figure 2.2, the deposition of fixed carbon and exudates by plants to their surroundings causes an influx of microbes thereby as a result increasing the microbial community because of the nutrients that are made available (Berendsen et al., 2012, Vorholt, 2012). Their interaction with the plant host is actually a gradual process that tends to be fully optimized as time goes on through having great impact on plant growth and development (Bakker et al., 2012). The rhizobiome composition, multifariousity, and abundance vary due to many factors which include the host plant, edaphic factors, and the microbial load. They all

determine the survival of the host plant (Sharma et al., 2010, Mendes et al., 2011, Lakshmanan et al., 2014).

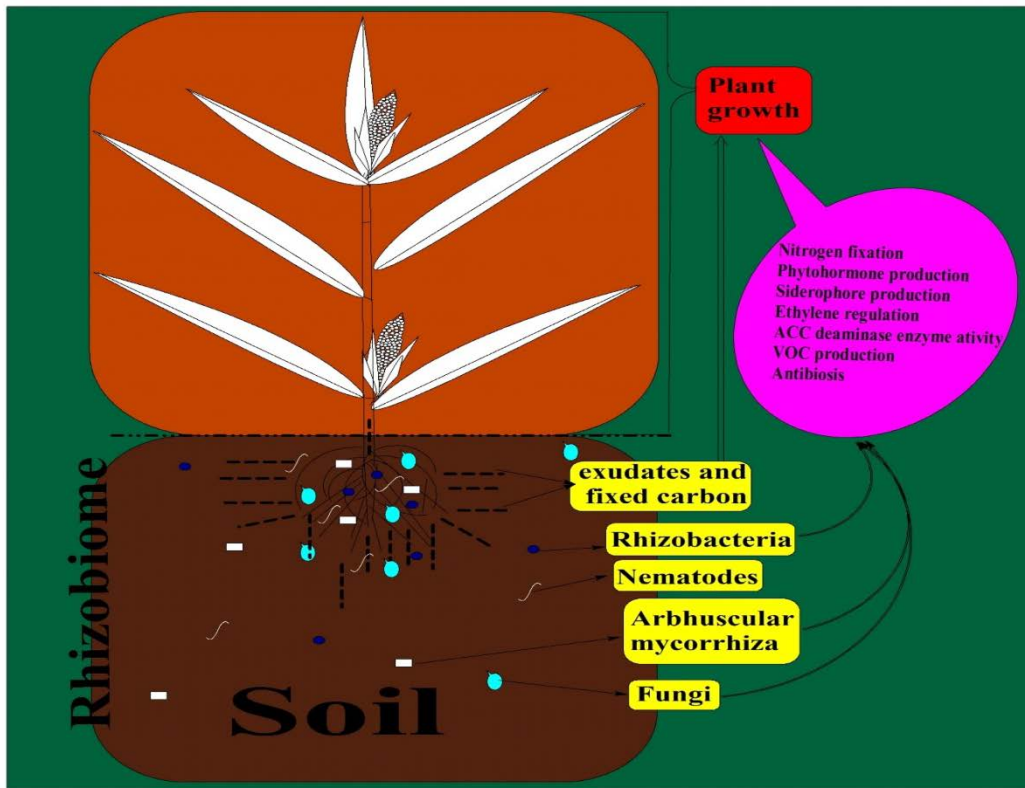


Figure 2.2: Rhizobiome diversity and how it affects plant health

The rhizobiome impact on plant health is more evident in disease suppression as the competition for an available nutrient is intense between beneficial microbes and pathogenic microbes (Lareen et al., 2016, Mommer et al., 2016). As most pathogens need to increase in population so as to overcome the rhizobacteria before they can invade the plant host, there is a great need and importance for the beneficial microbes to be more than the pathogenic microbes leading to nutrient starvation for the pathogens, thereby rendering them ineffective (Lareen et al., 2016). This phenomenon is referred to as “general disease suppression” and is attributed to the overall microbial activity (Berendsen et al., 2012). This gives the understanding of the diversity in the rhizobiome which can be beneficial, mutual or non-beneficial (pathogenic, predation). These levels of relationships between microbes in the rhizosphere have been greatly reviewed by Anderson et al. (2010) and Trdá et al. (2015).

2.4.1 Beneficial microbes

Beneficial microbes in the rhizobiome aid in plant growth, development, as well as control against pathogens using different mechanisms (Babalola, 2010a, Hayat et al., 2010, Saharan and Nehra, 2011). Some of these mechanisms include biofertilization, bioremediation, and biocontrol (Babalola, 2010a, Qiang et al., 2012, Ahemad and Kibret, 2014b). Examples of biofertilization include nitrogen fixation, phosphate solubilization, and production of plant growth hormones, while biocontrol involves curtailing of plant pathogens through the synthesis of siderophore, regulation of ethylene level, induced systemic resistance and acquired system resistance which is all well documented (Figure 2.2) (Bhattacharyya and Jha, 2012, Ahemad and Kibret, 2014b, Glick, 2014, Aznar and Dellagi, 2015).

Rhizospheric microbes are very much involved in the uptake of some trace elements like iron which exists primarily in the insoluble form making it inaccessible to the plants. This is where the use of siderophore come into the forefront (Barry and Challis, 2009, Aznar and Dellagi, 2015). Other notable mechanisms of biocontrol employed by beneficial microbes in addition to the ones mentioned earlier include quorum sensing interference, antibiosis, and competition for nutrients (Babalola, 2010a, Mela et al., 2011, Raaijmakers and Mazzola, 2012, Schenk et al., 2012b). Most rhizobacteria and rhizospheric fungi also produce metabolites which inhibit the growth of pathogens (Brakhage and Schroeckh, 2011, Saraf et al., 2014, Ludwig-Müller, 2015). *Trichoderma* species have been greatly credited with the production of these antimicrobial metabolites as discussed in the works of (Druzhinina et al., 2011, Hermosa et al., 2012, Mukherjee et al., 2012). Antibiotics can either act as growth inhibitors as well as mediators of cellular signals, depending on their concentration. They also act against biofilm formation and protozoa (Raaijmakers and Mazzola, 2012).

Volatile organic compounds (VOCs) are another major metabolites being produced by rhizosphere microbes. They are known to show plant growth activities and as signals between

host plants and the rhizobiome even though they are produced in small proportions compared to the other metabolites (Ali et al., 2015, Chung et al., 2015). *Burkholderia cepacia*, *Stenotrophomas maltophilia*, *Pseudomonas trivialis* and *fluorescens*, *Serratia plymuthica*, and *Bacillus subtilis* are among the VOCs-producing bacteria species (Trivedi et al., 2008, Saraf et al., 2014, Ali et al., 2015).

Plant immune system can also be triggered by some rhizospheric bacteria species and the system is regulated in most cases by jasmonic acid and ethylene (Berendsen et al., 2012, Nambara, 2013, Carvalhais et al., 2015). However, not all bacteria use the jasmonic acid/ethylene pathway. Instead, they use the salicylic acid pathway (Saikia et al., 2003, Nambara, 2013).

2.4.2 Non-beneficial microbes

These include the nematodes and the pathogenic fungi encompassing of the oomycetes. Their importance can also be related to the climatic conditions of the environment. Some bacteria are also pathogenic, examples are *Pectobacterium atrosepticum*, *Ralstonia solanacearum*, *Dickeya dadanthi*, *Dickeya solani*, *Agrobacterium tumefaciens*, and *Pectobacterium carotovorum* (Mansfield et al., 2012). Other pathogens include viruses which make use of nematodes and fungi as transport into the roots of plants (Rochon, 2009). Most of the nematodes are free-living while others can either be ectoparasites or endoparasites (Ali et al., 2011, Rasmann et al., 2012). The endoparasites can further be said to be migratory or sedentary depending on their location in the root. Nematodes' sensory organs aid them in the movement and location of nutrients (Rasmann et al., 2012). Human pathogens have also been discovered to affect plants as many reports have emerged in relation to this (Mendes et al., 2013, Morgan et al., 2013). Bacteria that cause human infections are also resident in the rhizobiome (Berg et al., 2005).

2.5 Conclusion

Plants, through the production of fixed carbon sources, help to maintain a stable rhizobiome while the beneficial microbes in turn help in aiding the growth of the plants by modifying the roots, nutrient acquisition, and protection against pathogens among other functions. This shows that there is a great role played by the rhizobiome in maintaining the health of the plants as they provide the full support needed by the plants for optimum growth and development. It is therefore recommended that more emphatically, work should be done on these rhizospheric microbes and their interactions with plant hosts to know how to make them more efficient for continuous crop production. Suggestions include the bioengineering of beneficial microbes either for dominance against pathogens in the area of nutrient acquisition, and superiority, which gives them more advantage in the rhizosphere. Another area to look at can also be the plant itself, plant modification to secrete useful exudates in the rhizosphere as well as toxic exudates to known pathogens will definitely maintain a high ratio of important microbes to pathogens in the rhizobiome.

2.6 Acknowledgements

North-West University is gratefully acknowledged for school bursary to OOS. OOB would like to thank the National Research Foundation, South Africa for a grant (Ref: UID81192) that have supported research in her laboratory.

2.7 Conflict of interests

There is no conflict of interest whatsoever from the authors.

CHAPTER 3

Functions and mechanisms of action of plant growth promoting rhizobacteria

Abstract

The idea of eradicating the use of hazardous fertilizers is becoming a reality because of the emergence of microorganisms that can serve the same purpose or even do better. Depletion of soil nutrients through leaching into the waterways thereby causing contamination are part of the hazardous effects of these chemical fertilizers which prompted the need for suitable alternatives. This brings us to the idea of using microbes which can be developed to biological fertilizers (biofertilizers). They are environmentally friendly as they are natural living organisms. As a result, they increase crop yield and production. In addition, they are less expensive compared to chemical fertilizers. These biofertilizers are generally called plant growth promoting rhizobacteria (PGPR). Although most PGPR are bacteria, some fungi have also been established to improve plant growth as well. Apart from improving crop yields, biofertilizers also control plant pathogens. For a more suitable and sustainable agriculture, the use of biofertilizers should be accepted over chemically synthesized fertilizers. It is of paramount importance to know that these PGPR definitely have specific ways and mechanisms in which they carry out these functions of plant growth, pathogen control, and other functions. These mechanisms need to be thoroughly worked on and understood to fully harness the potentials of these microbes. Perhaps the future of agriculture and food production is just at our fingertips.

Keywords: ACC deaminase, biocontrol, biofertilizer, bioremediation, phytohormones, siderophore

3.1 Introduction

Bacteria happen to be one of the most influential microbes known basically because of their importance in the health sector and disease causal agents. Bacteria are the most ubiquitous

organisms known. Apart from their use in health sectors for antibiotics production, bacteria have also emerged as good sources of fertilizer, agents of remediation and pathogen control. They have now been fully adopted in agricultural practices as they have been found to be more efficient plant growth promoters and biocontrol agents than chemical compounds. This discovery in plant growth promotion led to the coinage of the word “plant growth promoting rhizobacteria (PGPR)” (Kloepper and Schroth, 1978). However, other microbes such as fungi can also promote plant growth. Rhizobacteria is taken from the word rhizosphere which implies that these bacteria that promote plant growth are from the soil. Their functions are mainly source-dependent. Bacteria that are present on a farm land might not survive on a mining site due to changes in the environmental conditions. Rhizobacteria are predominantly located in the rhizospheric soil which is the soil around the root region of plants (Beneduzi et al., 2012). The rhizospheric soil is the most active part of the rhizobiome due to the activities of the microbes present. This is where most of the plant growth promoting activities take place. Today, several plant growth promoting rhizobacteria (PGPR) inoculum are currently being commercialized as biocontrol and biofertilizers.

This review aims to look at the different known mechanisms used by PGPR and other prospects into enhancing the role they can play for a sustainable agriculture and concurrently bring an end to the use of chemical fertilizers thus creating a safe environment as well as increased crop yield.

3.2 Overview of plant growth promoting rhizobacteria

The soil ecosystem consisting of bacteria (which are the most abundant), fungi, protozoa, virus and others are often separated into ‘microbes’ and ‘microfauna,’ with the latter also affecting plant growth as rhizobacteria (Abdul Khalil et al., 2015). This advantage in population helps the bacterial colonies to have more domineering effect in the rhizospheric ecosystem than others as they are able to colonize the rhizosphere more abundantly. The ability to effectively

colonize the rhizosphere is needed for dominating the ecosystem because the organisms with the higher presence will definitely be overwhelming for those with lesser counts. These factors majorly contribute to the ever presence state of bacteria as well as their power of survival in the ecosystem when competing with other organisms. For an organism to effectively colonize an ecosystem, many factors come into play majorly from the host plant and the immediate environment. Communications in the rhizosphere between plants and microbes as well as microbe-microbe likewise that of microbes and other organisms in the rhizosphere all have roles to play in shaping the rhizosphere, thereby determining the colonization ability of any organism in the rhizosphere. Due to their high colonizing power, rhizobacteria have a high impact in the rhizosphere and especially on the host plants. They have been discovered to aid plant growth and promote increased crop yield.

The emergence of growth-promoting microbes in form of rhizobacteria and some fungi have brought to focus, the importance of microbes in agriculture through nutrient availability improvement. These rhizobacteria are called plant growth promoting rhizobacteria (Kloepper et al., 1989). They are important free-living soil bacteria isolated from the rhizosphere, which has been shown to make crops healthy or increase crop productivity and output. These rhizobacteria are present in the soil in close proximity to the root of plants. The effects of PGPR on plants are majorly taken to be their plant growth and biocontrol activities (Saharan and Nehra, 2011, Ahemad and Kibret, 2014a). These effects are often promoted by the same organism. Their presence in the rhizosphere makes the entire plant more resistant to pathogens, thus giving them more advantage.

There have been reports depicting the significant increase in growth and yield in response to inoculation with PGPR (Chauhan et al., 2015, Grobelak et al., 2015). In recent years, PGPR have been greatly employed in sustainable agriculture and their use has increased rapidly in various parts of the world. In agriculture, PGPR are known for their impact on vascular plants

and are an integral part of crop yield management programs (Datta et al., 2015). It has been reported that the promoting ability of some bacteria may just be applicable to a particular plant species, cultivar, genotype and root exudates. Exudates are secreted by plants through the roots into the rhizosphere and these may support the activity of the PGPR inoculum or serve as substrates for the formation of bioactive substances by the inoculum (Babalola et al., 2003, Carvalhais et al., 2015). The presence of these exudates helps to a large extent to determine which organism colonizes or dominates the root more as the rhizospheric soil is more abundant in nutrients than the bulk soil. Besides root exudate production, the efficiency of rhizosphere bacteria is also attributed to their ability to use organic acids as carbon sources. As a result of the increasing concerns about the side effects of chemicals in crops, there is an increasing interest in understanding the mutualistic activities among rhizosphere microbial populations and how this relates to crop productions (Schenk et al., 2012a). Some PGPR are endophytic, meaning they reside inside the root forming an endophytic community, and adapting favorably to the role and function of the host plants, increasing root surface area which accelerates nutrient uptake, thereby increasing plant productivity (Adesemoye and Kloepper, 2009).

Researchers have majorly been focusing on the formulation of stable PGPR that can withstand natural conditions in the field when applied on seeds with or without chemical treatments by studying the different mechanisms involved. Criteria considered are among other things growth conditions before the formulation, development of carriers or transporters, and appropriate technology for application not forgetting issues involving registration and marketing of PGPR products (Figueiredo et al., 2011). Besides these criteria, focus should be shifted more to the biochemical aspects of plant growth using latest biotechnology means available. Most of these growth promoters are secretions which can be increased, reduced or even transferred to organisms that naturally would not produce them. This is what modern technology can achieve.

3.3 Mechanisms of actions of PGPR

PGPR promote plant growth by increasing root system uptake properties of rhizobacteria-colonized crops by aiding the uptake of nitrate ion, phosphate solubilization, iron chelation, increasing the availability of primary nutrients to the host plants, production of metabolites, antibiotics, and volatile compounds as well as inducing resistance to pathogens (Glick et al., 2007, Ali et al., 2015). Other genera like *Bacillus* and *Enterobacter*, produce siderophores, which function in sequestering iron for plant use, thus helping in delaying senescence and biological control (Babalola, 2010b, Ajilogba and Babalola, 2013). Another means is the production of plant hormones such as gibberellins, cytokinins, abscisic acid, and auxins, which influence plants physiological processes such as root respiration rate, metabolism, and root abundance (Ahemad and Kibret, 2014a, Ghorbanpour et al., 2015).

These mechanisms by which their function can be effected can be either directly or indirectly. There are many species of microbes colonizing the rhizosphere and each species has a higher activity for one or more of the mechanisms, meaning that no single organism have the ability to make use of all the mechanisms available (Saharan and Nehra, 2011). Several PGPR inoculants which seem to enhance growth through at least one of the mechanisms are currently being commercialized. Bacterial genera like, *Bacillus*, *Streptomyces*, *Pseudomonas*, and *Burkholderia* are the biological control agents which are consistently studied and sold (Pal and Tilak, 2012). It is of paramount importance to know that some of these mechanisms exhibited by each PGPR determine what they do and how they function. The most notable PGPR mechanisms are phytohormone production, nitrogen fixation and phosphate solubilization, ACC deaminase enzyme activity, siderophore production, antibiotics production, synthesis of hydrolytic enzymes, Induced Systemic Resistance (ISR) and System Acquired Resistance (SAR).

3.3.1 Phytohormone production

The ability of PGPR to produce and make use of phytohormones is one of the main mechanisms which have been employed in carrying out plant growth promotion. These phytohormones are auxin, gibberellin, cytokinin, ethylene and abscisic acid, and they are good plant growth regulators. As well as producing these phytohormones, PGPR also metabolizes and use them as nutrients (Dodd et al., 2010).

3.3.1.1 Auxin

Auxins are vital plant hormones that when not present can have a huge negative impact on plant growth. Patten and Glick (1996) reported that about 80% of rhizosphere microbes can synthesize and release auxin as secondary metabolites. The most naturally occurring and abundant class of auxin is indole-3-acetic acid also referred to as IAA (Spaepen et al., 2007). Others are indole-3-acetamide, indole-3-pyruvate, and indole-3-acetaldehyde shown in (Figure 3.1).

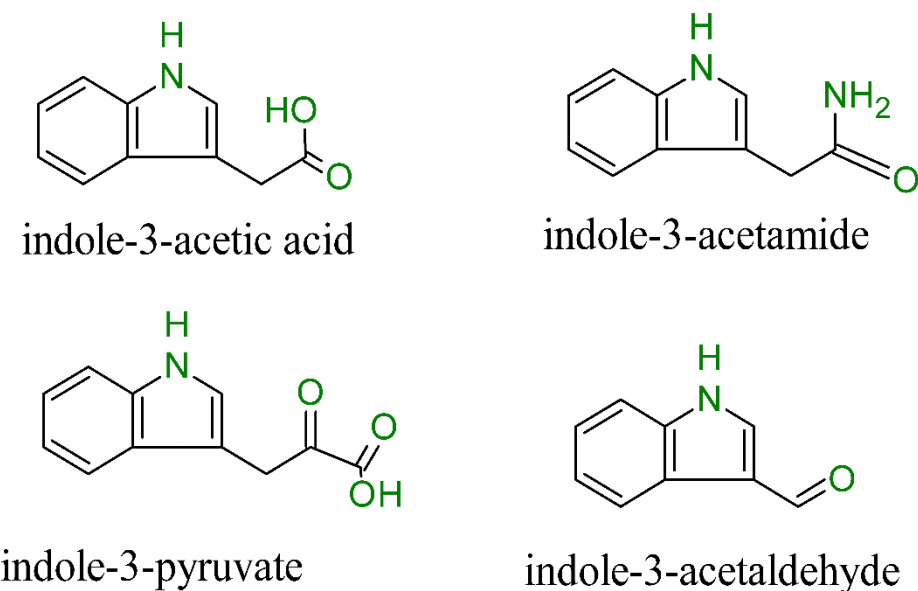


Figure 3.1: Some known derivatives of IAA

Inactive forms of auxin include 4-chloroindole-3-acetic acid and other conjugated forms which can be conjugated with sugars, alcohols, amino acids and glycoproteins (Korasick et al., 2013).

Auxins function in geotropism and phototropism, vascular tissue differentiation, apical

dominance, root initiation (lateral and adventitious), cell division, stem and root elongation (Grobela et al., 2015). Rhizobacteria IAA changes plant auxin pool, increases root length and surface area in the process increasing the uptake of root exudates by plants.

The precursor of IAA, which is tryptophan, is found in exudates at varying concentrations based on the plant genotype. The growth-promoting capacity of auxin-producing PGPR may require effective signals from the host plant (Vacheron et al., 2013). A schematic representation of IAA synthesis from tryptophan is shown in Figure 3.2.

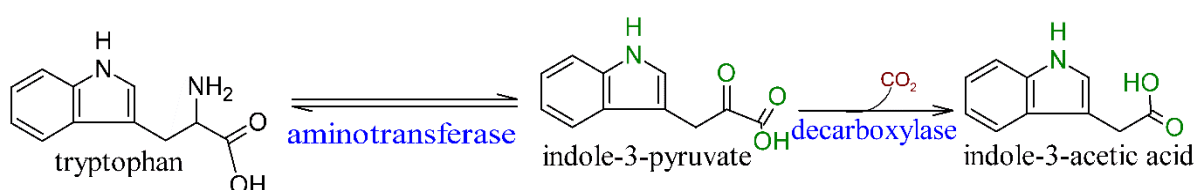


Figure 3.2: Schematic representation of IAA synthesis

3.3.1.2 Gibberellin (GA)

It is a large group of tetracyclic diterpenoid carboxylic acids having C₂₀ or C₁₉ carbon skeletons (Dodd et al., 2010, Hedden and Thomas, 2012). Presently 136 structures have been identified represented as GA₁ – GA₁₃₆ (Hedden and Thomas, 2012). Gibberellins are known for growth stimulation and activation of important growth processes like stem elongation, senescence, root hair abundance, sex expression, promotion of root growth, seed germination and flowering, the formation of fruits, regulation of vegetative, and reproductive bud (Kang et al., 2014, Zaidi et al., 2015). Along with other phytohormones, they are transducers of elicitor signals (Ghorbanpour et al., 2015). PGPR production of GAs has been observed in the following genera *Achromobacter xylosoxidans*, *Gluconobacter diazotrophicus*, *Acinetobacter calcoaceticus*, *Rhizobia*, *Azotobacter spp.*, *Bacillus spp.*, *Herbaspirillum seropedicae*, and *Azospirillum spp.* (Dodd et al., 2010, Deka et al., 2015). The biochemistry of gibberellins in bacteria is similar to that of plants with some few differences (Dodd et al., 2010). The absence of GAs is obvious in a decrease in lateral root number and length of roots and are either in the

active or inactive form (Dodd et al., 2010). More work on gibberellin in relation to its mechanisms, activation and deactivation processes are extensively discussed in the work of Hedden and Thomas (2012).

3.3.1.3 Cytokinins

Cytokinins are widely distributed in algae, bacteria, and higher plants but less information is available on the roles of bacteria-produced Cytokinins (Tirichine et al., 2007) which are synthesized by *Agrobacterium tumefaciens* and *Pseudomonas savastanoi* (Zhang et al., 2011). Cytokinins control cell differentiation in plant meristematic tissues. Cytokinin and auxin are very important in organogenesis (Jha and Saraf, 2015). There are two groups based on their structure which is the adenine type and the phenyl urea type. The adenine type includes kinetin and zeatin, while the phenyl urea type includes diphenyl urea and thidiazuron also shown in figure 3.3.

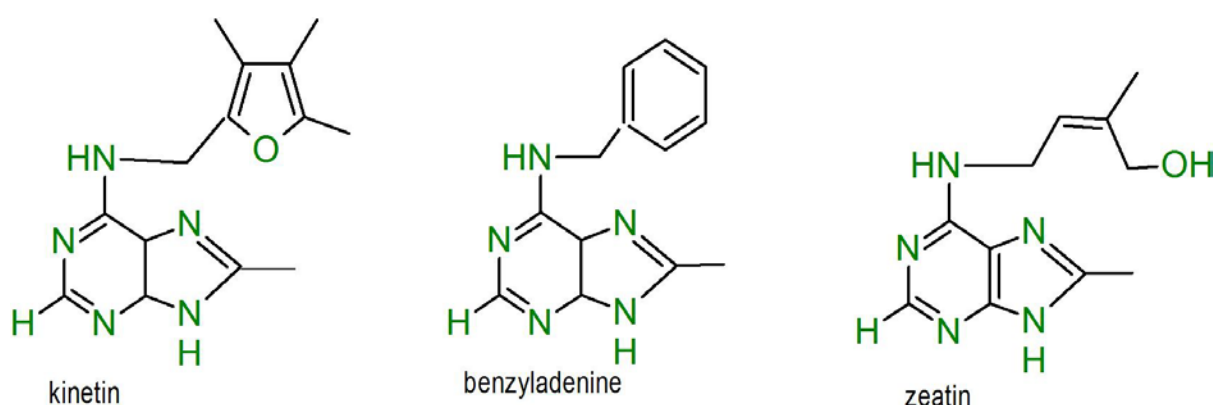


Figure 3.3a: Adenine-derived cytokinins

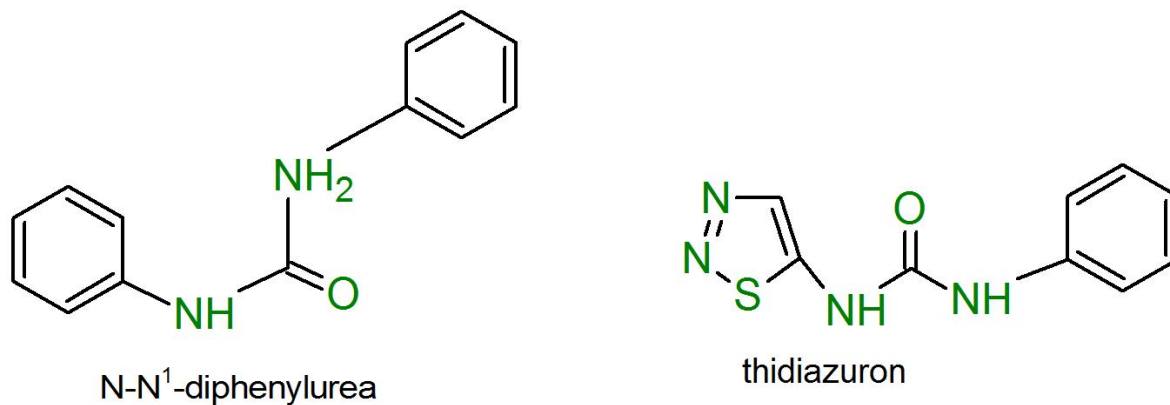


Figure 3.3b: Diphenylurea-type cytokinins

Cytokinins are known to take part in senescence delay by chlorophyll accumulation and organ formation in a wide range of tissues, root development, root hair formation, root elongation, stem initiation, and leaf expansion (Sakakibara, 2006).

3.3.1.4 Abscisic acid (ABA)

Abscisic acid is synthesized in the chloroplast from 9-*cis*- neoxanthin (Dodd et al., 2010).

It is released in response to abiotic stress conditions in plants carrying out homeostatic regulation (Nambara and Marion-Poll, 2005). ABA maintains elongation of roots which depend on the substrate–water potential (Dodd et al., 2010). It also supports water conservation in plants by regulating and controlling stomata water loss during drought stress conditions (Zhu, 2002). ABA is also responsible for the regulation of seed development in plants.

3.3.2 ACC deaminase enzyme activity on ethylene level

Ethylene became recognized as a plant hormone from its actions in leave shedding, seed geotropism, flowering and ripening of fruits after they have all been exposed to one form of ethylene or the other (Babalola et al., 2003). It should be noted that excessive production of ethylene in plants affects the growth as it causes stunted growth, does not allow for proper root elongation and this overproduction has been linked to the presence of pathogens (Babalola et al., 2002, Babalola, 2010b). Due to the fact that ethylene in normal quantity aids growth and

vice versa when in excess, there is a need for its production to be regulated so that plants will not be affected. It can be regulated by the action of ACC deaminase enzyme (Figure 3.4).

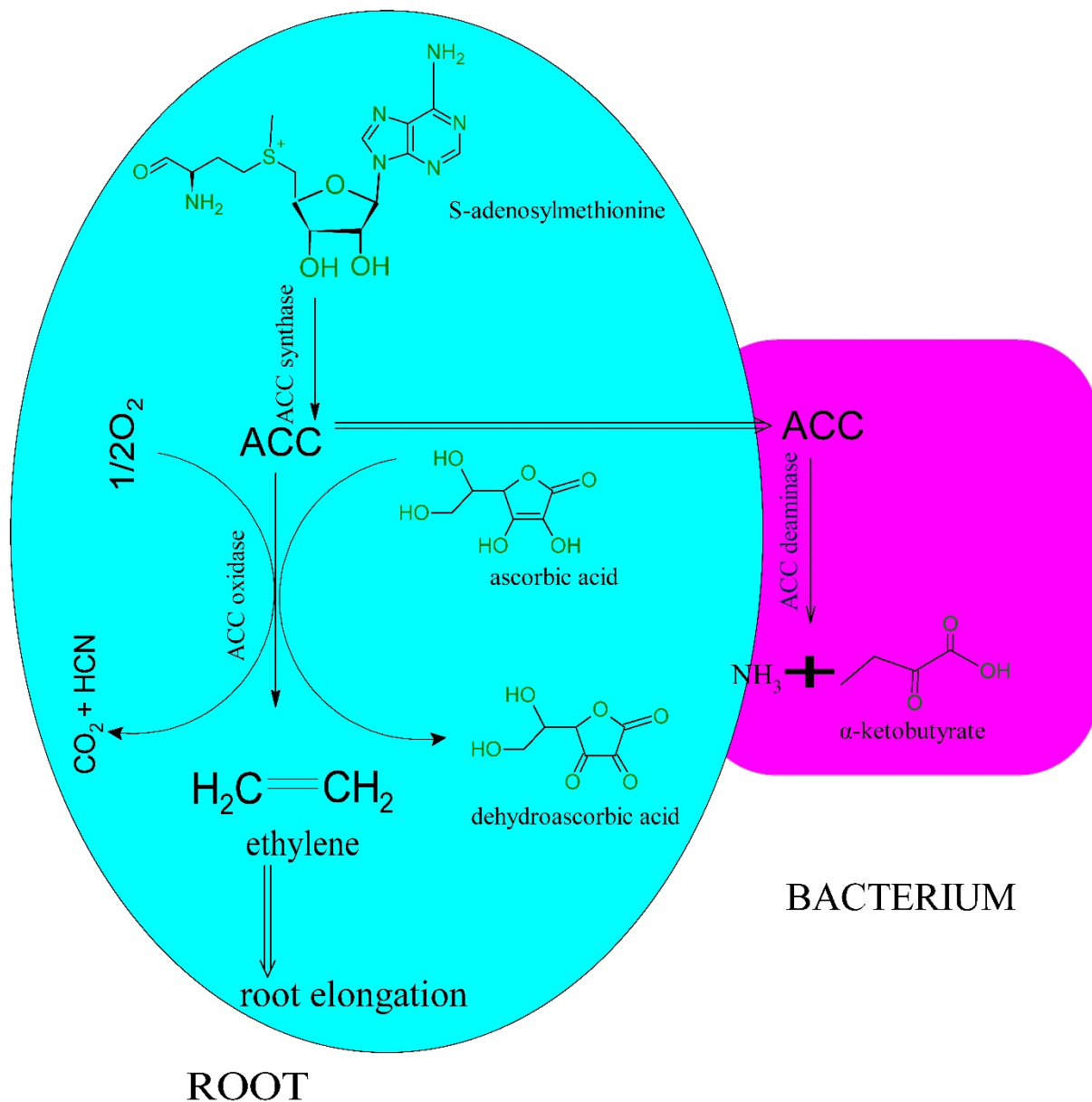


Figure 3.4: Functions and importance of the enzymes ACC deaminase, ACC oxidase and ACC synthase in the synthesis and regulation of ethylene both in the plants and bacterium

One of the most notable functions of ethylene is the alleviation of plant stress (Tak et al., 2013, Siddikee et al., 2015). Synthesis of ethylene is from its precursor ACC (1-Aminocyclopropane-1-carboxylic acid). The enzymes involved in the catabolism of ACC include S-adenosyl methionine (SAM) hydroxylase and decarboxylase, ACC synthase, oxidase, and deaminase

(Figure 3.5), all of which have been studied in numerous plant species (Babalola et al., 2003, Tak et al., 2013). The first four enzymes are produced in the plant while the last one, ACC deaminase, is produced by the PGPR possessing it. The enzyme ACC deaminase catabolizes ACC, resulting in the production of ethylene in plants and was first characterized in *Pseudomonas sp.* strain ACP after which it has been seen in many other organisms like *Penicillium citrinum*, *Hansenula saturnus*, *Rhizobium leguminosarum* *bv. viciae*, *Rhizobium hedysari*, and *Mesorhizobium loti* (Babalola et al., 2003). It has been reported that PGPR with ACC deaminase activity has helped in lowering and alleviating stress associated with pathogens in plants (Toklikishvili et al., 2010) and the down-regulation of ethylene levels in plants through the synthesis of the enzyme 1-amino-cyclopropane-1-carboxylate (ACC). Deaminase enzyme is a well-documented mechanism for growth promotion by PGPR (Babalola et al., 2003, Glick et al., 2007).

3.3.3 Nitrogen fixation

Nitrogen is one of the most important plant nutrients and its deficiency has a great effect on crop yield. Deficiency in nitrogen has led to the use of nitrogenous fertilizers in order to make up for the necessary plant requirements to achieve a maximum yield in most soils (Zhang et al., 2015). It is known that about 78% of nitrogen is present in the atmosphere, but this is not readily accessible to most organisms, as the form in which it is, is not suitable for plant assimilation (Baas et al., 2014). It was reported by Matiru and Dakora (2004) that the atmospheric nitrogen contributes about 65% of the nitrogen utilized by plants for growth; this will immensely contribute to the future of agriculture. It should be known that the atmospheric nitrogen has to be converted to the form that plants can assimilate through nitrogen fixation by nitrogen-fixing bacteria having the nitrogenase enzyme (Cooper and Scherer, 2012). Examples of symbiotic nitrogen fixers are *Rhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Allorhizobium*, *Mesorhizobium*, *Frankia*, *Azoarcus*, *Achromobacter*,

Burkholderia, and *Herbaspirillum* (Babalola, 2010b). It has been reported that diazotrophic PGPR, as well as rhizobia strains, promote cereal growth in synergy with other mechanisms through their nitrogen-fixing ability.

3.3.4 Phosphate solubilization

Bacteria that solubilize phosphorus are referred to as phosphate solubilizing bacteria. They supply phosphate in a more acceptable way to the plants and are a more safe way to the environment. They convert inorganic phosphate to organic phosphate which can be readily accessible to plants. The environmental condition, plant and soil conditions, and bacterial strains all affect the actions of phosphate solubilizers (Gupta et al., 2015). According to Banerjee et al. (2005), the most powerful phosphate solubilizers are of the genera *Bacillus*, *Rhizobium*, and *Pseudomonas* as well as non-symbiont nitrogen fixers such as *Azotobacter* and *Azospirillum* (Saharan and Nehra, 2011). On the other hand, it was made clear that phosphorus solubilizing bacterial activity is dependent on nitrogen source. There is an increase in phosphate when Ca^{2+} and EDTA are present in low amounts as the level of acidity and organic acids production also affect phosphate solubilization (Fankem et al., 2006).

3.3.5 Iron uptake

Iron has an important role to play in the plant photosynthetic system as it is an integral part of the light-absorbing chlorophyll and is also involved in different biosynthetic metabolisms (Tak et al., 2013, Jeong et al., 2014). The amount of soluble iron present is not always enough to have a great impact on crop yield. Catalase and peroxidase serve as biochemical markers for iron (Podile and Kishore, 2006). Pale green, yellowing or whitish leaves show unavailability of enough iron for the plant. Siderophores are produced by some bacteria which help in getting enough iron for plants (Saha et al., 2015).

These are the most widely attributed mechanisms to PGPR plant growth promotion. Many discoveries are being made as research becomes more advanced due to improvement in technology. Organisms have been engineered to produce more of these required molecules for plant growth as well as improving yield.

3.4 Functions of PGPR

Although the use of PGPR has not been embraced globally, the positive effects that they have on plant growth cannot be overlooked as it has been reported in many studies (Saharan and Nehra, 2011, Amprayn et al., 2012). PGPR function in different ways such as biofertilizer, biocontrol, and bioremediation.

3.4.1 PGPR as biofertilizer

Biofertilizers are simply organic fertilizers. According to Vessey (2003), biofertilizer is defined as a substance that contains living microbes and when applied to seed, plant surfaces, or soil, it colonizes the rhizosphere of the plant and promotes growth by increasing the availability of primary nutrients (N, P, Fe etc.) to the host plant. PGPR biofertilizers are alternatives to mineral and chemical fertilizers in increasing the yield and rate of plant growth for sustainable agriculture (Babalola, 2010b).

Biofertilizers are available for increasing crop nutrient uptake of nitrogen from nitrogen-fixers, iron uptake from siderophore-producers, sulfur uptake from sulfur-oxidizers, and phosphorus uptake from phosphate-mineral solubilizers in the rhizosphere (Stamford et al., 2008). They can also cater different needs of growing plant, and act mutualistically with other microbes in the rhizosphere. As a biofertilizer, PGPR can exist either in the rhizosphere or in the endosphere, root surface as well as intercellular spaces (Saharan and Nehra, 2011). In many cases, the PGPR is attached to the plant surface, but the means to which it is attached is rarely known, but there is a report on *Azospirillum* mechanism of attachment (Vicario et al., 2015). The best-expressed symbiosis reported are those involving host colonization by endophytic

PGPR, i.e., the legume-rhizobia symbiosis (Karmakar et al., 2015, Remigi et al., 2016). The chemotrophic attraction, attachment, and infection of the microbial symbiont and the growth of the root nodules that the bacteria inhabits is highly controlled (Babalola, 2010b). Biofertilizing PGPR may be present in all parts of plants (i.e. seed, roots, stems, leaves, fruit, etc.). Within the organs, the presence of endophytes living in apoplastic intercellular space within parenchyma tissue as well as xylem vessel apoplast is evident (Nogales et al., 2015). Some researchers consider xylem apoplast as an ideal location for endophytes, while others feel otherwise. It is important to also know that many plant pathogens cause plant death by colonizing the xylem apoplast (Upreti and Thomas, 2015).

The importance of PGPR produced hormones in root developments as it helps in root elongation has been established and shown in numerous studies (Aloni et al., 2006). Indole acetic acid (IAA), one of the hormones produced, can either have a positive or negative effect on plant growth depending on the concentration or amount present and how the plant tissues react to this concentration available to them (Dodd et al., 2010, Jha and Saraf, 2015). *Pseudomonas fluorescens* strains were reported to produce exudates in maize in response to IAA availability (Belimov et al., 2015). Auxin production by two strains of *Bacillus subtilis* was detected by Araujo et al. (2005), and the benefits they provided both as biofertilizer and biocontrol of fungal pathogens in soybean. *Azospirillum* have been discovered as good fixers of nitrogen, fixing nitrogen when in free-living which add value to the nitrogen contents of plants when inoculated with it (Kumar et al., 2014b).

With regard to phosphorous nutrition, *Pseudomonas*, *Bacillus* and *Rhizobium* are part of the bacterial genera with high phosphorus solubilization ability in the soil (Babalola, 2010b), which is due to their ability to produce siderophores, hydrolytic enzymes, and phytohormones (Vassilev et al., 2006). Most soil phosphorous are not readily accessible to plants as they are not in the form that plants can assimilate. This led to the use of phosphate fertilizers to make

up for the phosphorous demand of the plants (Saharan and Nehra, 2011). These fertilizers are expensive for most farmers, leading to the use of microbes that can solubilize phosphorus in poor soils. Other mechanisms imbibed by these microbes include the production of enzymes such as nitrogenase enzyme, chitinase, glucanase, ACC deaminase and the hydrolytic enzymes.

3.4.2 PGPR as biocontrol

Bacteria that have antagonistic activities against plant pathogens and prevent or suppress plant diseases are referred to as biocontrol agents. An effective biocontrol PGPR must be a vigorous root colonizer and have the ability to out-compete pathogens to be able to effectively suppress their actions. Synergism among PGPR against pathogens also works in favor of PGPR as they combine to combat the invading plant pathogen and diseases (Ali et al., 2015). Bacterial genera like *Bacillus*, *Streptomyces*, *Pseudomonas*, *Burkholderia*, and *Agrobacterium* are predominantly studied and marketed for this purpose (Berkelmans et al., 2003). Some notable works in pathogen biocontrol include biocontrol ability of alfalfa by *B. cereus* which enhanced the nodulation and seedlings of common bean, soybean, and cowpea (Figueiredo et al., 2008). Some PGPR have the ability to suppress plant diseases as well as to promote plant growth. PGPR, as biocontrol agents, can act through various mechanisms, such as: Competition for nutrients (Babalola, 2010b), Decrease of plant ethylene levels by the actions of ACC deaminase enzyme (Glick et al., 2007), synthesis of hydrolytic enzymes, such as chitinases, glucanases, proteases, and lipases, that can lyse pathogenic fungal cells (Maksimov et al., 2011), production of siderophores and antibiotics, induced systemic resistance (ISR) and systemic acquired resistance (SAR) (Saharan and Nehra, 2011, Goswami et al., 2016).

3.4.2.1 Siderophore production

Siderophores are small peptide molecules that have side chains and functional groups to which ferric ions are attached (Goswami et al., 2016). They are iron chelators that serve as iron carriers and have a high affinity for some ligands. Quite a large number of them have been

screened and used from microbes and they can also be species-specific (Sandy and Butler, 2009). Siderophore-producing microbes use them in fighting against pathogens by reducing the available iron and other useful ligands (Shen et al., 2013). Their high affinity to ferric ions makes it possible for their easy extraction from most mineral and organic complexes (Gupta et al., 2015). In the presence of oxygen and normal pH, the reduced form of Fe^{2+} is not stable so it is easily converted to the Fe^{3+} form. This form is slightly soluble and occurs as iron hydroxide which is not available to biological systems (Saha et al., 2015). Types of siderophores which are known so far include carboxylate, hydroxamates, phenol catecholate, pyoverdines, enterobactin, vibriobactin, mycobactin, acinetobactin, anguibactin, yersiniabactin, and pyochelin (Figure 3.6).

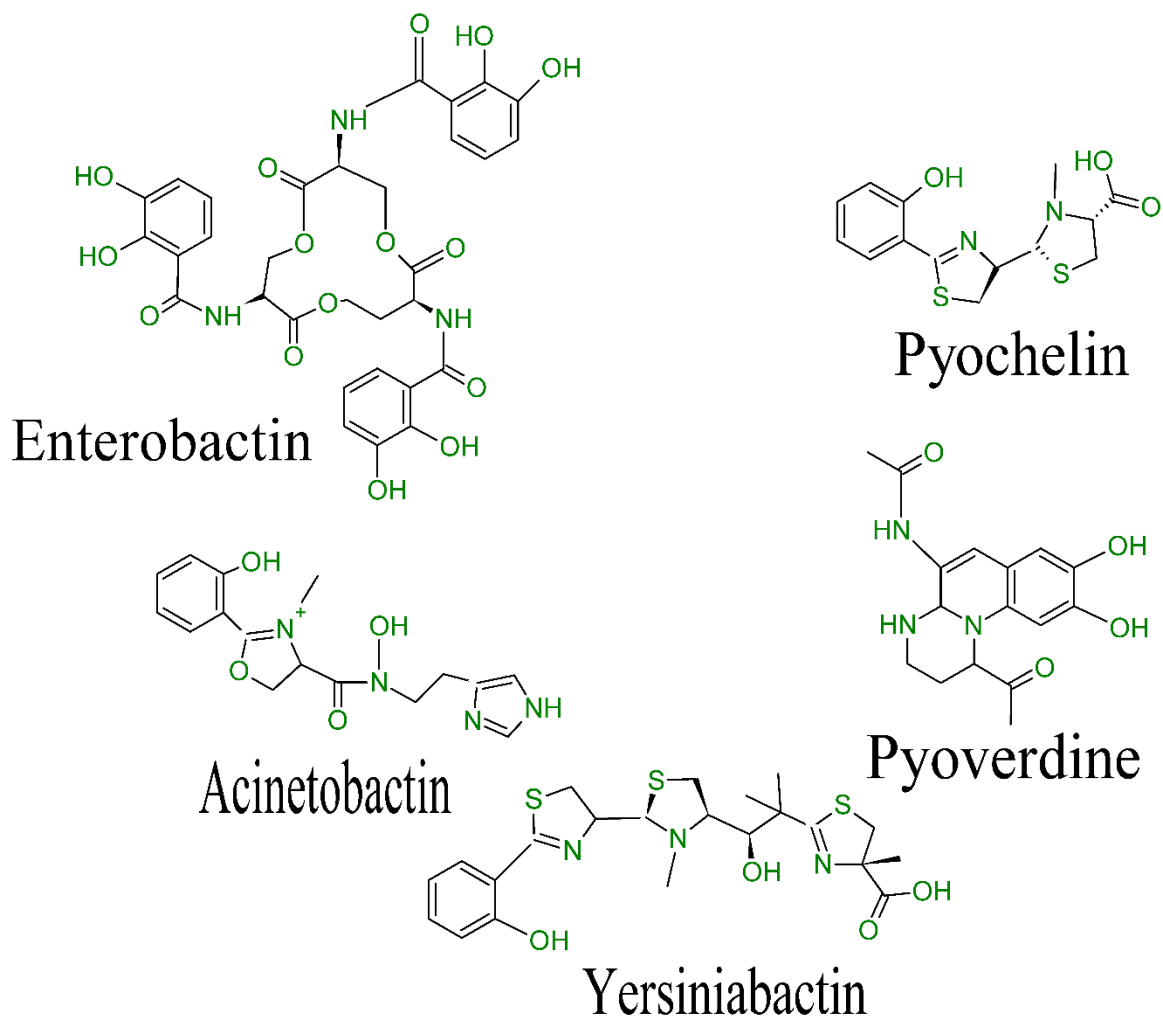


Figure 3.5: Some known siderophores which bacteria use in sequestering metals for plant growth and biocontrol

The activity of siderophores present on each microorganism determines their ability to improve plant development, with the plant also being able to recognize the bacterial iron-siderophore complex (Dimkpa et al., 2009). Another important factor is the presence of other metals such as nickel, lead, and cadmium (Dimkpa et al., 2008, Saha et al., 2015). It has not been verified yet if siderophores can actually provide plants with their necessary iron requirements from the siderophore complexes, but it is definitely an advantage for siderophore-producing PGPR that can colonize plant roots and shut out other microbes (Ahemad and Kibret, 2014a, Gupta et al., 2015). During competition by microbes for nutrients and minerals, the availability of siderophore may go a long way to determining which organism makes use of the carbon source

available from root exudates and rhizodeposition (Crowley, 2006). Among bacterial siderophores that have been studied are those of the *Pseudomonads* which are known for their high affinity to Fe²⁺.

3.4.2.2 Antibiotics production

Antibiotics are one of the most commonly used antagonists against plant pathogens (Glick et al., 2007). A large number of antibiotics have been isolated and characterized from fungal and bacterial strains (Maksimov et al., 2011). They contain a group of organic, low-molecular-weight compounds not favorable to the growth or metabolic activities of other microbes (Ali et al., 2015). Polymyxin, circulin, and colistin which were produced by *Bacillus* spp are active against Gram-positive and Gram-negative bacteria, as well as many pathogenic fungi (Maksimov et al., 2011). Some classes of antibiotic compounds which are understood to be related to the biocontrol of root diseases include phenazine, phloroglucinols, pyoluteorin, pyrrolnitrin, cyclic lipopeptides, hydrogen cyanide (HCN; which is volatile), quinolones, and resorcinol.

3.4.2.3 Induced systemic resistance (ISR)

Increasing the level of plant resistance to disease using external agents without altering the genetic make-up of the plant is known as induced resistance. It is also described as the process in which non-pathogenic microbes alleviate plant diseases by activating a resistance mechanism in the plants (Van Loon et al., 1998). Some PGPR have been established to carry out this induction. The inducing agents can be biotic or abiotic. Fengycin production by *Bacillus* strains is a strong case for ISR induction PGPR (Ali et al., 2015). ISR has been studied in many rhizobacteria-inoculated plants (Halfeld-Vieira et al., 2006) and was initially demonstrated by Van Peer and Schippers (1992) in plants, protected by the *Pseudomonas fluorescens* strain WCS417r against *Fusarium oxysporum* f. sp. *dianthi*. *Bacillus subtilis* also produces metabolites and enzymes like surfactin, iturin, and chitinase which all elicited ISR in

plants by acting on gene expressions as shown on oxylipin genes and genes in strawberry leaves (Cawoy et al., 2014, Yamamoto et al., 2015). Some studies have used the activity of the enzymes carrying out this induction to determine the mechanism involved. These activities are determined by the inducing agents, plant genotype, pathogen and physiological conditions (Waewthongrak et al., 2014, Yamamoto et al., 2015) . Many substances that help in disease suppression in plants have been attributed to the actions of PGPR. This shows how viable they can be when fully embraced in the modern agricultural system as they can help in increasing growth of plants as well as prevent plants from being attacked by phytopathogens. ISR helps plant to react faster to pathogenic attacks, and jasmonic acid and ethylene have also been shown to be involved (Ali et al., 2015, Farace et al., 2015).

3.4.2.4 Systemic acquired resistance (SAR)

Though much work has been done in this area, it was not until 1961 that it was really practiced and analyzed using tobacco plants with tobacco mosaic virus and was discovered to protect the plant against other viruses which led to the conception of the term “Systemic Acquired Resistance” (Ross, 1961). Fungi, viruses, nematodes and bacteria can all activate the plant’s defense mechanism which can be by a single organism or more organisms acting in synergy by secreting enzymes that carry out pathogenesis or aid pathogenesis (Ali et al., 2015). These pathogenesis-inducing enzymes include lipoxygenases, peroxidases, chitinases, and glucanases (Yamamoto et al., 2015). Peroxidases are expressed during the interaction of the pathogen and the host plants where it has been shown to oxidize phenols, plant protection, lignification and plant cells elongation (Abhayashree et al., 2016). Resistance in many plants such as rice and wheat has led to an increase in the activity of peroxidase and lipoxygenase metabolites aid in pathogen growth inhibition and phytoalexins induction (Bacon et al., 2015). Phytoalexins include secondary metabolites, antibiotics, low molecular weight substances, which are produced by plants in response to physical, chemical, or biological stress (Bacon et al., 2015).

They are able to prevent or reduce the activity of pathogens depending on the genotypes of host plants and the pathogen involved. The phytoalexin compounds are biocides and are directly related to the defense mechanism of plants (Kulkarni and Lingraju, 2015).

3.4.2.5 Synthesis of hydrolytic enzymes

Hydrolytic enzymes include glucanases, proteases, cellulases, and chitinases. Bacteria eliminate pathogens by the production of these enzymes which can be used to destroy oospores of phytopathogenic microbes affecting spore germination (Ali et al., 2015). Lytic enzymes secretion has been associated with biocontrol abilities of the producing bacteria because the enzymes hydrolyze the cell walls of invading pathogens (Pérez et al., 2016, Spadaro and Droby, 2016). Enzyme-producing PGPR were successfully used in consortium with other biocontrol agents which produce a co-inhibitory effect against the pathogen (Someya et al., 2007).

3.4.3 PGPR as bioremediators

Bioremediation involves the use of microbes to solve environmental issues caused by metals, spillage, and other factors. These issues affect agricultural practices as the soil is contaminated and might be made unsuitable for agricultural purpose. As useful as metals are to plants, they can also be hazardous to plants when they are in excess amount as these metals are oxidized to their unstable states. The metals have to remain in their stable state to be of help to plants. When unstable, they tend to disrupt both biological and physiological processes in plants (Masood et al., 2016); for example, they can attach to enzymes, biomolecules disrupting their activity thereby interfering with their functions (Wang et al., 2016). This increases the production of reactive oxygen species causing plant stress (Seth et al., 2008, Azarmi et al., 2016) as well as leading to toxicity in plants as they attack protein formations, this is evident in chlorosis and photosystems disruption (Wang et al., 2016). Three ways of dealing with these metals are physical, chemical and biological, the last is referred to as bioremediation. It has advantages over the other means of remediation as it has no hazardous effect on the

environment, unlike using chemicals. PGPR have been introduced in bioremediation and it has been shown that they help the plants in removing these heavy metals via the production of chelating agents like siderophores, while the plants in exchange provide nutrients for the survival of the microbes. According to Tak et al. (2013), microbes implore different mechanisms in their bioremediation processes, some of which are: Phytoextraction, phytostabilization, phytovolatilization, phytostimulation, phytodegradation, and rhizofiltration. In addition to controlling pathogens, it has been known that PGPR also helps in remediation activities (Wang et al., 2016). PGPR helps in degrading all these pollutants and making the soil less contaminated for crop production.

3.5 Conclusion and future prospects

PGPR are becoming increasingly important to food production in the ever-growing population of the world. The hazardous impact of chemical fertilizers on our environment is in no way reducing their popularity, making it necessary to source for an eco-friendly approach to improving crop yield. It is of great necessity to harness the growth promoting abilities of these bacteria. More techniques similar to the deep sequencing technology with thorough genomic analysis should be employed to adequately elucidate the root microbiome and their genes as this will make it possible to know exactly the different organisms present and the active genes. Presently no single technique can harness the whole genome present in the root microbiome. Harnessing the genes will give a better functioning of the rhizobacteria as this will help in determining what metabolites are produced by an organism and the gene producing them paving the way for easy rhizosphere engineering. Most of these functions are regulated by one gene or the other, so knowing the genes and the regulatory pathways involved will help to determine how to secrete more to decrease the synthesis of these metabolites. This brings metabolomics into the fold which will open up a totally different aspect of plant growth promotion and biocontrol as well as helping to probably constructing a stable complete

microbe. The invention of next generation sequencing coupled with thorough metabolomics and genomics studies will be key to advancing PGPR mechanisms and functions. Genetically modified organisms which have all the required genes can be the future of agricultural crops, and more work in this aspect will be helpful to the sustainability of food production and security.

3.6 Acknowledgements

North-West University is gratefully acknowledged for school bursary to OOS. OOB would like to thank the National Research Foundation, South Africa for a grant (Ref: UID81192) that have supported research in her laboratory.

3.7 Conflict of interests

There is no conflict of interest whatsoever from the authors.

CHAPTER 4

Development of bacterial strain for improved maize crop production

Abstract

The aim of this study was to enhance the growth and yield of maize crop through the activities of microbial strains inoculated alone or in combination with other strains as consortia. 6 isolates were selected after biochemical characterization and plant growth promotion assays have been carried out on all the initial isolates. The 6 selected isolates were further characterized by molecular methods before being tested on maize crops in the field and were designated as A1, A18, A29, NWU4, NWU14, and NWU198. The 6 isolates were tested against fungal and bacterial pathogens *Fusarium graminearum*, *Enterococcus faecalis*, and *Bacillus cereus*. All 6 were positive for ammonia, catalase and oxidase production, only A18 and NWU4 were positive for HCN production as well as A18 and A29 for IAA. Only A1 produced protease and phosphate solubilization was positive for A1, A18 and A29 isolates only. All 6 showed antagonistic activity against the fungal pathogen except A18 which showed no activity. All inhibited growth of *Enterococcus faecalis* at various rates while *Bacillus cereus* was only inhibited by A1 and NWU4. They were tested under field condition and they all showed great promise on the parameters such as plant height, root length, and leaf length etc. The result showed that combined PGPR as consortia is more effective in maize growth and yield. Some treatments also show great resistance to drought.

Importance of this study

This study was carried out in Mafikeng, North West Province of South Africa, which has harsh weather conditions. With this in mind, this study aim to evaluate the effect of the isolated rhizospheric bacteria in plant growth promotion as well as the combined effect of these bacteria in relation to single organisms compared to control. This study was also able to establish the colonization ability of these organisms in the natural environment provided by the study

location. Application of *Streptomyces* as plant growth promoters was also established as much work has focused on their biocontrol abilities. Drought tolerance ability of some isolates in consortia was also reported in this study.

Keywords

Antifungal, antibacterial, HCN, IAA, microbial consortia, phosphate solubilization, PGPR

4.1 Introduction

The rhizosphere depicts the region around the root system. The soil in this region is termed the rhizospheric soil (Vessey, 2003). This soil region is in direct contact with the root system of the plant hence making it more accessible to the nutrients released by the plants through the roots in the form of root exudates (Babalola, 2010a). Their actions in the soil can be beneficial or non-beneficial to the plants. The beneficial rhizobacteria are termed plant growth promoting rhizobacteria (PGPR) (Saharan and Nehra, 2011). It is important to know that it is not only bacteria that are found to aid plant growth as there are reports of some fungi and mycorrhizas as well as nematodes and other microbes all playing a role in plant growth promotion (Barea et al., 2004, Kumar et al., 2015). The rhizospheric system is very complex because of the interplay between the different rhizobacteria and other organisms; both prokaryotes and eukaryotes present. PGPR can impact plants directly or indirectly (Bhattacharyya and Jha, 2012). Direct effects include phytohormone production, ethylene regulation, action of ACC deaminase enzyme, N₂ fixation, and phosphate solubilization while indirect impacts can be through siderophore production, HCN and DAPG production, system acquired resistance and induced system resistance (Beneduzi et al., 2012, Bhattacharyya and Jha, 2012, Verma et al., 2013, George et al., 2015). Control of phytopathogens through antibiosis and production of some metabolites already mentioned makes the rhizobacteria good biocontrol agents (Ali et al., 2011, George et al., 2015). These actions of PGPR on plant growth have led to the insight of

using microbes as alternatives to fertilizers. This has brought about the name biofertilizer (Piromyou et al., 2011, Kumar et al., 2014a, Prasad et al., 2015, Vassilev et al., 2015).

Due to the ever increasing human population and the adverse effect chemical fertilizer is having on human health and the environment, there has been an urgent need to reduce the usage of these fertilizers (Ahemad and Kibret, 2014b). In this regard, biofertilizers are now being embraced to fill this gap as they do not only increase yield, but they also produce a safe environmental condition as they are not hazardous like chemicals (Lugtenberg and Kamilova, 2009, Kumar et al., 2014a). Cereal crops are one of the most important food crops in the world out of which maize is of high value (Jacobsen et al., 2013). It is one of the most consumed staple food crops in the world today from its use as food to paper and energy industries, as well as its use in livestock feeds (Bouffaud et al., 2012, Arruda et al., 2013). Its demand is increasing day by day and there is need to always meet up with the increasing demand. Its nutritional value and high carbohydrate content makes it one of the most sought after food crops (Hübner and Arendt, 2013, Tang et al., 2013). In time of drought, some PGPR are effective in helping the plants to withstand drought by reducing stress through ethylene regulation and the action of ACC deaminase enzyme (van Loon et al., 2006, Beneduzi et al., 2012).

The objective of our study is to evaluate the effect of indigenous microbes as single organisms, as well as in consortia on plant growth. Secondly, the objective is to access the effect of these microbes on maize plant growth in Mafikeng, South Africa.

4.2 Materials and methods

4.2.1 Field site and soil sampling

Mmabatho municipality field site's geographical coordinates are latitude 258470 S and longitude 258320 E, having 1281 m altitude. The average annual precipitation is 464 mm, while the average temperature range is from 68°C minimum to 308°C maximum. Soil properties of

Molelwane, the University farm are pH 5.7, organic carbon 0.69%, particle size distribution (Sand 59.9; silt 31.6; clay 8.5%) and silty loamy sand texture (Materechera, 2011). Sterile techniques were implord during each collection. Each sample was labelled immediately and placed in a dry cool place to prevent moisture accumulation and excessive drying. The samples were then taken to the North-West University's microbiology research laboratory for analysis.

4.2.1.1 Isolation of bacteria from rhizospheric soils

For the isolation of rhizobacteria, 10 g of rhizospheric soil was suspended in 90 mL distilled autoclaved water. Serial dilution method was used for further analysis of the prepared soil suspension. Suitable dilutions (10^{-2} , 10^{-4} , and 10^{-6}) were plated on Luria Bertani (LB) agar plates to isolate rhizobacteria and all plates (in three replicates) using standard microbiological isolation techniques. Well isolated colonies were purified by streaking on fresh LB agar plates.

Morphological traits

Morphological characteristics of the colony of each isolate present were examined on LB agar plates after 2 days of incubation.

4.2.2 *In vitro* screening of isolates for different plant growth promoting activities and biochemical traits

The isolated bacterial strains were screened for the following activities: IAA production, Phosphate Solubilisation, Catalase activity, oxidase production, protease production HCN production and Ammonia production.

4.2.2.1 Phytohormones production

The Phytohormones used by PGPR in crop growth and development include: IAA, Gibberellins, and Cytokinins etc.

Detection of IAA:

50 mL of LB broth containing 0.1% (D) L-tryptophan were inoculated with 500 μ L of 24 hours old bacterial cultures and incubated in a refrigerated incubator Shaker at $30\pm 0.1^\circ\text{C}$ and 180 revolution per minute for 48 hours in the dark. The bacterial cultures were centrifuged at 10,000 revolution per minute for 10 minutes at 4°C (Loper and Schroth, 1986).

4.2.2.2 Phosphate solubilization

Phosphate solubilization by the isolates was determined by spot inoculation on Pikovskaya agar medium plates. After incubation at 28°C for 7 days, the clear zone around the colonies was considered as a positive result for phosphate solubilization activity (Katznelson and Bose, 1959).

4.2.2.3 HCN production

Hydrogen cyanide (HCN) production by the isolates was carried out according to the method described by Castric (1975). Bacterial cultures were streaked on LB agar medium containing 4.4 g per liter of glycine. Whatman filter paper No. 1, which was soaked in 0.5% picric acid solution (in 2% sodium carbonate) was placed inside the lid of the plates which were then sealed with parafilm and incubated at $30\pm 0.1^\circ\text{C}$ for 4 days. The appearance of light brown to dark brown color indicates positive production of HCN.

4.2.2.4 Catalase activity

A drop of 3% hydrogen peroxide was added to 48 hours grown bacterial colony on a clean glass slide and mixed using a sterile tooth pick. The effervescence that follows indicates catalase activity.

4.2.2.5 Ammonia production

All isolates were tested for the production of ammonia according to the method described by Cappuccino and Sherman (1992). 0.5 mL of Nessler's reagent was added to overnight grown bacterial cultures that have been inoculated in 10 mL peptone broth and incubated at $30\pm 0.1^\circ\text{C}$

for 48 hours in an incubator shaker. The development of a faint yellow to dark brown color indicates ammonia production.

4.2.2.6 Protease production

Protease production was assayed according to Maurhofer et al. (1995). Each bacterial isolate spotted on skim milk agar plate and incubated for 24 hours shows halo zone around the bacterial colony and was considered as positive for protease production.

4.2.2.7 Oxidase activity

Oxidase activity of the isolates was determined by using filter paper spot method (Murray et al., 1981). Kovács oxidase reagent (1-2 drops) was added to 24-hour old culture on a small piece of filter paper. Change in color to dark purple within 60 to 90 seconds was considered positive.

4.2.2.8 Antagonism assay against phytopathogenic fungi and bacteria

All the 6 isolates were assayed for antifungal activities against *Fusarium graminearum* using Potato Dextrose Agar (PDA) medium. The isolates were streaked on PDA medium 3 cm in distance opposite to pathogenic fungi inoculated at the center of the medium. The barrier between isolates and fungi indicated antagonistic interaction between them. Antagonistic activity was investigated for 4 to 7 days after incubation at room temperature.

The percent growth inhibition (PGI) was calculated using the formula:

$PGI (\%) = \frac{KR-R1}{KR} \times 100$, where KR represents the distance (measured in mm) from the point of inoculation to the colony margin on the control dishes, and R1 the distance of fungal growth from the point of inoculation to the colony margin on the treated dishes in the direction of the antagonist. The PGI was categorized on a growth inhibition category (GIC) scale from 0 to 4, where 0 = no growth inhibition; 1 = 1-25% growth inhibition; 2 = 26-50% growth inhibition; 3 = 51-75% growth inhibition; 4 = 76-100% growth inhibition. The zone of

inhibition was recorded as the distance between the fungal pathogen and the area of antagonist growth after 7 days.

4.2.3 Molecular characterization of bacterial strains

Three isolates that showed the best PGPR traits were picked for further characterization and identification. The *Streptomyces sp.* that are also used have previously been identified.

4.2.3.1 DNA extraction

Genomic DNA of all isolates selected were extracted using ZR soil Microbe DNA MiniPrep™ (Zymo Research, USA) extraction kit. Bacterial cultures were grown in 10 mL of LB broth (Merck) at 25°C for 24 hours and centrifuged at 10,000 revolution per minute (Universal Z300K model centrifuge; HERMLE Labortechnik, Germany) for 5 minutes. The bacterial pellets were resuspended in 200 µL of distilled water and transferred to ZR Bashing Bead™ lysis tube and 750 µL lysis solutions were added to the tube. The bashing bead was secured in a bead beater fitted with a 2 mL tube holder FastPrep® 24 and processed at a maximum speed for 5 minutes. The ZR Bashing Bead™ lysis tube was centrifuged in a micro centrifuge at 10,000 xg for 1 minute. 400 µL of supernatant was transferred to a Zymo-Spin™ IV Spin Filter in a collection tube and was centrifuged at 7,000 xg for 1 minute and 1,200 bacterial DNA binding buffer to the filtrate in the collection tube. 800 µL of the mixture of the binding buffer and filtrate was transferred to a Zymo-Spin™ IIC Column in a collection tube and centrifuged at 10,000 xg for 1 minute. 200 µL of DNA Pre-Wash Buffer was added to the Zymo-Spin™ IIC Column in a new collection tube and centrifuged at 10,000 xg for 1 minute. 500 µL bacterial DNA Wash Buffer was added to the Zymo-Spin™ IIC Column in a new collection tube and centrifuged at 10,000 xg for 1 minute. The Zymo-Spin™ IIC Column was transferred to a clean 1.5 mL micro-centrifuge tube and 100 µL DNA Elution Buffer was added directly to the column matrix. The tube was centrifuged at 10,000 xg for 30 seconds to elute the DNA.

4.2.3.2 16S rDNA gene amplification

PCR was carried out in 25 μ L reaction volumes with each reaction containing 12.5 μ L PCR master mix, 0.5 μ L primer, 11 μ L nuclease free water and 1.0 μ L DNA template. The 16S rDNA gene was amplified using the universal bacterial primers 27f and 1525r (Forward 5'AGAGTTTGATCMTGGCTCAG, Reverse AAGGAGGTGWTCCARCC). PCR was performed using a DNA Engine DYADTM Peltier thermal cycler (Bio-Rad). The PCR program used was an initial denaturation at 95°C for 5 minutes, which was followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds and extension at 72°C for 1 minute, followed by a final extension at 72°C for 5 minutes.

4.2.3.3 Agarose gel electrophoresis and phylogenetic analysis

The PCR products were visualized by removing the gel slab from the tray and placing it on a UV trans-illuminator. The outcome of running the gel was recorded/ captured using ChemidocTM MP imaging system (Bio-Rad USA). Sequence alignment was performed with the Chromas software. Aligned sequences were analysed using the mega software, version 7.0.14. Phylogenetic analyses of the 16S rDNA sequences were performed by the maximum parsimony method.

4.2.4 Screening of rhizobacteria for plant growth promotion

4.2.4.1 Preparation of inoculum and seed treatment

Bacterial isolates were grown for 24 hours in LB broth and serial dilution was done to the level of 10^{-5} with sterilized distilled water. Maize seeds to be used for planting were surface sterilized with mercury chloride for 5 minutes and rinsed 5 times with sterilized distilled water. The seeds were subsequently inoculated with 1 μ L of the 10^{-5} bacterial dilution and left for 4 to 6 hours with occasional shaking. For controlled treatment, seeds were soaked in sterile distilled water.

4.2.4.2 Field study

Field trials were carried out using the selected isolates and some *Streptomyces sp.* This was done to ascertain the survival of the isolates in the natural environment when faced with the different adverse conditions of the natural habitat such as drought, competition with other microbes, stress etc. The design employed was a randomized complete block design with some isolates being used in consortia to examine the synergistic effect in respect to the control plant. Bacterial treated seed was sown to each in line. Irrigation was timely and all other agronomical intercultural processes were followed. All treatments were done in triplicate.

4.2.4.3 Data analysis

One-way analysis of variance (ANOVA) was performed to find out whether there was any difference between groups on specific treatments and the degree of significance of the differences among the variables (treatments) was determined using mean values considering the standard error of mean (SEM). Differences were calculated with Least Significant Difference (LSD) analysis ($p=0.05$). Data are reported as \pm standard error of the mean (SEM), which is a statistical parameter measuring the accuracy with which a sample represents a population. The smaller the standard error, the more representative the sample will be of the overall population.

4.3 Results

4.3.1 Screening, biochemical characterization and plant growth promoting ability of isolates

In the present study, different colonies differing in morphology were obtained from maize rhizosphere on LB agar media. Out of these, 31 isolates were achieved and classified according to their biochemical and plant growth promotion assay (Table 1 and 2). These isolates were labelled A1 to A31. Extra 3 *Streptomyces* species were acquired from Dr. Adegboye from the microbial biotech laboratory, North West University, South Africa and were used with the

isolated organisms. During the study, the potential plant growth abilities of the isolates were conducted *in vitro* based on their ability to produce ammonia, IAA, HCN and solubilization of phosphate as well as their antifungal and antibacterial antagonizing abilities.

About 93.5% of the isolates were able to produce ammonia which is related to nitrogen fixation. Nitrogen fixing microbes convert atmospheric nitrogen into ammonia. *Azospirillum* fix nitrogen under microaerobic conditions, as shown in wheat and sugarcane leaves (Malik et al., 2002, Kennedy et al., 2005). It also increases nitrogen content of cotton in the studies of Fayed and Daw (1987). All these show the production of ammonia.

A total of 35.5% produce IAA which is an important phytohormone for root elongation as well as coordinating hormonal balance making it important and necessary for plant growth (Kloepper, 2003). This supports the reports of Mendes et al. (2007) that endophytes produce more IAA than rhizosphere bacteria as all these are rhizosphere bacteria and few are IAA producers.

6 isolates (A1, A18, A29, NWU4, NWU14 and NWU198) were further characterized for their antifungal and antibacterial activities. The 6 isolates were selected based on their phosphate solubilization ability which is very essential for plant growth as well as their strong production of biochemical enzymes, and IAA production. Since the objective of this study is to work with consortia organisms, the isolates were selected in such a way that they will complement each other when acting in synergy for plant growth. They were further characterized for their antagonistic activity against selected fungal and bacterial pathogens. All 6 isolates showed strong antagonistic activity against the fungal pathogen *Fusarium graminearum* except A18 which did not show any inhibitory effect (Figure 4.1, Table 4.4). Both isolates showing more than 70% effect while NWU14 showed more than 50% with A29 and NWU4 showing more than 40% inhibitory effect. A1 and NWU198 were found to be most potent. In this study, it was observed that antifungal activity does not correlate to the protease production as thought

by (Grobelač et al., 2015). It was observed that the isolates that did not inhibit growth of the *Fusarium graminearum* as well as being negative was A18 only, others were all positive inhibitors but not all produce protease. HCN was produced by only A18 and NWU4.

4.3.2 Plant growth evaluation

Table 4.1 Biochemical characterization of isolates

Bacterial isolates	Catalase production	Oxidase production	Protease production
A1	+	+	+
A2	+	+	+
A3	+	++	-
A4	++	+	-
A5	+	+	-
A6	+	+	-
A7	+	-	+
A8	-	-	-
A9	+	-	+
A10	+	+	-
A11	+	++	+
A12	+	-	-
A13	++	-	-
A14	+	-	-
A15	+	-	-
A16	+	-	+
A17	++	-	-
A18	+	++	-
A19	+	-	-
A20	++	-	-
A21	++	-	-
A22	++	++	-
A23	+	++	+

A24	+	+	-
A25	+	-	+
A26	-	-	-
A27	++	+	+
A28	++	+	+
A29	++	++	-
A30	++	++	+
A31	+	-	-
NWU4	+	+	-
NWU14	++	+	-
NWU198	++	+	-

+, slight production; ++, significant production; -, no production

Table 4.2 Plant growth promotion assay

Bacterial isolates	Ammonia production	HCN production	IAA production	Phosphate solubilization
A1	++	-	-	+
A2	+	+	-	-
A3	+	+	-	+
A4	++	-	-	+
A5	+	-	-	-
A6	++	-	-	+
A7	+	-	-	-
A8	-	-	-	-
A9	+	-	-	-
A10	+	-	+	-
A11	++	-	+	-
A12	+	-	+	-
A13	+	-	-	-
A14	+	-	+	-
A15	++	-	+	+

A16	+	-	-	-
A17	++	-	+	+
A18	++	+	+	++
A19	+	-	-	-
A20	++	++	+	++
A21	+	-	-	-
A22	++	-	-	-
A23	++	-	+	-
A24	++	-	-	-
A25	+	-	-	-
A26	-	++	+	++
A27	++	-	-	-
A28	+	-	-	-
A29	++	-	+	+
A30	+	-	-	-
A31	++	-	-	-
NWU4	+	+	-	-
NWU14	+	-	-	-
NWU198	+	-	-	-

+, production; ++ significant production; -, no production

Table 3.3 Antifungal and antibacterial activity of selected isolates

Bacterial isolates	Antifungal activity		Antibacterial activity	
	<i>Fusarium graminearum</i>	<i>Enterococcus faecalis</i>	<i>Bacillus cereus</i>	
A1	+++	++	+	
A18	-	+	-	
A29	+	+	-	
NWU4	++	+	+	
NWU14	+	+	-	
NWU198	+++	++	-	

+++; significant inhibition; ++, moderate inhibition; +, slight inhibition; -, no inhibition

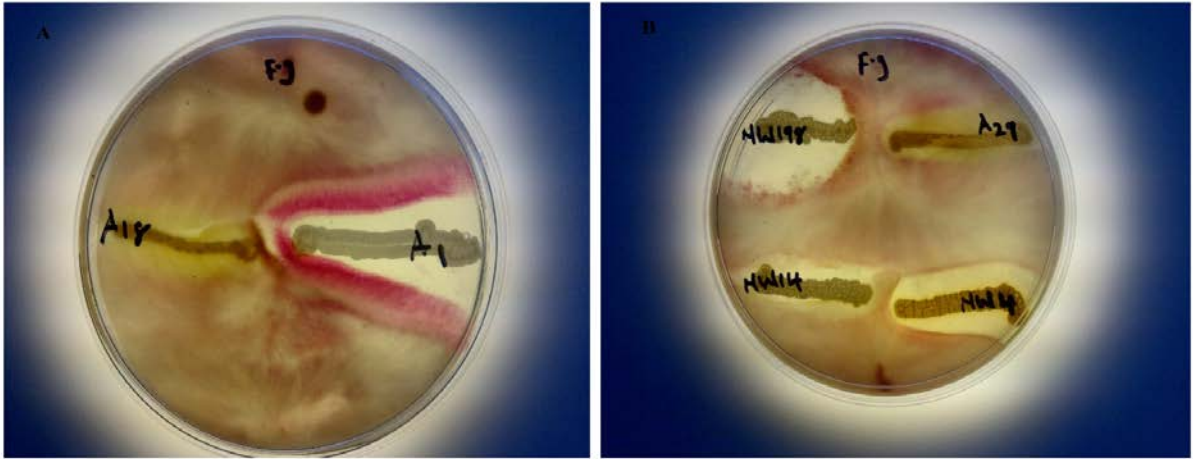


Figure 4.1: Antifungal activity against *F. graminearum* (F.g).

A1, NWU198, NWU4 show strong activity while NWU14 also show moderate activity level

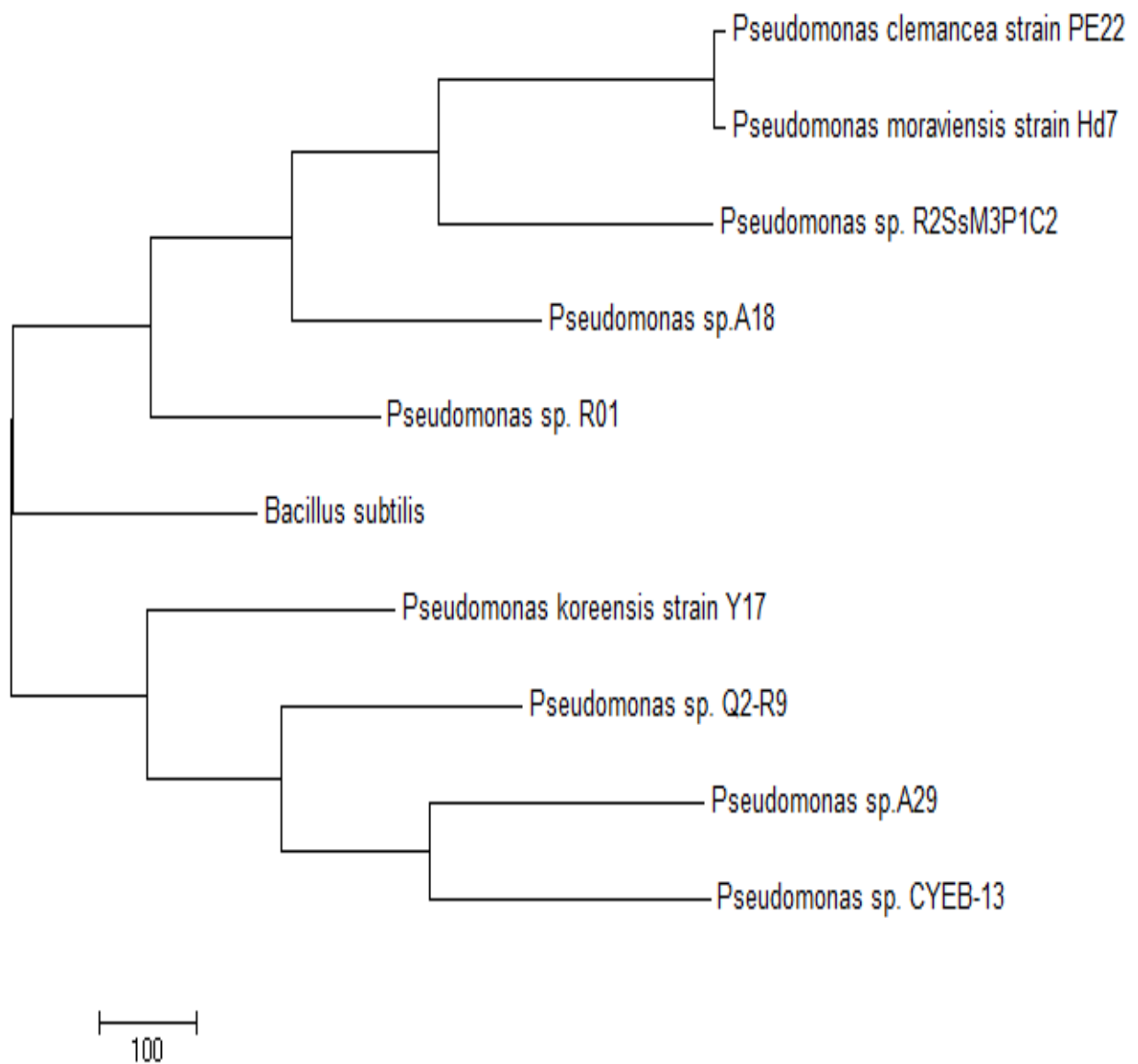


Figure 4.2: Evolutionary relationships of taxa of *Bacillus subtilis* and *Pseudomonas* sp.

The evolutionary history was inferred using the maximum parsimony method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. Evolutionary analysis were conducted in MEGA7.

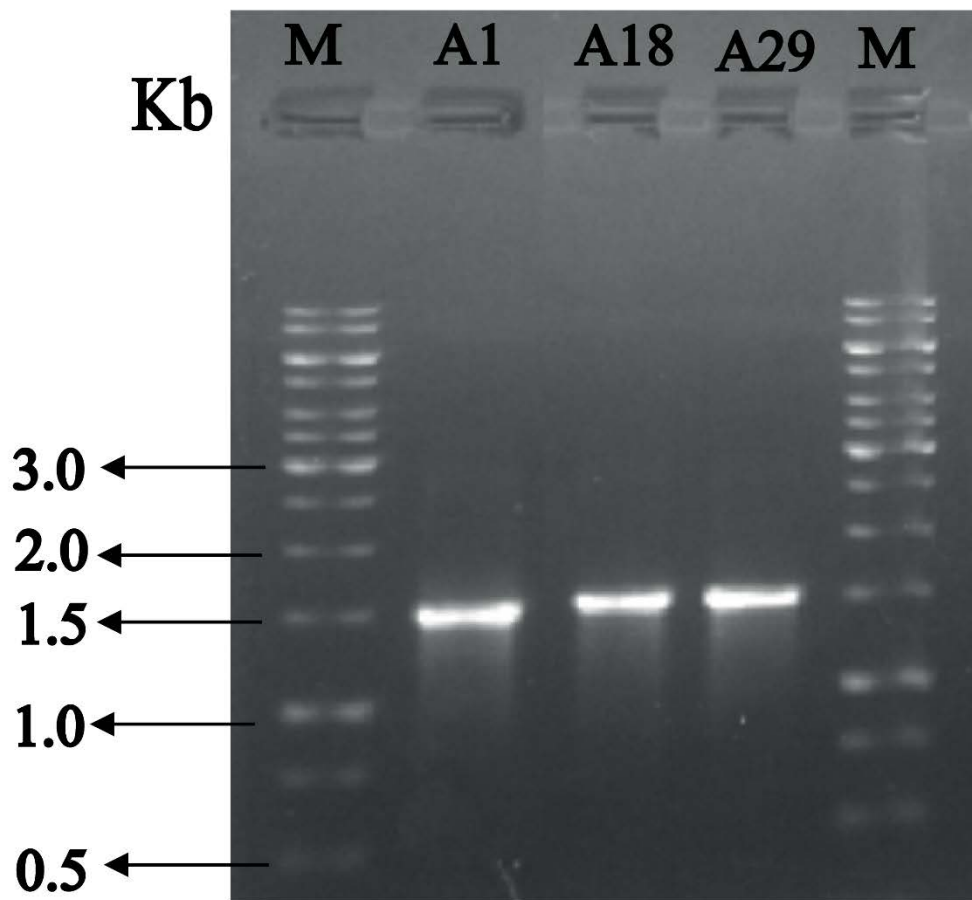
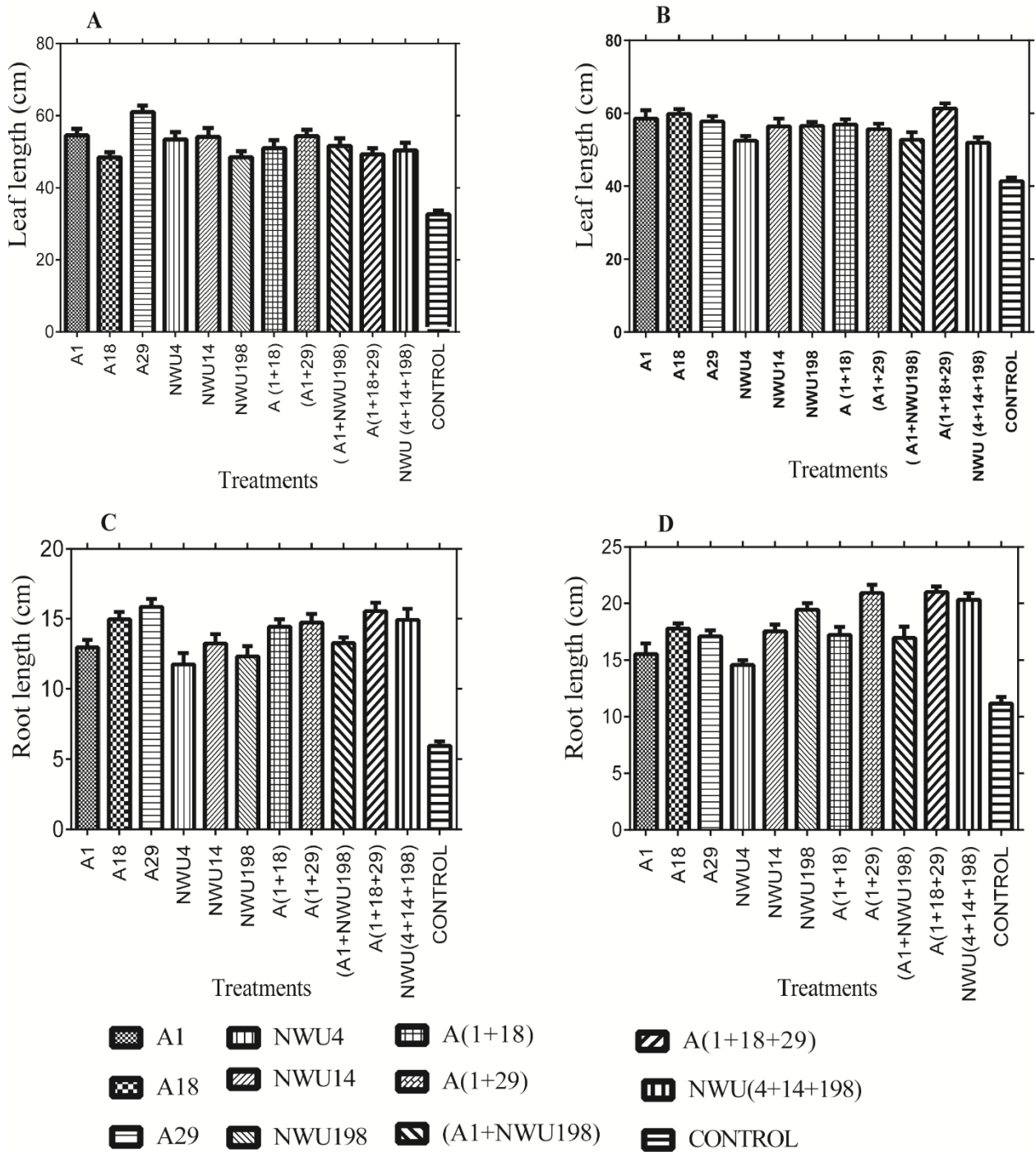


Figure 4.3: Agarose gel photograph indicating the positive band of approximately 1.5kb for 16S rDNA gene amplification

M = 1kb DNA marker, A1, A18 and A29 are PCR amplification of isolates.



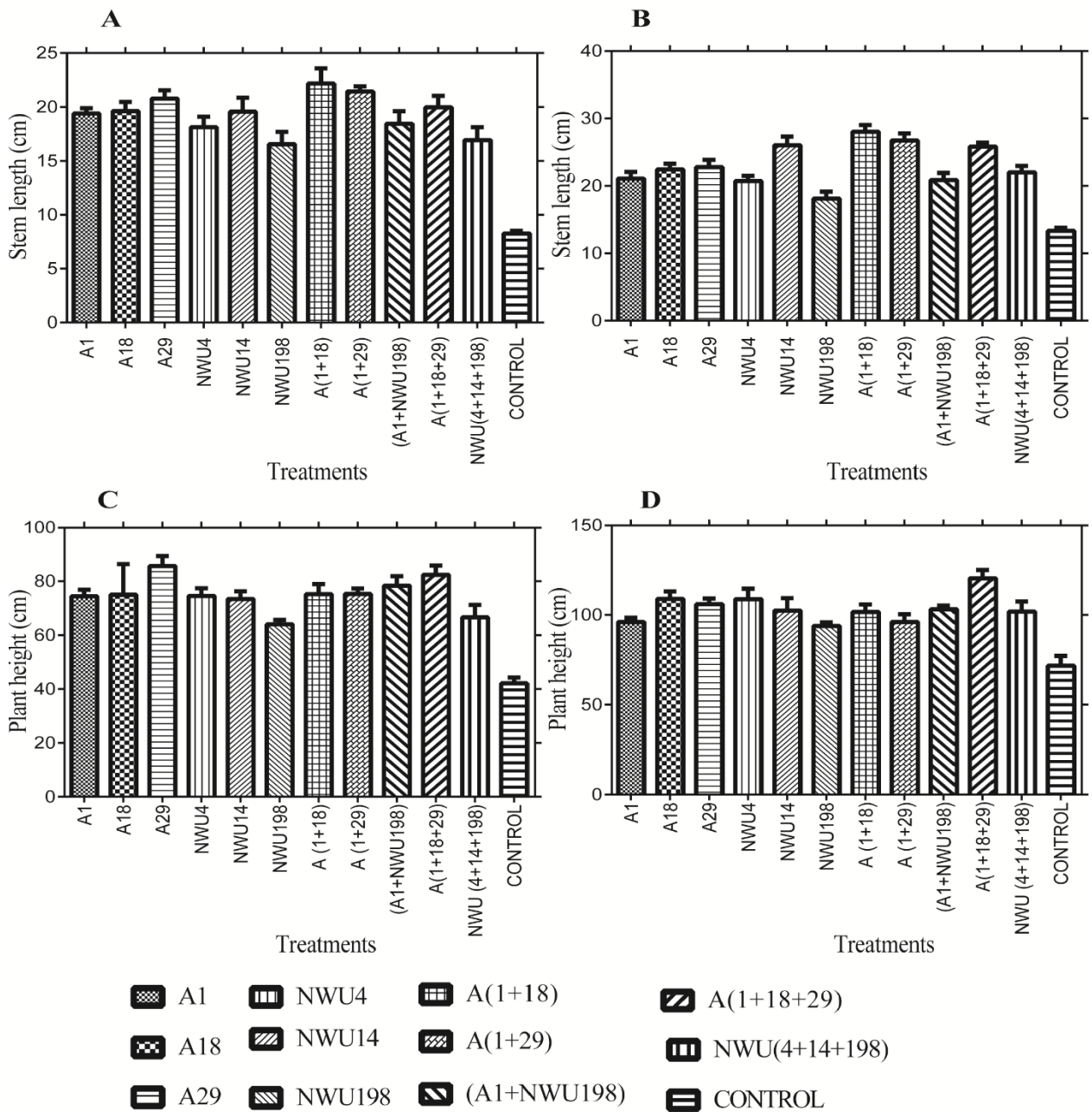


Figure 4.5: Plant growth promoting ability of isolates on maize plants

A and B- Stem length at 4 and 8 weeks respectively

C and D- Plant height at 4 and 8 weeks respectively

p>0.05 using the Geisser-Greenhouse test

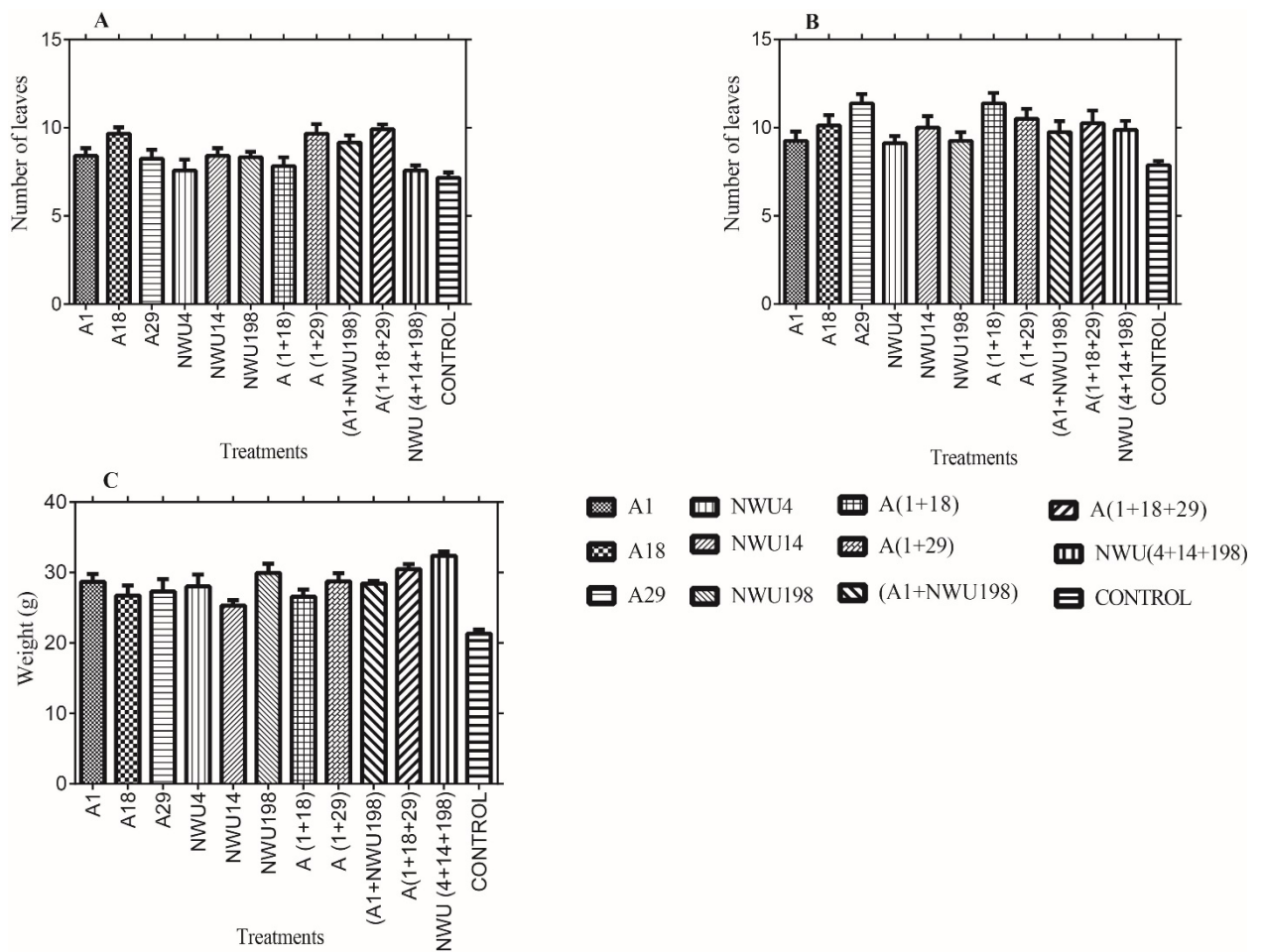


Figure 4.6: Plant growth promoting abilities of bacterial isolates

A and B-Number of leaves at 4 and 8 weeks respectively

C- Weight of 100 seeds

All measurements were taken in triplicates with the error bar representing standard error of the mean.



Figure 4.7: Drought resistance observed after 35 days without water

After 35 days, A, B, and C show drought resistance in maize which were inoculated with different PGPR. At this stage, the control plants are totally dried out and D shows maize plant with normal water content. A= A29, B=A1+A29, C=NWU4

4.4 Discussion

A variety of rhizobacteria such as *Streptomyces*, *Pseudomonas*, *Bacillus*, and *Enterobacter*, *Azospirillum* among others are present in the rhizosphere of different crops. Good root colonizing rhizobacteria strains have over the years proved to be good plant growth promoters. Their paramount use is aimed at maintaining a safe environment by reducing the use of chemical fertilizers in agricultural practices. Generally referred to as PGPR, their plant growth promoting abilities are elicited either directly on the plants or indirectly. They have proven to

improve yield and increase germination in various plants such as maize (Adjanohoun et al., 2011, Krey et al., 2013), rice (Gopalakrishnan et al., 2013a, Gopalakrishnan et al., 2013b, Guan et al., 2013), wheat (Guan et al., 2013, Islam et al., 2014, Kaur and Sudhakara Reddy, 2014), millet (Sharathchandra et al., 2004), and cucumber (El-Tarabily et al., 2010, Pii et al., 2015b). Root development and ability to promote nutrient availability are also key to the functions of PGPR Gopalakrishnan et al. (2015), as well as Sreevidya et al. (2016). Their non-toxicity, sustainable application as well as being environmentally friendly gives them an edge over chemical growth promoters. On the other hand, they are more sensitive to temperatures, pH, drought etc. Their efficacy on the field has not been generally consistent as is notable in the availability of more reports and data regarding activities of PGPR in the green house compared to the open field in most studies. The rhizospheric soil is the region in the root of plants and it serves as habitats to most of these PGPR, although they can also exist in other places like the endophytic PGPR which are found in the endophytes. These endophytes have also been involved in plant growth promotion as it is evident in some studies (Goudjal et al., 2013, Ali et al., 2014, Chen et al., 2014, Gholami et al., 2014, Gond et al., 2015). The rhizosphere is highly influenced by the root exudates as it determines the microbes present, thereby determining the rhizobiome of the plant. Interactions between the various rhizobiome constituents are key to the actions of the rhizobiome on plant growth. These interactions occur through various signals from the microbes or the exudates released by the plants. An organism is attracted to a specific signal which explains why the exudates can actually determine the rhizobiome constituent of any plant. It can be said from this that a plant inadvertently determines its own rhizobiome and that the rhizobiome of a maize plant will definitely differ from that of a cowpea because they will definitely produce different exudates. Apart from root exudates, nutrients such as phosphorous, nitrogen, iron, as well as sodium have all proven to be required for plant growth, especially nitrogen and phosphorous (Abou-el-Seoud and Abdel-Megeed, 2012, Pii et al.,

2015a). Nitrogen fixing rhizobacteria and phosphate solubilizers are regarded as important in PGPR classification. Depending on the degree of these traits exhibited by a rhizobacteria, their plant growth effect varies considerably from one organism to the other. It should be noted that no single organism exhibits a single plant growth promoting trait but have more than one trait working synergistically. The synergy actually helps the rhizobacteria to effectively be good candidates as a credible plant growth promoter as they complement each other. The rate at which the rhizobacteria colonizes the rhizosphere of plants determines its shelf life in that rhizosphere. A good colonizer will definitely exist for longer period than a poor one. The colonizing ability determines the survival rate against other microbes and the biome in general (Toumatia et al., 2016). The type of plant is another key determinant of the rhizobiome make up. For example, rhizobiome of a legume will definitely be different from the rhizobiome of a cereal as they are two different types of crops. A legume will definitely have more nitrogen fixers than cereals. This has been confirmed in many studies as is the case with *Rhizobium* which is not found in cereal rhizosphere but readily found in legumes (Joseph et al., 2012). This does not mean that they do not exist in cereals or other non-leguminous crops at all as revealed in the studies of Egamberdieva et al. (2014) and Cassán et al. (2014). The quality of the soil is another important determinant of plant growth as it determines and sharpen the rhizosphere constituents.

PGPR activities in biocontrol can be through stress alleviation, which are caused by environmental factors, drought or insufficient nutrients (Ali et al., 2014). They can also attack pathogens by limiting the nutrient available to these pathogens through better colonization as this gives them an edge in terms of population in regard to the invading pathogens (Beneduzi et al., 2012). With this, the pathogens do not stand a chance at all as they are out-numbered by the rhizobacteria. Emergence of PGPR has brought hope to achieving a safe environment with improved crop yield thereby giving hope for sustainable agriculture. PGPR has been

shown to efficiently elongate root growth, shoot length, leaf area, increase biomass and general aspects of plant growth through nitrogen fixation, siderophore production, ACC deaminase activity, phosphate solubilization, phytohormone production, as well as protecting these plants from foreign invaders through their biocontrol activities such as production of volatile compounds, HCN, induced system resistance and systemic acquired resistance among others (Jain et al., 2014, Dinesh et al., 2015, George et al., 2015, Jha and Saraf, 2015, Jog et al., 2016, Kumar et al., 2016).

This study isolated and identified rhizobacteria from maize planted at the North West University farm, Molelwane, South Africa. Three strains of *Streptomyces* which have already been identified in the works of Adegboye and Babalola (2013) were also used. Inoculation of PGPR strains increased all parameters determined in field experiment. After morphological characterization, plant growth promotion assay and biochemical characterization, 6 isolates that showed great promise as plant growth promoters were selected. Antagonistic assay against fungal and bacteria pathogens were carried out on the selected isolates and were used in this study. 5 of the 6 strains showed antagonistic activity against the fungal pathogen *Fusarium graminearum*. This antagonistic activity implies that these 5 isolates out of which are 3 *Streptomyces*, 1 *Pseudomonas* and 1 *Bacillus* definitely produce strong secondary metabolites and volatile compounds acting against the pathogen. Production of secondary metabolites is one of the main traits of recognised biocontrol agents. They can therefore be good control against the pathogen and possibly other fungal pathogens too. *Streptomyces* have been immensely used in this regard as they are good producers of secondary metabolites (Adegboye and Babalola, 2013). It is no surprise that they are effective antagonists. This result is supported by the works of several researchers (Lucy et al., 2004, Baysal et al., 2008, Ajilogba and Babalola, 2013, Cheng et al., 2014) and many strains have been tested for great biocontrol ability on different cereal crops such as observed in *Bacillus* sp and *Pseudomonas fluorescens*

which have antagonistic effect on soil borne pathogens such as *Fusarium oxysporum* that causes fusarium wilt in Chickpea (Saikia et al., 2009). Apart from fungal antagonism, all isolates were also tested for their antagonistic activity against bacterial pathogens *Enterococcus faecalis* and *Bacillus cereus*. It was observed that they all show antagonistic activity against *Enterococcus faecalis*, but only 2 were antagonistic against *Bacillus cereus*. All can be sources of antibiotics against these pathogens.

3 isolates are phosphate solubilizers meaning they can make phosphate available for plants use. Phosphorous is one of the important macronutrients in the soil and is important for its role in energy production in the form of ATP for various biological and chemical processes in the plant. It is present in soil as organic and inorganic phosphorous which are not readily accessible to plants. The ability of these organisms to solubilize these phosphorous into the form that can be used by plants makes them very essential for sustenance of plant growth. *Pseudomonas*, *Rhizobium*, *Bacillus*, *Flavobacterium*, *Erwinia*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Acinetobacter* and *Aerobacter* have the ability to solubilize insoluble inorganic phosphorous (Gupta et al., 2012, Behera et al., 2014). Inorganic soluble phosphorous are present as tricalcium and dicalcium phosphate, hydroxyl apatite as well as rock phosphates. *Pseudomonas*, *Bacillus* and *Rhizobium* are the most powerful phosphate solubilizers that have been identified (Browne et al., 2009, Kwak et al., 2015). The importance of phosphate and phosphate solubilizing rhizobacteria is supported in various works and it depend on plant host, soil conditions, and environmental conditions (Abou-el-Seoud and Abdel-Megeed, 2012, Behera et al., 2014).

All isolates were able to produce ammonia, but only 2 could produce HCN inferring that only these 2 isolates can actually take part in cyanogenesis. HCN is a secondary metabolite useful for fighting against pathogens through the production of cyanide which is toxic to the pathogens (Ali et al., 2011, Patel and Saraf, 2013, Dorokhov et al., 2014, Saraf et al., 2014, Ali

et al., 2015). Along with other metabolites, it makes the rhizobacteria good biocontrol agents. This is commonly produced by *Pseudomonas* (Ahmad et al., 2008). It is a phytotoxic agent inhibiting enzymes of major metabolic pathways and its applications as biocontrol agents are gaining importance (Jha and Saraf, 2015).

Indole acetic acid (IAA), a plant growth promoting hormone which functions through the elongation of the plant root as well as its synergy with ethylene biosynthesis helping in plant defense system (Bours et al., 2015, Di et al., 2016). IAA production is an important plant growth promoting trait and it has been shown to majorly have effect on plant root. More than 80% PGPR produce IAA. The effect of auxin is directly proportional to its concentration (Jha and Saraf, 2015). Two major modes of synthesis have been confirmed for auxin synthesis, the tryptophan dependent and tryptophan independent pathways. Other plant hormones secreted by rhizobacteria are cytokinin, gibberellin, and abscisic acid among others.

Beside the plant growth traits, the organisms displayed a broad range of morphological, biochemical and physiological characteristics. Consistent with this considerable phenotype heterogeneity, comparative gene sequencing clearly showed diversities in the genera. The phylogenetic relationship between the isolates was determined by 16S rDNA sequence analysis. The 16S rDNA gene of the isolates were PCR-amplified and sequenced. The partial nucleotide sequences of the 16S rDNA gene of the isolates were compared with nucleotide database of NCBI web server through BLAST tool. The BLAST search inferred that the isolates were members of the *Pseudomonas* and *Bacillus* genera. The 16S rDNA gene nucleotide sequence was obtained by BLASTN search; these reference sequences were selected based on high similarity (%) with good E value. The results of the 16S rDNA gene sequences analysis shows that query sequences were best pairwise aligned with 16S rDNA gene with sequence similarity and identity ranging between 90-100%, with E value of 0. Based on similarity criteria of the 16S rDNA gene sequences, the plant growth

promoting rhizobacteria were grouped into two main genera viz. *Bacillus* and *Pseudomonas*. They were identified up to the species level as 2 *Pseudomonas sp* and *Bacillus subtilis* along with the already identified *Streptomyces sp*. Previous findings showed that similar organisms to the isolates also possess plant growth promoting activities (Arshad et al., 2008, Avis et al., 2008, Beneduzi et al., 2012). Identified *Streptomyces* genera are also not left out in plant growth promotion majorly as biocontrol agents (Cheng et al., 2014, Al-Askar et al., 2015, Doolotkeldieva et al., 2015, Azura et al., 2016, Errakhi et al., 2016).

Upon inoculation of maize seeds with the organisms as treatments in single organism, double and triplicates, the effect of each treatment was assessed on the leaf length, root length, stem length, plant height, number of leaves and weight of 100 seeds from each treatment. The treatment combinations used are;

Single treatments – A1, A18, A29, NWU4, NWU14, NWU198

Double treatments – A (1+18), A (1+29), A1+NWU198

Triple treatments – A (1+18+29), NWU (4+14+198)

The control was treated with sterilized distilled water only.

The growth parameters were taken at 4 weeks and 8 weeks after planting at which all growth parameters of the inoculated plants taken were significantly more than the control plants. There were some tremendous differences from others which are discussed subsequently. The leaf length at 4 weeks shows the A29-treated plant to have the longest length while at 8 weeks, the consortia, A (1+18+29) have the longest length (Figure 4.4). One notable point to know is the somehow “regression” of growth observed in A29- treated plant compared to the 4 weeks while others increased, it seemed to have somehow retrogressed. Like the leaf length at 4 weeks, A29-treated plant showed the greatest effect on root length along with the triplicate treatments,

but at 8 weeks, both triplicate treated plants along with double treatment with A (1+29) showed tremendous increase in the observed root length of the maize plants (Figure 4.4). At both 4 and 8 weeks, the plants treated with two organisms, A (1+18) and A (1+29), have increased stem length compared to the other treatments with A (1+18+29) also showing great significance. A29 also showed tremendous increase in plant height at 4 weeks as well as (A1+NWU198) and A (1+18+29), which also have the highest value after 8 weeks of planting. All consortia organisms, especially those with three organisms, show tremendous increase in plant height compared to the two and single inoculated maize plants in respect to the control (Figure 4.5). Similar result was also established by Kumar et al. (2014a) on wheat plants, Egamberdiyeva (2007) on maize, Ajilogba and Babalola (2013) on Bambara plants. This result was also supported by other studies like Verma et al. (2014) and has been attributed to the aggregate production of phytohormones which help in root elongation, stem elongation and other important physiological processes in plant growth. The number of leaves both at 4 and 8 weeks are not very different from each other, but all the isolates showed increase in 100 seed weight compared to the control (Figure 4.6). Unlike other growth parameters that showed triple inoculations to be dominant over the others, some of the single inoculated maize plant seeds have higher weight than some double inoculations as shown by A1 and NWU198. The synergistic activities of the three *Streptomyces* isolates showed the greatest effect on seed weight followed by its second triplicate treatment. Increase in seed weight after rhizobacteria consortia inoculation was also reported by Yasari and Patwardhan (2007).

Another notable result in our work is the drought resistant ability shown by some treatments after 35 days without water (Figure 4.7). Low level of water or inadequate supply of water is known to significantly reduce the yield of any crop. The symbiotic relationship between plants and microbes in alleviating drought is key to maintaining drought tolerance in plants. This effect which was also supported by Armada et al. (2014) and Yandigeri et al. (2012) was

evident in some of the treatments. This is significant as the control is nowhere near being alive at this time, considering the hot and harsh climate of the location. Based on the partial 16S analysis, A18 and A29 were identified as *Pseudomonas* sp., while A1 was identified as *Bacillus subtilis* (Figure 4.2). Accession numbers KX453173 to KX453175 which have been deposited in the Genbank, have been assigned to isolates A1, A18 and A29. NWU 4, NWU 14 and NWU 198 are *Streptomyces globisporus*, *Streptomyces griseoflavus* and *Streptomyces heliomycini* which have previously been identified by Adegboye and Babalola (2013).

Efficient isolated microbial strains which are good root colonizers are also excellent plant growth promoters due to their excellent growth promoting traits (Montañez et al., 2012).

4.5 Conclusion

The use of consortia organisms in plant growth promotion has been evaluated on various crops. The working together of these organisms aggregate the majority of the plant growth traits exhibited by individual organisms and combine them to function as one. The effect of this was seen in this study.

This study also buttresses the importance of characterizing PGPR for multiple growth promoting traits and evaluating their potencies on field crops. The field exposes them to the natural environmental conditions and this help to identify the most suitable synergy and isolates that can survive in the natural habitat. The field provides a good platform to test their ability against competition and harsh weather conditions. It was expected that isolates A1, A18 and A29 show good promise to be excellent biofertilizers when in synergy as well as single isolates along with NWU4, NWU14 and NWU198 which are not as efficient, but have greater tendency to be equally effective if not more than the other isolates when combined as shown in some parameters but are generally known to be good biocontrol agents. This was demonstrated in their antagonistic activity against *Fusarium graminearum* (Figure 4.1). All isolates, depending on how they are used in the treatment either as single, double or triple, can effectively be used

for sustainable maize crop production but in all treatments, the triple treatment prove to be more effective. It is very important to know as it is shown in this study that not all combinations gave more increase than single treatments and not all triple combinations happen to be more effective which means that not all organisms can function well together as consortia to give maximum yield.

4.6 Acknowledgements

North-West University is gratefully acknowledged for school bursary to OOS. OOB would like to thank the National Research Foundation, South Africa for a grant (Ref: UID81192) that have supported research in her laboratory.

4.7 Conflict of interests

We declare that there is no conflict of interest.

CHAPTER 5

Metabolite profiling of *Bacillus subtilis* rhizobacteria isolated from maize rhizosphere

Abstract

Plant growth promoting rhizobacteria (PGPR) strain A1 which has already been identified previously in chapter four as *Bacillus subtilis* through 16S rDNA gene sequencing was assayed for its metabolites production using GC-MS technique. It was tested against fungal pathogen *Fusarium graminearum* and bacterial pathogens *Enterococcus faecalis* and *Bacillus cereus*. It showed considerable resistance these pathogens. The strain was also previously tested for its plant growth promoting ability on maize plants. The metabolites produced were isolated and analyzed with Gas chromatography-mass spectrometry (GC-MS) using eight solvents for extraction. The solvents used are chloroform, ethyl acetate, diethyl ether, n-hexane, methanol, butanol, petroleum ether, and benzene. Two metabolites were identified from butanol extract while benzene, ethyl acetate and hexane all show one notable bioactive metabolite. Petroleum ether and methanol have the largest return of metabolites with five and fifteen respectively. Chloroform and diethyl ether did not detect any hit in their GC-MS chromatograms. This result identifies the secondary bioactive metabolite produced by isolate A1 after successful trial in the field.

Keywords: *Bacillus subtilis*, biocontrol, GC-MS, PGPR, secondary metabolite

5.1 Introduction

Urbanization has increased the problem of land availability for agricultural purposes through its problems of pollution, environmental degradation and land use for settlements, not forgetting constant infestation by pathogens. Chemical fertilizers and pesticides are also not helping the environmental situation. This has called for the need for a more environmentally friendly approach towards solving these issues. The use of natural free-living microbes termed plant growth promoting rhizobacteria (PGPR) has been effective in this sense. *Bacillus* species

has been identified as good PGPR. They have been employed both in plant growth promotion and as biocontrol agent to increase crop yield.

Metabolites are formed from the end products of metabolism. Metabolism can be either catabolic or anabolic. Metabolites are categorized into primary and secondary metabolites based on their functions and metabolic pathways. Primary metabolites serve as a major source of energy while secondary metabolites are mostly excretory products which are used for protection (Ali et al., 2015). Secondary metabolites are bioactive compounds produced by organisms. These organisms can be bacteria, fungi, plants etc. Pharmaceutical, agricultural, and health sectors have all benefitted from these bioactive compounds (Brader et al., 2014). In agriculture, they are used as pesticides in biocontrol of pests. The use of these bioactive metabolites in biocontrol will help in reducing the dependence on chemical pesticides (Amoutzias et al., 2008). These form natural ways of controlling pathogenic organisms in farm land. Different techniques and approaches have been used to extract and identify these metabolites from their different sources. These techniques, among others, include NMR, GC-MS, LC-MS etc. These metabolites are derived from different compounds comprising of various functional groups including alkanes, alkenes, esters, amines, amides etc. Secondary metabolites are precursors of active agricultural biocontrol agents as well as antibiotics against drug resistant pathogenic microbes (Raaijmakers and Mazzola, 2012, Ali et al., 2015). Constant biosynthesis of active secondary metabolites has opened the way for discovery of more bioactive compounds that can be used in food production, pharmacy and health sector.

In the case of plant growth promoting rhizobacteria (PGPR) functioning as biocontrol agents, the secretion of secondary metabolites can be in the form of toxins which are harmful to pathogens causing diseases in food crops (Cavaglieri et al., 2005, Al-Askar et al., 2015). The secondary metabolites can also function as signal molecules for microbe communications in signaling such as quorum sensing (Dorokhov et al., 2014, Fischbach and Segre, 2016). Through

these activities, the secondary metabolites happen to be the dominant factor in PGPR serving as biocontrol agents and more are yet to be identified due to limited biochemical pathway elucidation in these microbes.

5.2 Materials and Methods

5.2.1 Field site and soil sampling

The rhizospheric soil samples were taken from Mmabatho municipality field site with geographical location of latitude 258470 S and longitude 258320 E, having 1281 m altitude. The average annual precipitation is 464 mm while the average temperature range is from 68 °C to 308 °C. Soil properties are pH 5.7, organic carbon is 0.69%, particle size distribution is (Sand 59.9; silt 31.6; clay 8.5%) and silty loamy sand in texture (Materechera, 2011). Sterile techniques were implored during each collection. Each sample was labelled immediately and placed in a dry cool place to prevent moisture accumulation and excessive drying. The samples were then taken to the North-West University's microbiology research laboratory for analysis.

5.2.2 Isolation of bacteria from rhizospheric soils

For the isolation of rhizobacteria, 10 g of rhizospheric soil was suspended in 90 mL distilled autoclaved water. Serial dilution method was used for further analysis of the prepared soil suspension. Suitable dilutions (10^{-2} , 10^{-4} , and 10^{-6}) were plated on Luria Bertani (LB) agar plates to isolate rhizobacteria and all plates (in three replicates) were incubated for 2 days at 28 °C (Aneja, 2001). Well isolated colonies were purified by streaking on fresh LB agar plates and further identified. Six actively growing isolates selected for metabolite profiling were used to inoculate 100 ml of Luria Bertani broth (Merck) in a 250-ml Erlenmeyer flask. After 48 h incubation at 30°C, the LB broth (10%) was used to seed culture in six 500-ml Erlenmeyer flasks each containing 100 ml of fermentation medium (glucose, oatmeal, yeast extract, NaCl, CaCO₃ at pH 7.0) The cultures were incubated at 25°C for 10 days under constant agitation of 220 rpm.

5.2.3 Antagonism assay against phytopathogenic fungi and bacteria

All the 6 isolates were assayed for antifungal activities against *Fusarium graminearum* using Potato Dextrose Agar (PDA) medium. The isolates were streaked on PDA medium 3 cm in distance opposite to pathogenic fungi inoculated at the center of the medium. The barrier between isolates and fungi indicated antagonistic interaction between them. Antagonistic activity was investigated for 4 to 7 days after incubation at room temperature.

5.2.4 Extraction and partial purification of the crude extracts from the isolates

The fermentation medium was centrifuged for 20 min at 8000× *g* to remove the mycelium. The supernatant was shared in 4 equal volumes of 60 ml, and then each was extracted with 60 ml of organic solvent. A range of extraction solvents was screened for effectiveness, including petroleum ether, *n*-hexane, chloroform, diethyl ether, ethyl acetate, benzene, methanol and *n*-butanol.

5.2.5 Gas chromatography-mass spectrometry (GC-MS) analysis

The partially purified active fractions were analyzed by GC-MS. The analyses of the compounds in the active fractions were run on a GC-MS system (Agilent GC: 6890, with a 7683B Autosampler). The fused-silica Rxi-5Sil MS capillary column (30 m 0.25 mm ID, film thickness of 0.25 mm) was directly coupled to an agilent variant. Oven temperature was programmed (35°C for 5 min, then 35-300°C at 10 °C/min) and subsequently, held isothermal for 20 min. The injector port; was 250°C, the transfer line: 290°C, splitless. Volume injected: 0.2 ml and the column flow rate was 1 ml/min of 1 mg/ml solution (diluted in chloroform). The peaks of components in gas chromatography were subjected to mass-spectral analysis. The MS was a LECO Pegasus 4D recording with a EI-source at -70 eV; the solvent delay was 9 min. scan time 1.5 s; acquisition rate 10 spectra/second mass Range 50-1000 amu, detector voltage 1800 V, and Ion source temperature: 250°C. Data were recorded in TIC mode. The software adopted to handle the mass spectra and chromatograms was an agilent chemstation software.

The constituents were identified after comparison with available data in the GC-MS library in the literatures.

The GC-MS mass spectrum data were analyzed using mnova 11.0.1 and interpreted using the database of National Institute Standard and Technology (NIST). The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were confirmed.

5.3 Results and discussion

Table 4.1 Antifungal and antibacterial activity of bacteria isolate

Bacterial isolate	Antifungal activity	Antibacterial activity	
	<i>Fusarium graminearum</i>	<i>Enterococcus faecalis</i>	<i>Bacillus cereus</i>
A1	+++	++	+

+++, significant inhibition; ++, moderate inhibition; +, slight inhibition

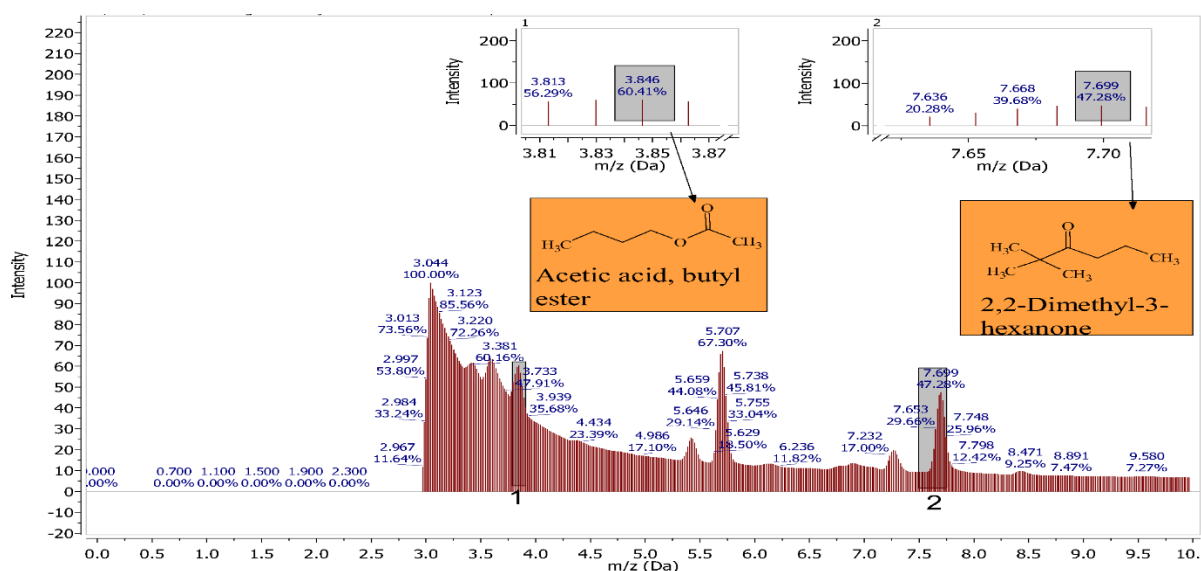


Figure 5.1: Butanol chromatogram with selected metabolites

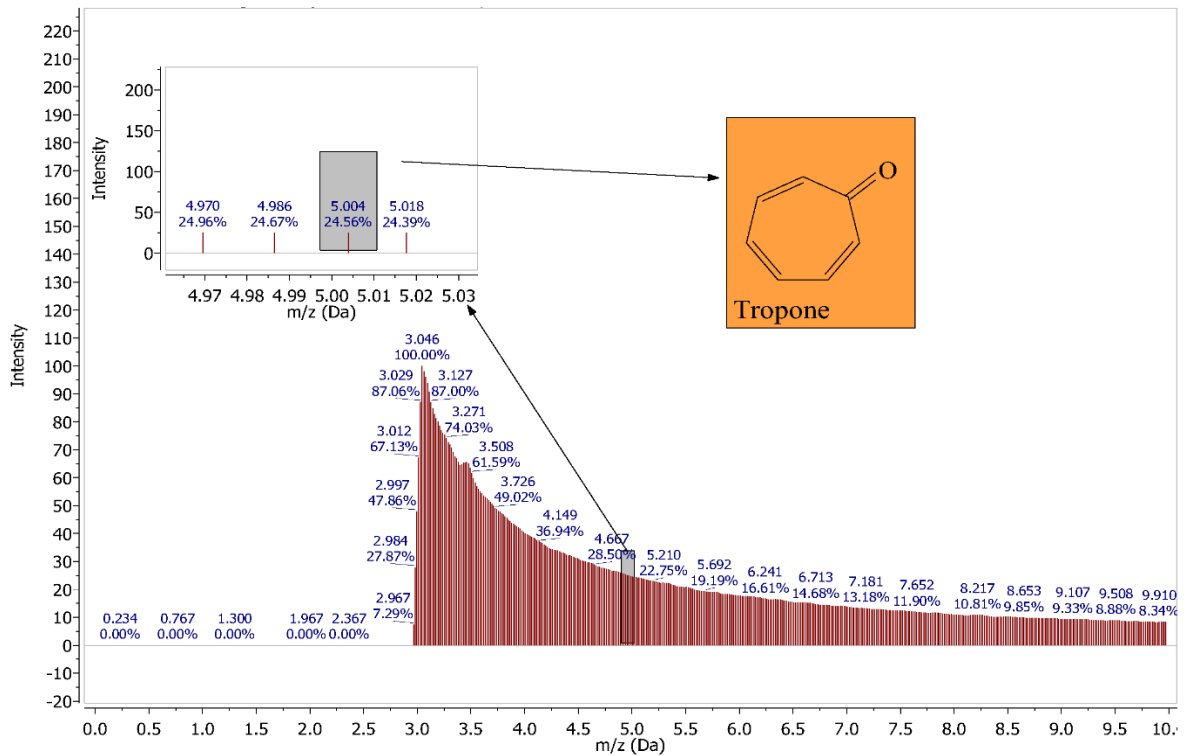


Figure 5.2: Benzene GC-MS chromatogram with selected metabolite

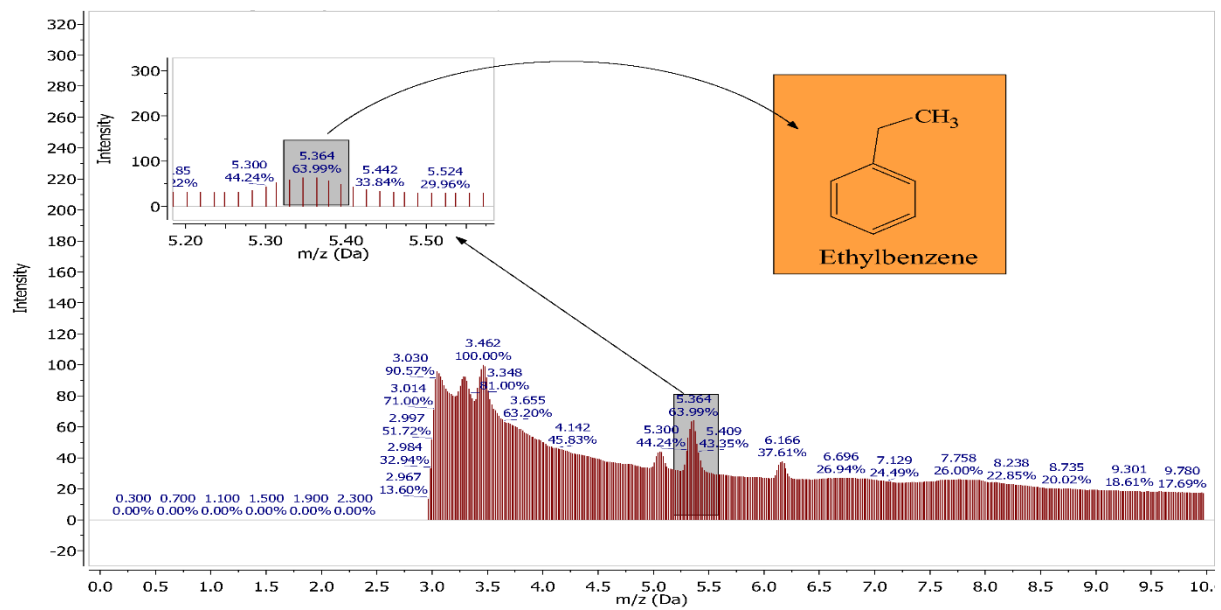


Figure 5.3: GC-MS chromatogram of ethyl acetate

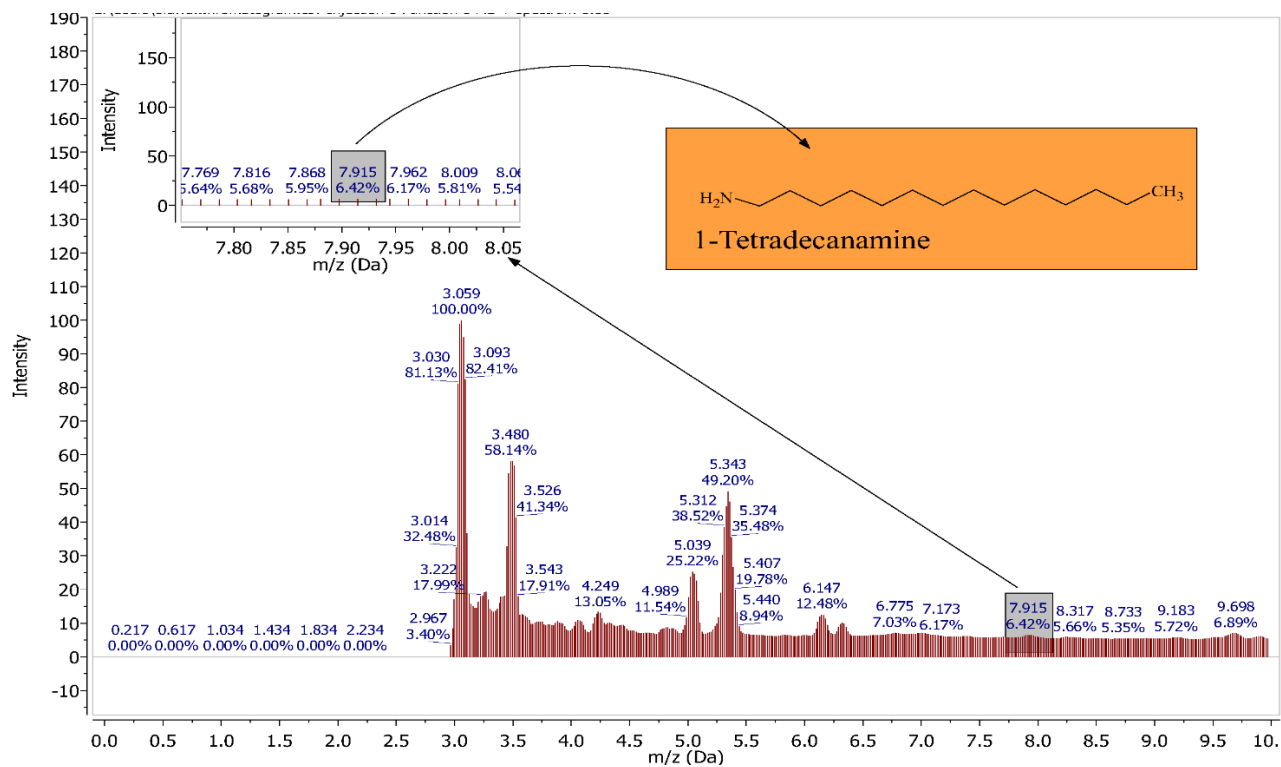


Figure 5.4: Hexane GC-MS chromatogram

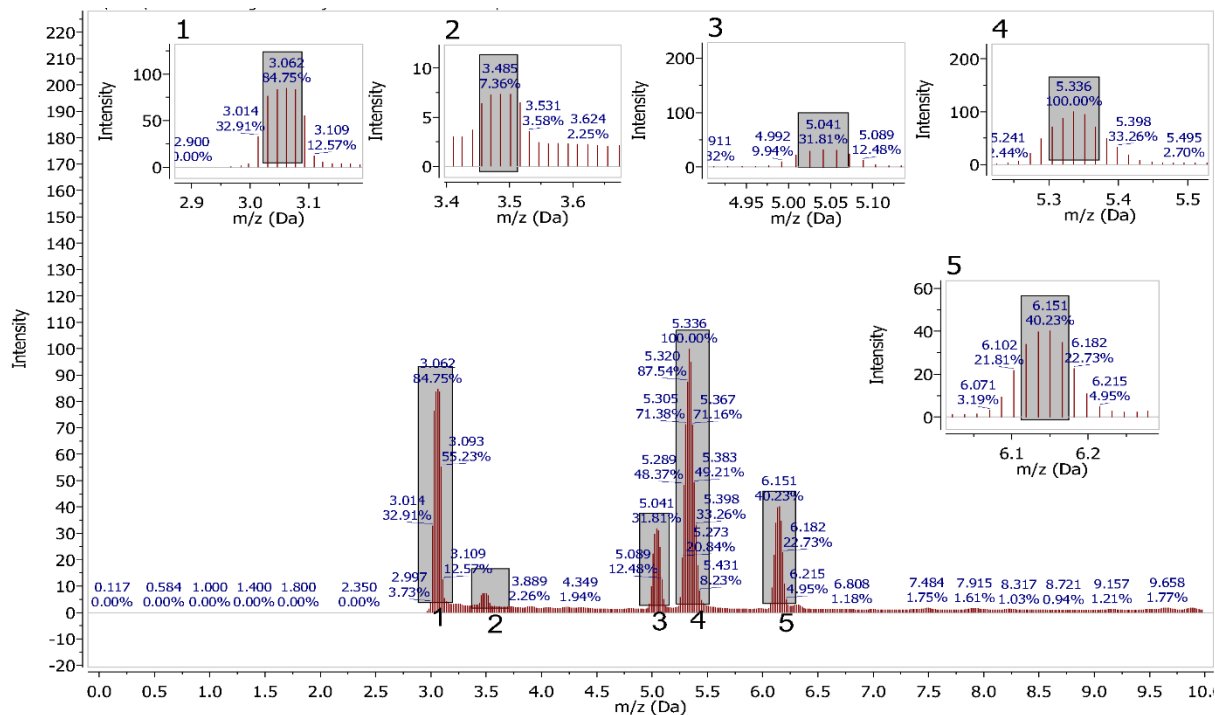


Figure 5.5: Methanol GC-MS chromatogram

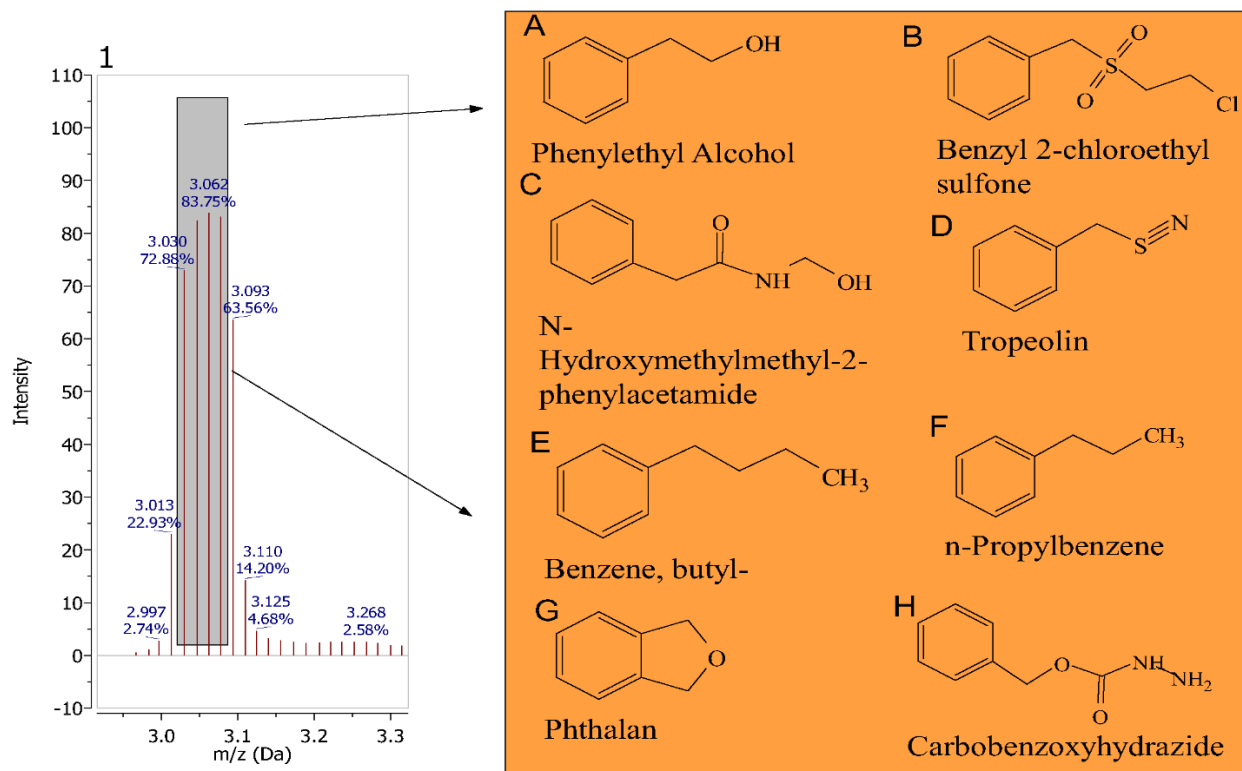


Figure 5.6: Metabolites detected at peak 3.062 from methanol solvent

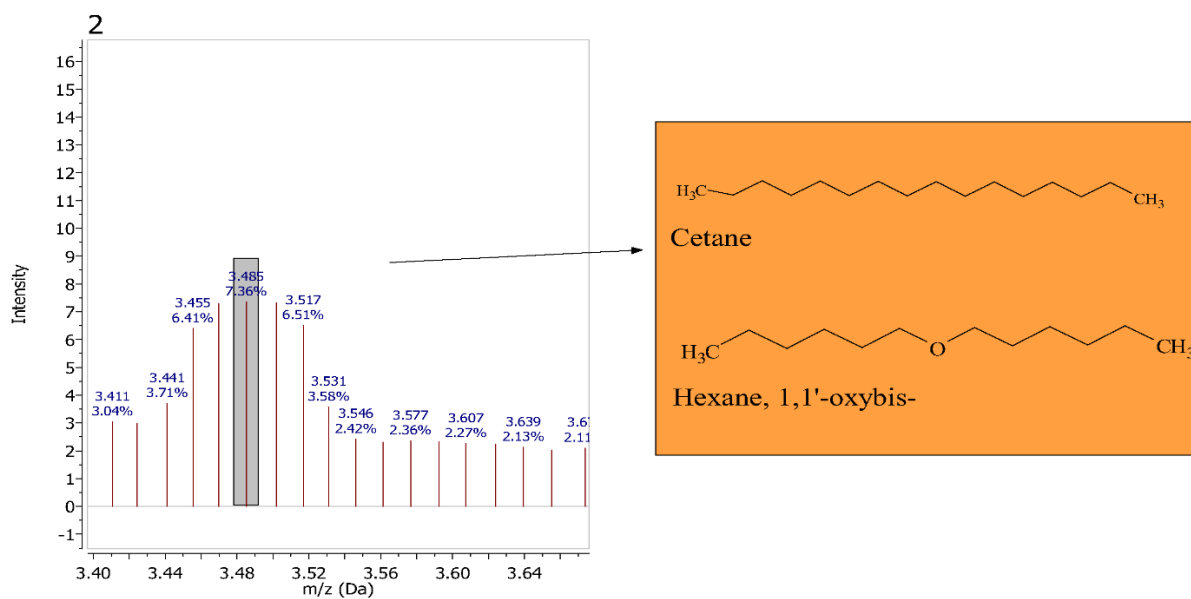


Figure 5.7: Metabolites detected at peak 3.485 from methanol solvent

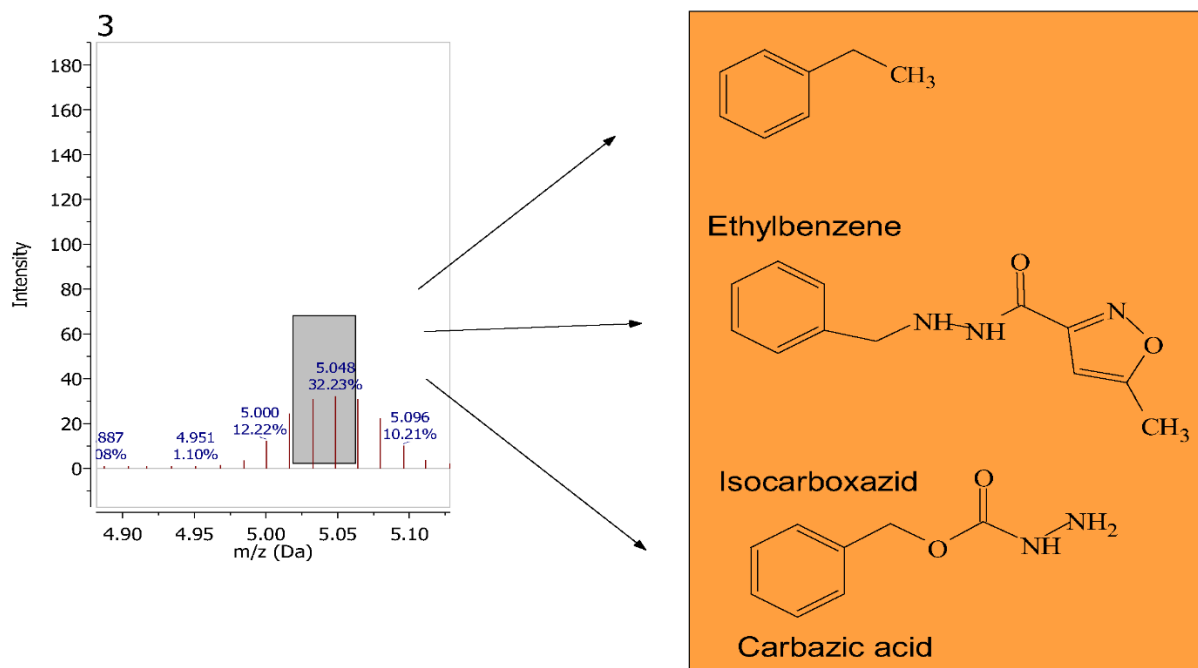


Figure 5.8: Metabolites detected at peak 5.048 of methanol solvent chromatogram

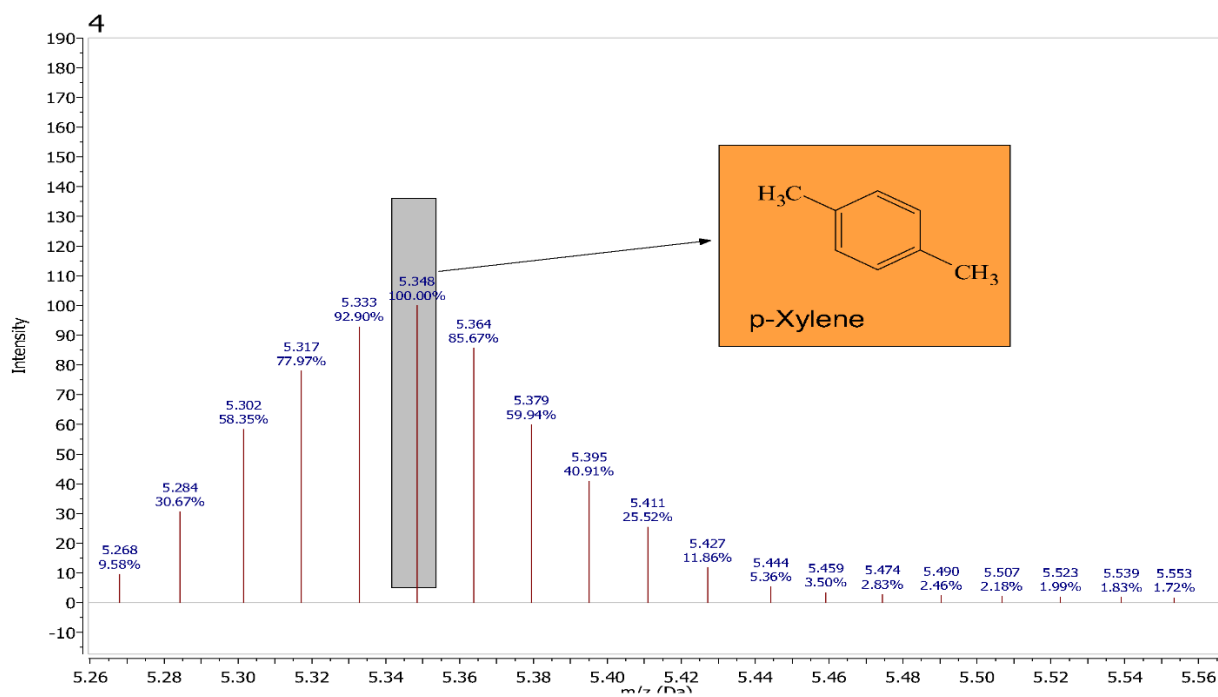


Figure 5.9: p-Xylene detected at peak 5.348 in the methanol chromatogram

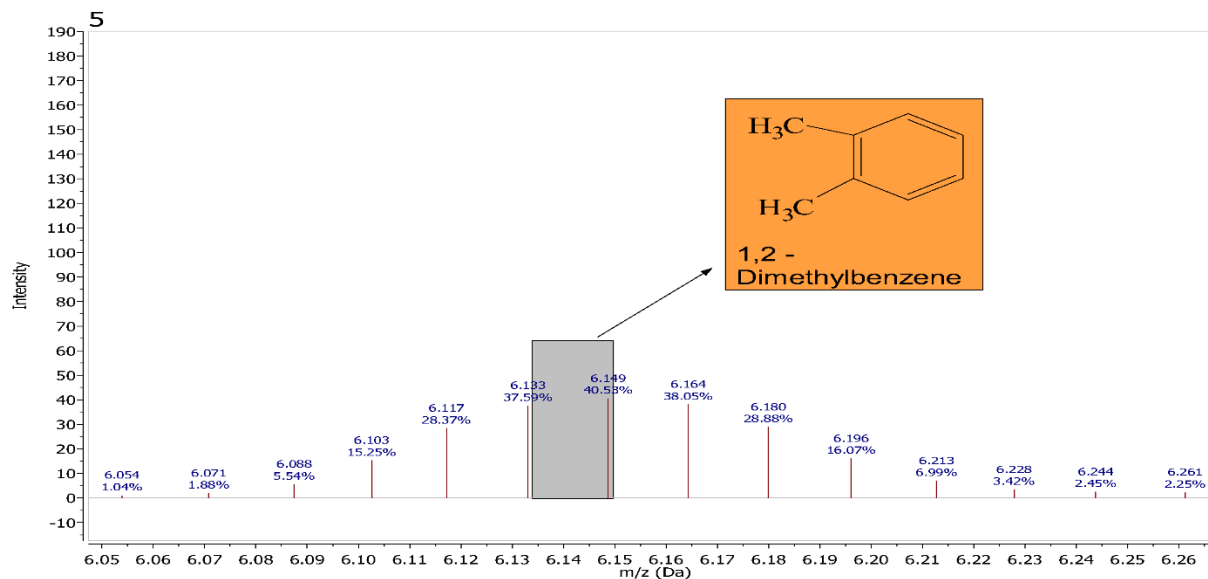


Figure 5.10: Metabolite detected between peak 6.133 and 6.149

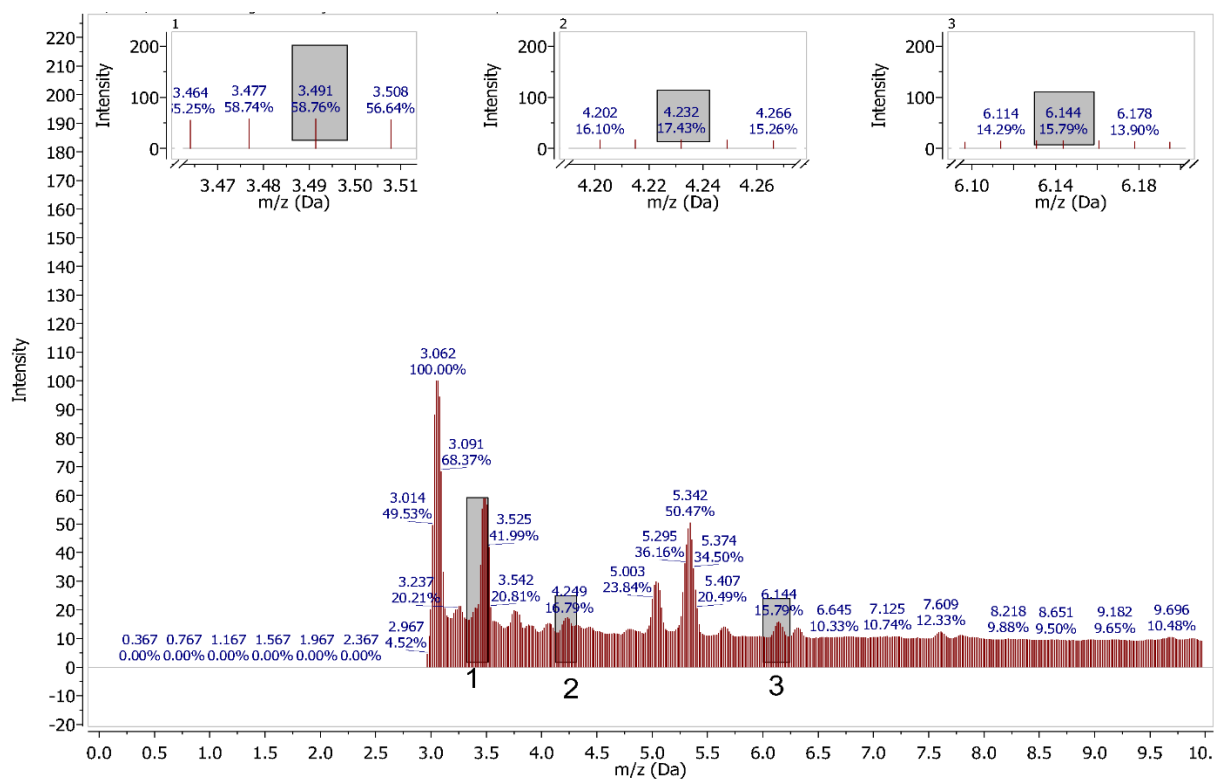


Figure 5.11: GC-MS chromatogram of petroleum ether and peaks at which solvents were detected

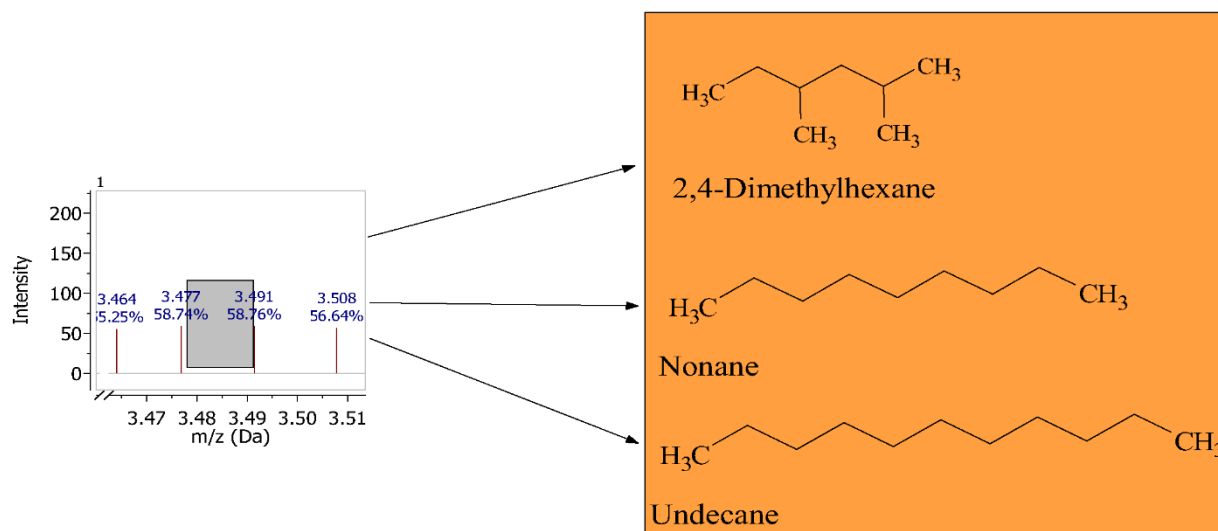


Figure 5.12: Detection between peaks 3.477 and 3.491

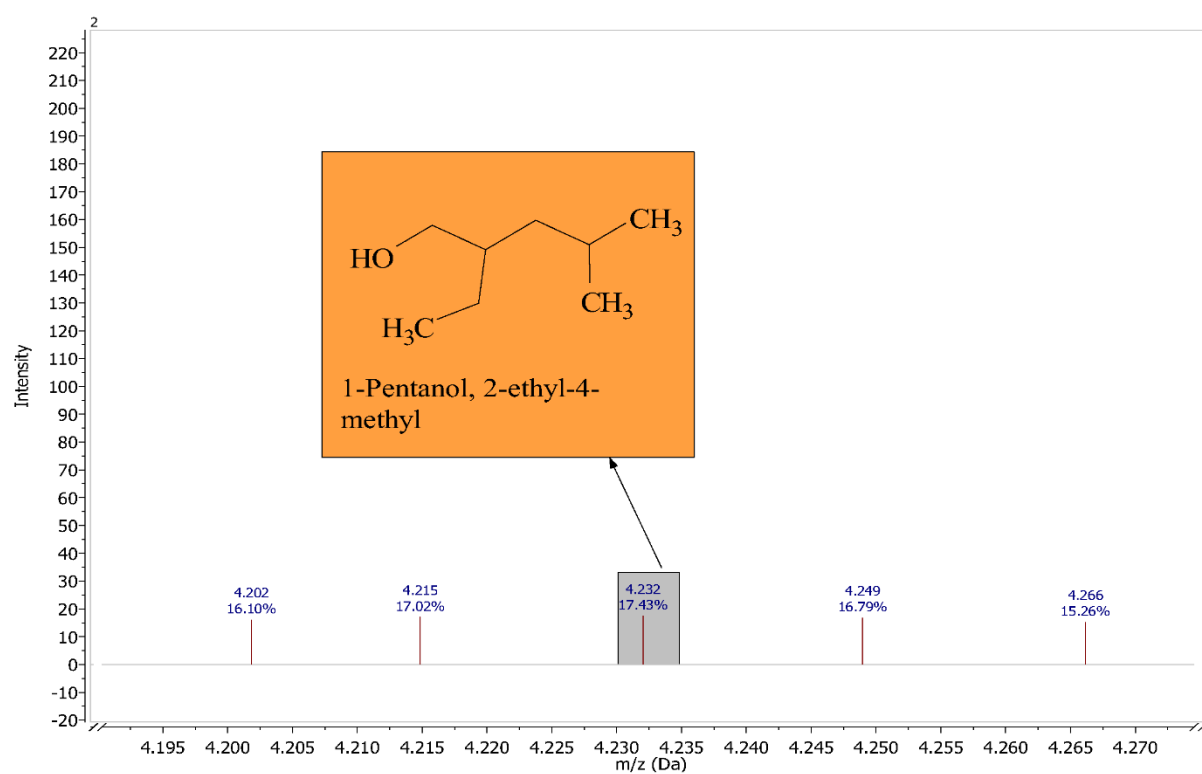


Figure 5.13: Metabolite detected at peak 4.232

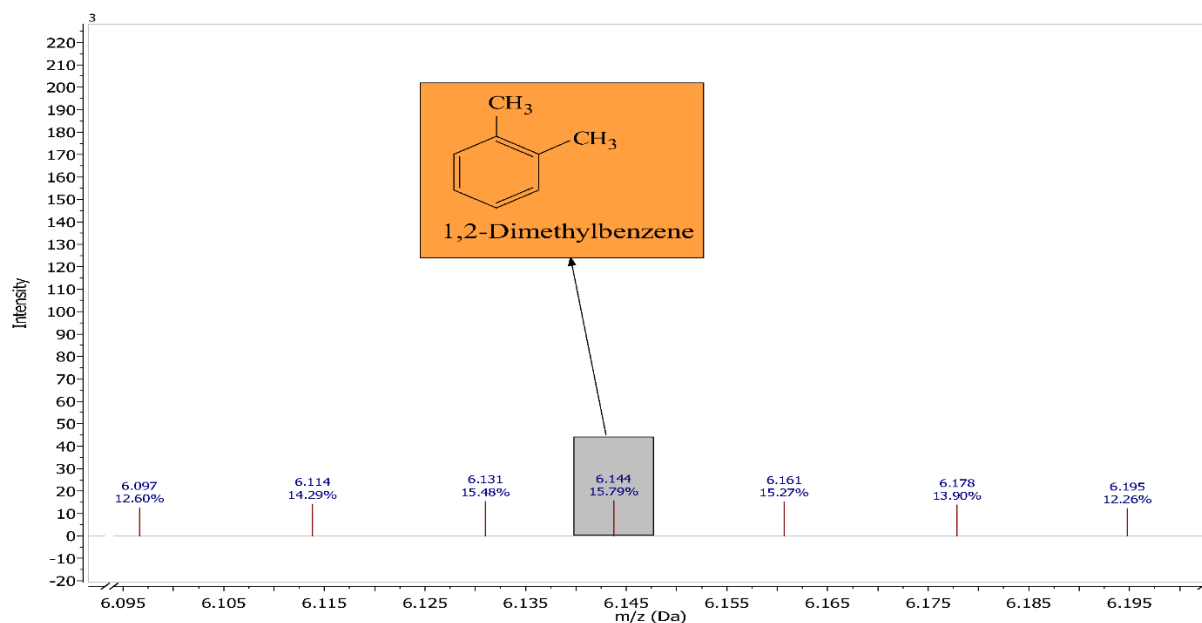


Figure 5.14: Metabolite detected at peak 6.144

The organism A1 has already been identified in previous work as *Bacillus subtilis*. It showed great antifungal and antibacterial ability as seen in table 5.1. *Bacillus subtilis* has been reported to be a good biocontrol agent which is evident in its use in producing some notable pesticides which is as a result of their secondary metabolites and production of volatile organic compounds (Compant et al., 2005). In this study, we decided to explore the bioactive secondary metabolites present in this organism using seven solvents for extraction.

GC-MS chromatogram of butanol solvent extracts showed two peaks at retention time 3.846 and 7.699 (Figure 5.1). Acetic acid, butyl ester was detected at 3.846 while 2,2-Dimethyl-3-hexanone was detected at 7.699. 2,2-Dimethyl-3-hexanone has previously been reported in the work of Hossain et al. (2013) with the retention time of 5.26. Benzene chromatogram identified one peak at 5.004 retention time with the metabolite 2, 4, 6-Cycloheptatrien-1-one (tropone) (Figure 5.2). This metabolite has been shown to be the main factor in iron scavenging by *Pseudomonas donghuensis* (Jiang et al., 2016). In their role in scavenging of iron, they will help the microbe to provide more iron and other nutrients for the plant use thereby improving crop yield in the process. Ethyl acetate chromatogram showed one peak at retention time 5.364.

At this retention time, ethyl benzene was identified (Figure 5.3). Hexane chromatogram also produces 1-Tetradecanamine at retention time of 7.915 (Figure 5.4). Methanol chromatogram showed five peaks with retention times 3.062, 3.485, 5.048, 5.336 and 6.151 (Figure 5.5). At 3.062, the major metabolites identified are Tropeolin, carbobenzoxyhydrazide and phthalan (Figure 5.6). Phthalans are highly bioactive compounds which are of importance in pharmaceutical and industrial sectors (Ghosh et al., 2015). They are used as pesticides in agriculture. At retention time of 3.485, cetane and hexane, 1, 1'-oxybis were detected (Figure 5.7) while ethylbenzene, isocarboxazid and carbazic acid were detected at retention time 5.048 (Figure 5.8). Isocarboxazid, also known as marplan, is an antidepressant with relatively little or no known application in agriculture. p-Xylene was identified at retention time 5.336 (Figure 5.9) and 1,2-Dimethylbenzene at 6.151 (Figure 5.10). Three peaks were identified in the petroleum ether chromatogram at retention time 3.491, 4.232, and 6.144 (Figure 5.11). At retention time 3.391, the compounds are 2, 4 -Dimethylhexane, nonane and undecane (Figure 5.12) while at 4.232, 1-Pentanol and 2-ethyl-4-methyl were deduced (Figure 5.13). 1, 2 - Dimethylbenzene was isolated at retention time 6.144. For the methanol chromatogram, the same 1, 2-Dimethylbenzene was detected at retention time 6.151.

In conclusion, new secondary metabolites that are produced by microbes can be used to optimize their availability by fermentation for further research and also for production in food production.

5.4 Conclusion and recommendation

Bacillus subtilis have been shown to be a good antipathogenic organism by many researchers but their metabolite production which is key to their activities against pathogens is not very known. This study has been able to show some of the metabolites produced by this organism which broadens the knowledge that is already known about them. In recommendation, more work should be carried out in this area. As technology continue to improve, there may be more

discoveries that might bring lasting solutions to the issue of pathogens as this is a good source of active metabolites.

5.5 Acknowledgements

North-West University is gratefully acknowledged for school bursary to OOS. OOB would like to thank the National Research Foundation, South Africa for a grant (Ref: UID81192) that have supported research in her laboratory.

5.6 Conflict of interests

There is no conflict of interest whatsoever from the authors.

CHAPTER 6

6.1 General discussion

This study have been able to show the efficacy of combination treatments in comparison with single treatments towards achieving a greater maize yield on the field. Most studies have been particular about using single treatments with few working on consortia organisms. A clue should be taken from the use of combination therapy in drugs for treatments which has been proven to be more effective than single treatment. With this in mind, it is expected before the study that consortia organisms will definitely have more effect than single treatments but this is not the case. The reason for this might not be fully known, at the same time it can be said that they are not synergistically cooperative enough to work together. The ability of these organisms to effectively cohabit is very important to their success as effective consortia PGPR. In the study, the all treated maize crops were better than the control in all parameters but not all consortia were better than single treatments.

Due to these findings, it is recommended that future researches should look into how to improve the synergy activities to improve maize yield and other crops generally taking into consideration the organisms involved and their abilities. Their metabolite production and types is also very important. In the actual fact, some metabolites being produced by some organisms might make other organisms to be redundant so careful examination should be carried out before deciding on which organisms to use as consortia.

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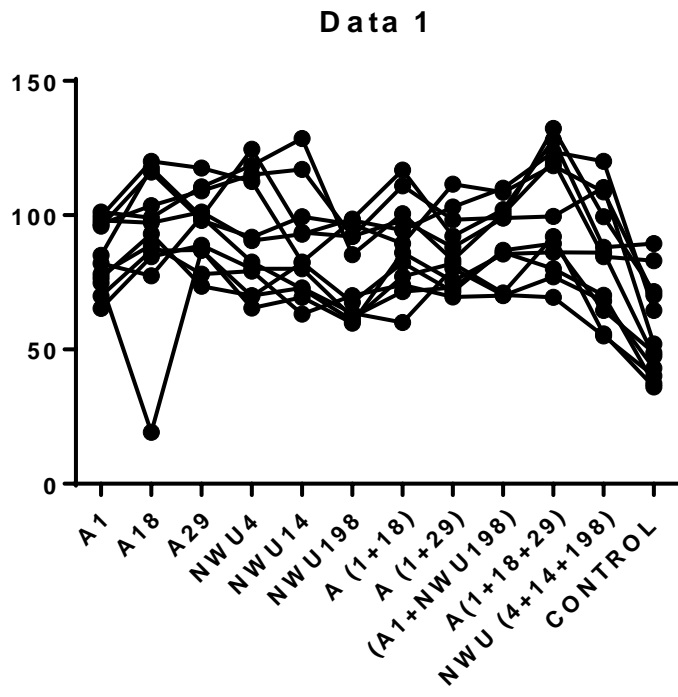


Figure 15