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ACTINOMYCETES IMPACTS ON DROUGHT STRESS IN MAIZE (*Zea Mays* L.).

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A dissertation submitted under the Department of Biological Sciences to the North-West University (Mafikeng Campus), in fulfillment of the requirement for the Degree:

Master of Science in Biological Sciences (Microbiology)

By

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DECLARATION

I declare that this dissertation titled "Actinomyccetes impacts on drought stress in maize (*Zea mays* L.)", is a true outcome of the research performed by me at the department of Biological Sciences, North-West University (Mafikeng Campus) under the supervision of Prof. Olubukola O. Babalola and Prof. Funso R. Kutu. I declare that the work has not been previously submitted by me for a degree at this or any other University, and that all information derived from the literature has been duly acknowledged in the text and a list of references provided.

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DEDICATION

This work is dedicated to Almighty God for His faithfulness, Unfailing Love and mercy upon my life.

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I am highly indebted to my maker for life, strength, wisdom and grace to carry out this project. My sincere gratitude goes to my Supervisor, Professor Olubukola Oluranti Babalola and my co-supervisor, Professor Funso Raphael Kutu for standing by me and for their continuous guidance, tolerance and encouragement through the period of this research.

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To you all, I say Thank you and God Bless!

OUTLINE OF DISSERTATION

This study consists of two major chapters submitted for publication in Accredited Journals. The Chapters contained therein are not projected to be individual articles but describe the research work that has been performed to achieve the aim and objectives of this study.

Chapter one presents the general introduction of the study, literature review, aim, objectives and outline of the research.

Chapter two describes the isolation, characterization and identification of drought tolerant actinomycetes from dry maize rhizosphere.

Chapter three studies the screening and quantification of plant growth promoting traits and the effects of inoculation of selected bacteria on growth and drought tolerance in maize plants.

Chapter four consists of the general conclusions from chapters 2 and 3 as well as future research prospects.

GENERAL ABSTRACT

Drought is a major cause of the present decrease in crop yield and agricultural productivity, accounting for the recent worldwide shortage in food availability. Drought results from the current change in climate conditions. The disastrous effects of drought on plants calls for a renewed concern on improved and effective strategies to improve plant growth and yield under drought stress. This work is therefore designed to identify rhizospheric actinomycetes and evaluate their potential to improve maize growth under drought stress condition.

In this study, seven actinomycetes strains were isolated from the rhizosphere of two maize fields. Biochemical and morphological characteristics, sequencing of 16S rDNA genes and phylogenetic analysis of nucleotide sequences obtained from the 16S rDNA genes of the bacterial isolates revealed that five of the isolates belong to the genus *Streptomyces*, one to *Arthrobacter* and the other *Microbacterium*. Isolates were screened for drought tolerance and it was observed that all isolates successfully grew in 5% polyethylene glycol (PEG) 8000 medium with outstanding growths observed in isolates *A. arilaitensis* and *S. pseudovenezuelae*. Bacterial growths at different pH values, temperatures, sodium chloride (NaCl) concentrations and drought tolerance potentials of isolates at different PEG concentration and time were examined and it was observed that isolates showed better growth between pH 5 and 9, temperatures 25 and 35°C and 0 to 4% NaCl concentration. Maximum growths for all bacterial isolates were observed at a PEG concentration of 5% and 120 h indicating that PEG concentration and time affected bacterial growth. The primers that amplified specific genes encoding proteins involved in drought tolerance: Glutathione peroxidase (*GPX*), glycine-rich RNA binding protein (*GRP*), desiccation protectant protein (*DSP*) and Gtp-binding protein (*GTP*) were designed and polymerase chain reaction (PCR) performed on them including the plant growth promoting (PGP) genes for ACC deaminase activity (*Accd*) and siderophore production (*Sid*) and amplifications were observed as follows: four isolates for *GPX* and *GRP*, two for *DSP*, one for *GTP*, seven for *Accd* and six for *Sid* genes. The amplification of these genes by some of the isolates is an indication of their drought tolerance and PGP potentials.

Isolates were screened for the production and amount of PGP traits and it was observed that the 7 isolates produced indole-3-actetic acid (IAA), 1-aminocycloprpane-1-carboxylate (ACC) deaminase, ammonia and siderophore, 5 solubilized phosphate and 1 produced hydrogen cyanide. *Streptomyces werraensis* produced the highest IAA of 10.12 ± 0.02 µg/ml followed by isolate *A.*

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arilaitensis (9.44 ± 0.01 µg/ml), *S. pseudovenezuelae* produced the highest ACC deaminase activity of 0.903 ± 0.024 µmol/min and *A. arilaitensis* produced the highest siderophore of 51.3%. Two (2) isolates (*A. arilaitensis* and *S. pseudovenezuelae*) were selected to evaluate the effect of bacterial inoculation on drought tolerance in maize plants at three soil moisture levels (field capacity, moderately watered and completely dry) and two inoculation methods (directly inoculated and vermiculite coated seeds). The results obtained showed that inoculated plants were not only protected from the deleterious effects of drought but also showed significant increase in the root and shoot lengths, chlorophyll contents, number of leaves, leaf area, and root and shoot dry weights. However, greatest growths were observed in the inoculated plants at field capacity. Significant growths were observed in plants whose seeds were coated with vermiculite compared to un-coated ones and also at field capacity. The results obtained on the trials also confirms the ability of the isolates to resist drought by growing on PEG 8000 medium and the amplification of protein encoding genes involved in drought tolerance.

The overall findings of this study indicates that the drought tolerant actinomycetes are important tools that can be developed into bio-inoculants for effective improvement of drought stress tolerance in plants.

KEYWORDS: Actinomycetes, drought stress, PEG 8000, maize, vermiculite.

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LIST OF ABBREVIATIONS

Abbreviations	Full names
2,4 DNP	2, 4-dinitrophenyl hydrazine
ABA	Abscisic acid
ACCD	1-aminocyclopropane-1-carboxyl deaminase
AIA	Actinomycetes isolation agar
bp	Base pairs
CAS	Chrome azurol S
CMC	Carboxylmethyl cellulose
DSP	Desiccation protectant protein
EPS	Exopolysaccharide
GPX	Glutathione peroxidase
GRP	Glycine-rich RNA binding protein
HDTMA	Hexadecytrimethyl ammonium
HSP	Heat shock protein
IAA	Indole-3-acetic acid
ISP	International Streptomyces Project
LB	Luria Bertani agar
ME	Malic enzyme protein
MES	2-(N-morpholino)ethanesulfonic acid
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGPB	Plant growth promoting bacteria
ROS	Reactive oxygen species
rpm	Revolutions per minute
Rubisco	Ribulose 1-5 bisphosphate carboxylase oxygenase
S-AdoMet	S-adenosylmethionine

spp	Species
TAE	Tris-Acetate-EDTA

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CHAPTER ONE: LITERATURE REVIEW AND OBJECTIVES

1.1 GENERAL INTRODUCTION AND STATEMENT OF PROBLEM

In a global concept, maize (*Zea mays* L.) ranks third as the most important and widely cultivated cereal food crop after rice and wheat while it is the first in South Africa. Its wide cultivation is mainly due to the fact that it constitutes large energy and protein sources in human and animal nutrition making it a very important element of global food security (Maazou et al., 2016). It has high rate of photosynthetic activity which brings about high grain and biomass yield. On the other hand, maize is very sensitive to drought stress. Therefore, its production requires the development of innovative management practices well adapted to drought stress.

Abiotic stresses of plants are drought (which may result in plant dehydration), cold, salinity, flood, radiation, nutrient deprivation, chemicals and heavy metals (Postolaaky et al., 2012). Drought stress results when plants are not able to meet up with evapotranspiration demand. It is prompted by unavailability of sufficient water for plants due to intermittent rainfall or insufficient irrigation. It can also be caused by some other factors like soil salinity, high temperature and soil physical properties. Soil salinity causes osmotic stress in plants by modifying soil water potential thereby making water unavailable for plants (Rauf et al., 2016). The structure and texture of the soil determine its properties like surface roughness and porosity which consequently affect water retention, water holding capacity and infiltration. The symptoms of water stress are often seen more easily in plants cultivated on sandy soils than clay textured soils. Increased transpiration rate as a result of high temperatures may also affect plant water availability which may result in temporary wilting of the plants, due to a higher rate of water loss than water absorption by plant roots (Istanbulluoglu et al., 2009; Rauf et al., 2016).

Drought or water shortage has been an issue of utmost concern across the globe. It is a major environmental factor that limits plant growth and yield. Most continents of the world are currently experiencing drought at different intensities and frequencies. An estimated 17% of the world cultivated area was reportedly affected by drought between 1980-2006 (Dai, 2013; Rauf et al., 2016). Crops planted under rain fed conditions are particularly affected by drought and this represents 80% of the total area cultivated worldwide (Dai, 2013). Pandey et al. (2007) reported that at least 23 million ha of rain fed rice, representing 20% of the total rice area in Asia, are

cultivated under drought-prone conditions. At the world level, the part of the cultivated area permanently affected by drought is projected to be around 19% for maize, 20% for wheat, 28% for sorghum and 19% for barley (Li et al., 2009; Gennari et al., 2015). Current changes in climate will probably increase drought occurrence and severity due to increased evapo-transpiration resulting from increasing temperatures (Feng et al., 2013). Gennari et al. (2015) estimated an increase in drought-prone areas as 129%, 70%, 67%, 55%, 60% and 76% between 1979 and 2006 for maize, wheat, barley, rice, soybean and sorghum respectively. Water scarcity has caused considerable world-wide crop loss and brought about more than 50% reduction in average yields of most major food crops. It is expected to result in 30% loss of land by the year 2021 and more than 50% by 2050 (Parry et al., 2007; Carrão et al., 2017 Schüler et al., 2017).

Drought is capable of impacting tremendously on the world's economy. World food prices increased by 3-4% in 2013 due to the drought that affected the US Great Plain grain belt in 2012 (Yuan and Quiring, 2014). In South Africa, drought affected the country's exchange rate in the food value chain as the CPI figures for May 2016 showed a lower than expected aggregate CPI value of 6.1% year-on-year (Lydia, 2017; Piesse, 2016). This figure was reported to be the lowest since December 2015 as prices of food increased while there was little or no reduction in high petrol prices (DPPME-SA, 2016). The increase in food security in the 21st century would largely depend on our ability to use modern biotechnological means to improve plant tolerance to drought stress. Drought effects on the yield of major food crops from different parts of the world are presented in Table 1.1.

Actinomycetes are considered the most economical and biotechnological prokaryotes responsible for the production of half of the bioactive secondary metabolites discovered (Yandigeri et al., 2012). Over 50 genera have been used in agriculture, human, medicine, industry and vertenary. One of the genera commonly responsible for the abundant percentage of the soil microflora is the Streptomyces. Streptomyces are particularly effective colonizers of plant-root systems and are also able to withstand unfavorable growth conditions by formation of spores (Yandigeri et al., 2012; Cruz et al., 2015). Recent reports have shown that bacterial volatiles are capable of promoting plant growth and induce systemic resistance in plants. The bacterial strain AOK-30 of the Streptomyces padanus volatile has also been proven to be associated with this induced drought tolerance in plants (Cruz et al., 2015). However, there are only few reports on the contribution of

actinomycetes in the phytohormonal regulation of plant growth under drought stress (Khamna et al., 2010). Furthermore, no attention has been paid on actinomycetes symbiotic relationship with maize plant neither in its drought improvement ability. An understanding of the function of actinomycetes communities in maize rhizosphere can be used in improving drought tolerance in maize plant.

1.2 DROUGHT STRESS: THE SOUTH AFRICAN EXPERIENCE

In early 2015, South Africa experienced the worst drought ever in history which led the South African Weather Service (SAWS) to declare the year 2015 as the driest year in South Africa since 1904 (Piesse, 2016). According to the SAWS, rainfall in the nine provinces of the country averaged 608 mm per annum since 1904, while in 2015; it received only an average of 403 mm which is only 66% of the annual average. Before this time, the lowest rainfall the country has ever received was 437 mm (72%) in 1945.

In South Africa, maize is the most cultivated field crop planted under diverse environmental conditions. In the African continent, South Africa is the main producer of maize as approximately 2.5 million ha of maize field is planted annually. In 2016, the annual maize crop production was estimated to be 7,161 million tons which represented a decrease of 2,794 tons (28.07%) from 2015. The decline in the production of maize was brought about by severe drought conditions in the maize producing areas of the country (DPPME-SA, 2016; Piesse, 2016; Lydia, 2017). This led to the estimation of maize importation of up to 3.30 million tons in 2016 to meet up with the increasing demand for the food crop. Hence, it was recorded as the second highest imports ever made in the history of South Africa. Not only did drought affect the growth and yield of maize crop in the country, it also had a very devastating impact on the general agriculture and economy of the nation although it doubled in 2017.

1.3 PLANT PHYSIOLOGY UNDER DROUGHT STRESS

Water deficiency is a major limitation to plant growth and yield. Its effects on plant growth include reduction in plant water potential and turgor, causing an abnormity in the physiological and biochemical plant functions (Almeselmani et al., 2011; Naveed, 2013). Upon plant exposure to drought, the major changes that occur include: changes in physiological and morphological

processes; and changes in ultrastructure of the subcellular organelles (Gorai et al., 2010). General effects of drought on plants growth are: stomatal closure, reduced germination, altered photosynthesis, inhibition in cell growth and enlargement, and inhibition in respiration, growth promoters, nutrient metabolism, carbohydrate, nutrient and ion uptake (Akinci and Losel, 2010; Naveed, 2013). In the event of mild water deficit conditions, plants tend to cope through both physiological and molecular mechanisms; however, this will lead to a lower biomass yield. Severe drought results in photosynthesis arrest, and water and turgor potential reduction causing a slowdown to leaf expansion and root elongation. It may also cause increase in extracellular matrices, inhibition in cell enlargement and concentration of solutes in the plant's cytosol (Bardi and Malusà, 2012). Subsequent effects are: constant accumulation of compatible osmolytes and abscisic acid (ABA) and the excessive production of reactive oxygen species (ROS) which eventually lead to the wilting and death of the plant.

1.4 PLANTS RESPONSES TO DROUGHT STRESS

Plants partially protect themselves against environmental stresses like drought by developing some means to deal with such stresses. They are able to overcome the severe effects of drought by either possessing certain traits that enable them to survive severe water stress situations (drought escape), or traits that help to reduce yield losses in crops exposed to mild drought (drought tolerance) (Basu et al., 2016).

1.5 DROUGHT ESCAPE

Drought escape occurs when plants are able to complete their life cycle before drought stress begins (Basu et al., 2016). This approach is usually beneficial in regions where drought only occurs when the growth cycle of the plants has already been completed. The flowering stage is the most critical time in a plant's life cycle. Therefore, water availability is very important at this time as it is not a proper time for drought stress introduction (Dai, 2013; Basu et al., 2016). Drought, however modifies plant phenology and there is a positive association between plasticity of yield and flowering time at different levels of water availability (Sadras et al., 2009). Therefore, an important trait influencing drought escape could be considered to be the plasticity of phenological development, including the plant's phenology (Sabadin et al., 2012).

1.6 DEHYDRATION AVOIDANCE

This phenomenon is the ability of the plant to maintain high cellular hydration or water status under the influence of drought (Rauf et al., 2016). These include morphological and physiological traits such as reduced leaf expansion, stomatal closure, osmotic adjustment, root elongation and alteration in photosynthesis.

1.6.1 REDUCED LEAF EXPANSION

Overall decrease in plant transpiration is a result of reduction in plant leaf which brings about smaller leaf area. This response facilitates the plant to limit the uptake of water without modifying its transpiration rate. This is done to enable the plant to maintain other important functions of leaf transpiration like leaf temperature regulation, and to preserve the forces that initiate the production of water flux which results in the uptake of nutrients from the soil (Bardi and Malusà, 2012). When a completely developed plant is affected by drought, reduced leaf area by abscission as well as induction of ethylene-mediated leaf senescence may occur.

1.6.2 ROOT ELONGATION

Reduction in cell turgor potential in the apices of plants' roots often located at the dry layers of the soil leads to a decrease in root elongation. This often makes plant roots to elongate towards the direction where there is more water availability. This process happens in deeper layers of the soil which usually is not subjected to drying. In determining the efficiency of plants' roots to extract water from the soil, its quality in terms of structure and distribution is considered more important than its volume (Farooq et al., 2009). Should there be a reduction in the leaf area index due to drought, the plant photosynthetic activity is less affected, bringing about a higher amount of assimilated carbon in leaves which becomes available to generate root biomass and increasing root to shoot ratio (Farooq et al., 2009; Basu et al., 2016).

1.6.3 STOMATAL CLOSURE

This is the most rapid response of plants to drought stress to protect them from desiccation which limits photosynthesis, plant growth as well as yield (Bardi and Malusà, 2012; Basu et al., 2016). This process may occur in two ways namely: passive (hydropassive stomatal closure) and active (hydroactive stomatal closure). Hydropassive stomatal closure occurs as a result of water loss from the guard cells through direct evaporation to the atmosphere while its hydroactive counterparts

occur as a metabolic response to general dehydration of plants' leaves and roots. In this case, stomata close as a reduction in turgor of guard cells brought about by a decrease in the concentration of the solute (Foley et al., 2011; Bardi and Malusà, 2012; Dai, 2013). Abscisic acid (ABA) is involved majorly in the active role as it plays a very important role in it. When a plant is dehydrated, the abscisic acid produced in the roots is moved to the shoot through the xylem (Sah et al., 2016). Normally, the concentration of ABA produced in the xylem is small, but under drought stress it tends to increase dramatically. Stomatal behavior is closely linked to soil moisture because of the ABA formed in the drying roots; although, stomatal closure is caused by soil dryness even when water potential is constantly maintained (Bardi and Malusà, 2012). In addition, ABA can also enhance drought tolerance in plants by stimulating the plant to produce more and deeper roots, inhibiting the expansion of leaf area as well as increasing the root to shoot ratio (Basu et al., 2016).

Table 1.1: Effect of drought on the reduction of yield among various crops

Crop types	% Yield reduction	Location	Reference
Maize (*Zee mays L*)	26.6% less than 2015 (severe drought)	South Africa	DPPME-SA (2016)
	43 % (moderate drought)	Iran	Khalili et al. (2013)
	48%	Czech Republic	Hlavinka et al. (2009)
	80% (severe drought)	Iran	Khalili et al. (2013)
Wheat (*Triticum aestivum* L)	18.6%	South Africa	Piesse (2016)
	57%	Czech Republic	Hlavinka et al. (2009)
Oat (*Avena sativa* L)	79%	Czech Republic	Hlavinka et al. (2009)
Potato (*Solanum tuberosum* L)	89%	Czech Republic	Hlavinka et al. (2009)
Rice (*Oryza sativa* L)	42% (moderate drought)	India	Raman et al. (2012)
	66% (severe drought)		
	65%	Thailand	Jongdee et al. (2006)
Barley (*Hordeum vulgare* L)	49% (moderate drought)	Jordan	Samarah (2005)
Common bean (*Phaseolus vulgaris* L)	78%	Romania	Szilagyi (2003)
Chickpea (*Cicer arietinum*)	50-80%	France	Leport et al. (1999)
Groundnut (*Arachis hypogea* L)	49% (severe drought)	South Africa	DPPME-SA (2016)
	55% (low temperature)-72% (high temperature)	India	Hamidou et al. (2013)
Sorghum	26.56% (drought)	South Africa	DPPME-SA (2016)

In the table, the percentages and levels of drought effects on major food crops as well as the country where the drought effect occurred were listed. For maize, the highest reduction of 80% was recorded in Iran. The highest reduction on wheat was recorded in Czech Republic with a 57% reduction. A 66% reduction was recorded for rice in India while in South Africa, the yield of Sorghum was reduced by 26.56% as a result of drought.

1.6.4 ALTERATION IN PHOTOSYNTHESIS

As previously stated in section 1.6.3, altered photosynthesis occurs in plants exposed to drought stress by stomatal closure and reduced leaf area (Xu et al., 2010; Basu et al., 2016). Plants respond immediately upon exposure to drought stress to prevent permanent alteration in their photosynthetic machinery. In this regard, stomatal closure primarily results in a decrease in the rate of photosynthesis (Mattos and Moretti, 2015). Cell turgor decrease, which is a direct negative effect of drought on plants and can lead to denaturation of plant enzymes or possibly hinder their activities. Severe drought stress also reduces the abundance of ribulose 1-5 bisphosphate carboxylase oxygenase (Rubisco, small subunits transcripts) leading to limited photosynthetic activity. Under drought conditions, the activities of the enzymes NADP-malic enzyme, pyruvate orthophosphate dikinase, fructose-1,6-biphosphate and phosphoenolpyruvate carboxylate also decline when there is a decrease in ATP synthesis and leaf water potential (Farooq et al., 2009; Hýsková et al., 2014; Chmielewska et al., 2016).

Photosynthetic electron chain activity is usually directed towards CO_2 availability in plants and photosystem II (PSII) which rapidly declines under drought conditions. Studies have shown that a decrease in the rate of photosynthesis in drought stress occurs mainly as a result of CO_2 deficiency, the reason being that photochemical efficiency could return to normal after a rapid movement of leaves to a CO_2 enriched environment (Farooq et al., 2009; Bardi and Malusà, 2012). Decrease in the level of intracellular CO_2 causes the over-reduction of components within the electron transport chain as the electrons get transferred to oxygen at photosystem I (PS I) which produces reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide and hydroxyl radicals (Roach and Krieger-Liszkay, 2014). The ROS needs to be scavenged by the plants as they may cause photo-oxidation. Redox signals act as a forewarning to the plants as they control the energy balance of the leaves. The detoxification systems of the plants which are ascorbate and glutathione pools control the plants' intracellular concentration of ROS. ROS function as second messengers

in redox signal transduction and are implicated in hormonal mediated events (Cruz de Carvalho, 2008; Sharma et al., 2012). Hydrogen peroxide functions as a signal for the closure of the leave stomata, acclimation of plant leaves to high irradiation and the induction of heat shock proteins (Shu-Hsien et al., 2005; Bita and Gerats, 2013; Hasanuzzaman et al., 2013).

Where water deficit becomes intense or prolonged, wilting can occur causing the cells to shrink, which can possibly lead to mechanical constraint on cellular membranes. This membrane strain is one of the serious effects of drought on plants' physiology (Chaves and Oliveira, 2004; Bardi and Malusà, 2012). Membrane strain causes damage to the ions functions and transporters as well as enzymes associated with membranes. Chloroplast membranes are very sensitive to oxidative stress damage caused by the production of excess amount of ROS in the membranes (Farooq et al., 2009; Roach and Krieger-Liszkay, 2014). ROS can cause mutation of nucleic acid and denaturation of proteins. They can also bring about extensive peroxidation and de-esterification of membrane lipids (Mahajan and Tuteja, 2005). Dehydration causes plant cells to shrink, leading to a decrease in cellular volume. When cellular volume decreases, cellular content becomes viscous, thus increasing the level of interactions between proteins and resulting in their aggregation and denaturation (Mahajan and Tuteja, 2005). During the period of water deficit recovery, the transcripts of some antioxidant genes are usually higher and may participate in protecting the cellular machinery against photo-oxidation caused by ROS.

1.6.5 OSMOTIC ADJUSTMENT

To be able to absorb water under drought stress, plant cells must maintain lower water potential than soil matric potential by accumulating solutes. Soil water potential is usually lower than plant osmotic potential at the permanent wilting point; it therefore cannot maintain the turgor pressure if transpiration is stopped completely. When soil dries, the ability of cells to increase the concentration of solutes by decreasing their water potential without losing cell volume or turgor pressure is known as osmotic adjustment (Bardi and Malusà, 2012). Inorganic ions like potassium can be accumulated in the vacuole while the concentration of compatible solutes is increased in the cytoplasm. Organic compounds like glycinebetaine, gamma-aminobutyric acid, proline, trehalose, dehydrins, citrulline, mannitol, polyols, fructans, sucrose, oligosaccharides and sorbitol are synthesized (Hayat et al., 2012; Signorelli et al., 2015). These compounds do not interfere with cellular metabolism but rather act as regulators of osmosis or stabilizers of cellular molecules and

structures. The functions of these compounds include among others to provide resistance to the plants against drought and dehydration by helping the cells to maintain their hydrated states. Osmotic adjustment in leaves is a well-studied phenomenon, however it also occurs in plants roots. Maintaining turgor pressure in root meristems enhances the maintenance of root growth (Farooq et al., 2009).

1.7 DEHYDRATION (DROUGHT) TOLERANCE

This is the relative ability to sustain the functions of the plant even in a dehydrated state (Rauf et al., 2016). The accumulation of molecular protectants that allows the plant to maintain its functions in a dehydrated state takes part in dehydration tolerance.

1.7.1 BUILD-UP OF OSMOPROTECTANTS AND STRESS RELATED GENES

Compatible solutes are responsible for the stabilization of key macromolecules and membranes from the damages caused by drought stress, as well as protecting them from the damage of ROS by the scavenging of free radicals.

Dehydrins are hydrophilic proteins that are formed during dehydration and late embryogenesis. They were first discovered by Hanin et al. (2011) in upland cotton (*Gossypium hirsutum* L.). Their concentration was up to 4% of the total protein in the seed and they were reported to have accumulated during late embryogenesis (Kleinwächter et al., 2014; Radwan et al., 2014). Dehydrins include a special class of proteins called LEA proteins or LEA-D11 proteins (Kosová et al., 2014). Some LEA-dehydrins are also called RAB proteins and are also responsive to ABA (Sharma et al., 2012). The functions of dehydrins include to protect the plants against all forms of abiotic stresses as well as to restore denatured proteins in plants. In cases of drought, they protect the hydrophobic regions of enzymes against solvent exposure, prevent structural changes in plants and help to maintain high ordered water molecules around the proteins. Genes enhancing the production of dehydrins are stress inducible and contain domains in their promoter regions that are responsive to water stress, ABA, heat stress and low temperature (Kovacs et al., 2008).

1.8 EFFECTS OF DROUGHT STRESS ON PLANT MORPHOLOGY

Drought stresses often cause morphological changes in plants, which can be seen in their cellular metabolism, growth and yield.

1.8.1 REDUCED CROP GROWTH AND YIELD

Impaired germination and poor establishment of strand are usually the first effects of drought on plants. One of the most drought sensitive physiological processes is cell growth due to decrease in turgor pressure. During severe drought, elongation of cells by higher plants can be repressed by interrupting the flow of water from the xylem through the surrounding elongating cells. The flow of water through plant xylem is guaranteed by the negative hydrostatic pressure produced by the leaves and also by the forces of cohesion applied by water into the vessels of the xylem (Bardi and Malusà, 2012). Plant resistance to water flow usually increases as a result of dryness, shrinking of roots due to dryness damages root hairs; root growth retards and the root surface is covered by suberin, making it water-impermeable (Bardi and Malusà, 2012). Drought causes cell expansion which consequently results in reduced growth and yield of plants as well as impaired mitosis. Water stress also reduces the number and size of leaves, and also the longevity of the leaf, by decreasing the soil water potential (Hoekstra et al., 2001; Istanbulluoglu et al., 2009; Basu et al., 2016). The reduction in leaf area due to drought is brought about by the suppression of leaf expansion through photosynthetic reduction. A major adverse effect of drought on plants is reduced nutrient uptake from the soil which lowers plant growth and yield resulting in lower fresh and dry biomass production (Zhao et al., 2006; Zhao et al., 2009). A study by Kamara et al. (2003) revealed that water stress imposed at silking, grain-filling period and at maturity of maize growth resulted in a decrease in total biomass accumulation of 37%, 34% and 21% respectively.

The result of the association and expression of various plant components is known as grain yield. The reduction in yield as a result of drought is dependent upon the duration of drought stress and how severe it is. Drought causes serious decrease in crop yield traits by disturbing leaf gas exchange properties which limits the size of the source and sink tissues, impairs the assimilate translocation and dry matter portioning (Farooq et al., 2009). Drought introduced at the flowering stage of plants, causes barrenness due to the reduction in assimilation of fluxes to the developing ear below the level needed to reach optimal growth yield (Yadav et al., 2004). Substantial reduction in yield and its components like kernel number, kernel grain yield per plant, 100 kernel weight, kernel rows, harvest index and biological yield per plant were observed when maize plants were exposed to drought at the tasseling stage (Anjum et al., 2011). Drought decreases plant growth and development, causing low flower production and grain filling resulting in the production of smaller

and fewer grains. Reduction in yield and its related components could be as a result of stomatal closure in response to low water of the soil, leading to reduced CO_2 intake and consequently decrease in photosynthesis (Flexas et al., 2004).

1.8.2 WATER AND NUTRIENTS RELATIONS

Stomatal resistance, leaf water potential, rate of transpiration, leaf and canopy temperatures and relative water content are all important characteristics that influence plant water relations. A study on *Hibiscus rosa-sinensis* showed a decrease in turgor potential, relative water content, transpiration, water-use efficiency and stomatal conductance under drought stress (Egilla et al., 2005). At the whole plant level, the ratio between the dry matter produced and water consumed is known as water-use efficiency (Monclus et al., 2006). A study on clover (*Trifolium alexandrinum*) conducted by Lazaridou and Koutroubas (2004) showed that water-use efficiency was increased by decreased rate of transpiration, leaf area and reduced yield due to lowered water loss under drought stress. Stomatal opening is strongly affected during water stress. However, a change in the temperature of the leaf may help in controlling the leaf water status under drought stress. Drought tolerant species aid in maintaining water-use efficiency by reducing loss of water but when there is a greater reduction in plant growth, water-use efficiency is significantly reduced (Farooq et al., 2009).

In the event of drought stress, decrease in availability of water leads to insufficient total uptake of nutrients and reduced tissue concentrations in crop plants (Farooq et al., 2009). A very significant effect of drought on plants is usually on the acquisition of nutrients by the roots, and their subsequent transport to the shoots. There may be variations in the response to mineral uptake among plant species and species genotypes. Generally, drought stress decreases phosphorus, increases nitrogen and has no obvious effect on potassium (Garg, 2003). Drought stress decreases the availability, uptake, metabolism and the translocation of nutrients in plants. This is because a reduction in the rate of transpiration brought about by drought reduces the absorption of nutrients and their utilization efficiency (Farooq et al., 2009).

1.8.3 PHOTOSYNTHESIS

Reduction in photosynthesis, which is one of the significant effects of drought on plants, results in decrease in leaf expansion, impaired photosynthetic machinery and premature senescence of the

leaves, leading to a reduction in food production (Wahid and Shabbir, 2005; Farooq et al., 2009). Water stress causes changes in photosynthetic pigments and components, damages photosynthetic apparatus and reduces the activities of Calvin cycle enzymes, which are very important causes of decreased crop yield (Farooq et al., 2009). The loss of balance between ROS and antioxidant production balance is another effect of drought stress that inhibits photosynthetic abilities and growth of plants (Reddy et al., 2004). This causes reactive oxygen species to accumulate, inducing oxidative stress in lipid membranes, proteins and other cellular machineries.

Stomatal closure, which is the first response of almost all plants upon exposure to severe water stress, helps to prevent loss of water through transpiration. This could lead to a decrease in water potential and leaf turgor or possibly low-humidity atmosphere. When the amount of soil water availability is moderate or limited, plants respond immediately by closing their stomata causing the inflow of CO_2 into the leaves to decrease and sparing more electrons to form ROS (Farooq et al., 2009). This is because a decrease in the rate of transpiration brings about an increase in the amount of heat that can be dissipated (Yokota et al., 2002). The environmental conditions that boost transpiration rate also increase the pH of leaf sap which is capable of promoting the accumulation of abscisic acid and lessening stomatal conductance.

Reduction in photosynthetic rate as a result of severe drought can lead to a decline in the activity of Rubisco (Bota et al., 2004). Under drought stress, the activity of photosynthetic electron transport chain depends on the amount of CO_2 available in the plants' chloroplast and a change in the photosystem II (Farooq et al., 2009). Dehydration in plants causes their cells to shrink, leading to a decline in cell volume which makes the contents of the cell become more viscous. Hence, when the probability of protein to protein interaction increases, they tend to aggregate and denature (Hoekstra et al., 2001). When the concentration of solutes increases, the viscosity of the cytoplasm also increases. This may be toxic and deleterious to enzyme functions including those responsible for photosynthesis (Hoekstra et al., 2001).

1.8.4 PARTITIONING OF ASSIMILATES

For proper seed development, the translocation of assimilate to plants' reproductive sinks is crucial. The setting and filling of seed can be incomplete due to either utilization (sink limitation) or availability (source of assimilate) (Asch et al., 2005). The allocation of dry matter to plant roots

is normally heightened by water deficit, thereby improving water uptake (Palta et al., 2007). The rate at which sucrose is exported from its source to sink organs is dependent on the concentration of sucrose in the leaves and its current photosynthetic rate (Komor, 2000). Decrease in the rate of photosynthesis, disruption in carbohydrate metabolism and level of sucrose in leaves, resulting in decrease in export rate, is usually as a result of drought stress (Kim et al., 2000). Plants' reproductive development can be highly affected by limited photosynthesis and accumulation of sucrose in the leaves which hampers the rate at which sucrose is exported to the sink organs. Besides limitation in source, the ability of the reproductive sinks to make use of incoming assimilates is usually affected by drought stress and may be a major cause of reproductive abortion. Carbohydrate deprivation, impaired ability to use the incoming sucrose by their reproductive sinks, and increase in the concentration of endogenous abscisic acid are potential causes of seed abortion in grains (Setter et al., 2001). In addition to limiting the size of the source and sink tissues, drought stress also causes impairment in plants' assimilate translocation, dry matter partitioning and phloem loading. This however depends on the plant species, duration, stage and severity of water stress.

1.8.5 OXIDATIVE DAMAGE

Plants' exposure to certain environmental stresses like drought often results in the generation of ROS like hydroxyl radicals (OH^-), hydrogen peroxide, alkoxy radicals (RO) and superoxide anion radicals (O_2^-). Oxidative damage and impairment of normal cell functions can be a result of the reaction between ROS and lipids, proteins and deoxyribonucleic acid (Cruz de Carvalho, 2008; Foyer and Fletcher, 2001). Reactive oxygen species such as hydrogen peroxide (H_2O_2) and hydroxides (OH) can be produced from downstream reactions. They can also be formed when oxygen interacts with the reduced components of electron transport chain in the mitochondria (Møller, 2001). On the other hand, during photorespiration, H_2O_2 is formed by peroxisomes when glycolate is oxidized to glycolic acid. Catalases and peroxidases also play major roles in the fine regulation of ROS in plant cells by the activation and deactivation of H_2O_2 (Sairam et al., 2005; Cruz de Carvalho, 2008). ROS can also be generated by many apoplastic enzymes under normal or stressful conditions (Cruz de Carvalho, 2008).

Reactive oxygen species are formed as by-products in plants mitochondria, plasma membrane (Sairam et al., 2005) and the electron transport chain of chloroplasts (Apel and Hirt, 2004). Several

reports have indicated the deleterious effects of ROS stimulated by drought on plants (Blokhina et al., 2003; Farooq et al., 2009). Reactive oxygen species causes peroxidation of lipids, thereby leading to membrane injuries, inactivation of enzymes and protein degradation (Sairam et al., 2005). Protein oxidation, formation of protease-resistant cross-linked aggregates and the loss of enzyme activity may also occur as a result of oxidative damage. The production of ROS, which is highly dependent on the severity of drought stress, causes the degradation of both functional and structural plant proteins, nucleic acids as well as enhanced degradation of membrane lipids. ROS produced during drought stress mainly target various plants organelles such as the chloroplasts, peroxisomes and mitochondria (Farooq et al., 2009).

1.9 METHODS TO IMPROVE PLANT DROUGHT TOLERANCE

Drought limits plant productivity worldwide causing an increase in desertification and food insecurity. Therefore, it is necessary to develop means to improve the plants for higher productivity as well as reducing the adverse effect of drought on plants. Below are some highly efficient strategies to improve drought stress tolerance in plants.

1.9.1 TRANSGENIC METHOD

Transgenic plants are plants formed as a result of the addition of a foreign gene. All around the globe, plant breeders are in active pursuit of genetic modification to develop cultivars or lines of crops that will be tolerant to stress factors (Ashraf, 2010; Naveed, 2013). This is because this method tends to carefully introduce the desired traits either from different varieties of the same crop or different species of the plant, making it much easier to eliminate undesired traits (Kim et al., 2012). The prospects of using genetic engineering to improve drought tolerance in plants look very promising because incorporating only the specific cloned genes and avoiding undesirable gene transfer is very possible and achievable. Through genetic engineering, joining genes with similar effects is possible (Gosal et al., 2009). Plants genetically modified to resist drought are able to more strongly withstand and produce higher plant yields under water deficit conditions than the wild types. Some crops genetically modified through successful incorporation of genes to improve drought tolerance are peanut (Bhatnagar-Mathur et al., 2009), maize (Quan et al., 2004), wheat (He et al., 2011), soybean (De Ronde et al., 2004), tomato (Naveed, 2013) and tobacco (Karim et al., 2007). Several laboratory and field studies have shown that the transgenic expression of stress-regulated genes causes an increase in plants tolerance to drought and other stresses (Naveed, 2013;

Basu et al., 2016). In order to identify less obvious genetic network that respond to drought stress, more sensitive and straight forward methods are required. Whole genomics and related technologies are helping in the identification of key genes that respond to drought stress and relating their regulation to adaptive events that occur during stress (Bruce et al., 2002).

1.9.2 GERMPLASM SCREENING

Living material possessing hereditary from which new plants can grow such as rootstock, leaf plant tissue and seeds is called germplasm (Marmar et al., 2013; Mwadzingeni et al., 2016). Germplasm constitutes the mining and introduction of traits from wild relatives and landraces to enhance drought tolerance in plants. Generally, superior germplasm is obtained by selecting cultivars with higher drought tolerance levels from plants that are well adapted to harsh conditions from different climatic zones (Naveed, 2013; Mantri et al., 2014). The germplasm selected passes through different forms of screening both under greenhouse and field conditions. The germplasm found to possess high levels of drought tolerance is tested under greenhouse conditions to determine its drought tolerance levels (Naveed, 2013; Mwadzingeni et al., 2016). Finally, the selected germplasm is propagated for multiplication and distribution to farmers (Mwadzingeni et al., 2016). The germplasm of several crop species has facilitated the discovery of traits that enhance drought tolerance in plants (Langridge and Reynolds, 2015). Recently in the United States, drought tolerant maize hybrids were released as a result of the introduction of traits from distant relatives (McKersie, 2015). However, the process of germplasm is very lengthy and time consuming. The prerequisite of this method is the establishment of proper, practical, cheap, reliable and fast methods of selection and multiplication system (Mantri et al., 2014; Hanin et al., 2016). This method also requires large and costly glasshouses and fields for screening out the desired drought-tolerant genotypes. It also requires multidisciplinary approaches in order to measure drought stress effects on the physiology, morphology and biochemistry of the plants (Mantri et al., 2014; Hanin et al., 2016). While increase in drought stress tolerance is achievable through this method, it is only to a small extent (Naveed, 2013).

1.9.3 BREEDING OF DROUGHT TOLERANT GENOTYPES

The development of tolerant genotypes that is resistant to drought can be achieved through plant breeding. Plant breeding represents a lucrative and competent way of manipulating plants for

efficient growth in drought-prone environments. The use of conventional plant breeding in dealing with the challenges of food insecurity is enormous and has been in existence since the last century (Kim et al., 2012; Naveed, 2013). A large number of drought tolerant cultivars of important plants have been developed during the last century. Through plant breeding, new varieties of plants with the desired traits are developed through purposeful crossing of distantly or closely related genes.

Manipulation through plant breeding is usually done by controlled pollination. Firstly, plant breeders intentionally create genetic diversity that would not exist in nature. Secondly, to produce new varieties, plants are repeatedly crossed over several generations which leads to a final artificial selection of progeny plants with the desired traits (Naveed, 2013). The selected resistant plants are eventually tested for their drought tolerance levels. The selected resistant plants with high yield are thereafter multiplied and distributed to farmers to be cultivated in fields in drought stressed environments. Among the world's major cultivars developed via traditional breeding methods are wheat N E01643 (Baenziger et al., 2008), soybean R 01-416F (Chen et al., 2007b), sunflower Morlin (Bergman et al., 2001) and peanut ICGV 87354 (Reddy et al., 2001).

However, only plants of the same species are used in this method to introduce traits. Due to the multigenic nature of drought tolerant traits, plants bred through this method have achieved only limited success in enhancing drought tolerance in crops. Plant breeding through this method could also result in inbreeding and breed deterioration. It also makes the plant become more prone to disease or mutation. Moreover, un-purposeful fixing of undesired traits through plant breeding could occur in this method thereby resulting in narrow genetic diversity and possible loss of some local species. Plant breeding via selection is usually cost and time intensive, and requires huge time investment making it mostly unfeasible (Ashraf, 2010). Also, the hybrids formed do not breed true offspring; they produce offspring with different traits, hence causing loss of the obtained traits.

1.9.4 USING MICROORGANISMS TO PROMOTE DROUGHT TOLERANCE IN PLANTS

This method uses biological products or substances containing living microorganisms to deal with plant stress. Recent studies have indicated that the inhibitory effects of drought stress on plants can be reduced by the use of appropriate tolerant plant growth-promoting rhizobacteria (Shakir et al., 2012; Yandigeri et al., 2012; Cao et al., 2016; Sathya et al., 2017). Several bacteria species

capable of enhancing plants' ability to withstand drought or water deficit conditions by increasing root elongation, seedling vigor and several plant biochemical and physiological responses have been isolated (Zahir et al., 2008; Naveed, 2013). Under drought stress, these microorganisms can survive through diverse mechanisms such as biofilm production (Chang et al., 2007), avoidance of water loss by production of osmolytes (Naveed, 2013) and the production of exopolysaccharides (EPS) (Nocker et al., 2012). Moreover, these microorganisms can also protect plants against dehydration by maintaining a moist environment favourable for root growth and development, acting as promoters of plant growth, aiding in the production of hormones and supply of nutrients (Kavamura et al., 2013).

The oxidative damage at cellular level caused by drought stress on plants can be suppressed by the plants' ability to produce antioxidant enzymes [catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD)] that scavenge free radicals (Simova-Stoilova et al., 2008). A study by Kohler et al. (2008), revealed that *Pseudomonas mendocina* inoculation improved the growth performance of lettuce (*Lactuva sativa* L.) by enhancing CAT under drought stress. According to a report by Fedonenko et al. (2010), inoculation by *Azospirillum brasilense* Sp245 improved relative leaf water content and elastic adjustment, which resulted in better growth, yield and mineral quality (K, Ca, and Mg) of wheat (*Triticum aestivum* L.) under drought stress. Treatment of plants with bacteria producing EPS induced resistance to drought stress, because EPS is responsible for the formation of biofilm on the surface of potato roots, thereby increasing yield (Naveed, 2013). Bacteria inoculated seedlings revealed an improvement in root adhering soil, soil aggregation and higher relative water content in the leaves of maize plant (Sandhya et al., 2010). From the work of Mayak et al. (2004), the bacteria strain (*Achromobacter piechaudii*) containing 1-aminocyclopropane-1-carboxyl (ACC) deaminase induced tolerance to drought stress in tomato and pepper. The production of ethylene was decreased in plants treated with bacteria, which resulted in significant increase in fresh and dried biomass when compared to the untreated controls (Naveed, 2013). A report from Zahir et al. (2008) revealed that under drought stress, *Pseudomonas* sp. enhanced the growth of pea (*Pisum sativum*) in axenic conditions and also in potted soil. They suggested that ethylene synthesis must have been reduced by the bacterial inoculation which resulted in a better root growth and total plant biomass under water deficit stress.

Drought resistance as a result of bacteria inoculation is a very important technology for use in arid and semi-arid regions of the world where plants are often prone to drought stress. Nonetheless, there is limited research on the interactions between rhizospheric actinomycetes with plants and drought resistance.

1.10 MECHANISMS OF DROUGHT STRESS ALLEVIATION BY PLANT GROWTH PROMOTING BACTERIA (PGPB)

In an agrobiologic system, the two types of plant growth promoting bacteria (PGPB) on the basis of the environment they live are: rhizospheric and endophytic bacteria (Bhattacharyya and Jha, 2012). Plant-growth-promoting bacteria are either isolated from the rhizosphere (rhizobacteria) or from the endosphere (endophyte). The inoculation of PGPB into the seeds or soil enhances plant growth via one or a combination of some functional mechanisms (Kim et al., 2012; Vurukonda et al., 2016). Growth improvement by PGPB can be as a result of production of phytohormones like indole-3-acetic acid (IAA), gibberellins and cytokinins (Vurukonda et al., 2016). They can also improve plant growth by beneficial nitrogen fixation (BNF), siderophore production, biocontrol of phytopathogens through the production of antibacterial or antifungal agents, induction of acquired host resistance, nutrient competition or mineral bioavailability enhancement (Mitter et al., 2013; Vurukonda et al., 2016). Drought resistance mechanisms by PGPB can be achieved through a process known as rhizobacterial-induced drought endurance and resilience (RIDER) involving several physiological and biochemical changes (Kaushal and Wani, 2016). These mechanisms include phytohormonal content modifications, antioxidant defense and production of osmolytes and EPS for drought stress tolerance (Vanderlinde et al., 2010). Recently, the production of heat-shock proteins (HSPs), volatile organic compounds and dehydrins have also been reported to induce drought tolerance in plants (Kaushal and Wani, 2016). Lately, molecular and biochemical methods are providing new insight into the genetic source of these biosynthetic pathways, their mode of regulation and significance in enhancing drought stress tolerance (Joshi and Bhatt, 2011). The main mechanisms involved in drought stress resistance and plant growth promotion by PGPB are discussed below.

1.10.1 MODIFICATIONS IN PHYTOHORMONES

Phytohormones are low molecular weight natural products that act at micro molar concentrations in regulating all the developmental and physiological processes throughout a plant's life cycle

(Naveed, 2013). Phytohormones are also known as plant growth regulators. Indole-3-acetic acid (IAA), ethylene, auxins, cytokinins, abscisic acid (ABA) and gibberellins are the commonly known bacterial phytohormones that aid in plant growth and development (Spaepen et al., 2007; Kaushal and Wani, 2016).

Modification in bacterial phytohormones is one of the mechanisms utilized by PGPR in ensuring the growth and survival of plants under drought stress. Indole-3-acetic acid has recently been reported to be successful in the impartation of osmotic stress tolerance in plants (Boiero et al., 2007; Kaushal and Wani, 2016). The production of IAA by PGPR results in the modification of a plant's architectural root system. This is done by increasing the plant's root surface area and number of root tips, thereby increasing the plant's acquisition of water and nutrients which helps the plant to cope with drought stress (Egamberdieva and Kucharova, 2009). Plants' inoculation with *Pseudomonas putida* reportedly survived drought stress as a result of IAA production (Marulanda et al., 2009). Reports have indicated that bacterial volatile organic compounds (VOCs) from *Bacillus subtilis* strain GB03 promoted the growth of *Arabidopsis* by upregulating the transcripts involved in auxin homeostasis (Zhang et al., 2007). A report by Pereyra et al. (2012) revealed that the inoculation of wheat seedlings with *Azospirillum* enhanced osmotic stress tolerance due to morphological modifications in the structure of the coleoptiles xylem. This was credited to the indole-3-pyruvate decarboxylase gene upregulation and improved synthesis of IAA in *Azospirillum.* Modifications in physiology were observed in soybean plants inoculated with the gibberellins producing rhizobacterium *Pseudomonas putida* H-2-3 as plant growth was increased under drought stress (Kang et al., 2014). According to Cohen et al. (2009), ABA and gibberellins production by *Azospirillum lipoferum* alleviated drought stress in maize plants. During water deficit, cellular dehydration occurs, bringing about the biosynthesis of ABA known as a stress hormone due to its abnormal accumulation in drought conditions. ABA regulates water loss by controlling stomatal closure and stress signal transduction pathways (Kaushal and Wani, 2016). Higher levels of ABA were observed in *Arabidopsis* plants inoculated with *Azospirillum brasilense* Sp245 than in the controls (Cohen et al., 2008). *Phyllobacterium brassicacearum* strain STM196, a PGPR isolated from the rhizosphere of *Brassica napus*, enhanced osmotic stress tolerance in inoculated *Arabidopsis* plants by raising the content of the ABA, resulting in a decrease in leaf transpiration (Bresson et al., 2013). In a study conducted by Liu et al. (2013), the

seedlings of *Platycladus orientalis* were inoculated with *Bacillus subtilis*, a cytokinin-producing PGPR, which interfered with shoot growth suppression, thereby conferring resistance to drought stress.

1.10.2 USING ACC DEAMINASE PRODUCING BACTERIA TO ENHANCE DROUGHT TOLERANCE

Plant activities are usually controlled by ethylene levels while ethylene biosynthesis is controlled by abiotic and biotic stresses (Hardoim et al., 2008). In ethylene biosynthetic pathway, 1-aminocyclopropane-1-carboxylate synthase converts S-adenosylmethionine (S-AdoMet) to 1-aminocyclopropane-1-carboxylate (ACC) which is the immediate precursor of ethylene (Glick et al., 2007; Glick, 2014). During drought stress, ethylene endogenously controls plant homoeostasis causing reduction in the growth of plant shoot and root (Glick, 2014; Vurukonda et al., 2016). ACC deaminase producing bacteria supply nitrogen and energy to plants by sequestering and degrading plants ACC (Shrivastava and Kumar, 2015; Vurukonda et al., 2016). Studies have shown that some PGPR possess the enzyme ACC deaminase that can cleave the ethylene precursor of the plant ACC to a-ketobutyrate and ammonia, hence, reducing the ethylene level (Kaushal and Wani, 2016). The removal of ACC decreases the toxic effect of ethylene thereby improving plant stress tolerance and promoting plant growth (Glick et al., 2007; Kaushal and Wani, 2016). Lim and Kim (2013) inoculated pepper with *Bacillus licheniformis* K11 and observed an increase in the production of ACC deaminase which imparted tolerance to cope with drought stress on the pepper plant. The relationship between IAA and the ethylene precursor ACC displays positive effects of IAA on plants' root growth by reducing the levels of ethylene (Lugtenberg and Kamilova, 2009). An ACC deaminase producing bacteria, *Pseudomonas* spp. inoculated into *Pisum* plant induced longer roots development which brought about an increase in water uptake from the soil under drought stress conditions (Zahir et al., 2008). A study performed by Hontzeas et al. (2004) revealed an increase in genes transcripts associated with cell proliferation, cell division and genes down regulation associated with stress in canola plants colonized by an ACC-deaminase-containing strain *Enterobacter cloacae* UW4. Also, the upregulation of auxin responsive genes and the down regulation of ethylene responsive genes were observed in *Arabidopsis* plants colonized by *P. fluorescens* FPT9601-T5 (Wang, 2005). Studies conducted *in-vitro* showed that inoculation of wheat plants with ACC deaminase producing bacteria enhanced

root and shoot length, lateral root number and root and shoot mass when compared to the control. Well-developed roots enhance water and nutrient uptake in plants which results in increased growth and yield under drought stress (Shakir et al., 2012).

1.10.3 ANTIOXIDANT DEFENSE

Under ideal growth conditions, the generation of ROS like hydroxyl radicals, super oxide and hydrogen peroxide is usually low in different plant organelles (Kaushal and Wani, 2016). Drought stress condition interrupts the plant's photosynthetic machinery and increases photorespiration, thereby changing the cells' normal homeostasis, consequently resulting in cumulative ROS production. Plants fortified with antioxidant defense systems are made up of enzymatic and non-enzymatic components that function in alleviating the oxidative damages that occur during drought by foraging of ROS (Miller et al., 2010). The enzymatic components of the plant's antioxidant defense system are catalase (CAT), glutathione reductase (GR), superoxide dismutase (SOD) and ascorbate peroxide (APX) while the non-enzymatic components contain glutathione, ascorbic acid and cysteine (Kaushal and Wani, 2016). Excessive ROS has been reported to cause increased lipid peroxidation and subsequent lipid, DNA and protein damage (Pompelli et al., 2010). Nevertheless, ROS also function as a signal activating stress-response and defense pathways (Kaushal and Wani, 2016). It is therefore necessary to regulate the levels of ROS by coordinating their production and scavenging systems in order to control oxidative damage and the concurrently modulating signally event (Kaushal and Wani, 2016). Drought stress is closely associated with antioxidant enzyme activity but the introduction of PGPR reduces the negative effects of drought stress on the activity of antioxidant enzymes (Han and Lee, 2005). In a study performed by Vardharajula et al. (2011), maize plants inoculated with *Bacillus* spp. were protected against drought stress by a reduction in the activity of the antioxidant enzymes APX and GPX. Also, an EPS-producing bacteria was reported to confer drought resistance to maize plants by decreasing the activities of the antioxidant enzymes GPX, CAT and APX (Naseem and Bano, 2014). These studies provide evidence for the use of plant growth promoting bacteria in improving drought tolerance in plants through the alteration in the activity of antioxidants under drought stress (Gusain et al., 2015).

1.10.4 OSMOLYTES ACCUMULATION

Under drought stress, plants have a greater obligation to adjust osmotically so as to reduce cell turgidity losses. Accumulation of osmolytes like glycine, trehalose, betaine and proline is the most common adjustment response seen in plants and bacteria towards drought stress (Rodríguez-Salazar et al., 2009; Kaushal and Wani, 2016). The prevention of protein denaturation by membrane integrity protection is very necessary under water deficit condition (Farooq et al., 2009). High levels of amino acids, considered to be a common indication of drought tolerance have been found in wheat, pepper and sorghum (Kaushal and Wani, 2016). Amino acid accumulation is brought about by protein hydrolysis that occurs in response to changes caused by osmotic acclimatization (Iqbal et al., 2011; Krasensky and Jonak, 2012). The synthesis of proline causes the scavenging of free radicals, osmotic adjustment and plants cells subcellular structures stabilization to overcome the damaging effects of drought on plants (Kaushal and Wani, 2016). Higher levels of proline have been reported to confer drought tolerance in plants (Chen et al., 2001; Chen et al., 2007a). Plants are able to escape oxidative damage by means of reserved catabolic pathway resulting in a massive increase in proline content (Mohammadkhani and Heidari, 2008). In response to drought stress, PGPR produce osmolytes that act synergistically with plants' osmolytes, hence stimulating plant growth (Paul and Nair, 2008). The presence of *Burkholderia* has been reported to increase proline synthesis in osmotically stressed plants (Barka et al., 2006). Under drought stress, plants inoculated with *Bacillus* strains showed significant increases in proline content, which was credited to the upregulation of genes for P5CS that function in proline biosynthesis and also inhibiting expression of the gene for proDH that function in proline metabolism (Kaushal and Wani, 2016). When proBA genes obtained from *Bacillus subtilis* were introduced into *Arabidopsis thaliana* plants, an increase in proline production was observed corresponding to osmotic tolerance acquisition in transgenic plants (Chen et al., 2007a). The inoculation of maize plants with *Bacillus* spp. revealed elevated levels of sugars, proline, and free amino acids, therefore increasing plants' relative water content, leaf water potential, biomass and root adhering soil/root tissue ratio (Vardharajula et al., 2011). Higher levels of leaf proline believed to have been generated during drought stress were observed in maize plants, which were further increased by inoculation with *P. fluorescens* (Ansary et al., 2012). A recent study by Bano et al.

(2013) demonstrated that *Azospirillum lipoferum* caused a significant increase in the growth of maize plant while accumulating soluble sugars and free amino acids during drought stress.

The accumulation of soluble sugars as osmolytes is also a mechanism of adaption for plants towards osmotic adjustment under water deficit condition. A report indicated that the hydrolysis of starch results in higher sugar levels (Kaushal and Wani, 2016). Higher sugar content as a result of degradation of starch was observed in maize seedlings inoculated with *Bacillus* strains, thereby imparting tolerance to plants during drought stress (Mohammadkhani and Heidari, 2008). Under un-inoculated condition, the negative effects of drought stress on plant growth may be as a result of decreasing sugar levels (Sandhya et al., 2010). Increased soluble sugar content was observed in maize seedlings inoculated with *Pseudomonas spp.* compared to un-inoculated ones, which indicated that inoculation results in starch hydrolysis, hence providing sugar for osmotic acclimatization to contradict the effect of drought stress (Bano and Fatima, 2009). It is an established fact that improved biosynthesis of glycine betaine such as quaternary compounds improves the adaptability of plants to different abiotic stresses (Chen and Murata, 2008). However, glycine betaine does not directly scavenge ROS, when it is synthesized; it produces H_2O_2 that stimulate ROS-scavenging enzymes thereby mitigating oxidative stress. Inoculation of *Pseudomonas pseudoalcaligenes* into *Oryza* has been reported to confer stress tolerance by rapid accumulation of glycine betaine (Jha et al., 2011). Inoculation of *Arabidopsis* plant with *B. subtilis* GB03 (VOC-emitting strain) induced higher levels of glycine betaine content and its precursor choline in the plants, thereby imparting drought tolerance in them (Kaushal and Wani, 2016). Conversely, drought tolerance induced by GB03 was lost in the *xipotl* mutant of *Arabidopsis* with a decrease in choline production (Zhang et al., 2010). A non-reducing disaccharide known as trehalose stabilizes dehydrated membranes and enzymes, thereby acting as an osmoprotectant. The biosynthesis of trehalose imparts osmoprotection in bacteria (Yang et al., 2010). Trehalose biosynthesis in *Arabidopsis brasilense* improved drought resistance and biomass production in maize plants (Rodríguez-Salazar et al., 2009). Mixed inoculation of both *Paenibacillus polymxa* and *Rhizobium tropici* improved the growth and nodulation of *Phaseolus vulgaris* L. subjected to 21 days of drought stress more than the plant inoculated with *Rhizobium* alone (Figueiredo et al., 2008). Other compatible osmolytes are N-acetyl glutaminyl glutamine amide (NAGGN), betaine and mannitol reported in *P. putida* (Kaushal and Wani, 2016). Polyamides are aliphatic compounds

of nitrogen present in bacteria, animals and plants and are involved in several hormonal and metabolic processes that regulate plant responses to drought stress as well as plant growth and development (Alcázar et al., 2011). Improvement in root growth as a result of the production of cadaverine (a polyamine) was observed in *Oryza* seedlings when inoculated with *A. brasilense* Az39 to reduce abiotic stress (Cassan et al., 2009).

1.10.5 EXOPOLYSACCHARIDES PRODUCTION

Exopolysaccharides known as bacteria biofilms are hydrated compounds containing 97% water in a polymer matrix that imparts protection against dehydration (Kaushal and Wani, 2016). Significant increment in the production of EPS by *Bacillus amyloliquefaciens* was observed in water deficit conditions compared to the controls (Kaushal and Wani, 2016). ESPs bring about increases in microaggregates production, which improves the growth of plants under drought stress by amplifying aggregate stability and root-adhering soil/root tissue (RAS/RT) ratio. Enhanced RAS aggregation increases water and nutrients uptake from the rhizosphere soil thereby guaranteeing the growth and survival of plants under drought stress (Vardharajula et al., 2011). The inoculation of wheat plant with an EPS-producing rhizobacteria *Pantoea agglomerans* led to an increase in RAS dry mass to RT dry mass (RAS/RT) and improved water stability of adhering soil aggregates (Kaushal and Wani, 2016). Cells subjected to stress activate guanine cyclases production, bringing about the production of cyclic-di-GMP, EPS and protein adhesins involved in the development of biofilm (Borlee et al., 2010). Inoculating plants with an EPS-producing bacteria leads to an extensive root system development that promotes shoot growth during drought stress (Awad et al., 2012). Naseem and Bano (2014) reported that EPS-producing bacteria increased drought tolerance in maize plant. Almost all *Pseudomonas* species are capable of producing alginate, a major EPS that is involved in maintaining hydration in biofilms and easing oxidative stress (Halverson, 2009; Kaushal and Wani, 2016). The ability of alginate to enhance drought tolerance might be as a result of its hydroscopic properties; it can also be as a result of the role it plays in biofilm architecture that reduces evaporation loss. Many recent research works conducted on several species of bacteria have opened an inventory of molecular determinants that took part during bacterial inoculation processes of surfaces and biofilm development (Kaushal and Wani, 2016). Large extracellular proteins that play a vital role in biofilm formation by *P. putida* are known as LapA and LapF. LapA is responsible for the initial attachment of individual bacteria

to a surface whereas LapF plays a role in the development of mature biofilms (Martínez-Gil et al., 2014). High levels of c-di-GMP increase the expression of LapA promoter while low levels increase the expression of LapF promoter. FleQ, a transcriptional regulator, is essential for modulation of LapA expression by c-di-GMP, while it has a minor effect on LapF expression. A report by Lahesaare et al. (2014) revealed that in *P. putida*, FIS (a small DNA binding and bending homo diametric protein) binds to LapF promoter *in-vitro* and represses the expression of LapF.

1.10.6 MOLECULAR TECHNIQUES IN DROUGHT STRESS ALLEVIATION BY PGPB

Studies on gene expression help in understanding and comparing the general responses of an organism to their environment (Schlauch et al., 2010). Gene expression as a result of drought stress has been characterized recently using molecular techniques (Kandasamy et al., 2009; Yuwono et al., 2005) as well as the physiological roles of PGPR in inducing drought tolerance in plants. Lim and Kim (2013) reported the identification of six differentially expressed stress proteins in pepper plants inoculated with *B. lichenformis* K11 under drought stress using 2-D polyacrylamide gel electrophoresis (2D-PAGE) and differential display polymerase chain reaction (DD-PCR). Specific genes of sHSP, VA, Cadhn and CaPR-10 among the stress proteins showed above 1.5-fold increase in treated plants when compared to untreated plants (Lim and Kim, 2013). The up-regulation of stress regulated genes APX1 and HSP17,8 in wheat leaves were identified using real-Time PCR (RT-PCR), and there was an increase in the enzymatic activities involved in the reaction of plant ascorbate-gluthatione redox cycle which conferred drought tolerance in the plant, while priming with *A. brasilense* NO40 and *B. amylolique-faciens* 5113, improved the toxic effects caused by drought stress (Kasim et al., 2013).

1.11 HYPOTHESIS, AIM AND OBJECTIVES

Drought is an abiotic stress which results from changes in climatic conditions with enormous toxic effects in plants. In curbing the deleterious effects of drought on plants, beneficial bacteria can be used as they have been found to be efficient, economical and environmentally friendly. This has led to the identification of bacteria species capable of inducing tolerance to drought stress in plants. More work is still required on the identification of potential bacteria capable of alleviating drought stress in plants for improved agricultural productivity to solve the current issues surrounding food

security in the future. Also, better ways of ensuring that the best is being obtained from these bacterial species including the mode of inoculation should be examined for efficient results.

In this research, we hypothesize that bacteria obtained from plant rhizosphere are capable of eliciting drought stress tolerance in plants and they also serve as plant growth promoters when inoculated on seeds. Therefore, this work is designed to determine if actinomycetes will be able to improve the growth of maize plants in the event of drought. The objectives of this study are to:

1. Isolate and identify actinomycetes from dry maize rhizosphere;
2. Determine the drought tolerance ability of selected actinomycetes;
3. Screen bacterial strains for the presence of drought tolerance and some plant growth promoting genes present by amplification with polymerase chain reaction (PCR);
4. Screen and quantify plant growth promoting traits like IAA, ACC deaminase, siderophores and ammonia (NH_3) present in bacterial isolates;
5. Evaluate the effect of inoculation of selected strains in improving maize growth under well-watered, moderately watered and drought stressed conditions;
6. Determine the effect of inoculation method on the growth parameters of maize plants under drought stress.

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CHAPTER TWO: SCREENINING AND IDENTIFICATION OF DROUGHT TOLERANT ACTINOMYCETES FROM DRY MAIZE RHIZOSPHERE

ABSTRACT

The identification of drought tolerant bacteria is very important as it gives more insight on their taxonomic and ecological positions. Actinomycetes species were isolated from the rhizosphere of two maize fields in Mafikeng, South Africa. Seven isolates showed varying degree of high tolerance to drought stress by growing on 5% polyethylene glycol (PEG) 8000. The physiological and morphological characteristics were similar to that of actinomycetales order. Screening for drought tolerance at different concentrations of PEG 8000 and culture time was performed and it showed that maximum growth was observed at 5% PEG concentration and at 120 h. Bacterial growth at different pH, NaCl concentration and temperatures were evaluated and optimum growth for all bacterial isolates was observed between pH 5 and 9, 0 to 4% NaCl, and 25°C and 35°C. Molecular identification of the seven drought tolerant actinomycetes was done using 16S rRNA gene sequence analysis which gave the targeted sizes of 1.5 Kb for 5 isolates and 350 bp for two isolates. These products were sequenced and computational analysis including BLAST search and phylogenetic analysis was performed to compare the isolates with other species in the GenBank library. Computational analysis performed revealed that five out of seven isolates were from the genus *Streptomyces* with 99-100% similarity while two were from the *Arthrobacter* and *Mycobacterium* genus with 99% similarity. The 16S rDNA sequences were submitted to the GenBank with the following accession numbers: MG547867, MG669347, MG547868, MG547869, MG547870, MG547870 and MG640369. The primers that amplified specific genes encoding proteins involved in drought tolerance [Glutathione peroxidase (*GPX*), glycine-rich RNA binding protein (*GRP*), desiccation protectant protein (*DSP*) and Gtp-binding protein (GTP)] were designed and amplified by PCR together with the two plant growth promoting genes for ACC deaminase activity and siderophore production (*Accd* and *Sid*). Amplifications were observed as follows: four isolates for *GPX* and *GRP*, two for *DSP*, one for *GTP*, seven for *Accd* and six for *Sid* genes. 16S rRNA gene sequence analysis is an important tool that aids in the identification of actinomycetes up to the species level. Moreover, the amplification of the drought tolerant and plant growth promoting primers indicates the presence of these genes in these bacteria and their possible use in drought tolerance and growth promotion in plants.

Keywords: Actinomycetes, amplification, drought tolerance, identification and plant growth promoting genes

2.1 INTRODUCTION

Plants are constantly subjected to different abiotic and biotic stresses like drought, salinity, flooding, high temperatures, toxic metals, radiation, insect, fungi, bacteria and viruses (Kim et al., 2012; Postolaaky et al., 2012). Drought is a major abiotic stress limiting plant growth and crop yield, resulting in substantial decrease in agricultural productivity. Reduction in growth and productivity are often observed in plants exposed to drought stress compared to non-drought stressed plants (Bardi and Malusà, 2012). Drought causes considerable loss of crops worldwide bringing about more than 50% reduction in the average yields of most major food crops, and is therefore a major threat to global food security (Tripathi et al., 2016). Plants respond to the deleterious effects of drought stress by synthesizing various defense-related proteins, and by modifying their physiological and metabolic activities (Paul and Nair, 2008; Vurukonda et al., 2016). Moreover, certain soil microorganisms known as plant growth promoting bacteria (PGPB) are also capable of assisting plants to overcome the drastic effects of different environmental stresses (Vardharajula et al., 2011; Bhattacharyya and Jha, 2012; Kaushal and Wani, 2016). This group of bacteria enhances plant growth directly by providing them with phytohormones (e.g. gibberellins, cytokinins and indole-acetic acid) or indirectly by reducing the level of plant ethylene production through the activity of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase and preventing or reducing the damage caused by fungi or bacteria on plants (Bardi and Malusà, 2012; Kim et al., 2012; Naseem and Bano, 2014). They also assist plants with the acquisition of essential nutrients like phosphorus, iron and nitrogen (Kim et al., 2012).

As the world's climatic conditions change as a consequence of massive increases in the world's population and global industrialization, more agricultural land is being lost to drought. Land loss due to drought is a problem that is becoming common in many regions of the world as it is expected to result in 30% loss of land by 2021 and greater than 50% by 2050 (Kasim et al., 2013; Tripathi et al., 2016). Current studies have shown that it is not limited only to arid areas but also occurs in temperate countries (Parry et al., 2007; Kaushal and Wani, 2016).

To eradicate the problem of water scarcity, modern agro-biotechnological strategies are being implemented to improve drought tolerance in plants. These technologies include genetic

engineering, germplasm screening, plant breeding etc., which have resulted in the development of hybrid products that are widely grown across different parts of the globe (Quan et al., 2004; Naveed, 2013; Rauf et al., 2016). However, abiotic stress tolerance mechanisms are complex, making the task of introducing new tolerant varieties very difficult and strenuous (Naveed, 2013). Other agricultural methods to reduce the negative effects caused by drought are conservation tillage, soil amelioration and mulching, but these methods are not only laborious but also time consuming (Bardi and Malusà, 2012). As agricultural activities spread to less fertile and marginal soils, more attention is being channeled to any intervention that is capable of increasing water use efficiency in plants through biotechnology and improved agronomic practices to satisfy the increasing demand for food (Foley et al., 2011; Flexas et al., 2013). Therefore, an understanding and improvement of plant growth and productivity under restricted water availability using bacteria is crucial. Bacteria have evolved several mechanisms to tackle the damages caused by drought on plants. These mechanisms may include: modifications of phytohormones which play a major role in helping plants escape or survive abiotic stresses (Skirycz and Inzé, 2010; Fahad et al., 2015), alteration in plant root morphology, accumulation of osmolytes, alteration of plant antioxidant defense mechanisms, production of exopolysscharides and the presence of drought resistant genes which is a molecular mechanism (Vurukonda et al., 2016). Bacteria possessing these traits can be isolated and used to improve plant growth under stress conditions. For successful application of this strategy, one should have a good knowledge of the ability of these organisms to resist drought. Many studies have revealed the successful isolation of bacteria for drought resistance in plants, but very few on actinomycetes genera. Also, only a few of these studies reported on the molecular mechanisms of these bacteria in drought tolerance. Identifying the genetic make-up of these bacteria as well as the evidence of drought tolerant genes may be of help in understanding the mechanism of bacteria-mediated tolerance to abiotic stress. Knowledge gained from this study will go a long way in helping to select beneficial bacterial strains that can be used to improve plant growth under drought stress. Therefore, the objectives of this study are to:

1. Isolate, characterize and identify actinomycetes from dry maize rhizosphere;
2. Determine the drought tolerance abilities of the identified actinomycetes;
3. Study the effect of temperature, salinity and pH on bacterial growth;

4. Design drought tolerance primers targeting drought tolerant genes;
5. Screen the actinomycetes for the presence of drought tolerance and plant growth promoting (PGP) genes.

2.2 MATERIALS AND METHODS

2.2.1 COLLECTION OF SOIL SAMPLES

Four (4) rhizospheric soil samples were collected from two different maize fields, (i) behind Animal Health Center, North-West University, Mafikeng Campus and (ii) North-West University Agricultural Farm, Molelwane. Soil samples were collected by carefully uprooting dry maize plants, and shaking the plants to remove soils loosely adhered to the plant roots. Soils tightly adhered to the roots were aseptically collected in sterile plastic bags and transported to the Microbial Biotechnology Laboratory in a cooler box. Collected samples were stored at -20°C for further analysis.

2.2.2 ISOLATION AND SELECTION OF BACTERIA

Bacterial isolation was carried out by suspending 1 g of each soil sample separately in 9 ml of sterile saline solution (0.85%) in 20 ml sterile test-tubes. The test-tubes were thoroughly shaken using a vortex machine and standard serial dilutions from 10^{-1} to 10^{-8} were made by transferring 1 ml of the soil suspension until the last test-tube. Aliquots of 200 µl from each dilution were spread using a glass rod on Actinomycete Isolation Agar (AIA) plates (pH, 7.2) supplemented with cycloheximide (20 mg/l) and nalidixic acid (100 mg/l) to minimize fungal and bacterial growths respectively (these were performed in triplicate). Plates were incubated at 25°C for 10 days for optimum growth. Isolated bacteria with varying colour and shape were randomly selected and repeatedly streaked on freshly prepared Yeast extract-malt extract agar (ISP-medium 2, see appendix pp. 140) plates to obtain pure actinomycetes cultures. Pure cultured bacterial strains were maintained on agar slants at 4°C.

2.2.3 MORPHOLOGICAL CHARACTERISTICS OF BACTERIAL ISOLATES

The morphological and cultural analysis of rhizospheric actinomycetes were performed according to the standard of the International Streptomyces Project (ISP) by Shirling and Gottlieb (1966). All the isolates were identified up to the genus level by checking the color of the aerial and substrate mycelium and other characteristics like colour of colonies on petri plates as well as spore mass color.

2.3.4 BIOCHEMICAL CHARACTRISTICS OF BACTERIAL ISOLATES

Biochemical properties like nitrate reduction test, starch hydrolysis, catalase activity and utilization of carbohydrate sources were also determined. Isolates were further characterized by following the keys of Bergey's Manual of Determinative Bacteriology (Holt, 1977).

2.2.4.1 NITRATE REDUCTION TEST

A loopful of spores from selected bacterial isolates was inoculated into 20 ml test-tubes (in triplicates) containing 5 ml of sterilized nitrate broth and incubated at 25°C for 8 days. Controls were test-tubes containing broth without inoculation. On the 8th day of the incubation period, broths were tested for presence of nitrate by addition of two drops of reagent A (α-naphthylamine solution) and two drops of reagent B (sulfanilic acid solution) into 1 ml of broth or 1 ml of control. A change in color of the broth to pink, orange or red indicated the presence of nitrate. Results were confirmed by the addition of a pinch of zinc dust after the reagents were added. A change in color from pink, orange or red to the original color of the broth confirms the result as positive.

2.2.4.2 UTILIZATION OF CARBOHYDRATE SOURCES

The seven selected actinomycetes strains were tested for their ability to utilize various carbon compounds as energy sources by the method recommended by Shirling and Gottlieb (1966) using the ISP-medium 9 (see appendix pp 140). The carbon sources used were: D-galactose, D-glucose, D-xylose, sucrose, D-mannitol, lactose and fructose. The carbon sources were filter-sterilized using a micro-membrane filter (20 μm/cm) to ensure they were free from various contaminants. All inoculated plates were incubated at 25°C for 8 days. A basal medium without a carbon source served as the positive control for this experiment. The positive utilization (+) of a carbon source was considered when growth of an isolate on a tested carbon source was significantly better than the growth on the basal medium without a carbon source, while growth similar or less than growth on basal medium without a carbon source was considered as a negative utilization (-).

2.2.4.3 CATALASE PRODUCTION TEST

Luria-Bertani (LB) agar slants were inoculated with each bacterial isolate in triplicates. Control for this experiment was an un-inoculated LB slant. All tubes were incubated at 30°C for 5 days. On the 5th day, a single colony from each inoculated tube was placed on a sterile glass slide and held at an angle while 2-3 drops of hydrogen peroxide (H_2O_2) was allowed to flow over the growth

of each culture on the glass slide. Catalase production is indicated by the production of oxygen bubbles within one minute after addition of H_2O_2.

2.2.4.4 TEST FOR HYDROLYSIS OF STARCH

A loopful of each bacterial isolate was streaked on starch agar plates (Sigmon, 2008) in triplicate, plates were incubated at 30°C for 8 days. On the 8th day, iodine solution was added to each culture plate. After about a minute, excess iodine was carefully poured out from the culture plates. Controls consisted of un-inoculated starch agar plates. A yellow zone around the colony in a dark blue medium is considered positive for starch hydrolysis while the absence of a yellow zone is considered as negative.

2.2.4.5 TEST FOR CASEIN HYDROLYSIS

The ability of selected isolates to hydrolyze casein was determined by spot-inoculating prepared skimmed milk agar plates with a loopful of each bacterial isolate in triplicates. Un-inoculated skimmed milk agar plates served as the control for this experiment and all experiments were performed in triplicate. All plates (inoculated and control) were incubated at 30°C for 5-8 days. Observation of a clear zone around the bacterial growth on the agar culture plates indicated an evidence of casein hydrolysis.

2.2.5 EFFECT OF PEG 8000 ON BACTERIAL GROWTH

Bacterial isolates were screened for drought tolerance in 250 ml conical flasks containing 50 ml sterile ISP medium-1 (yeast extract- malt extract broth) supplemented with 5% polyethylene glycol (PEG) 8000. Each flask was inoculated with 500 µl (optical density, OD = 0.2) of 3 day old bacterial cultures and incubated at 25°C under shaking conditions (150 rpm). Bacterial growth was determined at 8 days by measuring the OD of 2 ml of each culture at 600 nm using a UV spectrophotometer (Thermo Spectronic, Merck, SA). The controls for this experiment were PEG-free medium inoculated with bacteria. Growth of each bacterial isolate was represented graphically by plotting absorbance against time. Each test was performed in triplicate.

2.2.6 EFFECT OF TEMPERATURE ON THE GROWTH OF BACTERIA

Temperature effect on bacterial growth was done by growing 10 µl (OD = 0.1) of each bacterial isolate in 10 ml of sterilized ISP medium-1 containing 5% PEG-8000 and incubated at different temperatures (25°C, 30°C, 35°C, 40°C) under shaking conditions (150 rpm) for 7 days. The OD

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of each bacterial isolate was measured at 600 nm using a UV Spectrophotometer (Merck, SA). The experiment was performed thrice.

2.2.7 EFFECT OF pH ON THE GROWTH OF BACTERIA

The effect of pH on bacterial growth was determined by growing 10 µl of each bacterial culture in test tubes containing 10 ml of sterilized ISP medium-1 supplemented with 5% PEG 8000. The pH of the media was adjusted to 3, 5, 7, 9, and 11 using 1 N HCl and 1 N NaOH before autoclaving. Inoculated tubes were incubated at 25°C under shaking conditions (150 rpm) for 7 days after which the OD of each culture was measured using a UV spectrophotometer (Thermo Spectronic, Merck, SA). Bacterial inoculated tubes without PEG served as the control. Experiments were replicated thrice.

2.2.8 EFFECT OF NaCl ON THE GROWTH OF BACTERIA ISOLATES

Tolerance to NaCl by bacteria was assessed according to the method of Ndeddy Aka and Babalola (2017) by inoculating 20 µl of each bacterial isolate in 20 ml sterilized ISP medium-1 containing varying concentrations of NaCl (0.2, 0.4, 0.6, 0.8, and 1.0%). Inoculated tubes were incubated at 25°C for 5 days and the OD was measured at 600 nm using a UV spectrophotometer (Thermo Spectronic, Merck chemicals, SA). This experiment was performed in triplicate and pooled data was statistically analyzed.

2.2.9 DROUGHT TOLERANCE ABILITIES OF BACTERIAL ISOLATES

To study the drought tolerance capacities of bacterial isolates used in this study, a 5 ml (OD, 0.2) aliquot of freshly prepared cultures were inoculated into 150 ml cotton plugged flasks containing 50 ml sterilized ISP medium-1 supplemented with different concentrations (5, 10, 15 and 20%) of PEG 8000. The pH of the medium was adjusted to 7.2 before autoclaving at 121°C for 15 min. Inoculated flasks were incubated at room temperature with constant shaking (150 rpm) at different time intervals (24, 48, 72, 96 and 120 h). The growth of each bacterial isolate was obtained by measuring the OD at 600 nm using a UV Spectrophotometer (Thermo Spectronic, Merck chemicals, SA). The control for this experiment consisted of inoculated broth without PEG 8000 while un-inoculated broth served as blank. The experiment was performed in triplicate and pooled data were statistically analyzed.

2.2.10 MOLECULAR CHARACTERIZATION OF BACTERIAL ISOLATES

2.2.10.1 EXTRACTION OF GENOMIC DNA

For extraction of genomic DNA, bacteria were grown in 20 ml of ISP-1 medium in 50 ml Eppendorf tubes containing 5% PEG 8000. The cultures were grown with constant agitation (150 rpm) at a temperature of 25°C for 7 days for optimum growth. Bacteria were harvested by centrifuging at maximum speed for 10 min. Supernatants were discarded and cells were collected and re-suspended in sterile distilled water. The total DNA from each bacterium was extracted using a DNA extraction kit (ZR soil microbe DNA MiniPrep™ kit (Zymo Research, USA) according to the manufacturer's instructions.

2.2.10.2 DNA QUANTIFICATION AND PCR AMPLIFICATION OF 16S rRNA GENE

The concentration in ng/µl and purity of the extracted DNA was measured using a NanoDrop Spectrophotometer. Amplification of a 1500 and 350 bp fragment of the 16S rRNA gene was performed by polymerase chain reaction (PCR) using the universal 16S rRNA primers for bacteria fD1, rP2 and 341F, 907R (Teske et al., 1996; Weisburg et al., 1991) to confirm the identity of bacterial isolates. Details of primers are shown in Table 1. Amplifications were performed in a final volume of 25 µl consisting of 12.5 µl of 2x PCR Master mix (0.05 U/µl *Taq* DNA polymerase, 4 mM $MgCl_2$ and 0.4 mM dNTPs (Fermentas)), 1 µl of genomic DNA template, 0.5 µl of each of the forward and reverse primers and 11 µl of nuclease-free water. The PCR mixture was subjected to 30 cycles in a C1000 thermal cycler (BioRad) with the following conditions: initial denaturation step at 95°C for 3 min; denaturation at 96°C for 45 s; annealing temperature of 56°C for 30s; extension of 72°C for 2 min and final extension of 72°C for 5 min.

2.2.11 DETECTION OF DROUGHT TOLERANCE AND PLANT GROWTH PROMOTING (PGP) GENES IN BACTERIA USING POLYMERASE CHAIN REACTION (PCR) MACHINE

2.2.11.1 DROUGHT TOLERANT PRIMER DESIGN AND DEVELOPMENT

Drought tolerant primers used in this study were designed according to the protocol of Aremu and Babalola (2015). Database searching was through the National Center for Biotechnology Information (NCBI) at http://www.ncbi.nlm.nih.gov/. Retrieved sequences were glutathione

peroxidase, Desiccation protectant protein, Malic enzyme protein and glycine-rich RNA binding protein. The nucleotide sequences of these genes encoding for drought tolerance from NCBI were saved as FASTA files and copied into the program BioEdit Sequence Alignment Editor (Version 7.0.9.0) in BioEdit files. ClustalW 2.0 alogarithm (Larkin et al., 2007) was used to perform Multiple Sequence alignments (MSA). Stringency was varied in order to achieve as few gaps and mismatches as possible. The stringency was also altered to yield many regions with very high degree of sequence similarity. Consolidation of MSA was performed based on obvious discrepancies and lack of sequence similarity to the consensus which was subjectively measured on a percent similarity basis when needed. Consolidated trials were then aligned with each other and sequences with low similarity were discarded. To determine the highly conserved regions where primers can be designed for drought tolerance, these files were then opened in BioEdit. Primer design was performed using Primer3Plus interface (http://frodo.wi.mit.edu/) and the best primers were chosen using the standards for good primer design (Innis et al., 2012). The chosen primer sequences were tested for potential hairpins structures, cross homology, cross dimer and self-dimer. They were further tested using Gene Infinity Platform for binding similarities to the priming sites (delta G values). The primers specificity was determined through *in silico* PCR in Gene Infinity Platform. To check if the primers were able to target specific genes, NCBI Blast was used and the best primers were synthesized by WhiteHead Scientific Industries Limited South Africa.

2.2.11.2 PCR AMPLIFICATION OF SPECIFIC GENES ENCODING PROTEINS INVOLVED IN DROUGHT TOLERANCE

Primers were tested for varying annealing temperatures using a gradient PCR machine from 45 to 60°C. Reaction volumes were 25 µl which consisted of 12.5 µl of 2x PCR Master-mix (0.05 U/µl *Taq* DNA polymerase, 4 mM $MgCl_2$ and 0.4 mM dNTPs (Fermentas)), 1 µl of genomic DNA template (10 ng/ reaction mixture), 0.5 µl of each of the forward and reverse primers and 11 µl of nuclease-free water. The PCR started with a 95°C hot start for 10 min. The cycles consisted of 94°C denaturation temperature which lasted for 30 s/ cycle, a 45- 60°C annealing temperature for 30 s, 72°C elongation step for 1 min, a final elongation step of 72°C for 10 min and a holding period at 4°C for infinity.

2.2.11.3 PCR AMPLIFICATION OF PLANT GROWTH PROMOTING GENES

Siderophore (*Sid*) and ACC deaminase (*Accd*) genes which are associated with plant growth promotion in each bacterial isolate were screened by PCR amplification. Table 1 shows all the oligonucleotide sequences used as primers in this study. The PCR for siderophore and ACC deaminase genes were performed as follows: 2 µl (about 10 ng) of each DNA extract was amplified with 12.5 µl of 2x PCR Master mix (0.05 U of *Taq* DNA polymerase4 mM $MgCl_2$ and 0.4 mM dNTPs (Fermentas)), 1 µM of each primer and 8.5 µl of nuclease-free water, in a 25 µl reaction mixture in a C1000 thermal cycler (BioRad) with the following PCR conditions: 30 cycles of denaturation at 94°C for 1 min, annealing temperatures of 55°C for *sid* and 52°C *accd* for 45 s, extension of 72°C for 2 min and a final extension step of 72°C for 7 min.

Table 2.1 below show the details of primers used for the amplification of 16S rRNA (F1r2, 341F, 907R) and plant growth promoting genes (*Accd* and *Sid*) used in the present study.

Table 2.1: Oligonucleotide primers for PCR amplification of 16S and PGP genes

Gene	Forward sequence	Reverse sequence	Reference
F1r2	AGAGTTTGATCCTGGCTCAG	ACGGCTACCTTGTTACGACTT	Weisburg et al. (1991)
341F, 907R	CCTACGGGAGGCAGCAG	CCCCGTCAATTCCTTTGAGTTT	Teske et al. (1996)
Accd	GTGAACCACCTGAATGTA	AAACGAGATGATTTACTTGG	Raddadi et al. (2008)
Sid	GAGAATGGATTACAGAGGAT	TTATGAACGAACAGCCACTT	Raddadi et al. (2008)

2.2.12 AGAROSE GEL ELECTROPHORESIS

Genomic DNA and all PCR products were checked in 1% (w/v) agarose gel made by dissolving 1.5 g of agarose (Bi0-Rad, SA) in 150 ml of 1X Tris-acetate-ethylenediaminetetraacetate (TAE, pH 8). This mixture was heated in the microwave for 3 min after which it was allowed to cool. Upon cooling to a molten gel, 1 µl/ 10ml of Ethidium bromide (EtBr) was added and then poured in a gel casting tray with combs and allowed to solidify. When the gel had solidified, the combs were removed, and the gel was carefully placed in the electrophoresis tank containing 1X TAE buffer (40 nM Tris, 20 mM Acetic acid and 100 mM EDTA, pH 8.0). DNA samples were prepared by mixing 7 µl of DNA template and 3 µl of 6x DNA loading dye (Fermentas) while both PCR products and DNA ladders (1 kb or 100 bp used to estimate the size of bands for the genes) consisted of 5 µl volume. Samples were carefully loaded into the preformed wells in the gel. The gel was allowed to run for 60 min at 80 V. Results were visualized and photographed using a ChemDoc™ MP System (Bio-Rad Laboratories, Hercules, CA, USA).

2.2.13 DNA PURIFICATION, SEQUENCING AND PHYLOGENETIC ANALYSIS

Purification and sequencing of PCR products were performed by Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa using PRISM™ Ready Reaction Dye Terminator Cycle Sequencing Kit.

The analysis of sequences and construction of phylogenetic tree were performed according to the methods described by Aremu and Babalola (2015). Analysis of chromatograms resulting from sequencing reaction for assurance and good quality sequence was performed using ChromasLite version 2.3.3 software (Technelysium. Chromas). The chromatograms obtained were edited with BioEdit Sequence Alignment Editor (Hall) and consensus sequences were generated. The consensus sequences obtained were Blast in the NCBI database (www.ncbi.nlm.nih.gov) using the Basic Alignment Search Tool (BLSTn) for homology for identification of the bacteria. Sequences obtained were deposited in GenBank.

Analysis of phylogeny based on 16S rDNA gene was used to further characterize the bacterial isolates. Partial 16S rDNA sequences obtained were used to search for the reference nucleotide sequences in the NCBI GenBank database using BlastN algorithm (Altschul et al., 1997). MAFFT (version 7.0) was used to align multiple nucleotide sequences (Katoh and Toh, 2010) and the tree

was drawn using MEGA 7 (Kumar et al., 2016). Techniques used in drawing the tree include: The Neighbor-Joining (NJ) with cluster-based algorithm used to calculate pairwise distance between and group sequences that are most similar to each other and the Maximum Likelihood used to compare a set of data against set of evolution models in order to select the best model for the variation pattern of the sequences (Harrison and Langdale, 2006).

2.3 DATA ANALYSIS

All quantitative data obtained from this study were statistically analyzed by one way analysis of variance (ANOVA) using the Statistical Analysis Software (SAS), Version 9.4 (SAS, 2014). Significant mean differences were compared using New Duncan's Multiple Range Test (DMRT) at 5% level of significance.

2.4 RESULTS AND DISCUSSION

2.4.1 ISOLATION AND CHARACTERIZATION OF ACTINOMYCETES

From two rhizospheric soil samples, seven presumed actinomycetes strains were selected and characterized based on morphology of the colony, their ability to form aerial hyphae and substrate mycelium as well as their biochemical properties. Five isolates out of the seven were isolated from behind the Animal Health Center (AHC) while two were obtained from North-West University Agricultural Farm, Molelwane (NWUAFM). Most isolates showed slow growth rate with the exception of one (coded as R15) which showed moderate growth on the media. After 10 days' incubation period, colonies were observed to be white, brownish white and gray in colour (Table 2.2).

The biochemical properties showed that all tested bacterial isolates were positive for catalase activity, nitrate reduction test, starch hydrolysis and the utilization of glucose as a carbon source. Moreover, only two tested bacterial isolates (S4 and S7) hydrolyzed casein. From our results, four isolates (S12, S4, S11 and S20) utilized D-galactose, four (R15, S7, S4 and R11) utilized the D-xylose, five (R15, S12, S4, S11 and S20) utilized sucrose and two (S4 and R11) utilized D-mannitol. The results obtained also showed that all the isolates utilized fructose except isolate S7, while this isolate was the only one that utilized lactose among all tested bacterial isolates. Table 2.3 shows the results on biochemical and carbon utilization of bacterial isolates used in the study.

Based on the morphological, biochemical and physiological tests conducted on the bacterial isolates, it was established that amidst seven actinomycetes strains isolated from maize rhizosphere, five were from the genus *Streptomyces* while the other two were from *Arthrobacter* and *Microbacterium*. The tested bacterial isolates were compared with the description of the cultural, biochemical and physiological properties of other actinomycetes that fall on the same genus, hence the conclusion of the group (Shirling and Gottlieb, 1966; Holt, 1977). Similar studies have proven the dominance of *Streptomyces* sp. isolated from different hosts (Coombs and Franco, 2003). Akond et al. (2016) isolated the actinomycetes strains *Streptomyces* and *Nocardia* spp. from straw and compost samples of Savar, Bangladesh. Sreevidya et al. (2016) also isolated actinomycetes from vemicompost and chickpea rhizospheric soils for yield improvement in chickpea.

Table 2.2: Morphological properties of isolated rhizospheric actinomycetes

Isolate and Genbank accession No.	**Isolates Identification name**	**Growth and colony nature**	**Color of aerial mycelium**	**Color of substrate mycelium**	**Formation of pigment**
S4 (MG547867)	*Streptomyces werraensis*	Slow and smooth	gray	brown	No
S7 (MG669347)	*Streptomyces luteogriseus*	Slow and smooth	gray	Light brown	No
R11 (MG547868)	*Streptomyces indiaensis*	Slow and firm	gray	Light brown	No
R15 (MG547869)	*Arthrobacter arilaitensis*	Moderate and powdery	Brownish white	White	Yes
S20 (MG547870)	*Streptomyces pseudovenezuelae*	Slow and firm	White	Yellowish brown	Yes
S11 (MG640368)	*Microbacterium oxydans*	Slow and firm	White	Yellowish brown	No
S12 (MG640369)	*Streptomyces* sp.	Slow and firm	white	Light brown	No

Table 2.3: Physiological and biochemical properties of bacterial isolates

Biochemical and physiological properties	**Bacterial isolates**						
	S4	S7	S11	S12	S20	R11	R15
Catalase activity	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+	+
Casein hydrolysis	+	-	+	-	-	-	-
Starch hydrolysis	+	+	+	+	+	+	+
Utilization of carbohydrates							
D-galactose	+	-	+	+	+	-	-
D-glucose	+	+	+	+	+	+	+
D-xylose	+	+	-	-	-	+	+
D-mannitol	+	-	-	-	-	+	-
Sucrose	+	-	+	+	+	-	+
Lactose	-	+	-	-	-	-	-
Fructose	+	-	+	+	+	+	+

Note: S and R stand for bacterial isolates

2.4.2 EFFECT OF PEG 8000 ON BACTERIAL GROWTH

For bacteria to improve drought tolerance, high tolerance to PEG is required because tolerance to PEG medium is directly related to their ability to survive and grow in environments with low water availability. From this study, the effect of PEG on each bacterial growth was determined on ISP-1 medium in which 5% PEG was added. The growth pattern of all bacterial isolates on medium supplemented with PEG in comparison with their controls is shown in Figure 2.1. From the results, growth varied among the isolates and bacterial growth decreased in PEG supplemented medium for all the isolates compared to their respective controls. In PEG-free medium, maximum growths of 1.396 ± 0.11, 0.996 ± 0.04, 1.570 ± 0.08, 1.469 ± 0.12, 1.593 ± 0.07, 1.581 ± 0.03 and 1.341 ± 0.19 were reached by isolates S4, S7, S20, R11, R15, S12 and S11 respectively. On the other hand, the growths of isolate S4, S7, S20, R11, R15, S12 and S11 were 1.086 ± 0.12, 0.832 ± 0.16, 1.399 ± 0.15, 1.283 ± 0.15, 1.457 ± 0.21, 1.378 ± 0.08 and 1.174 ± 0.05 at OD_{600} respectively in PEG supplemented medium.

All bacterial isolates tested showed significant growth in the presence of PEG. This indicates their potential to be used as bio-inoculants in alleviating plant drought stress. Different bacteria species including actinomycetes have been reported to tolerate drought stress by growing in PEG supplemented medium. Marasco et al. (2012) reported that *Rhodococcus sp.* showed 100% tolerance to PEG. The result from this study is in agreement with that of Yandigeri et al. (2012) who reported that *Streptomyces olivaceus*, *Streptomyces coelicolor* and *Streptomyces geysiriensis* showed significant growth in PEG 6000 medium. Ali et al. (2014) also reported that the *Pseudomonas* isolates Rdgp10, SorgP3, SorgP4, SorgP12 and BriP15 showed outstanding growth in PEG supplemented medium.

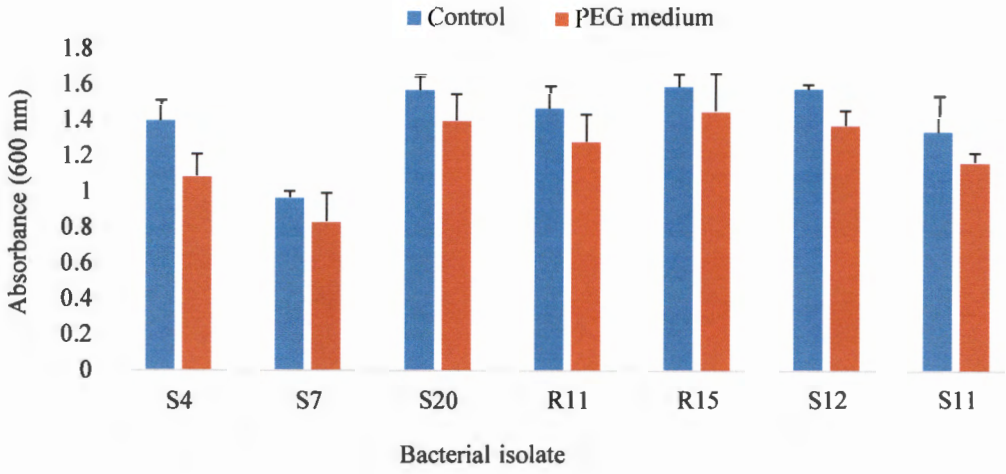

Figure 2.1: Effect of PEG 8000 on bacterial growth

2.4.3 EFFECT OF TEMPERATURE ON BACTERIAL GROWTH

The activity and growth of bacteria in soil depends on the soil temperature, which affects cellular enzymes. An increase in temperature leads to an increase in the activity of cellular enzymes. However, extremely high temperatures cause denaturation of protein structures while very low temperatures (closer to freezing point) cause the inactivation of enzymes and decrease in cellular metabolism (Akond et al., 2016). Most actinomycetes thrive better at temperature ranges between 25 and 30°C. However, certain bacteria require higher temperatures for growth and survival. Examples are the pathogenic bacteria which require 37°C for normal growth and the thermophiles that require a temperature range between 50 to 60°C or more to survive and grow (Akond et al., 2016). In the present study, seven actinomycetes isolates were grown under different temperatures. The results obtained for each of the isolates at various temperatures are shown in figure 2.2. Bacterial growths were determined by measuring the optical density (OD) of each bacterium at 600 nm. It was observed that all tested bacterial isolates grew at various temperatures, although there were variations in the growth pattern of each isolate. For isolates S4, optimum percentage growth (63.9 ± 0.09) were obtained at 30°C while its lowest percentage growth (52 ± 0.09 and) was observed at 35°C. Optimum growth percentages of 83.9 ± 0.08 and 76.2 ± 0.02 was observed in isolates S20 and S11 at 25°C whereas their lowest growth percentages were 29.8 ± 0.09 and

27.4 ± 0.01 observed at 40°C. The bacterial isolates S7, S12, R11 and R15 reached maximum growths percent of 74.5 ± 0.06, 77.1 ± 0.11, 78.7 ± 0.09 and 91.2 ± 0.03 respectively at 35°C. However, the lowest growth percentages for isolates S7, S12 and R15 were 19.4 ± 0.01, 35.1 ± 0.08 and 28.4 ± 0.02 respectively at 40°C while the lowest growth of 51.2 ± 0.17 was reached by isolate R11 at 30°C. Results obtained from this study showed that isolates S11 and S20 would thrive best at room temperature. On the other hand, 35°C favoured the growth of the rest of the isolates (S7, S12, R11 and R15). In this study, significant decrease in growth was observed for all isolates at 40°C. Most actinomycetes species are aerobic, indicating that oxygen is required for their growth. The effect of temperature on the growth of the tested bacterial strains showed that these bacteria can survive at varying temperature ranges. It indicates their potential to survive in temperate, harsh or hot climate conditions. The results obtained are in agreement with a study by Ndeddy Aka and Babalola (2017), who also reported a decrease in the growth of bacterial isolates when the temperature was raised to 40°C. According to them, the decrease in growth at this temperature could be due to a decrease in metabolic activity of the bacterial isolates caused by the high temperature increase. Everest et al. (2011) reported that *Nocardia* strains isolated from South African soil showed growth at the temperatures 30 and 37°C but did not grow at 45°C. In addition, Bhavana et al. (2014) reported that *Streptomyces carpaticus* obtained from the sea coast Bay of Vishakhapatnam, Bengal produced optimum mycelial growth and antibiotic at 30°C.

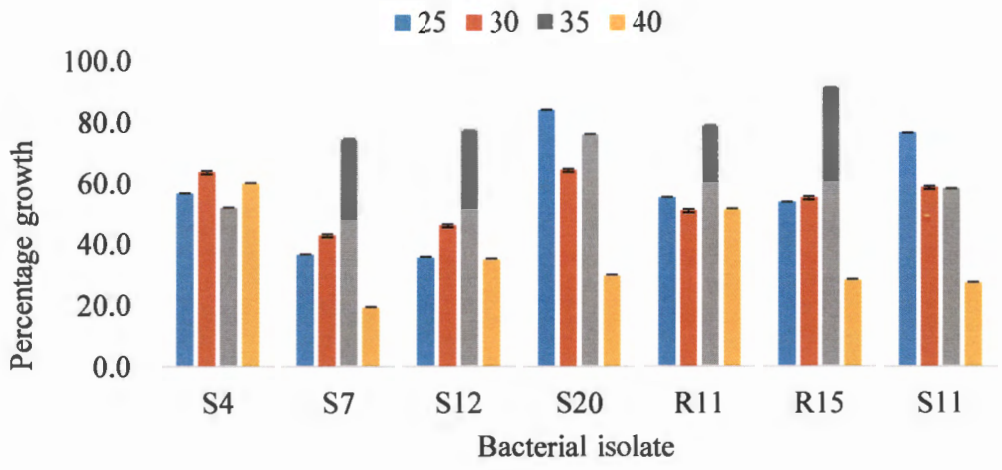

Figure 2.2: Effect of temperature on bacterial growth

2.4.4 EFFECT OF pH ON BACTERIAL GROWTH

The level of microbial activity in the soil is usually affected by the pH of the soil. The pH of the environment also influences bacterial survival and growth. For most soil bacteria, the specific pH range is usually between 4 and 9, with the optimum being 6.5 to 7.5 (Akond et al., 2016), although different bacteria have different pH ranges that favours their growth. In this study, isolated actinomycetes strains were grown at different pH ranges of 3, 5, 7, 9 and 11. The results obtained as shown in Figure 2.3 showed that optimum growth at OD_{600} for all tested bacterial isolates was observed between the pH of 5 and 9. Isolate S7 showed its optimum growth at pH 7 with OD_{600} value of 1.023 ± 0.04. Maximum growth of 1.045 ± 0.03 was observed at pH 9 for isolate S11. Isolates S20 and R15 reached maximum growths of 0.941 ± 0.12, and 1.085 ± 0.26 respectively at pH 7 while isolates S4, S12 and R11 reached maximum growth at pH 9 with the OD_{600} values of 1.030 ± 0.05, 1.201 ± 0.05 and 0.915 ± 0.11 respectively. Data on the lowest growth for each bacterial isolate showed that isolates S4 and S20 exhibited minimum growths of 0.286 ± 0.08 and 0.126 ± 0.02 respectively at pH 11 while isolates S7, S11, S12, R11 and R15 showed lowest growths (0.145 ± 004, 0.126 ± 002, 0.250 ± 0.04, 0.143 ± 0.04 and 0.128 ± 0.02 respectively) at pH 3. Results obtained indicate that the tested isolates are capable of adapting and surviving at a wide range of pH, which suggests their suitability for drought tolerance enhancement at various soil pH. Kim et al. (2004) also reported that maximum growth of new *Streptomyces* species was observed between the pH ranges of 4.3 to 7.3. Another study by Palanichamy et al. (2011) recorded maximum growth of *Streptomyces* spp. isolated from the Chennai coastal region at pH 7.6 to 8.0.

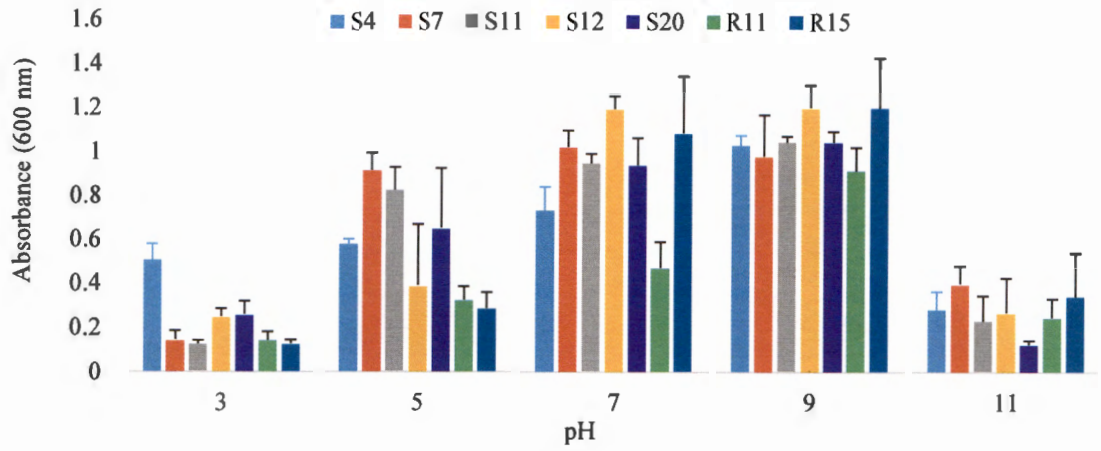

Figure 2.3: Effect of various pH on bacterial growth

2.4.5 EFFECT OF SODIUM CHLORIDE (NaCl) ON BACTERIAL GROWTH

Bacterial isolates S4, S7, R11, S11, S12, R15 and S20 were tested for their ability to withstand salinity stress by growing at different concentrations of sodium chloride (NaCl) 0, 0.2, 0.4, 0.6, 0.8, 1.0. Results shown in Figure 2.4 revealed that maximum growths of 0.814 ± 0.07 and 0.827 ± 0.10 at OD_{600} was observed for isolates S20 and S4 respectively at 0% NaCl while isolates R15, S11 and S12 on the other hand grew (1.012 ±0.02, 0.972 ± 0.05 and 1.113 ± 0.21 respectively) optimally at 0.2% NaCl. Best growth for S7 and R11 were respectively observed at NaCl concentrations of 0.4 and 0.8% with the OD_{600} values of 1.131 ± 0.09 and 0.594 ± 0.26. From the results obtained, all tested isolates were able to grow at all the NaCl concentrations used in this study. This confirms their ability to withstand various salinity stress conditions and suggests their applicability for salt tolerance improvement in plants. Results obtained also indicate that these bacteria strains are halotolerant because of their ability to withstand various levels of salinity stress. A study by Hamid et al. (2015) showed that three *Streptomyces* spp. from Malaysian soil grew at a preferred NaCl concentration of 3%. In addition, Krishnan and Sampath Kumar (2015) reported that a *Streptomyces grancidicus* strain isolated from an Indian soil had its optimum activity at 1.5% NaCl concentration. Small quantities of salts or metallic ions enhance microbial growth while large concentrations cause inhibitory effects on growth. High salt concentration also affects osmotic

pressure and causes protein denaturation, therefore halophilic bacteria possess specific enzymes in their active configuration that are only activated upon exposure to high salt concentrations (Akond et al., 2016). A new *Streptomyces* strain was recovered from Bangladesh soil in 2009 whose tolerance to salinity ranged from 0.5% to 3.0% (Ripa et al., 2009).

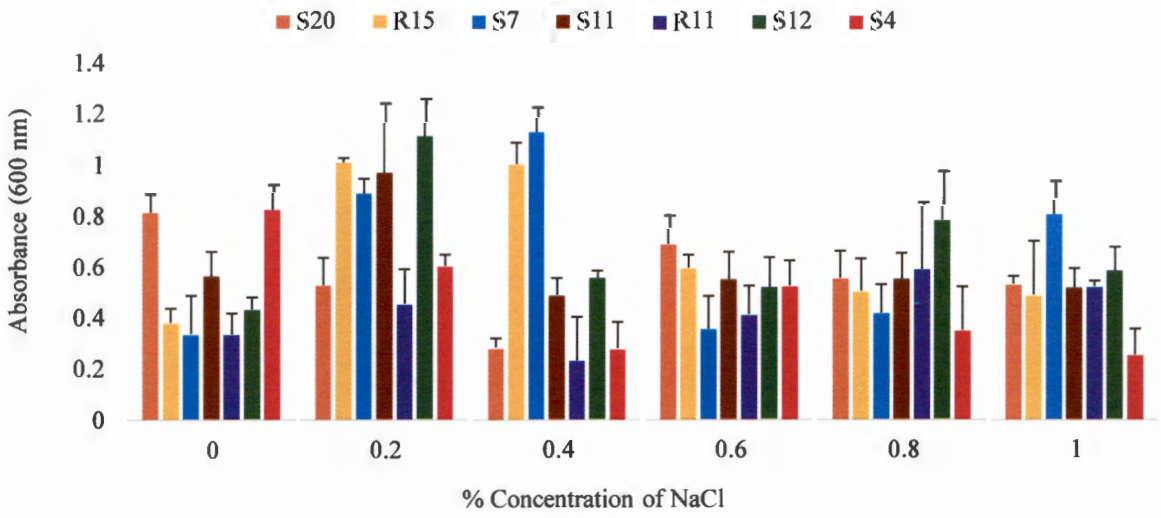

Figure 2.4: Effect of NaCl concentration on Bacterial growth

2.4.6 DROUGHT TOLERANCE ABILITIES OF BACTERIAL ISOLATES

The potentials of the selected bacterial isolates to tolerate drought was evaluated based on concentration and time. The level of tolerance to various concentrations of PEG 8000 by each bacterial isolate was determined as a function of time, after inoculation on PEG containing medium. Growth varied among the isolates and depended mainly on the concentration of PEG, as bacterial growth decreased in PEG supplemented medium for all the isolates, irrespective of the concentration, compared to their controls. Data obtained from the growth of most bacterial isolates was higher at lower concentrations than at higher concentrations. It was also observed that time influenced the tolerance capacities of these isolates as better growth on PEG medium was observed with time increase (Figure 2.5). The result obtained for each concentration was compared with that

of the control (without PEG). The maximum growth (1.068 ± 0.02) on PEG medium for the bacterial isolate S4 was reached at 5% concentration at 120 h while the lowest (0.237 ± 0.23) was reached at 20% concentration at 24 h. Bacterial isolate S7 exhibited its highest tolerance (1.202 ± 0.07) to drought also at 5% concentration at 120 h while the lowest tolerance (0.068 ± 0.33) was reached at 20% concentration at 24 h. Maximum tolerance values for the bacterial isolates S20, S12 and S11 were 1.449 ± 0.16, 1.522 ± 0.18 and 1.534 ± 0.19 respectively at 5% concentration and 120 h whereas their lowest tolerance values were 0.072 ± 0.33, 0.088 ± 0.32 and 0.097 ± 0.32 and also observed at 20% concentration at 24 h. However, for bacterial isolates R11 and R15, maximum tolerance was observed at 5% concentration at 96 h as 1.451 ± 0.16 and 1.526 ± 0.18 respectively while the lowest tolerance values of 0.067 ± 0.33 and 0.058 ± 0.33 were respectively reached at 20% concentration at 48 h for isolate R11 and 24 h for isolate R15. Results obtained were in agreement with previous studies on the effect of PEG 8000 on bacterial growth, which confirms that all tested bacterial isolates were able to grow on PEG medium. The ability of some bacteria to resist different concentrations of PEG has been reported. A study by Marasco et al. (2012) revealed that all bacteria used in the study tolerated 10 and 20% PEG. Moreover, Ali et al. (2014) also reported that nine out of seventeen fluorescent *Pseudomonas* tested grew at a minimum water potential of -0.30MPa. The result from this study is in agreement with that of Yandigeri et al. (2015) who reported that *S. olivaceus*, *S. coelicolor* and *S. geysiriensis* showed significant growth from -0.05 to -0.73 MPa of PEG6000. The tolerance capacities of these isolates to PEG 8000 show that they possess the potential to tolerate drought, and this indicates their suitability to be used in improving drought tolerance in plants.

Several mechanisms of drought tolerance by bacteria have been proposed. These mechanisms include: modification of phytohormonal activity, ACC deaminase activity, exopolysaccharide production and accumulation of osmolytes (Kaushal and Wani, 2016; Vurukonda et al., 2016). In these mechanisms, bacteria are able to enhance tolerance to drought by synthesizing phytohormones such as indole-3-acetic acid and gibberellins (Hayat et al., 2010; Glick, 2014). Certain bacteria that possess indole-3-acetic acid have been isolated and characterized (Etesami et al., 2015; Majeed et al., 2015; Khan et al., 2016). Indole-3-acetic acid has been found to play a major role in abiotic stress enhancement in plants, as it aids in root elongation and formation of lateral roots (Dimkpa et al., 2009), which helps plants to survive drought stress conditions.

Furthermore, bacteria possessing ACC deaminase facilitate the supply of nitrogen and energy to plants by the removal of ACC thereby improving drought stress tolerance (Glick et al., 2007; Choudhary et al., 2016).

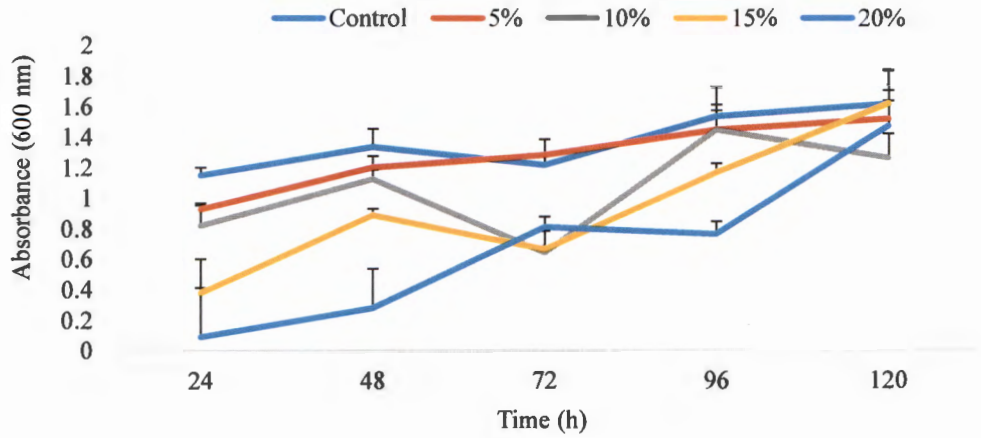

Figure 2.5a: Growth of isolate S12 at different time interval and PEG concentrations

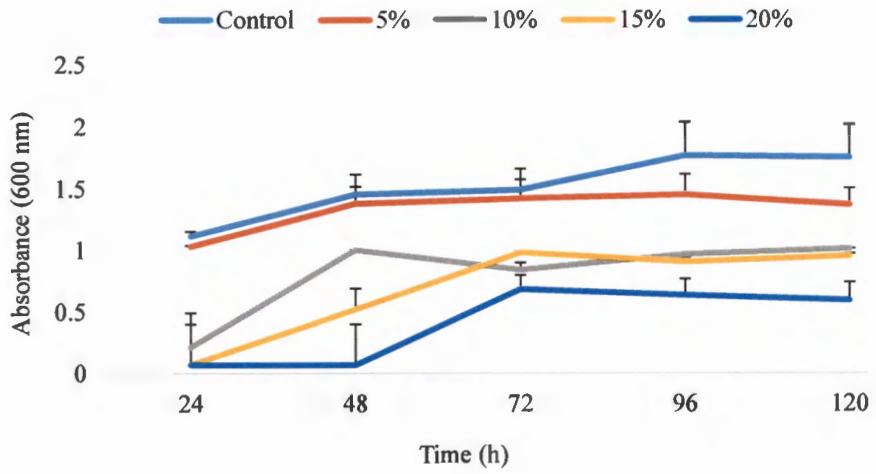

Figure 2.5b: Growth of isolate R11 at different time interval and PEG concentrations

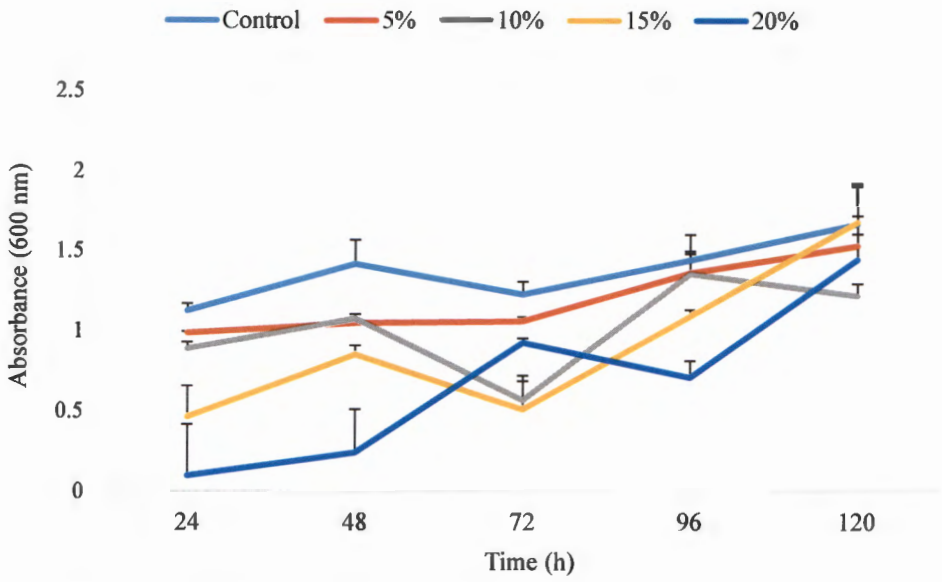

Figure 2.5c: Growth of isolate S11 at different time interval and PEG concentrations

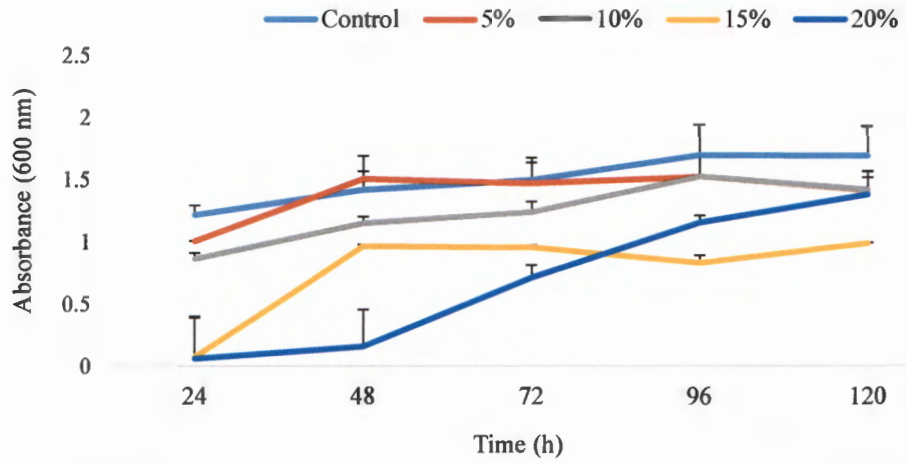

Figure 2.5d: Growth of isolate R15 at different time interval and PEG concentrations

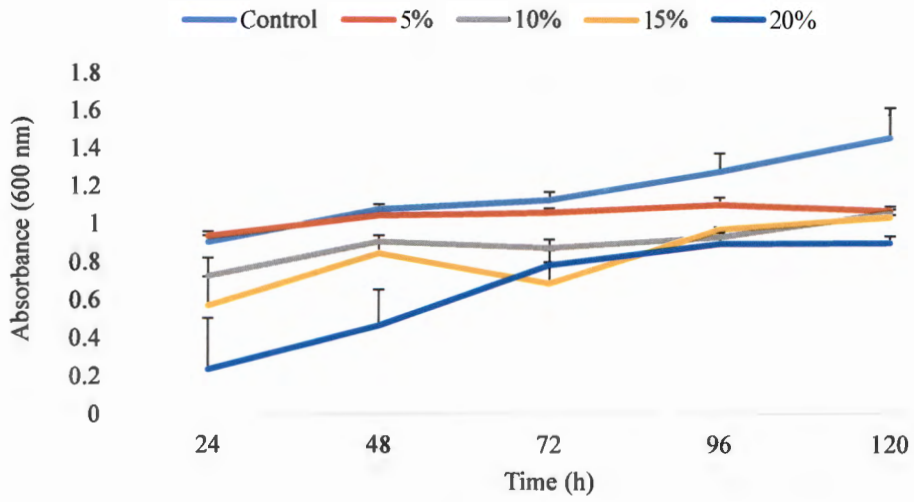

Figure 2.5e: Growth of isolate S4 at different time interval and PEG concentrations

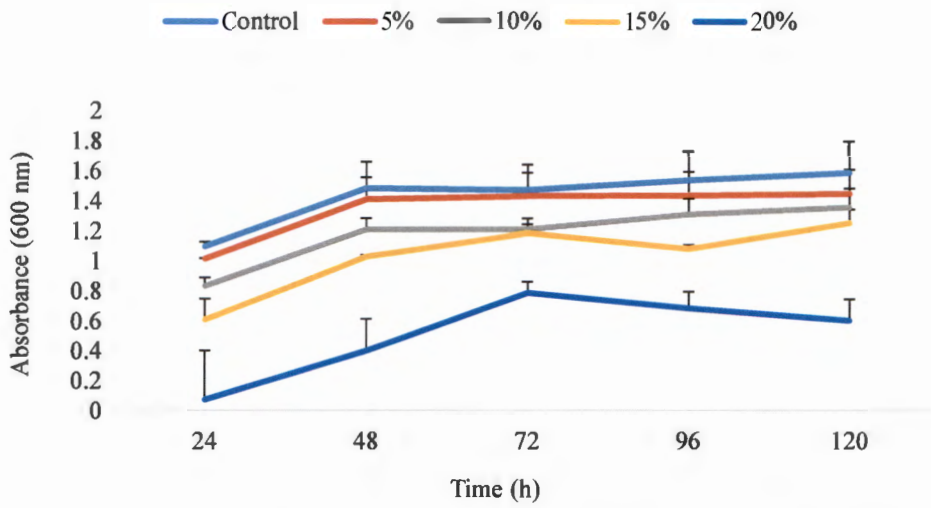

Figure 2.5f: Growth of isolate S20 at different time interval and PEG concentrations

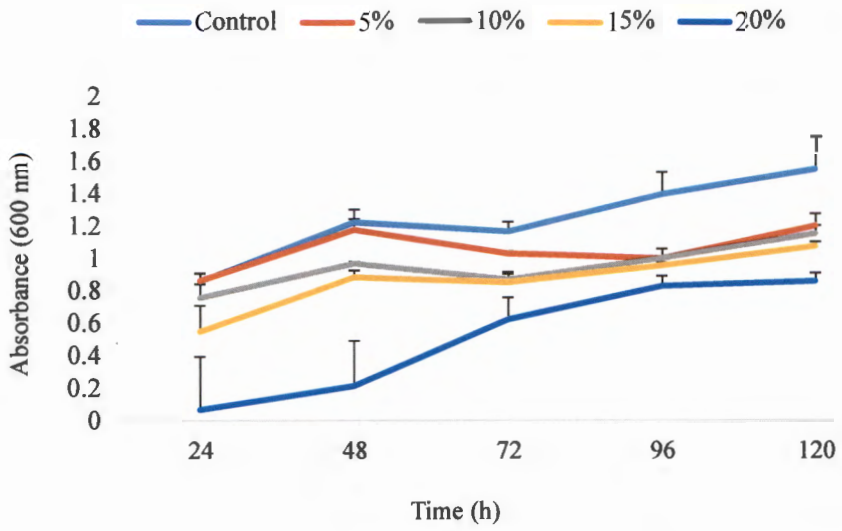

Figure 2.5g: Growth of isolate S7 at different time interval and PEG concentrations

2.4.7 MOLECULAR IDENTIFICATION OF RHIZOSPHERIC ACTINOMYCETES BASED ON 16S rRNA

Extraction of DNA using Zymo (ZR) soil microbe DNA MiniPrep kit yielded high quality DNA according to the NanoDrop readings (see appendix pp. 138). PCR amplification of the 16S rRNA genes was successfully performed using two sets of universal primers as shown in Table 2.1. The molecular marker (GeneRuler™ DNA Ladder) showing fragments of 1500 bp and 350 bp can be seen in the first Lanes in Figure 2.6a and b respectively. No non-specific amplification was observed.

The genomic DNA of bacterial isolates was amplified with 1.5 kb and 350 bp fragment of the universal primers F1r2 and 341F,907R respectively (Figure 2.6a and b). Bacterial identification was confirmed by computational analysis. The identification of actinomycetes isolates based on genus level was performed by partial sequences of their 16S rDNA gene. Partial 16S rDNA nucleotide sequences from all bacterial isolates used in this study were matched with similar sequences in the NCBI website using BLASTn program. The BLAST search inferred that the isolates belong to the GC-rich actinomycetales. The BLASTn homology search of NCBI for 16S rRNA gene sequences of 5 isolates among 7 were confirmed as *Streptomyces* spp. R15 and S20

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were confirmed as *Arthrobacter* and *Micobacterium* respectively. Isolates S7 had 100% similarity index with *Streptomyces luteogriseus* while isolates S4, R11, R15, S20, S11 and S12 showed 99% similarity to *Streptomyces werraensis*, *Streptomyces indiaensis*, *Arthrobacter arilaitensis*, *Streptomyces pseudovenezuelae*, *Microbacterium oxydans* and *Streptomyces* spp. respectively. The gene sequences of all isolates were submitted to GenBank (NCBI) with the following accession numbers: MG547867 (S4), MG669347 (S7), MG547868 (R11), MG547869 (R15), MG547870 (S20), MG547870 (S11) and MG640369 (S12) (Table 2.2).

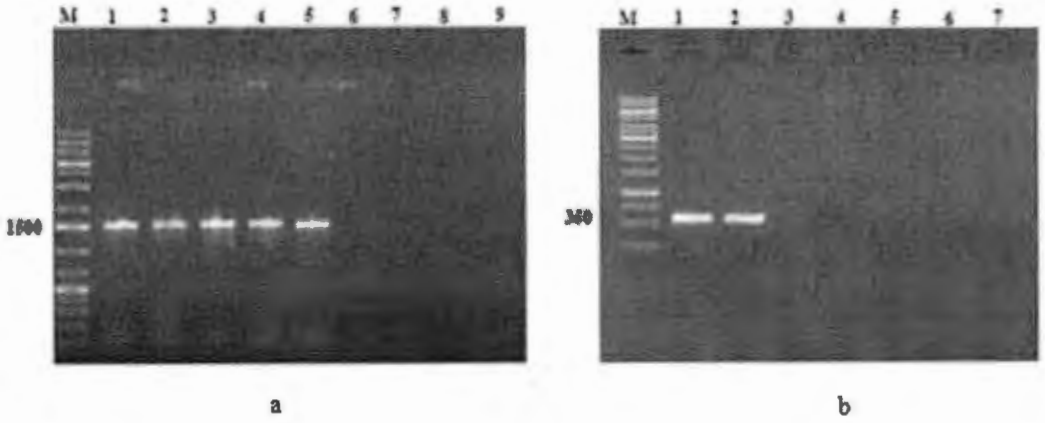

Figure 2.6a and b: Agarose gel showing amplified DNA sequences of 1500 bp and 350 bp respectively. In Figure 6a, Lane M= 1Kb molecular weight marker, Line 1: S4, Line 2: S7, Line 3: S20, Line 4: R11 and Line 5: R15. In Figure 6b, M= 1 Kb molecular marker, Line 1: S12 and Line 2: S11

Table 2.4: Partial 16S rRNA sequence alignment results from NCBI blast searches for the actinomycetes isolates

Isolate	Accession no	Blast ID (closest cultured representative)	Similarity (%)	E-value
S4	MG547867	*Streptomyces werraensis*	99	0
S7	MG669347	*Streptomyces luteogriseus*	100	0
R11	MG547868	*Streptomyces indiaensis*	99	0
R15	MG547869	*Arthrobacter arilaitensis*	99	0
S20	MG547870	*Streptomyces pseudovenezuelae*	99	0
S11	MG547870	*Microbacterium oxydans*	99	0
S12	MG640369	*Streptomyces sp.*	99	0

The BLAST query grouped the actinomycetes isolates into 5 *Streptomyces*, 1 *Arthrobacter* and 1 *Microbacterium* which is also in line with the result obtained from the biochemical tests performed on the various isolates.

2.4.8 PHYLOGENETIC ANALYSIS

The seven selected actinomycetes isolates used in this study were subjected to sequencing and phylogenetic analysis. As shown in Figure 2.7, the 16S rRNA sequences of the seven isolates were aligned to forty-three (43) reference sequences of the 16S rRNA of closely related taxa as retrieved from GenBank data library and *Brevibacterium* spp. is the out-group. The relationships of the isolates were based on evolutionary distances using the Neighbor-joining (NJ) and Maximum Likelihood method of Tamura et al. (2004). The distance based method inferred the evolutionary relationship using Neighbor Joining (NJ) clustered-based procedure (Saitou and Nei, 1987). The concatenated Neighbor Joining showed the optimal tree of 47956.95 branch length with 530 positions. Based on the cluster procedure, Neighbor Joining revealed the percentage of evolutionary relationship with the drought tolerant actinomycetes isolates according to the degree

of differences among the sequences. Evolutionary analyses were performed in MEGA7 (Kumar et al., 2016).

Bacterial isolate S7 possessed the highest similarity index of 100% with *S. luteogriseus*. The rest of the isolates (S4, R11, R15, S20, S11 and S12) also possessed very high similarity index of 99% with E-value = 0. These drought tolerant actinomycetes isolates expressed a high similarity value above the 70% borderline degree of relatedness as suggested by Wayne et al. (1987). Furthermore, the similarities expressed by these isolates with the reference taxa belonging to different species, is due to the high similarity value exhibited in the values of DNA reassociation which fall below the 70% threshold values (Stackebrandt and Goebel, 1994). According to Konstantinidis and Stackebrandt (2013), this shows a very high genetic relatedness that is progressively more reliable, as they cannot be wiped out overnight. In all, the high-level branching in the phylogenetic tree is in harmony with the traditional systematic divisions that classifies organisms belonging to the same family and genus into different species.

The molecular identification of bacterial isolates is very important because it identifies organisms up to the species level and provides information about the organisms, such as the compounds they produce and whether they are novel or not (Adegboye and Babalola, 2012). The analysis of 16S rDNA gene sequences have proven to be a very important method of phylogenetically characterizing microorganisms as it helps to explain the evolutionary relationship between organisms (Thenmozhi and Kannabiran, 2010). The phylogenetic relationship of the bacterial isolates used in this study was first estimated using a blast search in the GenBank database and closest related strains were chosen for pairwise sequence comparison, hence the construction of the phylogenetic tree. Most of the closest strains to the bacterial isolates in the present study have been associated with one stress tolerance or the other. An example is the study by Srivastava et al. (2015) which revealed that *Streptomyces rochei* alleviated the stresses caused by salt and *Sclerotinia. sclerotiorum* in chickpea. Kanini et al. (2013) also reported that *S. pseudovenezuelae* showed antagonistic activity against *rhizoctonia solani*. Aly et al. (2012) observed the PGP effect of *Streptomyces* spp. on wheat plant under saline conditions. *Streptomyces geysiriensis* and *Streptomyces coelicolor* were observed to tolerate water stress from −0.05 to −0.73 MPa and greatly increased the growth and yield of wheat plants subjected to drought stress (Yandigeri et al., 2012).

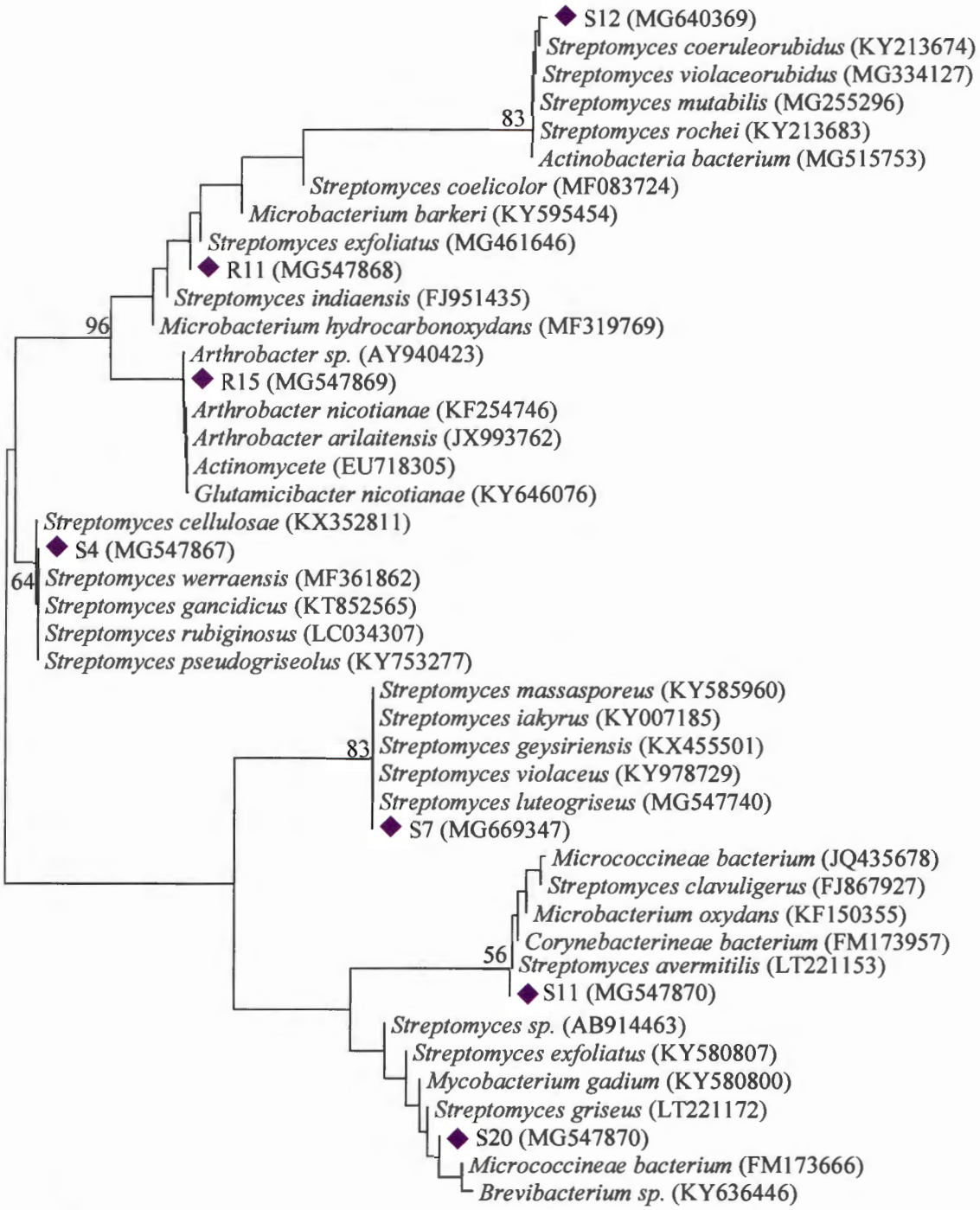

Figure 2.7: Neighbour-joining tree of the isolated actinomycetes isolates and representative species of actinomycetes bacteria based on partial 16S rRNA gene sequences. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. Only values greater than 50% are shown. The scale bar indicates 0.3 substitutions per nucleotide position.

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2.4.9 PRIMER DESIGN AND AMPLIFICATION OF DROUGHT TOLERANCE AND PGP GENES

The16S rDNA genes of bacteria were used to develop the various drought tolerant primers used in this study. This is because the 16S rDNA gene are the highly conserved regions of bacteria, and are therefore most reliable for primer design and development. These genes are possessed as a single copy per genome by target organisms and are very unlikely to undergo horizontal gene transfer (Aremu and Babalola, 2015). The importance of the alignment with several bacterial species was to develop primers that would amplify drought tolerance genes in all bacterial communities. This implies that our primers would include all bacterial species recognized by PCR analysis. The sets of primers used in this study as shown in Table 2.5 were developed around the species of bacteria that will be able to enhance abiotic stress tolerances in plants for their use in amplifying the DNA in bacteria. Four primers from 16S sequences of bacteria were developed successfully. The designed primers were tested for binding affinities to the priming sites (delta G values) in the Gene Infinity Platform. Tests showed that the designed primers as presented in Table 2.5 did not have cross-dimer, self-dimer, cross homology and potential hairpin structures. All forward primers possessed appropriate G/C clamp at the 3' ends, moderate melting temperatures and their location was past the 5' end of the coding sequence. The lengths of the primer sequences were moderate, aiding in specific binding to the target gene. *In silico* PCR was performed on the primers in Gene Infinity Platform which revealed that they were excellent in specificity. Also, primer specificity performed in the NCBI primer-BLAST gave the target drought tolerance genes. The properties of the designed primers in this study are in agreement with primer characteristics of the mercegens-specific PCR primers developed by Aremu and Babalola (2015). They also agree with the characteristics of primers as proposed by Innis et al. (2012) which yielded excellent results.

The primers glutathione peroxidase was designed to target antioxidant genes in the maize plants. Glutathione peroxidase is a protein that helps to protect plants against the damages caused by reactive oxygen species. Glycine-rich proteins target drought resistant genes in plants and they are involved in cell elongation, control of stomatal closure and promotion of stomatal opening. Hence, the GRP primer was developed for this purpose. Desiccation protectant protein and GTP-binding protein are smaller protein units that protect plants from desiccation effects therefore the primers

were developed. Studies on gene expression are important tools used to understand and compare the universal responses of bacteria to abiotic stress. Bacteria have been found to possess certain genes which enhance their tolerance to abiotic stresses. This study used designed primer-specific PCR amplifications on total genomic DNA fractions that targeted certain genes responsible for drought tolerance in bacteria. As shown in Figure 2.8, the PCR amplification of the GPX gene yielded the expected band size of 215 bp for isolates R15, S20, S7 and R11 while isolates S11, S12 and S4 yielded no amplification. In the case of GRP (Figure 2.9), isolates R11, S20, S12 and R15 yielded the expected band size of ~ 220 bp while isolates S4, S7 and S11 did not amplify. Isolates S20 and R15 amplified the DSP gene at the expected product size of 920 bp while no amplification was observed for the rest of the isolates at this size (Figure 2.10). For the GTP gene, only isolate R15 amplified at the expected band size of 668 bp (Figure 2.11).

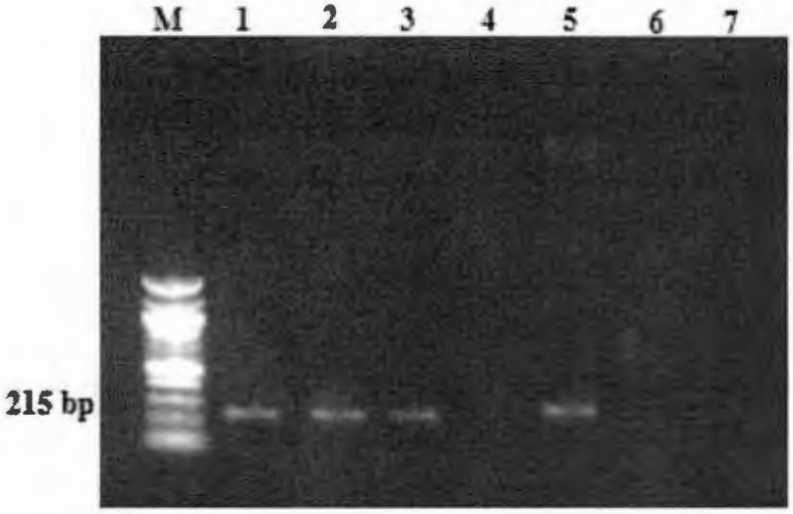

Figure 2.8: Agarose gel electrophoresis of GPX PCR products. Lane M = 120 bp molecular weight marker, Line 1: R15, Line 2: S20, Line 3: S7, Line 4: S11, Line 5: R11, Line 6: S12, Line 7: S4

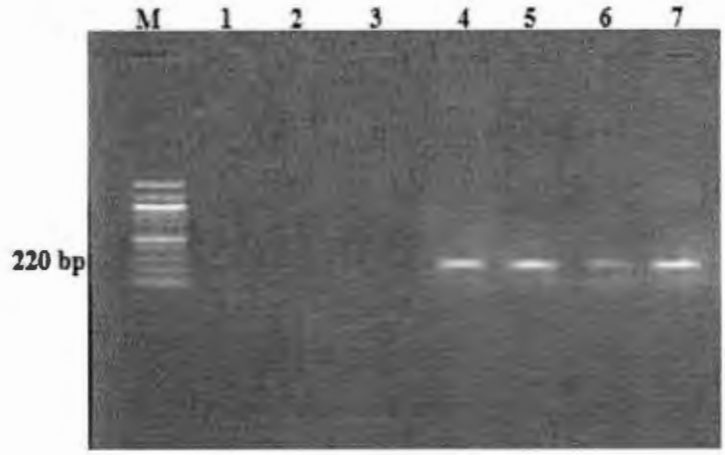

Figure 2.9: Agarose gel electrophoresis of GRP PCR products. Lane M = 100 bp molecular weight ladder, Line 1: S7, Line 2: R11, Line 3: S11, Line 4: S12, Line 5: S20, Line 6: R15, Line 7: R11

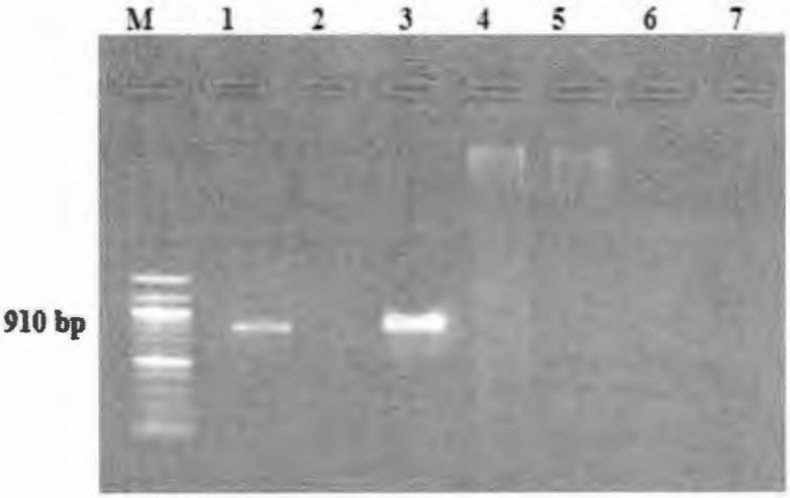

Figure 2.10: Agarose gel electrophoresis of DSP PCR products. Lane M = 100 bp molecular weight ladder, Line 1: S20, Line 2: S11, Line 3: R15, Line 4: S4, Line 5: S7, Line 6: S12, Line 7: R11

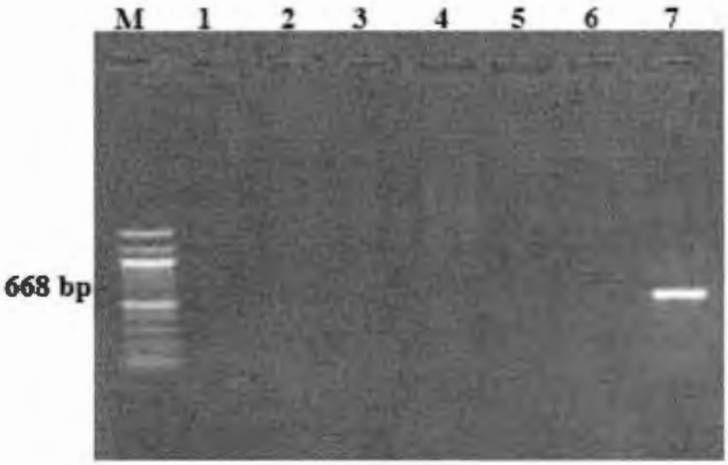

Figure 2.11: Agarose gel electrophoresis of GTP PCR products. Lane M = 100 bp molecular weight marker, Line 1: R11, Line 2: S4, Line 3: S20, Line 4: S12, Line 5: S11, Line 6: S7, Line 7: R15

The ACC deaminase (*Accd*) gene was amplified by PCR for all seven isolates (Figure 2.12) with the reference primers as shown in Table 2.1. Amplification of siderophore (*Sid*) gene (Figure 2.13) for the seven isolates was performed by PCR using reference primers (Table 1). It was observed that isolates S20, R11, S12, R15, S11 and S4 amplified this gene. However, no amplification was observed for isolate S7 for this gene.

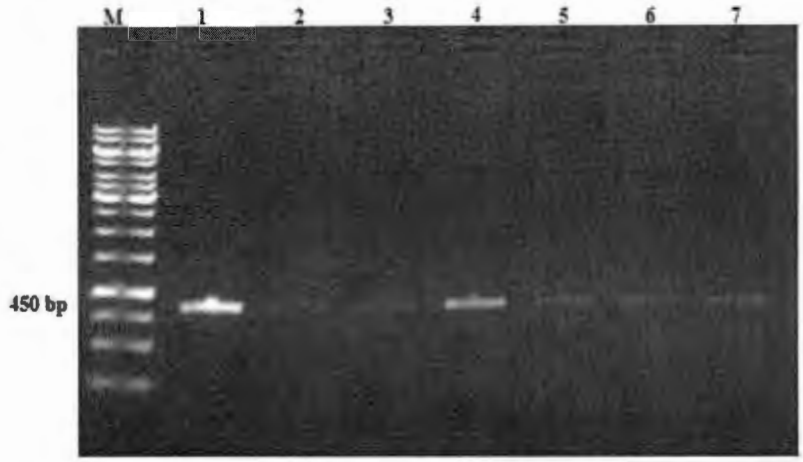

Figure 2.12: Agarose gel electrophoresis of *Accd* PCR products. Lane M = 1 Kb molecular weight ladder, Line 1: S11, Line 2: S12, Line 3: S7, Line 4: S20, Line 5: S4, Line 6: R15, and Line 7: R11

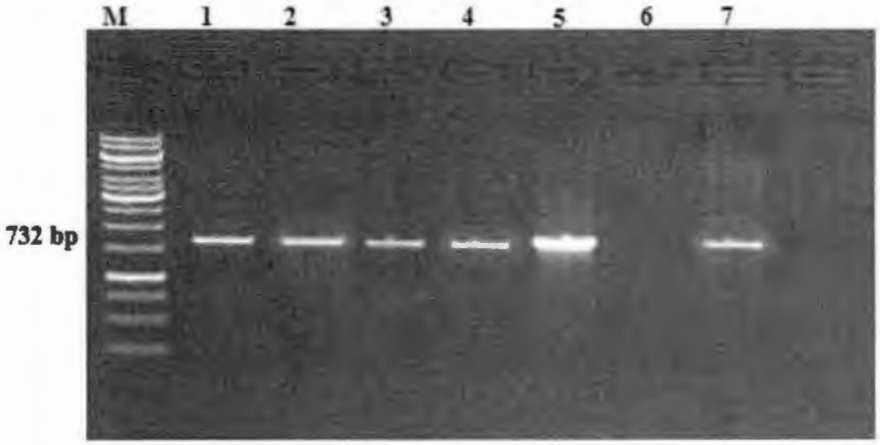

Figure 2.13: Agarose gel electrophoresis of *Sid* PCR products. Lane M= 1Kb molecular weight marker, Line 1: S20, Line 2: R11, Line 3: S12, Line 4: R15, Line 5: S11, Line 6: S7, Line 7: S4

The ability of some of the tested isolates to amplify at the target product sizes of drought tolerance and PGP genes used in this study could indicate the presence of these genes in their genome or

DNA chromosomes present in the nuclear regions of various bacterial cell walls. The present study is in harmony with the study of Raddadi et al. (2008) who reported a positive PCR for all bacterial strains tested for the *Accd* gene coding for ACC deaminase enzyme. Ali et al. (2014) also reported that isolate SorgP4 amplified the *acds* gene. Bacteria possessing ACC deaminase gene can help lower the ethylene levels in plants and also assist in the protection of plants against certain environmental stresses such as drought, flooding, phytopathogens and heavy metals (Grichko and Glick, 2001; Belimov et al., 2005; Vurukonda et al., 2016) which induce the synthesis of ethylene. The presence of the A*ccd* gene in the isolates in this study could be of great importance in field application under stress conditions. The presence of siderophore gene in bacterial isolates can enhance biocontrol of phytopathogenic fungi due to the competition for iron by plants, and also improve the availability of iron to plants. Masalha et al. (2000) reported the role of soil microorganisms in the acquisition of iron and plant growth promotion.

From the results obtained in the present study, all the isolates used in this study possessed one or more drought tolerant and PGP gene. This clearly indicates their potential for possible use as bio-inoculants, not only to improve drought tolerance in plants but also to serve as biofertilizers and biocontrol agents to facilitate plant growth.

Table 2.5: Properties of designed primers

Primer	Primer set	Oligonuclueotide sequence	GC content (%)	Tm	Length	Location
GPXF	1	CCGTTGACGTCGGTCTTCTC	60	56	20	16S
GPXR	2	GTGGTGAATGTGGCGTCCAA	55	54	20	16S
GRPF	1	GGGATTGGACGATTGTA	47.1	45	17	16S
GRPR	2	GAACTGGAGGAACTGAG	52.9	47	17	16S
DPF	1	TCAGCTTGTGATGTCTC	47.1	45	17	16S
DPR	2	GCACTCCTACTTCTAGC	52.9	47	17	16S
MEF	1	ACCGCTACAGGATAAGA	47.1	45	17	16S
MER	2	TGATGCCGTTGGATAAG	47.1	45	17	16S

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CHAPTER THREE: QUALITATIVE AND QUANTIFICATIVE SCREENING FOR PLANT GROWTH PROMOTING TRAITS OF ACTINOMYCETES ISOLATES AND THE EFFECTS OF *Arthrobacter arilaitensis* AND *Streptomyces pseudovenezuelae* ON DROUGHT TOLERANCE IN MAIZE

ABSTRACT

Screening of actinomycetes for plant growth promoting (PGP) characteristics is necessary as it helps to recognize and use certain species for growth promotion and stress tolerance in plants. The actinomycetes isolates *Streptomyces werraensis*, *Streptomyces luteogriseus*, *Streptomyces indiaensis*, *Arthrobacter arilaitensis*, *Streptomyces pseudovenezuelae*, *Microbacterium oxydans* and *Streptomyces* sp. were screened for the presence of PGP characteristics as well as the amounts produced. It was found that all isolates produced indole-3-acetic acid, ACC deaminase activity, ammonia and siderophore while five solubilized phosphate and one produced hydrogen cyanide. Quantitatively, *S. werraensis* produced the highest IAA of 10.12 ± 0.02 µg/ml followed by *A. arilaitensis* (9.44 ± 0.01 µg/ml), *S. pseudovenezuelae* produced the highest ACC deaminase activity of 0.903 ± 0.024 µmol/min and *A. arilaitensis* produced the highest siderophore of 51.3%. A greenhouse experiment was performed to evaluate the effect of two isolates *S. pseudovenezuelae* and *A. arilaitensis* on drought stress tolerance in maize at three soil moisture levels and two inoculation methods. Results showed that the greatest increase in physiological parameters were obtained when the two bacteria were combined in maize seeds. Increases in measurements were also observed in individually inoculated plants compared to the control plants. Higher growth was also observed in plants whose seeds were coated with vermiculite than the plants whose seeds were inoculated without coating. The ability of these isolates to produce PGP characteristics as well as drought tolerance potential is an indication that they can be effectively used to promote plant growth and improve drought tolerance in plants in either greenhouse or field conditions.

Keywords: Actinomycetes, drought tolerance, greenhouse, plant growth promotion, vermiculite.

3.1 INTRODUCTION

A major environmental problem facing most countries of the world today, regarding agricultural productivity and food availability, is drought. Drought has been a subject of concern as it has led to reduced plant growth and yield. It is therefore very important to seek means of reducing this menace, to increase food availability and sustain food security. At present, strategies like breeding and genetic modifications are being used to manage this problem (Langridge and Reynolds, 2015; Maazou et al., 2016). Also, agricultural practices including soil amelioration and mulching have been used (Jongdee et al., 2006). However, these strategies are not very efficient as they are not only time consuming but labour and cost intensive (Ashraf, 2010; Eisenstein, 2013). Often times, some desirable plants traits in the host plant gene pool can be unintentionally lost in the process of breeding (Philippot et al., 2013). Also, plant breeding transfers benefit to a single host specie and not to other crop systems, as it is usually difficult to identify the genetic component responsible for this improvement (Coleman-Derr and Tringe, 2014). The drawbacks mentioned above have made these technologies highly unreliable, leading to a quest for better and more efficient means to tackle this problem.

In recent times, the use of beneficial microbial species with plant growth promoting capabilities to relieve plants of the adverse effects of drought has gained interest in agriculture. Bacteria are important soil components, able to form mutualistic and beneficial associations with most plants. Symbiotic bacteria are capable of conferring stress tolerance to a wide variety of their plant hosts through phytohormonal modifications, production of exopolysaccharides, accumulation of osmolytes and acting as defense against reactive oxygen species (Coleman-Derr and Tringe, 2014; Zhang et al., 2008). These bacteria are also able to synthesize antibiotic substances, fix atmospheric nitrogen, produce soluble iron compounds (siderophore), and solubilize inorganic phosphates. In addition, they serve as plant growth regulators by producing the phytohormones indole-acetic acid (IAA), 1-aminocyclopropane-1-carboxylic acid (ACC), cytokinins and gibberellins (GA). These outstanding properties of the plant growth promoting bacteria (PGPB) facilitate the efficient stimulation of plant growth during unfavorable environmental conditions like drought. Several studies have revealed the successful application of isolated PGPB on drought stress improvement in plants (Figueiredo et al., 2008; Yandigeri et al., 2012; Gusain et al., 2015). However, most of these studies have concentrated on certain groups of bacteria species, mostly *Pseudomonas and*

Bacillus. The use of actinomycetes species to enhance stress tolerance in plants have received only little attention over the years. Actinomycetes, found mostly in soils, are widely known for their antibiotic production and their outstanding ability to survive in unfavorable environments (Passari et al., 2015). Their ability to produce certain plant growth promoting properties has also been identified, but with little information on the extent of these properties produced (Ali et al., 2014; Sreevidya et al., 2016). Hence, this study was conducted to:

1. Screen drought tolerant bacteria for the production of plant growth promoting (PGP) elements like ACC, IAA, siderophore, phosphate and ammonia (NH_3);
2. Determine the amount of certain PGP elements (IAA, ACC deaminase, siderophore) produced by the drought tolerant bacteria;
3. Evaluate the effect of bacteria inoculation on growth of maize (*Zea mays* L.) under well-watered, semi-watered and drought stressed conditions; and
4. Evaluate the effect of inoculation method on drought tolerance in maize.

3.2 MATERIALS AND METHODS

3.2.1 ISOLATION AND SELECTION OF DROUGHT TOLERANT BACTERIA

In the preceding study, seven drought tolerant bacteria were isolated from two maize plantations namely: *Streptomyces werraensis* MG547867, *Streptomyces luteogriseus* MG669347, *Streptomyces indiaensis* MG547868, *Arthrobacter arilaitensis* MG547869, *Streptomyces pseudovenezuelae* MG547870, *Microbacterium oxydans* MG640368, and *Streptomyces* sp. MG640369. These isolates were selected for this study based on their ability to withstand drought stress by growing at high concentrations of PEG 8000. In the present study, these bacteria were qualitatively and quantitatively screened for the presence of plant growth promoting traits. Due to their higher tolerance of higher concentrations of PEG 8000, *S. pseudovenezuelae* MG547870 and *A. arilaitensis* were chosen for greenhouse studies, to assess the effect of their individual and combined inoculation on the growth of maize plant under drought stress.

3.2.2 QUALITATIVE AND QUANTITATIVE ASSESSMENT OF PLANT GROWTH PROMOTING PROPERTIES OF BACTERIAL ISOLATES

3.2.2.1 AMMONIA PRODUCTION

The production of ammonia by actinomycetes isolates was tested by inoculating 10 µl (0.2 OD) of freshly prepared actinomycetes cultures into test tubes containing 10 ml of peptone water. An un-inoculated broth served as control for this experiment. The inoculated test tubes were incubated at 25°C for 96 h after which 1 ml of Nessler's reagent was added to each test tube, and any colour changes were observed. A change in the colour of the media to yellow or brown specifies a positive result for ammonia production. The experiment was done in triplicate.

3.2.2.2 PHOSPHATE SOLUBILIZATION ACTIVITY

To evaluate the ability of actinomycetes isolates to solubilize phosphate, 10 µl of freshly prepared culture were spot inoculated on Pikovskaya's agar plates containing 2% tri-calcium phosphate. Inoculated plates were incubated at 37°C for 72 h, plates were observed for the appearance of a clear zone around the actinomycetes colonies.

3.2.2.3 HYDROGEN CYANIDE ACTIVITY

Hydrogen cyanide activity was determined according to the protocol of Bakker and Schippers (1987). Bacterial cultures were separately streaked on Luria Bertani (LB) agar amended with 0.4%

(w/v) of glycine. A Whatman no. 1 filter paper soaked in 0.5% (w/v) picric acid in 2% (w/v) sodium carbonate was placed on the lid of the Petri dish. Thereafter, plates were properly sealed with parafilm and incubated for seven days. The change in color of the filter paper from yellow to deep orange when observed with the eyes indicates a positive result.

3.2.2.4 INDOLE-3-ACETIC ACID PRODUCTION

For qualitative determination of indole-3-acetic acid production by actinomycetes isolates the method of Bric et al. (1991) was employed. Freshly prepared bacterial cultures (20 µl) were inoculated in LB broth (20 ml) amended with 5 mmol tryptophan and incubated at 27°C for 4 days. After incubation, 1 ml of bacterial culture was transferred into sterile Eppendorf tubes and centrifuged at 5,000 g for 15 min. The supernatant was collected in a 15 ml centrifuge tube and 2-3 drops of orthophosphoric acid was added alongside 4 ml of Salkowsky reagent (50 ml of 35% perchloric acid in 1 ml of 0.5 M $FeCl_3$). The contents in the tubes were incubated at room temperature under dark conditions for 20 min, the development of a pink color indicated IAA production. The absorbance of the pink color was read using a UV spectrophotometer (Thermo Spectronic, Merck chemical, SA) at 530 nm. The control for this experiment consisted of an un-inoculated broth without tryptophan while the blank consisted of un-inoculated broth with L-tryptophan

The amount of IAA produced by each bacterial isolate was determined by the generation of a standard curve (appendix pp. 138). Standards were made in LB broth at 0, 5, 10, 20, 50 and 100 µg/l including a control consisting of LB broth only. 2 ml of Salkowsky reagent was added to 1 ml of each standard and incubated at room temperature for 20 min. Absorbance was read at 530 nm using a UV spectrophotometer (Thermo Spectronic, Merck chemical, SA).

3.2.2.5 SIDEROPHORE PRODUCTION

The production of siderophore by bacterial isolates was assayed according to a modified protocol described by Schwyn and Neilands (1987) using an indicator dye, chrome azurol S (CAS). Briefly, 60.5 mg of CAS was dissolved in 50 ml of distilled water and mixed with 10 ml of iron (III) solution (1 mM $FeCl_3$. $6H_20$ in 10 mM HCl). The mixture was slowly added while constantly stirring with a magnetic stirrer to 72.9 mg of hexadecyltrimethylammonium (HDTMA) bromide dissolved in 40 ml distilled water and then autoclaved at 121°C for 15 min. The final mixture (100

ml) was added while stirring to 900 ml of sterilized LB broth adjusted to pH 6.8 and poured into Petri plates. Upon solidification, freshly prepared bacterial cultures were spot inoculated on the Petri plates and incubated at 25°C for 7 days. A yellowish- orange halo around the bacterial colonies was considered a positive result for siderophore production (see appendix pp. 141).

The amount of siderophore produced by each bacterial isolate was estimated following the protocol of Alexander and Zuberer (1991) using a modified CAS assay solution. Hexadecyltrimethylammonium (HDTMA, 21.9 mg) was dissolved in 25 ml of distilled water with constant stirring under low heat. In a 50 ml flask, 1.5 ml of 1 mM $FeCl_3$. $6H_20$ in 10 mM HCl was added to 7.5 ml of 2 mM CAS. This solution was slowly added to the HDTMA solution and the resultant mixture transferred to a 100 ml flask. A buffer solution was prepared by dissolving 9.76 g of 2-(N-morpholino)ethanesulfonic acid (MES) in 50 ml distilled water and the pH adjusted to 5.6 with 50% KOH. This buffer solution was then added to the flask containing the dye solution while distilled water was added to get a final volume of 100 ml. A shuttle assay solution was prepared by adding 87.3 mg of 5-sulfosalicyclic acid to the above solution before use. All seven isolates to be tested for siderophore were inoculated in 5 ml sterilized LB medium without added Fe and incubated at 25°C for five (5) days. Bacterial cells were pelleted by centrifugation at 3000 g for 10 min and the supernatant was collected in tubes. The concentration of siderophore in the supernatant was obtained by mixing 100 µl of CAS assay solution with 100 µl of supernatant and allowing to equilibrate for 3-4 h, the absorbance of the 200 µl mixture was measured at 630 nm using a UV spectrophotometer (Thermo Spectronic, Merck chemicals, SA). The percentage siderophore produced was calculated by the equation:

$$\% \text{ Siderophore units} = \frac{Ar - As}{Ar} \times 100$$

Where Ar = Absorbance of reference at 630 nm (CAS reagent),

As = Absorbance of sample at 630 nm

3.2.2.5 ACC DEAMINASE ACTIVITY

The seven drought tolerant bacterial isolates used in this study were screened for ACC deaminase activity based on their ability to utilize ACC as sole nitrogen source. All bacteria were first grown on 5 ml of Tryptone-Soy broth (TSB, rich medium) and incubated at room temperature for 48 h. Bacterial cells were harvested by centrifugation at 5000 g for 5 min, washed twice with sterile 0.1 M Tris-HCl (pH 7.5) and resuspended in 1 ml of 0.1 M Tris-HCl (pH 7.5). Washed bacterial cells were spot inoculated on Petri plates containing modified Dworkin and Foster salts minimal medium (Dworkin and Foster, 1958). Minimal salts medium was composed of 2 g glucose, 2 g gluconic acid, 2 g citric acid, 4 g KH_2PO_4, 6 g Na_2HPO_4, 0.2 g $MgSO_4.7H_2O$ and 10 ml micro nutrient solution (200 mg $CaCl_2$, 200 mg $FeSO_4.7H_2O$, 15 mg H_3BO_3, 20 mg $ZnSO_4.7H_2O$, 10 mg Na_2MoO_4, 10 mg KI, 10 mg NaBr, 10 mg $MnCl_2$, 5 mg $COCl_2$, 5 mg $CuCl_2$, 2 mg $AlCl_3$, 2 mg $NiSO_4$ and 1000 ml distilled H_2O) in 990 ml distilled H_2O amended with 3 mM ACC as a sole nitrogen source. Negative control for this experiment was Petri plates containing only DF minimal salts medium without ACC while the positive control consisted of plates containing DF minimal salts medium + 0.2% (w/v) $(NH_4)_2SO_4$. Inoculated plates were incubated at 30°C for 5 days. The growth of bacterial isolates on DF minimal plates containing ACC was used to compare those of the positive and negative controls. Petri plates were selected based on bacterial growth by utilizing ACC as sole source of nitrogen. The experiment was performed thrice.

The activity of ACC deaminase was measured by growing all 7 actinomycetes isolates on TSB medium at 30°C for 5 days. The induction of ACC deaminase activity was achieved by collecting bacterial cells by centrifugation at 5000 g for 5 min and washing with 0.1 M Tris-HCl (pH 7.5). Washed cells were resuspended in 2 ml of modified DF minimal medium containing 3 mM concentration of ACC, then incubated under shaking at 30°C for 7 days. ACC deaminase activity was determined by measuring the production of α-ketobutyrate and ammonia generated when ACC cleaved to ACC deaminase (Penrose and Glick, 2003). Induced bacterial cells were harvested by centrifugation at 5000 g for 10 min, washed twice with 0.1 M Tris-HCl (pH 7.5) solution, then resuspended in 200 µl of 0.1 M Tris-HCl (pH 8.5). Toluene (5% v/v) was added to the cells to labilize and cells were vortexed at highest speed for 30 s. In sterile Eppendorf tubes, fifty (50) µl of labilized cells was collected and 5 µl of 3 mM ACC was added. Tubes were incubated at 30°C for 30 min. For this assay, the negative control consisted of 50 µl of labilized cell suspension

without ACC while the blank consisted of 50 µl of 0.1 M Tris-HCl (pH 8.5) with 3 mM ACC. Samples were then thoroughly mixed with 500 µl of 0.56 N HCl by vortexing and cell pellets were removed by centrifugation at 10, 000 g for 10 min. The supernatant (500 µl) was transferred into test tubes and mixed with 400 µl of 0.56 N HCl and 150 µl of 2, 4 DNP solution (0.1 g 2, 4-dinitrophenylhydrazine in 100 ml of 2 N HCl). The mixture was incubated at 30°C for 30 min. One ml of 2 N NaOH was finally added to the samples after which their absorbances were measured at 540 nm using a UV spectrophotometer (Thermo Spectronic, Merck chemicals, SA).

Alpha-ketobutyrate concentration in each sample was determined by comparing with a standard curve (appendix pp 139) generated as follows: alpha-ketobutyrate solutions (500 µl) of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 mM were mixed each with 400 µl of 0.56 N HCl and 150 µl of 2,4-DNP solution. One (1) ml of 2 N NaOH was then added and mixed. The absorbance was measured at 540 nm and the values obtained for the absorbance against concentration (mM) were used to generate a standard curve. Note: each standard was replicated 3 times.

3.2.3 GREENHOUSE EXPERIMENTS

Greenhouse experiments were conducted to: (i) evaluate the effect of bacterial inoculation on the growth of maize plant under three moisture levels [field capacity (FC), moderately wet (MW) and completely dry (D)], and (ii) evaluate the effect of the method of inoculation on drought stress tolerance in maize. Maize, known to be very susceptible to drought stress, was chosen based on its high nutritional and economic importance in South Africa. Knowledge gained from this will help to find suitable means of improving maize growth and yield, despite the ravaging drought.

3.2.3.1 ACTINOMYCETES INOCULUM PREPARATION

The two actinomycetes isolates *A. arilaitensis* MG547869 and *S. pseudovenezuelae* MG547870 were chosen and used in this experiment was based on their outstanding ability to resist drought stress *in-vitro* as well as their plant growth promoting potentials. The inocula were prepared by growing the bacterial strains in 250 ml conical flasks containing 100 ml of sterilized LB broth. Inoculated flasks were incubated at 30°C under constant shaking (120 rpm) for 7 days. Pellets were collected by centrifugation at 10000 rpm for 20 min and washed twice with sterile distilled water. Pelleted cells were resuspended in 0.01M phosphate buffer at pH 7 and adjusted to an

absorbance of 1.2 at 600 nm with a UV spectrophotometer (Thermospectronic, Merck, SA) (Ndeddy Aka and Babalola, 2016).

3.2.3.2 SOIL COLLECTION AND PREPARATION FOR POT EXPERIMENTS

Soil for the trial was collected from behind the Animal Health Centre of the North-West University, Mafikeng Campus at 25°S Latitude, 25°E Longitude and elevation of 1278.3 m. The soil was collected at the surface 0-20 cm depth, oven dried at 70°C for 48 h, passed through a 2 mm sieve and autoclaved at 121°C for 15 min. Sterilized soil was allowed to cool for 2 days after which 10 kg of soil was aseptically transferred into plastic pots.

3.2.3.3 SEED VIABILITY TEST IN PETRI PLATES

Seed germination tests were conducted to evaluate the effect of bacterial inoculation on the germination of the test seeds in the presence of 5% polyethylene glycol (PEG) 8000 (Rincón et al., 2008). Prior to the test, drought sensitive maize seeds of the variety S0/8/W/ I137TNW//CML550 obtained from the North-West University Agriculture Farm, Potchefstroom were washed first with tap water, then soaked in 2% sodium hypochlorite ($NaClO_2$) solution for 15 min and severally rinsed with sterile distilled water to remove the remains of the disinfectant (Madhaiyan et al., 2007). Thereafter, 4 clean Petri plates (replicated three times) were prepared by placing two filter papers at the bottom of each plate and subsequently 10 ml of each bacterial suspension or 10 ml of sterile tap water (in the case of the control) was pipetted in each Petri plate. Sterile seeds were immersed in 10 ml of bacterial suspension for 5 h in a rotary shaker at 150 rpm after which 20 seeds were placed in each petri plate and incubated at 25°C for 10 days. Germinated seeds in each Petri plate were counted and 5 seedlings per plate were randomly selected for growth parameter measurements (shoot length, root length and dry seedling weight). Percentage germination and vigor index were estimated according to the method of Ghorbanpour and Hatami (2014) as follows:

$$\text{Germination rate}(\%) = \frac{n}{\text{N}} \text{x } 100$$

Where n is the number of germinated seeds after 7 days and N is the total number of seeds

Vigor index = % germination x total length of seedling (shoot length + root length)

3.2.3.4 PREPARATION OF MAIZE SEEDS FOR GREENHOUSE STUDY

Drought susceptible maize seeds of the cultivar S0/8/W/ I137TNW//CML550 were firstly immersed in 70% ethanol for 15 min and washed three times with sterile distilled water. Thereafter, the seeds were soaked in 2% sodium hypochlorite ($NaClO_2$) solution for 10 min, and then thoroughly rinsed twice with sterile distilled water.

3.2.3.5 SEED INOCULATION WITH BACTERIAL ISOLATES

In this study, two methods of inoculation were employed to enable the bacterial isolates to adhere to the surface sterilized maize seeds. This was done to determine the effect of mode of inoculation on drought stress enhancement in maize plants. Firstly, surface sterilized maize seeds were inoculated by direct immersion in bacterial cultures (1.5 OD_{600}/ ml) or sterile distilled water for the control treatment for 12 h. Secondly, maize seeds were immersed in bacterial cultures (1.5 OD_{600}/ ml) or sterile distilled water in the case of the control for 12 h. Following immersion, seeds were resuspended in 1% carboxylmethyl cellulose (CMC, binder) in a 500 ml conical flask and finely ground and sterilized vermiculite was spread all over the seeds until they were completely coated. Both the directly inoculated and coated seeds were left to dry overnight in a sterile laminar flow chamber prior to being sown in the greenhouse.

3.2.3.6 GREENHOUSE EVALUATION OF BACTERIAL INDUCED TOLERANCE TO DROUGHT STRESS

In the factorial greenhouse experiment, a total of seventy-two (72) pots (23-cm diameter) were used, representing twenty-four (24) treatment combinations based on three (3) experimental factors (seed treatments, bacterial types and soil moisture levels); with three replicates each. The three experimental factors used in the present study include:

(i) Two seed treatments (inoculation method): directly inoculated seeds and vermiculite coated seeds as described in section 3.2.14 above

(ii) Four types of seed inoculation: without bacteria isolate (control), with *S. pseudovenezuelae*, with *A. arilaitensis* and combination of both bacterial isolates, and

(iii) Three moisture levels: Field capacity (FC), moderately wet (MW) and completely dry (drought stressed, DS).

All experimental pots containing treatments were arranged in a completely randomized design (CRD). Four seeds were sown per plastic pot containing 10 kg of sterilized soil at a depth of 5 cm. Ten days after germination, each pot was thinned and only one seedling was left in each pot. Bacterial suspension of 50 ml (1.5 OD per ml) was added near the plant root zone in each pot and this was done every 2 weeks till the end of the experiment. Pots were watered daily with 200 ml of water for the first 15 days after seed germination before drought stress was induced and lasted till the 35th day. During the period of drought stress, the treatments at field capacity received 200 ml of water per day. For moderately wet treatments, plants received 100 ml of water while completely dry treatments, plants received no water till the 35th day. All plants were watered again for 2 days after the 35th day and above ground data (chlorophyll content index, shoot length, number of plant leaves and leaf area) were collected before they were carefully uprooted from the pots. After uprooting, plants were washed with distilled water to remove adhering soil and root lengths were measured for each plant. Plant shoot and roots were packaged in aluminum foil papers and dried in an oven at 68°C for three days. Dry root and shoot weights were collected using a weighing balance. Thereafter, plant samples were stored in polyethylene bags at -4°C for further analysis. The greenhouse experiments were conducted twice and data were analyzed together. The 24 treatment combinations used in the present study are presented below as follows:

1. S (FC)	2. S (Mw)	3. S (DS)
4. S +V (FC)	5. S +V (Mw)	6. S +V (DS)
7. S + BS (FC)	8. S + BS (Mw)	9. S + BS (DS)
10. S+ BS + V (FC)	11. S + BS + V (Mw)	12. S + BS + V (DS)
13. S + BR (FC)	14. S + BR (Mw)	15. S + BR (DS)
16. S + BR + V (FC)	17. S + BR + V (Mw)	18. S + BR + V (DS)
19. S + BR + BS (FC)	20. S + BR + BS (Mw)	21. S + BR + BS (DS)
22. S+ BR + BS + V (FC)	23. S+ BR + BS + V (Mw)	24. S + BR + BS + V (DS)

where: S= Maize seed, V= vermiculite coated, BS = bacteria S20 (*S. pseudovenezuelae*), BR = bacteria R15 (*A. arilaitensis*), FC= field capacity, Mw = moderately wet and DS = drought stressed (completely dry).

3.3 DATA ANALYSIS

All experimental data obtained from this study were analyzed by One-way analysis of variance (ANOVA) using the Statistical analysis software (SAS), version 9.4 (SAS, 2014). For each treatment, generated data were presented as arithmetic means. Significantly different means were separated using New Duncan Multiple Range Test (DMRT) at 5% level of significance.

3.4 RESULTS AND DISCUSSION

3.4.1 DROUGHT TOLERANCE BY ACTINOMYCETES ISOLATES

Plants are continuously exposed to abiotic stresses such as drought, salinity and cold (Bardi and Malusà, 2012). Drought, being one of the most serious environmental problems affecting the growth and development of plants and subsequently agricultural yields and food supply, has gained research attention over the years. The ravaging effects of drought on plants can be reduced by the action of certain PGPB. These bacteria are capable of tolerating and surviving under harsh environments through the regulation of phytohormones, production of ACC deaminase activity, accumulation of osmolytes, production of volatile compounds and antioxidant defense (Vurukonda et al., 2016).

The actinomycetes strains used in this study were those isolated and identified in the preceding study due to their high tolerance to higher concentration of PEG 8000. *S. pseudovenezuelae* (MG547870) and *A. arilaitensis* (MG547869), chosen for greenhouse studies grew well at the highest PEG 8000 concentration of 20% with a growth of 0.786 ± 0.076 (OD_{600}) for *S. pseudovenezuelae* MG547870 and 1.379 ± 0.134 for *A. arilaitensis* MG547869.

3.4.2 PLANT GROWTH PROMOTING CHARACTERISTICS OF BACTERIAL ISOLATES

All tested isolates produced multiple plant growth promoting characteristics. The results of the qualitative plant growth promoting tests conducted are shown in Table 3.1. Results revealed that all seven isolates tested were positive for ammonia, Indole-3-acetic acid, siderophore and ACC deaminase activity, five isolates [*S. luteogriseus* (S7), *M. oxydans* (S11), *Streptomyces* spp. (S12), *S. indiaensis* (R11), and *A. arilaitensis* (R15)] tested positive for phosphate solubilization by showing clear zones around colonies on petri dishes while only *Streptomyces* spp. (S12) was positive for hydrogen cyanide activity.

Table 3.1: Qualitative plant growth promoting abilities of bacterial isolates

PGP traits	S4	S7	S11	S12	R11	R15	S20
Indole-3-acetic acid (IAA)	+	+	+	+	+	+	+
ACC deaminase activity	+	+	+	+	+	+	+
Siderophore	+	+	+	+	+	+	+
Ammonia production	+	+	+	+	+	+	+
Phosphate solubilization	-	+	-	+	+	+	+
Hydrogen cyanide	-	-	-	+	-	-	-

Note: S and R stand for bacterial isolates codes

3.4.3 INDOLE-3-ACETIC ACID PRODUCTION BY BACTERIAL ISOLATES

Plants' developmental processes are being regulated by the production of phytohormones in their various parts. The phytohormone, indole-3-acetic acid, plays a major role in plant development and its supply is capable of supporting its host during stress conditions like drought and pathogenic attacks (Bardi and Malusà, 2012; Sathya et al., 2017). It also improves seedling growth, and cell differentiation, as well as enhancing both elongation and development of lateral roots in plants (Vurukonda et al., 2016; Sathya et al., 2017). The results of IAA production by the tested bacterial isolates are shown in Table 3.1 and Figure 3.1. *Streptomyces werraensis* (S4) showed maximum indole-3-acetic acid production (10.12 ± 0.02 µg/ml) followed by *A. arilaitensis* (R15, 9.44 ± 0.01 µg/ml), *S. pseudovenezuelae* (S20, 8.96 ± 0.03 µg/ml), *S. luteogriseus* (S7, 8.68 ± 0.01 µg/ml), *S. indiaensis* (R11, 7.46 ± 0.02 µg/ml), *Streptomyces* spp. (S12, 6.98 ± 0.02 µg/ml), and *M. oxydans* (S11, 5.03 ± 0.01 µg/ml). Several rhizospheric bacteria have been documented for their ability to produce IAA as well as their different biosynthetic pathways of IAA production (Cassán et al., 2014; Duca et al., 2014; Vijayabharathi et al., 2016). Results from the present study revealed that all tested bacterial isolates produced indole-3-acetic acid. However, the amount of this acid produced varied, as there were significant differences in the amount produced by each of the bacterial isolates tested. The highest IAA production of 10.12 ± 0.02 µg/ml of IAA was achieved by *S. werraensis* while the lowest of 5.03 ± 0.01 µg/ml of IAA was obtained by *M. oxydans* (Figure

3.1). The increased amount of IAA produced by these bacteria in the medium used was due to the presence of L-tryptophan, as corroborated by Idris et al. (2007) who revealed that the secretion of IAA can be increased by the addition of tryptophan in medium. In this regard, inoculating maize plants with IAA producing bacteria can improve the growth and development of the plant under drought stress. The IAA result of this study is in agreement with previous reports of IAA production by bacteria. Studies have shown that the endophytic IAA producing *Streptomyces* species (*astrovirens, olivaceoviridis, rimosus, rochei and viridis*) improved seed germination, growth and root elongation in plants (Abd-Alla et al., 2013; El-Tarabily, 2008; Khamna et al., 2010). Matsukawa et al. (2007) also reported that the IAA triggered cell differentiation, sporulation and hyphal elongation in *Streptomyces atroolivaceus*.

3.4.4 PHOSPHATE SOLUBILIZATION BY BACTERIAL ISOLATES

Solubilization of phosphate is an important mechanism of plant growth promotion (Richardson, 2001). Bacteria are capable of increasing the availability of phosphorus (P) to plants through mechanisms such as the secretion of phosphatase to free P bound in organic matter and the production of organic acids/ chelating substances that helps to decrease rhizosphere pH (Rashid et al., 2004; Chen et al., 2006). From the results in Table 3.1, five out of the seven tested bacterial isolates solubilized phosphate by showing clear zones around colonies. These bacterial isolates include S20, R15, S12, R11 and S4 (*S. pseudovenezuelae*, *A. arilaitensis*, *Streptomyces* spp., *S. indiaensis* and *S. werraensis* respectively). Several bacterial species including *Pseudomonas* and *Bacillus* have also been reported to solubilize inorganic phosphates. In a study by Chabot et al. (1993), growth of lettuce and maize were enhanced by certain microorganisms capable of solubilizing mineral phosphate. Similarly, Almoneafy et al. (2012) reported the solubilization of phosphate in three strains of Subtillus D29, Am1 and H8. Rodríguez and Fraga (1999) also reported phosphate solubilization in *Pseudomonas striata* and *Bacillus polymyxa*. In this regard, the result from the present study conforms to other previous studies on phosphate solubilization.

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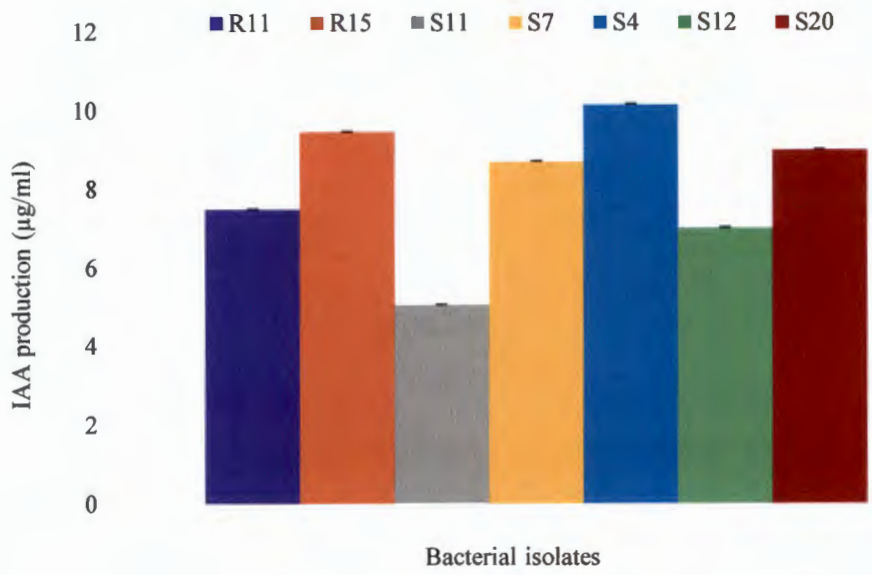

Figure 3.1: IAA production by bacterial isolates

3.4.5 ACC DEAMINASE ACTIVITY OF BACTERIAL ISOLATES

The introduction of drought tolerant ACC deaminase producing bacteria to drought stressed soils helps to improve stress tolerance in plants by lowering the production of stress induced ethylene. Several studies have reported the production of ACC deaminase activity bacteria (Glick et al., 2007; Hardoim et al., 2008; Rashid et al., 2012; Glick, 2014). Drought tolerant bacteria are capable of surviving in dry environments by adhering to the roots of developing seedlings or on seed coats of plants causing the deamination of ACC (the immediate precursor of ethylene in plants) by ACC deaminase which decreases the level of plant ethylene and consequently enhances plant growth and development (Ali et al., 2014; Glick, 2005). The mechanism of action of ACC deaminase producing bacteria in the improvement of both biotic and abiotic stresses is by the reduction of stress ethylene level through the activity of the enzyme ACC deaminase which breaks down ACC into α-ketobutyrate and ammonia instead of ethylene (Arshad et al., 2008). In the present study, drought tolerant bacteria were screened for ACC deaminase activity and all tested bacteria were positive (Table 3.1) though the levels of ACC deaminase activity produced varied among bacterial isolates (Figure 3.2). *Streptomyces pseudovenezuelae* produced the highest amount of ACC

deaminase activity (0.903 ± 0.024 µmol/min) followed by *A. arilaitensis* which produced 0.899 ± 0.023 µmol/min of ACC deaminase activity. *Streptomyces indiaensis, M. oxydans, Streptomyces* spp., *S. werraensis* and *S. luteogriseus* respectively produced 0.696 ± 0.028, 0.713 ± 0.003, 0.850 ± 0.032, 0.741 ± 0.004 and 0.671 ± 0.027 µmol/min of ACC deaminase activity. In the preceding chapter, PCR amplification of ACC gene revealed that the tested isolates amplified the *accd* gene primer when run on agarose gel. Bacteria producing ACC deaminase activity are known to improve the growth of a wide range of plants under stressful conditions like drought, salinity, heavy metals and flooding (Belimov et al., 2009; Shakir et al., 2012; Ali et al., 2014; Vurukonda et al., 2016). They also play major roles in plant nodulation processes in different leguminous plant species (Belimov et al., 2009).

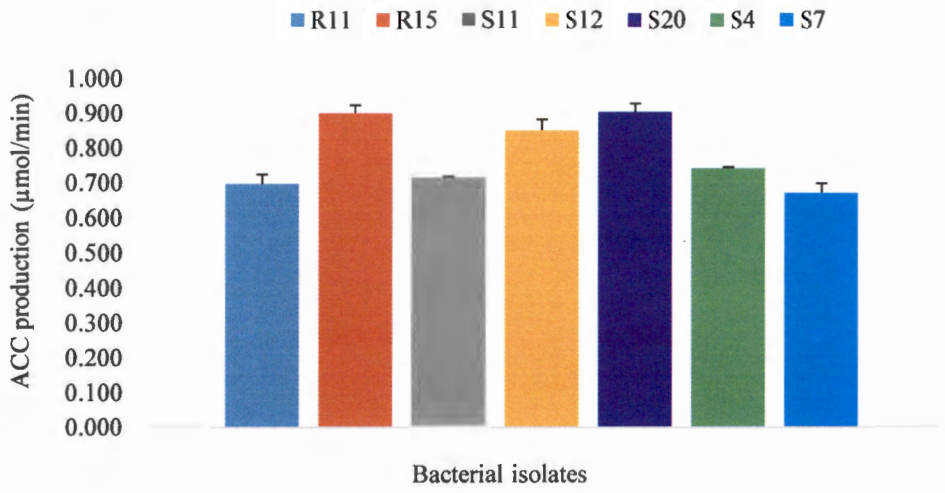

Figure 3.2: ACC deaminase activity of bacterial isolates

3.4.6 AMMONIA, SIDEROPHORE AND HYDROGEN CYANIDE PRODUCTION BY BACTERIAL ISOLATES

The role of bacteria in the control of phytopathogens in plants is another area that has gained attention in recent times. Some bacterial species are able to indirectly increase plant growth by inhibiting the growth of pathogens (Dobbelaere et al., 2003; Ndeddy Aka and Babalola, 2016).

The methods used by bacteria to inhibit pathogenic growth may include the secretion of volatile compounds like ammonia and other antifungal enzymes, the production of HCN, competitive secretion and the production of siderophores (Brimecombe et al., 2000). In the present study, all tested bacterial isolates produced ammonia. Plant growth promoting bacteria produce ammonia as secondary metabolite, playing a major role in antagonistic effects (Compant et al., 2005). Both qualitative and quantitative siderophore production was detected on all bacterial isolates tested (Table 3.1 and Figure 3.3). In the quantitative siderophore tests, statistically different values of percentage siderophore production were obtained for the tested bacteria. The highest percentage was produced by *A. arilaitensis* (51.3 ± 2.11%), followed by *Streptomyces* spp. (48.6 ± 2.04%), *S. pseudovenezuelae* (48.3 ± 1.41%), *S. werraensis* (44.5 ± 0.48 %), *M. oxydans* (42.7 ± 2.97%), *S. luteogriseus* (41.4 ± 2.57%) and the lowest produced was 38.3 ± 0.58% by *S. indiaensis*. Data obtained for siderophore production by bacterial isolates are shown in Figure 3.3. The production of low molecular weight metal chelators (siderophore) by the tested bacteria isolates offers them a competitive advantage to be used as biocontrol agents and to contribute to disease suppression in plants due to insufficient supply of essential trace minerals in natural environments (Laslo et al., 2012). A stimulated biosynthesis may cause these tested bacterial isolates to directly secrete antimicrobial compounds. In antagonism effect development, siderophore production and antifungal effect play major roles, although antifungal effects encompass other features (Selvakumar et al., 2008). Results obtained from the present study concur with the work of Laslo et al. (2012) who reported that 36.2% of tested isolates produced siderophore. Quan et al. (2010) detected different types of siderophores in *Pseudomonas* species. Among all tested bacterial isolates for HCN activity, only *Streptomyces* spp. produced HCN, indicating its potential for use as a biocontrol agent.

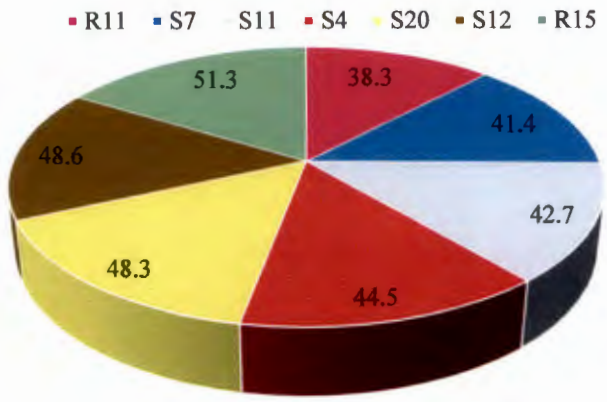

Figure 3.3: Percentage siderophore production by bacterial isolates

3.4.7 SEED GERMINATION TESTS

The results of the seed germination tests by *S. pseudovenezuelae* (MG547870) and *A. arilaitensis* (MG547869) are shown in Table 3.2. From the results, the highest germination percentage and vigor index of 94.44 ± 3.21 and 1825.93 ± 133.74 respectively was observed in seeds inoculated with both bacterial isolates without PEG 8000, followed by seeds inoculated with only *S. pseudovenezuelae* without PEG whose germination percentage and vigor index were 83.33 ± 3.12 and 1086.3 ± 105.60, respectively. The least germination and vigor index of 66.67 ± 3.21 and 675.56 ± 102.16 respectively were obtained in un-inoculated seeds. In the same manner, maximum shoot and root length of 5.3 ± 0.66 and 5.83 ± 0.80 cm respectively were observed in seeds co-inoculated with both bacterial isolates without PEG 8000 compared to individually inoculated seeds whose shoot and root lengths were 6.23 ± 0.72 and 6.9 ± 0.85 respectively for *S. pseudovenezuelae*; and 5.5 ± 0.80 and 6.23 ± 0.82 for *A. arilaitensis*, while the least shoot and root length of 4.8 ± 0.53 and 5.23 ± 0.57 was seen in the un-inoculated seedlings. Results also showed that maximum shoot and root length, germination percentage and vigor index of 5.3 ± 0.66, 5.83 ± 0.80, 61.11 ± 3.21 and 678.33 ± 88.06 respectively were observed in seeds co-inoculated with the two bacterial isolates in the presence of 5% PEG 8000 as compared to individual inoculated seeds whose shoot and root length, germination % and vigor index were 4.13 ± 0.61, 4.7 ± 0.56, 55.56 ± 3.21 and 492.78 ± 80.20 respectively for *S. pseudovenezuelae* and 3.73 ± 0.61, 4.57 ±

0.32, 50 ± 3.21 and 417.41 ± 65.00 for *A. arilaitensis* with the shoot and root length, germination % and vigor index (2.5 ± 0.21, 3.17 ± 0.24, 42.59 ± 3.70 and 243.7 ± 31.73) observed in un-inoculated seeds treated with 5% PEG 8000.

Plant growth promoting rhizobacteria capable of colonizing both the surface and inner parts of plant roots play essential roles that directly or indirectly influence plant growth and development (Gerhardt et al., 2009). In this study, maize seed treatment with the two selected bacterial strains *S. pseudovenezuelae* and *A. arilaitensis* significantly improved the emergence and growth of the seedlings. Different mechanisms have been proposed for the promotion of plant growth by PGPR, which include the indirect enhancement of seed germination and vigor index by reduction in the incidence of seed mycoflora that can negatively affect plant growth (Begum et al., 2012). In a study by Duarah et al. (2011), amylase activity was increased during rice and legume germination after treatment with PGPR. Starch is hydrolyzed by amylase to metabolizable sugars to provide the roots and shoots of germinating seeds with the energy to grow. The production of phytohormones such as IAA is another commonly reported mechanism of plant growth promotion (Patten and Glick, 2002). In this study, all tested isolates produced IAA. Studies have shown that several PGPR produce IAA (Banerjee et al., 2010; Ng et al., 2012; Zahid, 2015). IAA promotes root development and nutrient uptake making it a very important mechanism of plant growth promotion.

3.4.8 EFFECT OF BACTERIAL INOCULATION ON DROUGHT TOLERANCE IN MAIZE

Results from the greenhouse study are shown in Tables 3.3 and 3.4. The study showed that the plants inoculated with both bacterial isolates (*S. pseudovenezuelae* and *A. arilaitensis*) produced better chlorophyll content index (CCI) of 10.85 ± 0.87 µ/g compared to the un-inoculated bacteria whose CCI was 8.17 ± 0.52 µ/g at field capacity. For the plants that received moderate water, the inoculated plants also performed better than the un-inoculated ones as the highest CCI (8.27 ± 0.35 µ/g) at moderately wet category was observed in the plants co-inoculated with the two bacterial isolates while the lowest (7.37 ± 0.38 µ/g) was seen in the un-inoculated plants (Table 3.3). The results obtained for CCI on the completely drought stressed plants revealed that better CCI values were obtained by the inoculated plants than the un-inoculated plants as the co-inoculated plants produced CCI values of 7.13 ± 0.19 µ/g, the singly inoculated plants (BS and BR) produced CCI

values of 6.72 ± 0.19 μ/g and 6.37 ± 0.33 μ/g respectively while the un-inoculated plants produced a CCI of 5.65 ± 0.29 μ/g.

Table 3.2: Seed germination test

Treatment	S. length (cm)	R. length (cm)	Germ %	V. index
C1	4.8 ± 0.53^{cd}	5.23 ± 0.57^{de}	66.67 ± 3.21^{d}	675.56 ± 102.16^{d}
C2	2.5 ± 0.21^{f}	3.17 ± 0.24^{f}	42.59 ± 3.70^{g}	243.7 ± 31.73^{f}
M1	9.27 ± 0.99^{a}	10.17 ± 0.84^{a}	94.44 ± 3.21^{a}	1825.93 ± 133.74^{a}
M2	5.3 ± 0.66^{c}	5.83 ± 0.80^{cd}	61.11 ± 3.21^{de}	678.33 ± 88.06^{d}
R 15	$5.5 \pm 0.80b^{c}$	6.23 ± 0.82^{bc}	75.92 ± 1.85^{c}	888.33 ± 118.10^{c}
R15+PEG	3.73 ± 0.61^{e}	4.57 ± 0.32^{e}	50 ± 3.21^{f}	417.41 ± 65.00^{e}
S20	6.23 ± 0.72^{b}	6.9 ± 0.85^{b}	83.33 ± 3.12^{b}	1086.3 ± 105.60^{b}
S20+PEG	4.13 ± 0.61^{de}	4.7 ± 0.56^{e}	55.56 ± 3.21^{ef}	492.78 ± 80.20^{e}

Where: C1 = un-inoculated seeds; C2 = un-inoculated seeds + PEG 8000; M1 = seeds + bacterial isolates S20 + R15 (S20 = *S. pseudovenezuelae* and R15 = *A. arilaitensis*); M2 = seeds + bacterial isolates S20 and R15 + PEG 8000; S20 = seeds + bacterial isolate S20, S20 + PEG = seeds + bacterial isolate S20 + PEG 8000; R15 = seeds + bacterial isolate R15 and R15 + PEG = seeds + bacterial isolate R15 + PEG 8000, S. length = shoot length and R. length = root length. All values are means of triplicate determinations ± S.E. Means followed by the same letters are not significantly different at $P \leq 0.05$ according to New Duncan's Multiple Range Test (DMRT).

From Table 3.3, the greatest increases in plant heights (relative to the control) were observed in plants that received water at field capacity, followed by the moderately wet plants while the lowest increase was observed in those plants that did not receive water at all (completely dry). At field capacity, the highest height of 83.23 ± 2.37 cm was observed in the plants inoculated with the two bacterial strains while the lowest height of 71 ± 2.64 cm was observed in the uninoculated plants at field capacity. For the moderately wet category, the highest height of 70.87 ± 1.96 cm was observed in the co-inoculated plants followed by the individually inoculated plants whose heights

were 69.93 ± 2.0 cm for plants inoculated with *S. pseudovenezuelae* and 67.65 ± 2.26 cm for plants inoculated with *A. arilaitensis* while the lowest height of 58.80 ± 2.99 cm was observed in the uninoculated plants at this water level. Results at the no water level revealed that better plant height of 64.10 ± 2.40 cm was observed in plants whose seeds were co-inoculated with the two bacterial strains followed by plants whose seeds were singly inoculated by either *S. pseudovenezuelae* (63.07 ± 2.44 cm) or *A. arilaitensis* (62.80 ± 2.79 cm) while the lowest heights of 46.95 ± 2.52 cm was observed in the plants whose seeds were un-inoculated.

Results on the root lengths followed the same trend, as the longest root was observed in plants whose seeds were co-inoculated with *S. pseudovenezuelae* and *A. arilaitensis* with root length of 65.67 ± 11.12 cm at field capacity, 43.13 ± 7.16 cm at moderately wet level and 34.42 ± 4.86 cm at the completely dry level. On the other hand, lower root lengths were observed in the un-inoculated plants as lengths of 43 ± 7.63 cm was observed at field capacity, 34.86 ± 5.44 cm at moderately wet and 19.43 ± 1.69 cm at the completely dry level.

The number of leaves on each plant also varied according to treatment as more leaves were observed in the inoculated plants than in the un-inoculated ones. Plants inoculated with the combination of the two bacterial isolates produced approximately 11 ± 0.33 leaves per plant at field capacity, 10 ± 0.33 leaves per plant at moderately wet capacity and 10 ± 0.22 at drought stress capacity. On the other hand, significantly lower leaf number were observed per plant in the un-inoculated plants at moderately wet (10 ± 0.34) and drought stressed (8 ± 0.31) levels. However, there was no significant difference in the number of leaves obtained at field capacity for both inoculated and un-inoculated plants, as approximately 11 leaves per plant were observed at this water level for both treatments. Leaf area of 3991.3 ± 491.67 cm^2 was observed in the co-inoculated plants at field capacity, 2801.6 ± 362.7 cm^2 at moderately wet capacity and 2074.7 ± 258.17 cm^2 was observed in these plants when there was no water application at all. For the un-inoculated plants, total leaf area per plant was 2887.2 ± 422.10 cm^2 at field capacity, 2108.3 ± 323 cm^2 at moderately wet capacity and 1012 ± 218.23 cm^2 at zero water capacity.

Significant differences in dry shoot weights were observed in this study (Table 3.3). Co-inoculated plants produced higher shoot weights of 10. 77 ± 0.67 g at field capacity, 6.13 ± 0.41 g at moderate water application and 4.0 ± 0.31 g at zero water application while lower shoot weights were

observed in un-inoculated plants as weights of 5.92 ± 0.47 g were obtained at field capacity, 4.23 ± 0.37 g at moderately wet and 1.67 ± 0.16 g at zero water level. Higher dry root weights were also observed in the inoculated plants than the un-inoculated plants as the highest weight of 9.027 ± 1.99 g was observed in the co-inoculated plants at field capacity level while the lowest weights of 4.67 ± 0.54 g were observed in the un-inoculated plants at this capacity. At moderately wet capacity, 4.38 ± 0.60 g was observed in co-inoculated plants while 3.05 ± 0.61 g was observed in un-inoculated plants and at zero water level, the highest dry root weight was observed in co-inoculated plants as 2.75 ± 0.35 g while the lowest was observed in the un-inoculated plants as 1.15 ± 0.19 g.

All results obtained showed that physiological parameters were enhanced by the inoculation with both bacterial isolates, as well as with individual isolates, as better growths were observed in plants that were inoculated than the ones that were not inoculated. This confirms the effectiveness of bacteria inoculation on growth and drought tolerance in plants.

Table 3.3: Effect of bacterial inoculation on growth parameter measurement of well-watered (FC), moderately watered (MW) and drought stressed (DS) maize plants

Treatment	Chlorophyll content index (CCI) (µ/g)	Plant height (cm)	Root length (cm)	Number of leaves per plant	Leaf area (cm^2)	Dry shoot weight (g)	Dry root weight (g)
S (FC)	$8.17 \pm 0.52^{d-h}$	71.00 ± 2.64^{cde}	$43.00 \pm 7.63^{c-f}$	$11 \pm 0.34^{a-d}$	2887.2 ± 422.10^{de}	5.92 ± 0.47^{de}	$4.67 \pm 0.54^{d-g}$
S (Mw)	6.98 ± 0.38^{ijk}	58.80 ± 2.99^{ij}	$34.86 \pm 5.44^{f-i}$	10 ± 0.34^{fg}	2108.3 ± 323^{ghi}	$4.23 \pm 0.37^{g-k}$	$3.05 \pm 0.61^{g-k}$
S (DS)	5.65 ± 0.29^{l}	46.95 ± 2.52^{k}	19.43 ± 1.69^{jk}	8 ± 0.31^{i}	1012 ± 218.23^{k}	1.67 ± 0.16^{m}	1.15 ± 0.19^{m}
S+V (FC)	$8.45 \pm 0.38^{c-f}$	71.5 ± 2.02^{cde}	45.77 ± 7.29^{cde}	11 ± 0.17^{abc}	2952.3 ± 409.34^{cd}	6.12 ± 0.44^{de}	4.75 ± 0.53^{def}
S+V (Mw)	$7.37 \pm 0.18^{f-j}$	67.12 ± 2.47^{efg}	$37.98 \pm 4.92^{e-h}$	10 ± 0.17^{def}	2132.6 ± 399.87^{ghi}	$4.47 \pm 0.40^{f-j}$	$3.38 \pm 0.59^{f-k}$
S+V (DS)	6.03 ± 0.30^{kl}	54.88 ± 2.80^{j}	22.43 ± 1.49^{jk}	9 ± 0.21^{h}	1183.0 ± 235.03^{k}	2.4 ± 0.33^{lm}	1.57 ± 0.20^{lm}
S+BS (FC)	$8.77 \pm 0.59^{b-e}$	74.72 ± 2.52^{bc}	51.37 ± 9.49^{bcd}	11 ± 0.17^{abc}	3346.2 ± 350.36^{bc}	7.18 ± 0.42^{cd}	5.57 ± 1.03^{cde}
S+BS (Mw)	$7.5 \pm 0.43^{f-i}$	$66.4 \pm 2.05^{e-h}$	39.03 ± 6.46^{efg}	10 ± 0.17^{def}	244.3 ± 332.45^{efg}	$4.83 \pm 0.46^{e-i}$	$3.62 \pm 0.70^{f-j}$
S+BS (DS)	6.29 ± 0.32^{jkl}	60.27 ± 2.95^{hij}	26.05 ± 3.42^{ijk}	9 ± 0.21^{gh}	1710.2 ± 213.60^{ij}	3.0 ± 0.42^{kl}	$1.87 \pm 0.32^{k-m}$
S+BS+V (FC)	9.48 ± 0.50^{bc}	78.88 ± 2.31^{ab}	58.43 ± 9.84^{ab}	11 ± 0.17^{abc}	3613.6 ± 447.49^{ab}	8.05 ± 0.44^{bc}	6.78 ± 1.07^{bc}
S+BS+V (Mw)	$8.12 \pm 0.34^{d-h}$	$69.93 \pm 2.0^{c-f}$	41.42 ± 5.95^{def}	$10 \pm 0.17^{c-f}$	2646.3 ± 282.95^{def}	5.38 ± 0.42^{efg}	$3.98 \pm 0.64^{f-i}$
S+BS+V (DS)	6.72 ± 0.19^{ijk}	63.07 ± 2.44^{ghi}	$30.28 \pm 3.52^{g-i}$	10 ± 0.21^{fg}	1983.6 ± 196.65^{hi}	$3.55 \pm 0.32^{i-l}$	$2.45 \pm 0.36^{i-m}$
S+BR (FC)	$8.47 \pm 0.65^{c-f}$	73.98 ± 5.18^{bcd}	47.07 ± 8.88^{cde}	$11 \pm 0.50^{a-d}$	2.989 ± 532.92^{cd}	6.18 ± 0.77^{de}	4.80 ± 0.81^{def}
S+BR (Mw)	$7.32 \pm 0.44^{g-j}$	63.10 ± 4.04^{ghi}	$37.55 \pm 6.15^{e-h}$	10 ± 0.33^{efg}	2265.6 ± 417.39^{fgh}	$4.35 \pm 0.62^{g-j}$	$3.40 \pm 0.67^{f-k}$
S+BR (DS)	6.10 ± 0.38^{kl}	55.28 ± 4.18^{j}	23.10 ± 2.01^{jk}	9 ± 0.33^{h}	1378 ± 241.38^{jk}	2.431 ± 0.43^{m}	1.63 ± 0.311^{m}
S+BR+V (FC)	8.92 ± 0.66^{bcd}	75.30 ± 3.06^{bc}	52.27 ± 9.37^{bc}	11 ± 0.17^{abc}	3433.1 ± 446.95^{b}	7.1 ± 0.74^{cd}	5.93 ± 1.03^{bcd}
S+BR+V (Mw)	$7.68 \pm 0.43^{e-h}$	$67.65 \pm 2.26^{d-g}$	40.15 ± 6.62^{efg}	10 ± 0.26^{def}	2476.6 ± 349.10^{efg}	$5.13 \pm 0.45^{e-h}$	$3.75 \pm 0.70^{f-i}$
S+BR+V (DS)	6.37 ± 0.33^{jkl}	62.80 ± 2.79^{ghi}	$28.60 \pm 3.53^{h-k}$	9 ± 0.26^{gh}	1771.7 ± 271.21^{ij}	3.3 ± 0.35^{jkl}	$2.05 \pm 0.36^{j-m}$
S+BR+BS (FC)	9.77 ± 0.60^{b}	79.60 ± 2.30^{ab}	62.02 ± 9.97^{a}	11 ± 0.17^{ab}	3761.8 ± 521.12^{ab}	8.47 ± 1.22^{b}	7.37 ± 1.02^{b}
S+BR+BS (Mw)	$8.17 \pm 0.36^{d-h}$	$70.40 \pm 2.20^{c-f}$	$42.20 \pm 6.46^{c-f}$	$10 \pm 0.26^{c-f}$	2669.2 ± 326.88^{def}	5.72 ± 0.66^{ef}	$4.38 \pm 0.60^{d-h}$
S+BR+BS (DS)	6.83 ± 0.24^{ijk}	$63.70 \pm 2.09^{f-i}$	$33.43 \pm 4.38^{f-i}$	10 ± 0.22^{fg}	1986.7 ± 246.42^{hi}	$3.62 \pm 0.50^{i-l}$	$2.82 \pm 0.32^{h-k}$
S+BR+BS+V (FC)	10.85 ± 0.87^{a}	83.23 ± 2.37^{a}	65.67 ± 11.12^{a}	11 ± 0.33^{a}	3991.3 ± 491.67^{a}	10.77 ± 0.67^{a}	9.027 ± 1.99^{a}
S+BR+BS+V (Mw)	$8.27 \pm 0.35^{d-g}$	70.87 ± 1.96^{cde}	$43.13 \pm 7.16^{c-f}$	$10 \pm 0.33^{b-e}$	2801.6 ± 362.73^{de}	6.13 ± 0.41^{de}	$4.33 \pm 0.65^{f-i}$
S+BR+BS+V (DS)	7.13 ± 0.19^{ijk}	$64.10 \pm 2.40^{f-i}$	$34.42 \pm 4.86^{f-i}$	10 ± 0.22^{efg}	2074.7 ± 258.17^{ghi}	$4.0 \pm 0.31^{h-k}$	$2.75 \pm 0.35^{h-k}$

Legend: S= Maize seed, V= vermiculite coated, BS = bacteria S20 (*S. pseudovenezuelae*), BR = bacteria R15 (*A. arilaitensis*), FC= field capacity, Mw = moderately wet and DS = drought stressed (completely dry). All values are means of triplicate determinations ± S.E. Means followed by the same letters are not significantly different at $P \leq 0.05$ according to New Duncan's Multiple Range Test (DMRT).

The mean data on the effect of inoculation method on the growth of plants are presented in Table 3.4. The effect of the two inoculation methods used in the study showed that greater growth parameters were observed in plants whose seeds were bound with carboxylmethyl cellulose and coated with vermiculite than the directly inoculated plants. From the results, the co-inoculation of *S. pseudovenezuelae* and *A. arilaitensis* with vermiculite coating produced higher CCI value (μ/g) of 8.75 compared to 8.26 obtained when the two bacterial isolates were directly inoculated with both isolates. Similarly, plants whose seeds were inoculated with only *S. pseudovenezuelae* with vermiculite coating gave a CCI of 8.1 μ/g while plants inoculated directly with *S. pseudovenezuelae* gave a CCI value of 7.52 μ/g. Improved CCI was also observed in plants whose seeds were inoculated with *A. arilaitensis* with vermiculite coating (7.66 μ/g) compared to the plants whose seeds were directly inoculated with the isolate (7.2 μ/g). For the control seeds, results showed that better CCI value was observed in plants whose seeds were immersed in 1% CMC and coated with vermiculite as a CCI value of 7.28 μ/g was observed in them while the CCI value of 6.93 μ/g was obtained for plants whose seeds were only immersed in distilled water. The data on plant height, root length, number of leaves per plant, leaf area, dry shoot and root weight also revealed that for all the treatments, plants whose seeds were immersed in 1% CMC and coated with vermiculite were better in terms of growth and all the parameters measured than the plants whose seeds were either directly inoculated with bacteria or distilled water.

Table 3.4: Effect of inoculation method on growth parameter measurements of maize plants

Treatments	Chlorophyll content (CCI) (μ/g)	Plant height (cm)	Root length (cm)	No. of leaves/ plant	Leaf area (cm^2)	Dry shoot weight (g)	Dry root weight (g)
S	6.93^{e}	60.31^{e}	32.37^{d}	9^{e}	2002.5^{d}	3.94^{e}	2.96^{e}
S+V	7.28^{de}	62.78^{de}	35.39^{cd}	10^{d}	2101.5^{d}	4.30^{ed}	3.25^{de}
S+BS	7.52^{de}	66.64^{c}	38.82^{bc}	10^{bc}	2500^{c}	5.01^{cd}	3.68^{cde}
S+BS+V	8.11^{bc}	70.78^{ab}	43.38^{ab}	10^{ab}	2748.9^{ab}	5.77^{b}	4.41^{bc}
S+BR	7.2^{de}	65.46^{cd}	35.91^{cd}	10^{cd}	2198.7^{d}	4.35^{ed}	3.26^{de}
S+BR+V	7.66^{cd}	67.74^{bc}	40.34^{bc}	10^{cd}	2560.5^{bc}	5.18^{bc}	3.91^{cd}
S+BR+BS	8.26^{ab}	71.33^{a}	45.88^{ab}	10^{abc}	2804.9^{a}	5.82^{b}	4.8^{ab}
S+BR+BS+V	8.75^{a}	72.81^{a}	47.74^{a}	10^{a}	2955.8^{a}	7.0^{a}	5.36^{a}

where: S = maize seed, V = vermiculite coated, BS = bacteria S20 (*S. pseudovenezuelae*), BR = bacteria R15 (*A. arilaitensis*). All values are means of triplicate determinations. Means followed by the same letters are not significantly different at $P \leq 0.05$ according to New Duncan's Multiple Range Test (DMRT).

Drought stress is a serious environmental problem in agriculture as it causes severe loss in plant yield, depending on its severity. (Farooq et al., 2009). In this study, maize survival and growth was affected when drought stress was introduced. However, under drought stress conditions, better growth was observed in bacterial inoculated maize plants than the un-inoculated plants as better survival, dry root and shoot weight, root and shoot length and chlorophyll content were observed. Over the years, PGPR have been used mostly to promote plant growth because of their ability to stimulate plant growth through certain mechanisms such as the production of plant growth regulators and fixation of nitrogen (Lucy et al., 2004). Studies have demonstrated other beneficial effects of PGPR on plants including their ability to enhance tolerance toward several abiotic stresses such as drought (Yang et al., 2009; Wang et al., 2012; Yandigeri et al., 2012; Vurukonda et al., 2016). A study by Creus et al. (2004) demonstrated that there was significant increase in growth, water content, water potential, relative water content and apoplastic water function in roots and shoots of wheat plants primed with *Azospirillum brasilense* Sp245 compared to un-primed plants. In addition, Pereyra et al. (2012) reported that inoculation of *Azospirillum* on maize

seedlings under osmotic stress enhanced better water status of the seedlings indicated by the morphological modifications of the coleoptile xylem architecture. Their results were also attributed to *Azospirillum* ability to produce IAA and improved bacterial IAA synthesis.

A number of abiotic stresses are associated with ROS species accumulation in plant cells. Reactive oxygen species react with DNA, membrane lipids and proteins and are capable of causing severe oxidative damage to plant tissues (Reddy et al., 2004). For plants to be able to survive under drought stress, they need to avoid oxidative damage. These species can be removed by certain enzymes: catalase and peroxidases such as glutathione peroxidase (GPX) and ascorbate peroxidase (APX) (Gong et al., 2005). In the preceding study (chapter 2), *S. pseudovenezuelae* and *A. arilaitensis* amplified the specific gene primer glutathione peroxidase which encodes proteins involved in drought tolerance, indicating their capability of withstanding drought stress by the avoidance of oxidative damage. This could also be the reason behind the greener leaves of the inoculated plants over the un-inoculated ones. We also observed that the severity of drought stress showed more on the un-inoculated plants than the inoculated plants as the un-inoculated plants showed more signs of wilting than the inoculated plants. The increase in the severity of drought stress causes increase in enzyme (GPX and APX) activity. Koussevitzky et al. (2008) demonstrated that APX activity and APXI transcript levels were increased in *Arabidopsis thaliana* plants after their exposure to drought stress. They concluded that APX is necessary to protect plant chloroplast from increased levels of ROS during drought as APX helps in scavenging ROS. Omar et al. (2009) also recorded lower peroxidase and catalase activities in the leaves of barley plants primed with *Azospirillum brasilense* under salinity stress.

Damage to plant proteins often results from stress exposure, therefore it is necessary to maintain proteins in their functional forms to enable plants to survive under stress conditions (Wang and Huang, 2004). Plant proteins like the Heat-shock proteins (HSP), Malic-enzyme proteins (ME), glycine-rich RNA binding proteins (GRP) and desiccation protectant proteins are often synthesized during stress conditions and are recognized as mechanisms of stress tolerance in plants (Wahid et al., 2007). They play major roles in translocation, protein folding, degradation and assembly in several cellular processes. They can also assist in stabilizing and refolding of proteins under different conditions of stress (Wang and Huang, 2004). From the preceding study on drought tolerant genes, *S. pseudovenezuelae* and *A. arilaitensis* amplified the gene primers: glycine-rich

RNA binding protein, desiccation protectant protein and Malic-enzyme and this could have also contributed to their better survival under drought stress.

The growth of plants depends highly on differentiation, enlargement and cell division. Also, drought stress affects the physiological, morphological, ecological and genetic processes of plant growth (Taiz and Zeiger, 2002; Farooq et al., 2009; Vurukonda et al., 2016). According to Farooq et al. (2009), severe water limitation causes an inhibition in cell elongation as a result of water flow interruption from the xylem to surrounding elongation cells leading to cell mitosis and cell expansion; and finally resulting in reduced plant growth. The growth and formation of lateral roots may be stimulated by PGPB, thereby increasing the capacity of water uptake of inoculated plants. Studies have described the roles of PGPB in modifying plant metabolism under normal and abiotic stress conditions by mechanisms including indole-3-acetic acid production, ACC deaminase activity, nitrogen fixation and antioxidant production (Dimkpa et al., 2009; Bashan and De-Bashan, 2010). Plant growth promoting bacteria are also capable of producing compatible solutes (glycine-betaine and proline) that assist in the processes of osmoregulation (Dimkpa et al., 2009). In the present study, better tolerance to drought stress conditions was observed in bacterial inoculated plants than un-inoculated plants. This could be as a result of the high amounts of the plant growth promoting traits they produced which includes the IAA, ACC deaminase as well as the amplification of the genes encoding proteins involved in drought tolerance (GRP, DSP, GTP and GPX).

Besides the inoculation of plants with single strains of PGPR, co-inoculation or combination of two or more strains also induces drought stress tolerance in plants to an even greater extent (Wang et al., 2012). From the results obtained in the present study as shown in Table 3.3, better tolerance was observed in the co-inoculation of *S. pseudovenezuelae* (MG547870) and *A. arilaitensis* (MG547869) in maize plants as better shoot and root length, dry shoot and root weight, chlorophyll content and numbers of leaves were observed in the plants. Moreover, wilting of leaves was observed to be lower in the co-inoculated plants than those inoculated with either *S. pseudovenezuelae* or *A. arilaitensis*, which were much better than the control. The results from this study are in agreement with the study of Wang et al. (2012), who observed enhanced drought tolerance in cucumber plants when the seeds were inoculated with a Microbial consortia consisting of the PGPR *Bacillus subtilis* SM21, *Bacillus cereus* AR156 and *Serratia sp.* XY21 (BBS).

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According to Wang et al. (2012), darker green leaves, lighter wilting symptoms, relative electrical conductance, increased leaf proline and chlorophyll content and intension of root recovery were observed in BBS treated plants after water was withheld for 13 days. In a similar study, exopolysaccharide producing bacterial strains *Proteus penneri* (Pp1), *Pseudomonas aeruginosa* (Pa2) and *Alcaligenes faecalis* (AF3) exhibited better tolerance to drought stress in maize compared to individual PGPR strains (Naseem and Bano, 2014).

The effect of inoculation method on the growth and drought tolerance potential of *S. pseudovenezuelae* (MG547870) and *A. arilaitensis* (MG547869) in maize seeds are shown in Table 3.4. Results obtained revealed that plants whose seeds were co-inoculated with *S. pseudovenezuelae* and *A. arilaitensis* and coated with vermiculite showed better increase in growth parameter measurements compared to the plants whose seeds were directly co-inoculated with the bacterial isolates. Similarly, better growth was observed when seeds were un-inoculated but coated with vermiculite compared to the plants with seeds merely immersed in distilled water. In all, seed inoculation with the combination of the two bacterial strains was more effective than inoculation with individual bacterial strains. The better results obtained from the growth parameter measurements of the vermiculite coated seeds as compared to the un-coated seeds of both inoculated and un-inoculated seeds on the effect of inoculation method used in this study could have been due to the presence of 1% Carboxylmethyl cellulose (CMC), as this adhesive may have facilitated the better binding of the bacterial isolates to the seeds. Carboxymethyl cellulose is an adhesive that has also been used in drug and food industries (Williams and Phillips, 2004; Delcour and Poutanen, 2013; Ibarra et al., 2016). It plays major roles in binding inoculants to seeds and also protects the seeds from desiccation. It may also provide nourishment for the inoculated plants (Elegba and Rennie, 1984). Also, the coating of the seeds with vermiculite may have helped to protect the seeds from possible insect and pathogen attacks. From the results obtained from the present study, it is encouraged that for efficient tolerance to drought stress, inoculated seeds should be bound as well as coated with good binding and coating agents as this will reduce pest attacks, preserve seeds for longer periods and enhance easy delivery.

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CHAPTER FOUR

GENERAL CONCLUSION AND FUTURE RESEARCH PROSPECTS

Changing climatic conditions can bring about undesirable environmental conditions, including drought which is responsible for several physiological and morphological changes in plants and causing mass decrease in agricultural productivity. Soil harbours numerous bacteria that can be beneficial in agriculture to facilitate growth and abiotic stress tolerances in plants. Plant growth promoting rhizobacteria facilitate plants' growth and help them to resist and adapt to harsh and dry environmental conditions (drought), and could thereby play a major role in solving the problem of global food insecurity. Tolerance to drought stress by PGPB can be enhanced through a variety of mechanisms ranging from phytohormonal modifications, ACC deaminase activity, to alteration in root morphology and molecular techniques (Hardoim et al., 2008; Fahad et al., 2015; Kaushal and Wani, 2016). Several bacteria have been isolated and used to improve stress tolerance in a variety of crops, but with only little knowledge on the role of the genus actinomycetes in eliciting tolerance to drought stress in maize. This therefore made it necessary to isolate, characterize and identify actinomycetes from dry maize plant with the intention of evaluating their plant growth promoting characteristics as well as their ability to enhance drought tolerance in maize plants.

From the present study, seven actinomycetes strains were successfully isolated and characterized from maize rhizospheric soils using culture dependent techniques. The results obtained showed that all the bacterial isolates used in this study showed tolerance to 5% polyethylene glycol (PEG) 8000. As seen in the results, both concentration of PEG and time (culture age) affected growth of bacteria in PEG containing medium. The effect of pH, temperature and salinity on bacterial growth revealed that pH 5 and 9, temperature range of between 25 and 35°C, and 0% and 4% NaCl concentration favoured bacterial growth.

Malik et al. (2008) reported that only 1% of soil bacteria can be cultured using culture-dependent techniques. With the use of culture-independent techniques such as temperature gradient gel electrophoresis (TGGE) and *fluorescence in situ hybridization* (FISH), more actinomycetes species would have been isolated.

The growth of all tested bacterial isolates on different concentrations of PEG 8000, pH, temperatures and saline conditions indicates their potential for possible use as drought stress

alleviators in plants. However, there is need for further clarification on the precise tolerance mechanisms employed by the bacteria to resist both drought and salinity stresses. Furthermore, molecular techniques such as Scanning electron microscopy (SEM) should be used to further characterize the bacterial strains. There is also the need to evaluate the effect of bacterial combinations, such as bacteria from different genera or probably with fungi on drought stress tolerance.

The amplification of the drought tolerant genes *GPX*, *DP*, *ME* and *GRP* was performed from genomic DNA template fractions from all bacterial strains using conventional PCR machine. However, no sequencing analysis was performed to match them with available drought tolerant genes in GenBank database, hence the need for further investigation. Also, new technologies such as real time-polymerase chain reaction (RT-PCR) could be used for better results and also to quantify the levels at which these genes were expressed. The products obtained from drought tolerant genes expressions could also be used in the engineering of new drought tolerant bacteria as well as in the construction of biosensors.

Most drought tolerant bacterial isolates exhibited multiple PGP properties, both qualitatively and quantitatively. However, the bacterial isolates *Streptomyces pseudovenezuelae* (MG547870) and *Arthrobacter arilaitensis* (MG547869) were chosen for drought tolerance studies in the greenhouse due to their outstanding ability to grow both on the highest concentrations of PEG 8000 and their outstanding plant growth promotion abilities. The effects of individual and combined inoculation of these actinomycetes strains on growth and drought tolerance in maize plants were experimented. The inoculation of bacteria improved seed germination of maize with or without PEG 8000 as compared to un-inoculated seeds. Maximum germinated percentage was seen in seeds treated with a combination of the two bacterial strains (*S. pseudovenezuelae* and *A. arilaitensis*) while the lowest percent germination was seen in the un-inoculated seeds in 5% PEG 8000. A greenhouse experiment evaluated the effect of inoculation of *S. pseudovenezuelae* and *A. arilaitensis* on growth and drought tolerance in maize. Results obtained revealed that the combination of both bacteria strains produced better results compared to inoculation with individual strains. At field capacity, moderately wet and total drought levels, co-inoculated plants showed significant increase in chlorophyll content, shoot and root length, number of leaves and leaf area per plant, and in shoot and root dry weight. For individual inoculations, maximum growth

parameters were observed in plants inoculated with *S. pseudovenezuelae* compared to plants inoculated with *A. arilaitensis*, while there was a significant decrease in the growth of un-inoculated plants, especially at the total drought level when water application was withheld for 15 days. The effect of inoculation method on the growth and drought tolerance potential of these bacterial isolates on maize revealed that plants whose seeds were co-inoculated with *S. pseudovenezuelae* and *A. arilaitensis* and coated with vermiculite gave better increase in the measured growth parameters compared to the plants whose seeds were directly inoculated with the bacterial isolates. Similarly, better growth was also observed in those whose seeds were un-inoculated but coated with vermiculite compared to the plants whose seeds were merely immersed in distilled water. Based on the results obtained from the greenhouse study, it is therefore recommended that for effective drought stress tolerance, maize seeds inoculation with the two bacterial isolates and subsequent suspension in 1% CMC solution and vermiculite coating is necessary to ensure proper packaging of seeds as well as to protect the seeds from possible pathogen and insect attacks.

Drought stress tolerance by bacteria is an emerging technology that is cost effective and efficient to help solve the problem of low crop productivity and yield. Results of this study have proven that the inoculation of drought tolerant *S. pseudovenezuelae* (MG547870) and *A. arilaitensis* (MG547869) increases plant growth as well as reducing the undesirable effect of drought stress on the tested plant. Nonetheless, further studies need to be undertaken to understand the exact molecular mechanisms of plant-bacteria interactions in the rhizosphere for plant growth and drought tolerance, as further understanding of these mechanisms is necessary for efficient elicitation of drought stress in plants by soil bacteria. Furthermore, in spite of the recent progress in bacteria mediated tolerance to drought stress in plants, the mechanisms employed by PGPR to impart drought tolerance in plants are not clearly understood, therefore more research work is required in this area to give new insights for better agricultural productivity. Hence our recommendations for future research works are as follows:

1. To assess the root colonization of the maize plants by the bacterial strains *S. pseudovenezuelae* (MG547870) and *A. arilaitensis* (MG547869)

2. To test the effects of inoculation of the drought tolerant bacterial strains *S. pseudovenezuelae* (MG547870) and *A. arilaitensis* (MG547869) under field conditions in different agro-climatic zones
3. To study their effects on different plants species.

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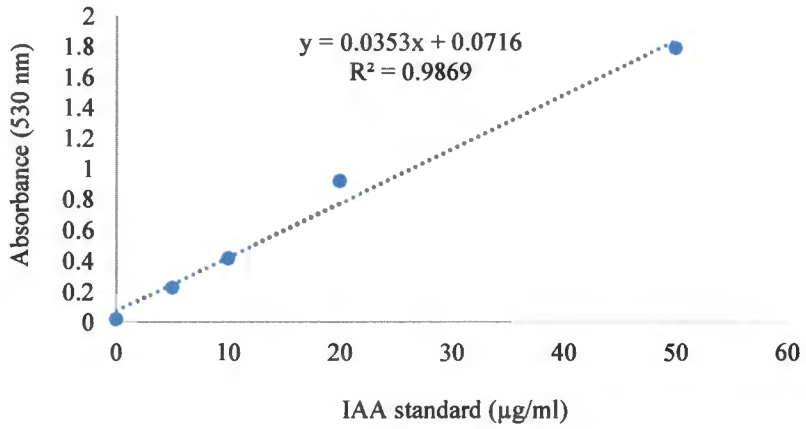
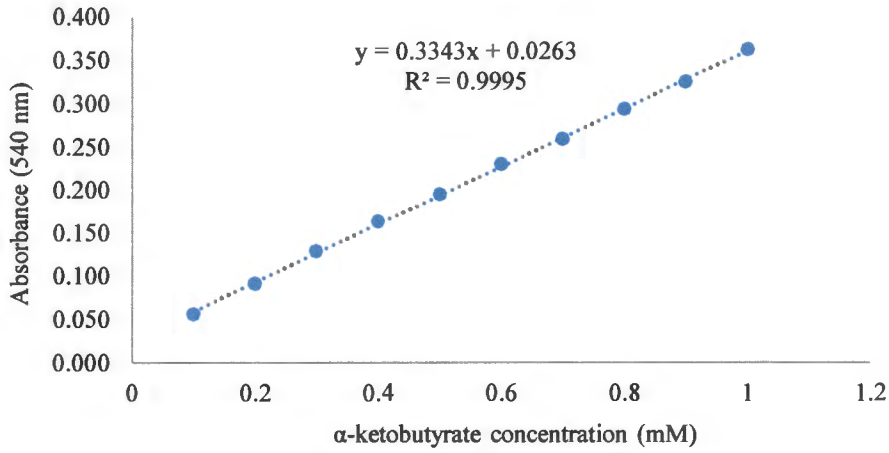
APPENDIX

Nanodrop readings of DNA concentrations of actinomycetes isolates

Isolate	DNA Quantity (ng/µL)
S20	213.1
R11	72.9
R15	101.2
S7	79.4
S11	230.2
S12	126.2
S4	191.0



Standard Curve for IAA Prduction by bacterial isolates



Concentration of α-ketobutyrate in samples

ISP Medium 1: Tryptone-yeast extract broth

Constituents	g/l
Bacto-Tryptone	5.0
Bacto-Yeast Extract	3.0
Distilled water	1.0 l

pH 7.0 - 7.2 before autoclaving

ISP Medium 2: Yeast extract-malt extract agar

Composition	g/l
Bacto-Yeast Extract (Difco)	4.0
Bacto-Malt Extract (Difco)	10.0
Bacto-Dextrose (Difco)	4.0
Distilled water	1.0
Adjust to pH 7.,3, then add	
Bacto agar	20.0
Liquefy agar by steaming at 100" C f o r 15-20 minutes.	

Pridham and Gottlieb trace and Basal Mineral salts (ISP-medium 9)

Composition	**g/l**
Pridham and Gottlieb trace salts	
$CuSO_4.5H_2O$	0.64
$FeSO_4.H_2O$	0.11
$MnCl_2.4H_2O$	0.79
$ZnSO_4.7H_2O$	0.15
Distilled water	100.0 ml
Basal mineral salts agar	
$(NH_4)2SO_4$	2.64
KH_2PO_4 anhydrous	2.38
$K_2HPO_4.3H2O$	5.65
$MgSO_4.7H2O$	1.00
Pridham and Gottlieb trace salts	
Distilled water	1.0 L
Dissolve ingredients and check pH, adjust to 6.8-7.0 if necessary with 1 N NaOH or 1 HCl	
Agar	15.0

Greenhouse evaluation of drought stress tolerance on maize by *S. pseudovenezuelae* and *A. arilaitensis*.

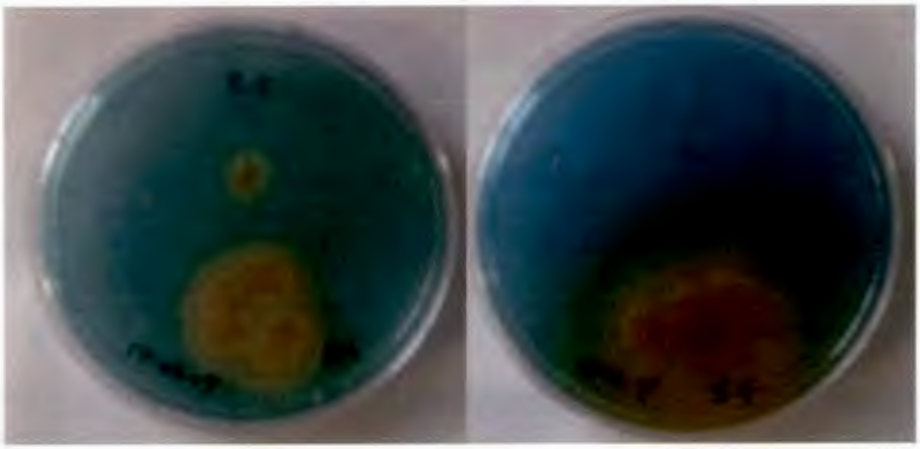


Image showing positive siderophore production by actinomycetes isolates

Image showing phosphate solubilization by actinomycetes isolates

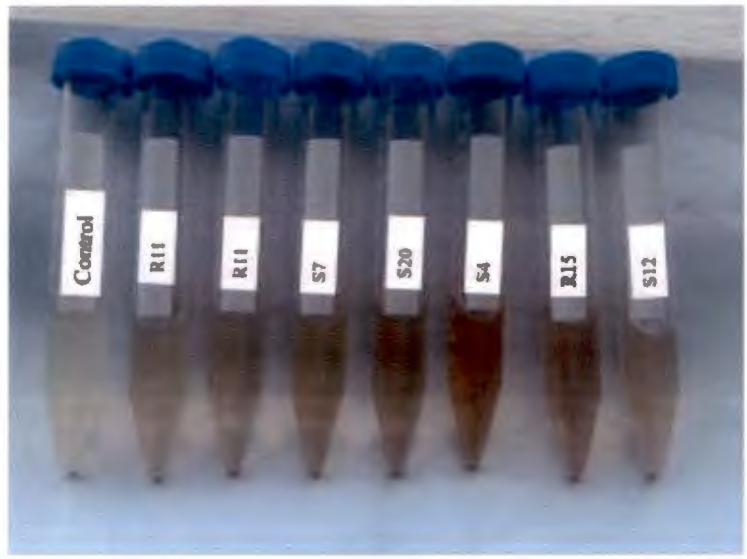


Image showing colorimetric IAA production by bacterial isolates