



Enhancement of soybean tolerance to drought using *Rhizobium* species and mycorrhizal fungi

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DECLARATION

I declare that this thesis titled “Enhancement of soybean (*Glycine max* L) tolerance to drought using *Rhizobium* species and mycorrhizal fungi” is an original output of the research conducted by me under the supervision of Professor Olubukola Oluranti Babalola. Therefore, I, the undersigned, declare that this thesis submitted to North-West University for the award of Doctor of Philosophy in Science, Biology in the Faculty of Natural and Agricultural Sciences has not been previously submitted by me or anyone else at any University in part or entirety for the award of any degree and that all the information obtained from published literatures have been acknowledged accordingly.

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DEDICATION

This work is dedicated to my Heavenly Father, the Giver of wisdom, strength and power.

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
ACC	1-aminocyclopropane-1-carboxyl
ADH	Arginine dihydrolase
AIDS	Acquired Immune Deficiency Syndrome
ALP	Alkaline phosphate
AM	Arbuscular mycorrhizal
AMF	Arbuscular mycorrhizal fungi
AMY	Amygdalin
ANOVA	Analysis of Variance
APX1	ascorbate peroxidase
ARA	Arabinose
ARC	Agricultural Research Council
ARISA	Automated Ribosomal Interspacer Analysis
BB	Beginning of blooming
BP	Beginning of pod development
Bp	Base pair
BGR	Bambara groundnut rhizosphere

BLAST	Basic Local Alignment Search Tool
BLASTn	Basic Local Alignment Search Tool n
BS	Beginning of seed development
CAS	Chrome azurol S
CCI	Chlorophyll content index
CDS	Coding sequences
CFU	Colony forming unit
CIT	Citrate utilization
CP	Continuous cropping
CPS	Capsular polysaccharides
CRD	Completely randomized design
2, 4-DAPG	2, 4-diacetylphloroglucinol
DF	Dworkin and Foster
DNA	Deoxyribonucleic acid
DGGE	Denaturing Gradient Gel Electrophoresis
DW	Dry weight
E	East
EPS	Exopolysaccharide

F	Forward
FAO	Food Agriculture Organization
FC	Field capacity
FISH	Fluorescent <i>in-situ</i> hybridization
FM	Full maturity
FS	Full seed
FW	Fresh weight
GC	Gas chromatography
GEL	Gelatinase
GC	Guanine cytosine
GLU	Glucose
HSPs	heat shock proteins
HTS	High throughput sequencing
IAA	Indole-3-acetic acid
IND	Indole production
INO	Inositol
ISR	Induced systemic resistance
Kb	Kilo-base

KEGG	Kyoto Encyclopedia of Genes and Genomes
LB	Luria Bertani agar
LDC	Lysine decarboxylase
LNF	Level of nitrogen fertilizer
LPS	β -1, 2-glucans and lipopolysaccharides
L0	Electrical conductivity after autoclaving
Lt	Electrical conductivity prior to autoclaving
MAN	Mannitol
MANOVA	Multivariate analyses of variance
Max.	Maximum
MEGA	Molecular Evolutionary Genetics Analysis
MEL	Melibiose
MF	Mycorrhizal fungi
MPN	Most Probable Number
mRNA	Messenger ribonucleic acid
MSIs	Multiple symbiotic impacts
NB	Nutrient broth

NCBI	National Center for Biotechnology Information
ND	Not determined
ND	No detected level
NGS	Next Generation Sequencing
<i>Nif</i>	Nitrogen fixing
Nm	Nanometer
No	Number
NRA	Nitrate reductase activity
NS	Non significant
OD	Optical density
ODC	Ornithine decarboxylase
ONPG	Ortho nitro-phenyl- β -D-galactopyranosidase
OTU	Operation taxonomic unit
P	Probability
PAMPs	Pathogen-associated molecular patterns

PAN	Pannar
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGP	Plant growth promoting
PGPR	Plant growth promoting rhizobacteria
PGPT	Plant growth promoting traits
PSC	Plant for sample collection
PT	Phosphate transporter
qPCR	Quantitative polymerase chain reaction
R	Reverse
R	Round-up Ready
RAST	Rapid Annotation using Subsystem Technology
RCBD	Randomize Complete Block Design
RDPII	Ribosomal Database Project

Rh	Rhizobacteria
RH	Relative humidity
RHA	Rhamnose
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
ROS	Reactive oxygen species
RWC	Relative water content
S	South
SA	South Africa
SAC	Saccharose
SAMS1	S-adenosyl-methionine synthetase
SAS	Statistical analysis system
SDA	Sabouraud dextrose agar
SE	Standard error
SH	Shoot height
SIP	Stable isotope probing
SLF	Standard level of fertilizer

SOR	Sorbitol
sp.	Species
spp.	Species
SPSS	Statistical Package for the Social Sciences
SRA	Sequence read archive
SS	Sewage sludge
STH	Starch hydrolysis
SW	Shoot width
SYM	Symbiotic
TAE	Tris-Acetate-EDTA
TDA	Tryptophan deaminase
Temp.	Temperature
TW	Turgid weight
tRNA	Transfer ribonucleic acid
TX	Texas
VP	Voges Proskauer
URE	Urease
USA	United State of America

UV	Ultra violet
VOCs	Volatile organic compounds
W	Weight
Wt	Weight
YEM	Yeast mannitol agar

GENERAL ABSTRACT

Food security is seriously under threat in many developing tropical countries as agriculture is becoming unsustainable due to drought stress. There is therefore an urgent need for a sustainable means to accomplish food availability in this region. Thus, the aim of this study was to evaluate the ability of new *Rhizobium* spp. and arbuscular mycorrhizal fungi (AMF) to enhance soybean (*Glycine max* L.) tolerance to drought.

In this present study rhizobial strains were isolated from Bambara groundnut (*Vigna subterranea*) rhizospheric soil and their mechanisms in relation to enhancement of plant growth and drought tolerance were evaluated. Isolates were characterized and identified by culture based and molecular techniques as *Rhizobium* sp. strain R1, *Rhizobium tropici* strain R2, *Rhizobium cellulosilyticum* strain R3, *Rhizobium taibaishanense* strain R4 and *Sinorhizobium meliloti* strain R5. Rhizobial strains were positive to almost all the plant growth promoting traits (such as exopolysaccharide, siderophore and indole-acetic-acid) tested. Rhizobial strains also survived and grew under stress conditions imposed by polyethylene glycol (PEG) but *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 showed the highest significant tolerance with optical density (OD) values of 1.35 and 0.32 respectively at a concentration of 30% PEG while *S. meliloti* had the lowest OD value of 0.17 at the same PEG concentration. *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 inoculation of soybean significantly ($P < 0.05$) enhanced seedling shoot dry weights under drought condition imposed by 4% PEG. Thus, genomic insights into *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 revealed the presence of some genes with their respective proteins involved in symbiotic establishment, nitrogen fixation, drought tolerance and plant growth promotion. In particular, *exoX*, *htrA*, *Nif*, *nodA*, *eptA*, *IAA* and siderophore-producing genes were found in the two rhizobial strains. A total of 5773 contigs

with a GC content of 61.91% and a total of 17,408,810 reads with a mean read length of 201.15 were obtained for *Rhizobium* sp. strain R1 while a total of 129 contigs with GC content of 43.59% and total of 17,794,094 reads with a mean read length of 214.18 were found in *R. cellulosilyticum* strain R3.

Rhizobium sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium (MY) were therefore used to inoculate soybean under drought conditions in the greenhouse. A gradient of watering levels ranging from 100 to 40% field capacity (FC) of soil retention capacity of water was tested on non-inoculated soybean plants and plants inoculated with rhizobia and AMF in pot cultures. It was observed that the inoculated soybean plants especially soybean dually inoculated with *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 (R1+R3) as well as *Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium (R1+R3MY) had significant impacts ($P < 0.05$) on soybean leaf relative water content (RWC) and electrolyte leakage respectively. Also, the levels of accumulated soluble sugars and proline revealed that their concentrations increased mainly in microbially amended soybean plants exposed to drought stress (70 and 40% FC) and similar results were observed for chlorophyll content. Plants inoculated with R1+R3MY showed the highest number of spore and % mycorrhization in all the water regimes. At 40% FC, R1+R3MY treatment was found to promote soybean growth since it had significant effects ($P < 0.05$) on soybean shoot width, branch number, and root dry weight compared to the non-inoculated plants. Similarly, under severe drought stress (40% FC), R1+R3MY inoculum had the greatest impacts on soybean pod number, seed number, seed fresh weight, highest seed number per pod and seed dry weight while under 70% water stress, significant impacts ($P < 0.05$) of *Rhizobium* sp. strain R1 and mycorrhizal (R1MY) co-inoculation were observed on pod number, pod fresh weight and seed dry weight.

Rhizobium sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal fungal inoculation of soybean in semi-arid field equally enhanced soybean tolerance to drought stress. Single and dual inoculation of the *Rhizobium* species and mycorrhizal fungi actually increased leaf and shoot RWC and decreased electrolyte leakage and increased soluble sugars and proline accumulation in soybean plants. Rhizobial and mycorrhizal inoculation also increased below-ground and above-ground plant components (such as shoot height and width, leaf number, taproot length, root number and pod number) at different stages of soybean growth but more significant increase ($P<0.05$) was observed in plants dually inoculated with R1+R3MY. In particular, soybean plants amended with R1+R3MY produced seeds with 34.3 g fresh weight, 15.1 g dry weight and 23% crude fat and soybean plants singly inoculated with *Rhizobium* sp. strain R1 (R1) produced more large seeds with 12.03 g dry weight. The non-inoculated (control) seeds contained higher percentage of moisture content compared to the microbially amended seeds. Increase in macro and micronutrient assimilation especially N, P, Mg, S, Ca, Co, Mo, Fe and B in soybean seeds inoculated with *Rhizobium* species and mycorrhizal fungi was also observed. However, co-inoculation with R1MY resulted in a significant increase ($P<0.05$) in almost all the macronutrients (N, P, Mg and Ca).

A study of the bacterial communities of soybean rhizosphere inoculated with R1+R3MY in the field using Next Generation Sequencing showed variations in the richness and abundance of bacteria during soybean growth. Stack bar/area plot and Heatmap clustering analysis unveiled variations among the rhizospheric bacterial communities at the class and order levels respectively. Particularly, *Actinobacteria* was the most abundant bacterial group with the highest reads counts observed at full seed (FS) stage followed by *Proteobacteria* which contained plant growth promoting bacterial species such as *Streptomyces* and *Rhizobium* species. Alpha-diversity

and Bray Curtis Index analyses at the family, genus, species and operational taxonomic units (OTUs) levels similarly showed that bacterial composition and abundance of the rhizosphere changed significantly during soybean growth. These results revealed that rhizosphere bacterial community structured varied at different growth stages of soybean in the field.

In conclusion, this study has revealed two *Rhizobium* species (*Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3) with *exoX*, *htrA*, *Nif*, *nodA*, *eptA*, *IAA* and siderophore-producing genes that were able to enhance soybean tolerance to drought stress even when co-inoculated with mycorrhizal fungi. It is therefore recommended that the availability of the whole genome sequences of *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 strains in public databases may further be exploited to comprehend the interaction of drought tolerant rhizobia with soybean and other legumes and the plant growth promoting ability of these rhizobial strains can also be harnessed for biotechnological application in the field especially in semiarid and arid regions of the globe.

KEYWORDS: biodiversity, biotechnology, drought, genomics, PGPR, soybean

CHAPTER 1

1.0 General Introduction

Two hundred and forty (240) million people in Sub-Saharan Africa, or one person in every four lack adequate food for nearly a healthy and active life, and food prices and drought are pushing many people into poverty and hunger (Bremner, 2012). Also, the world's population has now surpassed 6.9 billion (Tkacz and Poole, 2015) , and journalists in the past have asked “Can we feed the world?” (Bremner, 2012). In order to feed a present population of over 7.6×10^9 , the world will need a new vision for sustainable agriculture and the ultimate goal of sustainable agriculture is to develop farming systems that are productive, profitable, energy conserving, environmentally sound, conserving natural resources, and that ensure food safety and quality (Tkacz and Poole, 2015).

Therefore, the most promising strategy to reach this goal is to substitute hazardous agrochemicals (chemical fertilizers and pesticides) with environmentally friendly preparations of beneficial microorganisms, which can improve the nutrition of crops and also confer protection against biotic (pathogens and pests) and abiotic (drought) stresses (Tkacz and Poole, 2015). In particular, increasing the soil microbial species richness was shown to be useful in enhancing plant health and productivity (Babalola, 2010, Wagg et al., 2011, Hou and Babalola, 2013, Schnitzer et al., 2011) probably through the introduction or inoculation of alien microorganism into the soil. Besides the application of individual microorganism, identification of healthy and functionally diverse microbiome and their application for enhancing crop yield is another big and necessary challenge to venture. This is because, it has been found that the entire soil microbiome is an essential and an indispensable portion which represents the second genome of host plant (Bhattacharyya and Jha, 2012a, Chaparro et al., 2012, Wu et al., 2013).

Furthermore, plants are exposed to environmental stress such as drought during the period of growth and development (Alizadeh et al., 2011) and drought stress is one of the most important abiotic factors limiting plant nutrient uptake and growth (Alizadeh et al., 2011, Tkacz and Poole, 2015). Indeed, the effects of drought on legumes and other agricultural crops have been documented. Legumes such as soybean (*Glycine max* L.) have also been suggested as appropriate crops for the enhancement of plants productivity and the reclamation of low quality arable land because these plants do not only yield nutritious fodder, protein rich seeds and fruits, but they also enrich soil with nitrogen in symbiotic association with *Rhizobium* species (Younesi and Moradi, 2014) and mycorrhizal fungi (MF). Although, the tripartite interactions with arbuscular mycorrhizal fungi (AMF), *Rhizobium* species and soybean are mutualistic symbioses of high economic importance for increasing agricultural productivity (Igiehon and Babalola, 2018a), the question still arises: Are *Rhizobium* species and AMF able to enhance the tolerant ability of soybean under drought conditions?

Soybean is grown massively because of its potential to adapt to different soil and United State of America is the largest producer of this crop followed by Brazil. Soybean is a leguminous plant in the order *Fabales* and family *Leguminosae* with the ability to develop complex symbiotic associations with nitrogen fixing rhizobacteria in their nodules (Rascovan et al., 2016). Legumes are often considered to be the major nitrogen fixing systems as they are able derive about 90% of their nitrogen from atmospheric nitrogen (Rascovan et al., 2016). “Nevertheless, a deeper comprehension of the ecology and biology of their root microbiome could significantly contribute in developing new agronomic/biotechnological tools to promote crop health and growth (Coleman-Derr and Tringe, 2014, Lakshmanan et al., 2014)”.

Moreover, availability of nutrient is important to agricultural crop development such as soybean and this is affected by many factors such as soil physicochemical properties, climate change and crop cultivar. Crop cultivation in tropical environments needs high amount of conventional chemical fertilizers for plant growth (Miransari, 2011) and the usage of chemical fertilizer has negative effects on environmental receptors such as underground water and soil microorganisms. The challenge of sustainable agriculture is finding alternative for chemical fertilizers for plant growth and this feat may be achieved by the use of beneficial soil microorganisms (Cely et al., 2016) such as *Rhizobium* species and AMF. Microorganisms present in the rhizosphere of plant roots play a role in contributing towards nutrient availability for plant use. Indeed, some groups of these microbial candidates provide nutrients for plants by undergoing symbiotic interactions with plants. The symbiotic relationship between AMF/*Rhizobium* species and roots of plants is among the most beneficial associations that occur in the rhizosphere (Smith and Smith, 2011).

Specifically, AMF introduction in the field has been investigated previously (Pellegrino et al., 2011, Pellegrino et al., 2012) where it was shown to increase crop yield (Igiehon and Babalola, 2017). However, competition with indigenous fungi and host plant compatibility are some of the factors that militate against the successful establishment of AMF in the soil (Verbruggen et al., 2013b), but it is not clear whether these factors also influence *Rhizobium* species establishment. Therefore, this facets need to be investigated experimentally so as to assess the viability of *Rhizobium* and AMF utilization as biofertilizer in agriculture. The colonization potential of the AMF *Rhizophagus clarus* has been evaluated using soybean *in-vitro* and in greenhouse environments. It was found that, there was no significant difference in root colonization, plant growth and phosphorus absorption between the *in-vitro* and the pot

experiments indicating that *R. clarus* can be grown in pure culture and produce on a large scale (Cely et al., 2016) for field application.

Also, understanding the composition and role of root microbiome is crucial toward agricultural practices that are less dependent on chemical fertilization (Conway and Pretty, 2013, Rascovan et al., 2016, Igiehon and Babalola, 2018c). But, “The analysis of complex plant associated microbiome has been historically limited by technical constraints. Sequencing techniques were recently used to reveal root associated and rhizospheric soil microbiomes of *Arabidopsis thaliana* and *Hordeum vulgare* (Bulgarelli et al., 2012a, Lundberg et al., 2012, Bulgarelli et al., 2015, Schlaeppi et al., 2014). Regardless of these foundational works, little is known about root microbiome from crops and further research on root and rhizospheric soil microbiome of commercial crops and non - model plants is highly required (Babikova et al., 2014, De-la-Peña and Loyola-Vargas, 2014). For instance, no research to date has explored root associated microbiome of wheat (*Triticum aestivum*) and soybean in field conditions using high throughput sequencing techniques (FAOSTAT, 2015, Rascovan et al., 2016)”.

1.1 Aim of the Research Study

The aim of this present study is to examine the ability of *Rhizobium* species and mycorrhizal fungi (MF) to enhance soybean (*Glycine max* L.) tolerance to drought.

1.1.2 Objectives of the Research Study

The above aim will be accomplished by the following specific objectives:

- (i) To isolate and identify *Rhizobium* species by cultural and molecular approaches from a leguminous plant rhizosphere and determine their ability to enhance soybean tolerance to drought stress in a growth chamber.

- (ii) To determine plant growth promoting (PGP) traits of *Rhizobium* species including nitrogen fixing (*nif*), PGP, symbiotic and drought tolerance functional genes
- (iii) To determine physicochemical parameters of soil as well as aboveground and belowground parameters of soybean (inoculated with rhizobia and MF) grown under drought conditions in the greenhouse and field.
- (iv) To determine bacterial community changes in soybean rhizosphere (at different stages of growth) inoculated with *Rhizobium* and MF in the field using Next Generation Sequencing Technique.

List of Publication

Chapter 2: Below-Ground-Above-Ground Plant Microbial Interactions: Focusing on Soybean, Rhizobacteria and Mycorrhizal Fungi. *Published in The Open Microbiology Journal (2018).*

Authors: Nicholas Ozede Igiehon and Olubukola Oluranti Babalola

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

Chapter 3: Rhizosphere Microbiome Modulators: Contributions of Nitrogen Fixing Bacteria towards Sustainable Agriculture. *Published in International Journal of Environmental and Public Health Research (2018) 19, 513-519.*

Authors: Nicholas Ozede Igiehon and Olubukola Oluranti Babalola

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

Chapter 4: Biofertilizers and Sustainable Agriculture: Exploring Arbuscular Mycorrhizal Fungi. *Published in Applied Microbiology and Biotechnology (2017) 101:4871-4881.*

Authors: Nicholas Ozede Igiehon and Olubukola Oluranti Babalola

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Chapter 5: New *Rhizobium* Species Enhanced Soybean Tolerance to Drought. *This chapter has been submitted in this format for publication in Plant and Soil.*

Authors: Nicholas Ozede Igiehon and Olubukola Oluranti Babalola

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

Chapter 6: Genomic Insights into PGP Rhizobia Capable of Enhancing Soybean Germination under Drought Stress. *This chapter has been submitted in this format for publication in BMC Microbiology.*

Authors: Nicholas Ozede Igiehon, Olubukola Oluranti Babalola* and Bukola Rhoda Aremu

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

Chapter 7: Complete Genome Sequence of a Novel Drought Tolerant *Rhizobium* sp. Strain R1 Isolated from Bambara Groundnut Biome. *This chapter has been submitted in this format for publication in Microbiological Resource Announcements.*

Authors: Nicholas Ozede Igiehon, Olubukola Oluranti Babalola* and Bukola Rhoda Aremu

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Chapter 8: Whole genome sequence of a new plant growth promoting *Rhizobium cellulosilyticum* strain R3 isolated from *Vigna subterranea* rhizosphere. *This chapter has been submitted in this format for publication in Microbiological Resource Announcements.*

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

Chapter 9: The Panacea to Food Insecurity starts with Rhizobia, Mycorrhizal Fungi and Soybean Interactions in a Controlled Environment. *This chapter has been submitted in this format for publication in Frontiers in Microbiology.*

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Chapter 10: Growth and Mineral Nutrient Enhancement of Soybean by New *Rhizobium* Species and Mycorrhizal Fungi under Drought Conditions. *This chapter has been submitted in this format for publication in PLOS ONE.*

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Chapter 11: Effects of rhizobia and arbuscular mycorrhizal fungi on yield, size distribution and percentage crude fatty acid of soybean seeds grown under drought condition. *This chapter has been submitted in this format for publication in Microbiological Research.*

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Chapter 12: Microbial Dynamics of Soybean Rhizosphere at different Growth Stages in the Field. *This chapter has been submitted in this format for publication in The ISME Journal.*

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CHAPTER 2

Below-Ground-Above-Ground Plant-Microbial Interactions: Focusing on Soybean,

Rhizobacteria and Mycorrhizal Fungi

Abstract

Introduction:

Organisms seldom exist in isolation and are usually involved in interactions with several hosts and these interactions in conjunction with the physicochemical parameters of the soil affect plant growth and development. Researches into below and aboveground microbial community are unveiling a myriad of intriguing interactions within the rhizosphere and many of the interactions are facilitated by exudates that are secreted by plants roots. These interactions can be harnessed for beneficial use in agriculture to enhance crop productivity especially in semi-arid and arid environments.

The Rhizosphere:

The rhizosphere is the region of soil close to plants roots that contain large number of diverse organisms. Examples of microbial candidates that are found in the rhizosphere include the arbuscular mycorrhizal fungi (AMF) and rhizobacteria. These rhizosphere microorganisms use plant root secretions such as mucilage and flavonoids which are able to influence their diversity and function and also enhance their potential to colonize plants root.

Natural Interactions between Microorganisms and Plant:

In the natural environments, plants live in interactions with different microorganisms, which thrive belowground in the rhizosphere and aboveground in the phyllosphere. Some of the plant-microbial interactions (which can be in the form of antagonism, amensalism, parasitism and

symbiosis) protect the host plants against detrimental microbial and non-microbial invaders and provide nutrients for plants while others negatively affect plants. These interactions can influence below-ground-above-ground plants' biomass development thereby playing significant role in sustaining plants. Therefore, understanding microbial interactions within the rhizosphere and phyllosphere is urgent towards farming practices that are less dependent on conventional chemical fertilizers, which have known negative impacts on the environments.

Belowground Rhizobacteria Interactions Alleviate Drought Stress:

Drought stress is one of the major factors militating against agricultural productivity globally and is likely to further increase. Belowground rhizobacteria interactions could play important role in alleviating drought stress in plants. These beneficial rhizobacterial colonize the rhizosphere of plants and impart drought tolerance by up regulation or down regulation of drought responsive genes such as ascorbate peroxidase, S-adenosyl-methionine synthetase, and heat shock protein..

Insights into Below and Aboveground Microbial Interactions via Omic Studies:

Investigating complex microbial community in the environment is a big challenge. Therefore, omic studies of microorganisms that inhabit the rhizosphere are important since this is where most plant-microbial interactions occur. One of the aims of this review is not to give detailed account of all the present omic techniques, but instead to highlight the current omic techniques that can possibly lead to detection of novel genes and their respective proteins within the rhizosphere which may be of significance in enhancing crop plants (such as soybean) productivity especially in semi-arid and arid environments.

Future Prospects and Conclusions:

Plant-microbial interactions are not totally understood and there is therefore the need for further studies on these interactions in order to get more insights that may be useful in sustainable agricultural development. With the emergence of omic techniques, it is now possible to effectively monitor transformations in rhizosphere microbial community together with their effects on plants development. This may pave way for scientists to discover new microbial species that will interact effectively with plants. Such microbial species can be used as biofertilizers and/or bio-pesticides should to increase crop yield and enhance global food security.

Keywords: drought, microbial interactions, rhizosphere, soybean, omic studies

2.0 Introduction

Organisms seldom exist in isolation and are usually present in associations with several hosts and the interactions could be in different forms namely: virus versus virus, bacterium versus bacterium, protozoan versus protozoan, fungus versus fungus, bacterium versus fungus, fungus versus plant or animal, bacterium versus plant or animal, virus versus plant or animal, protozoa versus plant or animal, bacterium versus fungus versus plant or animal, as well as other parasitic and symbiotic associations with unique mechanisms that consolidate the associations which can lead to enhanced host plant growth. The recruitment of a foreign species into a new ecosystem is dependent on the type of host and indigenous microbial community. Generally, an ecosystem with lost species diversity has a high tendency to accept new species or invaders and the invaders have to interact with species in the new ecosystem in order to occupy the niche (Braga et al., 2016).

Research into below and aboveground microbial community are unveiling a myriad of intriguing interactions within the rhizosphere and many of the interactions are facilitated by exudates (Fig. 1) that are secreted by plant roots (Philippot et al., 2013). Root exudates play a role in regulating biotic and abiotic functions within the rhizosphere. Some of the functions of the root exudates include: altering soil physicochemical properties, suppressing the proliferation of competing plants and influencing microbial community structure. Particularly amazing are root exudate compounds that are known to mediate symbiotic interactions within the soil. These compounds include monosaccharide (e.g. glucose), disaccharide (e.g. sucrose), polysaccharide, different types of amino and organic acids such as arginine and benzoic acids. It is also possible for plant roots to secrete “higher-molecular-weight-compounds” such as fatty acids, nucleotides, tannins, alkaloids and vitamins which are known to enhance interactions in the soil (Rasmann and Turlings, 2016) particularly those involving rhizobacteria and arbuscular mycorrhizal fungi (AMF).

Rhizobacterial and mycorrhizal fungal interactions have been studied extensively (Song et al., 2015, Igiehon and Babalola, 2017). The main roles of these microorganisms are to provide nutrients to plants, plant growth stimulation, inhibition of phytopathogens growth and soil structure enhancement. In particular, plant symbiotic association involving these bacteria and fungi is a subject of scientific debate. However, interactions involving archaea are not well understood, although they are present in soil rhizosphere where they are involved in bioleaching especially heat loving archaea (Song et al., 2015). Host interactions with viruses are similarly important since viral particles cause several disease conditions in many host and change the bacterial richness and diversity by attacking dominant strains. This means that plant interactions with microorganisms could be positive or negative depending on the species involved.

Considering the enhancement of nutrient status, rhizosphere microorganisms have the capacity to fix nitrogen, obtain iron using siderophores and increase the bioavailability of phosphorus especially by MF (Hardoim et al., 2015). In addition, the introduction of a symbiotic bacterium into the rhizosphere could also impact plant health by enhancing the process of photosynthesis, chlorophyll content as well as assimilation of carbon (IV) oxide (Naveed et al., 2014, Dourado et al., 2013). Root endophytes can produce phytohormones in the form of auxins as well as gibberellins which have plant growth promoting traits (Babalola, 2010). There are several host/rhizobacterial interactions which may have beneficial and or disease-causing effects on the host plant. Microbial interactions with plants either in the rhizosphere or plant tissue have also the potential to enhance plant development by releasing phytohormones, cleaning up pollutants and enhancing tolerance to biotic and abiotic stress (Mendes et al., 2013b).

Drought stress which may either be short, moderate or very severe, is one of the most detrimental abiotic stresses that have increased in intensity, currently having negative impacts on global food security (Vurukonda et al., 2016). By 2050, drought is anticipated to cause severe plant problems for over 50% of the agricultural land (Kasim et al., 2013). To overcome the effects of drought, some rhizobacteria produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase which breakdown ethylene precursor ACC to 2-oxobutanoate and ammonia thereby reducing ethylene content and eventually minimizing plant stress (Babalola et al., 2003). Thus, belowground microbial communities can be manipulated towards the selection of microbial assemblages that can tolerate abiotic stress and enhance drought tolerance and health of plants.

This review therefore focuses on recent progresses in microbial interactions with plants. The discussion is largely targeted to AMF and rhizobacteria and their effects on below and aboveground plant community. Similarly, plants' tolerance to stress by these microbial

interactions, insights from omic analyses of the components of these interactions and prospects of harnessing these interactions for agricultural sustainability are highlighted.

2.1 Soil: the Site for plant Microbial Interactions

Soil is the region below the earth's surface composed of both living and nonliving entities for which structure is influenced by both biotic and physicochemical parameters. Biotic factors such as plant nature and plants root enhanced soil structure when bringing soil organic matter (Δ C/N) piercing through the soil matrix (aeration) and the constituent microbial species similarly affect the fertility of the soil, thus may modulate soil pH, structure and other related soil properties. Rhizobacterial existence in the soil might support plant growth by releasing growth enhancing compounds, helping to form firm soil structure, liberating mineral elements from the degradation of organic compounds as well as by forming mutualistic association with plant's root (Fig 1a). Bacteria and MF for instance contribute to the improvement and maintenance of soil structure (Babalola and Glick, 2012). It was reported that soil parameters (such as soil pH) are involved in the establishment of microbial community and structure in soil rhizosphere (Miethling et al., 2000, Babalola and Glick, 2012) and that changes in microbial community composition could cause transformations in their functions (Cristina et al., 2017) most especially in the rhizosphere.

2.2 The Rhizosphere: a Zone of Microbial Interaction

The rhizosphere (Fig. 1a) is the thin zone of soil close to plant roots and harbor a great deal of microorganisms as well as invertebrates (Philippot et al., 2013, Mendes et al., 2014a). These organisms constantly interact with one another for survival resulting in the establishment of different associations such as commensalism, parasitism, amensalism, saprophytism and

symbiosis. Organisms that inhabit the rhizosphere can greatly influence plants' growth and development (Berendsen et al., 2012a, Mendes et al., 2011) thereby playing significant role in sustaining the plants. Some of the microorganisms that are of great benefit to plants in the rhizosphere are rhizobacteria and MF. Taxonomic investigation demonstrated that the rhizosphere species are a subset of bulk soil species (Mendes et al., 2014a). Many studies on microbial communities of the rhizosphere have shown the influence of plants on rhizosphere microbiota. Examples of such studies include soybean (Mendes et al., 2014a), *Arabidopsis* (Bulgarelli et al., 2012b, Lundberg et al., 2012) and rice (Knief et al., 2012).

However, it is not totally clear to what degree plants can choose a consistent rhizosphere microbial composition from profoundly varying deposits of bulk soil microbial composition particularly under tropical environments (Mendes et al., 2014a). There are two mechanisms used to explain the choice of plants' microbial diversity in rhizospheric soil namely neutral and niche based mechanisms. The neutral based mechanism explains that since most species have the same abilities in exploiting niches, microbial composition is influenced by spatial distance between plants due to low recruitment realization and dispersal restriction. The niche based approach shows that environmental changes are related to variation in microbial community composition. Mendes, *et al.*, 2014 [19] reported that soybean chooses a particular microbial group in the rhizosphere based on functional characteristics that might be useful to the plant in relation to nutrient uptake and growth enhancement. This selection ability possessed by soybean plant could be explained by niche based hypothesis, showing the strong ability of the plant to determine the richness and abundance of microorganisms in the rhizosphere and other ecological changes that influence microbial diversity.

Species in the rhizosphere mainly migrate from the bulk soil (Fig. 1a) and environmental changes in the bulk soil could affect the structure and composition of microbial community in the rhizosphere. Rhizosphere microorganisms use nutrients such as mucilage and exudates liberated by plant roots and these nutrients influence the microbial diversity and function within the rhizosphere (Mendes et al., 2013b). Root released flavonoids, different organic acids and cutin monomers that are involved in regulation of plant root-microbial interactions as well as microbial gene expression (Venturi and Keel, 2016). It is very possible that many other chemical signals produced and secreted by the root will be recognized and possibly deployed to improve microbial colonization of the root for sustainable agricultural development. It was recently reported that methyl salicylate secreted from the root could trigger root colonization by beneficial *Bacillus subtilis* (Kobayashi, 2015). Beneficial microorganisms in the rhizosphere vigorously respond to root exudates by tuning their transcriptional machinery toward traits associated with mobility, detoxification, chemotaxis, secondary metabolism, biofilm formation and polysaccharide degradation (Zhang et al., 2015, Balsanelli et al., 2016). Once beneficial microorganisms are established in the rhizosphere, the root exudates might serve as environmental signals to enhance biofilm formation on the surface of the root (Beauregard et al., 2013). Many published articles have excellently described the mechanisms, functions and communication signals involved in root-microbial interactions (Bednarek et al., 2010, Rasmann and Turlings, 2016, Rosier et al., 2016, Huang et al., 2014, el Zahar Haichar et al., 2014).

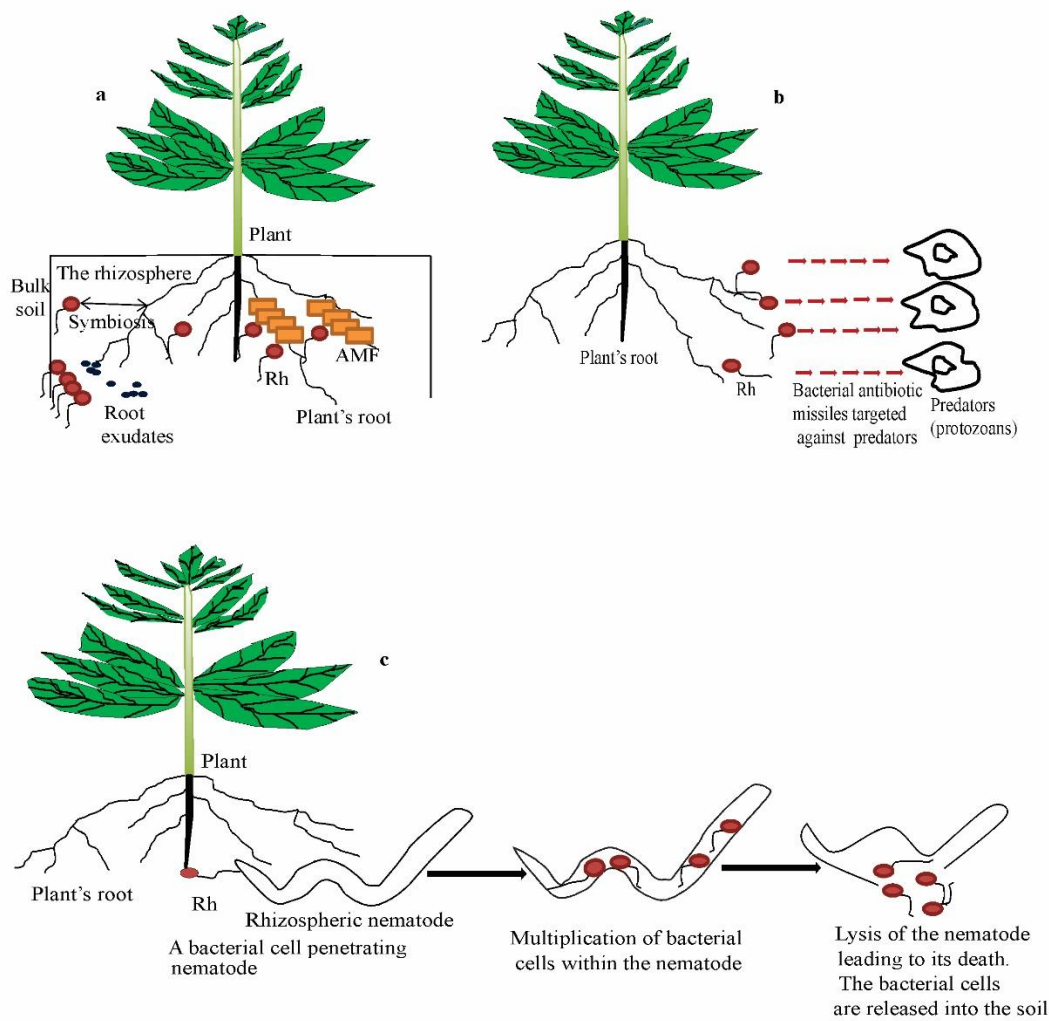


Fig. 2. 1 Natural interactions in the rhizosphere. (a) symbiotic interactions between plant's root and Rh/AMF (b) amensalistic interaction between antibiotic-producing rhizobacteria and protozoans (c) parasitic interaction between the bacterium *Pasteuria penetrans* and root-knots nematode in the rhizosphere. Rh stands for rhizobacteria and AMF stands for arbuscular mycorrhizal fungi.

2.3 Natural Interactions between Microorganisms and Plants

Microorganisms interact with plants in the natural environment and such interactions are important in ecosystem functioning. Some of the interactions protect the host plants against pathogens and provide nutrients for the hosts while others are detrimental to the plant. Beneficial microbial interactions in the soil have been found useful in enhancing and improving agricultural crops development (Table 1). Soybean (*Glycine max* L) which is a leguminous plant belonging to the order Fabales, family Leguminosae has the potential of forming symbiotic associations with rhizosphere microbiota (Rascovan et al., 2016) including MF. It has been reported that these microbial symbionts support their hosts in the environment.

Therefore, understanding microbial interactions within the rhizosphere is urgent towards farming practices that are less reliant on conventional chemical fertilizers, which have known negative impacts on environmental receptors (Rascovan et al., 2016). Some of the interactions that occur in nature among organisms are highlighted below:

2.3.1 Antagonism: This is an interaction between two species in an ecosystem in which one species has effect on the other. Some antagonists synthesize enzymes such as cellulases, chitinases, protein and lipid degrading enzymes that have the potential to lyse the cell walls of fungal pathogens (Kundan et al., 2015). Rhizobacteria are able to reduce the adverse effect of pathogenic microorganisms in the rhizosphere through this interaction. For instance, Firmicutes, Actinobacteria and Proteobacteria play essential role in suppressing the disease caused by root-infecting fungal pathogen *Rhizoctonia solani* and this led to the suggestion that certain interactions are fundamental to disease suppression (Schenk et al., 2012). A study of antagonistic bacteria to eradicate the effect of *R. solani* on lettuce demonstrated that the bacteria had temporary effects on indigenous rhizobacteria and endophytic fungi (Scherwinski et al., 2008).

Another report highlighted that the use of antagonistic bacteria on lettuce minimize the disturbance to resident fungal and bacterial species caused when *R. solani* alone was present (Adesina et al., 2009). Negative effects on symbiotic AMF would explain the unpredictable outcomes of field application of this antagonist (Frey-Klett et al., 2011). However, it was reported that beneficial bacteria *Pseudomonas fluorescens* F113, which releases antifungal compound 2, 4-diacetylphloroglucinol, was not harmful to AMF *Glomus mosseae* but basically enhanced root colonization by the fungus symbiotic species (Barea et al., 1998). Besides bacteria, some fungal species may contribute to mycorrhizal establishment while they inhibit other fungal species. This implies that, there are certain fungi that inhibit other fungal species. Hence, it is essential to establish “fungus specificity of mycorrhizal helper bacteria” since some helper fungal species have been found to enhance mycorrhizal establishment and inhibit other non-helper fungi (Garbaye, 1994). Antifungal specificity can therefore be a criterion for a better selection of antagonists as biocontrol agents in the future.

Other rhizobacteria such as *Rhizobium* and *Bacillus* produce siderophores (Kundan et al., 2015) which deprive pathogens of iron acquisition from the environment, thus affecting the existence of the pathogens. This eventually culminates in enhancing plant growth and productivity.

2.3.2 Amensalism: This is an interaction between dissimilar organisms in which one of the organisms leaves detrimental effects on another one. In this type of interaction, chemical released by an organism could damage or kill another organism. Amensalism also known as antibiosis leads to the production of antimicrobial substance by one microorganism which may be detrimental to the other microorganisms. The release of antibiotics is the most commonly used mechanism of amensalism in bacteria against their host pathogens. These antibiotics are

“low molecular weight compounds” capable of destroying or hampering the enzymatic and metabolic reactions of pathogenic microorganisms thereby retarding their growth (Kundan et al., 2015). Since many microorganisms are confronted with several predators; the production of metabolites detrimental to these predators is crucial. Since many beneficial microorganisms such as *Pseudomonas* species are confronted with several predators (Weller et al., 2007); the production of metabolites detrimental to these predators is crucial. Indeed, the elimination of these bacteria from the soil by these nematodes and other predators will result in loss of diversity of plant growth promoting rhizobacteria and such biodiversity loss may affect plant growth and productivity. However some microorganisms have developed escape strategy by producing secondary metabolites. The bio-production of secondary metabolites might be important in reducing vulnerability of bacteria to bacterivorous (organisms that feed on bacteria) predators (Fig1b). Studies have shown that *Pseudomonas fluorescens* produced, 2, 4-diacetylphloroglucinol (2, 4-DAPG) that lyses protists (Weller et al., 2007). However, abuse of antibiotic releasing rhizobacteria has the shortcoming of causing the emergence of antibiotic resistant pathogens. In another study, 2, 4-DAPG improved the survival of *P. fluorescens* by enhancing its tolerance to attack by the predatory protozoan *Acanthamoeba castellanii*. On the contrary, Pf-5 and Q8r1-96 strains of *P. fluorescens* deficient in 2, 4-DAPG were resistant to attack by *Naegleria americana* and *Colpoda* species while the *gacA*-mutants of the bacterial species were susceptible to predation by these predatory protists. These complementary findings indicates that the ability of 2,4-DAPG to serve as escape mechanism from predatory enemy is bacterial strain and/or predator specific (Raaijmakers and Mazzola, 2012).

Bacillus, *Streptomyces* and *Pseudomonas* produced bioactive lipopeptides able to distort cell membrane integrity resulting in death of microbial pathogens including oomycetes and

trophozoites of *N. americana*. Viscosin and massetolide lipopeptide produced by two different strains of *P. fluorescens* protect the bacterial strains from predation by *N. Americana*. Bacterial strains with lipopeptide production potential are described as more tolerant to protozoan enemy than lipopeptide deficient strains. However, both strains have the same growth rate in soil where *N. American* species are absent (Raaijmakers and Mazzola, 2012).

2.3.3 Parasitic Interaction: In this interaction, one organism benefits at the expense of the other (Table 1). Metabolomics investigation of the parasitic association between the fungal parasite *Stachybotrys elegans* and *R. solani* revealed the expression of different concentrations of secondary metabolites. In the study, overexpression of parasitic and pyridoxal reductase enzyme gene was observed for *S. elegans* and *R. solani* respectively. In addition, *S. elegans* released enzymes involved in cell wall degradation including trichothecenes and atranones toxins. The trichothecenes produced by *S. elegans* have been reported to have effect on *R. solani* metabolism and development (Chamoun et al., 2015, Braga et al., 2016). Trichothecenes are also known to hinder protein synthesis and induce oxidative stress in eukaryotes (Braga et al., 2016).

The impacts of AMF and bacterial species on nematodes have also been studied. The bacteria *Pasteuria penetrans* was reported to reduce root-knots nematodes growth via a parasitic interaction (Fig. 1c). The bacteria multiply within the infected nematodes resulting in their death and infertility in those that are able to survive. There was also a reduction in the mobility and penetration of young nematodes into plant roots when a large number of bacterial spores glued to the young nematodes in the soil (Flor-Peregrín et al., 2014).

Plants have also developed their own defense mechanisms to combat pathogens. RNA-seq was recently used to detect plant genes involved in defense responses to the plant root pathogen *Verticillium dahliae* (Xu et al., 2011). Additionally, plant-based microarray

transcriptomics analyses have unveiled a web of signal transduction pathways activated upon recognition of elicitors with defense signaling compounds and pathogen-associated molecular patterns (PAMPs) (Sana et al., 2010) as microorganisms naturally interact with plant roots in the rhizosphere. It would be valuable to encourage the interactions between all organisms in the greenhouse and to search for beneficial rhizosphere microorganisms that may have parasitic and antagonistic impacts on plant pathogens.

2.3.4 Symbiotic Interaction: Symbiotic interaction is a mutualistic association in which the two organisms involved in the association benefit as exemplified in the relationship between leguminous plants (Table 1) and nitrogen fixing bacteria (Tajini et al., 2012). The conversion of atmospheric nitrogen by nitrogen-fixing bacteria to plant's utilizable ammonia form is termed nitrogen fixation.

Rhizobacteria do not only assist plant with nitrogen fixation and nutrient acquisition in symbiotic interactions, they also produce different signaling compounds to impact their hosts for improving plant development and tolerance to abiotic and biotic stress. Some rhizobacteria species release different compounds that elicit ISR in plant and these compounds include 2, 3-butanediol (volatile organic chemical), diffusible signal factor diketopiperazines, antibiotics produced by rhizosphere associated pseudomonads, polyketides and lipopeptides produced by rhizosphere associated bacilli, biosurfactants and siderophores (Pieterse et al., 2014, Schenk et al., 2014, Sieper et al., 2014). The mutualist *B. subtilis* released volatile compounds such as butanediol which was reported to elicit induced systemic resistance by modulating the transcription of sodium ion transporter 1 in plants (Pieterse et al., 2014, Zhang et al., 2008). Rhizobacteria also release compounds that specifically influence root development by inhibiting elongation of primary root and enhancing lateral root formation. Some of these bacteria

including fungi produce auxins which interfere with the signaling of this compound in the root (Spaepen et al., 2014). But, auxins derivatives released by *Piriformospora indica* (root endophyte) do not play role in root enhancement of barley plant; instead, they are needed for biotrophic infection of the plant root (Lee et al., 2011, Hilbert et al., 2012). Root zone-associated bacteria and fungi also dimethyl disulfide and pyocyanin (Meldau et al., 2013, Ortiz-Castro et al., 2014) that regulate plant root development by modulating auxins signaling process.

In addition, AMF interactions with plant roots have been reported to influence the aboveground interaction between plant and herbivore, a process that eventually leads to plants' protection (Babikova et al., 2013a, Gilbert and Johnson, 2015). AMF establish symbioses with over 80% terrestrial plants (Fig 1a) unlike rhizobia which are limited to leguminous plants and *Parasponia* species belonging to the *Cannabaceae* family (Philippot et al., 2013). AMF symbioses are mainly important to plants cultivated in phosphorus deficient environment where they aid the plants to access inorganic phosphorus that ordinarily are not bioavailable to plants. Increased productivity of soybean (*Glycine max*) that was symbiotically attached to AMF was attributed to enhanced uptake of phosphorus (Abdel-Fattah et al., 2014). AMF can also transport nitrogen and sulfur to host plants, and high level of sulfur content which regulated the “sulfate transporter gene” in a host plant has been previously reviewed (Igiehon and Babalola, 2017).

Belowground symbiosis does not only occur between microorganisms and plant, but also there are symbioses that involve interaction between one microorganism and another. The association between beneficial bacteria and AMF is symbiotic since the bacteria aid the mycorrhizal in establishing symbiosis while AMF promote bacterial invasion potential and diversity. However, there are other benefits accrued to such interactions. Symbiotic relationship has also been shown to exist between the bacterium *Burkholderia* and *Rhizopus* (fungus) and it

was reported that in the absence of *Burkholderia* species, the fungus *Rhizopus* was unable to sporulate indicating that the fungus rely on compounds produced by the bacterium to survive (Braga et al., 2016). This interaction is not well comprehended with respects to the metabolites and processes involved.

Table 2. 1 Benefits of microbial interactions on plants under well-watered and drought stress conditions.

Biotic factor	Interaction type/benefit	References
<i>Pseudomonas putida</i> and soybean (<i>G.max</i>)	Mutualistic interaction between the bacterium <i>P. putida</i> and soybean (<i>G. max</i>) under drought and saline conditions. Secretion of gibberellins by the bacterium enhanced soybean growth	(Kang et al., 2014)
<i>Rhizobium etli</i> 12a3 and common bean (<i>Phaseolus vulgaris</i>)	Mutualistic interaction between <i>R. etli</i> 12a3 and common bean (<i>P. vulgaris</i>) under drought stress condition. The bacterium enhanced plant tolerant ability of the bean plant by causing increase in proline accumulation	(Tajini et al., 2012)
<i>Bradyrhizobium diazoefficiens</i> and <i>Aeschynomene afрасpera</i>	Symbiotic interaction with plant under well-watered condition. The microorganism enhanced host plant growth by fixing atmospheric nitrogen	(Kulkarni et al., 2015, Braga et al., 2016)
<i>Rhizobium</i> spp. and soybean (<i>G. max</i>)	Symbiotic interaction with plant under well-watered condition. <i>Rhizobium</i> inoculation with AMF prevented red crown rot disease of soybean (<i>G. max</i>) during symbiotic	(Gao et al., 2012)

interaction

AMF and host plant	Symbiotic relationship with the host. Host plant protection against herbivore attack through common mycelial network in well-watered soil. The AMF's common mycelial network led to the production of volatile organic compounds that attract parasitoid wasps (herbivore enemies) that destroyed the herbivore	(Babikova et al., 2014)
<i>Bacillus licheniformis</i> K11 and pepper plant	Mutualistic interaction between the bacterium <i>B. licheniformis</i> and pepper plant under drought stress conditions. Accumulation of drought stress proteins and RNA in the bacterial inoculated plants made them to survive better than the non-inoculated pepper plants	(Lim and Kim, 2013)
Rice and species of <i>Burkholderia</i> and <i>Rhizopus</i>	Symbiotic and pathogenic interaction with plant under well-watered condition. The fungus was unable to produce spores in the absence of the endosymbiont. The endosymbiont produces rhizoxin (the causative agent of seedling blight of rice) and phytotoxin. The fungus	(Ross et al., 2014)

stimulates the endosymbiont growth

<i>R. solani</i> and <i>S. elegans</i>	Parasitic relationship with the host under well-watered condition. The toxins released by the parasite (<i>S. elegans</i>) caused changes in <i>R. solani</i> growth and metabolic process leading to down regulation in the biosynthesis of several antimicrobial substances by <i>R. solani</i>	(Chamoun et al., 2015)
Mycorrhizal fungus <i>Glomus mosseae</i> and soybean (<i>G. max</i>)	Mutualistic interaction between the fungus <i>G. mosseae</i> and soybean (<i>G. max</i>) under drought stress condition. The fungus protected the plant against premature nodule senescence induced by drought stress by inducing higher glutathione reductase (GR) activity in soybean root and nodules which may have contributed to decreased oxidative damage to biomolecules involved in early nodules senescence	(Porcel et al., 2003)
<i>Erysiphe pisi</i> and <i>Pisum sativum</i>	Pathogenic interaction under well-watered condition. The fungus (<i>E. pisi</i>) upregulated the gene coding for invertase and decreased the starch content of the host plant (<i>P. sativum</i>)	(Storr and Hall, 1992)

<i>Azospirillum</i> sp.	Mutualistic interaction between <i>Azospirillum</i> sp. and wheat	(Arzanesh et
And wheat plant	plant under drought condition. IAA improved wheat root	al., 2011)
	development and uptake of nutrients and water under	
	drought stress	
<i>Blumeria</i>	Pathogenic interaction. The fungus (<i>B. graminis</i>) caused	(Swarbrick et
<i>graminis</i> and	change in sugar content and down-regulation of	al., 2006)
<i>Pisum sativum</i>	photosynthesis in the host plant (<i>P. sativum</i>). under well-	
	watered condition ‘The depression in photosynthesis was	
	not due only to cell death and the effective removal of green	
	leaf area, but also to an alteration in host metabolism’	
A consortium of	Mutualistic interaction between the bacterial consortium	(Hussain et
<i>Mesorhizobium</i>	and wheat plant under drought stress condition. The wheat	al., 2014)
<i>ciceri</i> (CR-30 and	plant growth, drought tolerance index and biomass were	
CR-39),	improved by IAA produced by the bacterial consortium	
<i>Rhizobium</i>		
<i>phaseoli</i> (MR-2),		
and <i>Rhizobium</i>		
<i>leguminosarum</i>		
(LR-30) and		
wheat plant		

<i>Bacillus subtilis</i> QST713	Amensalistic interaction under well-watered condition. The bacterium produced antibiotic (iturin A) that helps to control damping off disease caused by <i>Botrytis cinerea</i> and <i>R. solani</i> in host plants	(Kloepper et al., 2004)
A Consortium of AMF and <i>Bacillus thuringiensis/Pseudomonas</i> and <i>Trifolium repens</i>	Mutualistic interaction between the microbial species (fungi and bacteria) and the plant <i>T. repens</i> in a natural arid soil under drought conditions. AMF/bacterial inoculation significantly enhanced the plant growth by increasing plant's nutrient uptake and relative water content	(Ortiz et al., 2015)
<i>Pantoea agglomerans</i> C9-1	Amensalistic interaction under well-watered condition. <i>P. agglomerans</i> produced herbicolin (antimicrobial substance) effective against <i>Erwinia amylovora</i> , the causative agent of fire blight in plants	(Wright et al., 2001)
A consortium of exotic <i>Glomus intraradices</i> , <i>G. deserticola</i> and <i>G.</i>	Mutualistic interaction between the exotic mycorrhizal fungal consortium and the plant <i>Juniperus oxycedrus</i> under drought stress conditions. Mycorrhizal fungal consortium increased root and shoot nitrate reductase by 38 and 188%	(Alguacil et al., 2006)

<i>mosseae</i> and the plant <i>Juniperus oxycederus</i>	respectively with respect to the plant neither treated with composted sewage sludge (SS). ‘Both the plants inoculated with exotic AM fungi and the plants grown with composted SS developed additional mechanisms to avoid oxidative damage produced under water-shortage conditions’
<i>Pseudomonas aeruginosa</i> Migula 7NSK2 and tomato plants	Mutualistic/antagonistic interaction. The bacterium (Buysens et al., 1996) produced pyochelin siderophore that protect tomato plants from the parasitic oomycetes (<i>Pythium</i> spp.) under well-watered condition
<i>Heteroconium chaetospira</i> and Chinese cabbage	Mutualistic interaction between the fungus <i>H. chaetospira</i> (Narisawa et al., 1998) and the host plant Chinese cabbage under well-watered condition. <i>H. chaetospira</i> was found to suppress club-root disease (caused by soil-borne protozoan <i>Plasmodiophora brassicae</i>) on <i>Chinese cabbage</i> and enhanced the above-ground plant biomass of the plant in non sterile soil. The hyphae of the fungus colonized the root tissues of the plant without causing any external effects on the plant

<i>Azospirillum</i>	Mutualistic interaction between the bacterium <i>Azospirillum</i> (Cohen et al.,
<i>lipoferum</i>	<i>lipoferum</i> and maize under drought condition. Gibberellins 2009)
<i>and maize</i>	produced by the bacterium increased Absciscic acid levels
	and alleviated drought stress

2.3.4.1 Below and Aboveground Tripartite Symbiotic Impacts-Cost and Benefit: Symbiosis between two different species does not take place in isolation in a natural environment. Symbionts are rather embedded in midst of multiple symbiotic interactions. Multiple interactions are reported to change the health benefit of different plant cultivars involved in the interaction as well as environmental ratio of cost to benefit within a focal interaction. Multiplayer impacts in symbiotic interactions also known as multiple symbiotic impacts (MSIs) are widespread. However, the effects of the impacts differ and depend on several factors. In a symbiotic interaction involving plant host and two microbial partners, these factors include intersection of benefit flowing from the host and microbial symbionts, mutualistic interaction cost, the extent to which the host can sustain the interaction cost and the conserved regions in host genomes that regulate the mutualists (Afkhami et al., 2014, Heath and McGhee, 2012, Wise and Rausher, 2013).

Studies have shown how several mutualistic microorganisms interacting with the same plant host impact one another. Plants generally undergo symbiotic interactions with several belowground and aboveground symbionts like rhizobacteria, AMF and different endophytic microorganisms. Abd-Alla et al. (2014) reported that leguminous plants establish tripartite mutualistic interactions with rhizobacteria species and AMF (Fig. 2). Plant interactions with several symbionts allow them to obtain enormous benefits which enhance their growth and productivity, resistance to different diseases, pest attacks and abiotic stresses (Gao et al., 2012, Barber, 2013, Redman et al., 2011, Philippot et al., 2013). Investigating the mechanisms on how the diversity of these mutualists impact each other including the plant host is not well understood, but such investigation is important to get insights into how hosts are able to simultaneously sustain several mutualistic partners in a MSIs (Afkhami et al., 2014).

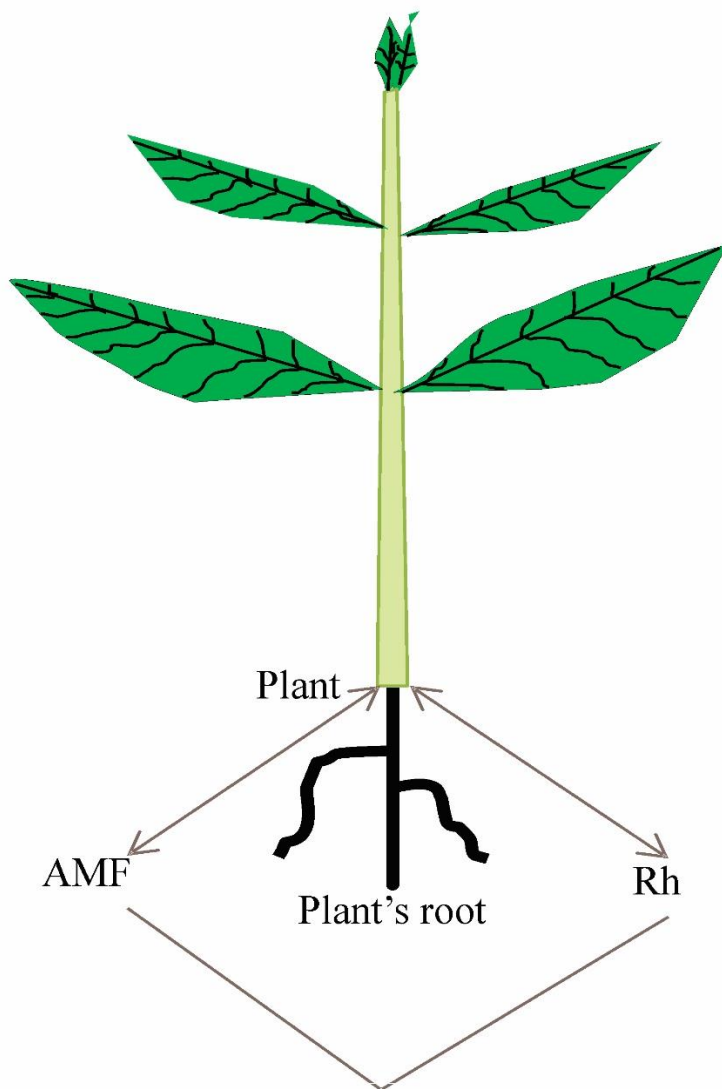


Fig. 2. 2 Tripartite interaction involving a leguminous plant, Rh and AMF. Rh stands for rhizobacteria and AMF stands for arbuscular mycorrhizal fungi.

The cost of sustaining organisms involved in symbiotic relationships could result in tradeoffs among host input (Ossler et al., 2015). Indeed, it has been observed that in plants that interact with AMF and protective endophytic fungi, the existence of the endophytic fungi triggered a reduction in the level of host colonization by the AMF. If such interactions are important, in a MSI involving plant host in the field, negative correlation will arise between the levels of abundance of the microbial symbionts; that is, increase in the colonization level of one symbiont will lead to a corresponding decrease in the colonization level of the second symbiont. However, if the existence of one of the symbionts enhances nutrient uptake by the plant host and/or increases the amount of carbon substrate release to the second symbiont, then the correlation of the colonization levels between the two symbionts interacting with the same plant will be positive (Afkhami et al., 2014). For instance, ant and coccoid scale are mutualists of *Macaranga* trees; increase in the richness of ant occurs with concomitant increase in the richness of coccoid scales. Perhaps, the high amount of honeydew released by the coccoid scales enhances ant protection (Heath and McGhee, 2012, Ossler et al., 2015).

The existence of multispecies interactions involving leguminous plants, rhizobacteria and AMF is critical to understanding the importance of mutualisms in a terrestrial environment (Fig. 2). Each of the microbial mutualists plays a key role in such interaction by enhancing nutrient uptake, plant yields and community structure (Bauer et al., 2012). Legumes develop structures in their roots termed nodules which harbor rhizobial species that convert atmospheric nitrogen to the form that can be utilized by the plants. In addition, leguminous crops such as soybean associate with AMF, which have the potential of infecting plant roots and establishing complex intraradical assemblies made up of arbuscules, vesicles, spores and hyphae that assist plants with nutrient uptake. The rhizobacteria and AMF on the other hand utilize the plant's

photosynthetically produced carbon substrates which may determine the extent of root colonization by the microbial symbionts. Co-inoculation of both the rhizobacterial species and AMF results in a synergistic impact on host diversity, development and health, which can be greater than the impact expected from a single inoculation (Larimer et al., 2014). It is noteworthy that genetic investigations have revealed commonality in plants genome involved in symbiotic relationship and control of AMF and rhizobacteria indicating that such plant-microbial interactions may have evolutionary linkage (Takeda et al., 2013). Leguminous plants possess unique receptors for discriminating *Rhizobium* and AMF symbionts; even though the symbionts however have common SYM genes that regulate the establishment of symbiotic association.

AMF and rhizobacterial colonization of densely infected plant roots can be controlled in a process called autoregulation (Sakamoto et al., 2013). It is evident that AMF influence the persistence and performance of rhizobacterial nodulation and vice versa. AMF have been reported to enhance nodule biomass, reduction in acetylene activity and plant root nodulation richness which may have occurred as a result of increased uptake of phosphorus by the plant (Larimer et al., 2014, Abd-Alla et al., 2014, Wang et al., 2011, Sakamoto et al., 2013). Rhizobacterial inoculation could also affect AMF by either increasing or decreasing their colonization level.

2.4 Influence of Belowground Microbiotas on Aboveground Interactions

There are reports on how interactions in the rhizosphere affect aboveground interactions with pathogens, decomposers, carnivores, mutualists and herbivores (Van der Heijden et al., 1998). These ‘below-ground-above ground interactions’ in plant can be triggered by changes in water and nutrient uptake or modification in plant defenses (Staley et al., 2007). Rhizospheric fungi such as AMF, as well as rhizobacteria (e.g. species of *Rhizobium*, *Bacillus* and

Pseudomonas) can elicit the systemic host immune responses that naturally help the plant to resist to pathogens and pests from the aboveground plant biomass (Zamioudis and Pieterse, 2012). In particular, the plant response to the aboveground herbivore may be due to the interaction of the plant with belowground AMF (Fig. 3).

On the other hand, induced defense response in the phyllosphere could spread to the roots and affect rhizosphere microorganisms (de Roman et al., 2011). Rhizobacteria trigger systemic resistance in plants by activating defense responses further exemplified upon pathogen attack. These immune responses are modulated by plant-derived jasmonic acid, ethylene and salicylic acid leading to secondary metabolites production, oxidative burst as well as cell wall reinforcement. For instance, the type and concentration of secondary metabolites that are detrimental to herbivores were modulated in the plant by rhizosphere microbiotas (Joosten et al., 2009). Rhizobacteria do not only facilitate the production of defense metabolites such as glucosinolates, they also enhance plant metabolites production with unknown structures and functions (van de Mortel et al., 2012).

2.4.1 Belowground AMF Interactions Trigger Phyllosphere Protection: The phyllosphere is the part of the plant that is above the ground level mainly the region around the leaves. Studies have shown how belowground associations can affect aboveground (phyllosphere) interactions with herbivores and carnivores (Philippot et al., 2013). AMF, *Bacillus* and *Pseudomonas* are able to elicit in plants a systemic resistance response that is detrimental to various disease-causing microorganisms (Zamioudis and Pieterse, 2012, Philippot et al., 2013). A study has shown that interplant associations by means of common mycelial systems resulted in high level of resistance to disease, protective enzyme responses and expression of genes that encodes defensive proteins in un-infected or healthy tomato plants (*Lycopersicon esculentum* Mill) linked to diseased plants

(*Alternaria solani*) with leaf early blight. This implies that there was possibly transfer of fungal disease protective signals (volatile organic compounds) through the mycelial network to the tomato plant. (Babikova et al., 2013b). The systemic resistance (regulated by the host plant hormones) induced by many non-pathogenic rhizobacteria results from elicitation of different defense reactions upon attack by pathogens (Philippot et al., 2013).

If common mycelial networks could facilitate signaling compound's availability, there is therefore the possibility of AMF facilitating plant defenses to herbivore. Similar effects on these insects' enemies are also possible since the insects and their enemies react differently to volatile organic compounds (VOCs) released by plants. Herbivores (such as aphids) use VOCs as signals for detecting host plants. However, upon attack, the compositions of subsequent VOCs liberated change which repel other herbivores and attract parasitoid wasps (the herbivore enemies) that destroy the insect (Babikova et al., 2013a, Gilbert and Johnson, 2015). VOCs released by attacked plants are always generated systemically and transferred aerially between plants and transported to the rhizosphere through the roots. Common mycelial networks therefore enhance the transfer of signaling compounds from herbivore attacked plant to un-infested plant resulting in its protection from the insects (Fig. 3).

Such microbially induced plants protection against herbivore attack guarantees the aboveground biomass and therefore this microbially induced resistance enhances the aboveground plant biomass and could help to minimize the use of expensive and less ecofriendly conventional chemical insecticides. Therefore, from a pragmatic view point, more insights into mycorrhizal functions in plant defense immunity might help to enhance food security (Babikova et al., 2013a).

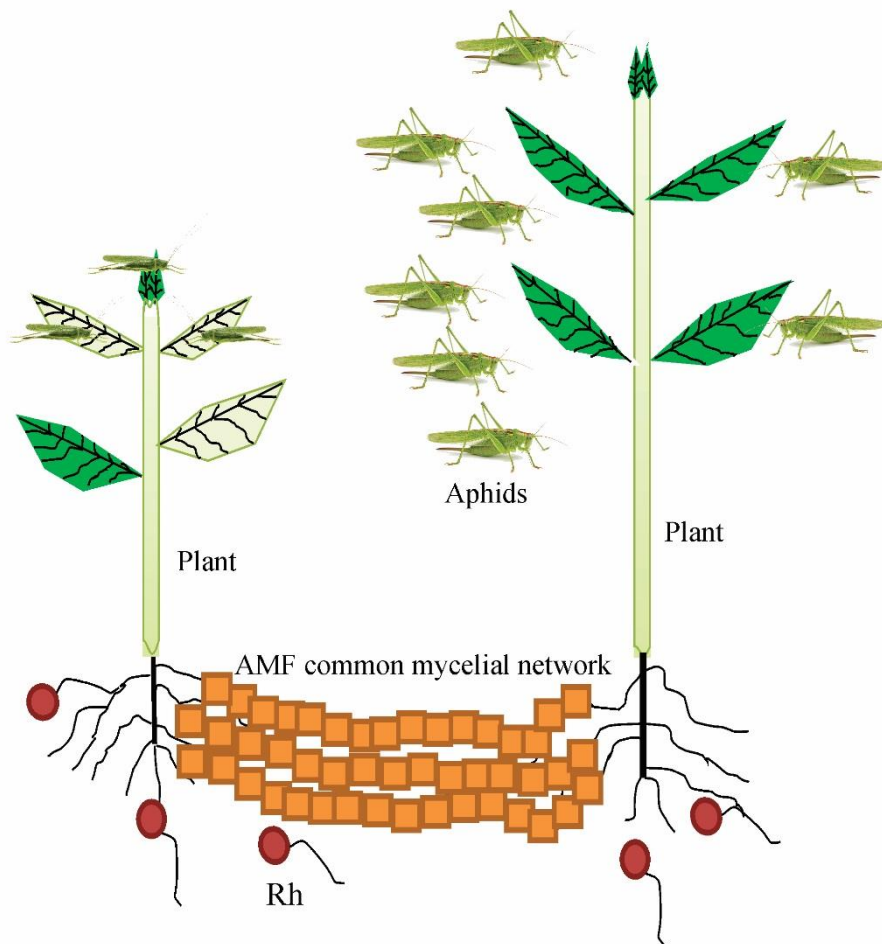


Fig. 2. 3 Phyllosphere protection by AMF common mycelial network. Aphids attack on the stunted plant (left) led to the release of modified signaling compound (volatile organic compound) via common mycelial system thus preventing their attack on the other plant (right).

2.5 Belowground Rhizobacteria Interactions Alleviate Drought Stress

Abiotic stresses are some of the factors militating against crop cultivation which may affect their development and yield. The effects of these stresses can be curtailed by belowground microbial interactions since some microorganisms influence the physical and chemical parameters of rhizospheric soil while others may enhance agricultural crops protection to environmental stresses including drought, heavy metal pollutants, salinity and heat . Examples of these microorganisms are bacteria in the rhizobacterial group. The specificity and level of establishment of these plant-microbial interactions are not absolutely explicit and more research is therefore needed to decipher the interactive mechanisms, determinant factors of specificity and how microorganisms confer/amplify plant tolerance to drought environments.

One of the ways to understand the impacts of abiotic stresses on plants is through omic studies which help to elucidate plant microbial processes that can be harnessed for biotechnological purposes. At the transcriptomic level, it was observed that the bacterium *Paenibacillus polymyxa* B2 enhanced the drought tolerance of *A. thaliana* in good correlation with overexpression of some drought responsive genes compared to un-inoculated plants (Timmusk and Wagner, 1999). With the aid of differential display polymerase chain reaction and 2-D polyacrylamide gel electrophoresis, six drought stress proteins (pathogenesis-related protein, adenosine kinase , vacuolar H⁺-ATPase, dehydrin-like protein, S-adenosylmethionine synthetase, early nodulin ENOD18) were expressed in pepper plants amended with *Bacillus licheniformis* under drought stressed condition (Lim and Kim, 2013). Also, quantitative polymerase chain reaction (qPCR) revealed the *Bacillus amyloliquefaciens* 5113 and *Azospirillum brasilense* priming effects on the expression of drought responsive genes [such as ascorbate peroxidase (APX1), S-adenosyl-methionine synthetase (SAMS1) and heat shock protein (HSP17)]and on

some enzyme activities involved in enhancing drought stress of wheat leaves (Kasim et al., 2013). Illumina sequencing (HiSeq 2000 system) revealed drought responsive genes in sugar cane inoculated with the diazotroph *Gluconacetobacter diazotrophicus* PAL5. Enhanced plant acclimatization to arid environment through AMF interaction is connected to the fungal ability to enhance plant nutrient- and water- uptakes resulting in an increase in the plant tissue water content. Thus, AMF interactions enable plants to acclimatize to drought conditions through different mechanisms which comprise nutritional, morphological, and physiological mechanisms (Kong et al., 2014). Although findings have shown how these fungi confer drought tolerance to plant, more information is needed to unveil the whole mechanisms associated with such process.

In addition to single inoculation of rhizobacterial species, a further inoculation with AMF enhances plant drought tolerance. Inoculation of common bean (*Phaseolus vulgaris* L.) with *P. polymyxa*-DSM36, *P. polymyxa*-Loutit strain and *Rhizobium tropici*-CIAT 899 resulted in greater growth compared to single inoculation with *Rhizobium* species. Moreover, combined inoculation increased the number and weight of nodules of the plant under drought stress environment compared to inoculation with *Rhizobium* alone (Figueiredo et al., 2008). Under arid and semi-arid conditions, AMF and *Pseudomonas mendocina* significantly improved root phosphatase activity and proline accumulation in lettuce leaves (Kohler et al., 2008). Subsequent co-inoculation with *Pseudomonas* species and *Azotobacter chroococcum* reduced drought stress in wheat through increased modifications of the anatomical structures such as mesophyll and phloem tissues, thickness of epidermis and diameter of xylem vessel whereas water deficiency had negative effects on the anatomical tissues of the control (El-Afry et al., 2012). Bacterial consortium of *Bacillus cereus* AR156, *Serratia* species and *B. subtilis* SM21 enhanced drought resistance in cucumber plants. After exposing the plant to drought for 13 days, the plants treated

with the bacterial isolates showed darker green leaves with mild symptoms, increased chlorophyll and proline content and decreased relative electrical conductivity. The bacterial consortium treated cucumber plants also enhanced the superoxide dismutase activity and alleviated the drought-activated down-regulation of genes coding for ribulose-1, 5-bisphosphate carboxyl/oxygenase (Rubisco) large and small subunits and ascorbate peroxidase in the leaves of cucumber plants (Wang et al., 2012a). Microbial consortium containing exopolysaccharide producing bacterial isolates exhibited more potential to drought tolerance in maize compared to single bacterial strain (Naseem and Bano, 2014). Rhizobacteria consortia reduced drought stress by minimizing oxidative damage and increasing proline content in rice plants cultivated under drought condition which resulted in enhanced plants growth. These findings show that beneficial plant-microbial interaction under drought stressed condition can be encouraged by the introduction of drought resistant microbial isolates that have the potential to enhance plant metabolic plasticity and to maintain the growth of plants.

2.6 Influence of Positive and Negative Interactions on Plant and Microbial Diversity

The associations between plants and rhizosphere microorganisms are dynamic and complicated (Philippot et al., 2013, Turner et al., 2013a). Although, belowground interaction can maintain or increase aboveground productivity, compounds generated in the aboveground biomass can have significant positive or negative effects on the belowground microbial diversity and vice versa. In particular, the immune defense structure of plants is believed to play a role in shaping microbial structure. Mutant strain of *A. thaliana* that lacked acquired immunity exhibited microbial community structure different from that of the wild type, but chemical activation of the acquired immune system did not cause noticeable change in the bacterial community in the rhizosphere. Also, reduction in the richness and abundance of endophytes was observed as a

result of defense mechanism triggered by salicylic acid in the phyllosphere of *A. thaliana* while plants without jasmonate-triggered defense revealed greater diversity of epiphytes. These results suggest that the distinct plant immune pathways differentially impact soil microbial community and structure especially certain groups of bacteria (Philippot et al., 2013).

The production of plant hormones such as indole-3-acetic acid and gibberellins are common among rhizobacteria species and they are known to enhance plant growth. *Pseudomonas syringae* releases compounds similar to hormones that hinder ethylene and jasmonate signaling thereby allowing pathogen to gain access into the plant through the resultant effect of stomatal opening (Philippot et al., 2013). On the other hand, many chemical compounds generated by plants enhance specific interactions in certain microbial groups. In particular, flavonoids cause different responses in MF, rhizobacteria, root pathogens and some plants (Hassan and Mathesius, 2012) while strigolactones produced in low concentrations from plant roots stimulate MF hyphal branching and parasitic plant (e.g. *Orobancha* spp.) seed germination (Philippot et al., 2013, Turner et al., 2013a). Glucosinolates generated by transgenic *Arabidopsis* changed the microbial communities in the root and rhizosphere. Alkaloids, terpenoids and phenolics are commonly produced by plants. As an illustration, the order Brassicales and *Arabidopsis* produce glucosinolates, but *Avena strigosa* commonly known as oat produces avenacins which are effective against a wide range of fungi. Mutant oat deficient in avenacins exhibits different rhizosphere culturable fungal species which are more vulnerable to pathogenic fungi than the wild type (Turner et al., 2013a). Upon analytical investigation, there was amazingly no significant variation in the rhizosphere fungal diversity between the mutant oat and the wild type. However, mutant genotype deficient in avenacins seriously affected eukaryotic community of Amoeba including Alveolata without having any effects on bacteria (Turner et al.,

2013a). This suggests that precise changes in plants metabolism could have serious impacts on plant associated microorganisms.

2.7 Insights into Below and Aboveground Microbial Interactions via Omic Studies

Plants establish interactions with several different microorganisms and a large proportion of these exceedingly complex microbial groups have not been characterized. In particular, it was recently reported that only 7% of all terrestrial fungi have been characterized (Mora et al., 2011) and a significant fraction of the unidentified fungi interact with plant in different ways. Investigating complex microbial community in the environment is a big challenge. Therefore, omic studies of microorganisms that inhabit the rhizosphere are crucial because this is the region where most plant-microbial interactions occur and this technology could help to discover novel beneficial plant-microbial interactions that may be useful to alleviate drought stress of plants.

Real-time application of metagenomics, metatranscriptomic and transcriptomic to rhizosphere microorganisms may possibly provide more insights into complex microbial interactions (Gust et al., 2010, Kikirde et al., 2010, Schenk et al., 2012). A technical method that merges PhyloChip metagenomics of the rhizosphere microbiota with culture-based transcriptomic analyses unveiled over 33000 archaeal and bacterial species in the rhizosphere of plants cultivated in a disease-suppressive soil (Mendes et al., 2011). In addition, data from structural genes, functional genes and protein analyses and metabolomics investigations may be combined statistically that will help to detect vital biological functions and make projections via modeling. This strategy was recently utilized to gain insights into interaction involving ectomycorrhizal *Laccaria bicolor* and *Populus tremuloides* roots. Transcriptomic profiling of matured ectomycorrhizal via next generation sequencing technique resulted in the detection of transcripts which were matched to definite metabolic channels and used to establish the fungal

metabolome model. The model was used to project that other metabolites such as glycine and glutamate produced by *L. bicolor* might be utilized by *P. tremuloides* which in return, supplies carbon substrates such as glucose and fructose to the AMF (Schenk et al., 2012).

Furthermore, omic will be useful in detecting the roles of different genes, RNA, proteins and metabolites involved in plant-microbial interactions which would eventually unveil important processes that strengthen these interactions. This approach could simply be accomplished by establishing ‘genome-scale models’ that are appropriate for metabolic flux analyses in which microbial groups might be seen as a single interacting super-organisms while interacting plant’s ‘genomic-scale models’ integrate a certain level of division and differentiate metabolic processes (Oberhardt et al., 2009, de Oliveira Dal'Molin et al., 2010). This type of model has been constructed for *Arabidopsis* and some bacterial spp.

2.8 Future Prospects

According to Babalola (Babalola and Glick, 2012), interactions between plant and microbial species are not exhaustively understood. Hence, the need for more studies on plant microbial interactions is becoming more pressing as this will give useful insights that may be important in sustainable agriculture development. Also, knowledge of such interactions can be useful in the development of microbial inoculants (Alori et al., 2017) with growth enhancing potentials. To achieve this feat and discover other plant microbial interactive benefits, several facts have to be understood since considerable numbers of factors are involved in interactions relating to microorganisms, host plant, soil matrix and other environmental components. In addition, the formation of common mycelial network by AMF which have insecticidal potential is an emerging area of study that could be harnessed for the production of bio-insecticides

(Babikova et al., 2013a, Johnson and Gilbert, 2015). This will help to minimize the detrimental effects of chemical insecticidal compounds in the environment.

Studies have also depicted that plant-microbial interactions could also play a role in sustainable agriculture by helping to control a wide range of pathogens thereby enhancing crop health, productivity, yield and consequently boosting global food security (Igiehon and Babalola, 2018c). However, more knowledge on the physiology of these useful interactive microorganisms, microbial-cultivar acclimatization relationships, and microbial inoculant introduction or delivery techniques is needed. Also required is how inoculated beneficial microbial populations and their activities could be maintained in the soil rhizosphere (Babalola, 2010).

Researches into microbial ecological interactions have resulted in significant outcomes biotechnologically (Braga et al., 2016). Thus, there is the prospect of developing more useful and beneficial compounds from complex microbial interactions in the ecosystem that could be relevant in agriculture. More knowledge is similarly needed as regards the whole mechanisms involved in interactions between microorganisms and host plants and for this to be accomplished; the establishment of *in vitro* and *in vivo* techniques or models is paramount (Schenk et al., 2012).

2.9 Conclusions

Plant roots are extremely important for mineral nutrient uptake and productivity, and there is therefore the need to continue study on plant interactions with soil microorganisms. Similarly, more attention should be placed on field application of these microorganisms as biofertilizers and/or bio-pesticides so as to abate the negative effects of conventional chemical

fertilizers and pesticides, increase crop yield and enhance global food security. With the emergence of advanced omic technologies, it is now possible to effectively monitor transformations in rhizosphere microbial community together with their effects on plants development. This may pave way for scientists to develop discover microbial species that will interact effectively with plants.

2.10 Abbreviations

AMF	=	Arbuscular mycorrhizal fungi
MF	=	Mycorrhizal fungi
ACC	=	1-aminocyclopropane-1-carboxylate
2, 4-DAPG	=	2, 4-diacetylphloroglucinol
RNA	=	Ribonucleic acid
PAMPs	=	Pathogen-associated molecular patterns
ISR	=	Induced systemic resistance
MSIs	=	Multiple symbiotic impacts
IAA	=	Indole-3-acetic acid
SS	=	Sewage sludge
VOCs	=	Volatile organic compounds
Rh	=	Rhizobacteria
qPCR	=	Quantitative polymerase chain reaction

APX1	=	ascorbate peroxidase
SAMS1	=	S-adenosyl-methionine synthetase
HSP17.8	=	heat shock protein
Spp.	=	Species

2.11 Conflict of Interest

Author 1 declares that he has no conflict of interest. Author 2 declares that she has no conflict of interest.

2.12 Acknowledgements

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CHAPTER 3

Rhizosphere Microbiome Modulators: Contributions of Nitrogen Fixing Bacteria towards Sustainable Agriculture

Abstract

Rhizosphere microbiome which has been shown to enhance plant growth and yield are modulated or influenced by a few environmental factors such as soil type, plant cultivar, climate change and anthropogenic activities. In particular, anthropogenic activity, such as the use of nitrogen-based chemical fertilizers, is associated with environmental destruction and this call for a more ecofriendly strategy to increase nitrogen levels in agricultural land. This feat is attainable by harnessing nitrogen-fixing endophytic and free-living rhizobacteria. *Rhizobium*, *Pseudomonas*, *Azospirillum* and *Bacillus*, have been found to have positive impacts on crops by enhancing both above and belowground biomass and could therefore play positive roles in achieving sustainable agriculture outcomes. Thus, it is necessary to study this rhizosphere microbiome with more sophisticated culture-independent techniques such as next generation sequencing (NGS) with the prospect of discovering novel bacteria with plant growth promoting traits. This review is therefore aimed at discussing factors that can modulate rhizosphere microbiome with focus on the contributions of nitrogen fixing bacteria towards sustainable agricultural development and the techniques that can be used for their study.

Keywords: microbiome; next generation sequencing; plant yield; rhizobacteria; rhizosphere; sustainable agriculture

3.0 Introduction

The rhizosphere is the zone of the soil environment that is endlessly regulated by exudates, border cells and mucilages released by plant roots (Moe, 2013, Babalola, 2010, Igiehon and Babalola, 2017). “Rhizodeposit nutrients, border cells, mucilages, and exudates produced by plants attract and serve as food for microorganisms that are present in the rhizosphere (Igiehon and Babalola, 2017)”. It therefore means that plant’s root exudates can influence the diversity of resident microorganisms and invertebrates in the rhizosphere and these organisms can as well influence the plants by releasing regulatory substance. Hence, rhizosphere organisms are considered as a well-developed external functional environment for plants (Philippot et al., 2013, Spence and Bais, 2013, Turner et al., 2013a, Spence et al., 2014) and they are regarded as plant’s second genome (Berendsen et al., 2012a). Since plants are regarded as metaorganisms (East, 2013), understanding the actual contributions of rhizosphere microbiome (especially nitrogen (N) fixing bacteria) towards plant health and productivity is necessary.

Imbalance in nitrogen cycling, soil physicochemical properties, changing climatic conditions and abiotic stresses are the interwoven factors threatening sustainable agriculture. Sustainable agriculture depends on fertile soil, but land degradation and rapid desertification cause estimated loss of 24 billion tons of fertile arable land globally (Dubois, 2011). Recent investigations have demonstrated that plant host and its developmental stages play a role in shaping rhizosphere microbiome structure (Hou and Babalola, 2013, Peiffer et al., 2013, Chaparro et al., 2014a). Also, comparison of the microbial diversity in different maize rhizosphere provided indication of host genetic influences on the richness of the rhizosphere microbiomes (Peiffer et al., 2013, Bouffaud et al., 2012), but whether these disparities differentially have effects on plant health and development of the corresponding plant cultivar is yet to be known. This finding indicates

that it may be possible to harness host plant genetic variation in a way that will allow the incorporation of rhizosphere microorganisms into plant breeding programmes so as to stimulate beneficial interactions between plants and microorganisms.

Moreover, factors such as soil type determine the composition of both endophytic and free-living rhizosphere microorganisms (Lundberg et al., 2012, Bulgarelli et al., 2012b, Lakshmanan et al., 2014, Babalola et al., 2007). Many studies have shown that soil has a great effect on bacterial and mycorrhizal fungal structure in the rhizosphere (de Ridder-Duine et al., 2005, Santos-González et al., 2011b, Andrew et al., 2012). In one of the studies, soil and cactus rhizospheric microbial communities were surveyed using culture independent techniques and it was discovered that microbial communities were shaped mainly by soil properties from different geographical locations followed by the cactus rhizosphere (Andrew et al., 2012). In a similar study, the actinobacterial populations in the rhizosphere of strawberry plants grown in different soils were more alike than the bulk soil were to each other, suggesting that plant is a stronger modulator of microbial richness than the soil type (Costa et al., 2006).

Other extrinsic factors like climate change and anthropogenic activities can also modulate the microbial communities in a specific plant host (Vacheron et al., 2015). In particular, anthropogenic activity such as the use of N-based chemical fertilizer is associated with environmental destruction (Isobe and Ohte, 2014). This factor has led to the reduction in land mass that can be cultivated for agricultural purpose. Yet, there is increase in food consumption as a result of the rising human population and this poses a great demand on large hectares of viable land for agriculture. This challenge can be overcome by harnessing rhizosphere microbiome (such as N fixing bacteria) for different agronomic purposes, especially as microbial inoculants. The idea behind this agricultural practice is to expressly decrease the use of chemical fertilizers

which have a lot of negative impacts on the environment. Thus, the use of plant microbiome has the potential of minimizing environmental pollution, the occurrence of disease out-break, enhancing plant development, health and productivity. These microorganisms which can be applied on agronomic seeds singly or in consortium include endophytic and free-living root microbiome especially N-fixing bacteria that are known to fix atmospheric N for plant use. N is among the most important mineral nutrients needed for plant growth (Isobe and Ohte, 2014) and modern agriculture currently needs a safe and sustainable source of N and this can be achieved by the use of microbial inoculants.

Researchers have consequently been giving attention to endophytic bacteria because of their role in increasing N input to the soil (Gallego-Giraldo et al., 2014). These efforts have demonstrated that root nodules of crops contain endophytic bacteria including *Rhizobium* spp., non-nodulating strain of *Endobacter medicaginis*, *Micromonospora* spp., *Microbacterium trichothecenolyticum* and *Brevibacillus choshinensis* (Ramírez-Bahena et al., 2013, Trujillo et al., 2010). Taxonomic distinctions among bacteria associated with the stem, leaves and nodules of legumes have also been unveiled using culture independent techniques (Pini et al., 2012) nevertheless little is known about endophytic bacteria associated with some agricultural crops (Khalifa et al., 2016).

Hence, the fundamental questions to better comprehend plants and their associated microbiomes are “who is there?”, “how do they respond to variation in environmental conditions?” and “do they impact plant health and growth?” Proffering solutions to the above questions will result in better understanding of how environmental changes modulate plant microbial composition; a prerequisite to detect if and how such microorganisms might be utilized in the future to enhance plant growth and crop yield especially in arable lands faced with

environmental perturbations. Genomic studies of the plant associated microbiome are of utmost importance to provide answers to the abovementioned posers. DNA based analyses of each microbial species or meta-genomic profile of entire microbial communities reveal the composition and functional potential of plant rhizosphere microbiome (Knief, 2014). Among the currently used molecular techniques, NGS techniques have the greatest impact on DNA and RNA based analysis techniques. NGS techniques help to find solutions to problems that could not be solved previously, due to financial and technical constraints. Hence, plant associated microbiome can currently be studied at a speed cum depth as never before.

This review is therefore aimed at discussing factors that can modulate rhizosphere microbiome with focus on the contributions of N fixing bacteria towards sustainable agricultural development and the techniques that can be used for their study (Mendes et al., 2011, Knief et al., 2012, Rincon-Florez et al., 2013, Schlaeppi et al., 2014, Yergeau et al., 2014).

3.1 Modulators of Rhizosphere Microbiome of Agricultural Crops

Various factors such as biotic and abiotic parameters modulate or influence the microbial diversity and composition in the region surrounding the root (Figure 1). The level to which microbial communities are influenced by biotic and abiotic factors is not totally understood. Biotic factors such as soil type, plant host genotype/cultivar, climate change and anthropogenic activities are some of the abiotic factors that determine the rhizosphere microbiota composition (Berendsen et al., 2012a, Chaparro et al., 2012, Philippot et al., 2013, Turner et al., 2013b, Spence and Bais, 2013, Minz et al., 2013).

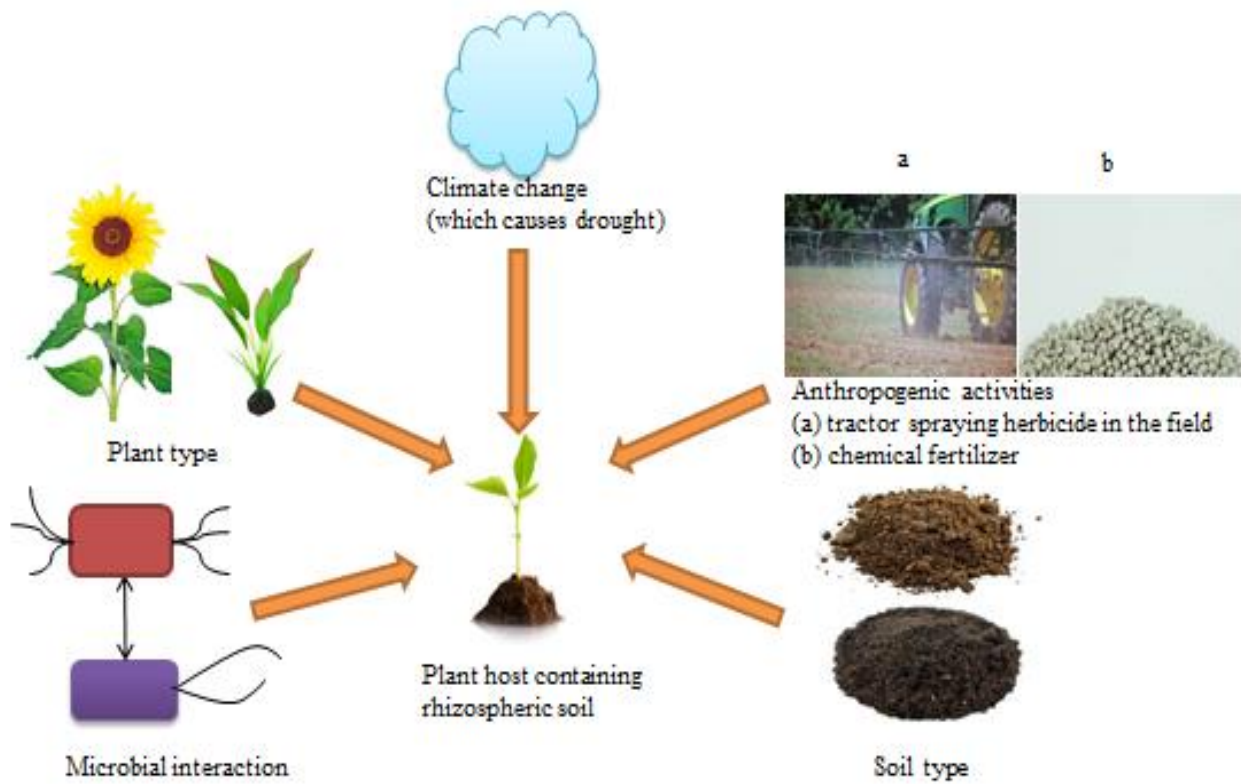


Fig. 3. 1 Rhizosphere microbiome modulators in agricultural system

Several studies have revealed that soil has a great effect on the structure of mycorrhizal fungal communities in the rhizosphere (Santos-González et al., 2011a, Andrew et al., 2012, İnceoğlu et al., 2012). The physicochemical qualities of the soil influence plant health and type of root exudate release with the consequential effect on the structure of the rhizosphere microbial community. Sequence analysis of the bacterial community of the rhizosphere of different *Arabidopsis thaliana* cultivars revealed that soil type greatly affects rhizosphere microbial diversity (Bulgarelli et al., 2012b, Lundberg et al., 2012). The differences and resemblances observe in several studies could best be understood by viewing the microbial structure of the rhizosphere as emanating from a cascade of events. Firstly, the soil can be viewed as a seed bank of microorganisms (Lennon and Jones, 2011) and secondly, the physicochemical characteristics of the soil coupled with bio-geographical activities determine the microbial assembly of the soil environment.

Soil can differ in structure, organic matter, pH, texture and nutrient status. These soil properties can select specific microorganisms by creating conducive environments that favour certain types of microorganisms and regulate the availability of roots exudates affecting selection of microorganisms by plants. In particular, soil pH and availability of nutrient such as carbon have been observed to affect the diversity of crop pathogenic nematodes, bacteria, fungi and beneficial microorganisms (Rotenberg et al., 2005, Toljander et al., 2008, Dumbrell et al., 2010). In some cases, soil properties may lead to soil type-specific composition of rhizosphere microbiome (Garbeva et al., 2004). This was further confirmed by Gelsomino et al. (1999) who demonstrated that bacterial community structures were alike in soils of the same type and Latour et al. (1996) found that soil type affected the abundance and composition of *Pseudomonas* species in flax and tomato rhizosphere. This suggests that soil properties and soil type can

determine the types of microorganisms that colonize the rhizosphere, and that different soil types can contain different microbial species (Figure 1).

Plant cultivar/genotype (Figure 1) influences the indigenous microorganisms present in the plant rhizosphere (Inceoğlu et al., 2012, Hardoim et al., 2012, Bouffaud et al., 2012). While physicochemical characteristics of the soil can influence the composition of soil microorganisms, plant root exudates are able to modify the rhizosphere environment that slowly alters the soil microorganisms to support the establishment of a rhizobiome (Lareen et al., 2016). These root exudates together with plant root immune system would further select those microorganisms that have the ability to colonize root surface (rhizoplane) and inner root tissue (endosphere). Microorganisms that colonize root tissues (endophytes) can have harmful or positive effects on plant species which eventually have feedback effects on rhizosphere microbiome (Bever et al., 2012). Furthermore, certain metabolites liberated into the root region can elicit several responses in various soil microorganisms. In particular, flavonoids release from plants attract symbiotic microorganisms such as *Bradyrhizobium japonicum*, and disease-causing microorganisms e.g., *Phytophthora sojae*. The flavonoid naringenin produced by legumes activates germination of mycorrhizal spore and hyphal branching while the flavonoid catechin produced by *Combretum albiflorum* regulates quorum sensing (Hassan and Mathesius, 2012, Pérez-Montaña et al., 2011). Similarly, some defense metabolites (e.g., pyrrolizidine alkaloids) can affect the rhizosphere microbial structure by enhancing the growth of microorganisms that are able to break down these metabolites.

Recent study shows that variations between plant cultivar in a single gene could significantly affect the rhizosphere microbiome. The release of a single exogenous glucosinolates changed the microbial population on transgenic *Arabidopsis* roots (Bressan et al., 2009) in which

fungi and α -proteobacteria were predominantly affected, as revealed by denaturing gradient gel electrophoresis (DGGE). A study has also shown that the ABC transporter mutant of *Arabidopsis*, *abcg30*, produced root exudates containing high amounts of phenolic compounds and low amounts of sugars, which also gave rise to a unique rhizosphere microbiome (Badri et al., 2009).

Rhizosphere bacterial analysis using PhyloChip technique of three different cultivars of potato grown at two separate sites revealed 2432 operational taxonomic units (OTUs) and 40% of the OTU abundance was site-specific (Weinert et al., 2011). However, the abundance of 9% of the OTUs was cultivar-contingent in one or the other site, whereas only 4% of the OTUs had cultivar-contingent abundance in both sites. These outcomes demonstrate not only the significance of the soil in shaping rhizosphere microbial community structures, but also that certain microorganisms have a special affinity for specific plant cultivars. Amazingly, variations in abundance on the potato cultivars were observed for microorganisms belonging to the order *Pseudomonales* and families *Streptomycetaceae* and *Micromonosporaceae*, which have been broadly studied for their capacity to control plant pathogenic microorganisms. This result further suggests that plant cultivar can influence the accumulation of bacteria that protect the plant against pathogens. Similarly, reports have revealed differences in the ability of wheat genotypes to assemble *Pseudomonas* species that produce antifungal metabolite 2, 4-diacetylphloroglucinol (DAPG), bringing about differences in disease suppressiveness (Meyer et al., 2010, Gu and Mazzola, 2003). Additionally, the quantity of antimicrobial substance produced on roots by certain biocontrol species differs between wheat genotypes (Okubara and Bonsall, 2008). Certain wheat genotypes were also found to differentially support biocontrol bacterial species indicating

that there is a level of specificity in the association between plant cultivar and the microbial species in the rhizosphere soil environment (Meyer et al., 2010).

It has been suggested that the modern way of plant breeding has probably not taken into consideration traits that are important for plants to serve as hosts to mutualistic microorganisms (Philippot et al., 2013). In an attempt to know the genetic components in plants responsible for establishing symbiosis with rhizobacteria, three “quantitative trait loci” in the genome of a tomato plant involved in suppressing disease caused by *Bacillus cereus* were detected. Furthermore, research on microbial diversity in the rhizosphere of maize showed that host genetic factor influences the composition of the rhizosphere microbiome (Peiffer et al., 2013, Bouffaud et al., 2012).

In addition, microorganisms in the soil are a possible factor that influences the structure of the rhizosphere microbiome (Figure 1). Several studies have examined microbial colonization of the rhizosphere by intentionally coating crop seeds with certain microorganisms (Philippot et al., 2013). Previous investigations revealed that bacterial community on cucumber seedlings roots are more similar to the soil bacterial community than the seed coat bacterial biofilm, indicating that bacterial flora of the seed surface have little or no effect on the rhizosphere microbial structure. This may not be true for microorganisms present within the seeds, since study of endophytic bacteria of maize seeds showed the presence of one of the groups of the endophytic bacteria in maize rhizosphere (Blee et al., 2013). Seed endophytic bacteria introduction into the rhizosphere suggests that plants could transfer certain microorganisms from generation to generation. Such carry-over effect on the structure of the rhizosphere microbiome has a significant effect on co-evolution of plant-microbial interactions in the environment (Philippot et al., 2013).

3.1.1 Other Rhizosphere Microbiome Modulators: Climate Change and Anthropogenic

Activities

Microorganisms play important functions in all environments and so it is significant to understand how they respond to anthropogenic activities and climate change (Singh et al., 2010, Crowther et al., 2015). Climate change has different effects, ranging from local cooling to global warming, shifting vegetation zone and augmented extreme weather events and all these effects have indirect impacts on rhizosphere microbiome (Figure 1). Increase in carbon dioxide levels, a component that is alleged to be the key driver of climate change, could also directly affect rhizosphere interactions by changing root exudation patterns and soil food web structure and functioning (Haase et al., 2008, Stevnbak et al., 2012, Drigo et al., 2013). The structure of the soil food web can play a significant role in ameliorating the impacts of extreme weather events (De Vries et al., 2012). Experimental modification of carbon dioxide, precipitation and temperature has also indicated that climate changes have significant effects on microbial composition and abundance in the soil (Castro et al., 2010, Sheik et al., 2011). Such transformations in microbial structure can similarly change the biogeochemical cycles/processes mediated by these microorganisms. Alterations in natural ecosystems caused by climate and anthropogenic changes can augment the impact of previously trivial biogeochemical processes or introduce new processes to the ecosystem (Singh et al., 2010).

An unraveled problem now is how microbial communities of the rhizosphere will respond to different facets of climate change. Rhizosphere microorganisms may have a greater ability to evolve than their host plants. In addition, as a result of their vast biodiversity, rhizosphere microorganisms may comprise of taxa that are acclimatized to warmer environmental conditions. The dispersal of soil microorganisms may also permit the immigration of microbial species from

warmer environments into relatively cooler soil environments. Whether rhizosphere microbial composition under a particular climate change is dependent on spread of microorganisms or on their genetic acclimatization is an important question that needs to be addressed so as to know whether the microbial communities of the rhizosphere can cope with changes caused by global climate change.

In particular, climate change can cause abiotic stresses and some of the stresses associated with it are drought, cold and heat. It has been found that *Rhizobium* species can survive in dry environments, but their diversities are significantly lower in dry soil environments. Moreover, drought does not only affect the ability of *Rhizobium* species to fix N but also the growth and development of plant legumes. At times, it is difficult to separate the consequences of drought stress from heat stress as they both occur at the same time in semi-arid and arid regions of the world (Giller, 2001, Serraj and Adu-Gyamfi, 2004). Suitable rhizobial species that can survive under drought environments in symbiosis with leguminous crops are of utmost importance (Giller, 2001) and therefore more research has to be focused in this area using modern state-of-the art techniques.

Furthermore, since the inception of the industrial revolution, anthropogenic activities in natural ecosystem have escalated as a result of the rising human population, pollution and ecosystem degradation and these have greatly affected soil microbial community structures. For instance, anthropogenic transformation of forest to farm lands resulted in the homogenization of the indigenous soil bacterial structure (Rodrigues et al., 2013). Thus, during the last 4 decades, the increasing awareness of the destructive impacts of human activities on the environments has triggered the enactment of laws to checkmate human behavior towards the environment. Though, the emphasis of the positive change in human behavior has been on the preservation of animals

and plants while the negative impact of anthropogenic activities on microbial community has been relegated. This is a serious issue because microorganisms are the first responders to ecosystem disturbance and can either improve or buffer ecosystem shift (Labbate et al., 2016). Such ecosystem disturbance can occur as a result of herbicide and chemical fertilizer applications (Figure 1).

Herbicides are chemical compounds commonly used to control weeds in agriculture and many of them cause environmental contamination cum human health problems due to their toxicity and persistence in the soil (Gyamfi et al., 2002). Like chemical fertilizer, non-target impacts of herbicides on soil microbiome can be positive, negative or neutral (Griffiths et al., 2008, Haney et al., 2002, Lupwayi et al., 2004). reported that since herbicides kill plants, they can similarly kill or influence the activity of soil microbiome (Haney et al., 2002). Soil microbial community is a crucial component of soil quality and thus, the effect of herbicides in the soil is very important.

The rhizobacterial community can be affected by the presence of herbicides such as glyphosate, frequently used where ‘genetically modified herbicide-tolerant’ crops are grown. The impact of glyphosate on rhizosphere microorganisms from agricultural crops like soybean and maize has been reviewed (Kremer and Means, 2009). Certain herbicides like glyphosate move slowly in the soil and may adhere to organic matter and gradually degrade in soil and water (Pessagno et al., 2008, Barja and dos Santos Afonso, 2005) while others remain as recalcitrant in the soil or are leached into groundwater (Boyd, 2000). Although some herbicides persist in the soil, the herbicides glyphosate, atrazine and bentazon can be degraded by microorganisms. Atrazine-degrading microbiome might accumulate in the soil as a result of repeated applications and degradations of the herbicide (Satsuma, 2009). But Toyota, Ritz (Toyota et al., 1999)

observed that atrazine and glyphosate might reduce soil microbial populations and enzyme activity while Ratcliff et al. (2006) observed changes in fungal and bacterial communities upon application of different herbicides.

Herbicides do not only affect microbial diversity, they also affect microbial functions (e.g., N fixation) in the soil. N fixation in legumes such as soybean can be triggered by symbiotic interaction between *Rhizobium* species and soybean roots and there is indication that the presence of sub-lethal amounts of glyphosate in growth media results in the accumulation of shikimate and decrease in *Bradyrhizobium japonicum* growth (Hernandez et al., 1999). But, different *B. japonicum* strains exhibit different sensitivities to the herbicide (Hernandez et al., 1999, Moorman et al., 1992). Some species in the family *Rhizobiaceae* might breakdown glyphosate and utilize it as the main source of phosphorus in the presence of aromatic amino acids (Liu et al., 1991). The impacts of glyphosate on nitrogen fixation by *Rhizobium* spp. in soybean have been studied in field and greenhouse experiments (Bohm et al., 2009, Zobiole et al., 2010) even though the results as regards the effects of the herbicide on N fixation have been inconsistent. For instance King et al. (2001) observed significant decrease in nitrogenase activity in glyphosate-resistant soybean when the herbicide was added three weeks after emergence and recommended that N fixing activity was more susceptible to glyphosate in the early stages of soybean growth. However, Powell et al. (2009) reported that there were no negative effects of the herbicide on N fixation under greenhouse experimental set-up. Also, soil microbial activity was affected when pure and formulated herbicides were applied to soils at rates greater than the recommended rate (Crouzet et al., 2010) and these effects of herbicides on microbial communities can either be short-term or long-term (Dick et al., 2010). Furthermore, the level of chemical fertilizer application is also another important anthropogenic factor that modulates

rhizosphere bacterial diversity of plants in the field (Ikeda et al., 2010a). Sequence analysis of bacterial communities of rice crops in fields amended with low and standard levels of N fertilizer showed that the rhizosphere microbiome was strongly affected by the level of N fertilizer. The abundance of OTUs in the genera *Bradyrhizobium*, *Methylosinus* and *Burkholderia* were higher in the rhizosphere microbiome from the field of the low level N fertilizer than standard level N fertilizer. On the contrary, the relative abundance of methanogenic archaea was higher in the field amended with standard level of fertilizer (SLF) than low level N fertilizer (LNF) field (Ikeda et al., 2010b). The genes *pmo/mmo* and *acdS* responsible for methane oxidation and plant interaction respectively were more abundant in rice rhizosphere microbiome grown in LNF field. Similarly, functional genes for the metabolism of sulphur (S), iron (Fe), aromatic compounds and N were significantly higher in the LNF rhizosphere microbiome (Ikeda et al., 2014), but, ¹³C-labeled methane experiment and quantitative polymerase chain reaction (qPCR) analyses for *mcrA* and *pmoA* genes coding for methyl coenzyme-M reductase and methane monooxygenase respectively indicated that methane oxidation was more active in the rice roots cultivated in LNF field than in those grown in SLF field (Ikeda et al., 2014). These outcomes indicate that low-N fertilizer management is a crucial factor that modulates rhizosphere microbiome community structure and these coupled with other negative impacts of N based fertilizers necessitate the need for a more ecofriendly means of enhancing N level of agricultural land. This feat can be achieved by harnessing N-fixing endophytic and free living rhizobacteria.

3.2 Plant Endophytes and Their Ability to Fix Atmospheric Nitrogen

Nodule formation, which is a very efficient process of N uptake, has been reviewed extensively (Oldroyd et al., 2011, Udvardi and Poole, 2013). However, not all bacterial species can initiate nodulation because nodulation of plants such as legumes requires a complicated

plant-microbe interaction. Certain bacteria penetrate roots via cracks initiated by “lateral root emergence” as well as wounds caused by “movement through the soil” (Gaiero et al., 2013). These bacteria enhance plant development and play roles in N fixation (Santi et al., 2013). Though there is no direct evidence that endophytes fix N in their plant hosts, the possibility of the process is broadly accepted. For instance, mutant strain of *Gluconacetobacter diazotrophicus* which lacks the capacity to fix N is not able to enhance its host plant growth as much as the wild type. The most studied endophyte that has the ability to fix N is *Pseudomonas stutzeri* A1501, which was first isolated in China (Tkacz and Poole, 2015). The bacterium has possibly obtained genes coding for nitrogenase and enzyme that are adapted to different environmental conditions. The bacterium was studied so as to understand how nitrogenase activity and N fixation process are regulated and it was found that addition of ammonia to culture media stops the process of N fixation by N fixing bacteria. This is because the gene responsible for the expression of nitrogenase is down-regulated in that condition. For instance, the transcription of *nif* genes which are needed by free living organisms is repressed in the presence of ammonia. “Interestingly, *P. stutzeri* can switch between denitrification, nitrification, and N fixation under anaerobic, aerobic, and micro-aerobic conditions, respectively (Yan et al., 2010)”. Transcriptomic study has also unveiled a formerly unknown gene that plays role in N fixing process termed *pnfA*. *PnfA* is controlled by similar sigma factors as *nifHDK* (which code for nitrogenase). The expression of *PnfA* genes is not directly affected by mutation; however the mutant strain exhibits reduced nitrogenase activity particularly in micro-aerobic environment (Yan et al., 2010).

Azoarcus species are N-fixing plant growth promoting rhizobacteria (PGPR) and the wild type of *Azoarcus* sp. BH72 which inhabits kallar grass roots was able to enhance the dry weight of the grass cultivated in an environment deficient in N when compared with “*nifK* mutant strain

of BH72". Surprisingly, the bacterium can transform irreversibly from free living to endophytic forms and vice versa and as such it is not always feasible to re-isolate *Azoarcus* sp. BH72 endophytic colonies from roots (Sarkar and Reinhold-Hurek, 2014).

Rhizobium sp. IRBG74 as well as *Azorhizobium caulinodans* which colonize rice roots have been isolated respectively from *Sesbania aculeata* and *Sesbania rostrata*. *Rhizobium* sp. IRBG74 has similarly been recovered from *Sesbania cannabina*, but being an endophyte, it does not have the potential to fix N since it lacks certain *nif* genes like *nifV*. *Rhizobium* sp. IRBG74 has now been re-categorized from the genus *Agrobacterium* to *Rhizobium* since it does not possess Ti plasmid, *fusA*, *rpoB* and 16S rRNA gene sequences. This bacterium possesses a sym-plasmid having *nifH* together with *nodA* genes (Crook et al., 2013) and it colonizes a wide range of *Sesbania* plants. Similarly, *A. caulinodans* ORS57 is able to colonize rice and fix N in endophytic form, however this bacterium should be tested with other plants species for its endophytic infection and N fixing abilities so as to know if this potential is unique to this plant sp. or is a common characteristic. "In order to determine whether it is the plant that initiates the N₂-fixation in its bacterial symbiont (as regards nodulation), a common SYM pathway rice mutant should be tested for its ability to form endophytic symbiosis with ORS571 (Chen and Zhu, 2013, Venkateshwaran et al., 2013)". It is also essential to distinguish identified multitude of genes of *Azorhizobium* responsible for plant infection, tolerance to stress and nodulation in order to ascertain those involved in *Azorhizobium-Sesbania* mutualistic interaction and rhizobial-legume associations.

Herbaspirillum seropedicae is an additional endophyte of plant roots that have been studied extensively and it infects the roots of rice, sugarcane and sorghum. It enhances the growth of its host plant by fixing N even in soil deficient in nitrogen and oxygen content (Pedrosa et al.,

2011). *H. seropedicae* perhaps acquired its N fixing capability via “horizontal gene transfer” like other non-rhizobial strains. Like other disease-causing microorganisms of the genus *Herbaspirillum*, it is fascinating to know that *H. seropedicae* which is non-pathogenic has the entire genetic make-up for type I, II, III, V, VI and IV pili which it uses to facilitate communication with its host plant (Schmidt et al., 2012). The type III is now known to be involved in the “initial signal communication” of *Bradyrhizobium elkani* and *Rhizobium* sp. NGR234 with their hosts (Okazaki et al., 2013).

Scientists’ attention on endophytes and their contributions to plant health and productivity should not only be theoretical and thus, these microorganisms can probably be utilized to improve nutrient absorption and plant diversity.

3.3 Rhizobiome as Plant Growth Promoters

Rhizobiome (which can be regarded as rhizobacteria) is a group of bacteria found in the rhizosphere that help to enhance the growth of their host plants. The rhizosphere has abundant microbial diversity and nutrients such as carbon substrates than the bulk soil. Microorganisms in the rhizosphere can be manipulated by wide range of complicated and highly monitored cell to cell interaction and by exploring signaling molecules to monitor their habitat and modify their activities. Rhizobacteria can affect nutrients absorption by plant roots directly or indirectly through nutrient mobilization/immobilization or alteration in root structure/physiology respectively. They can also enhance host plant nutrient status by increasing bioavailability of rhizospheric nutrients and improving other symbiotic interactions of the host (Vessey, 2003). Many microorganisms excellent at oxidizing manganese in the rhizosphere could therefore alleviate the toxic level of manganese in plants cultivated in oxygen deficient and saturated soils

or enhance the manganese deficiency level in aerated soil containing high amount of calcium carbonate (Babalola, 2010).

Rhizobacteria are a major component of PGPR, a terminology that was coined over 30 years ago (Kloepper and Schroth, 1978). PGPR are non-pathogenic bacteria that colonize plant roots and promote plant development and health by helping the plant to absorb more nutrients and control the proliferation of pathogens that would have been detrimental to the host plant (Babalola, 2010). Besides plant growth promotion, inoculation of rhizobacteria as a biofertilizer may enhance indigenous soil microbial diversity without leaving any negative effects in the soil unlike the conventional chemical fertilizers that have been reported to contaminate agricultural land upon application (Conway and Pretty, 2013, Rascovan et al., 2016). For instance, nitrates from chemical fertilizer can contaminate underground water and increase the risk of blue baby syndrome in newborn babies as well as stomach cancer in adults. Chemical fertilizer and pesticides can also have adverse effects on other environmental components such as surface water and soil fauna and flora.

It should be noted that some PGPR cannot be considered as biofertilizers. PGPR that enhance plant growth by eradicating pathogens are bio-pesticides and not biofertilizers. Amazingly, some PGPR seem to promote the growth of plants by functioning as biofertilizer and bio-pesticides. For instance, *Burkholderia cepacia* can destroy the pathogen *Fusarium* spp. and also promote maize growth under iron-deficient environments through siderophore production (Bevivino et al., 1998). Moreover, siderophores as well as antibiotics produced by some species of rhizobacteria are pathogen-suppressing factors which could also be utilized for agricultural purpose. Both factors have microbial antagonizing properties and are able to stimulate systemic resistance (Beneduzi et al., 2012).

However, some microorganisms such as arbuscular mycorrhizal fungi (AMF) present in the rhizosphere have been reported to have pesticidal traits (Babikova et al., 2014, Philippot et al., 2013); a potential that could also help to nullify the negative impacts of chemical pesticide application and indirectly improve above and belowground plants' biomass.

In particular, studies have shown how several rhizobiome influenced both above and below-ground biomass (Table 1) (Wani and Khan, 2013, Gopalakrishnan et al., 2015b, Uma et al., 2013). It is therefore desirable to harness a more ecofriendly, cost-effective and natural biological entities such as rhizobacteria and mycorrhizal fungi with soil-enriching, pesticidal and antimicrobial potentials for sustainable agricultural development (Philippot et al., 2013, Hou and Babalola, 2013).

Table 3. 1 Selected rhizobiome and their contributions towards sustainable agriculture development

Rhizobacteria Species	Contributions towards Sustainable Agriculture	References
<i>Azospirillum amazonense</i>	Enhanced grain yield by increasing dry matter, panicle number and nitrogen content at maturation.	(Rodrigues et al., 2008)
<i>Pseudomonas aeruginosa</i>	Enhanced the remediation capacity of broad bean plants cultivated in soil environment containing oil contaminants. It also helps to control plant pathogens.	(Philippot et al., 2013, Radwan et al., 2005, Zamioudis and Pieterse, 2012)
<i>Serratia liquefaciens</i>	Enhanced the remediation capacity of broad bean plants cultivated in soil environment containing oil contaminants.	(Babalola, 2010)
<i>Bradyrhizobium</i> spp.	Improved nodulation in leguminous plants as well as shoot and root growth. They also enhance plants resistance to drought and production of indole-3-acetic acid	(Babalola, 2010, Gopalakrishnan et al., 2015b, Uma et al., 2013, Shaharoona et al., 2007)

<i>Azospirillum</i> spp.	Enhanced N content in <i>Vicia sativa</i> .	(Mfilinge et al., 2014)
<i>Rhizobium</i> spp.	Enhanced significantly the height, pod number and length as well as seed weight of <i>Vigna mungo</i> and <i>Vigna radiate</i> .	(Ravikumar, 2012)
<i>Bacillus</i> spp.	Help plants to develop resistance against pathogens and pest.	(Philippot et al., 2013, Zamioudis and Pieterse, 2012)
<i>Sinorhizobium meliloti</i>	Improved biomass diversity in black madic plant that was subjected to copper stress.	(Gopalakrishnan et al., 2015a, Fan et al., 2011)
<i>Rhizobium</i> RL9	Increased lentil plant development, nitrogen content, seed protein content and seed produced under heavy metal stressed environment.	(Wani and Khan, 2013)
<i>Rhizobium</i> MRPI	Promoted nodule formation, leghaemoglobin concentration, seed protein and seed harvest in pea plant.	(Ahemad and Khan, 2011)

The effects of rhizosphere microorganisms on agronomic crops are highlighted below.

3.4 The Effects of Rhizosphere Microbiome on Sustainable Agriculture and Food Security

The global world requires novel ideas for farming so as to be able to generate farm produce that can cater for world population of 6.9 billion. Actualizing food security which is the process of producing sufficient food and enhancing its quality to sustain the ever increasing population without undermining environmental protection is termed global green revolution (Gupta, 2012). Sustainable development in the area of agriculture is required to alleviate these issues. Development of agricultural practices (that are eco-friendly), natural resources and energy conservative strategy that guarantee food security are the critical aims of sustainable agriculture as reported by National Research Council (Lakshmanan et al., 2014). It is the view of scientists that the most likely approach to actualize this objective is to replace hazardous inorganic fertilizers and pesticides with ecofriendly formulations of symbiotic microorganisms (such as *Bradyrhizobium* spp.) that have the potential to improve crop growth while providing protection from biotic stresses (such as plants pathogens and pests) and abiotic stresses (climate change/drought and environmental pollution).

Several studies on isolation, identification and application of microorganisms as an alternative method for chemical fertilizer utilization have been reported (Bhattacharyya and Jha, 2012a, Doornbos et al., 2012). Enhancing the richness and abundance of soil microorganisms by this alternative method has also shown to improve plants health and yield (Lau and Lennon, 2011, Schnitzer et al., 2011, Wagg et al., 2011). In this case, the microbial cultures are mixed with chemical carriers using solid or liquid fermentation techniques. The microbial isolates are either incorporated to the plant in pure or mixed culture either via seed application, seedling dip, bio-priming or soil application. In addition to the use of individual microorganism, identifying

suitable and functionally different microbiome and their usage for improving crop productivity is another huge and fundamental task to embark on since the entire microbiome is important, as it is described as the plant host second genome, “the metaorganisms” (Lakshmanan et al., 2014).

Hence, accomplishing food security is dependent on the enhancement of plant growth and diversity including seed yield right from the field and one of the ways this feat can be attained is through inoculation of agricultural crops (such as soybean) with rhizospheric rhizobacteria (e.g., *Rhizobium* spp.) as briefly discussed below.

3.4.1 Impact of *Rhizobium* Inoculation on Leguminous Crops Productivity

Rhizobium species are bacteria with the potential to reduce atmospheric N to ammonia for their host use through the formation of nodules on the roots or stems of leguminous plants (Mfilinge et al., 2014). This group of bacteria has been greatly studied due to their significance in agriculture and environment (Karaman et al., 2013, Nyoki and Ndakidemi, 2014). The amendment of seeds with *Rhizobium* species enhances seed protein, nodules formation and N absorption. In a review reported by Mfilinge et al. (2014), soybean (*Glycine max* L.) inoculated with *Rhizobium* significantly increased the crop growth and yield constituents such as number of branches bearing pod per plant, total number of pod per plant and seed number per pod. Amendment of *Vicia sativa* L. (vetch) with *Azospirillum* in greenhouse and field experiments increased significantly N fixation activity, percentage N and N content. *Rhizobium leguminosarum* introduced into pea and lentil seeds was able to enhance pea nodulation, shoot/root diversity and yield of pea seed. Also, seedling height, nodule and shoot biomass of lentil were enhanced. There was also enhancement on nodulation of peanut treated with *Rhizobium* species while chicken pea treated with same species in greenhouse and field experiments resulted in significant increase in plant growth, root dry weight and number of

nodules. Ravikumar (2012) discovered that there was significant increase in the height, fresh weight, roots, nodules, leaves, shoots and pods number, pods length and seed weight of *Vigna mungo* and *Vigna radiate* inoculated with *Rhizobium* when compared to control experiments (Table 1). Height of soybean inoculated with *Rhizobium* in field experiment significantly increased and stem girth was also increased in greenhouse cum field experiments (Tairo and Ndakidemi, 2013). Similarly, in a study carried out by Nyoki and Ndakidemi (2014), cowpea treated with rhizobial inoculants significantly increased the height of the crop when compared to the control counterparts.

These plant growth effects of *Rhizobium* species are enhanced when co-inoculated with other microorganisms. In co-inoculation, certain microorganisms function as helper microorganisms to enhance the effectiveness of the other microorganisms. Therefore, some bacteria can be co-inoculated with *Rhizobium* spp. that will not only enhance the effectiveness of the rhizobial spp. but also improve crop productivity.

Bacillus, *Azotobacter*, *Pseudomonas*, *Enterobacter* and *Serratia* are some bacteria that have been successfully co-inoculated with *Rhizobium* spp. *Azospirillum* was observed to improve the productivity of legumes after inoculation (Remans et al., 2008). Co-inoculation of *Azospirillum lipoferum* and *Rhizobium leguminosarum* bv. *trifolli* was observed to improve nodulation in white clovers (Tchebotar et al., 1998). *Azospirillum* was reported to increase the infection site for *Rhizobium* spp. leading to enhanced nodulation while inoculation of *Rhizobium* and *Azospirillum* was found to increase siderophore, vitamins and phytohormones production (Cassan et al., 2009, Dardanelli et al., 2008). Amendment of common bean with *Rhizobium* and *Azospirillum* was similarly observed to improve N fixation in the crop (Remans et al., 2008). *Azotobacter* was

reported to be a prospective co-inoculant with *Rhizobium* that improved vitamins and phytohormones production resulting in increased nodulation (Akhtar et al., 2012).

Bacillus sp. was as well found to stimulate plant growth promoting (PGP) ability, nutrient uptake and crop yield (Mishra et al., 2009, Stajkovic et al., 2011, Singh et al., 2011) as increase in yield and root weight of chickpea was observed upon inoculation of *Rhizobium* with *Bacillus* spp. (Elkoca et al., 2007). Improved N fixation and nodulation was detected when *Azospirillum* and *Bacillus* spp. were inoculated with *Rhizobium* in pigeon pea (Rajendran et al., 2008, Remans et al., 2008). *Streptomyces lydius* WYEC108 interaction with *Rhizobium* was noticed to promote pea growth including nodule number, perhaps by *Streptomyces* colonization of root and nodule. Furthermore, *Enterobacter* species are PGP bacteria that enhanced the yield of nodules in plant upon co-inoculation with a species of *Bradyrhizobium*. There was increase in infection sites area and the number of root hair formed when *Medicago truncatula* was amended with *Pseudomonas fluorescens* WSM3457 and *Sinorhizobium* (Fox et al., 2011). Also, shoot and root dry weight, nodulation, grain and straw yield, nodulation and phosphorus uptake were significantly enhanced in chickpea as a result of co-inoculation with *P. aeruginosa* and *Mesorhizobium* sp. (Verma et al., 2013). Similar effects together with the antagonistic activities against *Rhizoctonia solani* and *Fusarium oxysporum* have been noticed on chickpea amended with a bacterial consortium of *Azotobacter chroococcum*, *Trichoderma harzianum*, *Mesorhizobium* and *P. aeruginosa* (Verma et al., 2014). Although, different combinations of microorganisms have been explore, there is still a need for further research in this area (Gopalakrishnan et al., 2015b). *Rhizobium* introduction in leguminous crops is known for growth stimulation and is used as a substitute to the expensive conventional chemical fertilizers (Mfilinge et al., 2014). The use of suitable species as an inoculant in N depleted environments might be a better means to enhance the

development and growth of legumes. Considering the relatively cheap rate of inoculation and the possible agricultural benefits, farmers are admonished to take advantage of these inoculants as bio-fertilizers on leguminous crops.

3.4.2 Impact of *Rhizobium* Inoculation on Mineral Nutrients Absorption by Leguminous Crops

The availability and absorption of mineral nutrients like nitrogen (N), phosphorus (P), magnesium (Mg), sulphur (S), calcium (Ca) and potassium (K) are very essential for the growth of plants especially in Africa where various cropping systems involving leguminous crops such as soybeans are practiced.

In particular, symbiotic interactions between legumes and *Rhizobium* spp. may be a major source of N in most cropping systems with about 80% of the needed N emanating from biological N fixation (Vance et al., 2000). Indeed, Rudresh et al. (2005) reported that the amendment of leguminous seeds with rhizobial cultures enhances nodulation and N uptake. Studies on *Phaseolus vulgaris* indicated that N fixation from rhizobial inoculation contributed N equivalence of 20–60 kg of N per hectare in Brazil while Clayton et al. (2004) found that *Rhizobium* increased plant N from 19–42 mg per plant.

Also, P absorption is important for the development and proper functioning of plants and there is a large reserve of P in the soil environments, mainly in non-soluble forms that cannot be absorbed by plants and thus limiting plants' growth. PGPR can solubilize the non-soluble forms of P through chelation, enzymatic activities and acidification (Richardson et al., 2009, Hameeda et al., 2008). The uptake of these mineral nutrients relies majorly on their concentrations, activities in the soil around the root region and their replacement ability in the soil. In legumes, this challenge can be surmounted by introducing *Rhizobium* species and essential mineral

nutrients into the rhizospheric soil since *Rhizobium* and even other bacterial species such as *Bacillus*, *Burkholderia*, *Azospirillum*, *Pseudomonas* and *Erwinia* have been reported to have phosphate solubilizing potential (Sudhakar et al., 2000, Mehnaz and Lazarovits, 2006).

Besides deficiencies in major nutrients, micronutrients such as zinc (Zn), boron (B), molybdenum (Mo) and iron (Fe) are also limiting nutrients that work against legumes productivity. It was reviewed by Mfilinge et al. (2014), that the statistical significant increase observed for K intake was linked to *Rhizobium* inoculation into Pigeon pea (*Cajanus cajan* L. Millsp) and increase in the amount of nutrient in the environment increased the chance of plants uptake. Study carried out by Makoi et al. (2013) on the impact of *Rhizobium* strains on mineral nutrient absorption by *Phaseolus vulgaris* showed significant increase in the uptake of P, K, Mg, Ca and S in the entire plant parts. It was reported by the author that even though the concentration of P and K skyrocketed in the root region due to *Rhizobium* introduction, the increase was only significant in the greenhouse experiment and not in the field condition. *Rhizobium* inoculation enhancement of micronutrient (such as Mn, Fe, Cu, Zn, B and Mo) uptake in the shoots, roots, pods and the entire plant with the exception of Mo intake in the roots has been reported. *Rhizobium* has also been shown to cause significant increase in Ca and sodium (Na) content and the pH of the soil (Kisinyo et al., 2012). The uptake of Zn, Fe, Mn and Cu by cowpea was significantly different between treatments amended with *B. japonicum* and P under greenhouse and field conditions (Nyoki and Ndakidemi, 2014). *B. japonicum* caused significant increase in the intake of Zn, Fe, Cu and Mn in soybean (*G. max* L.) under greenhouse condition while uptake of Fe, Cu and Mn increased significantly and that of Zn decreased under field condition (Tairo and Ndakidemi, 2013).

There is however limited information on the roles of different rhizobial species of legumes on the bioavailability of other mineral nutrients in bean cultivars especially in Africa. Similarly, the impacts of *Rhizobium*, P and K interaction have not been studied in detail and it is therefore important to study the potential role of *Rhizobium*, P and K on the bioavailability of other mineral nutrients in leguminous crops such as soybean grown in each ecological zone in Africa.

3.4.3 Impact of *Rhizobium* Inoculation on Chlorophyll Concentration and Photosynthetic

Activities of Leguminous Crops

The crucial regulating element for plant growth is N due to its limited availability and also beans require this element more than other mineral nutrients. N is a component of most organic compounds such as proteins, growth regulators and chlorophylls. N deficiency can lead to stunted growth, yellowing of leaves and reduced branching in beans. N is one of the monomeric units of proteins and is greatly required for the entire enzymatic process in the cells of plants. It is also present in many vitamins and chlorophyll and plays a role in photosynthetic process (Tairo and Ndakidemi, 2013). In a study carried out in greenhouse and field environments, the chlorophyll content of the leaves of common beans significantly increased upon inoculation with rhizobial species. There was also significant increase in the photosynthetic activities of plant amended with rhizobial strains by 140 and 80% for greenhouse and field experiments respectively compared to control experiments (Mfilinge et al., 2014).

It has similarly been reported that soybean amended with *B. japonicum* showed increase in chlorophyll content and growth indices such as height of plant, leaves number on a plant, stem width, day's number to 50% flower and pod development as against the control counterparts (Tairo and Ndakidemi, 2013). Upon studying the impacts of P and *B. japonicum* on cowpea (*Vigna unguiculata* L.) growth, it was discovered that the chlorophyll content of the leaf of

cowpea significantly skyrocketed for treatment inoculated with *B. japonicum* in the field. There is however little information on the impacts of K, P and *Rhizobium* inoculation and their interactions on formation of chlorophyll in *Phaseolus vulgaris* in Tanzania which is a gap that needs to be covered research wise (Mfilinge et al., 2014).

3.5 Nexus of PGPR, Fe Acquisition, Plant Productivity and Pathogens Eradication

Besides inorganic P and N, Fe is an additional mineral nutrient that plants can obtain through symbiotic interactions with soil rhizobacteria. Although, ferric ion (Fe^{3+}) is more available in the soil, plants usually prefer to absorb Fe in the form of reduced ferrous ion (Fe^{2+}) (Salisbury and Ross, 1992). Plants release phyto-siderophores and chelators which adhere to Fe^{3+} and helps to retain it in soil solution. The chelators release the Fe^{3+} to the surface of the root where it is converted to Fe^{2+} and absorbed immediately while phyto-siderophores secreted by grasses are absorbed with Fe^{3+} across plasma membrane (von Wirén et al., 2000).

On the other hand, some PGPR are able to sequester the insoluble form of Fe from the soil with the aid of siderophores making it available for the host plant (Tkacz and Poole, 2015) and there is indication that a couple of plants can absorb bacterial Fe^{3+} -siderophore complexes (Bar-Ness et al., 1991) for their growth. However, the importance of bacterial Fe^{3+} -siderophore absorption to plants' iron nutrition is controversial. Some researchers reported that the contribution of Fe from this source to plants' overall Fe requirement is small (Glick, 1995) while others gave credence to their important and vital contributions especially in calcareous soils (Duijff et al., 1994, Masalha et al., 2000). Bar-Ness et al. (1992), who previously accepted the idea of bacterial siderophore uptake by plants (Bar-Ness et al., 1991), reported that two bacterial siderophores namely ferrioxamine B and pseudobactin did not effectively provide iron for plants.

The sequestration or acquisition of Fe through PGPR siderophores decreases the bioavailable Fe in the rhizosphere and as such affect the growth of fungi that might be pathogenic to the plant (Traxler et al., 2012, Bal et al., 2013). In Fe-deficient soil, plant is more productive in microorganism-rich soil than in soil devoid of microorganisms, buttressing the fact that PGPR help the host plants in acquiring this limited mineral nutrient (Tkacz and Poole, 2015).

3.6 New PGPR that are Related to Human Opportunistic Pathogens

There are some rhizospheric microorganisms that have the potential to cause opportunistic infections in humans. Berg et al. (2005) suggested that different bacterial genera such as *Enterobacter*, *Staphylococcus*, *Herbaspirillum*, *Pseudomonas*, *Ralstonia*, *Stenotrophomonas*, *Burkholderia* and *Ochrobactrum* have rhizosphere-associated species that can interact with both human and plant hosts. The mechanisms by which these bacteria colonize plants' rhizosphere and control plants' pathogens are similar to those responsible for colonization of human cells. Multiple antibiotic resistances are not only common with clinical isolates of these bacterial groups but also with isolates from the rhizosphere. These antibiotic resistances are caused by presence of different antibiotics, high competition and horizontal gene transfer in the rhizosphere. Several PGPR are phylogenetically related to some of the human opportunistic microorganisms and their potential to cause disease can easily be assessed by their ability to survive at 37°C (Alavi et al., 2014). The distinctions between pathogens and PGPR can be unveiled via comparative genomics. *Stenotrophomonas maltophilia* and *S. rhizophila* DSM14405T (PGPR) are genomically similar to *S. maltophilia* K279a (a human pathogen) but the former possess genes involved in the breakdown of cell walls of bacteria and plants, Fe sequestration, tolerance to salinity and spermidine synthase production (Alavi et al., 2014).

Irrespective of their origin, both rhizosphere-associated and clinical *Stenotrophomonas* species produce indole-3-acetic acid but environmental species seem to produce more of the phytohormones (Suckstorff and Berg, 2003). Moreover, plant's innate immune response stimulated by bacterial components like lipopolysaccharide or flagella is similar to the response of human immune system towards pathogens and this systemic acquired resistance was reported by Van Loon et al. (1998).

Many other N fixing endophytes are also closely related to some human pathogens. In particular, *Klebsiella pneumoniae* Kp342 is an endophytic N-fixer of some agricultural crops and has human pathogenic close relative (strain MGH78578). Strain Kp342 is different from strain MGH78578 since it is able to fix N and lack the genes that encode “global secondary messenger cdi-GMP” known for control of virulent components and biofilm formation. “In total, 4205 proteins (putative orthologues with the average identity of 96%, based on coding sequence prediction) were shared between these two strains, and 1107 proteins were unique to the plant associated Kp342”. Surprisingly, none of the projected coding sequence of Kp342 was similar to that of the already sequenced *Azoarcus* sp. BH72 (Tkacz and Poole, 2015).

In addition, detection of virulence factors in most of these rhizosphere related opportunistic human pathogens is difficult because of the absence of animal models. However, alternative models (often non-mammalian in nature) have been developed for some of these bacteria. For instance, the nematode *Caenorhabditis elegans* and the slime mould fungus *Dictyostelium discoideum* have been used as models to study the pathogenesis of *Pseudomonas*, *Burkholderia* *Stenotrophomonas* infection (Alonso et al., 2004). Studies have also shown that similar functions are involved in beneficial associations with plants and virulence in humans. In particular, siderophore uptake is common to plant beneficial bacterial and human pathogens (Tan et al.,

1999). Dörr et al. (1998) suggested that the type IV pili of plant-associated *Azoarcus* sp. BH72 responsible for adhesion on fungal and plant cells have amino acid sequence that is similar to the human pathogens *P. aeruginosa* and *Neisseria gonorrhoeae* indicating that the plant-associated bacterium may be able to cause opportunistic infections in humans.

The category of humans that are susceptible to opportunistic pathogens are the immuno-suppressed patients including AIDS patients, the aged with chronic diseases and those that have been subjected to long-term antibiotic therapy. According to Denton and Kerr (1998), long-term antibiotic therapy, prolonged hospitalization, corticosteroid therapy, catheter and admission to intensive care units are some of the risk factors for *Stenotrophomonas* infections. There is even a projection that in future, opportunistic infections will increase because of the increasing numbers of at-risk patients (Berg et al., 2005). Therefore, there is an urgent need for management strategies to curb opportunistic pathogens so as to minimize the challenge of environmentally acquired infections.

3.7 Nexus of Rhizobia, Nodule Formation and SYM Pathway

Some members of the Rhizobiales such as *Bradyrhizobium* species and Beta-proteobacteria are capable of forming nodules on the roots of leguminous crops where they transform atmospheric N to ammonia for plant use and gain carbon substrate from the plant in exchange (Gyaneshwar et al., 2011, Oldroyd et al., 2011). Within the root nodules, the bacteria are confined in specialized cells in the compartments christened symbiosomes and the symbiosome membranes originate from plant cell plasma membrane. The symbiotic interaction between leguminous crops and rhizobia is a good example of prolonged intracellular lifestyle of bacteria within specialized membrane-bound compartments. But the mechanisms by which the symbiotic cells are modified to contain the intracellular bacteria are elusive (Gavrín et al., 2015).

Actinobacteria such as species of *Frankia* also form nodules in interaction with plants like Casuarina and Alder. Several bacteria are free living in the environment or may exist as endophytes in plant roots, and similarly have the capacity to fix N (Turner et al., 2013a). Nodule formation in legumes was first observed approximately 100 million years back (Doyle, 2011) long after observing mycorrhizal colonization of plant, indicating that alteration in mycorrhizal pathway gives rise to nodulation. The existence of symbiotic common pathway (SYM pathway) in microbial association with plants raises the question of whether it serves as a route for soil microbiome to gain access to plant root tissues. Oomycetes for instance use this pathway to gain access to plant and cause havoc (Wang et al., 2012b). Although, mutant strains of rice lacking SYM pathway reveal that certain endophytic microorganisms like *R. leguminosarum* are still able to infect plant roots (Chen and Zhu, 2013) suggesting that this is not the only pathway available for microorganisms to gain entry into plant tissues. However, plants might be able to detect certain pathogens via the SYM pathway.

3.8 Techniques Use for Investigation of Rhizosphere Microbial Community Structure

Changes in plants' rhizosphere microbiome caused by the aforementioned rhizosphere modulators can be detected by various techniques. Therefore, researchers can gain insight into the composition and abundance of microorganisms present in the rhizosphere of plants in natural and agricultural ecosystem by using either what is broadly classified as traditional (Lakshmanan et al., 2014) or molecular (Philippot et al., 2013) techniques.

3.8.1 Traditional Techniques

Plant microbiome is rich and abundant and they composed of pathogens, endophytes and mutualistic symbionts. In the soil environment, bacterial count could be up to 10^6 – 10^7 cells/cm²;

this count was obtained by culturing soil samples in nutrient media under different growth conditions (Figure 2).

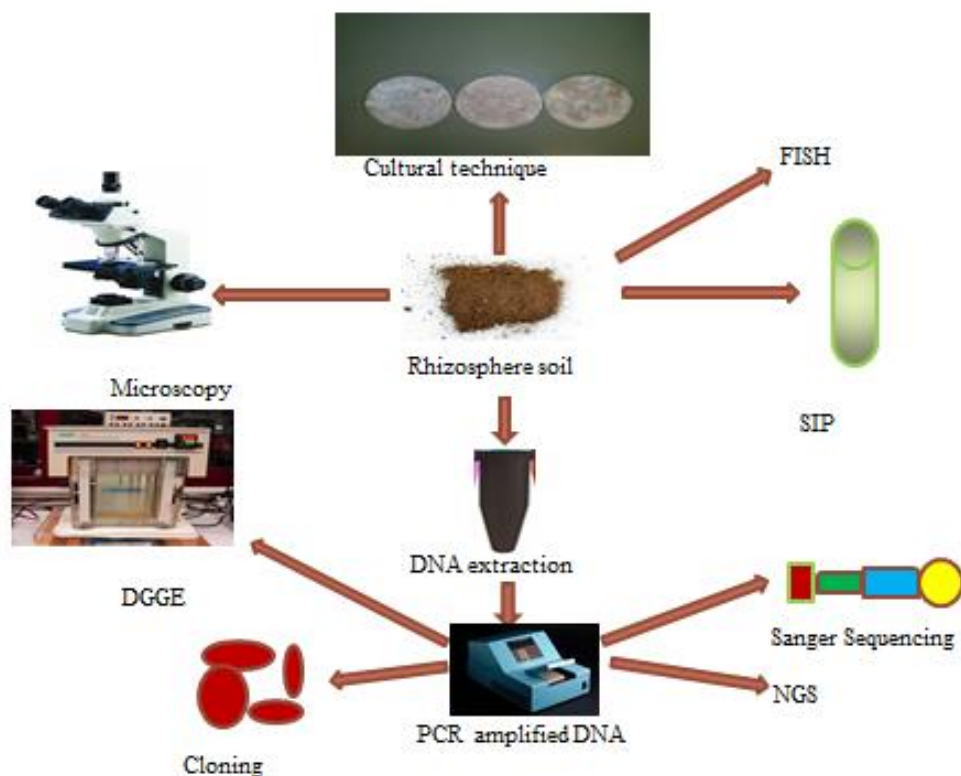


Fig. 3. 2 Schematic representation of collective techniques describing culture and un-culture based techniques for the study of rhizosphere microbiomes. DNA-deoxyribonucleic acid, SIP-Stable isotope probing technique, PCR-polymerase chain reaction technique, DGGE-denaturing gradient gel electrophoresis, NGS-next generation sequencing, FISH - Fluorescent *In-situ* hybridization

Plants obtain different nutrients from the soil with the aid of microorganisms in the rhizosphere. These nutrients include majorly N, Fe and P. These elements have the potential to influence plant growth by activating the production of plant growth regulators. Hence, the traditional techniques or culture dependent techniques are routinely used to assay for bacteria responsible for plant growth promotion (Taulé et al., 2012). These methods involve culturing microorganisms in culture plates or broth to isolate and study PGP traits of PGPR. Some of these PGP traits include siderophores, hydrogen cyanide, phosphate solubilization and exopolysaccharide test. The traditional techniques are also used to isolate and characterize the genetic materials associated with these microorganisms and unfortunately the culture based techniques are not able to capture majority of the unculturable microorganisms in the rhizosphere microbiome (Lakshmanan et al., 2014) as they can only isolate approximately 1% of the entire microbiome in environmental soil samples while the remaining 99% can be studied via molecular techniques or culture independent technologies.

3.8.2 Molecular Techniques

Attempt to profile whole microbiome commenced with the identification and application of 16S RNA gene and the use of PCR for characterization of microorganisms. This had metamorphosed into advanced techniques such as metagenomics use to explore entire microbial community. The drawbacks of these culture independent techniques were recently reviewed (Berlec, 2012, Rincon-Florez et al., 2013, Dini-Andreote and van Elsas, 2013) and these technologies involve metagenome sampling, purification, separation, sequencing, analysis of data and interpretations. The sequencing method is rapidly advancing and currently there is NGS or high throughput sequencing (HTS). The HTS techniques comprises of Roche 454 Genome Sequencer, HiSeq 2000, and AB SOLiDTM System (Lundberg et al., 2012, Peiffer et al., 2013,

Rincon-Florez et al., 2013, Yergeau et al., 2014). NGS techniques differ from other culture independent techniques like Sanger sequencing in aspects of data analysis and cost. Although NGS has the advantage of making genome sequences handy, data analysis and explanations are currently the challenge associated with the techniques (Liu et al., 2012a). Moreover, experimental work involving NGS increases the chances of better citation when published. Other molecular techniques involving DNA/RNA stable isotope probing (SIP) and DNA arrays are also used in microbial analysis of environmental samples (Rincon-Florez et al., 2013, Uhlik et al., 2013). Indeed, SIP technique (Figure 2) revealed that the root exudates emanating from maize and wheat play a role in shaping microbial community of the soil surrounding the root regions (Lakshmanan et al., 2014). The microorganisms of the rhizosphere utilizing root exudates were done by analyzing only the denaturing gradient gel electrophoresis (DGGE) profile of ^{13}C DNA fixed by plants amended with $^{13}\text{CO}_2$ soil while microorganisms using organic matter from the soil were evaluated using ^{12}C DNA. This investigation demonstrated that some classes of bacteria e.g., *Sphingobacteriales* and *Myxococcus* could use root exudates emanating from all plants while microorganisms from the order *Sphingomonadales* can utilize carbon substrates of root exudates and soil organic matter.

There is presently transition from the metagenomics approaches to metatranscriptomics techniques. Metatranscriptomics give information about the diversity and functional molecules of the microbial community unlike the metagenomics that only show diversity. It was recently noted that functional diversity of microbiome is probably more dominant in ecological niches than genomic diversity (Chaparro et al., 2012, Barret et al., 2011, Turner et al., 2013b). Metatranscriptomics methods such as qPCR, help to give information about the functional state of microbiome in the rhizosphere (Uhlik et al., 2013). However, culture independent techniques

have a lot of challenges even though the general difficulty of qPCR use for detecting gene expression of microbial community has been surmounted. The challenges entail detecting either ribosomal RNA (rRNA) or messenger RNA (mRNA), accomplishing broader analysis of an environmental RNA pool, designing effective probe and enhancing the performance of sequencing.

Metaproteomics on the other hand, deals with the analysis of protein which involves the extraction of metaproteome from environmental samples and carrying out protein fingerprinting of the extract using mass spectrometry (Keiblinger et al., 2012, Kolmeder and de Vos, 2014). Metatranscriptomics and metaproteomics which are somewhat nascent are confronted with the challenge of sampling and data procurement (Keiblinger et al., 2012).

Recently, researchers have studied the rhizosphere microbial community of soybean (*G. max* L.) in soil ecosystems in the USA (Mendes et al., 2014a). Yet, much is not known about microbiome associated with the root of most agricultural crops and more research on soil rhizospheric microbiome of crops is therefore needed (Tkacz and Poole, 2015, De-la-Peña and Loyola-Vargas, 2014, Blee et al., 2013). For example, no study has investigated rhizosphere associated microbiota of soybean in field conditions using HTS, even though this is the fourth most produced crops in the globe (FAOSTAT, 2015).

Nowadays, virtually all investigations involve molecular analyses which are necessary for detailed characterization of species and investigation of microbial interactions with host plants in the rhizosphere. “Though there are many technical innovations in HTS that lead to insightful and better understanding of the microbiome phylotypes and functions; Dini-Andreote and van Elsas (2013) have emphasized its hindrance on testing ecological hypotheses and the current need of a ‘paradigm shift’ from HTS to studies on fundamental questions about yet

unexplored plant soil microbiota systems, especially towards phenotypic diversity of rhizospheric microbiome on a spatial and temporal level”.

3.9 Future Direction

“The future trend needs to be in developing genetically modified PGPR over transgenic plants for boosting plant performance, as it is simpler to modify a bacterium than complex higher organisms. Moreover, instead of engineering individual crops, a single, engineered inoculant can be employed for several crops, especially when using a nonspecific genus like *Azospirillum*. PGPR strains development is hampered mainly by the fact that these organisms are sometimes unable to survive harsh environmental conditions, including high concentrations of environmental contaminants, salts, extremities of pH and temperature. Genetic engineering can be used to develop PGPR strains that are effective at low inoculum doses and under a variety of environmental conditions. It is urgent to develop more effective PGPR strains with longer shelf lives to achieve sustainable crop production in dry land production. Recent advances in the fields of microbiology, biotechnology, molecular biology and bioinformatics have opened up the way to identify novel genes involved in drought tolerance. Concepts of micro biotechnology application in agriculture should be employed to isolate indigenous PGPR from the stress affected soils, and screening on the basis of their stress may be useful in rapid selection of efficient strains that could be used as bio-inoculants for crops grown in dry lands (Kaushal and Wani, 2016)”.

3.10 Conclusions

There are myriads of microorganisms including rhizobacteria found in the ecosystem of the rhizosphere and these bacterial interactions with the plants root have been declared beneficial

to sustainable agricultural development. This group of bacteria, among other merits can enhance plant development and diminish the occurrence of plant disease. New as well as uncultured microbial candidates in the rhizosphere can better be captured and studied using culture independent techniques which have the potential to analyze broad spectrum of microbial species unlike the culture based techniques. The mechanism by which these microorganisms achieve the mutual benefits on their hosts is not completely comprehended; however, it has been observed that virtually all of their traits enable them to accomplish these benefits. To add to this, rhizospheric microorganisms must be competent in the sense that they should be able to strive within rhizospheric soil that is being influenced by several factors including soil type, plant cultivar and agricultural practices. It is advisable to properly match suitable PGPR with compatible host plant cum environmental condition so as to accomplish better benefits on the plant. This feat will help to reduce the usage of conventional chemical fertilizers as well as pesticides most especially if these microbial inoculants are delivered effectively to the target plant and environment.

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3.12 Authors Contribution: N.O.I. wrote the first draft. O.O.B. provided academic input and thoroughly critiqued the article. Both authors approved the article for publication.

3.13 Conflicts of Interest: The authors declare no conflict of interest.

CHAPTER 4

Biofertilizers and Sustainable Agriculture: Exploring Arbuscular Mycorrhizal Fungi

Abstract

Agricultural food production has to double in 2050 globally so as to feed the global increasing population while reducing dependency on conventional chemical fertilizers plus pesticides. To accomplish this objective, there is the need to explore several mutualistic interactions between plant roots and rhizosphere microbiome in a process termed biofertilization. Biofertilization is the process of boosting the abundance of microorganisms such as arbuscular mycorrhizal fungi (AMF) in the natural plant rhizosphere which depicts a beneficial alternative to chemical fertilization practices. Mineral nutrients uptake by AMF are plausible by means of transporters coded for by different genes and example include phosphate transporter. These fungi can be produced industrially using plant host and these, including the possibility of AMF contamination by other microorganisms are factors militating against large scale production of AMF. AMF isolates can be inoculated in the greenhouse or field; and it has been shown that AMF survival and colonization level were enhanced in soybeans grown on land that was previously cultivated with the same plant. Next Generation Sequencing (NGS) is now used to gain insight into how AMF interact with indigenous AMF and screen for beneficial microbial candidates. Besides application as biofertilizers, novel findings on AMF that could contribute to maintenance of agricultural development include AMF roles in controlling soil erosion, enhancing phytoremediation and elimination of other organisms that may be harmful to crops through common mycelia network. The combination of these potentials when fully harnessed under agricultural scenario will help to sustain agriculture and boost food security globally.

Keywords: arbuscular mycorrhizal fungi, biofertilizer, chemical fertilizer, food security, next generation sequencing, soybean

4.0 Introduction

The use of microbial inoculants (Alori et al., 2017) termed “biofertilizer” in sustainable food production allows plants to effectively utilize mineral elements such as nitrogen and phosphorus. Phosphorus is a significant mineral needed by plants and its deficiency could diminish plant development. Arbuscular mycorrhizal (AM) associations are means by which several plants obtain sufficient supply of phosphorus from rhizospheric soils. AM symbiotic relationship is especially essential for plants growing in environment deficient in phosphorus as it enhances plant growth and phosphorus level in plants. Alkaline phosphatase (ALP) through gel electrophoresis and ultracytochemistry has been recognized as a functional and useful enzyme for AM. ALP is responsible for phosphorus acquisition by plants since its presence in the vacuoles of highly developed arbuscules was revealed by ultracytochemical studies (Abdel-Fattah et al., 2014). Growing agricultural crop such as soybean (*Glycine max* L.), one of the oil seed in order to increase yield and extend the cultivated area in recently reclaimed soil is one of the ways to achieved agricultural sustainability. Enhanced productivity of soybean interacting with AM fungi has been linked to improved absorption of phosphorus (Abdel-Fattah and Asrar, 2012). Added to phosphorus and nitrogen acquisition, sulfur could also be transported to host plant via AM fungal interaction. AM interactions enhance the sulfur nutritional level of the host thereby upregulating the expression of the plant sulfate transporter gene.

Despite the significance of potassium to plant, the role of AM fungi in the acquisition of potassium by host plants has hardly been investigated. Potassium is very rich in soil but its bioavailability to plant is low and its buildup has been shown in spores, hyphae and vesicles. The

over expression of potassium transporter protein in plant has been reported in the roots of *Lotus japonicas* colonized with AM fungi (Berruti et al., 2015). Potassium gotten from AM interaction could be connected to an enhanced plant resistant to stressors such as drought and salinity. Metaanalysis investigations which recently emphasized on the function of AM interaction in acquisition of micronutrients by plants have been reported (Lehmann et al., 2014). AM fungi could be harnessed to enhance micronutrient level in agricultural crops and agricultural biofortification. In addition to crop yield, AM association can similarly influence crop quality as a result of macro cum micronutrient enhancement. For instance, AM interaction positively impacted Zn levels in different agricultural crops grown under different environmental conditions (Lehmann et al., 2014) while copper, iron, manganese increased uptake in AM symbiosis has also been reported. Pellegrino and Bedini (2014) revealed that inoculation of AM fungi in the field can be an effective way to enhance chicken pea as it could enhance productivity. Regulation of AM infection level by nutrient availability is an important means of limiting or enhancing AM establishment by plants in order to meet their needs. It was reported that bioavailability of phosphorus is an environmental issue that could affect AM symbiosis. The inhibition of AM establishment triggered by high phosphorus concentrations has been reported severally. Besides high phosphorus concentration, nitrate can also negatively affect AM fungal colonization while magnesium, iron and calcium ions including sulfate do not have negative impact on the fungal colonization potential (Nouri et al., 2014, Berruti et al., 2015).

The production and application of microbial fertilizers is gaining global increase owing to the negative impacts of over use or improper usage of chemical based fertilizer and the increased awareness about the association between rhizosphere microorganisms and plants (Babalola et al., 2007, Babalola, 2010, Hou and Babalola, 2013, Alori et al., 2017). This has motivated scientists

to search for microorganisms exhibiting plant growth promotion ability by improving directly or indirectly the nutrient status of plants. The beneficial agricultural impacts of microbial products and rhizobacterial based products which have been commercialized for the past six decades have unveiled a global market of a novel fertilizer termed biofertilizers. This reviewed is therefore aimed at highlighting the possibility, production and limitations of using arbuscular mycorrhizal fungi as a biofertilizer with a view of undermining the use of chemical fertilizer and achieving food security. In addition, novel potentials of these microbial candidates will also be xrayed.

Biofertilizers have gained usefulness in agriculture because of their interactions with biotic and abiotic components of the rhizosphere. Therefore what is rhizosphere?

4.1 The Rhizosphere

The rhizosphere (Fig. 1) , which is an ecological niche for a great number of invertebrates and microorganisms, is the thin layer of soil close to the root and is affected by the roots of plants (Philippot et al., 2013). Rhizospheric microorganisms can have direct or indirect impacts on plant biodiversity in an ecosystem. Many organisms are involved in these processes resulting in myriads of interactions among plants, symbionts and antagonists within below and above ground environments (Behie et al., 2012, Vannette and Rasmann, 2012, Bever et al., 2012, Hou and Babalola, 2013, Babalola, 2010). At present, much awareness about the associations and functions in the root region have emanated from investigations on agronomic crops as well as model plants such as *Arabidopsis thaliana* and *Medicago truncatula*. Furthermore, biotic agents that are found in the rhizosphere microbiome have great impacts on plant health, growth and nutrition; arbuscular mycorrhizal fungi including *Glomus intraradices* and *Rhizophagus irregularis* are a few examples of such agents. The arbuscular mycorrhizal fungi of the phylum *Glomeromycota* form symbiosis with over two third of plants. From ecological and agricultural

perspective, this symbiotic interaction involving fungi is the most significant mutualism in terrestrial environment (Tisserant et al., 2013). Rhizodeposits nutrients, border cells, mucilages and exudates produced by plants attract and serve as food for microorganisms (including bacteria, fungi, archaea, Oomycetes and viruses) that are present in the rhizosphere. Several studies have demonstrated the impacts of plant type on the microbial diversity in the rhizosphere of cultivated and non cultivated plants spp. (Dias et al., 2012). It has been reported that, there exist less microbial richness and abundance in the root region compared with the bulk soil (that is, the soil environment that is far away from the root region or the rhizosphere). Even with this, it is hard to give a generalized picture of the rhizosphere microbiota due to huge disparity between various studies, which may have resulted from biological variation and practical facts such as sampling of the soil in the root region (Philippot et al., 2013).

Rhizosphere community composition and structure is influenced by living and nonliving components. Numerous works have revealed that the diversity of bacteria and mycorrhizal fungi in the root zone is influenced greatly by soil type. To put this in context, soil physical and chemical parameters have impacts on plant structure and functioning as well as release of root exudate which consequentially affect the microbial assembly in the rhizosphere.

4.2 Mineral Nutrients Transporters involved in AM Interactions with Host Plants

The nutrient uptake processes in AM associations have been well studied since there has been description of high affinity phosphate transporter (PT) in the fungi. It is renowned that AM interaction particularly stimulate plant gene to express inorganic phosphorus (pi) transporter proteins. In addition to enhanced uptake of pi in plants, AM fungi is also responsible in influencing the development of arbuscule structure and shape as well as maintaining symbiotic association (Yang et al., 2012, Xie et al., 2013). It was recently shown that *Medicago truncatula*

and *Lotus japonicas* expressed *MtPT4* and *LjPT4* Pi transporters as a result of AM interaction which were present in the root tips of plants that were not colonized by AM fungi and a function of *PT4* genes as parts of the Pi sensing machinery has been reported (Volpe et al., 2015). There are reports on the role of AM interaction in the transmission of nutrients in many plant hosts. Besides Pi transporters, ammonium transporters induced by AM fungi have similarly been discovered. Surrounding the arbuscule is the periarbuscular membrane gotten from plant where mutualistic inorganic nutrient transmission occurs. Plant transporter proteins present in the above mentioned membrane are able to obtain and transfer inorganic nutrients from the periarbuscular apoplast to the cortical cell (Bapaume and Reinhardt, 2012). Ammonium transporters induced by AM fungi have been shown to be located in soybean periarbuscular membrane indicating a function in the transfer of ammonium to the cortical cell (Breuillin-Sessoms et al., 2015). It has been suggested that transfer of inorganic phosphorus or ammonium via the transporters not only supplies nutrient to the root cells but also play a role in the maintenance of arbuscule (Breuillin-Sessoms et al., 2015, Berruti et al., 2015). Sulfate transporter responsible for the uptake of sulfate from the arbuscules was recently identified.

4.3 Industrial Production of Mycorrhizal Fungi

The production method of AMF has emerged years back from inconsequential methodologies which involved AMF multiplication on plant roots grown in the field or pots. Besides the advantages of cost effectiveness and ease of production of AMF via interaction with a host in non axenic environments, AMF inoculum produced from such technology has limited usage and can get contaminated easily (Berruti et al., 2015, Vosátka et al., 2012). AMF production in greenhouse with plants amended with pure or mixed fungal species in pots is very common because greenhouse experiments are easy to set up and control. However, achieving

AMF strains purity is the major issue of concern considering large scale production of this microbial group. The purity of multiplied AMF can be ascertained by Next Generation Sequencing (NGS) technique which can also be used for AM abundance and composition studies (Pellegrino et al., 2012, Thonar et al., 2012, Rodriguez and Sanders, 2015). More advanced multiplication techniques *in vitro* such as altered root hair cultures have been lately constructed which allow AMF production in pure conditions (Vosátka et al., 2012). There are presently three small scale companies involved in *in vitro* production of AMF in European countries while two large scale ones are present in other continents. The production capacity of the small and large scale companies is cumbersome to estimate universally since the commercial statistical figures are not accessible publicly. Few years back, there has been a significant increase in the number of firms producing mycorrhizal inoculants. More than 15 small medium firms have been shown to produce mycorrhizal fungi but most time, it is not easy to differentiate primary manufacturers from suppliers of repackage inocula. Initially each manufacturer supplies its own products mainly within its territory but lately markets are beginning to overlap and many chains of distributors are now being formed. A couple of companies have acquired multinational companies and are currently marketing ectomycorrhizal fungal internationally even though most of the firms are small in size. The firms are partly embracing horticulture business like production of tree, shrub and precious fruit trees which are also targets for mycorrhizal inoculation. Forest and ornamental trees nursery as well as phytoremediation sites where plants are subjected to abiotic stress such as organic contaminants are similarly used for inoculations. However, it should also be noted that mycoremediation of organic compounds exist (Iggehon, 2015). Generally, inoculation output is more obvious and economically practicable in less nutrient-rich and dry soils. Using mycorrhizal inoculants for crops in extensive farming is

controversial as a result of unknown effects since mycorrhizal introduction could be efficient but unattainable economically. Some companies are making move to establish commercial niche in developing nations. Information on commercial mass production of inocula with mycorrhizal technology is scarce, however it is known that application of mycorrhizal fungi abound in Africa and there are substantial markets establishment in India.

4.4 Limitations to AM Fungal Propagules Production and Introduction

The use of AMF as a biological fertilizer for sustainable agronomic development is becoming very pressing since proper harnessing of these mutualistic fungi can possibly diminish the utilization of agrochemicals which are hazardous to environmental receptors. The means to accomplish this objective is through the introduction of AMF inoculum into the soil of interest. AMF are obligate mutualists and therefore cannot be grow in culture media in the absence of host plant and this makes mass production of AMF inocula unrealistic and cumbersome. Soil from the rhizosphere of AMF colonized plants, AMF hyphae and AMF spores are the major kinds of AMF inocula. Examples of such AMF grown in the soil are *Gomus* spp. (Table 1) and *Gigaspora rosea*. The multiplied AM fungal inoculum is a mixture of soil and AMF propagules and thus it is impossible to accomplished pure inoculum that is devoid of extraneous microorganisms; and the cost of transportation of AMF is outrageously high in developing nations because of the weight of the soil containing the AMF propagules and this can limit large scale use of these fungi as a biofertilizer (Ceballos et al., 2013, Rodriguez and Sanders, 2015). Unless facts about biodiversity and infectivity are known, soil inocula are risky because of the possibility of transferring disease-causing microorganisms and weed seeds. Crude inocula could be achieved by growing AMF with the host plant in an optimized medium that can support AMF proliferation and this is the most frequently utilized kind of inoculum for massive crop

inoculation. Lastly, AMF colonized plant root fragments separated from trap plant culture could as well serve as inocula. Large scale production of crude AMF inoculum is still very perplexing despite the recent development of seed coating method and new ways for large scale production (Vosátka et al., 2012, Heijden et al., 2015).

Furthermore, the establishment of AMF reference collection centres is also challenging because it needs technologies that are different from those used for other microbial collection. This is because AMF are obligate symbionts, a feature that also limit their mass production. Lack of quick technique for evaluating the extent to which the plant host is populated by AMF also limits these species use in agriculture. The control of large amount of inoculum needed for large scale usage is also a challenging factor. AMF inoculation is done easily for agricultural systems involving transplant stage which requires little quantity of inoculum. Inoculating an extensive agricultural land appears technically and economically impossible, but once AMF biomass is reinstated and established, and if control system such as cover cropping and conservation tillage is adopted, the AMF diversity will survive (Lehman et al., 2012, Säle et al., 2015). Alternatively, cost effective small scale production of AMF is also feasible while employing the approach of “fertility islands”, in which small area of land is utilized leading to healthy AMF biodiversity. However, AMF inoculation is cheaper than chemical fertilization and in order to provide more credence to AMF inoculation, farmers should apply AMF to crops so as to assess the AMF cost effectiveness and positive influence on plant health. World economic recession is currently prompting farmers to understand the possibility of sustainable agronomic development and phosphorus introduction using AMF inocula. Unluckily, solid inoculation methods are yet to be employed and research emphasized on getting the finest inoculum preparation methods (Verbruggen et al., 2013a). AMF potential has now made the commercial world to produce and

vend AMF as a biofertilizer by preparing formulations containing little components of AMF. Some formulations contain single AM fungal species while others contain different species of AMF that normally have broad host and environmental conditions spectrum. Though marketable inocula are always advertised for a broad variety of environmental conditions and plants, the actual gains to the host might not always be positive (Faye et al., 2013). In order to consolidate AMF inoculum commercialization, researchers should fortify the nexus between research and commercial firms possibly by evaluating the purity of commercial products as regards AMF composition. To place this in context, the list of species on commercial AMF label does not invariably correspond precisely to the real inoculum composition (Berruti et al., 2013). Moreover, as obligate symbionts, AM fungal mycelia are commonly produced by means of containerized soil medium in greenhouses or fields and therefore, cannot be devoid of extraneous microorganisms. To prevent contamination, several manufacturers are currently adding agrochemicals to commercial AMF products.

4.5 Comparison between AMF Inoculation in Greenhouse and Field Experiments

Most researches involving agricultural sustainable development of crops such as soybean using mycorrhizal fungi are either done in the greenhouse or open field environments. In a greenhouse experiment, a containerized soil is used unlike in the open field experiment where soil is not boundary bound. In both cases (greenhouse and open field experiments), inoculation of the mycorrhizal of interest into the targeted soil is necessary. In a study carried out by Berruti et al. (2015); it was shown that mycorrhizal fungal colonization of uninoculated control experiments was more in the field experiments than in the greenhouse experiments (Fig. 1). It was suggested by the author that the reason for such observation may be due to the fact that the uninoculated plant from the field experiments usually contains AM fungal spores or hyphae

while control greenhouse experiments usually contain sterilized soil devoid of AMF propagules. There was no significant difference in the effects of AMF inoculation on crop yield, shoot biodiversity, plant nutrition in both green house and field experiments unlike in the root biomass.

Knowledge of whether or not alien AMF interact with the indigenous AMF population and if such interaction affects crop productivity is strategic to effective use of AMF propagules in agronomy. Surprisingly, few field investigations have attributed yield increases with successful establishment of alien AMF in the rhizosphere. One field experiment where increased productivity was recorded with equivalent increase in AMF establishment showed that one of the inoculated AMF might have effectively colonized and survived two years after inoculation (Pellegrino et al., 2012). The question which is yet to be unraveled is whether that specific AM fungal isolate was responsible for the yield increase or increased level of AMF colonization observed in the plant roots. It is yet unknown if inoculation of agricultural crops with AM fungus will result in successful colonization by introduced or alien fungus; this however becomes irrelevant if the impacts are indirect through the local AMF population (Rodriguez and Sanders, 2015).

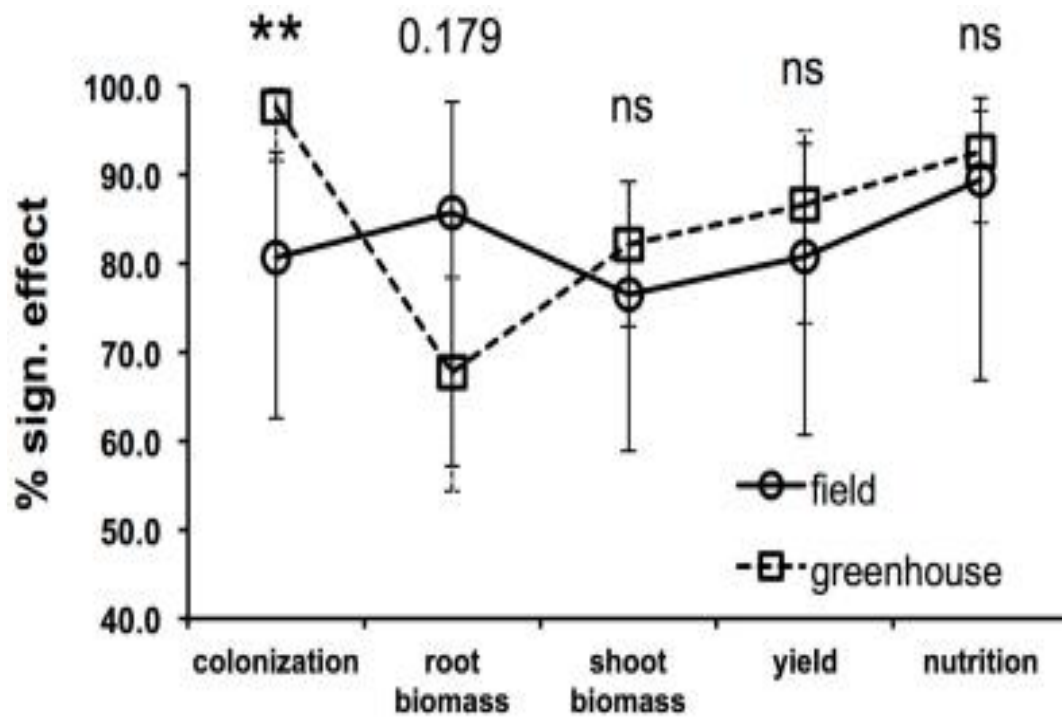


Fig. 4. 1 Field and green house experiments of fungal colonization, root and shoot biomass, yield and plant nutrition. Ns-non significant, **- level of significance

Source: (Berruti et al., 2015)

4.6 Screening for Beneficial Microorganisms and/or AM Fungi

Conventional cultivation, isolation and characterization techniques have only targeted a small segment of copiotrophic microbial isolates as candidates for inoculation of plants for sustainable development. Culture independent studies have demonstrated the existence of substantial populations of new microbial groups (e.g. *Verrucomicrobia* and *Acidobacteria*) in the soil region surrounding plant roots that were not captured using culture based techniques (Gupta, 2012, Babalola, 2014). Pyrosequencing aiming at the surface protein that code for *cpn60* can help to determine microbial diversity (that is, the richness and abundance of species) *in situ*, which could complement the isolation strategy using culture dependent technologies. Many molecular technologies in the past few years have been employed to characterize AMF inocula and entire AMF communities in complicate soil matrix (Borriello et al., 2012, Davison et al., 2012, Berruti et al., 2013). These technologies also monitor inoculated AMF in the plant throughout the cultivation phase (Pellegrino et al., 2012, Thonar et al., 2012). High tech Next Generation Sequencing (NGS) is a sensitive and formidable technique that helps to temporally and spatially trace the inoculated AMF. This technique also aid in verifying whether the introduced AMF favor substantial levels of colonization, however this might not be necessary if the impacts on crop yield and quality are indirect through the resident fungal community. NGS is similarly employed to gain insight into how the inoculated AMF interact with the indigenous AMF community.

In addition, tracing inoculated AMF in field experiments is difficult and it has therefore been cumbersome practically to determine the establishment of an inoculated AMF. Also, similar fungal strains might be present already in the local AMF community suggesting that a great deal of molecular markers used for identifying the fungus might already be present in the local

community. It has been suggested by Rodriguez and Sanders (2015) that it is now possible to investigate the persistence and invasiveness of *R. irregularis* by carrying out genomic analysis of the population even though there is already the existence of the fungus in the soil. There exists powerful and dependable sequencing technique for detecting polymorphisms within genome of microbial population termed “random amplified polymorphic DNA sequencing”. With this technology, it is currently easy to carry out partial genomic sequencing of *R. irregularis* strains followed by discrimination of inoculated fungus from the indigenous ones; this is however possible as a result of the discovery of *R. irregularis* isolate DAOM 197198 reference genome (Tisserant et al., 2013). Prospective marker detection will rely on polymorphisms between the inoculated *R. irregularis* isolate and multitude of the same species with sequenced genomes (Rodriguez and Sanders, 2015).

4.7 Why AMF can Enhance Food Security in Tropical Countries

That it is possible for AMF to help in increasing food security is based on the fact that vital food crops undergo symbiosis with these microbial agents and AMF effectively provide plants with more phosphate from the soil. The two major factors that pose a threat to global food security are that phosphate fertilizers are rapidly going out of supply and there is a parallel increase in demand for these fertilizers to produce foods that will be enough to feed the increasing population. This threat can be minimized by enhanced phosphate absorption via the AM fungal associations however. The ability of AMF to help to enhance crop productivity has been acknowledged for years. Despite the great research on AMF symbiosis, there are few studies showing that massive inoculation of food crops in an agronomic setting leads to noticeable increase in crop yield (Rodriguez and Sanders, 2015). This is due to the fact that AMF are naturally present in the soil and yield increase in the AMF inoculated crop should be more

than that observe for the uninoculated plant for such inoculation to be regarded as being effective. In addition, majority of the AMF open field tests have been done in temperate environments with high nutrient content unlike the tropical agro ecosystems with relatively low nutrient availability, thus reducing the likelihood of an AMF impact. AMF inoculation of crops in tropical agricultural soils would have a great potential in boosting food production and reducing the use of conventional chemical fertilizer since tropical soils are characterized with low phosphate bioavailability and high phosphate retention ability. Unfortunately these soils have been the most ignored (Ceballos et al., 2013, Rodriguez and Sanders, 2015).

4.8 Novel Aspects of Arbuscular Mycorrhizal Fungi

Before now, much attention has been on the nutrient absorption potentials of mycorrhizal fungi for plant growth and development most especially the assimilation of phosphorus (Vosátka et al., 2012). It is largely acknowledged that mycorrhizal fungi grow and strive more efficiently for plant development and resistance to abiotic stress such as deficiency of phosphates in the soil. This is very crucial nowadays since phosphates from rocky environment will be exhausted few years from now and ban will be placed on chemical fertilizer production. Mycorrhizal fungi however have non-nutritional impacts on host plants including their processes in the environment which may help to maintain ecological services. One example of ecological or ecosystem services provided by mycorrhizal fungi is the control of soil erosion by enhancing soil structure and return of plant photosynthetic products into MF biomass is also an important “carbon sink” globally (Vosátka et al., 2012). There are also evidences of AM fungi playing roles in growth inhibition or death of other organisms via common mycelial networks. Soil microorganisms’ manipulation aiming at achieving sustainable agriculture involves the use of mycorrhizal fungi or mixed microbial isolates. Utilization of AM fungal potential to enhance phytoremediation has

been reported (Bhargava et al., 2012, Meier et al., 2012). There are many findings that some AM fungal have the ability to minimize the adsorption of heavy metals into plant above-ground structures and so guarantee the edibility of food crops. Other fungi can enhance phytoextraction and therefore aid in remediation of contaminated soils. However, in this regard, AM fungal inoculants should be carefully chosen before applying on a large scale so as to prevent using species with opposite or undesirable effects. Recently applied AM fungi have been reported to enhance food crop quality such as increase in the amount of sugar in plant, elements such as zinc, magnesium, other useful elements and antioxidants have (Albrechtova et al., 2012). Several studies have been tilted towards unveiling the associations between host plants infected with mycorrhizal fungi and disease causing microorganisms and pests. Modification of plant defense system caused by mycorrhizal invasion leads to local and systemic plant immune response to diseases (Jung et al., 2012, Hao et al., 2012, Philippot et al., 2013, Babikova et al., 2013a). An amazing fact about the relationship between mycorrhizal associated plant and parasite is the recently reported partial protection of *Striga*. “Recently defined phytohormones strigolactones induce suicidal germination of *Striga* seeds, control shoot branching and seems to be part of host recognition signals for mycorrhizal fungi” (Vosátka et al., 2012). This mechanism needs further investigations since the function of strigolactones in plant communication and as allelochemicals is not fully understood. Other bioactive compounds such as lipochitooligosaccharides involved in the establishment of AM association have been reported recently and could contribute to AMF potential for effective enhancement of crop productivity.

Also, synergistic introduction or coinoculation with other beneficial microorganisms provides benefits to crop production, and therefore they are currently needed. For instance, AMF fungal inoculation with rhizobacteria (Babalola, 2010) improves plant development even more

than when single inoculation is done. Coinoculation of AM fungi and saprophytic fungi could also exert positive impacts on crop development as well as food quality which were recently reported in onions (Albrechtova et al., 2012). Species of *Trichoderma* are emerging group of beneficial microbial biofertilizer since genotypes of this genus have been found to support plant growth. In a null shell, besides application as biofertilizers, novel findings on AM fungi that could contribute to maintenance of agricultural sustainability include AM fungi roles in controlling soil erosion, enhancing phytoremediation and elimination of other organisms that may be harmful to crops through common mycelial network.

4.9 Soil Type and Crop Species Influence on Successful Establishment of AMF

Much is not known about acclimatization of AMF to ecological conditions (Johnson et al., 2013). It is commonly assumed that for AMF field application to be effective; it has to be pre conditioned to a particular type of soil or crop. As high concentration of nutrient can negatively influence AMF colonization, AMF establishment in nutrient rich agroecosystems could be challenging. Some AM fungal strains such as *R. irregularis* seem to possess broad geographical range, indicating that such species are not environment specific. *R. irregularis* obtained from Spanish environment was effectively used to inoculate cassava plant (that had not been previously exposed to the fungus) in an acidic tropical field soil deficient in nutrient (Ceballos et al., 2013). The issue of AMF environmental adaptability has only been peripherally addressed. Laboratory finding has recently suggested AMF mechanisms involved in adaption to a new environment (Angelard et al., 2014). *R. irregularis* that was sustained for about 24 months on the roots of carrot plant demonstrated prompt genetic transformation upon inoculation on potato plant with variation in phenotypic traits as a result of host change. It is therefore necessary to get understanding of the mechanisms that can support AMF adaptation to a foreign environment so

as to ascertain whether these fungi have to be acclimatized to a particular soil environment. However, there exist little research data to ascertain whether AMF from a particular agro-soil would be more efficient in that soil so as to determine their potency if use as a biofertilizer. it is also important to determine the role of selective adaptability as regards the viability of AMF in a particular soil/crop (Rodriguez and Sanders, 2015).

4.10 Nexus of Continuous Cropping of Soybean, AMF, Microbial Diversity and Ecosystem Community Structure

Brazil is the second largest producer of soybean, which is also the fourth biggest grain produce in China. Additionally, soybean is an oil crop having high nutritional content with high amount of soy proteins which is more nutritious than cereal proteins and could therefore be regarded as a good source of protein for humans. Soybean contains bioactive compounds such as isoflavones, oligosaccharides and saponins that are of health benefits to humans. Majority of soybean crop has been cultivated severally via continuous cropping (CP) system especially in China. This agronomic practice can be harmful since it makes crops vulnerable to pests and can adversely influence the physical and chemical parameters of the soil. This can result in low yield and complete crop failure (Liu et al., 2012b). Yield and quality reduction of soybean triggered by CP is now a universal issue and many investigations have diverted attention to knowing the mechanisms associated with the effects of this agronomic practice (Gesch and Archer, 2013). In addition, many of the sterilization technologies for eradicating spores circuitously cause fungal dominance in soils subjected to CP.

It was recently reported that growing soybean on a particular piece of land year after year causes transformations to the rhizospheric soil microbial community. This will gradually cause a shift in the soil from bacterial rich community to fungal rich community. Similarly, CP has the

potential of changing the pH of the soil from neutrality to acidic condition which is favorable to fungal development while obstructing the growth of actinomycetes and bacteria. This can make a particular soil environment to be fungal dominant (Tanaka et al., 2012). Furthermore, CP for several years triggers the fortification of exudates from plant roots such as phenolic acids in the soil. It has been reported that there is an exponential increase in fungal abundance as a result of artificial increase in the concentrations of phenolic acids in the soil. CP of soybean similarly causes a rise in the concentration of organic substance such as sugars, amino and organic acids and enhances the proliferation of root rot causing pathogens. It can also cause deficiency in some essential nutrients needed for soybean growth as a result of nutrients unevenness in the rhizosphere. This environment circuitously upsurges soybean roots colonization rate by AMF. In a study carried out by Jie et al. (2013), AM fungi in the rhizosphere of soybean cultivated under distinct CP schemes expressed dissimilar colonization rates. The level of colonization of AM fungi in soybean grown under three years of CP environment was greater than that grown under two years of CP environment, but AM fungi from the two environments had similar diversity index indicating that CP of soybean favors AM fungal survival and colonization rate. The colonization rate also differs among the various soybean cultivars used with resultant differences in the fungal rhizosphere community structure among the cultivars.

AM fungus being an oligotrophic organism can attack plant root cells and form mutualistic association with plants to enhance plant growth. AM fungi benefit plants in many ways including promotion of community succession and environmental stability making these microorganisms to function as a biofertilizer. Studies have recently focused on the use of AMF on agronomic crops (Hart and Forsythe, 2012). The impacts of AMF on legumes have been studied with sterile pot experimentations and by investigating plant development under

environmentally impaired situations. Investigations have highlighted that AMF have the potential to facilitate the nutrient uptake capacity of soybean, nitrogen fixation by *Rhizobium* and microbial colonization structure in the rhizosphere ecology and thereby skyrocketing yields cum “economic efficiency of soybean” (Tian et al., 2013).

4.11 Scientific and Legal Perspective of Biofertilizer

The terminology biofertilizer (Babalola, 2010) has been described in several ways for the past two decades and the meaning is extracted from the excellent understanding of the interactions existing between rhizosphere microbiome and host plant. It has been projected that microorganisms (such as mycorrhizal fungi and plant growth promoting rhizobacteria) around the root region which enhance nutrients absorption by plant and do not replenish such nutrients cannot be classified as biofertilizers. The main mechanistic process of a microbial isolate in improving plant development has given rise to the suggestion of describing them as biofertilizers. Those that have the potential of enhancing plant hormones are termed biological enhancers and those mineralizing organic contaminants detrimental to plant health are called rhizoremediators (Malusà and Vassilev, 2014).

Scientific articles usually refer to biofertilizers as microbial isolates (Table 1) having the potential to promote plant growth and development (Bardi and Malusà, 2012, Alori et al., 2017). For biofertilizer to be suitable for agricultural purposes, the beneficial microbial isolates need to be incorporated in formulations that will prolong its shelf life, that is, the timeframe between production and application in the field. Biofertilizer like conventional chemical fertilizer is a commercial product which contains useful species, carrier substance and possibly additives that could enhance the efficiency of microbial activity. Thus, whether the microbial isolate enhances plants’ growth by adding nutrients to the rhizosphere (that is, fixing atmospheric nitrogen into

the form that can be used by plants), or by increasing the bioavailable portion of soil nutrient to plant, in as much as the plant nutrient provision has been improved upon (Alori et al., 2017), the formulated mixture containing the microbial isolates that is added to soil will be called biofertilizer. Also, the word biofertilizer legally should be used to describe biostimulants.

According to the reviewed by Malusá and Vassilev (2014), the legal meaning of commercial products like biofertilizer is important for manufacturers having the interest to market such products. There are no legal description of biofertilizer or definite provision stating their qualities in Europe and United State of America. In the Europe, microorganisms are incorporated into the “European Union Commission Regulation on organic production” for biocontrol of plant diseases and pests only. They are therefore within the legal purview of protective products for plant and also the United State of America only see biological products or biocontrol agents as living organisms that can offer protection for plants. The disadvantages of not getting legal meaning for biofertilizers could be seen considering arbuscular mycorrhizal products. In years back, a commercial product with AMF claiming growth enhancement and stimulating capabilities was not categorized as a product that offer protection for plants by European General Directorate for Health and Consumers. In 2003 and 2008, dual products containing AMF claiming to possess antifungal and root protective characteristics were classified as biocontrol agents and therefore demand registration with strict adherence to established regulations for biocontrol compounds. Also, India perhaps has the most detailed legal structure for biofertilizers.

Table 4. 1 Contributions of biofertilizers to plant productivity

Mycorrhizal fungi	Contributions towards plant productivity	References
<i>Glomus etunicatum</i>	Enhanced vegetative growth, chlorophyll and nutrient level in maize more than non mycorrhizal maize plant	(Sadhana, 2014)
Alien arbuscular mycorrhizal fungi	Enhanced root and shoot biomass of <i>Juniperus oxycedrus</i> than non mycorrhizal fungi even in the presence of environmental stress	(Alguacil et al., 2006)
<i>Vigna radiata</i>	The fungus had positive impact on the nitrogen, potassium, phosphorus and protein content of the green grain	(Bhat et al., 2010)
Necrotrophic mycorrhizal species	Increase the nutrient status and secure plant against herbivore (such as aphids) attack	(Gilbert and Johnson, 2015)
AM fungi	Reduce the concentration of cadmium in plant grown under cadmium contaminated site thereby enhancing its productivity.	(Janoušková et al., 2006)
<i>Gomus intraradices</i>	Mycoremediate zinc from <i>Medicago truncatula</i> environment with the resultant effect of improved growth and development	(Hildebrandt et al., 2007)

<i>Glomus mosseae</i>	Improved wheat yield even under more efficient in enhancing crop yield under stress	(Daei et al., 2009)
<i>Glomus</i> spp. inoculated with <i>Mortierella</i> sp.	<i>G. aggregatum</i> or <i>G. mosseae</i> when inoculated with <i>Mortierella</i> sp. had significant impacts on the dry weights of shoot and root of <i>Kosteletzkya virginica</i> in the presence of salt stress.	(Zhang et al., 2011)
<i>Acaulospora lacunosa</i> and <i>Glomus</i> <i>constrictum</i>	Improved foliar nutrient status of onions (<i>Allium porrum</i>)	(Hart and Forsythe, 2012)
AMF	Enhanced nutrient uptake capability of soybean (<i>Glycine max</i>) and consequently increased yield	(Liu et al., 2012b, Tian et al., 2013)

4.12 Future Direction in Accomplishing Agricultural Sustainability

Sustainable agriculture development relies partially on reduced utilization of conventional inorganic chemical fertilizers and pesticides (Philippot et al., 2013, Babalola, 2014, Alori et al., 2017). One main area of research depends on choosing and cultivating plant cultivars that capitalize on both biotic cum abiotic resources in the soil. This relates to “major paradigm shift” which entails a change towards acclimatizing plant cultivars to the habitat instead of the habitat to plant cultivars, in which, for instance high productivity depends on high amount of chemical input. This choice or selection could be accomplished by genetically manipulating plants to become highly resistant to disease causing microorganisms through different mechanisms. In particular, great resistance was demonstrated to *Pectobacterium carotovorum* disease by plants producing bacterial kind of acyl homoserine lactonase, a protein that breakdown quorum sensing compounds together with plants that produce a great deal of glucosyl transferase. High cost of fitness might be a probable drawback to this approach (Zeller et al., 2013). A second research approach to improve agronomic sustainable development is the detection of plant characters that affect either greenhouse gas emission by microorganisms present in the soil or nitrogen losses via leaching. This idea could be utilized to raise novel plant cultivars that are able to alleviate these release or emissions. Scientists’ knowledge of root exudation process is inadequate and therefore a better insight into the chemistry of exudates is required in order to detect new aspects for genetic engineering and plant breeding. A sacrosanct challenge is how to make use of the myriads of exudates or rhizodeposits released by plant to enhance microbial richness and abundance as well as their activities that can benefit ecosystem function and consequentially reduce the use of chemical fertilizers.

Considering the problem of climate change, more research should be geared towards enhancing AMF role in conferring increased resistance to environmental stresses and as such fundamental and applied systematic investigation should be emphasized on. Ample knowledge on the mechanistic pathways that enable AMF host plants to resist or tolerate environmental stresses should also be studied. Add to this, genomic approaches including transcriptomics, metabolomics and proteomics will be relevant in detecting expressed genes in AMF plants involved in nutrient uptake, mitigation of abiotic stresses including drought and disease resistance. AMF management in future research and the significance of maintaining AMF inoculants diversity in the soil rhizosphere for efficient and realistic applications in agriculture should be prioritized. Mycorrhizal application should not be left out in future sustainable agriculture development policies since AMF application can reduce energy inputs and the use of chemical fertilizer while promoting plant health. The use of AMF inoculants will possibly become more relevant as a result of the detrimental effects of agrochemicals on the environment which needs to be avoided or reduced to increase food yield, food quality and ecological protection. More studies should verify the aftermath effects of the interaction between microorganisms in the rhizosphere containing AMF so as to evaluate their (AMF) environmental impacts as a feasible substitute for agriculture and land reclamation (González-López, 2013).

Additionally, there are four aspects where ecologists can help in the effective utilization of AMF for enhanced agricultural sustainability (Rodriguez and Sanders, 2015) and these include: knowing the persistence and infection potential of inoculated AMF amidst already existing indigenous AMF community; getting more insight into the adaptability of alien AMF to a foreign environment; the significance of genetic variability among AM fungal species and its

impact on plant growth and finally determining whether there is a direct or indirect impact on crop productivity by inoculated AMF.

4.13 Concluding Remarks

It is important to improve on the activities of microorganisms (such as arbuscular mycorrhizal fungi) that contribute positively on plant nutrition via absorption of scarce nutrients, plant resistance to diseases, and tolerance to different abiotic stressors to sustain and increase worldwide food production while preserving environmental health. Several beneficial mutualistic microorganisms have been discovered, however their dependable utilization as biofertilizers in field conditions is yet to be fully harnessed. New insights into rhizosphere microbial diversity could lead to the detection of new inoculants and improve persistence and performance of valuable microorganisms *ex situ* upon their inoculation into soil environments.

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4.15 Compliance with Ethical Standards

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4.16 Conflict of Interest

Author 1 declares that he has no conflict of interest. Author 2 declares that she has no conflict of interest.

4.17 Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

CHAPTER 5

New *Rhizobium* Species Enhanced Soybean Tolerance to Drought Stress

Abstract

Background and Aim Food security is seriously under threat in many developing tropical countries as agriculture is becoming unsustainable due to drought stress. There is therefore an urgent need for a sustainable means to accomplish food availability in this region. Thus, the aim of this study was to isolate new *Rhizobium* spp. from Bambara groundnut rhizosphere (BGR) and evaluate their ability to enhance soybean tolerance to drought.

Methods New rhizobial strains were isolated from BGR and their mechanisms in relation to enhancement of plant growth and drought tolerance were evaluated. Isolates were characterized and identified by culture based and molecular techniques. Soybean seeds inoculated with the microbial isolates were grown under drought stress imposed by 4 % polyethylene glycol (PEG).

Results Isolates were identified as *Rhizobium* sp. strain R1, *Rhizobium tropici* strain R2, *Rhizobium cellulosilyticum* strain R3, *Rhizobium taibaishanense* strain R4 and *Sinorhizobium meliloti* strain R5. Strains were positive to almost all the plant growth promoting parameters tested. Rhizobial isolates grew under stress conditions imposed by PEG but *Rhizobium* sp. and *R. cellulosilyticum* showed the highest significant tolerance with OD values of 1.35 and 0.32 respectively at a concentration of 30 % PEG while *S. meliloti* had the least OD value of 0.17 at the same PEG concentration. *Rhizobium* sp., *R. cellulosilyticum*, and *S. meliloti* inoculation of soybean significantly enhanced seedling shoot and root dry weights especially in treatments amended with *Rhizobium* sp. and *R. cellulosilyticum*.

Conclusions These findings showed that the new *Rhizobium* sp. and *R. cellulosilyticum* can further be harnessed to enhance the growth of soybean and other legumes under drought conditions.

Keywords: drought stress, plant growth promoting parameters, *Rhizobium* spp., root, soybean, 16S rRNA

5.0 Introduction

Food security is seriously under threat in many developing tropical countries as agriculture is becoming unsustainable due to the increasing human population. The world population is projected to double by 2040 and much of the population increase shall occur in these regions where hunger is already a problem. Also, most agricultural land in this region cannot satisfy current food demands as a result of low soil water content and loss of fertility caused by drought. In such conditions, crop yield might not be significantly enhanced even by applications of chemical fertilizers (Masciarelli et al., 2014, Joint, 1998).

The application of beneficial microorganisms has been demonstrated to be an “environmentally sound option” to improve crop productivity. Plant growth-promoting bacteria (PGPR) which are found within the root regions might directly or indirectly enhance plant growth and productivity (Igiehon and Babalola, 2018c). Several species of bacteria in the genera of *Rhizobium*, *Bradyrhizobium*, *Pseudomonas*, *Azotobacter*, and *Burkholderia* have shown to promote plant growth (Ahmad et al., 2008). There are many ways in which these bacteria enhance plant growth directly. Firstly, although this is a negligible aspect of the benefit provided by PGPR, some of these bacteria can help plants fix atmospheric nitrogen. Others synthesize siderophore which sequester iron from the environment and make it available for plant use. They also produce phytohormones like gibberellins, auxins and cytokinins which are able to regulate

plant growth and solubilize minerals to the form that can be used by plants (Glick, 1995, Glick et al., 2007, Bashan and De-Bashan, 2010). Bacteria might directly affect plant development by taking advantage of one or more of the above-mentioned plant growth promoting traits (PGPT). Since many PGPR possess many of these traits at the same time, different traits at different stages of plant growth may be used. On the other hand, PGPR indirectly improve plant growth by acting as biocontrol agents against pathogenic bacteria in the soil (Bhattacharyya and Jha, 2012b, Senthil Kumar et al., 2009). The most studied bacteria used as biocontrol candidates are *Bacillus* and *Pseudomonas* species. Nodule-forming crops are notable reservoirs of these PGPR as they harbor a large number of these bacteria including nitrogen fixing *Rhizobium* species.

Legumes are very nutritious harboring reasonable amounts of minerals and vitamins such as calcium and folic acid respectively. Soybean (*Glycine max* L.) is an upright annual subtropical crop of the leguminosae family and is among the most well-known oil seed (18-22%) globally containing proteins (40-42%) which adequately supply eight amino acids required for human health and as such it is therefore among the best source of plant protein (Masciarelli et al., 2014). Soybean is generally used to produce biscuits, milk, vegetable cheese, oil and other edible products.

Soybean micro-symbionts in the rhizobial group are nitrogen fixers capable of forming symbiotic interactions with the soybean. However, just as some plant cultivars are better in fixing atmospheric nitrogen so also with some rhizobial species. The amounts of nitrogen fixed in kilograms per hectare by soybean was revealed to be 450kg N/ha. So, soybean depends on beneficial micro-symbionts for a significant proportion of its nitrogen requirements for survival and increased productivity. Added to this, some of these micro-symbionts have the ability to solubilize phosphate for plant growth. It has been demonstrated that inoculation with phosphate

solubilizing bacteria resulted in enhanced nodulation, straw and grain yield as well as phosphorus and nitrogen uptake by plants (Linu et al., 2009).

Some of the leguminous crops such as Bambara, soybean and cowpea are essential plants (Barea et al., 2005, Esitken et al., 2006) which are sometimes cultivated in drought-prone environments. Growths of these crops are reduced as a result of biochemical and physiological changes caused by drought (Marschner, 1995). However, many factors trigger the plants' tolerance response to drought and physiological, nutritional, biochemical and hormonal changes can reduce or elevate damaging effects of drought (Ortiz et al., 2015). In addition, rhizospheric microorganisms can stimulate a great variety of physiological and nutritional plant responses to drought stress (Benabdellah et al., 2011, Marulanda et al., 2006a, Medina and Azcón, 2010). Microorganisms dwelling in water stressed soils are usually adapted to such deleterious conditions and PGPR can be present under harsh environments and can get acclimatized to these extreme conditions (Kohler et al., 2009, Marulanda et al., 2007, Wilde et al., 2009). Indeed, water limitation is not only harmful to plants but also to microorganisms, affecting growth and activity. Nevertheless, adapted drought-resistant microorganisms alleviate the drought effect and can be valuable in enhancing plant establishment. The usage of adapted PGPR to sustain valuable crops such as soybean in semi-arid and arid environments is therefore a fascinating possibility (Medina and Azcón, 2012). Hence, identification of PGPR and manipulation of their interactions with agronomic crops is currently considered a strategy to sustain agriculture in developing countries.

The useful effects of microbial interactions under drought and sterile environmental conditions have been reported previously (Marulanda-Aguirre et al., 2008, Medina and Azcón, 2012) and as such it would be worthwhile to further investigate the beneficial effects of new

PGPR under sterile stressed conditions as this will serve as a baseline study for accurate selection of PGPR inoculants for biotechnological purpose. One of the ways to achieve this feat is by inducing stress on the PGPR and plant using stress inducer such as polyethylene glycol (PEG) (Ortiz et al., 2015).

Thus, in this study new *Rhizobium* spp. from Bambara groundnut rhizosphere in semi-arid region of South Africa were isolated and identified using culture-based and culture dependent techniques. The ability of three genotypes and/or cultivars of soybean to tolerate drought under laboratory conditions in the absence of bacteria was also assessed. Finally, the possibility of the new *Rhizobium* spp. to enhance soybean under stress condition through production of plant growth regulators was evaluated.

5.1 Materials and Methods

5.1.1 Bacterial isolation from Bambara groundnut rhizosphere

Soil was collected from Bambara groundnut rhizosphere by destructive sampling in North-West Province, South Africa and taken to the laboratory in an ice box. One g (1 g) of the soil was weighed on a weighing balance (Radwag weighing machine by Lasec, made in Poland) and placed in a test tube containing 9 ml of sterile distilled water. One ml (1 ml) of 10^{-6} and 10^{-7} dilutions was plated on nutrient agar prepared according to manufacturer's instruction. Plates were incubated at 27 °C for 5 days. The mixed culture isolates were purified by streaking on yeast mannitol agar (YEM) (Ahmad et al., 2008). Isolates were tested for Gram reaction using crystal violet, iodine, 70 % alcohol, safranin and stained smear was examined under oil immersion objective lens. Motility test of bacterial culture was done by stab-inoculating pure bacterial isolates in YEM in test-tubes and incubated at 27 °C. Catalase and oxidase tests were done by conventional methods following the method employed by Masciarelli et al. (2014) with

little modifications. Briefly, for catalase test, 30 % hydrogen peroxide was placed on cleaned sterile slides. Pure isolates were added to the hydrogen peroxide and formation of oxygen gas indicated positive result. Oxidase test was done by soaking sterile filter paper with oxidase reagent and pure cultures of the isolates were mixed with the oxidase reagent on the filter paper. Formation of a blue colour indicated positive result. API 20 E strips were used to determine other biochemical tests such as β -galactosidase (Ortho nitro-Phenyl- β -D-galactopyranosidase), arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H₂S production, urease, tryptophan deaminase, indole production, acetoin production (Voges Proskauer), gelatinase, glucose, mannitol, inositol, sorbitol, rhamnose, saccharose, melibiose and amygdalin and arabinose.

5.1.2 Determination of PGPT of the Bacterial Isolates

5.1.2.1 Ammonia (NH₃) Production Test

Isolated bacteria were tested for NH₃ production in peptone water by the methods of Ahmad et al. (2008) and Cappuccino and Sherman (1992). Freshly grown pure cultures were inoculated in tubes containing 10 ml of peptone water while control tubes were left uninoculated. Four (4) replicates were done per isolate. Tubes were incubated in a shaking incubator at ambient temperature (28±2 °C) for 4 days. Nessler's reagent (0.5 ml) was dispensed into each tube and the development of yellow to orange colour was taken as positive test for NH₃ production.

5.1.2.2 Phosphate Solubilization Test

Phosphate solubilization test was determined as described by Gaur (1990) with little modifications. Pikovskaya's agar supplemented with tri-calcium phosphate was inoculated with

Luria Bertani (LB) broth culture of each isolate. Plates were incubated at 27 °C and observed for 4 days and clear zones or halos around the wells indicated positive result.

5.1.2.3 Siderophore Production Test

Isolated bacteria were tested for production of siderophore as described by Masciarelli et al. (2014) and Ahmad et al. (2008). Chrome azurol S agar plates were divided into equal parts and spot inoculated with freshly grown pure bacterial isolates and incubated at 27 °C and observed for 4 days. Yellow colour development around the spot of bacterial inoculation was a positive test.

5.1.2.4 Hydrogen Cyanide (HCN) Production Test

Bacterial isolates were screened for HCN production according to Ahmad et al. (2008) with little modifications. Five hundred ml (500 ml) of freshly prepared YEM was amended with 2.2 g of glycine and plates were streaked with pure bacterial isolates. Sterile Whatman filter paper no.1 were soaked in 2 % sodium carbonate and 0.5 % picric acid and placed on top of the plates. Plates sealed with parafilm were incubated at 27 °C and observed daily for 4 days for the formation of orange to red colour which indicates production of HCN.

5.1.2.5 Indole-acetic acid (IAA) Test

Qualitative IAA was determined according to the methods of Loper and Schroth (1986); Ahmad et al. (2008) and Gaur (1990) with little modifications at different tryptophan concentrations of 0, 100 and 200 mg in LB broth. Pure bacterial isolates were inoculated in the broth and incubated in a shaking incubator at ambient temperature (28±2 °C) for 5 days. Bacterial growth suspensions were subjected to centrifugation at 3000 rev/min for 1800 sec. Two ml (2 ml) of the bacterial supernatant was mixed with 4 ml of Salkowski reagent (prepared by

mixing 49 ml of 35 % perchloric acid in 1 ml of 0.5 M ferric chloride solution) and 2 drops of orthophosphoric acid. Development of pink colour indicated the production of IAA.

5.1.2.6 Antifungal Test

Antifungal assay was done according to the methods of Mehmood et al. (1999) and Ahmad et al. (2008). One hundred μ l (100 μ l) of spores of fungi *Aspergillus niger*, *Mucor*, *Penicillium* and *Fusarium* strains mixed with 100 ml of normal saline solution was spread on Sabouraud dextrose agar (SDA) prepared according to manufacturer's instruction (60g/l). Wells of 8 mm in diameter were made in the inoculated SDA plates. Forty μ l (40 μ l) of freshly grown bacterial broth was aseptically inoculated into each well and incubated at ambient temperature (28 ± 2 °C) for 5 days. The diameter of zones of inhibition was obtained.

5.1.2.7 1-aminocyclopropane-1-carboxylate (ACC) Deaminase Activity

Qualitative ACC deaminase activity for the bacterial isolates were determined according to the method of Ali et al. (2014). "Screening for ACC deaminase activity of drought tolerant bacterial isolates was done based on their ability to use ACC as a sole nitrogen source. All the bacterial isolates were grown in 5 ml of LB medium incubated at 28 °C at 120 rpm for 4 days. The cells were harvested by centrifugation at 3000 *g* for 5 min and washed twice with sterile 0.1 M Tris-HCl (pH 7.5) and re-suspended in 1 ml of 0.1 M Tris-HCl (pH 7.5) and spot inoculated on Petri plates containing modified DF (Dworkin and Foster) salts minimal medium (Dworkin and Foster, 1958), glucose, 2.0 g; gluconic acid, 2.0 g; citric acid, 2.0 g; KH_2PO_4 , 4.0 g; Na_2HPO_4 , 6.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; micro nutrient solution (CaCl_2 , 200 mg; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 200 mg; H_3BO_3 , 15 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mg; Na_2MoO_4 , 10 mg; KI, 10 mg; NaBr, 10 mg; MnCl_2 , 10 mg; COCl_2 , 5 mg; CuCl_2 , 5 mg; AlCl_3 , 2 mg; NiSO_4 , 2 mg; distill water, 1000 ml), 10 ml and distilled water, 990 ml; supplemented with 3 mM ACC as sole nitrogen source. Plates containing

only DF salts minimal medium without ACC as negative control and with $(\text{NH}_4)_2\text{SO}_4$ (0.2% w/v) as positive control. The plates were incubated at 28 °C for 72 h. Growths of isolates on ACC supplemented plates was compared to negative and positive controls and was selected based on growth by utilizing ACC as nitrogen source”.

5.1.3 Identification and Selection of Drought Tolerant Microorganisms under increasing Concentrations of PEG in Nutrient Broth

One hundred μl (100 μl) of each bacterial broth was inoculated and grown in nutrient broth (NB) under increasing PEG concentrations of 0 %, 10 %, 20 % and 30 % in test tubes for 8 days. PEG is a chemical product that is able to induce osmotic stress and isolates showing highest growths at the highest PEG concentration were chosen as the greatest osmotic stress tolerant bacteria (Ortiz et al., 2015, Hamayun et al., 2010, Türkan et al., 2005). The optical density of the bacterial NB was taken at days 0, 2, 4 and 8 at 600 nm using UV spectrophotometer (Merck).

5.1.4 DNA Extraction and Genotypic Identification

5.1.4.1 DNA Extraction

Bacterial isolates were fully grown in LB agar plates and bacterial DNA was extracted using Zymo DNA extraction kit following manufacturer’s instruction. The presence of DNA in the extracts was determined by agarose gel electrophoresis. Briefly, 4 μl of bacterial DNA samples and 10 μl of Hyper Ladder™ 1kb were run in 1.0 % agarose prepared by microwaving 1 g agarose powder in 100 ml 1 × TAE for 4 min at 65 °C, followed by the addition of 10 μl of ethidium bromide. DNA bands were examined in a Gel Doc machine. The concentration of the

extracted DNA samples was determined in a NanoDrop spectrophotometer at a wavelength of 260 nm as described by (Abbaszadegan et al., 2007).

5.1.4.2 Polymerase Chain Reaction (PCR) Amplification of Bacterial DNA

Genotypic identification was done by PCR amplification and partial nucleotide sequences of 16S rDNA. “The partial nucleotide sequences” of the bacterial isolate 16S rRNA genes were obtained by direct sequencing of the amplified PCR products. The 16S rRNA samples were amplified in the presence of *Taq* polymerase and primers specific to conserved regions of the 16S rDNA genes. The nucleotide sequence of the forward (27f) and reverse (1492r) primers used were 5'-AGAGTTTGATCCTGGCTCAG-3' and 3'-GGTTACCTTGTTACGACTT-5' respectively. The 25 µl PCR reaction mixture for each bacterial isolate was composed of: 12.5 µl, 0.25 µl forward primer, 0.25 µl reverse primer, 1 µl DNA template and 11 µl PCR water. Negative control was made by replacing 1 µl DNA by 1 µl of PCR water in the 25 µl PCR reaction mixture. PCR amplification was carried out in a PCR thermal cycler machine (BIO-RAD Laboratories, C1000 Touch) according to the following program conditions: 3 min at 94 °C, followed by 25 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min and 72 °C for 10 min. Amplified DNA was detected by running and examining DNA samples in 1 % agarose gel and in a Gel Doc machine (BIO-RAD Laboratories, California, U.S.A) respectively as described above. Twenty µl (20 µl) of DNA samples for each bacterial isolate were sent for sequencing (INQABA, PRETORIA, SA).

5.1.4.3 Sequence Alignment and Construction of Phylogenetic Tree

BLAST program cum GenBank database on the National Center for Biotechnology information (NCBI) were used to determine bacterial isolate sequence similarities and identities. Identified bacteria received accession numbers after depositing sequences on GenBank on the

NCBI web server. Isolated sequences were further subjected to multiple sequence alignment by ClustalX using a Bio-Edit program. MEGA7 program was used to construct the phylogenetic tree from the ClustalX sequence outcomes and Maximum Parsimony Analysis of taxa with Kimura 2-parameter model. Bootstrap consensus tree was used as the phylogeny test (Masciarelli et al., 2014).

5.1.5 Soybean Seeds

Soybean seeds (PAN1532 R, PAN1614R and PAN1623R cultivars) provided by Agricultural Research Council–ARC (Potchefstroom, North-West Province, SA) were used. Seeds were confirmed to be viable by the quality control personnel.

5.1.6 Selection of Soybean Cultivar that is moderately sensitive to PEG-Imposed Drought Stress

PAN1532 R, PAN1614 R and PAN1623 R soybean seeds were surface-sterilized in 75 % ethanol and 1 % (v/v) sodium hypochlorite for 10 min and rinsed severally in sterile distilled water (Ndeddy Aka and Babalola, 2016). Soybean seeds were exposed to stress induced by PEG by placing 30 seeds per cultivar in sterile Petri dishes containing sterile filter paper soaked in 15 of ml 10 and 0 % PEG solutions. Plates were sealed with parafilm and incubated for 8 days at ambient temperature (28 ± 2 °C). Growth parameters such as shoot and root height, shoot and root width, shoot and root fresh weight, shoot and root dry weight, seedling fresh weight and number of lateral roots were measured using the entire soybean seedlings from each replicate as described by He et al. (2009). Seedling shoots and roots were separated and their fresh weights were weighed and recorded. Shoot and root samples were oven dried at 60 °C for 24 h to obtain the dry weight. Fresh and dry weights were expressed as grams per replicate (g replicate⁻¹). The

sensitivity of the cultivars to PEG was classified as follows: extremely sensitive > moderately sensitive > slightly sensitive.

5.1.7 Bacterial Growth and Preparation

From the outcomes of PGPT and drought tolerant tests, 3 bacterial isolates were selected for soybean inoculation. Bacterial growth and preparation were performed as described by Prakamhang et al. (2015) with little modifications. Briefly, freshly grown bacterial cultures were inoculated in 500 ml LB broth and incubated in a shaking incubator at ambient temperature (28 ± 2 °C). The exponential phase of bacterial broths were centrifuged at 5000 rpm for 5 min, residual medium was removed by washing cell pellet in 0.85 % (w/v) sterile NaCl. Cell pellet was then homogenized in 0.85% (w/v) saline solution before seed inoculation.

5.1.8 Petri dish Assay of Soybean Growth

Petri dish assay of soybean growth was conducted by the methods of Huang et al. (2013) with little modifications. The bacterial titers were adjusted to 20×10^5 , 11×10^5 and 21×10^5 CFU (colony forming unit) ml^{-1} for selected bacteria R1, R3 and R5 respectively. Bacterial suspension (0.5 ml for each isolate) was added to a layer of sterile Whatman filter paper no.1 in Petri dishes with a diameter of 90 mm. PAN 1532 R soybean seeds were surface sterilized for 10 min in 75 % ethanol, soaked in 1 % sodium hypochlorite and rinsed severally in sterilized distilled water. Thirty (30) seeds were transferred to the surface of the inoculated filter paper in the Petri dishes containing sterile filter papers soaked in 15 ml of 4% PEG solution. Plates were gently swirled severally to glue the bacteria to the seeds. Four (4) treatments were set-up: (1) soybean seeds inoculated with bacterium R1 and amended with 4 % PEG solution, (2) soybean seeds inoculated with bacterium R3 and amended with 4 % PEG solution, (3) soybean seeds inoculated with bacterium R5 and amended with 4 % PEG solution, (4) non-inoculated soybean

seeds (control) amended with only 4 % PEG solution. Each treatment was replicated three times. Plates were sealed with parafilm and incubated for 8 days in a growth chamber (GC-300TL JEIO TECH, South Korea) adjusted to 23/16 day/night for periods of 8/16 hours night/day at 10,000 light lux for 8 days. Ten (10) seedlings were randomly selected from each replicate and growth parameters such as shoot and root height, shoot and root width, shoot and root fresh weight, shoot and root dry weight, seedling fresh weight and number of lateral roots were measured as described by He et al. (2009) and Masciarelli et al. (2014). Seedling shoots and roots were separated and their fresh weights were weighed and recorded. Shoot and roots samples were oven dried at 60°C for 24 h to obtain the dry weight. Fresh and dry weights were expressed as grams per replicate (g replicate⁻¹).

5.1.9 Statistical Analysis

Data were cleaned in excel sheet and normality test was done. The mean and standard errors were obtained and graphs were drawn in Microsoft excel sheet. Raw data were imported to SPSS sheet where analysis of variance (ANOVA) including Duncan Multiple test was done. Probability value less than 0.05 ($P < 0.05$) was considered significant.

5.2 Results

As indicated in Table 1, microscopic test showed that the new bacterial strains were Gram negative rod s. Similarly to certain *Rhizobium* species well-known for their catabolic traits and breakdown of macromolecules, all isolated bacteria were positive for gelatinase and have the ability to hydrolyze starch. Bacterial strains R1, R3 and R4 were positive for catalase and all the bacterial strains except R4 (could not be determined-ND) were observed to be motile. Additionally, strains R1, R3 and R5 were positive for glucose test. All the isolated bacterial strains did not have the ability to produce indole and were negative for Voges Proskauer

including oxidase activity (Table 1) as evident by the absence of purple colour formation suggesting that these strains lack cytochrome c oxidase enzyme complex.

Table 5. 1 Morphological and biochemical characteristics of the isolated bacteria

Strain	Morphological and biochemical characteristic																			
	Gram reaction	Shape	Oxidase	Catalase	ONG	ADH	LDC	ODC	CIT	H ₂ S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA
R1	-	Rod	-	+	+	+	+	+	+	-	+	-	-	-	+	+	+	+	-	+
R2	-	Rod	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
R3	-	Rod	-	+	-	+	-	+	+	-	+	-	-	-	+	+	+	-	+	-
R4	-	Rod	-	+	-	+	-	-	-	-	-	+	-	-	+	-	-	-	-	-
R5	-	Rod	-	-	+	+	+	+	+	+	+	+	-	-	+	+	-	-	-	+

Legend: - = negative, + = positive, ONPG = Ortho nitro-phenyl- β -D-galactopyranosidase, ADH = Arginine dihydrolase, LDC = Lysine decarboxylase, ODC = Ornithine decarboxylase, CIT = Citrate utilization, H₂S = Hydrogen sulphide production, URE = Urease, TDA=Tryptophan deaminase, IND = Indole production, VP = Voges Proskauer, GEL = Gelatinase, GLU = Glucose, MAN = Mannitol, INO = Inositol, SOR = Sorbitol, RHA = Rhamnose, SAC = Saccharose, MEL = Melibiose, AMY = Amygdalin, ARA = Arabinose

Furthermore, all the isolated strains were able to produce siderophore, NH_3 , IAA and solubilize phosphate (Table 2). Similarly, all the strains were positive for ACC deaminase activity and able to reduce nitrate but only strains R2, R3 and R5 were able to produce HCN (Table 2). Antifungal activities of all the isolated new *Rhizobium* spp. were tested against *Fusarium*, *Aspergillus*, *Penicillium* and *Mucor* strains. All the test fungal strains with the exception of *Mucor* were sensitive to one or more bacterial strains (Table 3). The antifungal activity of the tested fungal strains differs with zones of inhibition ranging from 12.7 to 33.3 mm. Bacterial strains R2 and R5 showed antifungal activity against three fungal strains, R1 and R3 against two fungal strains and R4 showed antifungal activity against one fungal strain.

Table 5. 2 Plant growth promoting traits (PGPT) of bacterial strains

PGPT of bacterial strain								
Strain	HCN production	Siderophore production	NH ₃ production	IAA production	Phosphate solubilization	ACC deaminase	NRA	STH
R1	-	+++	+	++	+	+	+	+
R2	++	+	+	+	+	+	+	+
R3	++	++	+	++	+	+	+	+
R4	-	+	+	+	+	+	+	+
R5	+	++++	+	+	+	+	+	+

Legend: - = negative, + = positive, HCN = hydrogen cyanide, NH₃ = ammonia, IAA = indole-acetic-acid, ACC = 1-aminocyclopropane-1-carboxylate, NRA= nitrate reductase activity, STH = starch hydrolysis

Table 5. 3 Antifungal activity of the bacterial strains

Strain	Zone size in mm ($\times \pm$ SE)			
	<i>Fusarium</i> sp.	<i>Penicillium</i> sp.	<i>Aspergillus</i> sp.	<i>Mucor</i> sp.
R1	26.7 $\pm$ 4.9	-	18 $\pm$ 3.6	-
R2	27.7 $\pm$ 3.7	15.3 $\pm$ 1.9	12.7 $\pm$ 1.2	-
R3	26.7 $\pm$ 1.2	-	15.7 $\pm$ 2.3	-
R4	-	-	12 $\pm$ 0.0	-
R5	33.3 $\pm$ 2.2	18.3 $\pm$ 5.5	20.0 $\pm$ 1.0	-

Legend: $\times \pm$ SE = Mean $\pm$ standard error, Number of replicates (n) = 4, - indicates no inhibition

The new bacterial strains exhibited different tolerance levels to the osmotic stress stimulated by PEG. The strains showed high optical density (OD) values at lower concentrations of PEG, particularly R1 and R3. Strains R2 and R5 (Fig. 1a; 2) were less tolerant to PEG-stimulated stress specifically at the highest PEG concentrations (3%) and they showed less OD values in PEG amended broth medium than R1 and R3 (Fig. 1a, c). Strains R1 and R3 showed the highest OD values of 1.35 and 0.32 respectively under the highest PEG-stimulated stress medium and thus the two *Rhizobium* strains including R5 (Fig. 2) which had the least OD value (0.17) at 30% PEG medium were used for soybean seed inoculation.

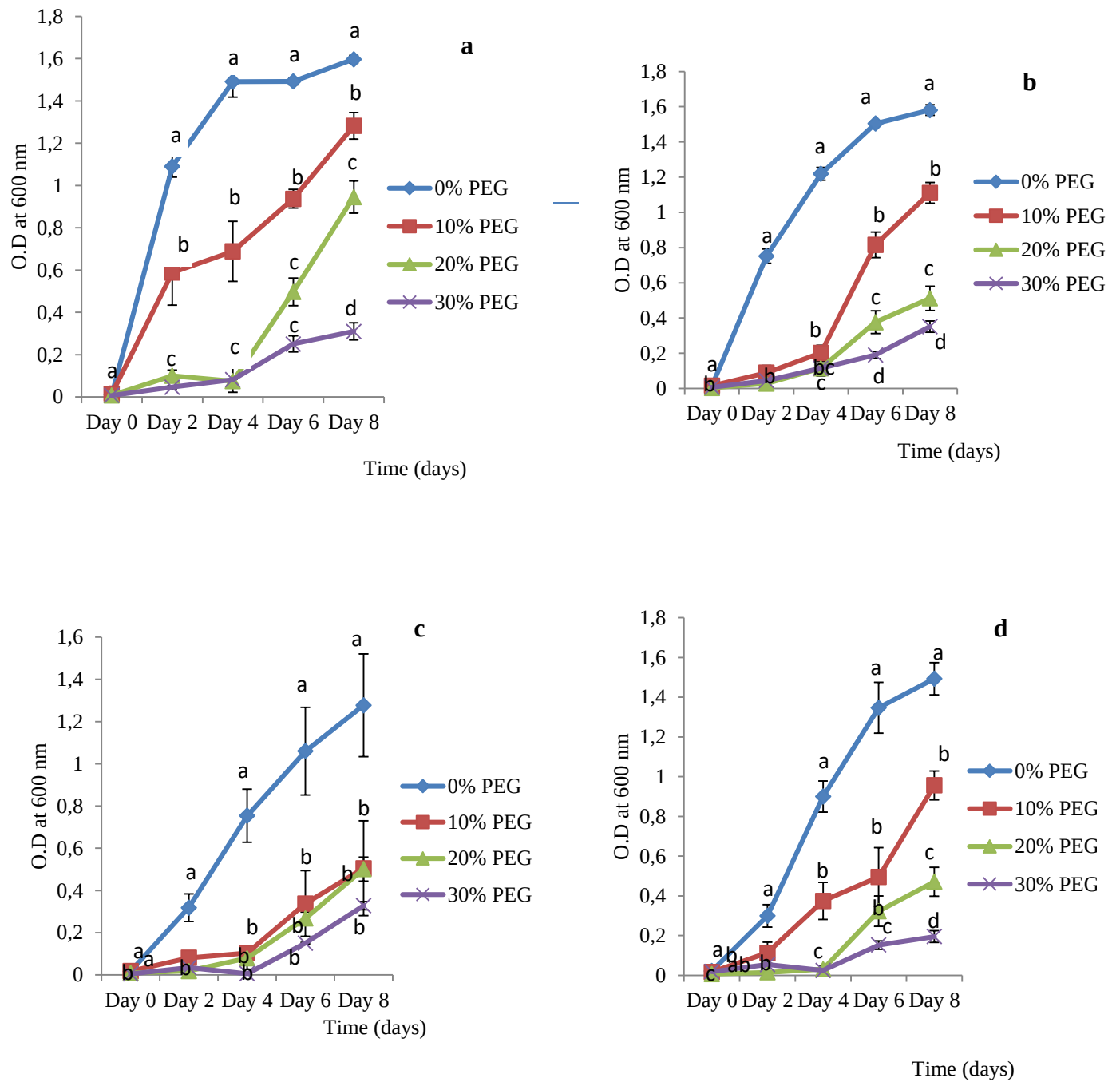


Fig. 5. 1 Growth of (a) R1 (b) R2 (c) R3 and (d) R4 bacterial strains exposed to PEG stimulated osmotic stress. Number of replicates (n) = 4. Data represent mean $\pm$ SE

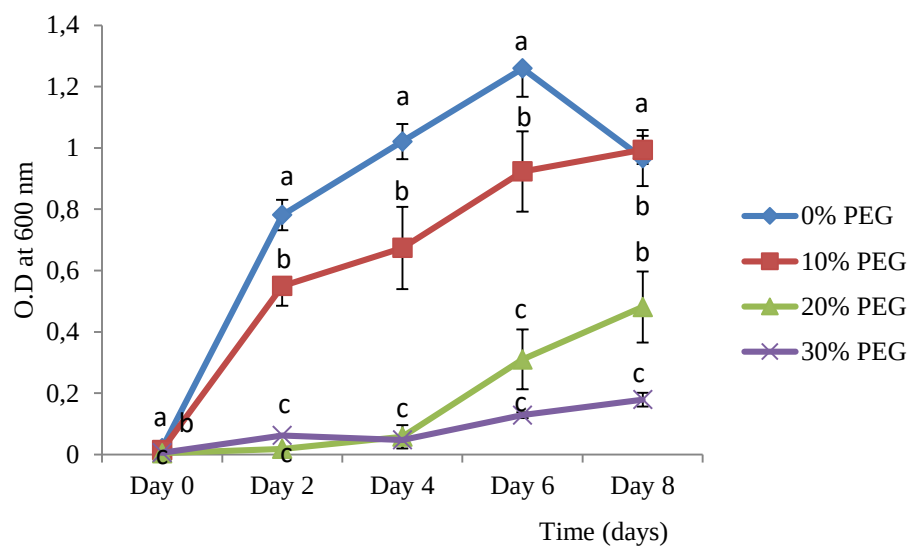


Fig. 5. 2 Growth of R5 bacterial strain exposed to PEG-stimulated osmotic stress. Number of replicates (n) = 4. Data represent mean $\pm$ SE

Identification of putative PGPR strains isolated from Bambara groundnut rhizosphere through 16S rRNA amplicon sequence analyses showed that the new bacterial strains R1, R2, R3, R4 and R5 with their accession numbers are *Rhizobium* sp. (accession no. MG309875), *Rhizobium tropici* (accession no. MG851722), *Rhizobium cellulosilyticum* (accession no. MG309874), *Rhizobium taibaishanense* (accession no. MG851723) and *Sinorhizobium meliloti* (accession no. MG851724) respectively. The new *R. taibaishanense* strain R4 has high homology (97 %) with *Rhizobium cellulosilyticum* (KF439811) while *R. tropici* strain R2 has homology of 100 % with *Rhizobium tropici* (MG851722) obtained from GenBank database. The constructed phylogenetic tree of these bacterial strains using the 16S rRNA amplicon sequences and bacterial sequences of related genera recovered from GenBank database is shown in Fig. 3. There are five major clades of Rhizobial species classified in the tree; the major clade is the Rhizobial group containing the new *R. taibaishanense* strain R4 and has strong bootstrap support (>70 %). The other four new *Rhizobium* species determined by phylogenetic assigning were properly classified in the other groups but *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 were classified in the same clade.

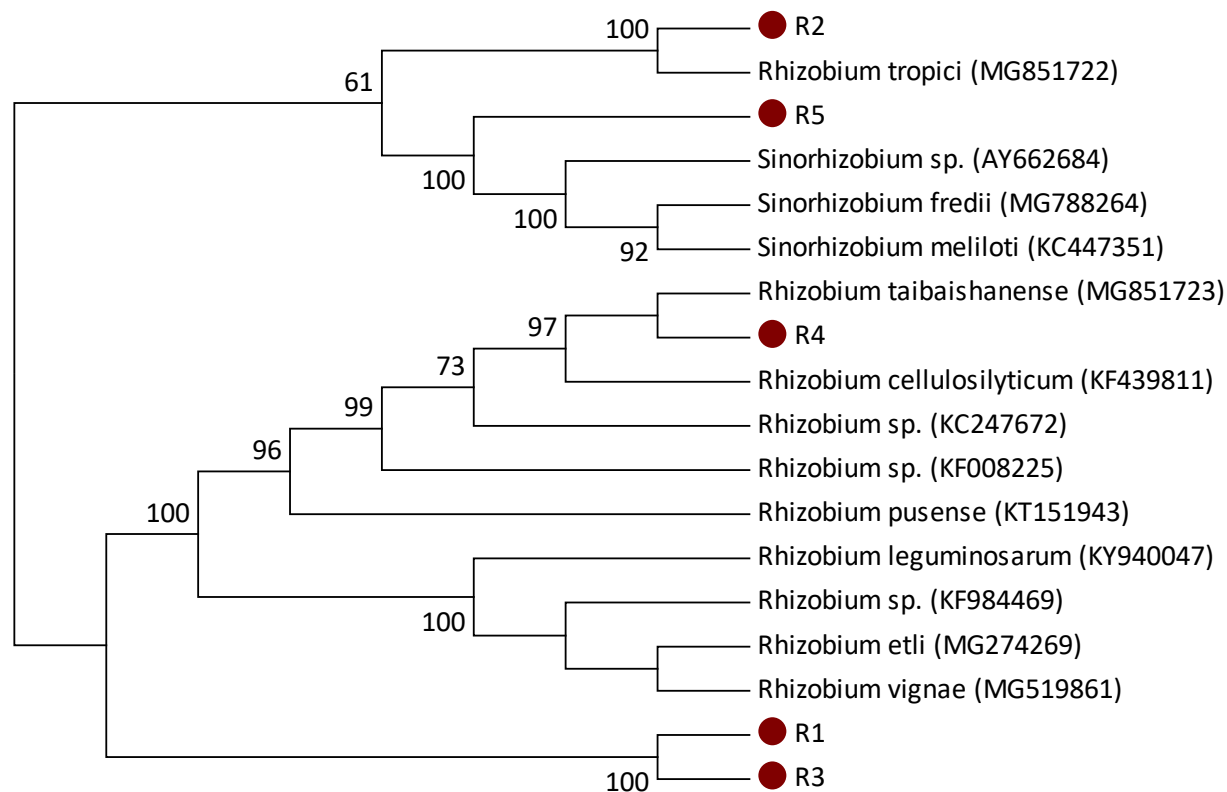


Fig. 5. 3 Phylogenetic tree of partial 16S rRNA sequences of *Rhizobium* strains recovered from Bambara groundnut rhizosphere. *Rhizobium* sp. strain R1 (accession no. MG309875), *Rhizobium tropici* strain R2 (accession no. MG851722), *Rhizobium cellulosilyticum* strain R3 (accession no. MG309874), *Rhizobium taibaishanense* strain R4 (accession no. MG851723) and *Sinorhizobium meliloti* strain R5 (accession no. MG851724) were the new strains isolated in this study

The results of soybean cultivar tolerance to 10 % PEG solution showed that the different components of soybean measured were affected by PEG as compared to the control (Fig. 4, 5, 6). At this concentration, PAN 1623 R had the highest shoot height followed by PAN 1532 R and PAN 1614 R (Fig. 4). But soybean seedlings in the control experiments (0 % PEG) had relatively higher shoot heights compared to the PEG amended seedlings. The higher shoot heights of soybean cultivars in the control were due to the absence of PEG in the treatment. Presence of PEG poses osmotic stress capable of altering the morphological and physiological functions of plants. In this present research, the responses of the different soybean cultivars to PEG solution were observed to be different. Also, stress induced by PEG decreased the shoot width, root height and width, shoot fresh weight and dry weight, root fresh and dry weight, number of lateral roots and seedling fresh weight. However, PAN 1623 R and PAN 1614 R had the highest and lowest outcome in virtually all the measured soybean growth components respectively while PAN 1532 R was in-between. As a result of this outcome, the sensitivity of soybean cultivars to PEG-imposed drought stress was arranged in a decreasing order as follows: PAN 1614 R > PAN 1532 R > PAN 1623 corresponding to extremely sensitive > moderately sensitive > slightly sensitive cultivar respectively. The moderately sensitive cultivar (PAN 1532 R) was selected for soybean inoculation experiment.

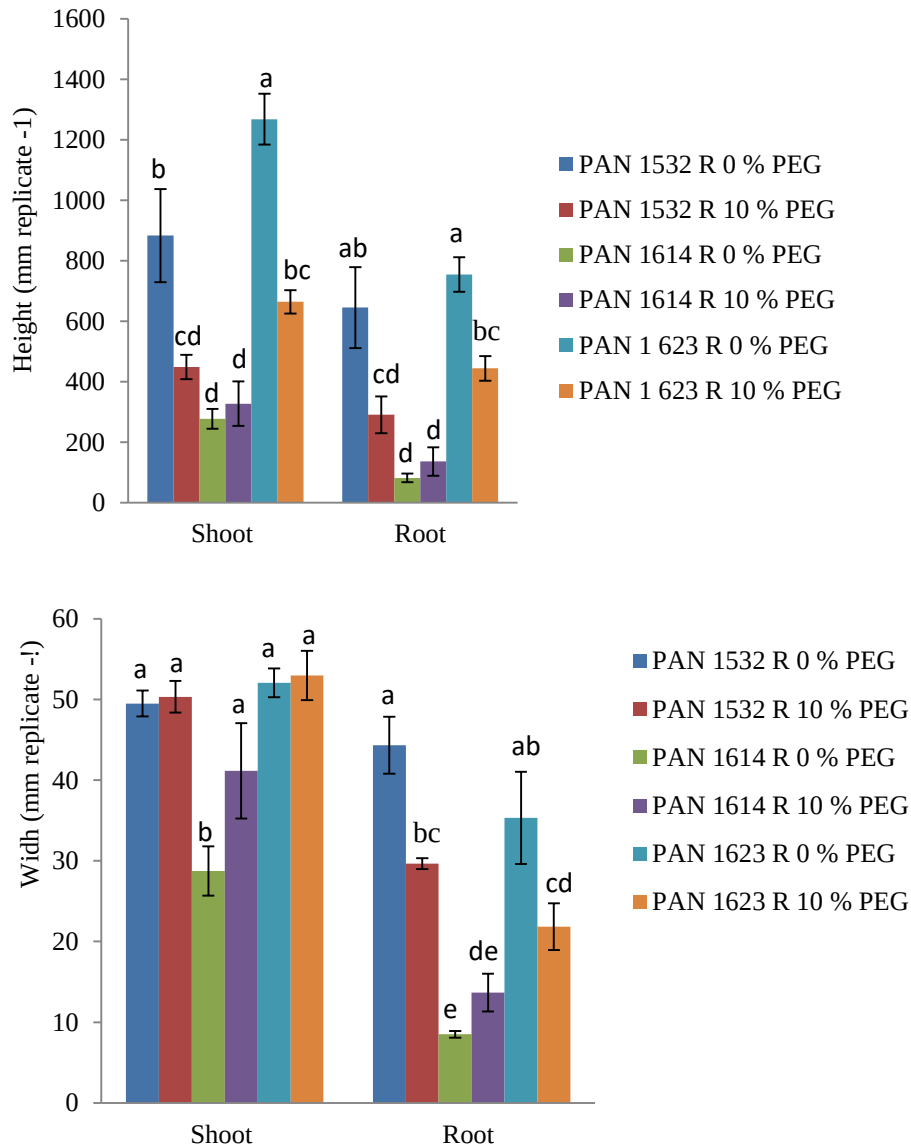


Fig. 5. 4 Height and width of shoot and root of soybean seedlings at day 8 after sowing under PEG-stimulated osmotic stressed condition. Number of replicates (n) = 3. Data represent mean $\pm$ SE. Data were collected from all the seedlings plate⁻¹ or replicate⁻¹

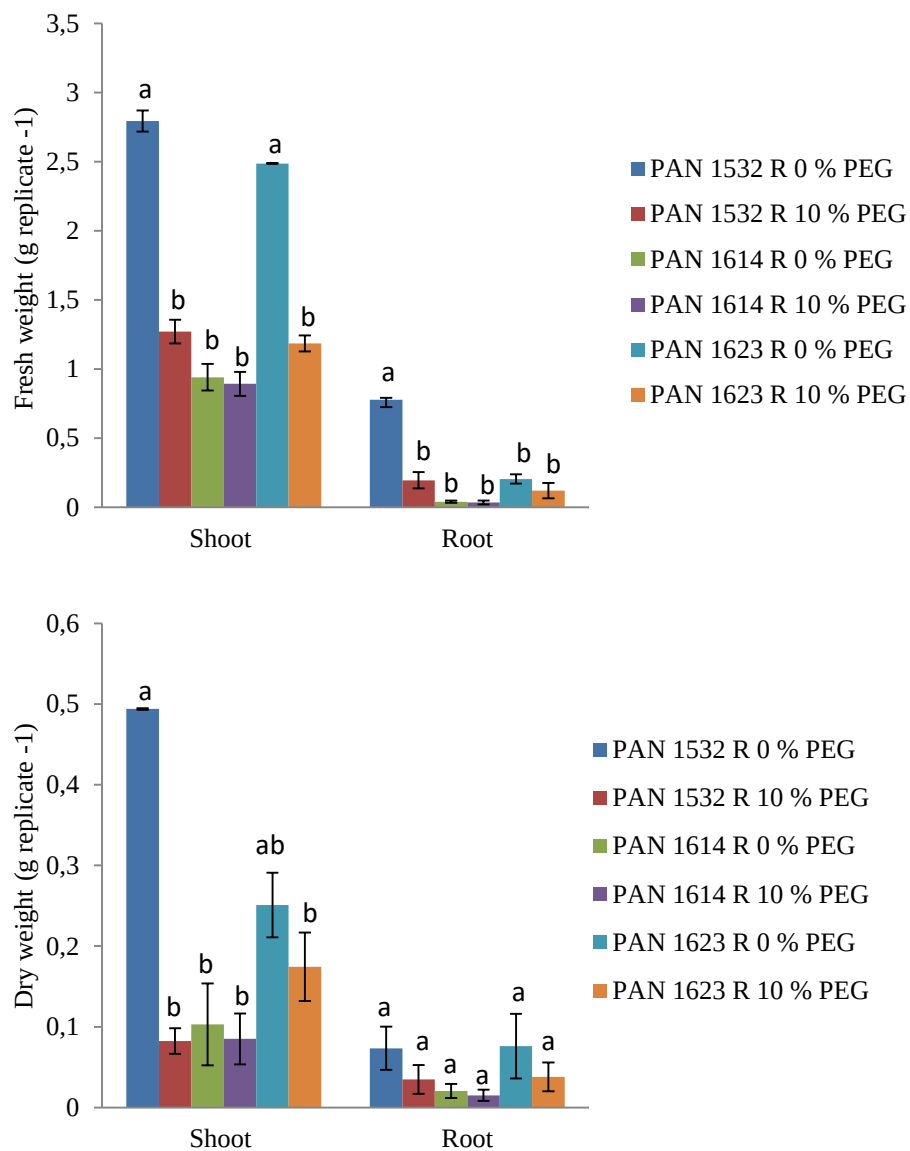


Fig. 5. 5 Fresh and dry weight of shoot and root of soybean seedling at day 8 after sowing under PEG-stimulated osmotic stressed condition. Number of replicates (n) = 3. Data represent mean $\pm$ SE. Data were collected from all the seedlings plate⁻¹ or replicate⁻¹

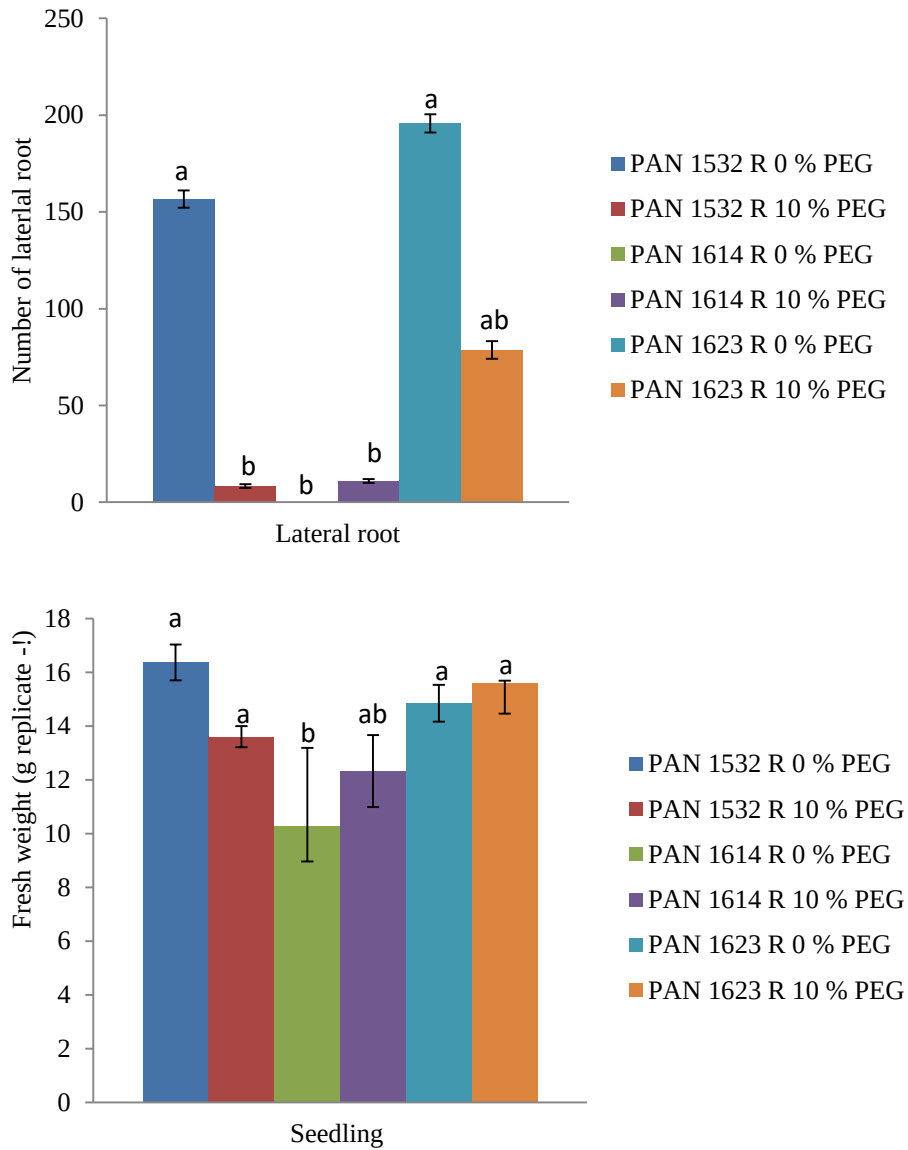


Fig. 5. 6 Number lateral roots and fresh weight of soybean seedlings at day 8 after sowing under PEG-stimulated osmotic stressed condition. Number of replicates (n) = 3. Data represent mean $\pm$ SE. Data were collected from all the seedlings plate⁻¹ or replicate⁻¹

The plant growth promoting effects of individual inoculation with *Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and *S. meliloti* strain R5 on soybean PAN 1532 R growth was assessed under stress condition induced by 4 % PEG (Fig. 10). Fig. 7 shows shoot and root (height and width) of soybean seedlings. Height and width increased in shoots of inoculated soybean seedlings compared to the non-inoculated (control) treatments. Remarkable significant differences ($P < 0.05$) were observed in shoot height and width of controls and bacterial strains R1 and R3. Similarly, significant differences were observed in shoot and root fresh weights of bacterial strains (R1 and R3) and controls but the shoot and root fresh weights of strain R5 inoculated seedlings were not significantly from non-inoculated seedling (Fig. 8). *R. cellulosilyticum* strain R3 also showed significant higher shoot dry weight ($P < 0.05$) than those amended with *Rhizobium* sp. strain R1, *S. meliloti* strain R5 and the non-inoculated (controls) seeds. Significant differences ($P < 0.05$) were also observed in soybean seedling fresh weights inoculated with *R. cellulosilyticum* R3 and controls (Fig. 9). Similar result was noticed for *Rhizobium* sp. strain R1 but no significant difference ($P > 0.05$) was observed in seedling fresh weights inoculated with *S. meliloti* and the non-inoculated (control) seedlings. With the exception of *S. meliloti* inoculated seedlings, the number of lateral roots increased in all the inoculated soybean seedlings compared to the control with strain R1 inoculated seedlings having the highest number.

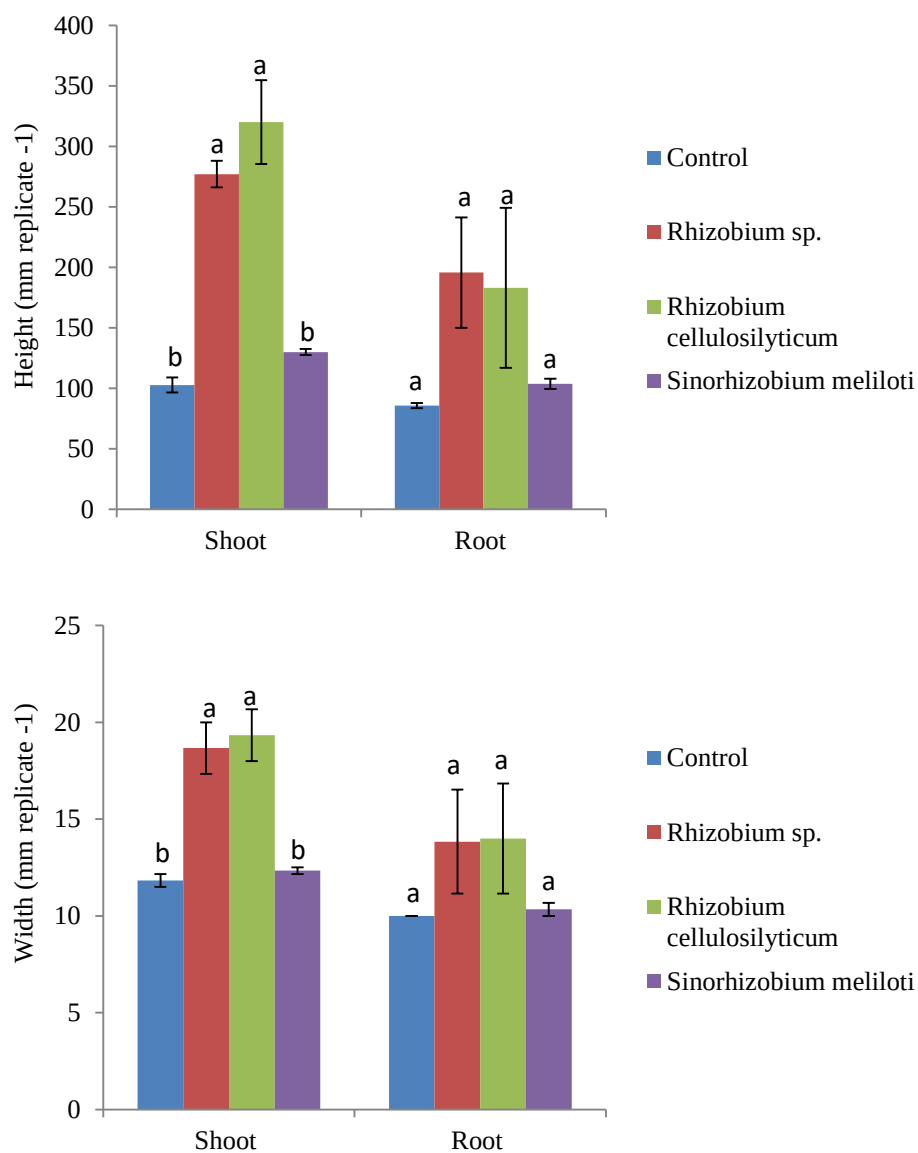


Fig. 5. 7 Height and width of shoot and root of soybean seedlings at day 8 after sowing under PEG-stimulated osmotic stressed condition. Number of replicates (n) = 3. Data represent mean $\pm$ SE. Data were randomly collected from 10 seedlings plate⁻¹ or replicate⁻¹

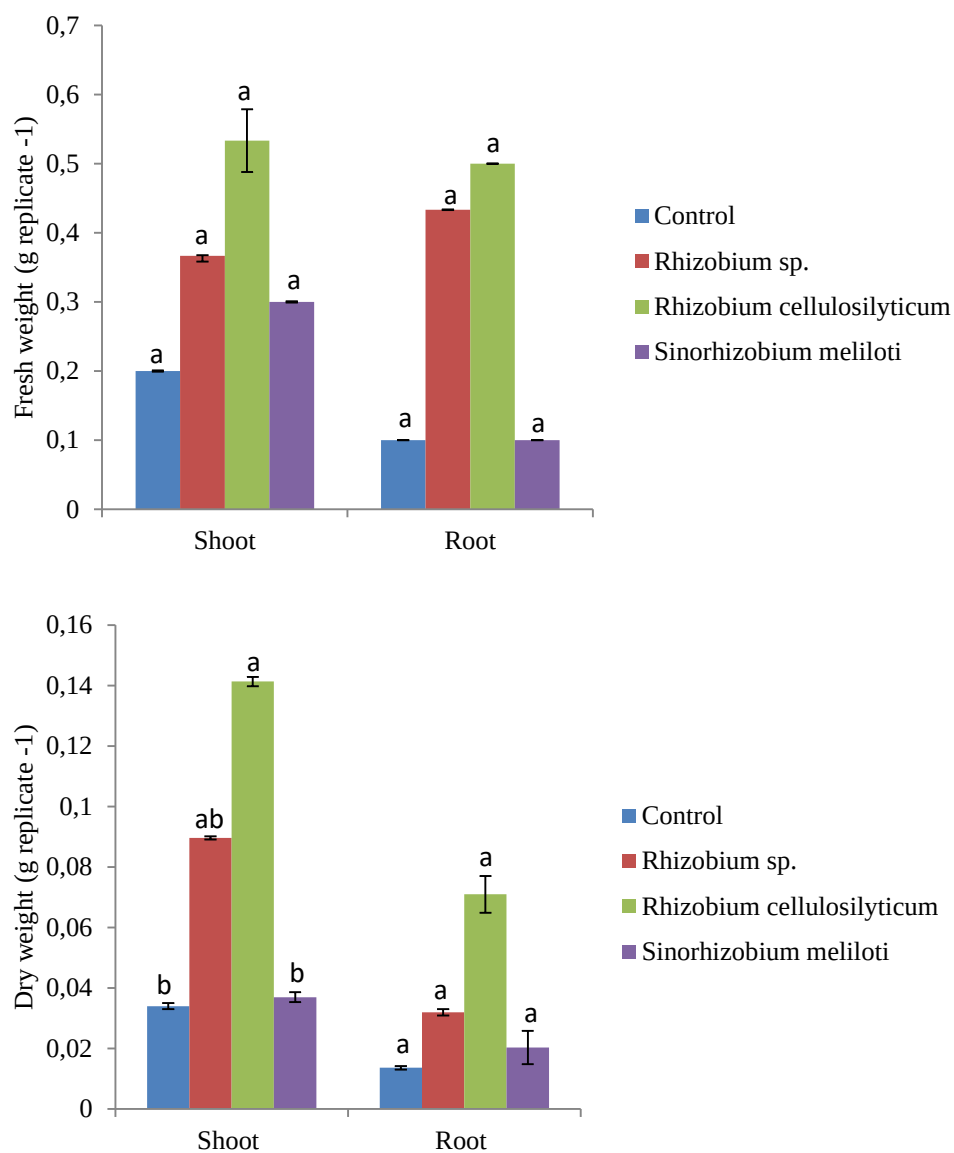


Fig. 5. 8 Fresh and dry weight of shoot and root of soybean seedlings at day 8 after sowing under PEG-stimulated osmotic stressed condition. Number of replicates (n) = 3. Data represent mean $\pm$ SE. Data were randomly collected from 10 seedlings plate⁻¹ or replicate⁻¹

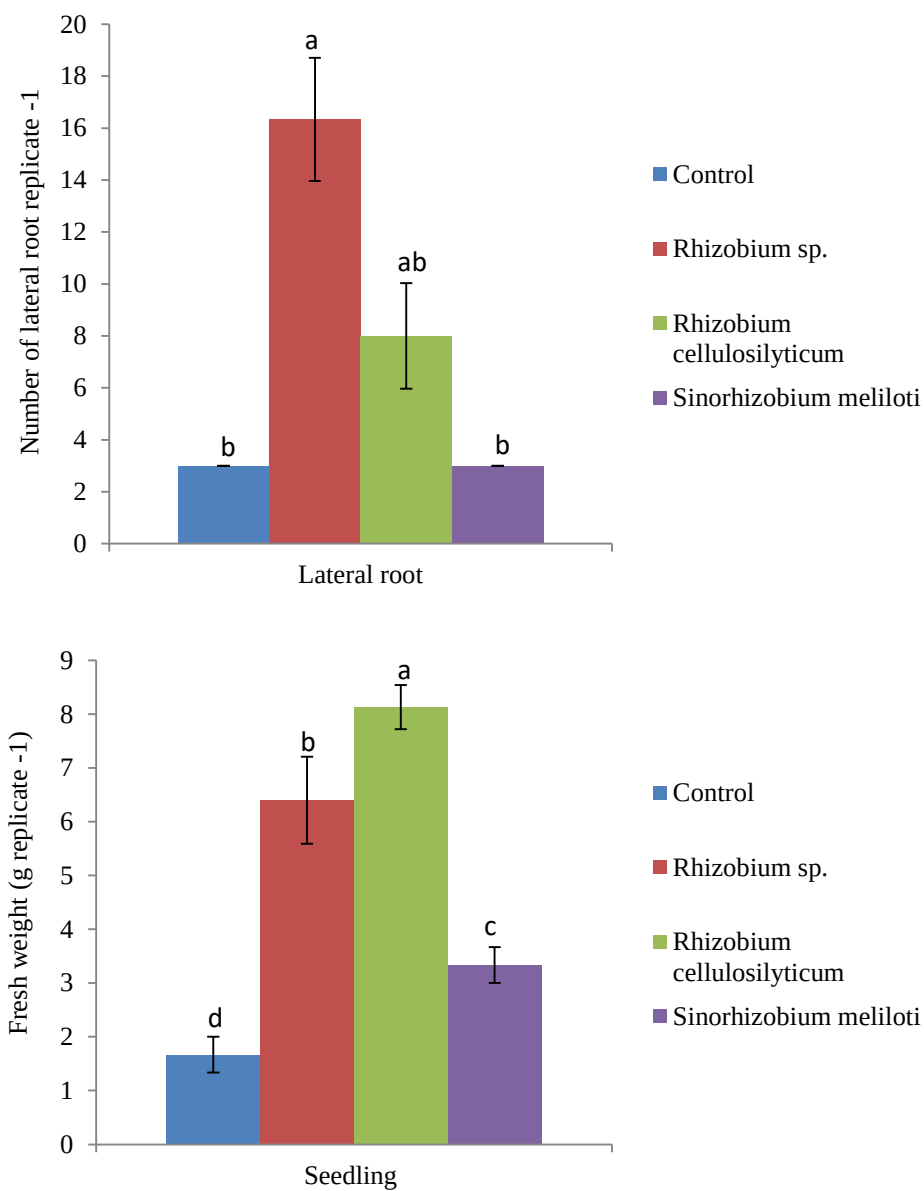


Fig. 5. 9 Number of lateral roots and fresh weight of soybean seedlings at day 8 after sowing under PEG-stimulated osmotic stressed condition. Number of replicates (n) = 3. Data represent mean $\pm$ SE. Data were randomly collected from 10 seedlings plate⁻¹ or replicate⁻¹

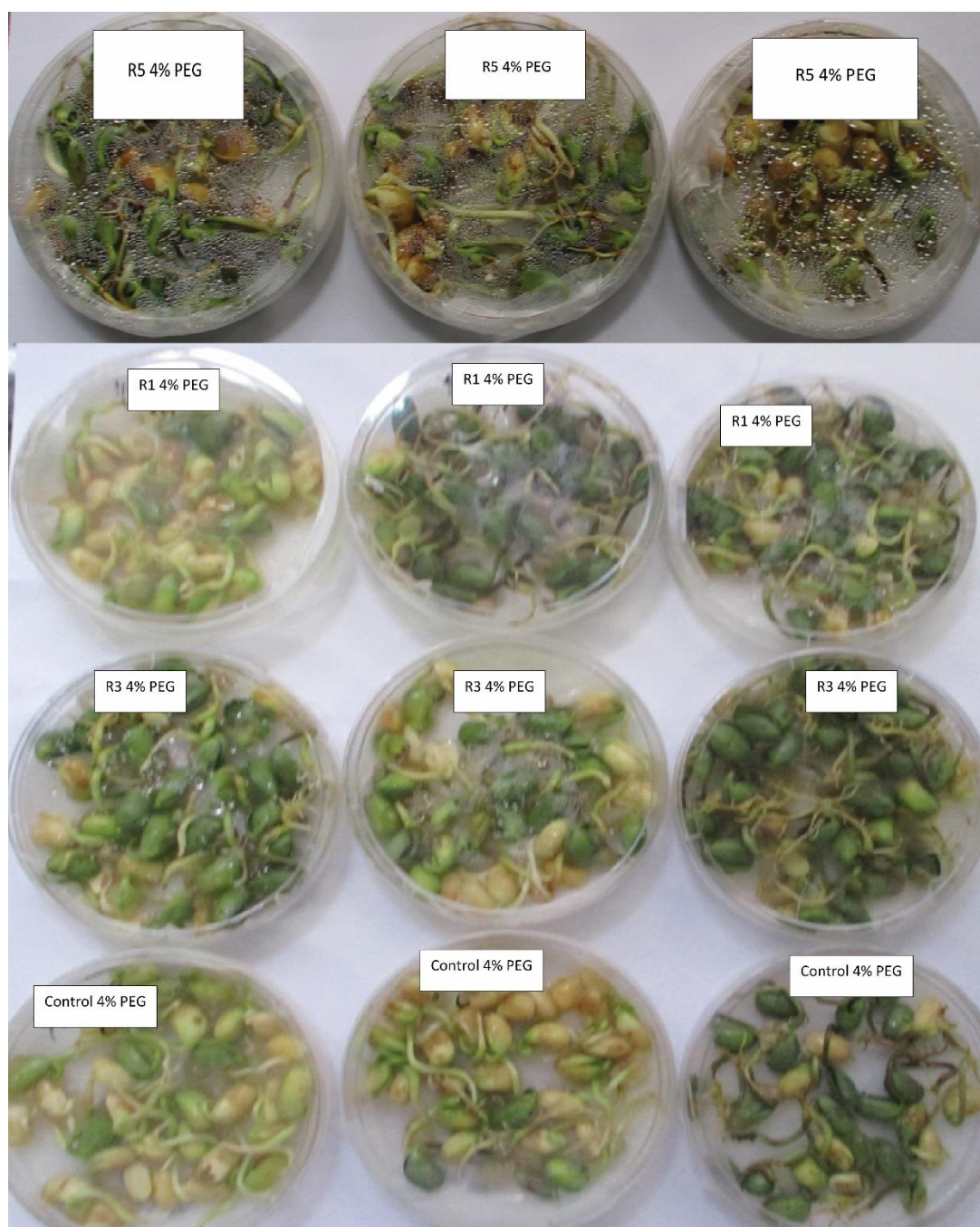


Fig. 5. 10 Soybean seedlings inoculated with new *Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3, *S. meliloti* strain R5 and grown under 4 % PEG-stimulated osmotic stressed condition. Control represents non-inoculated seedlings. Number of replicates (n) = 3

5.3 Discussion

Plant rhizosphere is well known to be a favourite reservoir for different types of microorganisms because of its rich nutrient status. It has been anticipated that inoculation with PGPR like *Rhizobium* species improved plant growth because of their plant growth promoting properties (Ahmad et al., 2008, Glick, 1995). Therefore in this current study, new bacterial strains were isolated from Bambara groundnut rhizosphere and identified by molecular techniques as *Rhizobium* sp. strain R1 (accession no. MG309875), *Rhizobium tropici* strain R2 (accession no. MG851722), *Rhizobium cellulosilyticum* strain R3 (accession no. MG309874), *Rhizobium taibaishanense* strain R4 (accession no. MG851723) and *Sinorhizobium meliloti* strain R5 (accession no. MG851724). Different molecular techniques have been used for subtyping microbial isolates (Wiedmann et al., 2000, Stafford et al., 2005) which are capable of differentiating bacterial strains into groups. In this study, PCR-amplified 16S rRNA has confirmed that all the PGPR used in this research work belong to rhizobial group. The nucleotide sequence of *R. tropici* strain R2 showed 100 % homology to *R. tropici* (accession no. MG851722) indicating that 16S rRNA can be used to characterize and identify *Rhizobium* isolates which allows predicting the microbial isolate at the species level and this is in conformity with the report of Rai et al. (2012). Amazingly, the phylogenetic tree of partial 16S rRNA sequences of *Rhizobium* strains shows that *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 that were used for soybean inoculation in this study are closely related suggesting that they have similar nucleotide sequences. However, the *Rhizobium* spp. isolated from Bambara groundnut rhizosphere in this present study are different from the rhizobial species reported by Habibi et al. (2017) who isolated mainly *Bradyrhizobium* and *Ensifer* spp. from soybean environment grown in Afghanistan. The reason for this difference could be that the occurrence of

a leguminous crop in an environment selects for specific *Rhizobium* species due to the rhizodeposits that exude from the host crop (Hartmann et al., 2009, Venkateswarlu et al., 1997).

The 5 strains of *Rhizobium* spp. were also characterized morphologically, biochemically and then screened for PGP activities. Microscopic examination confirmed pure isolates Gram negative rods and biochemical result showed that all the strains are aerobic and possess catalase enzyme since they were able to breakdown hydrogen peroxide to form oxygen (Table 1). These findings are in conformity with the results of Masciarelli et al. (2014) who reported the isolation of Gram negative and catalase positive rhizobial species from soybean seeds.

Siderophore and NH_3 production were observed in all the bacterial species. IAA production was similarly detected in all the *Rhizobium* species and this is in agreement with the finding of Masciarelli et al. (2014) and Boiero et al. (2007) who reported production of high level of IAA and other phytohormones by *Bradyrhizobium japonicum*. The production of IAA by the new isolated bacterial strains indicates that their plant growth enhancing abilities do not only include helping plants to tolerate drought stress, but also enhance enzymatic activities and phytohormone production.

Three of the isolated bacterial strains in this study revealed all the PGPTs which might enhance plant growth either directly or indirectly or synergistically and they include *R. tropici* strain R2, *R. cellulosilyticum* strain R3 and *S. meliloti* strain R5. Similar multiple plant growth promoting activities among rhizospheric bacteria have been reported previously (Ahmad et al., 2008, Gupta et al., 1998). Another essential trait of rhizospheric bacteria that can indirectly affect plant growth is siderophore production. Siderophores bind to bioavailable form of Fe^{3+} in the soil, thereby limiting its availability to plant pathogens and defending plant health (Igiehon and Babalola, 2018c). In this study, all the bacterial strains were observed to produce siderophore

(Table 2) but at different levels and antifungal activities (Table 3) against at least one test fungal species. The strain R5 showed better antifungal activity than other bacterial strains as it was most effective against *Fusarium*, *Penicillium* and *Aspergillus* spp. with zones of inhibition of 33.3, 18.3 and 20.0 mm respectively. This finding corresponds with the highest level of siderophore production by this bacterium (Table 2). Based on the data obtained from this study, susceptibility of the test fungal strains was in the order of *Fusarium* sp. > *Aspergillus* sp. > *Penicillium* sp. Bacterial strains R2 and R5 revealed ‘broad spectrum antifungal activity’ against three of the four tested fungal strains. The antifungal activity of the bacterial strains suggested a closed relationship between siderophore and HCN production and such antimicrobial activity may be due to either the production of HCN or siderophore or synergistic interaction of the two metabolites or with other metabolic compounds.

Also, all the bacterial strains were screened for ACC deaminase by spot inoculating modified DF salt minimal medium supplemented with ACC as sole nitrogen source, DF salt minimal medium only (negative control) and DF medium containing 0.2 % w/v $(\text{NH}_4)_2\text{SO}_4$ (positive control). The new bacterial strains grew in ACC supplemented DF medium and DF medium containing $(\text{NH}_4)_2\text{SO}_4$ and this finding is in agreement with the finding of Ali et al. (2014). The growth of the bacteria in DF containing ACC indicated that they possess ACC deaminase activity and can aid plants to tolerate stress.

Plants are usually confronted with abiotic stress (e.g. drought) which is one of the severe challenges associated with plant development affecting their productivity and/or yields (Ali et al., 2014, Igiehon and Babalola, 2018c, Igiehon and Babalola, 2017). According to Ali et al. (2014), amendment of soils facing drought with ACC-producing microorganisms can reduce stress in crops by minimizing ethylene (C_2H_4) production which is known to induce stress in

plants. Drought tolerant bacteria can survive in stressed soils and bind to seed coat or seedling roots, and trigger deamination of ACC (the immediate precursor of C_2H_4 in plants) to α -ketobutyrate and NH_3 (instead of C_2H_4) resulting in low concentration of C_2H_4 in plant environment and thus enhancing plant growth and development (Ali et al., 2014, Arshad et al., 2007, Glick et al., 1998).

To further buttress the ACC deaminase activity of bacterial strains, each strain was grown in medium amended with different concentrations of osmotic-stimulant (PEG). All the bacterial strains grew at the different PEG concentrations but the highest growths were observed in the 0 % (control) PEG medium (Fig. 1a, b, c, d; 2). The present outcomes concur with the results of Burd et al. (1998), who highlighted that the plant growth-enhancing bacterium *Kluyvera ascorbata* SUD165 was resistant to environmental stress caused by heavy metal and exhibited ACC deaminase activity that reduced the level of C_2H_4 . In addition, the finding of Ortiz et al. (2015), who indicated increase in viable cell counts of PGPR in nutrient broth amended with 0, 10, 20, 30 and 40 % PEG further gives credence to the results obtained from this study. The bacterial counts from their study decreased as the PEG concentrations increased which is similar to what we observed in this current study. But, strains R1 and R3 (Fig. 1a and d) demonstrated the highest level of tolerance to osmotic stress (since they had the highest OD values at 30 % PEG) while strain R5 (Fig 2) showed the lowest level of tolerance in the PEG experiment. Hence, R1 and R3 (the most tolerant strains) and R5 (the least tolerant strain) were selected for soybean inoculation and growth under stress condition so as to ascertain whether the most stress tolerant bacterial strains will have the same effect(s) as the least tolerant strain on soybean growth.

Prior to soybean inoculation, 3 soybean cultivars were subjected to PEG-imposed drought stress and the responses of the cultivars to 10 % PEG concentrations were significantly different. This condition triggered substantial interaction between cultivars and PEG treatment. This indicates that there are genetic variations among the soybean cultivars as regards osmotic stress tolerance. However, PEG solution decreased the measured growth components in all the cultivars but PAN 1623 R was more tolerant than PAN 1532 R and PAN 1614 R. In particular, shoot and root heights of PAN 1623 R were significantly higher than the heights observed in the other two cultivars. As a result, sensitivity of cultivars to 10 % PEG was arranged in the following decreasing order: PAN 1614 R > PAN 1532 R > PAN 1623 R. This finding is in agreement with previously reported works (Gill et al., 2002, Almodares et al., 2007, Blanco et al., 2007).

Furthermore, cultivars showed different responses in terms of lateral root number at 10 % PEG and PAN 1623 R cultivar had the highest number of lateral root (78.6 replicate⁻¹) followed by PAN 1614 R (11 replicate⁻¹) and PAN 1532 R (8.3 replicate⁻¹). Amazingly, PAN 1614 R did not develop any root at 0 % (control) PEG unlike PAN 1623 R (195.6 replicate⁻¹) and PAN 1532 R (156.6 replicate⁻¹). Like every other growth parameter, application of PEG decreased the number of lateral root in almost all the cultivars. However, considering all the parameters measured (Fig 4, 5 and 6), PAN 1623 R was the most productive cultivar under PEG-induced stress seconded by PAN 1532 R.

In addition, PAN 1623 R showed higher shoot and root dry weights at 10 % PEG compared to the other cultivars. The mean shoot dry weights of PAN 1623 R, PAN 1532 R and PAN 1614 R at 10 % PEG were 0.174, 0.103 and 0.085 g replicate⁻¹ respectively (Fig. 5). Also, PAN 1623 R was observed to have the highest root dry weight (0.039 g replicate⁻¹) in the PEG-

amended treatment. Similar results were obtained for the seedling fresh weights of cultivars and PAN 1623 R showed the highest weight (which was even higher than that of the control treatment) under PEG condition compared to PAN 1532 R and PAN 1614 R. On the basis of these findings, the sensitivity of the cultivars was classified as follows: PAN 1614 R > PAN 1532 R > PAN 1623 R corresponding to extremely sensitive cultivar > moderately sensitive cultivar > slightly sensitive cultivar. Then, the moderately sensitive soybean cultivar (PAN 1532 R) was selected for microbial inoculation and growth under 4 % PEG condition.

One amazing result found in this study is the inherent ability of the new *Rhizobium* species assayed to cope with stressful conditions. Moreover, not only *R. cellulosilyticum* strain R3 but also *Rhizobium* sp. strain R1 proved effective in aiding inoculated soybean PAN 1532 R to alleviate the detrimental osmotic stress effects on seedling growth (Fig. 10); nevertheless, the major effects were noticed in soybean shoot and root heights, shoot and root weights (fresh and dry weights) especially in seedlings inoculated with the new *R. cellulosilyticum*.

In this present study, shoot and root heights were increased by the microbial inocula applied. Similar result was observed in shoot and root width especially in soybean seedlings inoculated with *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3. The microbial inoculants applied may have induced different natural processes that assisted the soybean seedlings to grow and develop under drought condition (Ortiz et al., 2015).

An essential plant growth promoting parameter ACC, helps plants to respond and attenuate drought stress (Ali et al., 2014, Saravanakumar and Samiyappan, 2007). The lowest values obtained for virtually all the measured components of soybean seedlings (Fig. 7, 8, 9) in the control treatments suggested their poor capability to cope effectively with osmotic stress induced by the 4 % PEG. In particular, soybean seedlings colonized with specific bacterial

species inocula enhanced the seedling fresh weight (Fig. 9) by 80.32 % (seedling fresh weight inoculated with *R. cellulosilyticum* strain R3) or by 75 % (seedling fresh weight inoculated with *Rhizobium* sp. strain R1) or by 51 % (seedling fresh weight inoculated with *S. meliloti* strain R5). Perhaps, the increase in the seedling fresh weight noticed in the soybean seedlings amended with microbial inoculants is partly attributed to the production of ACC deaminase an enzyme that plays a pivotal role in microbial and plant adaptation to stressed conditions (Saravanakumar and Samiyappan, 2007) by reducing the production of C₂H₄ (a stress inducer) and enhancing the generation of α -ketobutyrate and NH₃ from ACC (C₂H₄ precursor). This mechanism of ethylene reduction is most important to dicotyledonous crops that are more vulnerable to the effects of C₂H₄ (Hall et al., 1996) particularly under environmental stress conditions such as drought (Mayak et al., 2004b), phytopathogens (Wang et al., 2000) and flooding (Grichko et al., 2000). These findings are in conformity with the results of this current study since the dicotyledonous soybean seedlings responded positively to the applied microbial inocula.

In conclusion, the new *Rhizobium* spp. isolated from Bambara groundnut rhizosphere in this study can be used as bio-inoculants to enhance osmotic stress tolerance and growth of different legumes especially in semi-arid and arid environments. *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 can be ‘exploited in sustainable agriculture’ since these strains showed significant growth promotion in this study. Moreover, modification of hormonal status of crops to reduce the production of the senescence-inducing and growth-impeding C₂H₄ (or its precursor ACC) or to sustain photosynthesis cum biomass production and allocation (by modifying IAA biosynthesis) appears to be promising strategies to improve crop tolerance to stress by soil microbiome. However, many of the fundamental molecular and physiological mechanisms have

to been identified so as to optimize the agricultural applications of soil PGPR (Dodd and Pérez-Alfocea, 2012).

5.4 Acknowledgements

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5.5 Conflict of interest

Author 1 declares that he has no conflict of interest. Author 2 declares that she has no conflict of interest.

5.6 Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

CHAPTER 6

Genomic Insights into PGP Rhizobia Capable of Enhancing Soybean Germination under

Drought Stress

Abstract

Background

The role of soil microorganisms in plant growth, nutrient utilization, drought tolerance as well as biocontrol activity cannot be over-emphasized especially in this era where food crisis is a global challenge. This research was therefore designed to gain genomic insights into plant growth promoting (PGP) *Rhizobium* species capable of enhancing soybean (*Glycine max* L.) seeds germination under drought condition.

Results

Rhizobium sp. strain R1, *Rhizobium tropici* strain R2, *Rhizobium cellulosilyticum* strain R3, *Rhizobium taibaishanense* strain R4 and *Sinorhizobium meliloti* strain R5 were found to possess the entire PGP traits tested. Specifically, these rhizobial strains were able to solubilize phosphate, produce exopolysaccharide (EPS), 1-aminocyclopropane-1-carboxylate (ACC), siderophore and indole-acetic-acid (IAA). These strains also survived and grew at a temperature of 45°C and in an acidic condition with a pH 4. Consequently, R1, R3 and R5 strains enhanced the germination of soybean seeds (PAN 1532 R) under drought condition imposed by 4% PEG; nevertheless, *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 inoculations were able to improve seeds germination more than R5 strain. Thus, genomic insights into *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 revealed the presence of some genes with their respective proteins involved in symbiotic establishment, nitrogen fixation, drought tolerance and plant growth

promotion. In particular, *exoX*, *htrA*, *Nif*, *nodA*, *eptA*, *IAA* and siderophore-producing genes were found in the two rhizobial strains.

Conclusions

Therefore, the availability of the whole genome sequences of R1 and R3 strains may further be exploited to comprehend the interaction of drought tolerant rhizobia with soybean and other legumes and the PGP ability of these rhizobial strains can also be harnessed for biotechnological application in the field especially in semiarid and arid regions of the globe.

Keywords: drought stress, nitrogen fixation, PGP, symbiotic establishment, soybean, whole genome sequences

6.0 Background

To start with, the symbiotic interaction between leguminous plants and nitrogen (N) fixing bacteria, generally called rhizobia, has been the focus of research for over 12 decades. Recently, ‘a renewed interest’ in this area of research has been noticed due to its importance in sustainable agriculture, minimizing cost for the agriculturalists, enhancing soil fertility, alleviation of greenhouse-gas emissions (Ormeño-Orrillo et al., 2012) and improving plant’s tolerance to drought stress (Igiehon and Babalola, 2018c).

In addition, the role of soil microorganisms in plant growth, nutrient utilization, drought tolerance as well as biocontrol activity is well known and these beneficial microorganisms inhabit plant rhizosphere. In the rhizosphere, these microorganisms promote plant growth via ‘direct and indirect mechanisms’ (Grover et al., 2011). Additionally, the role of these beneficial microorganisms in biotic and abiotic stresses is gaining relevance and the mechanisms by which they enhance plant tolerance to drought include: Production of ACC deaminase to minimize the quantity of ethylene produced in the roots, microbial exopolysaccharide (EPS), induced systemic

resistance and phytohormones production such as indole-3-acetic acid (IAA) (Yang et al., 2009, Dimkpa et al., 2009, Maheshwari, 2011, Kim et al., 2012).

Indeed, plant growth can be regulated by ethylene (C_2H_4) content and the biosynthesis of this compound is regulated by biotic and abiotic stressors (Hardoim et al., 2008). In the synthetic pathway of C_2H_4 in plants, S-adenosyl methionine (S-AdoMet) is transformed to the immediate precursor of C_2H_4 1-aminocyclopropane-1-carboxylate (ACC) by aminocyclopropane-1-carboxylate synthase (ACS). Under drought stress conditions, plant homeostasis are regulated by C_2H_4 leading to a decrease in shoot and root growth and even seed germination. Plant ACC is confiscated and disintegrated by ACC-producing rhizobia to release and supply energy and nitrogen. Thus, the disintegration and consequential removal of ACC by rhizobia alleviate the effects of C_2H_4 , thereby minimizing plant stress and enhancing plant growth (Glick, 2005). Therefore single and dual inoculation of plants with ACC-producing rhizobia can results in improved seed germination even under drought stress conditions. In particular, dual inoculation of ACC deaminase producing *Pseudomonas* and *Bacillus* with *Mesorhizobium ciceri* enhanced seed germination, shoot height, root length and seedling fresh weight of chickpea grown under stressed condition when compared to non-inoculated plants (Sharma et al., 2013).

In addition, drought stress affects water availability to plant and water availability influences the production and utilization of polysaccharides by rhizobia (Roberson and Firestone, 1992). Example of such polysaccharides is exopolysaccharide (EPS) and production of EPS by rhizobia protects them from harsh conditions which enhances their survival under such conditions. Amendment of wheat with EPS and catalase producing *Rhizobium leguminosarum* (LR-30), *Rhizobium phaseoli* (MR-2) and *Mesorhizobium ciceri* (CR-30 and CR-39) benefited the plant by improving its growth, drought tolerance index and biomass under drought condition

using polyethylene glycol (PEG) 6000 as the drought factor. Thus, there is further need to x-ray the effects of new strains of *Rhizobium* on growth parameters (such as percentage seed germination) of other agricultural crops such as soybean (*G. max* L.) under drought condition stimulated by PEG.

Again, it has been reported that soil bacteria offer benefits to their host plants by suppressing plant pathogens and facilitate nutrient assimilation (Mantelin and Touraine, 2004, Yang et al., 2009, Pérez-Montaña et al., 2014). In our previous study (Igiehon and Babalola, 2018c), it was reported that some rhizobacteria mop up the insoluble form of iron from the soil environment and make it available to plant ‘with the aid of siderophore’ (Tkacz and Poole, 2015) and there is evidence that some plants can use bacterial iron (III)-siderophore complexes for their growth (Bar-Ness et al., 1991) even though the phytorelevance of these complexes is controversial. On the other hand, the removal of iron from the soil by siderophore-producing rhizobia reduces the bioavailability of iron in the root region and consequentially suppresses the growth of fungal pathogens (Traxler et al., 2012, Bal et al., 2013).

Similarly, just like siderophore-producing bacteria, some rhizobia contribute to plant growth by helping to mineralize insoluble phosphate compounds to release phosphorus needed for plant growth (Ahmad et al., 2008). Phosphorus in di-calcium phosphate, hydroxyapatite, rock phosphate and tri-calcium phosphate in soil can be released by phosphate solubilizing bacteria such as *Rhizobium*, *Bacillus*, *Burkholderia* and *Agrobacterium* while other rhizospheric rhizobia have the ability to produce indole-acetic acid (IAA) which helps in root elongation and production of lateral roots and root hairs involved in nutrient absorption (Mohite, 2013). Elongated and increase in the number of root produced by plants as a result of IAA production by plants can serve as a survival strategy to plants under drought stress condition and may even

contribute in some other ways to plant development since it was reported by Masciarelli et al. (2014) that the increased production of IAA by *Bradyrhizobium japonicum* shows that, in addition to plant promotion, the bacterium could have other beneficial traits needed for plants (such as soybean) survival. In short, considering these benefits, the interaction between plants, especially legumes and rhizobia is key to plant productivity.

Actually, rhizobia-legume symbiotic relationship commences with a molecular dialogue between the partners. The legumes produce flavonoids (Hungria and Stacey, 1997) that elicit the production of Nod factors (lipochitin oligosaccharides), that in turn, stimulate the development of the root nodule (Oldroyd and Downie, 2008). Rhizobial species enter and colonize the root nodules where they metamorphose to bacteriods that fix atmospheric N (Perret et al., 2000). Admittedly, other bacterial systems are involved in root colonization, efficient nodulation and N-fixation, ‘including surface polysaccharide and secretion systems’ (Perret et al., 2000, Fauvart and Michiels, 2008, Ormeño-Orrillo et al., 2012). These processes in addition to PGP and drought tolerance ability of rhizobia are regulated by myriads of genetic components which can further be exploited to gain insights into legume –rhizobial interactions.

Therefore this study was designed to gain genomic insights into selected PGP rhizobia capable of promoting soybean seed germination under drought stress condition

6.1 Materials and Methods

6.1.1 Source of Rhizobial Species used in this Study

The rhizobial species used in this study were isolated from Bambara groundnut rhizospheric soil at North-West University campus (25.82080S: 025.61382E), Ngaka Modiri Molema District, Mahikeng, North-West Province, South Africa (Fig. 1) and the physicochemical analysis showed that the soil has the following properties: 7.65 pH, 1.62 mg/kg

Fe, 24.1 mg/kg Mn, 1.06% organic carbon, 4.01% organic matter, 285 mg/kg K, 397 mg/kg mg and 0.066% total N. The Rhizobial species were sequenced by Sanger sequencing technique and identified in our previous study as *Rhizobium* sp. strain R1 (accession no. MG309875), *Rhizobium tropici* strain R2 (accession no. MG851722), *Rhizobium cellulosilyticum* strain R3 (accession no. MG309874), *Rhizobium taibaishanense* strain R4 (accession no. MG851723) and *Sinorhizobium meliloti* strain R5 (accession no. MG851724).

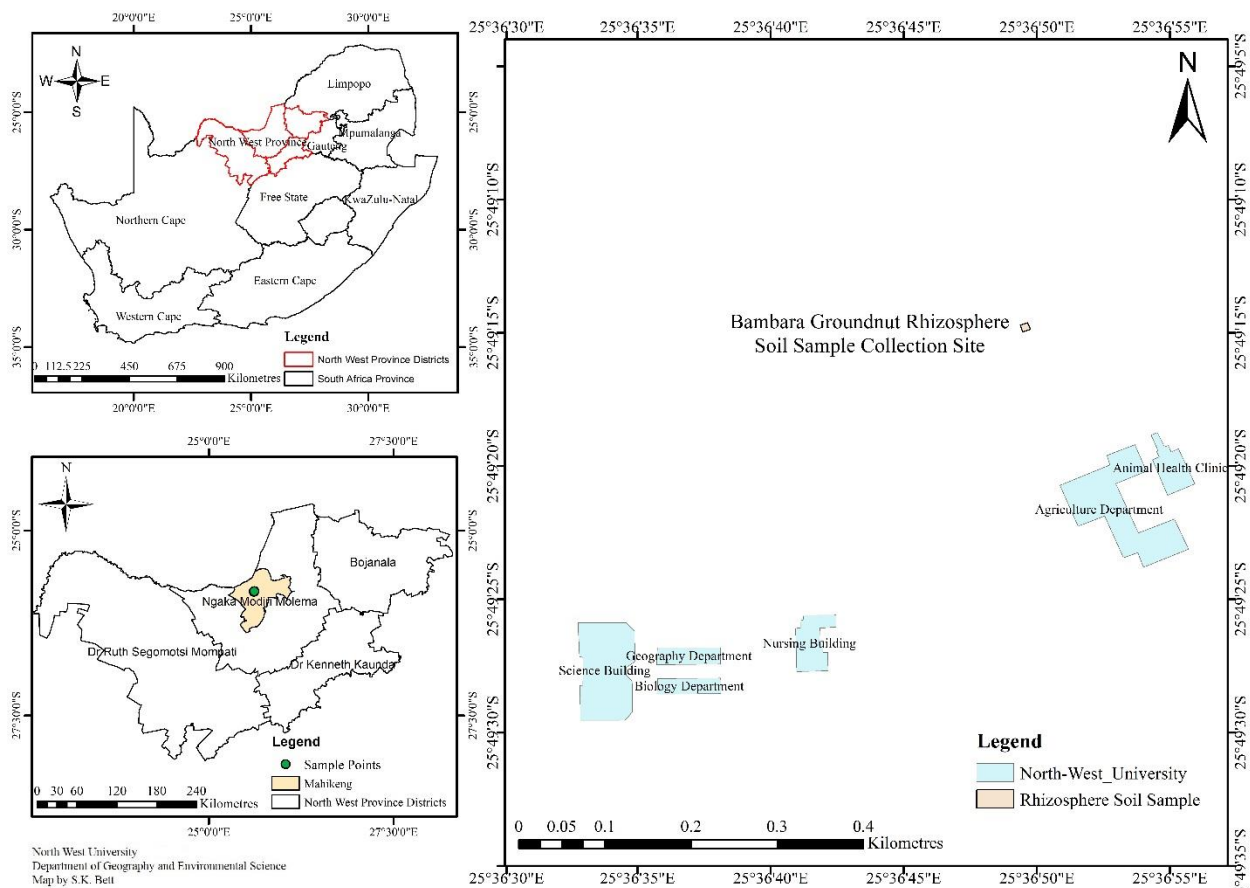


Fig. 6. 1 Geographical location of Bambara groundnut rhizospheric soil used for rhizobial species isolation. To the left, the upper sketch represents a map of South Africa showing North-West Province (red sketch) and below is a map of North-West Province showing a map of Mahikeng (the light-yellow region) which encompasses Ngaka Modiri Molema district (the

green spot) the site of North-West University where Bambara groundnut rhizospheric soil was collected for bacterial isolation. To the right, is a sketch showing Bambara groundnut rhizospheric soil sample collection site

6.1.2 ACC Activity Quantification

Rhizobial strains were grown in 5 ml Luria Bertani (LB) at ambient temperature. Then ACC deaminase activity was determined according to method described by Ali et al. (2014) as follows: “To induce ACC deaminase activity under non-stress and drought stress conditions, the cells were collected by centrifugation, washed twice with 0.1 M Tris-HCl (pH 7.5), suspended in 2 ml of modified DF minimal medium supplemented with 3 mM final concentration of ACC without PEG for non-stress condition and with PEG 6000 (-0.30 MPa) for drought stress respectively, and incubated at 28°C with shaking for another 36-72 h. ACC deaminase activity was determined by measuring the production of α -ketobutyrate and ammonia generated by the cleavage of ACC by ACC deaminase (Honma and Shimomura, 1978, Penrose and Glick, 2003). The induced bacterial cells were harvested by centrifugation at 3,000 *g* for 5 min, washed twice with 0.1 M Tris-HCl (pH 7.5), and re-suspended in 200 μ l of 0.1 M Tris-HCl (pH 8.5). The cells were labilized by adding 5% toluene (v/v) and then vortexed at the highest speed for 30 s. Fifty μ l (50 μ l) of labilized cell suspension was incubated with 5 μ l of 0.3M ACC in an Eppendorf tube at 28 °C for 30 min. The negative control for this assay included 50 μ l of labilized cell suspension without ACC, while the blank included 50 μ l of 0.1 M Tris- HCl (pH 8.5) with 5 μ l of 0.3 M ACC. The samples were then mixed thoroughly with 500 μ l of 0.56 N HCl by vortexing and the cell debris was removed by centrifugation at 12, 000 *g* for 5 min. A 500 μ l aliquot of the supernatant was transferred to a glass test tube and mixed with 400 μ l of 0.56N HCl and 150 μ l of DNF solution (0.1 g 2,4-dinitrophenylhydrazine in 100 ml of 2N HCl); and

the mixture was incubated at 28 °C for 30 minutes. One ml of 2N NaOH was added to the sample before the absorbance at 540 nm was measured. The concentration of α -ketobutyrate in each sample was determined by comparison with a standard curve generated as follows: 500 μ l α -ketobutyrate solutions of 0, 0.01, 0.05, 0.1, 0.2, 0.5, 0.75 and 1 mM were mixed respectively with 400 μ l of 0.56 N HCl and 150 μ l DNF solution. One ml of 2N NaOH was added and the absorbance at 540 nm was determined as described above. The values for absorbance versus α -ketobutyrate concentration (mM) were used to construct a standard curve”.

6.1.3 Exopolysaccharide Test

To start with, rhizobial strains were qualitatively screened for exopolysaccharide production according to the method described by Paulo et al. (2012) with little modifications. In brevity, sterile Whatman filter paper discs (6 mm in diameter) were aseptically placed in Petri dishes containing nutrient agar and 2 μ l of freshly grown cultures of each rhizobial species was directly inoculated on the surfaces of the discs in the plates. The nutrient agar used in this study was amended with 10% sucrose adjusted to pH of 5.5 and 7.5. Upon inoculation, plates were incubated at 28 $\pm$ 2 °C, 37°C and 45 °C for 7 days, 2 days and 1 day at the respective temperatures. Then, EPS production was evaluated on the basis of formation of mucoid colonies around the discs.

Alternatively, the quantity of EPS produced was determined according to the method described by Putrie et al. (2013) with little modifications. In summary, the four isolates were grown in nutrient broth amended with PEG (10% and 5%) and 0% PEG 800 to induce drought stress. Cultures were incubated in a rotary incubator at room temperature for 4 days and were thereafter centrifuged to obtain the supernatant. Three ml (3 ml) of cold absolute alcohol was mixed with 5 ml of each rhizobial supernatant and incubated for 12 hrs at 4 °C. Then EPS was

gotten by centrifuging the cold alcohol-rhizobial supernatant mixture at 10000 rpm for 15 min and the resultant supernatants were discarded. Then, the optical density of the EPS that settled at the bottom of the tubes were determined using spectrophotometer (ThermoSpectronic, Merck) at 490 nm.

6.1.4 Quantitative Determination of Siderophore Produced by Rhizobial Species

The quantitative determination of siderophore produced by rhizobial species was done according to the method described by Sasirekha and Srividya (2016) with little modifications. Briefly, freshly grown rhizobial species were inoculated into King B broth (10 g/l glycerine, 20 g/l peptone, 1.5 g/l MgSO_4) and iron-free succinic acid broth (6 g K_2HPO_4 , 3 g KH_2PO_4 , 1 g $(\text{NH}_4)_2\text{SO}_4$, 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 4 g succinic acid) in tubes while controls were amended with chrome azurol S (CAS) solution and incubated ambient temperature in a shaking incubator at 120 rpm. Rhizobial broths were centrifuged at 10000 rpm for 10 min. The quantity of siderophore produced was assessed by measuring the optical density of the supernatant at 400 nm.

6.1.5 Indole-Acetic-Acid (IAA) Test

Quantitative determination of IAA produced by rhizobial species were performed according to the method described by Ahmad et al. (2008) with little modifications. In summary, each rhizobial strain was inoculated in 0.2 L LB broth and incubated in a rotary shaker at ambient temperature for 96 hrs. One ml (1 ml) of the rhizobial broth was centrifuged at 3000 rev/min for 30 min and thereafter, 2 ml of the supernatant was mixed with 2 drops of orthophosphoric acid and 4 ml Salkowski reagent. Optical density of the pink broth was taken at 530 nm using a spectrophotometer (ThermoSpectronic, Merck) and the actual concentration of

IAA produced by rhizobial was estimated from a standard IAA curve in the range of 0 – 120 µg/ml.

6.1.6 Phosphate Solubilization Test

Phosphate solubilization test was determined as described by Gaur (1990) with little modifications. Pikovskaya's agar with the following composition per litre was prepared: tricalcium phosphate (5 g), potassium chloride (0.2 g), magnesium sulphate (0.1 g), manganese sulphate (0.0001 g), yeast extract (0.5 g), glucose (10 g), agar (15 g), ammonia sulphate (0.5 g), ferrous sulphate (0.001 g) and the medium was autoclaved at 121 °C for 15 min after adjusting the pH of the final composition to pH 7.0. Autoclaved medium was poured on Petri dishes and allowed to solidify. Wells of 8 mm in diameter were made in the medium and inoculated with 25 µl of broth culture of each isolates. Three (3) wells per isolate were used. Plates were incubated at 27°C for 4 days and cleared zones around the wells indicated positive result. Diameters of zones were obtained by measuring the diameter of zone of inhibition minus the diameter of the wells.

6.1.7 Rhizobial Growth Response to Different Temperature

LB broth was prepared according to the manufacturer's guidelines and autoclaved. Five microliters of each rhizobial strain was inoculated in 25 ml of LB broth and gently vortexed. Each rhizobial treatment was replicated 3 times for the different temperatures. Inoculated broth was incubated at 28, 35 and 45°C. The OD of the rhizobial growths was taken using a spectrophotometer at 630 nm at day 4, 8, 12, 16 and 20.

6.1.8 Rhizobial Growth Response to Different Alkaline Environments

LB broth was prepared according to manufacturer's guidelines and the pH of the broth was adjusted to acidic (4), neutral (7) and alkaline (10) pH and autoclaved. Five microliters of each rhizobial strain was inoculated in 25 ml of LB broth and gently vortexed. Each rhizobial treatment was replicated 3 times. Inoculated broth was incubated at 28 °C. The OD of the rhizobial growths was taken using a spectrophotometer at 630 nm at day 5, 10, 15 and 20.

6.1.9 Bacterial Growth and Preparation

Three (3) of the rhizobial strains were selected for soybean inoculation. Rhizobial spp. were harvested as described by Prakamhang et al. (2015) with little modifications. Freshly grown culture of the rhizobial spp. were centrifuged at 5000 rpm for 300 s and the pellets were washed in 0.85% (w/v) normal saline solution and thereafter homogenized in saline solution prior to solution.

6.1.10 Seed Germination Test

The colony counts of the rhizobial spp. were 20×10^5 CFU (colony forming unit) ml^{-1} (for R1 strain), 11×10^5 CFU ml^{-1} (for R3 strain) and 21×10^5 CFU ml^{-1} (for R5 strain). Rhizobial suspension (0.5 ml) of each strain was pipetted into Petri dishes containing Whatman filter paper while 0.5 ml of sterile distilled water was transferred to the non-inoculated (control) plates. Soybean seeds (PAN 1532 R) obtained from Agricultural Research Council, SA were surface sterilized in 75% alcohol and 1% sodium hypochlorite for 600 s and rinsed in sterile distilled water. Then 30 seeds were placed in the Petri dishes containing inoculated filter papers and 4% PEG and plates were gently swirled. Each treatment was done in triplicate. Parafilm was used to seal the plates incubated for 8 days in a growth chamber (GC-300TL, JEIO TECH, Korea) adjusted to 23/16 day/night for periods of 8/16 hours night/day at 10,000 light lux for 8

days. The number of germinated seeds was counted afterwards and percentage seed germination rate was estimated using the following formula:

$$\text{Percentage seed germination (\%)} = \frac{n}{N} \times 100$$

Where n is the number of germinated seeds after 8 days and N is the total of seeds.

6.1.11 DNA Extraction for Whole Genome Sequencing

Fresh cultures of *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 were obtained by taking inocula from 50% glycerol and streaking on freshly prepared nutrient agar. Plates were incubated at 28°C for 4 days and thereafter bacterial DNA was extracted from the fresh isolates using Zymo DNA extraction kit following manufacturer's instructions. The success and relative band thickness of the extracted DNA was checked in 1% agarose. Again, DNA concentration was evaluated using a NanoDrop spectrophotometer (Thermo Scientific, NANODROP LITE Spectrophotometer) and the concentration of the DNA was found to be 40 ng/μl (for R1 strain) and 45 ng/μl (for R3 strain). The DNA extracts were store at -20°C until use. Afterwards, 40 μl of DNA of extract of each bacterium was sent in an ice pack to Molecular Research Laboratory (Mr. DNA), Texas, USA for HiSeq system (illumina) sequencing.

6.1.12 Sequencing, Quality Check, Trimming and Assembly

Following manufacturer's instructions, DNA libraries were made from 25-50 ng of extracted DNA using KAPA HyperPlus kits (Roche). Upon library preparation, DNA concentration was determined using the Qubit® dsDNA HS Assay Kit (Life Technologies) and average library size was evaluated using Agilent 2100 Bioanalyzer (Agilent Technologies). 'The workflow combines enzymatic steps and employs minimal bead-based cleanups'. DNA samples were enzymatically degraded into ds DNA fragments and thereafter end repair cum A-tailing

were performed to obtain ‘end-repaired, 5'-phosphorylated, 3'-dA-tailed ds DNA fragments.’ Adapter ligation was performed by ligating ds DNA adapters with 3'-dTTP overhangs to 3'-dA-tailed DNA molecules and thereafter, DNA libraries amplification were performed by using high fidelity and low-bias PCR. The DNA libraries were then assembled and diluted to 10.5pM and ‘sequenced paired end for 500 cycles using the HiSeq system (Illumina).

Illumina data were extracted and uploaded into Kbase, reads quality was done by performing quality check of the illumina sequence using FastQC (v1.0.4) and low quality sequence and adapter were trimmed off using trimmomatic (Bolger et al., 2014). Illumina sequence reads were *de novo* assembled using both SPAdes and ARAST to create contigs.

6.1.13 Annotation

The genomes of R1 and R3 strains were annotated using Kbase Prokka (V1.12) annotation pipeline and rapid annotation using subsystem technology (RAST) server (Aziz et al., 2008). The aforementioned systems permit the identification of introns, functional annotations as well as ‘manual curation of gene annotations’. They also possess platforms for metabolic construction with the aid of Kyoto encyclopedia of genes and genomes (KEGG) for comparing sequences using BLAST and functional comparisons using KEGG and/or FIGfam. The data for R1 and R3 were both given Bioproject number PRJNA496421 while R1 and R3 data were assigned Biosample numbers SAMN10240937 and SAMN10245972 respectively upon submission to the GenBank database. In addition, R1 has SRA Accession number: SRR8060784 and R3 has SRA Accession number: SRR8061690.

6.1.14 Statistical Analyses

Data obtained for the plant growth promoting and seed germination tests were analyzed using Microsoft Excel and SAS platforms. Analysis of Variance (ANOVA) was performed for

the data followed by Duncan test to determine differences between mean and $P < 0.05$ was considered significant (Igiehon, 2015, Dytham, 2011). With respect to sequenced data, mean read length and standard deviation of read length were computed using Kbase pipeline.

6.2 Results

6.2.1 Plant Growth Promoting Traits of Rhizobial Species

In this study, all rhizobial species produced EPS. In particular, the rhizobial species incubated at 37°C produced EPS at pH 5.5 and 7.5 but R1 and R4 strains did not produce EPS when incubated at 45°C, while R2 and R3 strains produced EPS under all the environmental conditions considered (Table 1).

Table 6. 1 Qualitative response of bacteria towards exopolysaccharide (EPS) assay

Bacterial strain	Response at pH 5.5			Response at pH 7.5		
	28°C ±2	37°C	45°C	28°C ±2	37°C	45°C
R1	+	+	-	+	++	-
R2	+	++	+	+	++	++
R3	+	++	+	+	++	++
R4	+	++	-	+	++	++
R5	ND	++	+	ND	+	++

Legend: + = positive, - = negative, + + = strongly positive, ND = not determined. R1 - *Rhizobium* sp. strain R1, R2 - *Rhizobium tropici* strain R2, R3 - *Rhizobium cellulosilyticum* strain R3, R4 - *Rhizobium taibaishanense* strain R4 and R5 - *Sinorhizobium meliloti* strain R5

6.2.2 1-aminocyclopropane-1-carboxylate (ACC) production by Rhizobial Species

With regard to ACC production, R5 strain produced the highest concentration of ACC followed by R1 strain while R2 strain produced the lowest concentration of ACC (Fig. 2) under stress condition imposed by PEG. These rhizobial strains were further screened for other plant growth promoting traits such as EPS, siderophore production, IAA and phosphate solubilization.

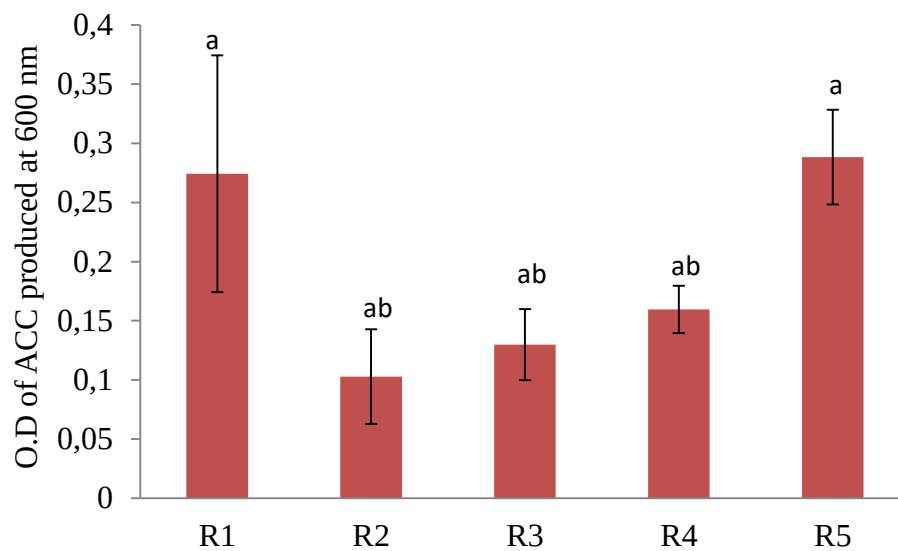


Fig. 6. 2 The concentration of ACC produced by rhizobial species under drought stress induced by polyethylene glycol (PEG). R1 - *Rhizobium* sp. strain R1, R2 - *Rhizobium tropici* strain R2, R3 - *Rhizobium cellulosilyticum* strain R3, R4 - *Rhizobium taibaishanense* strain R4 and R5 - *Sinorhizobium meliloti* strain R5. ACC=1-aminocyclopropane-1-carboxylate. Number of replicates (n) = 3. Data represent mean $\pm$ SE

6.2.3 EPS Production by Rhizobial Species

Similarly, the ability of rhizobial strains to produced polysaccharides such as EPS under osmotic stress conditions induced by PEG was tested and it was observed that all the rhizobial strains produced EPS but at varying concentrations. To be specific, among the rhizobial treatments, R1 strain produced the highest concentrations (0.7 and 0.6 O.D respectively) of EPS at 0 and 10% PEG concentrations and R2 produced the highest EPS at 5% PEG concentration (Fig. 3) followed by R1 and R3 strains.

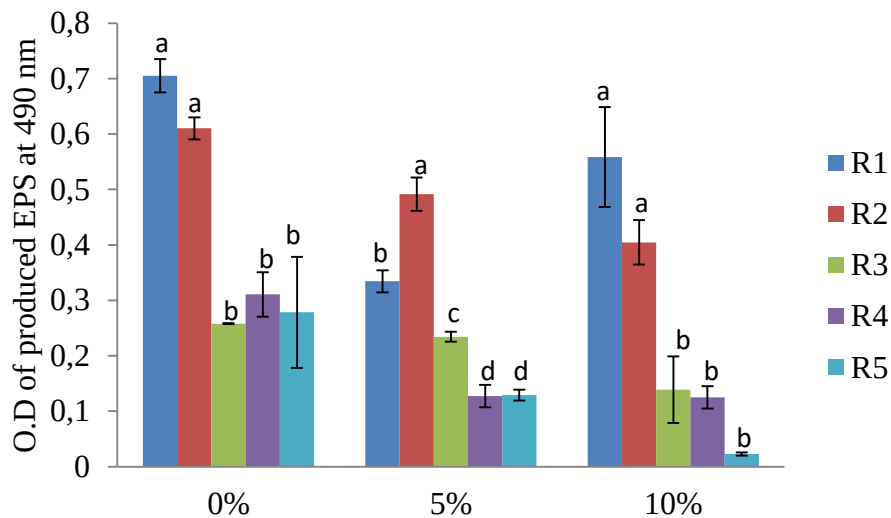


Fig. 6. 3 The concentration of EPS produced by rhizobial species under drought stress induced by polyethylene glycol (PEG). R1 - *Rhizobium* sp. strain R1, R2 - *Rhizobium tropici* strain R2, R3 - *Rhizobium cellulosilyticum* strain R3, R4 - *Rhizobium taibaishanense* strain R4 and R5 - *Sinorhizobium meliloti* strain R5. EPS – exopolysaccharide. Number of replicates (n) = 3. Data represent mean $\pm$ SE

6.2.4 Siderophore Production by Rhizobial Species

The ability of rhizobial species to produce siderophore in different media (succinic acid broth and King B broth) showed that all the rhizobial species produced more siderophore in King B broth compared to succinic acid broth (Fig. 4). R1 strain obviously produced the highest concentration of 0.9 O.D in King B broth while R1 and R2 strains produced more siderophore than R4 and R5 strains in succinic acid broth (Fig. 4). Conversely, the control treatments amended with CAS solution showed the lowest values for both media.

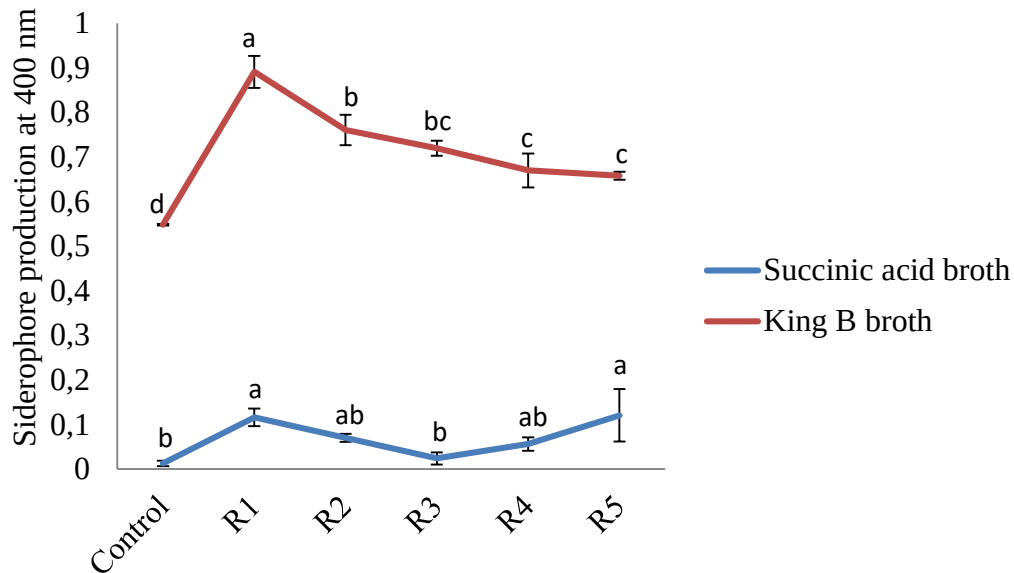


Fig. 6. 4 The concentration of siderophore produced by rhizobial species under drought stress induced by polyethylene glycol (PEG). R1 - *Rhizobium* sp. strain R1, R2 - *Rhizobium tropici* strain R2, R3 - *Rhizobium cellulosilyticum* strain R3, R4 - *Rhizobium taibaishanense* strain R4 and R5 - *Sinorhizobium meliloti* strain R5. Number of replicates (n) = 3. Data represent mean $\pm$ SE

6.2.5 IAA Production by Rhizobial Species

The ability of rhizobial species to produce IAA under different tryptophan concentrations revealed higher concentrations of IAA production by R1 and R3 strains. In particular, R3 strain produced the highest concentrations of IAA (22.19 and 23.155 μ l) at 0.5 and 1 mg/ml of tryptophan respectively followed by R1 strain but the lowest concentrations were produced by R5 strain.

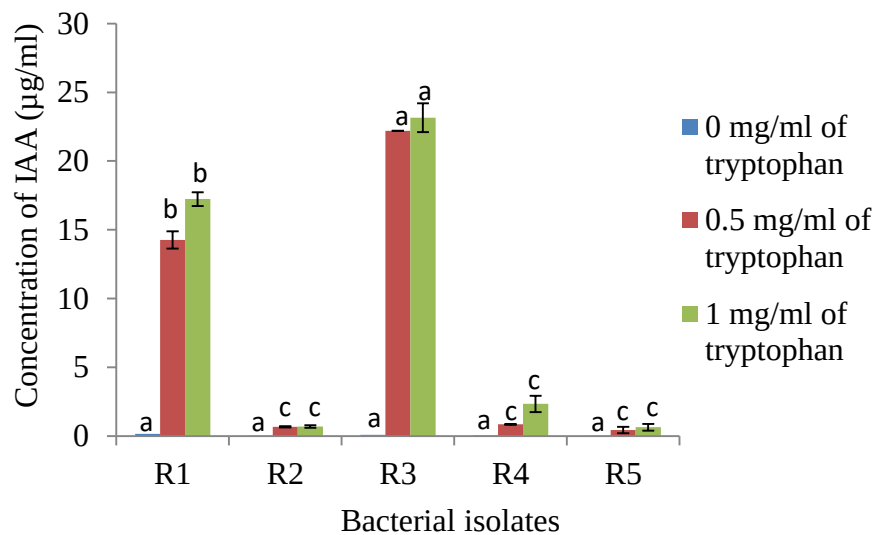


Fig. 6. 5 The concentration of IAA produced by rhizobial species. R1 - *Rhizobium* sp. strain R1, R2 - *Rhizobium tropici* strain R2, R3 - *Rhizobium cellulosilyticum* strain R3, R4 - *Rhizobium taibaishanense* strain R4 and R5 - *Sinorhizobium meliloti* strain R5. Number of replicates (n) = 3. IAA = Indole-acetic-acid. Data represent mean $\pm$ SE

6.2.6 Phosphate Solubilization by Rhizobial Species

As regards phosphate solubilization, R2 strain comparatively showed bigger halo-zone in Pikovskaya's agar with a mean diameter of 17.3 mm while R1 strain showed a mean diameter of 16.7 mm. The diameter of the halo-zone produced by R5 strain was lowest in this study (with a mean value of 10.7 mm). Nevertheless, R3 and R4 strains produced halo zones with the same diameter (13.7 mm) (Fig. 6).

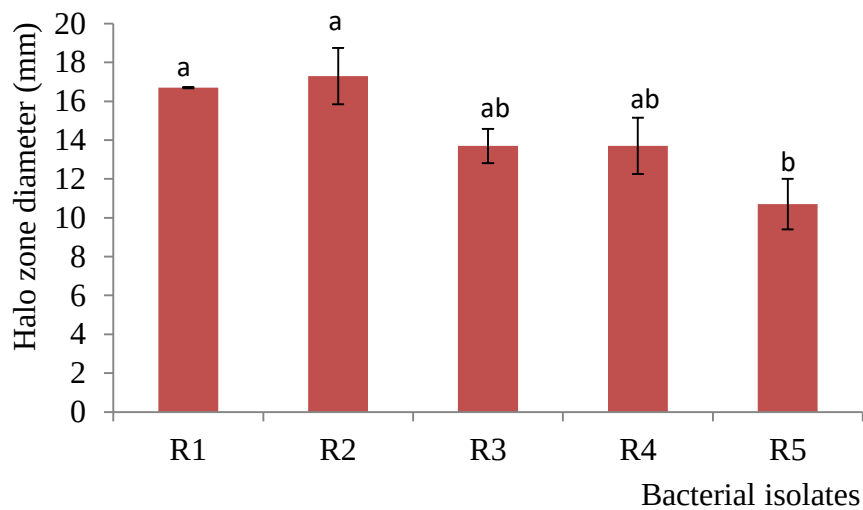


Fig. 6. 6 Diameter of clear (halo) zones produced by phosphate solubilizing rhizobial species.

R1 - *Rhizobium* sp. strain R1, R2 - *Rhizobium tropici* strain R2, R3 - *Rhizobium cellulosilyticum* strain R3, R4 - *Rhizobium taibaishanense* strain R4 and R5 - *Sinorhizobium meliloti* strain R5.

Number of replicates (n) = 3. Data represent mean $\pm$ SE

6.2.7 Rhizobial Growth Response under Environments with different Temperatures

Considering the response of rhizobial species towards different environmental temperatures, we observed that R1 strain showed the highest growth at 28°C as depicted by O.D values-throughout the experimental period. However, at 45°C, rhizobial growth response modulates throughout the experimental period. As an illustration, R1 strain showed the highest O.D values of 0.4 and 0.3 on day 4 and 8 respectively but R5 and R4 strains showed the highest growths of 0.05 and 0.7 O.D corresponding to day 12 and 16 while R2 strain had the highest O.D value of 0.98 on day 20. The same pattern of rhizobial growths was observed at 37°C (Fig 7 a, b, c, d and e).

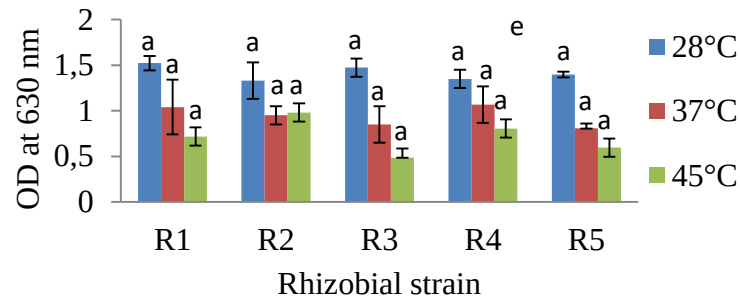
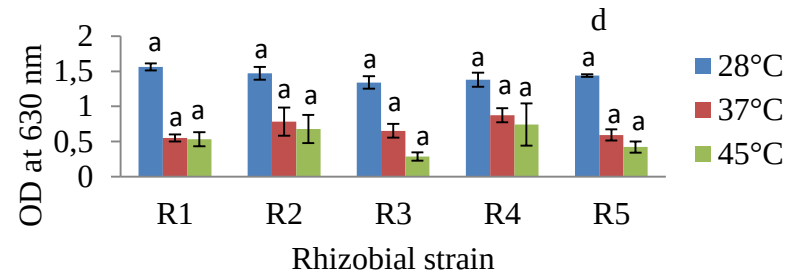
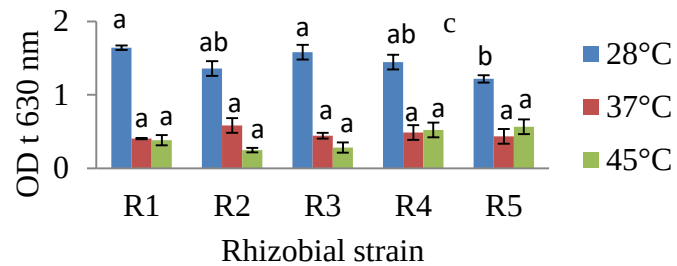
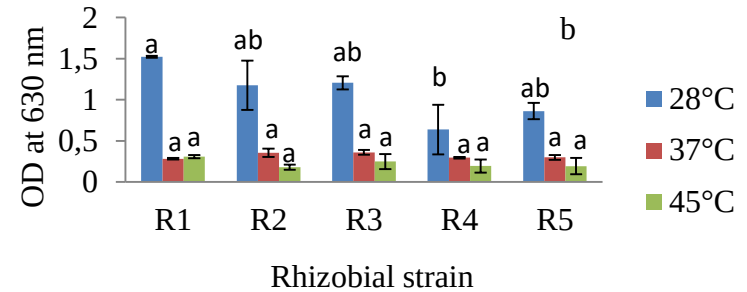
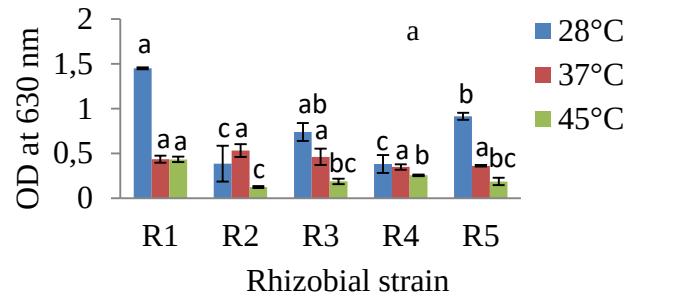


Fig. 6. 7 Bacterial growth response to different environmental temperatures on day (a) 4, (b) 8, (c) 12, (d) 16 and (e) 20. R1 - *Rhizobium* sp. strain R1, R2 - *Rhizobium tropici* strain R2, R3 - *Rhizobium cellulosilyticum* strain R3, R4 - *Rhizobium taibaishanense* strain R4 and R5 - *Sinorhizobium meliloti* strain R5. Number of replicates (n) = 3. Data represent mean $\pm$ SE

6.2.8 Rhizobial Growth Response under Environments with different pH

Furthermore, R1 strain tended to show better growth at a pH of 4 at the onset of rhizobial growth response to pH experiment (Fig. 8a) and later decreased as the experiment progressed. However, R1 strain responded more positively at pH 7 throughout the experimental sampling period (Fig. 8a,b,c and d) but R3 grew better at pH 10 on day 5 (Fig. 8a) while R5 strain was more abundant (with a cell biomass of 0.79 O.D) on day 20 this pH (Fig. 8d)

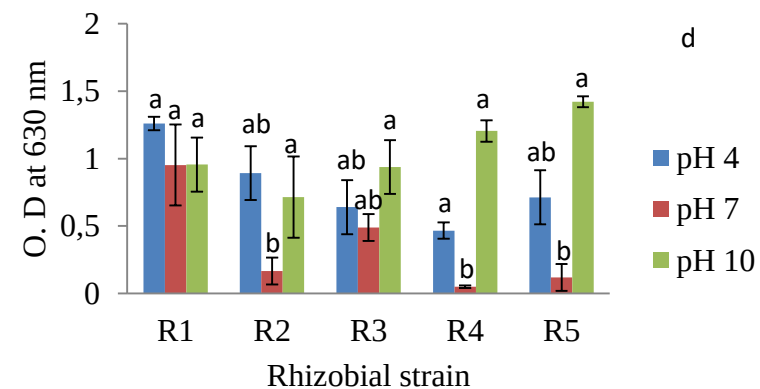
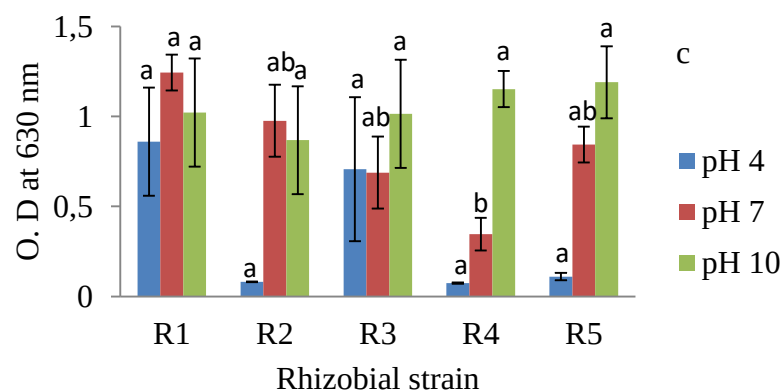
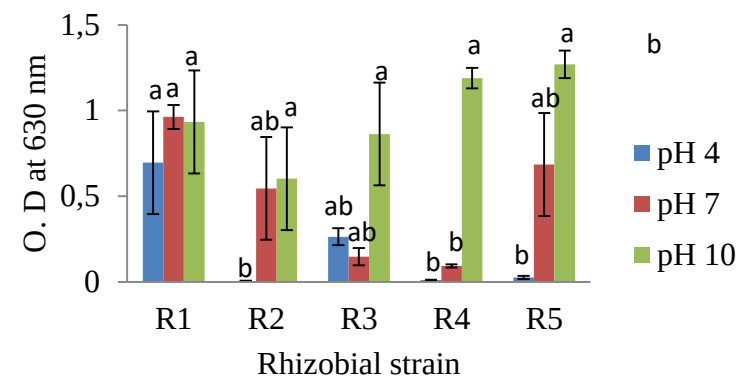
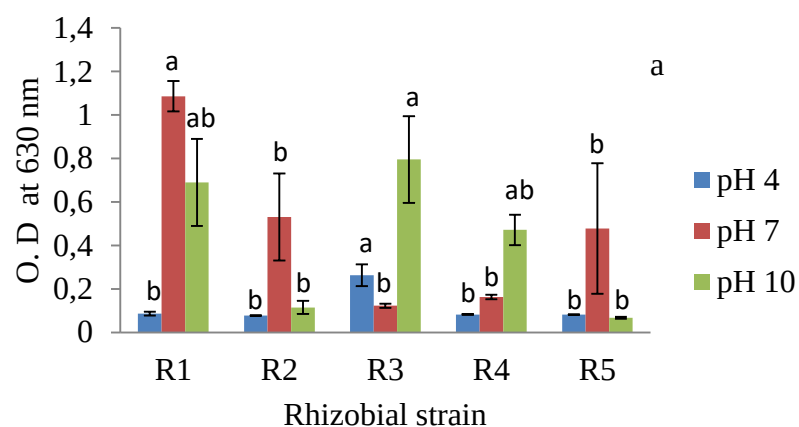


Fig. 6. 8 Rhizobial growth response to different environmental pH on day (a) 5, (b) 10, (c) 15 and (d) 20. R1 - *Rhizobium* sp. strain R1, R2 - *Rhizobium tropici* strain R2, R3 - *Rhizobium cellulosilyticum* strain R3, R4 - *Rhizobium taibaishanense* strain R4 and R5 - *Sinorhizobium meliloti* strain R5. Number of replicates (n) = 3. Data represent mean $\pm$ SE

6.2.9 Soybean Seed Germination

The effects of R1, R3 and R5 inoculation on soybean seeds germination under drought stressed condition imposed by 4% PEG revealed that R1 and R3 strains had a better effect on percentage soybean seed germination when compared to R5 strain. However, the non-inoculated (control) experiment showed the lowest percentage seed germination (Fig. 9). Thus, whole genome sequencing was performed for R1 and R3 strains in order to gain genomic insights into some of the functional genes that may be involved in drought tolerance, symbiotic establishment as well as plant survival and growth promotion.

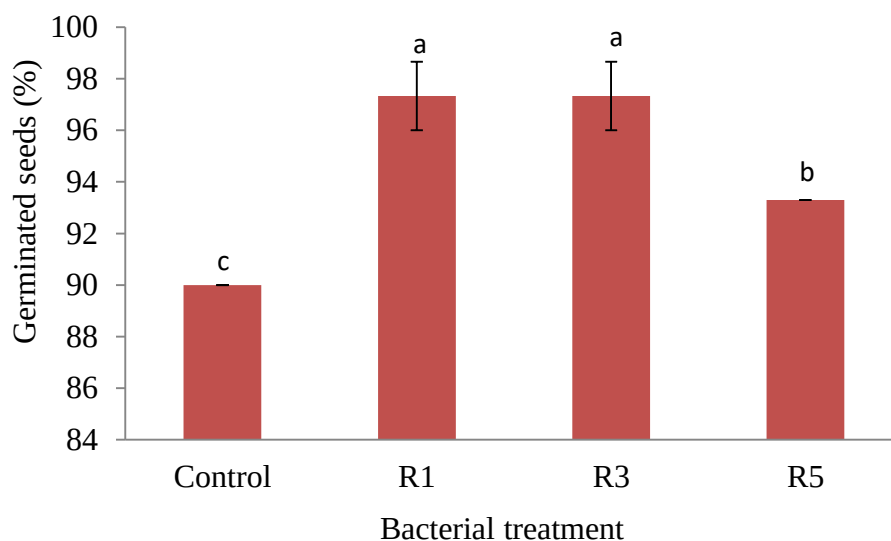


Fig. 6. 9 Percentage of soybean seeds inoculated with rhizobial species that germinated in Petri dishes. R1 - *Rhizobium* sp. strain R1, R3 - *Rhizobium cellulosilyticum* strain R3 and R5 - *Sinorhizobium meliloti* strain R5. Number of replicates (n) = 4. Data represent mean $\pm$ SE

6.2.10 Genomic Overview of R1 and R3 strains

Prior to illumina sequencing, DNA concentration of 35.6 ng/μL, DNA library concentration of 81.60 ng/μL with an average size of 647 bp were generated for R1 strain while R3 strain yielded 50.2 ng/μL, 90.40 and 661 bp corresponding to DNA concentration, final DNA library concentration and average library size (Table 2).

Table 6. 2 DNA final library concentration and average library size

Rhizobial species	DNA concentration (ng/μL)	Final DNA library concentration (ng/μL)	Average Library size (bp)
R1	35.6	81.60	647
R3	50.2	90.40	661

Legend: R1 - *Rhizobium* sp. strain R1 and R3 - *Rhizobium cellulosilyticum* strain

Upon *de novo* assembly, R1 strain was found to have 17,408,810 reads with a mean length of 201.15 and a total of 5773 contigs. The number of genes predicted was 29842 with a GC content of 61.91%. The N₅₀ value of 936 was obtained for the scaffold. On the other hand, R3 strain had 17,794,094 reads with a mean read length of 214.18 and 129 contigs. The genome size of the strain was 4,114,542 with GC content off 43.59%. The N₅₀ value of 57294 was obtained for the scaffold.

6.2.11 EPS Producing Genes

Whole genome sequencing revealed 78 *exoX* genes in R1 strain and 99 *exoX* genes in R3 strain and these genes are responsible for the production of exopolysaccharide in the bacterial species. Two (2) of the 78 *exoX* genes found in R1 strain code for signal transduction histidine-

protein kinase BaeS and exodeoxyribonuclease III proteins with the corresponding baeS_1, 2.7.13.3 and xthA 3.1.11.2 aliases (Table. 3). The location of the signal transduction histidine-protein kinase gene was between 474 – 762 contigs (Fig. 10 a) and that of BaeS and exodeoxyribonuclease III proteins gene was between 47 – 389 contigs (Fig. 10 b). On the other hand, of the 99 *exoX* genes found in R3 strain, 2 encode signal transduction histidine-protein kinase ArlS and response regulator aspartate phosphatase J with arlS 2.7.13.3 and rapJ_2 3.1 aliases respectively (Table 4). Also, the location of the signal transduction histidine-protein kinase ArlS gene was between 3,808 – 5,173 contigs (Fig. 12 a) while that of response regulator aspartate phosphatase J gene was between 26,133 – 27,255 contigs (Fig. 12 b).

6.2.12 High-Temperature Stress Response Genes

Again, 5 *htrA* and 6 *htrA* genes were found in R1 and R3 strains. *HtrA* genes are involved in tolerance to high temperature and therefore the survival and growth of R1 and R3 strains observed at 45°C (Fig. 7 a, b, c, d, e) may be due to the high temperature tolerant proteins produced by these microorganisms (Table 3 and 4). Notably, 2 of the *htrA* genes found in R1 strain are responsible for the production of extracellular serine protease and microbial serine proteinase (Table 3) and they were located between 16 – 271 and 1,372 – 2,164 contigs respectively (Fig. 10 c, d). In the same way, R3 strain had 2 genes coding for serine protease Do-like HtrA (Table 4) but with different contigs locations (Fig. 12 c, d).

6.2.13 Nitrogen Fixing Genes

Nitrogen fixing (*nif*) genes are involved in the conversion of atmospheric N to the form that can be utilized by plants. Two (2) of the *nif* genes noticed in R1 strain are involved in the production of cysteine desulfurase SufS and cysteine desulfurase IscS with the corresponding sufS 2.8.1.7 and iscS 2.8.1.7 aliases (Table 3). The locations of these genes were between 11 –

338 contigs for cysteine desulfurase SufS gene and 1,202 – 1,664 (Fig. 10 e) for cysteine desulfurase IscS gene (Fig. 10 f). Regarding R3 strain, 2 of its *nif* genes are involved in the production of cysteine desulfurase IscS and Putative cysteine desulfurase NifS proteins (Table 4). These protein producing genes with different aliases also had different contigs locations (Fig. 12 e, f).

6.2.14 Nodulation Genes

Nodulation genes play a key role in nodule formation in plant roots where *Rhizobium* species establish symbiosis with host plants. As for R1 strain, 23297 *nodA* genes were found and 2 of the genes possess putative MFS-type transporter YcaD and riboflavin transporter protein potential (Table 3) with contigs locations between 93 – 735 and 734 - 1,091 respectively (Fig. 10 g, h). On the contrary, 2 of the 12242 genes found in R3 strain code for Beta-N acetylglucosaminidase and Teichoic acid poly (ribitol-phosphate) polymerase situated between 4,582 – 7225 and 734 - 1,091 contigs respectively (Fig. 12 g, h).

6.2.15 Siderophore-Producing Genes

At the same time, R1 strain was found to have 13 siderophore-producing genes and 2 of the genes evidently produce Catecholate siderophore Receptor Fiu and 2, 3-dihydro-2,3 dihydroxybenzoate dehydrogenase. The Catecholate siderophore receptor Fiu gene had *fiu* aliases while 2, 3-dihydro-2, 3 dihydroxybenzoate dehydrogenase gene had *dhbA* 1.3.1.28 aliases (Table 3) situated between contigs 662 - 1,424 (Fig. 11 a) and 59 – 572 (Fig. 11 b) within the genome. With respect to R3 strain, it had 33 siderophore-producing genes and 2 of the genes and their respective contigs locations within the genome are shown in Table 4 and Fig. 13 a, b accordingly.

6.2.16 IAA Producing Genes

Unlike R3 strain, 1 CDS and 1 IAA producing-gene were found in R1 strain with the biological function of Isoaspartyl peptidase (Table 3) and they were both located within 536 – 704 contigs (Fig. 11 c, d). But, R3 strain had 6 IAA-producing genes and 2 of the genes produce inner membrane protein YiaA and tRNA dimethylallyltransferase proteins with the corresponding yiaA and miaA 2.5.1.75 aliases. The locations of their respective gene were also different within the genome (Fig. 13 c, d). In reality, proteins produced by these genes can be involved in root elongation and lateral root production in plants.

6.2.17 Low-pH Stress Response Genes

As a matter of fact, R1 and R3 strains were also found to possess genes that are involved in tolerance to low pH environments and one of them reported in this study is collectively called *eptA*. Indeed, R1 strain had 12 *eptA* genes and 2 of the genes were found to have the biological functions shown in Table 3. But, R3 strain also had several of the *eptA* genes and 2 of the genes had heptaprenyl diphosphate synthase component 1 and septation ring formation regulator EzrA biological functions (Table 4). These genes were found to be located within different contigs locations in the genome of R1 (Fig. 11 e, f) and R3 (Fig. 13 e, f) strains.

Online data availability

R1 strain: Bioproject number PRJNA496421, Biosample number SAMN10240937, SRA Accession number: SRR8060784.

R3 strain: Bioproject number PRJNA496421, Biosample number SAMN10245972, SRA Accession number: SRR8061690.

Table 6. 3 Selected stress tolerance, symbiotic and plant growth promoting functional genes found in the genome of *Rhizobium* sp.

strain R1

Feature ID	Type	Function	Aliases	Start	length	Location
Exo X						
JKFNCFJO_00944	Gene	Signal transduction histidine-protein kinase BaeS	baeS_1, 2.7.13.3	762	288	Contig: NODE_1409_length_750_cov_2.449333 474 - 762 (- Strand)
JKFNCFJO_01811	Gene	Exodeoxyribonuclease III	xthA, 3.1.11.2	47	342	Contig:NODE_2692_length_718_cov_3.898329 47 - 389 (+ Strand)
HtrA						
JKFNCFJO_06099	Gene	Extracellular serine protease	3.4.21.-	16	255	Contig: NODE_11432_length_1141_cov_2.421560 16 – 271 (+Strand)
JKFNCFJO_02038	gene	Microbial serine proteinase	aspA, 3.4.21.-	2164	792	Contig:NODE_3069_length_2224_cov_3.164568 1,372 – 2,164 (-strand)
Nif genes						
JKFNCFJO_00231	gene	Cysteine desulfurase SufS	sufS, 2.8.1.7	11	327	Contig: NODE_344_length_1119_cov_2.287757 11 – 338 (+Strand)
JKFNCFJO_04114	gene	Cysteine desulfurase IscS	iscS, 2.8.1.7	1,664	462	Contig: NODE_6900_length_1667_cov_2.808638 1,202 – 1,664 (-Strand)
Nod A						
JKFNCFJO_00001	gene	putative MFS-type transporter YcaD	ycaD_1	93	642	Contig: NODE_1_length_1071_cov_2.464052 93 – 735 (+Strand)
JKFNCFJO_00002	gene	Riboflavin transporter	ribN	1,091	357	Contig: NODE_1_length_1071_cov_2.464052 734 - 1,091 (- Strand)
Siderophore						
JKFNCFJO_00628	gene	Catecholate siderophore Receptor Fiu	fiu	1,424	762	Contig: NODE_980_length_1476_cov_2.882791

						662 - 1,424 (- Strand)
JKFNCFJO_07774	gene	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase	dhbA, 1.3.1.28	59	513	Contig: NODE_18194_length_629_cov_2.839428 59 - 572 (+ Strand)
IAA						
JKFNCFJO_03956_CDS	CDS	Isoaspartyl peptidase	iaaA, 3.4.19.5	704	168	Contig: NODE_6586_length_704_cov_2.566761 536 - 704 (- Strand)
JKFNCFJO_03956	gene	Isoaspartyl peptidase	iaaA, 3.4.19.5	704	168	Contig: NODE_6586_length_704_cov_2.566761 536 – 704 (-Strand)
EptA gene						
JKFNCFJO_06547	gene	UDP-N-acetylmuramate--L-alanyl-gamma-D-glutamyl-meso-2,6-diaminoheptandioate ligase	mpl, 6.3.2.45	782	1,365	Contig: NODE_12707_length_2871_cov_2.986416 782 - 2,147 (+ Strand)
JKFNCFJO_05747	gene	Phosphoethanolamine transferase EptA	eptA_3, 2.7.-.-	42	534	Contig: NODE_10489_length_1026_cov_2.685185 42 – 576 (+Strand)

Table 6. 4 Selected stress tolerance, symbiotic and plant growth promoting functional genes found in the genome of *R.*

cellulosilyticum strain R3

Feature ID	Type	Function	Aliases	Start	length	Location
Exo X gene						
LKJIOFBO_00637	gene	Signal transduction histidine-protein kinase ArlS	arlS, 2.7.13.3	5,173	1,365	NODE_12_length_14500_cov_30.995518 3,808 – 5,173 (-Strand)
LKJIOFBO_01604	gene	Response regulator aspartate phosphatase J	rapJ_2, 3.1.-.-	26,133	1,122	NODE_32_length_40656_cov_31.105864 26,133 – 27,255 (+Strand)
htrA						
LKJIOFBO_03117	gene	Serine protease Do-like HtrA	htrA_1, 3.4.21.107	6,151	1,203	Contig:NODE_64_length_16935_cov_30.627930 6,151 - 7,354 (+ Strand)
LKJIOFBO_04119	gene	Serine protease Do-like HtrA	htrA_2, 3.4.21.107	24,021	1,362	Contig:NODE_203_length_47982_cov_29.809929 24,021 - 25,383 (+ Strand)
Nif gene						
LKJIOFBO_00593	Gene	Cysteine desulfurase IscS	iscS_2, 2.8.1.7	40,060	1,146	NODE_11_length_78586_cov_30.601507
LKJIOFBO_01424_CDS	CDS	Putative cysteine desulfurase NifS	nifS, 2.8.1.7	72	264	NODE_29_length_80092_cov_31.155697
Nod A						
LKJIOFBO_00005	gene	Beta-N acetylglucosaminidase	lytD, 3.2.1.96	4,582	2,643	Contig: NODE_1_length_64013_cov_32.129894 4,582 – 7225 (+Strand)
LKJIOFBO_00006	gene	Teichoic acid poly (ribitol-phosphate) polymerase	tarL, 2.7.8.-			Contig: NODE_1_length_64013_cov_32.129894 7,268 - 9,128 (- Strand)
Siderophore						
LKJIOFBO_02039	gene	putative siderophore transport system permease protein YfiZ	yfiZ_1	14,769	1,002	Contig: NODE_38_length_39997_cov_29.245344 14,769 - 15,771 (+ Strand)

LKJIOFBO_02038	gene	putative siderophore-binding lipoprotein YfiY	yfiY	14,769	978	Contig: NODE_38_length_39997_cov_29.245344 13,660 - 14,638 (- Strand)
IAA						
LKJIOFBO_01515	gene	Inner membrane protein YiaA	yiaA	11,965	264	Contig: NODE_30_length_44008_cov_32.195396 11,701 - 11,965 (- Strand)
LKJIOFBO_02665	gene	tRNA dimethylallyltransferase	miaA, 2.5.1.75	10,955	945	Contig: NODE_48_length_27057_cov_29.404369
EptA						
LKJIOFBO_03929	gene	Heptaprenyl diphosphate synthase component 1	hepS, 2.5.1.30	8,080	765	Contig: NODE_102_length_18176_cov_29.706535 7,315 - 8,080 (- Strand)
LKJIOFBO_00595	gene	Septation ring formation regulator EzrA	ezrA	43,376	1,689	Contig: NODE_11_length_78586_cov_30.601507 41,687 - 43,376 (- Strand)

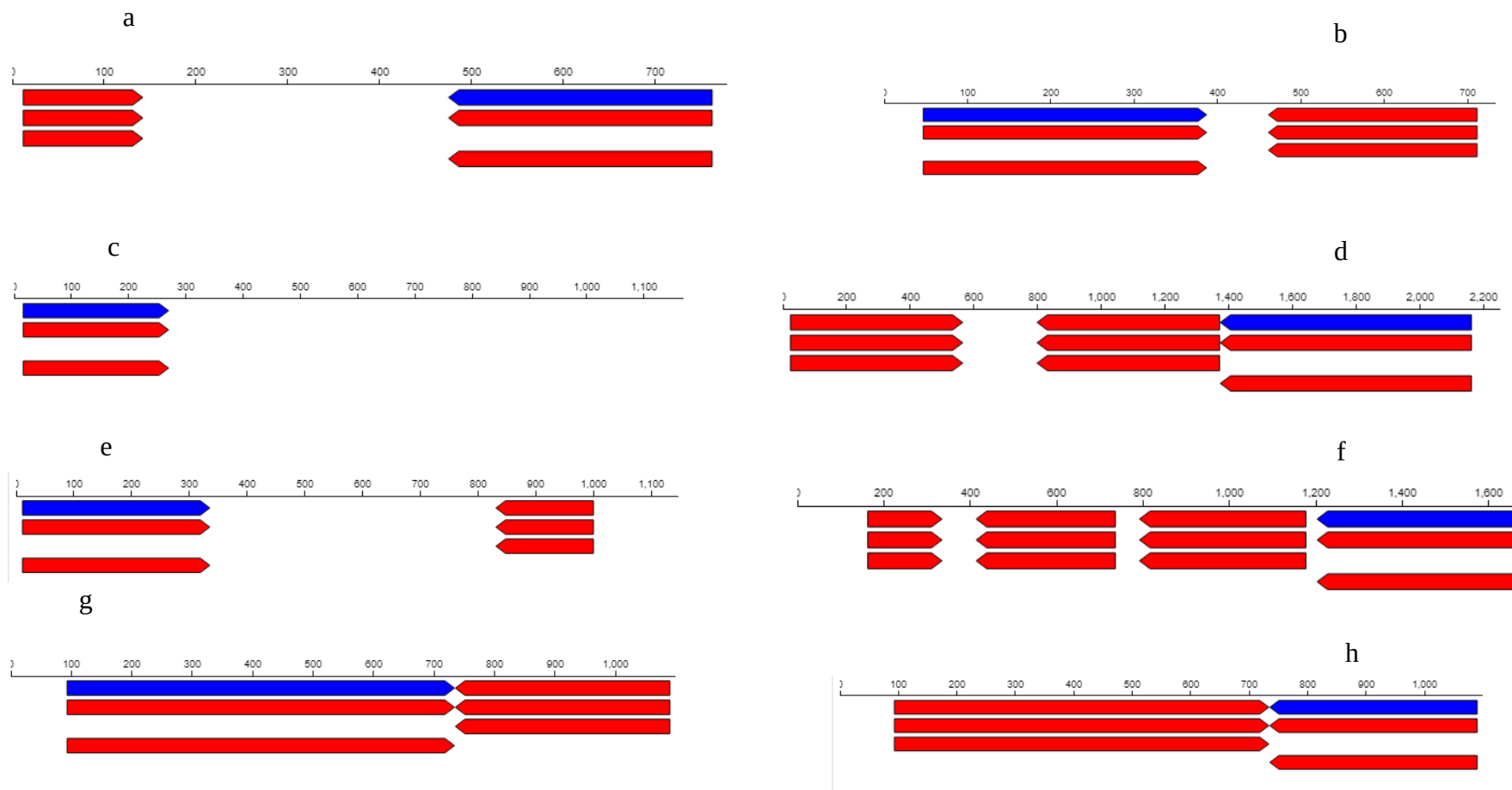


Fig. 6. 10 Feature context of (a) Signal transduction histidine-protein kinase BaeS (b) Exodeoxyribonuclease III (c) Extracellular serine protease (d) Microbial serine proteinase (e) Cysteine desulfurase SufS (f) Cysteine desulfurase IscS (g) putative MFS-type transporter YcaD and (h) Riboflavin transporter genes found in R1 strain. The blue bars represent the gene locations

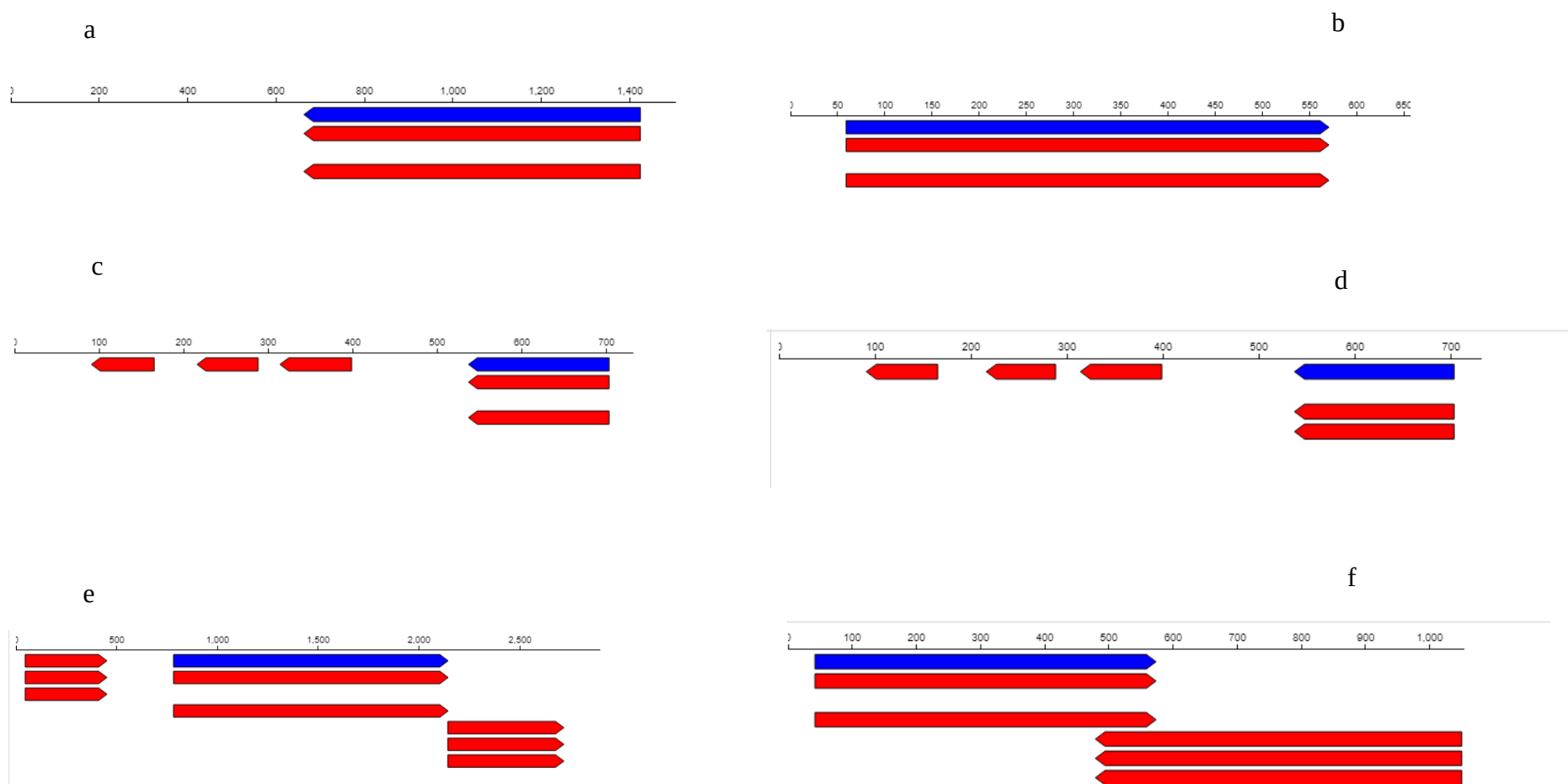


Fig. 6. 11 Feature context of (a) Catechol siderophore Receptor Fiu (b) 2, 3-dihydro-2,3-dihydroxybenzoate dehydrogenase (c) Isoaspartyl peptidase (d) Isoaspartyl peptidase (e) UDP-N-acetylmuramate--L-alanyl-gamma-D-glutamyl-meso-2,6-diaminoheptandioate ligase (f) Phosphoethanolamine transferase EptA genes found in R1. The blue bars represent the gene locations

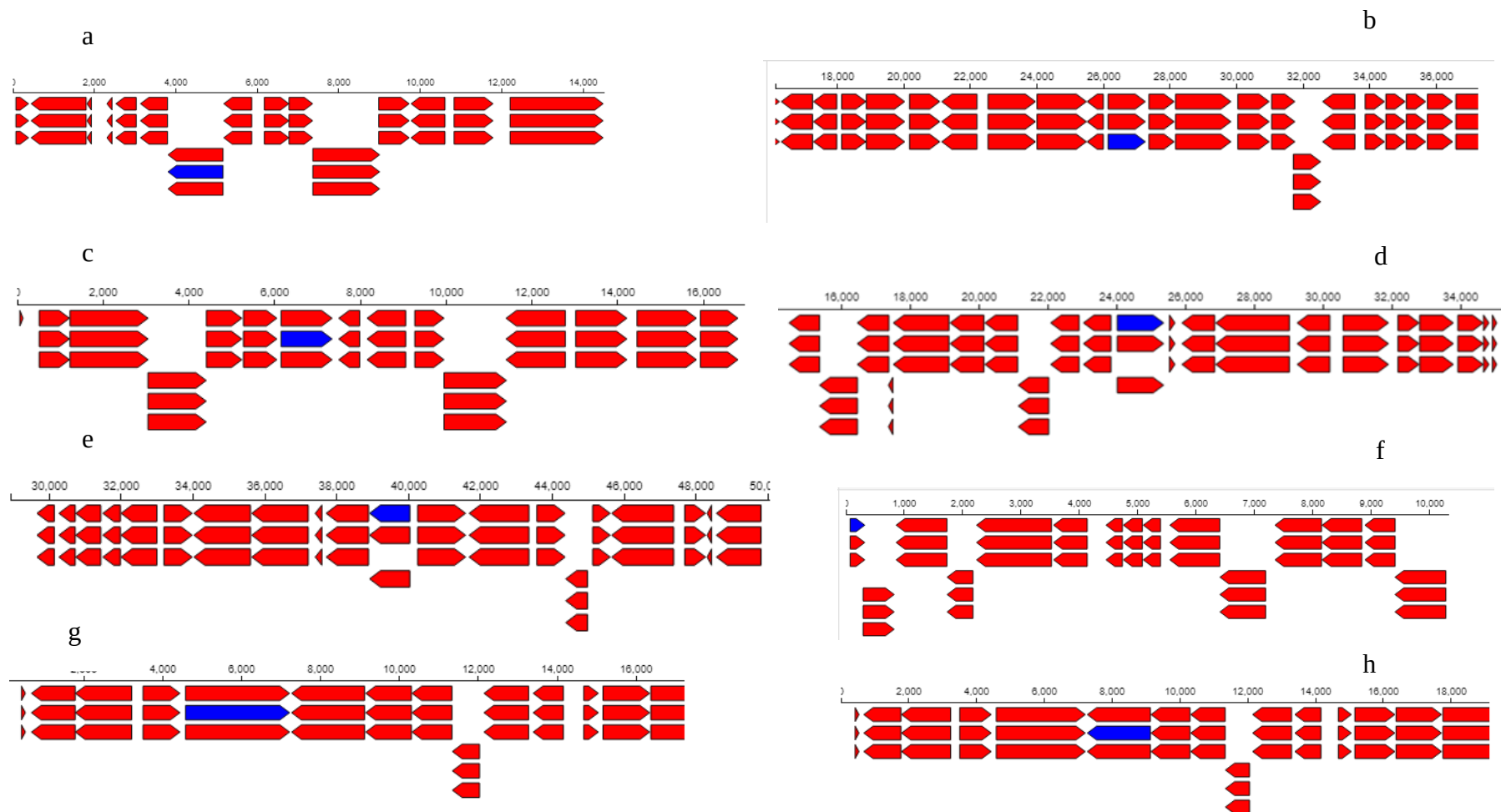


Fig. 6. 12 Feature context of (a) Signal transduction histidine-protein kinase ArlS (b) Response regulator aspartate phosphatase J (c) Serine protease Do-like HtrA (d) Serine protease Do-like HtrA (e) Cysteine desulfurase IscS (f) Putative cysteine desulfurase NifS (g) Beta-N acetylglucosaminidase (h) Teichoic acid poly (ribitol-phosphate) polymerase genes found in R3 strain. The blue bars represent the gene locations



Fig. 6. 13 Feature context of (a) putative siderophore transport system permease protein YfiZ (b) putative siderophore-binding lipoprotein YfiY (c) Inner membrane protein YiaA (d) tRNA dimethylallyltransferase (e) Heptaprenyl diphosphate synthase component 1 (f) Septation ring formation regulator EzrA genes found in R3. The blue bars represent the gene locations

6.3 Discussion

In this present study, the plant growth promoting traits of rhizobial species were determined. To be specific, the ACC experiment revealed the production of ACC by the rhizobial species (Fig. 2) but the highest concentration of ACC was produced by R5 strain followed by R1 strain while the lowest concentration of ACC was produced by R2 strain. The production of ACC by these microorganisms shows that they have the potential to increase plant tolerance to drought stress since it was reported by Ali et al. (2014) that application of ‘ACC deaminase-producing microorganisms’ into water stressed soil environments can reduce stress in the local plants by minimizing stress triggered by C_2H_4 . Indeed, a study carried out by Arshad et al. (2008) showed that ACC deaminase-producing *Pseudomonas* species partially eradicated the detrimental effects of water stress on pea (*Pisum sativum* L.) growth and/or productivity.

In a study performed by Putrie et al. (2013), bacterial tolerance to water stress conditions was characterized by EPS production and therefore in this study, rhizobial tolerance to drought stress was determined by their ability to produce EPS in medium amended with different concentrations of PEG (drought stress stimulant). Based on the qualitative results, we found that environmental stresses such as pH and temperature stimulated the production of EPS. In particular, R2, R3 and R4 strains produced EPS under the different pH and temperature conditions (Table 1). Quantitatively, our findings showed that R1 strain was more effective in EPS production under severe drought condition (10% PEG) while R2 strain produced more EPS at 5% PEG (Fig. 3). Other rhizobial species produced EPS at different concentrations in this study. Indeed, EPS production by bacteria protect them from water stress, heavy metals and other environmental stresses (Ozturk and Aslim, 2010, Putrie et al., 2013) and therefore, it is possible for these rhizobial species to survival, multiply and harness other plant growth

promoting traits to promote plants when applied under drought conditions - as evident in the soybean germination experiment (Fig. 9)- even in a complex soil environment in the field.

Furthermore, these rhizobial species were able to produce siderophore in King B and Succinic acid broth (Fig. 4) and siderophore production by different microorganisms has been reported by many researchers (Sayyed et al., 2005, Omidvari et al., 2010). In this present study, the ‘maximum siderophore production’ was found in King B broth. The highest amount of siderophore was produced by R3 strain in King B broth followed by R5 strain, but in succinic acid broth, the reverse was the case – R5 strain produced the highest siderophore concentration followed by R1 strain. These quantitative results for siderophore production further validate the qualitative plate test for siderophore production for these rhizobial species in our previous study (data not shown). However, the results of this study contradict the findings of Sasirekha and Srividya (2016) who reported maximum siderophore production by *Pseudomonas* species in succinic acid broth.

The rhizobial species used in this study showed other plant growth promoting traits. In particular, all the rhizobial species produced IAA but at different concentrations. This is in agreement with the report that IAA production can differ among different bacterial species which could be influenced by culture condition, nutrient availability and growth stage (Mohite, 2013). In addition, bacteria from plant rhizosphere are more effective producer of IAA than those from bulk soil (Sarwar and Kremer, 1995). In this study, we observed that R3 strain was more efficient in producing IAA at both concentrations of tryptophan (Fig. 5) and this could be the reason for the high root biomass observed in soybean treated with this species in our previous study (data not shown) since IAA production has been implicated in root elongation and development of lateral roots (Mohite, 2013).

Another strategic mechanism that can be used by rhizospheric bacteria to support the growth of agricultural crops lies in their capacity to solubilize phosphate and it has been stated that phosphates always occur in bound forms in the soil (Putrie et al., 2013). Indeed, we found that R2 strain was more effective in solubilizing tri-calcium phosphate in Pikovskaya's agar followed by R1 strain, but R3 and R4 strains showed similar phosphate solubilizing potential while R5 strain demonstrated the least ability to degrade phosphate as shown in Fig. 6. Thus, these rhizobial species have the tendency to solubilize bound phosphates and make them available for plant uptake in the soil.

Additionally, rhizobial growth response towards different environmental temperatures showed that these species possess the capacity to strive and survive at relatively high temperature (45°C) as indicated in Fig. 7 a, b, c, d and e. At 45°C, R1 strain showed the highest growth on day 4, 8 but its growth declined on day 12 in which R5 strain had the highest growth. Furthermore at the same temperature, R4 and R2 strains showed the highest cell biomass on day 16 and 20 respectively. Conversely, R1 strain showed the maximum cell biomass at 28°C throughout the experimental period (Fig 7 a, b, c, d and e). It has been shown that the optimal growth temperature for so many rhizobial species is 25 – 30°C (Zhang et al., 1995) which agrees with our findings, since in this study, rhizobial species grew best at 28°C. But the ability of these species to grow and survive at 37 and 45°C indicate that they may be able to help agricultural crops such as soybean to tolerate and survive in most tropical countries currently facing drought and/or high temperature problem and further contradict the reports of Alexandre and Oliveira (2013); Chen et al. (2002) that soybean rhizobial species grow poorly at 40°C and none of the species is 'able to grow' at 42°C. On the contrary, some rhizobial species capable of nodulating common bean (*Phaseolus vulgaris*) can survive at 47°C but do not have the ability to form

nodules at such high temperature (Karanja and Wood, 1988). But other rhizobial species from *Phaseolus vulgaris* are able to survive and remain infective at 40°C (Alexandre and Oliveira, 2013).

Additionally, the ability of rhizobial species to grow and survive at different pH values was experimented on in this study since real-life biotechnological application of microbial inoculants under drought condition in the field would demand that these species should possess the capacity to adapt to pH fluctuations inherent in complex soil ecosystem. Thus, all the rhizobial species in this current study were able to grow and survive in acidic (pH 4), neutral (pH 7) and alkaline environments (pH 10) (Fig. 8 a, b, c, d) indicating that these species possibly have broad environmental adaptability with respect to environmental pH. Such trait can help these microorganisms to function actively (without interference) in their symbiotic interactions with crops since it was reported by Abd-Alla et al. (2014) that nodulation of faba bean treated with *Rhizobium leguminosarum* was inhibited significantly by soil alkalinity.

The selection of R1, R3 and R5 strains for *in vitro* enhancement of soybean germination under drought condition stimulated by 4% PEG showed that R1 and R3 strains were able to effectively enhance the germination of soybean than R5 strain. This finding is in agreement with the results of Jalili et al. (2009) who reported that ‘ACC deaminase – producing fluorescent pseudomonads’ improved canola (*Brassica napus* L) seed germination under osmotic stress.

Regarding genomic insights into R1 and R3 strains, annotation of R3 genome revealed the presence of 99 different genes (*exo* genes) responsible for EPS production. One of the genes with aliases *yjcG* had the biological function of putative phosphoesterase while *ArIS* gene had the biological function of signal transduction histidine-protein kinase. These EPS genes are known to empower microorganisms to survive under harsh environmental conditions (Roberson and

Firestone, 1992). Although, it has been reported that EPS production is a survival strategy needed by microorganisms under drought stress condition, Ormeño-Orrillo et al. (2012) reported that production of EPS is connected to acid tolerance. In reality, EPS has also been produced by *Agrobacterium tumefaciens* and *S. meliloti* in acidic environments (Yuan et al., 2008, Hellweg et al., 2009, Cunningham and MuNNS, 1984).

Under certain circumstances, surface polysaccharides such as EPS, capsular polysaccharides (CPS), β -1, 2-glucans and lipopolysaccharides (LPS) which are essential molecules for symbioses establishment (Becker et al., 2005, Skorupska et al., 2006) might have other functions such as defence against antimicrobial substances and oxidative stress (Ormeño-Orrillo et al., 2008, D'Haese and Holsters, 2004, Ingram et al., 2010). Different types of *exoX* genes have been reported (Zhan and Leigh, 1990), but in this study, we are reporting only *exoX* genes produced by R1 and R3 strains. It was also reported by Zhan and Leigh (1990) that *exoX* gene regulates the production of exopolysaccharide (such as succinoglycan) by a 'new *Rhizobium meliloti*'. The 2 *exoX* genes reported for R1 strain were located between contigs 474 – 762 and 47 – 389 (Fig. 10 a, b) with signal transduction histidine-protein kinase BaeS and exodeoxyribonuclease III biological functions respectively (Table 3). Similarly, R3 strain had its 2 *exoX* genes located between 3,808 – 5,173 and 26,133 – 27,255 (Fig. 12a, b) with signal transduction histidine-protein kinase ArlS and response regulator aspartate phosphatase J functions respectively (Table 4).

As previously mentioned, R1 and R3 strains were able to survive and grow at 45°C (Fig. 7) and this trait is thought to be essential for their success as semiarid and/or arid inoculant species (Ormeño-Orrillo et al., 2012, Martínez-Romero et al., 1991, Hungria et al., 2000). A number of proteins are induced upon exposure to relatively high temperatures and these proteins

are generally termed heat shock proteins (HSPs). A couple of HSPs were present in R1 strain and *htrA* genes were found in both R1 and R3 strains. Specifically, 5 *htrA* and 14 *htrA* genes were correspondingly found in R1 and R3 strains. Besides, so many different *htrA* homologues are similarly found in the genome of other bacteria (Clausen et al., 2002). However, it was reported that alterations in one ‘*htrA* paralogue’ of *Brucella abortus* and *S. meliloti* had only a little impact on growth at high temperatures (Glazebrook et al., 1996, Phillips and Roop, 2001), perhaps as a result of functional redundancy. Furthermore, Ormeño-Orrillo et al. (2012) also reported *htrA* gene as one of the components of heat shock response in *Rhizobium tropici* CIAT 899 and *Rhizobium* sp. PRF 81. In reality, besides high temperatures, some HSPs also offer protection against other stressful conditions such as the DnaK machinery which protects against salt stress (Nogales et al., 2002) and even the HtrA that proffers fortification against oxidative damage (Phillips and Roop, 2001). Among the *htrA* found in the rhizobial strains in this present study are the R1 strain extracellular serine protease and microbial serine proteinase *htrA* (Table 3) and the R3 strain serine protease Do-like *htrA* and serine protease Do-like *htrA* (Table 4). Again, both *htrA* genes for R1 strain (Fig. 10 c, d) and R3 strain (Fig. 12 c, d) have their unique contigs locations within the genome.

R1 and R3 strains harboured nitrogen fixing (*nif*) genes with 6 different biological functions and 2 of the genes are presented in Table 3 and 4 respectively. With respect to location, *iscS_1*, 2.8.1.7 gene was located between contigs 38, 300 – 39,443 (Fig. 12 e) while *nifS* was located between contigs 72 – 336 (Fig. 12 f) in R3 strain but the *nif* genes in R1 were found at contigs locations (Fig. 11 e, f) different from that of R3 strain. IscS has the biological function of providing sulphur for the synthesis of iron-sulphur cluster *in vitro*; nevertheless, *in vivo* role of IscS in iron-sulphur formation is yet to be established (Schwartz et al., 2000). Studies of the

Azotobacter vinelandii nitrogen fixation gene cluster revealed that there are activities that enhance the effectiveness of iron-sulphur cluster assembly (Dean et al., 1993). To be specific, study of *nifS* led to the detection that the protein produced by *IscS* gene is a pyridoxal 5-phosphate-haboring cysteine desulfurase that helps to transfer the sulphur moiety from cysteine to cysteinyl active site of *nifS* leading to the formation of enzyme-bound persulphide (Zheng et al., 1994). After reduction and incorporation of an Fe source, the sulphur can be released and effectively integrated into the iron-sulphur protein cluster of the nitrogenase enzyme complex (Zheng et al., 1994).

Also, we found 23297 and 12242 *nodA* genes in R1 and R3 strains respectively but only 2 of the genes are reported for each strain in this study. Particularly, we observed *NodA* genes encoding putative MFS-type transporter YcaD and riboflavin transporter in R1 strain (Table 3) and Beta-N acetylglucosaminidase and Teichoic acid poly (ribitol-phosphate) polymerase in R3 strain (Table 4). The location of putative MFS-type transporter YcaD was between contigs 93 – 735 (Fig. 10 g), riboflavin transporter was between contigs 734 – 1091 (Fig. 10 h), Beta-N acetylglucosaminidase was between contigs 4582 – 7225 (Fig. 12 g) and Teichoic acid poly (ribitol-phosphate) polymerase was between contigs 7268 – 9128 (Fig. 12 h). It is a common knowledge that *Nod* genes help in the formation of nodules, the site of nitrogen fixation by nitrogen fixing bacteria such as *Rhizobium* species. Moreover, it was suggested that the kind of ‘Nod factor acyl group attached by *NodA* can contribute to the determination of host range’ (Debellé et al., 1996, Ormeño-Orrillo et al., 2012). *NodA* gene was also reported by Ormeño-Orrillo et al. (2012) as one of the nodulation genes found in *Rhizobium* species.

R1 and R3 strains were found to have 13 and 33 siderophore-producing genes respectively. To be specific, Catecholate siderophore receptor *fiu* and 2,3-dihydro-2,3-

dihydroxybenzoate dehydrogenase genes were noticed in R1 strain (Table 3) while putative siderophore transport system permease protein YfiZ and putative siderophore-binding lipoprotein YfiY genes were detected in R3 strain (Table 4). It is presumed that these siderophore protein-producing genes in R1 strain located between contigs 662 - 1,424 and 59 – 572 (Fig. 11 a, b) and in R3 strain located between 14769 – 15,771 and 13,660 – 14,638 (Fig. 13 a, b) could have contributed to the quantitative siderophore production (Fig. 4) observed in this study which can help these rhizobia strains to chelate Fe and make it available for plant use. Naturally, the role of siderophore producing-rhizobia under drought conditions is highly appreciated since it was reported by Hungria et al. (2013) that plant are highly prone to pathogenic attack under water stress conditions. Siderophore is obviously antimicrobial in nature (Igíehon and Babalola, 2018c) and thus application of siderophore-producing rhizobia will certainly help to improve plant health under drought stress and consequently increase agricultural productivity (Igíehon and Babalola, 2018a).

Furthermore, IAA genes involved in the production of Isoaspartyl peptidase protein was detected in R1 strain (Table 3). Similarly, 2 of the 6 IAA genes found in R3 strain are responsible for the production of inner membrane protein YiaA and tRNA dimethylallyltransferase (Table 4) and the locations of the respective genes were between 536 – 704 for the R1 strain (Fig. 11 c, d) and between contigs 11,701 – 11,965 and 10,955 – 11,900 for the R3 strain (Fig. 13 c, d).

Based on the results of the growth response to different environmental pH, R1 and R3 strains can be considered acid-tolerant strains since they were able to survive and grow at a low pH of 4. Again, upon application in the field, these strains may be confronted with acid stress in acidic soils and within the symbiosome. According to Ormeño-Orrillo et al. (2012), the

mechanisms involved in rhizobial survival and growth in acidic environments are yet to be understood. *EptA* genes coding for UDP-N-acetylmuramate-L-alanyl-gamma-D-glutamyl-meso-2,6-diaminoheptandioate ligase and Phosphoethanolamine transferase were found in R1 strains while those coding for heptaprenyl diphosphate synthase component 1 and septation ring formation regulator EzrA were found in R3 strains. Ormeño-Orrillo et al. (2012) also found genes in *R. tropici* CIAT 899 and *Rhizobium* sp. PRF 81 coding for ‘putative lipid A Phosphoethanolamine transferase’ similar to that found in R1 strain in this study . In *Salmonella typhimurium* and *Escherichia coli*, *eptA* gene is activated under mildly acidic environments and it was reported that *eptA* imposed acid tolerance in *Shigella flexneri* 2a (Martinić et al., 2011) . Also, *eptA* genes were reported in *R. rhizogenes* K84, sinorhizobia, agrobacteria, but not in other *Rhizobium* spp. (Ormeño-Orrillo et al., 2012).

6.4 Conclusions

It was found that *Rhizobium* sp. strain R1, *Rhizobium tropici* strain R2, *Rhizobium cellulosilyticum* strain R3, *Rhizobium taibaishanense* strain R4 and *Sinorhizobium meliloti* strain R5 isolated from Bambara groundnut rhizosphere possess the PGP traits considered in this study. In particular, these rhizobial strains produced EPS, ACC and in addition were able to survive and grow at a temperature of 45°C and in an acidic condition with a pH of 4. Consequently, R1, R3 and R5 strains enhanced the germination of soybean seeds (PAN 1532 R) under drought condition imposed by 4% PEG; nevertheless, *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 inoculations were able to improve seeds germination more than R5. Thus, genomic insights into *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 revealed the presence of some genes with their respective proteins involved in symbiotic establishment, drought tolerance and plant growth promotion. In particular, *exoX*, *htrA*, *Nif*, *nodA*, *eptA*, *IAA* and siderophore-

producing genes were found in the two rhizobial strains. Therefore, the PGP ability of these rhizobial strains can further be harnessed for biotechnological application in the field especially in semiarid and arid regions of the globe.

6.5 Acknowledgements

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6.6 Conflict of Interest

Author 1 declares that he has no conflict of interest. Author 2 declares that she has no conflict of interest.

6.7 Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

6.8 Authors Contribution

Igiehon NO performed the experiment and wrote the article; Babalola OO secured funding, provided academic input in writing the manuscript and thoroughly critiqued the article while Igiehon NO and Aremu BR did bioinformatics analyses of the genomic data. All authors approved the article for publication.

CHAPTER 7

Complete Genome Sequence of a Novel Drought Tolerant *Rhizobium* sp. Strain R1 Isolated from Bambara Groundnut Biome

Abstract

The strain of *Rhizobium* sp. R1 was isolated from Bambara groundnut rhizosphere and possessed plant-growth promoting traits and capacity to enhance soybean (*Glycine max* L.) growth under drought stress. A low nucleotide similarity to database species supports its description as a novel *Rhizobium* strain. Sequencing unveiled genes involved in plant growth promotion, stress response and nodulation. These genomic traits may help to protect plants against the detrimental effects imposed by drought stress.

Drought stress is one of the main challenges on agricultural yield globally and is projected to further increase. Thus, some adaptations and alleviation strategies are needed to cope with drought stress and rhizobial species could play a vital role in mitigation of this stress in agriculture (Vurukonda et al., 2016).

Therefore, *Rhizobium* sp. strain R1 was originally isolated from the rhizosphere of Bambara groundnut (*Vigna subterranea*) in a semiarid environment of South Africa (25.82080S: 025.61382E). This species showed plant-growth promoting traits and capacity to enhance the growth of soybean (*G. max* L.) under drought conditions (Igiehon and Babalola, 2018c). The rhizobial DNA was extracted from a pure culture grown for 4 days on nutrient agar (NB) medium using the Zymo DNA extraction kit (Zymo-Research, USA). HiSeq system (illumina) was used to perform whole-genome sequencing using an average DNA library size of 647 bp. The genome sequence was subjected to quality check using FastQC (v1.0.4), trimming and *de*

novovo assembly using Kbase pipeline. A total of 5773 contigs with a GC content of 61.91% and a total of 17,408,810 reads with a mean read length of 201.15 were obtained. The number of genes predicted was 29842, genes with non-hypothetical function were 21450, genes with EC-number were 10726 and genes with seed subsystem ontology were 8378. Protein coding sequences (CDS) of 29354 with an average protein length of 308 aa were obtained. The scaffold outcome showed N₅₀ value of 936, N₇₅ value of 686, L₅₀ value of 1855 and L₇₅ value of 3514.

The Prokka (V1.12) and RAST online pipeline annotation was performed for assembled sequence and the total genome size of 31,661,774 was obtained without using a known reference strain (Seemann, 2014, Tejerizo et al., 2017, Aziz et al., 2008). A couple of heat shock proteins involved in response to stress including 5 *htrA* genes and were found. Seventy eight (78) genes that play role in biosynthesis of extracellular polysaccharide (*ExoX* genes) involved in tolerance to drought and other environmental stresses (Ormeño-Orrillo et al., 2012) were also found. In addition, 1 CDS and 1 gene for indole-acetic-acid (IAA) production and 23297 *nodA* genes related to nodule formation were noticed in the species. In short, these genomic traits can be linked to the ability of this species to strive and survive under osmotic stress induced by polyethylene glycol (PEG) and enhance soybean productivity under drought conditions in a controlled and semiarid field.

7.0 Accession number(s): The whole genome of the new *Rhizobium* sp. strain R1 was deposited in NCBI GenBank and has been assigned SRA accession number SRR8060784, Bioproject number PRJNA496421 and Biosample number SAMN10240937.

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CHAPTER 8

Whole Genome Sequence of a New Plant Growth Promoting *Rhizobium cellulosilyticum*

Strain R3 Isolated from *Vigna subterranea* Rhizosphere

Abstract

We report in this study the whole genome sequence of *Rhizobium cellulosilyticum* strain R3, isolated from Bambara groundnut (*Vigna subterranea*) rhizosphere. It has *nod* genes and *nif* genes for nitrogen fixation. The species was able to enhance the growth of soybean (*Glycine max* L.) under controlled and complex soil ecosystem.

Nitrogen (N) availability in agricultural land is one of the major ‘concerns for crop cultivation in agriculture’ and N is important for the synthesis of vitamins, proteins, amino acids and vitamins (Tejerizo et al., 2017). Thus, N-fixing rhizobia and plant growth-promoting rhizobacteria (PGPR) are beneficial alternatives for conventional chemical fertilizers in legume plants (Souza et al., 2015, Igiehon and Babalola, 2018a, Igiehon and Babalola, 2018c). These bacteria are able to fix atmospheric N through conversion to ammonia. Rhizobia are a group of bacteria under the class *Alphaproteobacteria* and/or *Betaproteobacteria* found in the soils where they enter into N-fixing symbiosis with legumes (Jones et al., 2007). Thereafter, they trigger the formation of root nodules resulting in the development of bacteriods that fix atmospheric N. They also enhance legume root system and accessibility to phosphate by producing indole-acetic-acid (IAA) (Mohite, 2013) and solubilizing bound phosphates in the soil.

In particular, *R. cellulosilyticum* strain R3 was isolated from Bambara groundnut rhizosphere and enhanced soybean growth as a result of phosphate solubilization, IAA

biosynthesis and its inherent N-fixing genes (Igiehon and Babalola, 2018c). We also found that R3 is not a fast-growing microorganism.

R. cellulosilyticum strain R3 was isolated from Bambara groundnut (*V. subterranea*) rhizospheric soil using Yeast Mannitol Agar (YEM) (Ahmad et al., 2008) and the rhizobial DNA was extracted using Zymo DNA extraction kit (Zymo-Research, USA). The R3 'genome was sequenced' using HiSeq illumina platform and an average library size of 661 bp were sequenced. The quality of the sequence was checked using FastQC (v1.0.4), then trimmed with trimmomatic to remove the adapter and low quality base. These resulted in 17,794,094 reads, estimated genome size of 4,114,542 and mean read length of 214.18 with 129 contigs as well as GC content of 43.59%. The N₅₀ value of 57294, N₇₅ value of 32492, L₅₀ value of 23 and L₇₅ value of 45 were obtained for the scaffold. Finally, annotation was performed with Prokka (V1.12) and RAST online pipeline. The protein coding sequences (CDSs) was 4417 and tRNA predicted gene was 27.

The Kbase Prokka annotation pipeline predicted one CDS/five genes for N-fixation and three CDS/three genes for IAA with different biological functions and contigs locations. One of the nitrogen fixing genes was the IscS genes that encode cysteine desulfurase similar to that reported by Schwartz et al. (2000). These genes including others such as siderophore genes could be responsible for the soybean growth promotion potential of this new strain.

8.0 Accession number(s): The complete genome of this new species has been deposited at NCBI GenBank with a SRA accession number SRA8061690, Bioproject number PRJNA496421 (<https://www.ncbi.nlm.nih.gov/sra/PRJNA496421>) and Biosample number SAMN10245972 (<https://www.ncbi.nlm.nih.gov/biosample/10245972>).

8.1 Acknowledgements

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CHAPTER 9

The Panacea to Food Insecurity starts with Rhizobia, Mycorrhizal Fungi and Soybean

Interactions in a Controlled Environment

Abstract

Food insecurity is a serious threat due to the increasing human population particularly in developing countries and may be remedied by the use of microbial inoculants. Therefore, in this study, the ability of rhizobial species and arbuscular mycorrhizal fungi (AMF) to enhance soybean (*Glycine max* L.) tolerance to drought stress in a controlled environment was investigated. A gradient of watering levels ranging from 100 to 40% of 'soil retention capacity of water' was tested on non-inoculated soybean plants and plants inoculated with rhizobia and AMF in pot cultures. It was observed that the inoculated soybean plants especially soybean dually inoculated with *Rhizobium* sp. strain R1 and *Rhizobium cellulosilyticum* strain R3 (R1+R3) as well as *Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium (R1+R3MY) had significant impacts ($P<0.05$) on soybean leaf relative water content and electrolyte leakage respectively. The levels of accumulated soluble sugars and proline revealed that their concentrations increased mainly in microbially amended soybean plants exposed to drought stress (70 and 40% FC). Similar results were observed for chlorophyll content. Plants inoculated with R1+R3MY showed the highest number of spore and % mycorrhization in all the water regimes. At 40% FC, R1+R3MY treatment was found to promote soybean growth since it had significant effects ($P<0.05$) on soybean shoot width, branch number, and root dry weight compared to the non-inoculated plants. Similarly, under severe drought stress, R1+R3MY inoculum had the greatest impacts on soybean pod number, seed number, seed fresh weight, highest seed number per pod and seed dry weight while in 70% water stress,

significant impacts ($P < 0.05$) of R1MY inoculation were observed on pod number, pod fresh weight and seed dry weight. These results revealed that co-inoculation of rhizobia and mycorrhizal fungi can be harnessed biotechnologically for field application to proffer solution to food insecurity intrinsic in developing countries especially in semi-arid regions.

Keyword: Biotechnology, drought stress, food insecurity, *Glycine max* L., microbial inoculants, soybean seed number

9.0 Introduction

Food security and food insecurity are terminologies that are usually used in global food related discussions. “The Food and Agriculture Organization (FAO) definition holds that food security (FS), at the individual, household, national, regional and global levels (is achieved) when all people, at all times, have physical and economic access to sufficient, safe and nutritious food to meet their dietary needs and food preferences for an active and healthy life (Pérez-Escamilla et al., 2017, Stewart and Roberts, 2012)”. Food insecurity on the other hand occurs when people do not have access to adequate amounts of high quality and nutritive food and thus are not utilizing sufficient food for “an active and healthy life” (Stewart and Roberts, 2012). FAO evaluated that in 2030, the demands for agronomic products would be 60% higher than current time and more than 85% of the extra demand would emanate from developing nations and there is therefore an urgent need for a sustainable strategy to enhance productivity of agricultural crops (e.g. soybean) mainly in arid and semi-arid environments since over the years, mankind has depended on increasing farm yields to meet the endless demand for food (Abd-Alla et al., 2014).

Enhanced production of soybean seeds (*G. max* L.) can basically help to cushion the alarming impacts of food insecurity since it is among the most economically essential legumes

that supply more than a ¼ of food for mankind and the bulk of plant proteins (Graham and Vance, 2003). Unfortunately, the growth of this legume can be limited in soils exposed to drought. Soil is a major factor that determines the agricultural sustainability of an ecosystem and semi-arid and arid environments are characterized by nutrient-deficient soil, high temperatures, drought and low precipitation. These factors have adverse effects on plant development and yield (Zarik et al., 2016, Chaves et al., 2003). It is well-known that mankind has tried to enhance the fertility of nutrient-deficient soil by chemical fertilizer application. However, chemical fertilizers are not only detrimental to the environments, they are also relatively expensive and as such most small-scale farmers may not be able to afford them. Hence, it becomes necessary to explore a more ecofriendly, cheaper and natural biological agents (such as rhizobia and mycorrhizal fungi) with the potential to supply plant nutrients by establishing symbiosis with the host plant. Such agronomic practices could be used to enhance agricultural crops development and productivity especially under drought conditions (Hou and Oluranti, 2013, Philippot et al., 2013).

Rhizobial species are the most studied plant growth promoting rhizobacteria (PGPR) because of their ability to fix nitrogen in legumes. Rhizobia is the generic name given to a set of Gram negative, rod-shaped bacteria with the ability to produce nodules on leguminous plant roots and classified in the family Rhizobiaceae under the phylum proteobacteria (Abd-Alla et al., 2014). The symbiotic interactions of these bacteria with legumes under drought conditions were previously highlighted in our previous work (Igihon and Babalola, 2018a, Igihon and Babalola, 2018c). Drought is one of the factors that can decrease the nitrogen fixing ability of legume-rhizobial symbiosis. As a result, several attempts have been made on microbial enhancement of plant traits to tolerate unfavorable environmental conditions (Dwivedi et al., 2006). Also, the effects of temperature and drought including other environmental stressors have

been well studied in isolating rhizobia in ecosystem that are tolerant towards these environmental stressors which could be utilized as inoculants on specific plant (Rupela et al., 1991, Hungria et al., 1993, Hungria and Vargas, 2000, Provorov and Tikhonovich, 2003, Roumiantseva, 2009, Elboutahiri et al., 2010) and the ability of these bacteria to interact and enhance the growth of legumes such as soybean have been studied (Masciarelli et al., 2014). However, the specificity and extent of these legumes-rhizobial interactions are poorly comprehended (de Zelicourt et al., 2013). Consequently, further study is required to understand the effect of the host-rhizobial interactive mechanism(s) on the morphological and/or productivity of legumes. The resultant information from such study according to de la Peña and Pueyo (2012) would help in choosing and genetically manipulating rhizobia and leguminous plants with better adaptation to extreme and stressful conditions.

Indeed, the tripartite symbiotic interaction between legumes-rhizobia and AMF is one of the most crucial natural mutualisms (Remy et al., 1994, Igiehon and Babalola, 2018a). AM fungi associate with over 80% terrestrial plant roots (Igiehon and Babalola, 2017, Abd-Alla et al., 2014) and play an important role in the regulation of ion homeostasis of host plants (Ramos et al., 2011, Song and Kong, 2012). AM fungi are found in stressed soil and contribute to plant development and also enhance plants' tolerance to abiotic and biotic stress (Igiehon and Babalola, 2017, Igiehon and Babalola, 2018c, Abd-Alla et al., 2000, Berta et al., 2005, Abdel-Fattah et al., 2010) by adjusting their biochemical and physiological processes (Evelin et al., 2009, Covacevich et al., 2012).

Furthermore, several studies involving the use of valuable soil microorganisms (e.g. AMF and rhizobia) to reduce drought stress have been investigated (Zarik et al., 2016, Igiehon and Babalola, 2017, Igiehon and Babalola, 2018a, Igiehon and Babalola, 2018c). Other studies

have demonstrated that inoculation with AMF enhanced water absorption in numerous plants that were submitted to drought stress (Augé, 2001, Augé et al., 2004). Specifically, under harsh conditions, AMF mycelia can penetrate larger volume of soil than host plant roots, therefore increasing water absorption and transport to the root and other plant parts (Marulanda et al., 2003, Marulanda et al., 2006a, Marulanda et al., 2006b, Al-Karaki et al., 2004, Augé, 2001, Augé et al., 2003) which eventually improves the osmotic regulation, cellular and physiological effects in plants (Wu et al., 2007, Caravaca et al., 2003, Ruiz-Lozano, 2003). In addition, studies have also showed the effectiveness of co-inoculation of specific rhizobia and mycorrhizal fungi in a controlled environment (Igiehon and Babalola, 2018c, Aliasghar zad et al., 2006) but more work with different rhizobial and mycorrhizal combinations is needed in this aspect.

Thus, the aim of this study is to determine the effects of single and co-inoculation of rhizobia and AMF on soybean growth and productivity exposed to drought stress under a controlled environment with the prospect of harnessing the effective microbial inoculants for field application. This biotechnological approach will not only minimize the use of expensive and hazardous chemical fertilizer but also help to abate the problem of food insecurity in semi-arid and arid regions of developing countries.

9.1 Materials and Methods

9.1.1 Description of Experimental and Soil Collection Sites

Soil samples for pot experiment were collected from two different locations (namely site 1 and site 2) within North-West University, Mahikeng campus. Soil collection site 1 and 2 and greenhouses (Figure 1) were located at the following geographic coordinates: 25.82090S:025.61389E, 25.81934S:025.61419E, 25.82396S:25.60877E respectively in North-West University, Mahikeng campus located at Ngaka Modiri Molema district in Mahikeng town,

North-West Provinces, South Africa (Figure 1). The greenhouse experiment was also conducted in North-West University, Mahikeng campus (Figure 1).

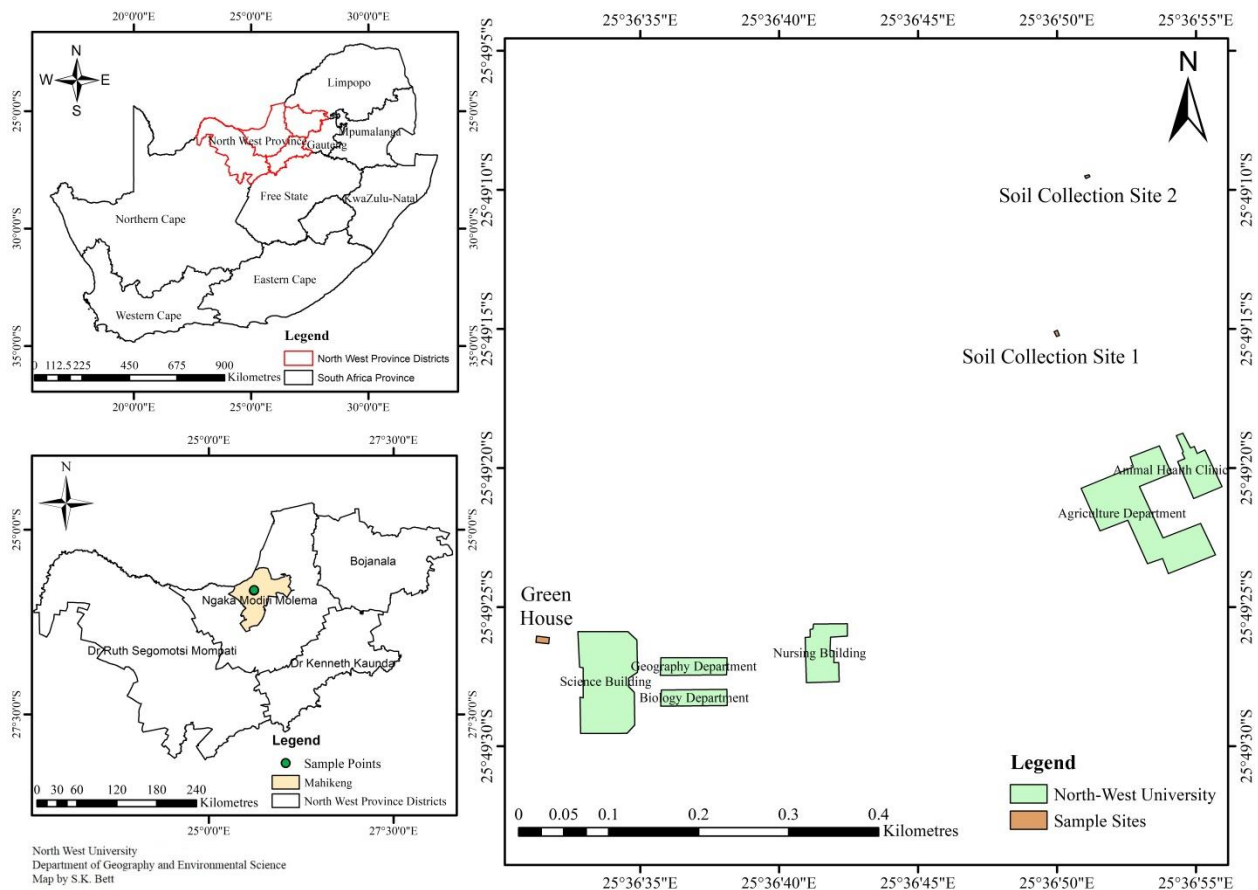


Fig. 9. 1 Experimental and soil collection sites in South Africa. The top left sketch depicts a map of South Africa enclosing North-West Province (the region in red sketch) and at the bottom left is map of North-West Province enclosing a map of Mahikeng (light yellow area) which encompasses Ngaka Modiri Molema district (the green spot) where North-West University Mahikeng campus is located which was the location of experimental and soil collection sites. To the right, is a sketch depicting the two soil (used for the greenhouse experiment as depicted in figure 2 below) collection sites and greenhouse location within North-West University, Mahikeng campus. The sketches in green are departmental buildings within the campus



Fig. 9. 2 Soybean plants in pot cultures within the greenhouse. (a) pot experiment containing both PSC (used for % mycorrhizal colonization, physiological and biochemical analyses) and plants used for below-ground-above-ground and/or morphological evaluation and (b) pot experiments containing plants grown to maturity used only for below-ground-above-ground and/or morphological evaluation

9.1.2 Experimental Set-up and Soybean Growth Conditions

Soil samples for pot experiment were collected from topsoil (0-25 cm) with a shovel in plastic bags close to North-West University garden, Mahikeng campus (Figure 1). Soil samples were collected between July, 2017 and February 2018, homogenized and sieved using a 5.6 mm diameter round sieve. Sieved soil samples were packed in autoclavable plastics and sterilized at 121°C for 30 min in an autoclave (SA-300VL Autoclave, Taiwan). Autoclaving of soil was done three times to ensure complete elimination of microorganisms including spore-forming microorganisms from the soil since the first run would kill microbial vegetative cells, the second

run would kill vegetative cells of growing microbial spores present in the soil and the third run would eliminate the entire microbial cells. Sterility test was carried out on autoclaved soil by plating 1 ml of serially diluted soil sample using pour plate technique in potato dextrose agar (for fungi) and Luria Bertani agar (for bacteria) and incubated at 28±2°C and 37°C respectively. Absence of microbial growth in the culture plates confirmed that the autoclaved soil samples were sterile.

The experimental trial was set-up using 3 factorial completely randomized design (CRD) and the three factors included rhizobia, arbuscular mycorrhizal fungi (AMF) consortium and drought or water stress. ‘The experiment was conducted in a greenhouse under natural light’.

Three drought or water regimes were used in this study namely: 100, 70 and 40% field capacity (FC). The water stress levels (70 and 40% FC) were determined by the method described by Zarik et al. (2016) with little modifications. Six (6) small plastic pots were weighed (W1), the plastic pots + 100 g of soil were weighed (W2) and weight of 100 g of soil only (W3) was obtained by subtracting W2 from W1. The first plastic pot with soil was watered with 40 ml of water until ‘soil saturation’, the second, third, fourth, fifth and sixth pots were watered with 30, 20, 15 and 10 and 5 ml of water respectively. Watered pots were sealed with plastic and kept on the lab shelf. The sixth pot watered with 5 ml of water was not waterlogged after observing for 3 days and was weighed again in order to obtain its saturated weight and water holding capacity of the soil was subsequently calculated from this pot using the formula:

$$\text{Water holding capacity} = \frac{\text{Saturated weight} - \text{dry weight}}{\text{dry weight}} \times 100\%$$

Then, the water holding capacity (which represents the amount of water needed to saturate the soil) of the 8 kg of soil (used for the greenhouse experiment) was obtained from the water holding capacity obtained for the 6th pot above by direct proportionality.

The two rhizobial species (*Rhizobium* sp. strain R1 and *Rhizobium cellulosilyticum* strain R3) used in this study were isolated in our previous work from Bambara groundnut rhizosphere and identified by molecular methods. In the previous work, these bacteria were also found to possess some plant growth promoting traits and were tolerant to *in-vitro* osmotic stress induced by stress-stimulant [polyethylene glycol (PEG)] while the AMF consortium [consisted of *Paraglomus occulum* (molecular determination), *Gigaspora gigantea*, *Funneliformis mosseae* (previously *Glomus mosseae*), *Claroideoglomus etunicatum* (previously *Glomus etunicatum*) and *Rhizophagus clarus* (previously *Glomus clarum*)] was obtained from Department of Biochemistry and Microbiology, Rhodes University, Grahamstown, South Africa. The colonization ability of the AMF consortium was assessed by culturing for twelve (12) weeks in pot cultures using sorghum plant as a host. Sorghum was used because it has fine root system. The AMF consortium had 8-10 per gram and 50-100 per gram corresponding to spore number and most probable number (MPN). The rhizobia and AMF consortium used in this study have not been genetically modified in any way. *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 were grown and harvested according to the method of Prakamhang et al. (2015) with little modifications. Fresh cultures of the bacteria were grown in flasks containing 1000 ml nutrient broth in a shaker incubator (FMH200 Instruments) at 180 rpm for six days. Fully grown culture was centrifuged at 1000 g for 10 min and washed twice in a sterile 0.85% saline solution. The initial optical density (OD) of the bacteria was adjusted to 1.3 OD and the bacterial titer was

adjusted to 30×10^9 and 31×10^9 CFU (colony forming unit) ml^{-1} for *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 respectively.

The experiment was conducted in plastic pots (30 cm diameter, height 29 cm) containing 8 kg of dried and sterile soil. Twenty four (24) treatments with eight (8) replicates per treatment were set-up, giving rise to a total of 192 replicates (two plants per pot culture). Soybean seeds (PAN 1532 R cultivar) were washed in sterile distilled water, surface sterilized in 75% ethanol and then 1% sodium hypochlorite and rinsed severally to totally remove the chemicals. For single Rhizobial inoculation, approximately 240 surface sterilized soybean seeds were inoculated with 200 ml of *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 suspension each, co-inoculation was done by amending approximately 240 surface sterilized soybean seeds with 100 ml of *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 suspension each and 200 ml of sterile 0.85% saline water was added to approximately 480 surface sterilized soybean seeds for AMF consortium and control treatments. Flasks containing seeds were agitated (to thoroughly mix the seeds with the inocula) in a shaker incubator at 180 rpm at $28 \pm 2^\circ\text{C}$ for 24 hr. Thereafter, the liquid suspension was decanted and air-dried on a sterile aluminium foil paper in a sterilized laminar flow cabinet (Filta Matix Laminar Flow Carbinet) prior to sowing. Inoculated seeds were air-dried to allow the inocula to stick to the surface of the seeds (Seed coating). All the greenhouse pots containing 8 kg of sterile soil were watered with 360 ml of water (100% FC) before sowing. Five seeds were sowed per pot. Treatments involving AM fungi were inoculated by placing a tea spoonful (approximately 5 g) of AMF consortium in the soil before adding the seeds. Seed germination in Petri-dish was not carried out prior to sowing in this study because the viability of the soybean seeds was confirmed by Agricultural Research Council, Pretoria, SA (source of soybean seeds). Soybean bean plants were thinned to two plants per pot two weeks

after emergence. One of the plants was marked and termed as ‘plant for sample collection (PSC)’ used for assessment of physiological, biochemical and % mycorrhization parameters while the second plant was allowed to grow to maturity which was used to assess the below-ground-above-ground and/or morphological parameters. All the water treatments (100, 70 and 40% FC) were fully watered to the field capacity after every 72 hr for approximately three weeks after germination and thereafter, drought or water stress was initiated in the 70 and 40% FC treatments and were watered after every 48 hr with 252 and 144 ml of water respectively but we continued watering the 100 % FC treatments (the watering control) with 360 ml of water. Three weeks after initiating drought stress, pots were watered after every 72 hr until termination of the experiment so as to increase drought stress.

9.1.3 Physicochemical Parameter Analyses of Soil Samples

Prior to autoclaving, 500 g of homogenized and sieved soil was collected from the bulk of soil used for the pot experiment and kept at 4 °C for future analyses. Soil physical parameters such as silt (%), clay (%), clay (%) and chemical parameters such as iron, manganese, organic carbon, organic matter, potassium, magnesium content, pH and total nitrogen were determined as described by Rascovan et al. (2016) and Tajini et al. (2012) with little modifications.

9.1.4 Parameters Measured

9.1.4.1 Relative Water Content

The leaf relative water content was determined according to the methods described by Aroca et al. (2003) and Ortiz et al. (2015) with little modifications and the ‘youngest fully developed leaves of each plant’ were used. Fresh leaf samples were weighed (Fresh weight-FW) and placed in test tubes saturated with water and kept at 4°C for 48 h. Thereafter, the leaf

samples were weighed again to obtain the turgid weight (TW) and oven-dried at 60 °C for 24 h and dry weights (DW) were obtained. The leaf relative water content was calculated as follows:

$$\text{Leaf relative water content (\%)} = \frac{(FW-DW)}{(TW-DW)} \times 100$$

9.1.4.2 Electrolyte Leakage

The youngest leaves of approximately the same size from the youngest branch ‘toward the distal end’ were collected from the sampling plants and thoroughly rinsed with de-ionized water to remove electrolytes attached to the leaf surfaces. Leaf samples were placed in 45 ml falcon tubes containing 10 ml deionized of H₂O and incubated on a shaker incubator at 28±2°C for 24 h. Subsequently, electrical conductivity of the liquid suspension (Lt) was obtained using conductivity meter (PL-700AL, Taiwan) and samples were autoclaved at 120 °C for 1200 sec, cooled to 25°C and electrical conductivity of the liquid (L0) was obtained. The electrolyte leakage was calculated as follows:

$$\text{Electrolyte leakage (\%)} = \frac{L_t}{L_0} \times 100\% \text{ (Ortiz et al., 2015)}$$

9.1.4.3 Determination of Soluble Sugar Content

One hundred mg (100 mg) of leaf samples were homogenized in 4 ml of 80% ethanol and centrifuged for 10 min at 5000 rpm. One ml (1 ml) of the supernatant was mixed with 5 ml of concentrated sulphuric acid (H₂SO₄) and 1 ml of 5% phenol. Samples were vigorously agitated, allowed to cool for 30 min and absorbance was measured at 485 nm using spectrophotometer (ThermoSpectronic, South Africa). The concentration of soluble sugar was calculated from a standard curve ‘established with a reference glucose solution’ as described by Zarik et al. (2016); Dubois et al. (1956) with little modifications. Briefly, stock solutions of glucose were prepared by weighing 10, 20, 30, 40, 50, 60 and 70 mg of D (+)-glucose in 10 ml of sterile distilled water.

Two ml (2 ml) of each stock solution was pipetted into 3 cleaned test-tubes. One ml (1ml) of 5% phenol was added. Five ml (5 ml) of concentrated H₂SO₄ was quickly added. 'The tubes were allowed to stand for 10 min', then shaken and placed in a water bath for 10 min at 51-57°C. Tubes were allowed to cool for 30 min and absorbance was measured at 485nm using spectrophotometer and a standard curve was plotted from the values obtained.

9.1.4.4 Determination of Leaf Proline Content

Proline concentration was determined according to the methods of Ortiz et al. (2015) and Bates et al. (1973) with little modifications. Briefly, 1.25 g of ninhydrin was dissolved in 20 ml of 6 M phosphoric acid and 30 ml of glacial acetic acid by heating on a hot-plate with agitation. The solution was allowed to cool and kept at 4°C and the solution became stable after 24 hr. Approximately 500 mg of fresh soybean leaf sample was ground in 10 ml of 3% aqueous sulfo-salicylic acid and centrifuged at 10000 g for 10 min. Two ml (2 ml) of the supernatant was reacted with 2 ml of glacial acetic acid and 2 ml of acid-ninhydrin solution in 45 ml falcon tubes at 100°C in a water bath for 60 min and the reaction was stopped in an ice box. Four ml (4 ml) of toluene was added to extract the mixture and agitated vigorously for 15-20 sec in a shaker incubator at 250 rpm. The mixture was kept in the dark for 30 min and the 'chromophore containing toluene was aspirated from the aqueous phase and the absorbance was read at 520 nm using toluene for a blank'. The concentration of proline was estimated from a standard curve 'established with a reference proline solution'. Briefly, 1 mg/ml stock solution of proline was prepared by weighing 10 mg of proline (DL-Proline, China) in 10 ml of sterile water. 0, 50, 100, 150, 200, 250, 300 µl of the stock solution was pipetted into seven tubes containing 300, 250, 200, 150, 100, 50 and 0 µl of sterile water respectively. The mixtures were then reacted with 2 ml of glacial acetic acid and 2 ml of acid-ninhydrin solution in 45 ml falcon tubes at 100°C in a

water bath for 60 min and the reaction was stopped in an ice box. The mixture was vigorously agitated using a vortex (Vortex Genie, U.S.A) after adding 4 ml of toluene. The mixture was kept in the dark for 30 min and the absorbance of the proline-containing upper layer 'was read at 520 nm using toluene for a blank' and proline standard curve was plotted from the absorbance values.

9.1.4.5 Chlorophyll Content

Chlorophyll content was taken from the youngest fully developed leaf at the distal end of PSC using a chlorophyll content meter (CCM-200 plus).

9.1.4.6 AMF Spore Estimation

AM fungal spores were estimated by wet sieving and decanting methods as described by Pacioni (1992) with little modifications. Fifty g (50 g) of soil close to soybean root region was collected and mixed in 500 ml of sterile distilled water. The mixture was passed through a series of sieves of different sizes stacked together in an increasing order from the base to the top: 53, 63, 106 and 212 μm . The trapped fungal spores on the 53 μm were rinsed into a 1.5 ml vials and centrifuged at 1800 rpm for 5 min. The supernatant (1 ml) was mixed with 60% sucrose (0.5 ml) in a 1.5 ml vial and centrifuged at 1800 rpm for 5 min. The supernatant-containing AMF spores was decanted in a cleaned Petri-dish and examined under a stereomicroscope at X3 and X4 magnification.

9.1.4.7 Percentage Mycorrhizal Colonization

Soybean roots were cut into pieces of 1 cm long and cleared in 10% KOH at 121°C for 15 min in an autoclave (SA-300VL, Taiwan). Roots were covered with 2% HCl for 30 min after rinsing severally with distilled water. Root samples were subsequently covered with trypan blue solution (trypan blue solution consisted of 0.82 g trypan blue powder, 640 ml distilled water, 520

ml lactic acid and 480 ml glycerol) and autoclaved at 121°C for 15 min. Root samples were then de-stained in 50% glycerol and examined under a stereo-microscope (Ortiz et al., 2015, Phillips and Hayman, 1970).

9.1.4.8 Below-Ground-Above-Ground/Morphological Parameters

After harvesting soybean plants grown to maturity, data such as shoot height, shoot width, branch number, leaf number, taproot length, lateral root number, plant fresh weight, shoot dry weight and root dry weight were obtained according to the method described by Masciarelli et al. (2014). Soybean plant roots were rinsed severally and the plant fresh weights were immediately obtained by weighing the whole plant (including the yield components) on a weighing machine (Wagi Elektroniczne, Poland). The roots were subsequently separated from the shoot. The shoot height and taproot length were measured using a measuring tape; branch, leaf number, lateral root number were obtained by counting the number of fully developed branches, leaves lateral root (excluding the taproot) respectively. The separated shoots (excluding the yield components) and roots were oven-dried at 65°C for 48 hrs and weighed on a weighing machine to obtain the dry weights.

9.1.4.9 Above-Ground Yield Parameters

In this study, the following yield parameters were considered such as pod number, pod fresh weight, seed number, seed fresh weight, highest seed number per pod and seed dry weight. The pods were separated from soybean plants and pod number was determined by counting the number of fully developed pods. The pod with the highest number of seeds per treatment was identified and seed number was recorded and this was done for all the treatments. Then, pods were opened, seeds were separated from the pods and seeds were counted. Seed fresh weight was

determined by weighing seeds on a weighing machine. Seeds were oven-dried at 65°C for 48 hrs and weighed on a weighing machine to obtain the dry weights.

9.1.5 Statistical Analyses

The results of the experiment were cleaned and analyzed using Microsoft excel and SAS software package. Outliers were removed and data were transformed when necessary. General linear model analysis of variance (ANOVA) was used to evaluate the impact of each treatment and differences between mean (post hoc analysis) was determined by Duncan's multiple-range test (Duncan, 1955) and differences were significant at $p \leq 0.05$ (Igiehon, 2015, Dytham, 2011).

9.2 Results

The soil that was used in this study contain more of sandy soil (70%) and less of silt (8%) and the percentage of total nitrogen was low compared to 1.260 and 3.190% observed for organic carbon and organic matter respectively (Table 1). Also, the soil pH (7.390) was within the pH range that supports bacterial growth. Potassium (K) content was 406 mg/kg and K is known to play role(s) in plant tolerance to drought.

Table 9. 1 Physicochemical parameters of homogenized soil samples used for the greenhouse experiment

Parameter	Result
pH (H ₂ O)	7.390
Fe (mg/kg)	1.950
Mn (mg/kg)	45.400
Organic carbon (%)	1.260
Organic matter (%)	3.190
Phosphorus	N/D
Potassium (mg/kg)	406.000
Magnesium (mg/kg)	639.00
Total nitrogen (%)	0.095
Sand (%)	70.000
Silt (%)	8.000
Clay (%)	22.000

Generally, the non-inoculated soybean plants were negatively affected with drought stresses (70 and 40% FC) compared to soybean plants inoculated with rhizobia and mycorrhizal fungi. In all the microbial treatments, soybean plants exposed to 70 and 40% FC maintained high relative water content in their leaves compared to the non-inoculated (control) plants. Amazingly, at 40% water stress, the soybean plants that were dually inoculated with *rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium-MY (R1+R3MY) showed less percentage relative water content (29.562%) than what was observed in the control

treatments (1.42%). However, the increase was highly significant ($p < 0.05$) in the plants co-inoculated with *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 (R1+R3) at 40% FC (Figure 3).

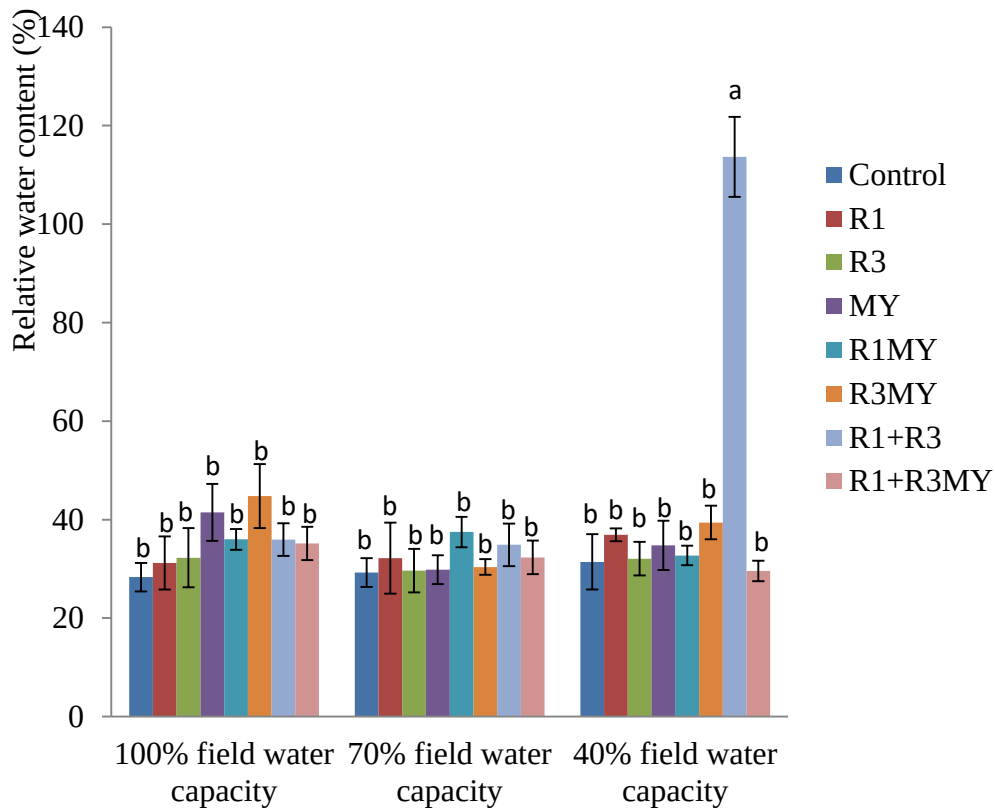


Fig. 9. 3 Relative water content of leaves of inoculated and non-inoculated soybean plants exposed to a 4-week period of drought stress. Control – non-inoculated treatment, R1 – *Rhizobium* sp. strain R1, R3 – *R. cellulosilyticum* strain R3, MY – mycorrhizal consortium, R1MY – *Rhizobium* sp. strain R1 and mycorrhizal fungal consortium, R3MY – *R. cellulosilyticum* strain R3 and mycorrhizal consortium, R1+R3 – *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3, R1+R3MY – *Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium. Number of replicates (n) = 8. Data represent mean $\pm$ SE

As regards electrolyte leakage, with the exception of soybean plants amended with R1+R3MY, soybean plants inoculated with *R. cellulosilyticum* strain R3 (R3) showed a better result at 70% FC than other inoculated soybean plants. But at this level of water stress, significant increase ($P<0.05$) was observed in non-inoculated treatment; the electrolyte leakage however decreased in 100% FC (Figure 4). In all the water stress levels, the control treatments were the most negatively impacted particularly at 40% FC which showed electrolyte leakage of 94.9%. Similarly, soybean plants inoculated with R1+R3MY showed the lowest electrolyte leakage values of 16.6, 17.3, and 16.2% at 100, 70 and 40% FC respectively compared to other treatments.

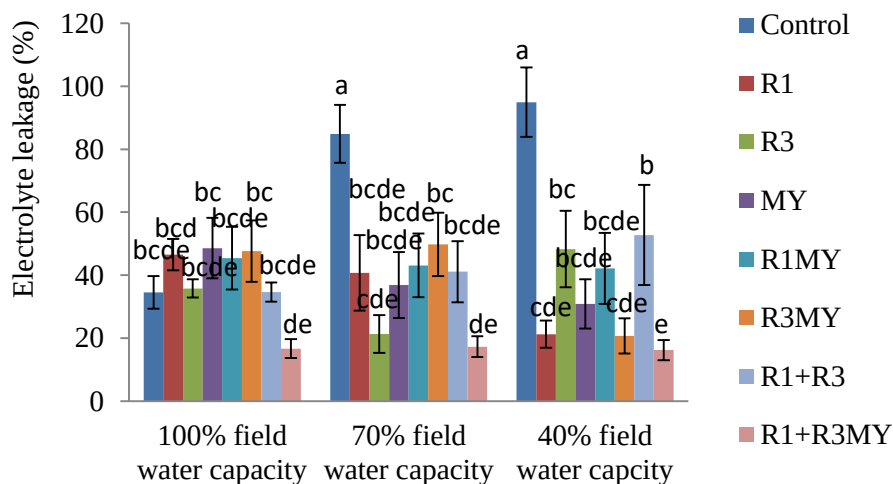


Fig. 9. 4 Electrolyte leakage of leaves of inoculated and non-inoculated soybean plants exposed a 4-week period of drought stress. Control – non inoculated treatment, R1 – *Rhizobium* sp. strain R1, R3 - *R. cellulosilyticum* strain R3, MY – mycorrhizal consortium, R1MY - *Rhizobium* sp. strain R1 and mycorrhizal fungal consortium, R3MY - *R. cellulosilyticum* strain R3 and mycorrhizal consortium, R1+R3 - *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3,

R1+R3MY - *Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium.

Number of replicates (n) = 8. Data represent mean $\pm$ SE

Furthermore, accumulation of soluble sugars was found to be more in soybean plants amended with single inoculation of *Rhizobium* sp. strain R1 (R1), dual inoculation of R1 and MY (R1MY), dual inoculation of R3 and MY (R3MY) as well as R1+R3 grown in 70% water stress compared to the same microbial treatments grown in 100% well-watered condition. At 40% FC, water stress and microbial inoculation were found to cause increase in the concentrations of soluble sugars in soybean plants inoculated with R1MY and R1+R3MY (Figure 5). Mycorrhizal consortium (MY), R1+R3 and R1+R3MY induced accumulation of the highest concentrations of soluble sugars in soybean plants exposed to 100% FC.

Accumulation of proline in soybean leaves was highest in plants exposed to 40% water stress. Soybean plants dually inoculated with R3MY was found to accumulate the highest concentration of proline which was significantly different ($P < 0.05$) from the non-inoculated (control) plants that produced the lowest amount of proline under 40% FC (Figure 6). Also, all the microbially treated soybean plants produced more proline than their counterparts that were not exposed to drought stress (100% FC). However, the non-inoculated soybean plants grown in 100% FC accumulated more proline than the non-inoculated soybean plants subjected to 70% FC. Furthermore, contrary to what was observed in the 40% water stress, soybean plants inoculated with R3 and R1+R3MY accumulated the highest concentration of proline in the 70% field water capacity. Also, R3 treatment accumulated more proline under well-watered condition (100% FC)

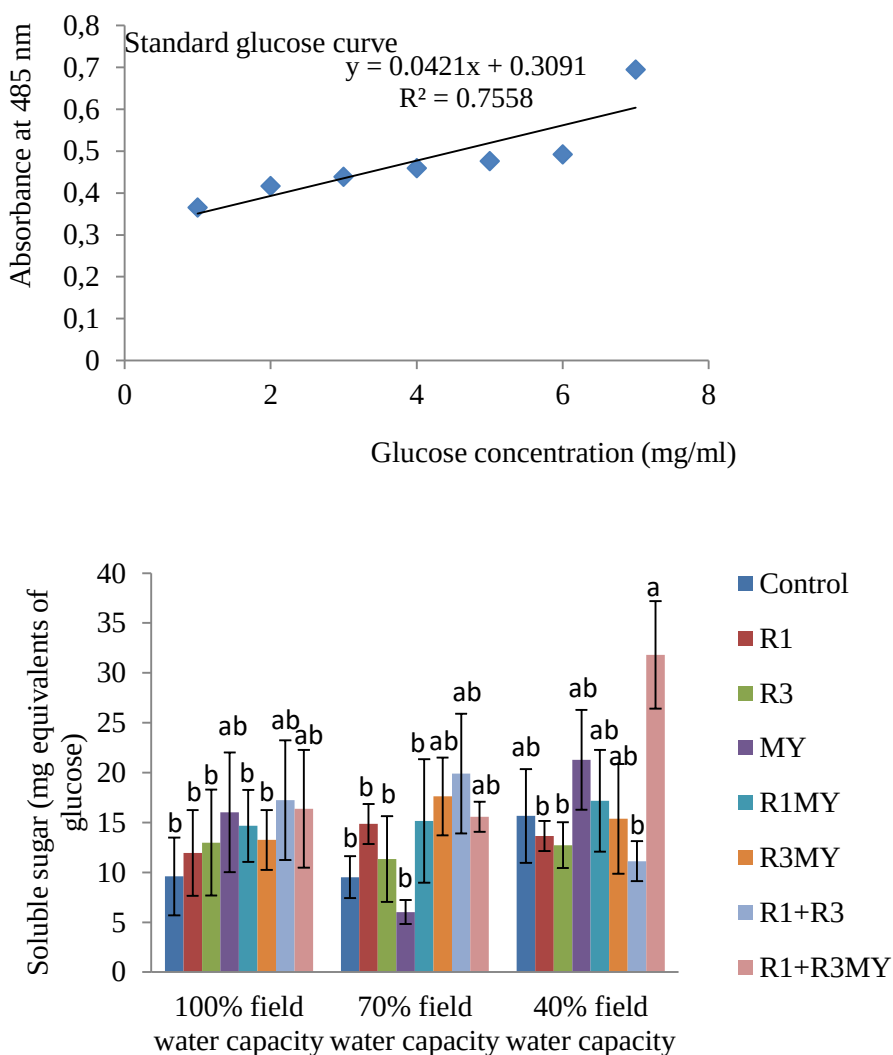


Fig. 9. 5 Soluble sugar accumulation in leaves of inoculated and non-inoculated soybean plants exposed to a 10-week period of drought stress. Control – non-inoculated treatment, R1 - *Rhizobium* sp. strain R1, R3 - *R. cellulosilyticum* strain R3, MY – mycorrhizal consortium, R1MY - *Rhizobium* sp. strain R1 and mycorrhizal fungal consortium, R3MY - *R. cellulosilyticum* strain R3 and mycorrhizal consortium, R1+R3 - *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3, R1+R3MY - *Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium. Number of replicates (n) = 8. Data represent mean $\pm$ SE

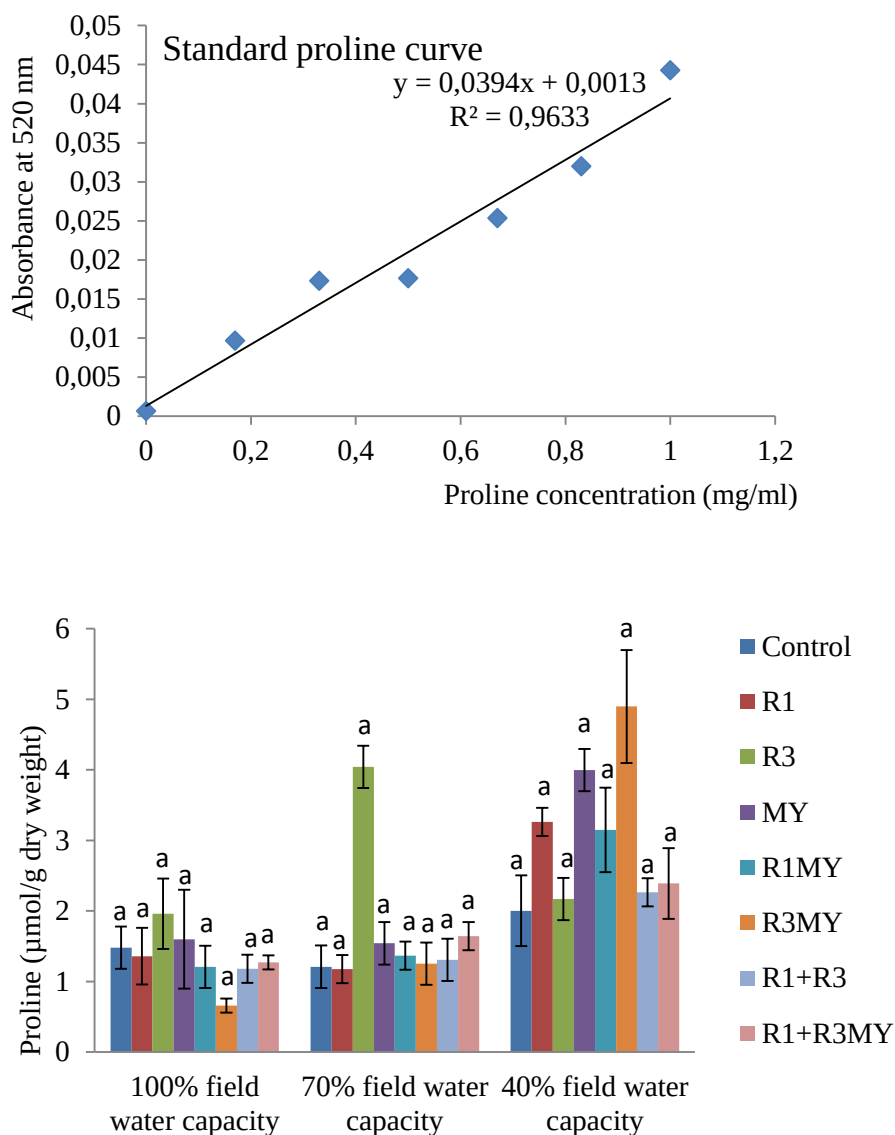


Fig. 9. 6 Proline accumulation in leaves of inoculated and non-inoculated soybean plants exposed to a 6-week period of drought stress. Control – non-inoculated treatment, R1 - *Rhizobium* sp. strain R1, R3 - *R. cellulosilyticum* strain R3, MY – mycorrhizal consortium, R1MY - *Rhizobium* sp. strain R1 and mycorrhizal fungal consortium, R3MY - *R. cellulosilyticum* strain R3 and mycorrhizal consortium, R1+R3 - *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3, R1+R3MY - *Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium. Number of replicates (n) = 8. Data represent mean $\pm$ SE

Amazingly, in soybean plants subjected to 40% water stress, R1, MY, R1MY and R1+R3MY inoculants were observed to increase the chlorophyll content of soybean leaves more than the control plants (exposed to 40, 70 and 100% FC) and their counterparts subjected to 70 and 100% water stress (Figure 7). The non-inoculated (control) soybean plants grown in 70% FC showed the lowest chlorophyll content (11.375 CCI-chlorophyll content index) among all the plant treatments subjected to 70% water stress. At this water stress level, the greatest impact on chlorophyll content was detected in soybean plants dually amended with R1MY and R1+R3MY. In addition, in 100% FC, the control plants had the lowest chlorophyll content of approximately 10.9 CCI and this increased in all the inoculated plant treatments at this water level. Specifically, approximately 11.6, 12.0, 11.8, 11.6, 15.1, 11.8 and 13.3 CCI were recorded in plants treated with R1, R3, MY, R1MY, R3MY, R1+R3 and R1+R3MY respectively in 100% FC.

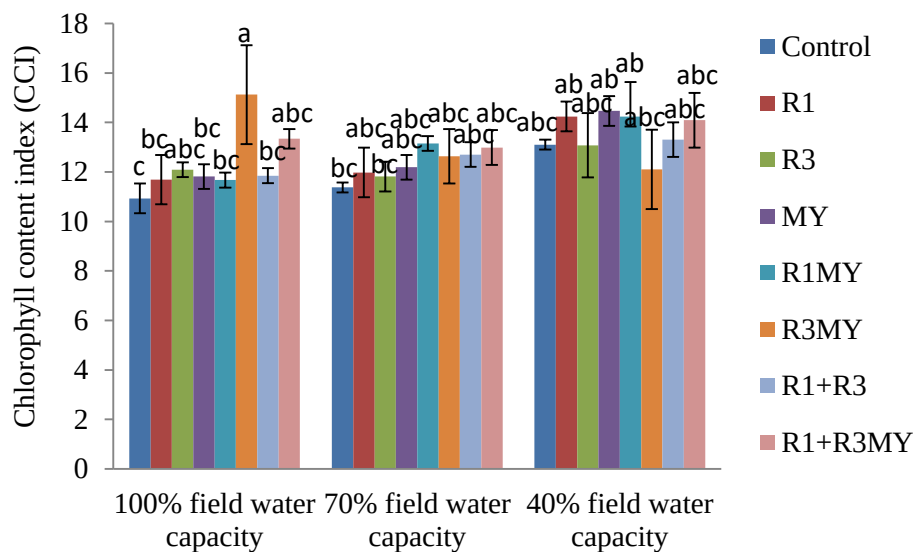


Fig. 9. 7 Chlorophyll content of leaves of inoculated and non-inoculated soybean plants exposed to a 3-week period of drought stress. Control – non-inoculated treatment, R1 - *Rhizobium* sp. strain R1, R3 - *R. cellulosilyticum* strain R3, MY – mycorrhizal consortium, R1MY - *Rhizobium* sp. strain R1 and mycorrhizal fungal consortium, R3MY - *R. cellulosilyticum* strain R3 and

mycorrhizal consortium, R1+R3 - *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3, R1+R3MY - *Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium.

Number of replicates (n) = 8. Data represent mean $\pm$ SE

The treatment with the microbial combination R1+R3MY showed the highest number of spore in all the water levels (100, 70, 40% FC) while the lowest spore number was recorded for treatment solely inoculated with mycorrhizal consortium (MY). We observed increase in spore number in soybean plants dually inoculated with rhizobia and mycorrhizal fungi compared to the plants that were solely treated with mycorrhizal consortium. This increase was significant ($P < 0.05$) particularly in treatments dually inoculated with R3MY and R1+R3MY. In addition, treatments exposed to 40% water stress had the least spore number compared to the 70 and 100% FC. Expectantly, control, R1, R3 and R1+R3 treatments did not show presence of fungal spore (Figure 8a) since these treatments were not amended with mycorrhizal fungi (Figure 8).

Similar results were also observed for percentage (%) mycorrhization and the highest colonization levels were revealed in soybean plants co-inoculated with R1+R3MY in all the water regimes. Mycorrhizal colonization (Figure 8a) was observed to be more in the treatments co-inoculated with rhizobia and mycorrhizal fungi and coincidentally, colonization level in soybean plants inoculated with R1MY was approximately 58% in treatments subjected to 70 and 40% FC. Also, R3MY inoculant was able to colonize soybean plants exposed to 70% FC to the same level revealed in plants subjected to 100% FC (Figure 8). The non-inoculated soybean plants and plants inoculated with only rhizobial species were not colonize with mycorrhizal since these treatments were not inoculated with mycorrhizal fungi (Figure 8)

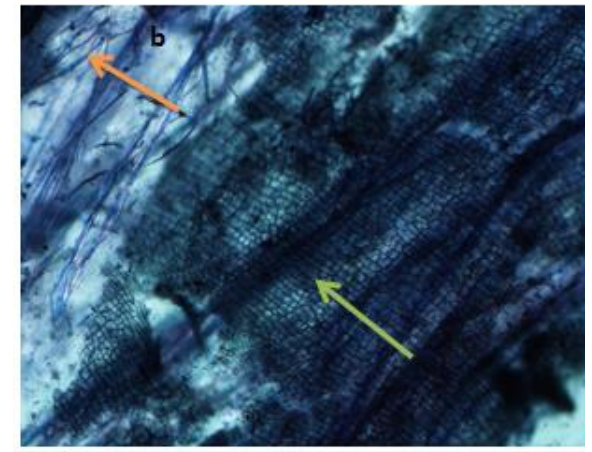
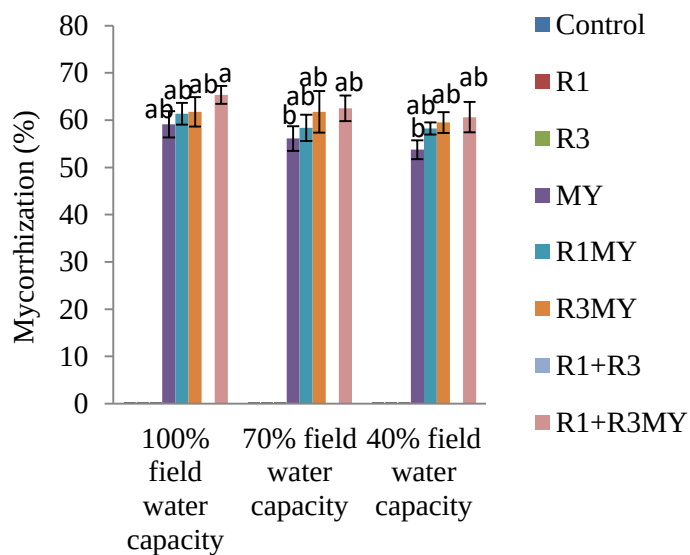
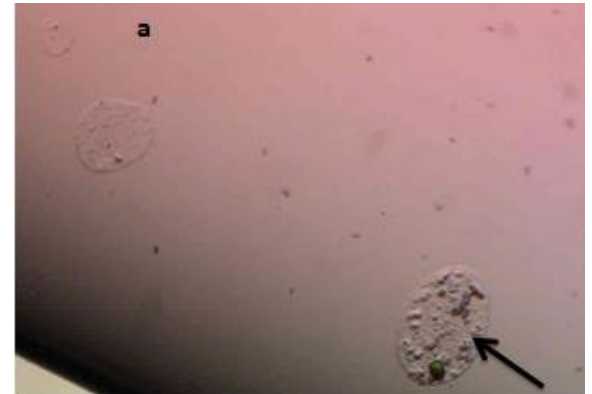
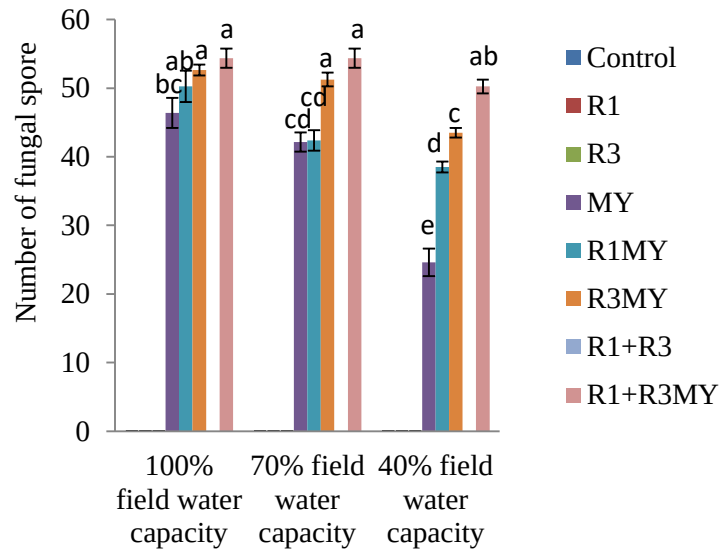


Fig. 9. 8 Spore number and percentage colonization level of mycorrhizal of inoculated and non-inoculated soybean plants exposed to a 10-week period of drought stress. Control – non-inoculated treatment, R1 - *Rhizobium* sp. strain R1, R3 - *R. cellulosilyticum* strain R3, MY – mycorrhizal consortium, R1MY - *Rhizobium* sp. strain R1 and mycorrhizal fungal consortium, R3MY - *R. cellulosilyticum* strain R3 and mycorrhizal consortium, R1+R3 - *Rhizobium* sp. strain

R1 and *R. cellulosilyticum* strain R3, R1+R3MY - *Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium. (a) Represents AMF spores (b) represents root colonized with AMF. Black, green and orange arrows point towards fungal spores, arbuscule and hyphal respectively. Number of replicates (n) = 8. Data represent mean $\pm$ SE

Microbial (rhizobia and mycorrhizal) inoculation enhanced the growth of soybean plants virtually in all the water regimes compared to the non-inoculated (control) treatments (Table 2). We noticed that under different water regimes, inoculated soybean plants maintained higher below-ground-above-ground biomass (such as shoot height, shoot width, branch number, leaf number, taproot length, taproot width, lateral root number, plant fresh weight, shoot dry weight and root dry weight) compared to the non-inoculated plants (Table 2).

Particularly, at 40% field water capacity, soybean plants amended with R1+R3MY had significant effects ($P < 0.05$) on the shoot width and branch number of soybean plants. Mycorrhizal consortium (MY) had the greatest impact on shoot height with a mean height of 185.9 mm while the lowest shoot height was observed in non-inoculated soybean plants with an average height of 171.4 mm. We noticed positive effects of synergistic interaction on leaf number in treatments amended with R3MY and R1+R3 which showed mean leaf number of 10.8 and 9.1 respectively. MY, R3 and R1MY had the greatest impacts on the below-ground parameters taproot length, taproot width and lateral root number respectively while more effects were seen in root dry weight in plants treated with R1MY and R1+R3MY with a mean of 0.2 g (Table 2). Also, at 40% water stress, the below-ground-above-ground plant fresh weights of all the treatments ranged from 1.03-1.5 g with the smallest and largest weight corresponding to non-inoculated and R1MY treatments.

For 70% field water capacity, the rhizobial and mycorrhizal amended plants revealed an increase in virtually all the below-ground-above-ground parameters measured compared to the 40% water stressed soybean treatments. Interestingly, highly significant effect ($P < 0.05$) of R1+R3MY inoculation was observed on the above-ground shoot height (Table 2). Also, the R1MY microbial treatment had the greatest effects on the above-ground parameters shoot width, branch number, leaf number and shoot dry weight corresponding to the mean values of 3.7 mm, 8.1, 19.5 and 1.1 g. On the other hand, the below-ground-above-ground biomass of non-inoculated (control) plants received the greatest negative impacts of the 70% water stress since this treatment showed the lowest values for shoot height, shoot width, branch number, leaf number and shoot dry weight (Table 2). The below-ground parameters taproot length was positively and maximally impacted by R3MY, taproot width by MY and R1+R3MY and lateral root number by R3. But, the greatest impact on root dry weight was observed in all the dually inoculated plants and R1 treatments.

For 100% field water capacity, the non-inoculated plants were least productive as shown in all the above-ground and half of the below-ground components (taproot length and lateral root number) measured (Table 2). Mycorrhizal consortium (MY) treatment showed a better effect on all the above-ground components with the exception of shoot width which was enhanced significantly ($P < 0.05$) by R1+R3 and R1+R3MY treatments. Considering the below-ground components, maximal impacts were observed in plant taproot treated with R1MY, taproot width treated with R3MY, lateral root number treated with MY and root dry weight treated with R1, R3, MY and R1MY. The greatest below-ground-above-ground plant fresh weight (4.9 g) was observed in plants inoculated with R1 which was significantly different from other plant treatments grown in 100% FC including those exposed to 70 and 40% FC (Table 2).

Table 9. 2 Effects of rhizobial and mycorrhizal inoculation on below-ground-above-ground parameters of soybean plants exposed to a 16-week period of drought stress

Treatment	Shoot height (mm)	Shoot width (mm)	Branch Number	Leaf number	Tap root length (mm)	Tap root width (mm)	Lateral root number	Plant fresh weight (g)	Shoot dry weight (g)	Root dry weight (g)
Control 40	171.4±14.48 ^{gh}	1.8±0.16 ⁱ	2.8±0.33 ^{gh}	4.6±0.46 ^e	49.9±19.66 ^d	1.3±0.31 ^{abcde}	12.9±3.57 ^f	1.03±0.22 ^e	0.3±0.02 ^d	0.1±0.02 ^{bc}
R1 40	195.9±16.69 ^{defgh}	3.1±0.21 ^{abc}	2.6±0.63 ^h	4.5±1.27 ^e	64.3±14.12 ^{cd}	1.1±0.28 ^{bcde}	14.3±2.05 ^f	1.2±0.22 ^e	0.4±0.05 ^d	0.1±0.02 ^c
R3 40	174.6±6.21 ^{fgh}	3.0±0.19 ^{abcd}	5.0±0.46 ^{cdefgh}	7.4±0.82 ^e	121.0±20.29 ^{abc}	1.5±0.31 ^{abcd}	13.9±2.21 ^f	1.3±0.15 ^e	0.6±0.05 ^d	0.1±0.02 ^{abc}
MY 40	185.9±9.65 ^{efgh}	1.9±0.23 ^{hi}	3.0±0.42 ^{gh}	8.6±1.43 ^e	123.1±25.92 ^{abc}	1.4±0.17 ^{abcd}	27.1±4.36 ^{abcde}	1.3±0.18 ^e	0.6±0.07 ^d	0.1±0.02 ^{abc}
R1MY 40	174.7± 14.59 ^{fgh}	2.2± 0.19 ^{fghi}	3.0± 0.78 ^{gh}	8.9± 2.84 ^e	91.6± 8.27 ^{bcd}	1.3± 0.24 ^{abcd}	29.6± 3.69 ^{abcd}	1.5± 0.24 ^e	0.6± 0.05 ^d	0.2± 0.03 ^{abc}
R3MY40	177.0± 12.72 ^{fgh}	1.9± 0.13 ^{hi}	3.9± 0.81 ^{efgh}	10.8± 2.48 ^{de}	97.3± 19.41 ^{abcd}	0.9± 0.27 ^{cde}	19.9± 3.94 ^{def}	1.4± 0.23 ^e	0.5± 0.05 ^d	0.1± 0.03 ^{abc}
R1+R3 40	182.3± 12.91 ^{fgh}	2.0±0.00 ^{hi}	3.4± 0.37 ^{fgh}	9.1± 1.13 ^e	102.6± 13.57 ^{abcd}	1.2± 0.21 ^{bcde}	25.8± 2.58 ^{bcde}	1.4± 0.15 ^e	0.5± 0.04 ^d	0.1± 0.02 ^{abc}
R1+R3MY 40	159.0± 11.38 ^h	3.1± 0.07 ^{abc}	5.1± 0.63 ^{cdefgh}	6.7± 1.78 ^e	105.9± 12.70 ^{abcd}	1.4± 0.14 ^{abcd}	23.1± 1.42 ^{abcdef}	1.3± 0.28 ^e	0.5± 0.09 ^d	0.2± 0.02 ^{abc}
Control 70	206.0± 10.24 ^{cdefgh}	1.9± 0.06 ^{hi}	4.4± 0.43 ^{cdefgh}	12.6± 1.32 ^{de}	118.9± 21.31 ^{abcd}	1.4± 0.18 ^{abcd}	26.6± 3.13 ^{abcde}	1.4± 0.13 ^e	0.5± 0.12 ^d	0.1±0.01 ^{abc}
R1 70	237.6±8.45 ^{bcd}	3.6±0.18 ^{ab}	8.1±0.72 ^{abc}	16.1±2.17 ^{cde}	97.4±26.80 ^{abcd}	1.3±0.28 ^{abcde}	22.4±3.55 ^{cdef}	2.0±0.24 ^{bcde}	0.7±0.07 ^d	0.2±0.02 ^{abc}
R3 70	209.9±11.08 ^{bcdefgh}	2.4±0.26 ^{defghi}	5.4±0.75 ^{cdefgh}	14.6±3.04 ^{cde}	134.6±13.05 ^{ab}	1.2±0.12 ^{abcde}	34.3±2.14 ^{ab}	1.5±0.22 ^e	0.6±0.13 ^d	0.1±0.01 ^{bc}
MY 70	240.3±10.24 ^{bcd}	2.3±0.17 ^{efghi}	5.4±0.42 ^{cdefgh}	17.1±1.94 ^{cde}	139.1±21.31 ^{ab}	1.6±0.18 ^{abc}	33.4±3.13 ^{abc}	1.7±0.24 ^{de}	0.6±0.09 ^d	0.1±0.01 ^{abc}
R1MY 70	209.6±13.12 ^{bcdefgh}	3.7±0.14 ^a	8.1±0.67 ^{abc}	19.5±3.57 ^{cde}	113.1±11.15 ^{abcd}	1.4±0.11 ^{abcd}	27.1±1.33 ^{abcde}	4.2±0.98 ^e	1.1±0.26 ^{dc}	0.2±0.26 ^{abc}
R3MY 70	222.9±12.35 ^{bcdefg}	2.6±0.25 ^{cdefgh}	5.4±0.49 ^{cdefgh}	16.1±2.35 ^{cde}	168.0±12.50 ^{ab}	0.5±0.07 ^e	16.6±0.88 ^{ef}	1.4±0.23 ^e	0.8±0.14 ^d	0.2±0.02 ^{abc}
R1+R3 70	225.6±10.17 ^{bcdef}	2.1±0.29 ^{ghi}	6.3±1.56 ^{bcdef}	15.9±2.94 ^{cde}	142.0±27.94 ^{ab}	1.3±0.21 ^{abcde}	27.4±3.16 ^{abcde}	1.9±0.55 ^{cde}	0.7±0.14 ^d	0.2±0.07 ^{abc}
R1+R3MY70	259.8±17.91 ^{ab}	2.3±0.16 ^{defghi}	5.0±0.42 ^{cdefgh}	17.5±3.01 ^{cde}	143.3±23.14 ^{ab}	1.6±0.171 ^{abcd}	30.4±2.95 ^{abcd}	1.9±0.34 ^{cde}	0.7±0.16 ^d	0.2±0.02 ^{abc}
Control 100	214.4±33.71 ^{bcdefgh}	2.1±0.21 ^{ghi}	6.1±0.85 ^{bcdefg}	18.5±5.15 ^{cde}	89.75±16.75 ^{bcd}	0.8±0.13 ^{de}	2.0±0.57 ^{ef}	0.8±0.25 ^f	0.8±0.25 ^d	0.2±0.03 ^{abc}
R1 100	240.4±20.33 ^{bcd}	2.8±0.23 ^{cdefg}	9.1±1.75 ^{ab}	37.5±10.92 ^{ab}	107.5±20.98 ^{abcd}	1.4±0.36 ^{abcd}	33.0±4.95 ^{abc}	4.9±1.58 ^a	2.3±0.81 ^{ab}	0.3±0.06 ^{ab}
R3 100	247.1±21.39 ^{abcd}	2.3±0.29 ^{fghi}	6.3±1.72 ^{bcde}	21.9±9.73 ^{bcde}	96.0±30.16 ^{abcd}	1.2±0.39 ^{abcde}	29.3±5.35 ^{abcd}	3.6±1.95 ^{abcde}	1.3±0.78 ^{bcd}	0.3±0.12 ^{abc}

MY 100	291.3±13.19 ^a	2.9±0.35 ^{bcdef}	9.5±0.93 ^a	47.8±8.99 ^a	155.9±15.64 ^{ab}	1.8±0.25 ^{ab}	37.5±3.53 ^a	4.4±1.19 ^{ab}	2.5±0.73 ^a	0.3±0.06 ^a
R1MY 100	260.9±20.42 ^{ab}	2.8±0.31 ^{cdefg}	8.8±1.66 ^{ab}	39.0±13.54 ^{ab}	166.9±19.76 ^a	1.3±0.15 ^{abcde}	31.1±4.14 ^{abcd}	3.4±0.9 ^{abcde}	2.0±0.71 ^{abc}	0.3±0.06 ^{abc}
R3MY 100	236.5±15.66 ^{bcde}	3.0±0.41 ^{abcde}	7.4±1.13 ^{abcd}	28.5±9.75 ^{bcd}	94.4±17.27 ^{bcd}	1.9±0.17 ^a	30.9±4.16 ^{abcd}	4.3±1.29 ^{abc}	1.3±0.42 ^d	0.1±0.05 ^{abc}
R1+R3 100	251.8±19.56 ^{abc}	3.7±0.29 ^a	6.8±0.90 ^{abcde}	22.1±4.79 ^{bcde}	149.8±30.93 ^{ab}	0.9±0.12 ^{cde}	29.8±4.69 ^{abcd}	2.8±0.62 ^{abcde}	0.9±0.30 ^{dc}	0.2±0.03 ^{abc}
R1+R3MY 100	246.8±13.14 ^{abcd}	3.8±0.31 ^a	8.1±0.58 ^{abc}	33.0±4.72 ^{abc}	128.9±16.2 ^{abc}	0.8±0.12 ^{cde}	25.5±1.94 ^{bcde}	4.1±0.51 ^{abcd}	1.3±0.18 ^{bcd}	0.2±0.04 ^{abc}

Legend: Control – non - inoculated treatment, R - *Rhizobium* sp. strain R1, R3 - *R. cellulosilyticum* strain R3, MY - Mycorrhizal consortium, R1MY - *Rhizobium* sp. strain R1 and mycorrhizal fungal consortium, R3MY - *R. cellulosilyticum* strain R3 and mycorrhizal consortium, R1+R3 - *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3, R1+R3MY - *Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium, mm – millimeter, g – gram. Data represent mean ± SE. According to Duncan's multiple range test (n = 8), values that have common letters are not different significantly (P<0.05)

Irrespective of the water treatment, rhizobial and mycorrhizal inoculation increased soybean yield components such as pod number, pod fresh weight, seed number, seed fresh weight, highest seed number per pod and seed dry weight. For 40% FC, R1+R3MY inoculum had the greatest impact on pod number, seed number, seed fresh weight, highest seed number per pod and seed dry weight (Table 3). But R1MY and R1+R3 were observed to have the greatest effects on pod fresh weight with a mean weight of 0.16 g each at this level of water stress. However, the non-inoculated plants and plants that were singly inoculated with R1 and R3 did not produce seeds even though they had pods. Significant effects ($P<0.05$) of R1+R3MY inoculation were not only observed on seed number, seed fresh weight and seed dry weight but also on pod number and highest number per pod compared to other microbial and non-microbial or control treatments subjected to the 40% FC.

On the other hand, we observed that the effects of R1+R3MY treatment decreased at 70% FC, but the decrease was only significant for highest seed number per pod and seed dry weight (Table 3). At this level of water stress, significant impacts ($P<0.05$) of R1MY inoculation were observed on pod number, pod fresh weight, seed number and seed dry weight (Table 3). Unlike in 40% FC, the control, R1 and R3 treatments produced seeds at 70% FC. Overall, 70% water stress negatively affected the control soybean plants compared to the microbially amended plants (Table 3). In particular, the mean pod fresh weight, seed number, seed fresh weight, highest seed number per pod and seed dry weight observed in the control treatment corresponds to 0.12 g, 0.5, 0.05 g, 0.05 and 0.01 g but only the highest seed number per pod was significantly less than all inoculated treatments while others were not significantly less ($P>0.05$) than some of the inoculated treatments (Table 3).

A similar pattern was noticed in inoculated and non-inoculated plants grown in 100% water level. R1 treatment produced more seed with the greatest pod and seed fresh weight. Generally, under this water regime, the non-inoculated plants were less productive compared to the other treatments as depicted by the yield components measured (Table 3).

Table 9. 3 Effects of rhizobial and mycorrhizal inoculation on the yield components of soybean plants exposed to a 16-week period of drought stress

Treatment	Pod number	Pod fresh weight (g)	Seed number	Seed Fresh weight (g)	Highest seed number per pod	Seed dry weight (g)
Control 40	0.50±0.33 ^{de}	0.01±0.00 ^d	0.00±0.00 ^d	0.00±0.00 ^c	0.00±0.00 ^f	0.00±0.00 ^b
R1 40	0.25±0.25 ^e	0.03±0.03 ^d	0.00±0.00 ^d	0.00±0.00 ^c	0.00±0.00 ^f	0.00±0.00 ^b
R3 40	0.13±0.44 ^e	0.01±0.13 ^d	0.00±0.00 ^d	0.00±0.00 ^c	0.00±0.00 ^f	0.00±0.00 ^b
MY 40	0.88±0.38 ^{bcde}	0.13±0.20 ^{cd}	0.510±0.59 ^d	0.04±0.09 ^c	0.75±0.38 ^{bcdef}	0.03±0.03 ^b
R1MY 40	0.50±0.87 ^{de}	0.16±0.61 ^{cd}	0.63±1.36 ^d	0.05±0.31 ^c	0.50±0.31 ^{def}	0.01±0.12 ^b
R3MY 40	0.38±0.35 ^{de}	0.05±0.33 ^d	0.25±0.74 ^d	0.01±0.07 ^c	0.25±0.41 ^{ef}	0.02±0.02 ^b
R1+R3 40	0.25±0.25 ^e	0.16±0.11 ^{cd}	0.75±0.49 ^d	0.07±0.05 ^c	0.50±0.33 ^{def}	0.03±0.02 ^b
R1+R3MY 40	0.57±0.43 ^{bcde}	0.10±0.08 ^{cd}	1.14±0.73 ^{bcd}	0.10±0.07 ^{bc}	1.14±0.73 ^{abcd}	0.093±0.08 ^{ab}
Control 70	1.12±0.35 ^{bcde}	0.12±0.04 ^{cd}	0.50±0.19 ^d	0.05±0.19 ^c	0.50±0.19 ^{def}	0.01±0.00 ^b
R1 70	1.25±0.37 ^{bcde}	0.27±0.09 ^{cd}	0.88±0.39 ^d	0.06±0.03 ^c	0.75±0.31 ^{bcdef}	0.04±0.02 ^b
R3 70	0.88±0.44 ^{bcde}	0.25±0.13 ^{cd}	1.13±0.58 ^{dc}	0.11±0.07 ^{bc}	0.75±0.37 ^{bcdef}	0.03±0.02 ^b
MY 70	1.00±0.38 ^{bcde}	0.53±0.21 ^{bcd}	1.50±0.59 ^{bcd}	0.19±0.09 ^{bc}	1.00±0.38 ^{abcdef}	0.06±0.03 ^b
R1MY70	2.50±0.87 ^{abcde}	1.17±0.61 ^{abc}	2.88±1.35 ^{abcd}	0.50±0.31 ^{abc}	1.25±0.31 ^{abcde}	0.19±0.12 ^{ab}
R3MY 70	1.13±0.35 ^{bcde}	0.95±0.33 ^{abcd}	1.13±0.74 ^{dc}	0.07±0.07 ^c	0.63±0.42 ^{cdef}	0.03±0.02 ^b
R1+R3 70	2.13±0.58 ^{abcde}	0.65±0.24 ^{bcd}	3.00±0.96 ^{abcd}	0.28±0.11 ^{abc}	1.25±0.37 ^{abcde}	0.14±0.05 ^{ab}
R1+R3MY 70	1.88±0.39 ^{abcde}	0.56±0.25 ^{bcd}	2.25±0.77 ^{bcd}	0.26±0.15 ^{bc}	1.25±0.31 ^{abcde}	0.08±0.05 ^b
Control 100	0.17±0.11 ^{cde}	0.17±0.11 ^{cd}	0.50±0.27 ^d	0.03±0.02 ^c	0.50±0.28 ^{def}	0.01±0.01 ^b
R1 100	4.38±1.99 ^a	1.82±0.89 ^a	6.25±2.96 ^a	0.78±0.41 ^a	1.75±0.49 ^{abc}	0.23±0.12 ^{ab}
R3 100	2.75±1.19 ^{abcde}	0.79±0.37 ^{abcd}	2.63±1.05 ^{bcd}	0.29±0.13 ^{abc}	1.13±0.39 ^{abcdef}	0.08±0.04 ^b
MY 100	3.38±1.71 ^{abc}	0.99±0.53 ^{abcd}	2.51±1.54 ^{abcd}	0.27±0.19 ^{abc}	2.88±0.39 ^{abcdef}	0.37±0.07 ^{ab}

R1MY 100	3.13±0.58 ^{abcd}	0.76±0.21 ^{bcd}	2.25±0.90 ^{bcd}	0.22±0.09 ^{bc}	0.88±0.29 ^{abcdef}	0.06±0.03 ^b
R3MY100	3.50±1.28 ^{ab}	1.17±0.46 ^{abc}	5.00±2.33 ^{ab}	0.61±0.32 ^{ab}	2.00±0.38 ^a	0.35±0.28 ^a
R1+R3 100	2.88±0.95 ^{abcde}	0.80±0.24 ^{abcd}	2.88±0.74 ^{abcd}	0.31±0.09 ^{abc}	1.75±0.31 ^{abc}	0.09±0.03 ^{ab}
R1+R3MY100	4.13±1.16 ^a	1.38±0.46 ^{ab}	4.63±1.31 ^{abc}	0.62±0.28 ^{ab}	1.88±0.48 ^{ab}	0.23±0.12 ^{ab}

Legend: Control – non-inoculated treatment, R1 - *Rhizobium* sp. strain R1, R3 - *R. cellulosilyticum* strain R3, MY - Mycorrhizal consortium, R1MY - *Rhizobium* sp. strain R1 and mycorrhizal fungal consortium, R3MY - *R. cellulosilyticum* strain R3 and mycorrhizal consortium, R1+R3 - *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3, R1+R3MY - *Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium, 40 – 40% field water capacity, 70 – 70% field water capacity, 100 – 100% field water capacity, g - gram. Data represent mean ± SE. According to Duncan's multiple range test (n = 8), values that have common letters are not different significantly (P<0.05)

9.3 Discussion

The drought experiment in this present study was conducted in two adjacent greenhouses located in North-West University, North-West Province, South Africa (Figure 1). North-West Province is known to be semi-arid and this fact was confirmed by our previous study which demonstrated that the highest amount of rainfall recorded in one of the months within the planting season of soybean in this region was 3.88 mm which is less than the optimal amount of rainfall (31.07 mm) needed for soybean growth as reported by (Prakamhang et al., 2015).

In this study, physiological, biochemical and below-ground-ground or morphological parameters connected to drought tolerance in soybean plants amended with rhizobia and mycorrhizal consortium under water stress conditions were investigated. It was observed that water levels (40 and 70% FC) had impacts on virtually all the physiological (% relative water content and % electrolyte leakage), biochemical (soluble sugars and proline content) and below-ground-above-ground or morphological (shoot height, shoot width, branch number, leaf number, taproot length, taproot width, lateral root number, plant fresh weight, shoot dry weight, root dry weight, pod number, pod fresh weight, seed number, seed fresh weight, highest seed number per pod, seed dry weight, number of fungal spore and % mycorrhizal) parameters of soybean plants grown in a controlled or greenhouse environment (Figure 2).

Our results indicated that virtually all the singly and dually inoculated soybean plants showed higher percentage relative water content (% RWC) under 40 and 70% FC. However, water stress reduced the RWC in non-inoculated soybean plants exposed to both water stressed treatments and plants inoculated with R1+R3MY grown in 40% FC (Figure 3). Overall, this current study unveiled that microbial inoculations enhanced RWC in soybean plant leaves under water stress.

RWC is used to evaluate water level balance in plants and it is a dependable strategy by which the level of osmotic stress is determined (Zarik et al., 2016). Indeed, RWC helps to determine the level of water in plants which is pointer to the metabolic state in plant tissues. Under water stress, RWC is usually considered an excellent indicator of drought stress tolerance in different plants (Anjum et al., 2011, Shaw et al., 2002, Gholami et al., 2012, Rostami and Rahemi, 2013). Thus, in this study, MY treatment was able to increase RWC from 29.8 (in 70% FC) to 34.8% (in 40% FC). This confirms the reports that mycorrhizal fungi increased leaf RWC (Baslam and Goicoechea, 2012, Aroca et al., 2008). In addition, some single and dual inoculations similarly led to increase in RWC and this increase was pronounced in plants treated with R1+R3. The mechanisms associated with this increase in RWC by microbial isolates is linked to as reported by Zarik et al. (2016) “stomatal regulation through hormonal signals (Aroca et al., 2008); higher stomatal conductance and transpiration fluxes (Zhu et al., 2012); indirect effect of improved phosphate and other nutrient uptake (Goicoechea et al., 1997b); greater osmotic adjustment (Wu and Xia, 2006) and/or higher root hydraulic conductivity (Augé, 2001)” than non-inoculated (control) plants.

Plant responses to water stress have also been evaluated based on physiological values derived from electrolyte leakage (Ortiz et al., 2015) which in this present study were lowest particularly in soybean plants dually inoculated with rhizobial spp. and mycorrhizal consortium (R1+R3MY) and highest in the non-inoculated plants (Figure 4). The low level of electrolyte leakage is a pointer to “cell membranes stability” and drought stress tolerance (Berglund et al., 2004). Cell membrane is very crucial in monitoring the efficacy of plant root-microbial interactions (Bashan et al., 2004). Most of the microbial inocula used in this study reduced plant membrane damaged in drought stressed soybean plants since they showed relatively low %

electrolyte leakage compared to the non-inoculated plants. This result is in agreement with the results obtained by Ortiz et al. (2015) who reported low electrolyte leakage values in *Trifolium repens* amended with selected (PGPR) and AMF in arid soil environment.

In addition to the physiological approach, plants also use biochemical strategy to endure or tolerate water stress by accumulating high level of soluble sugars and proline which help to regulate cell osmotic potential under water deficit conditions (Zhang et al., 2010). According to the results obtained in this study, under 40% FC, leaf soluble sugars increased in soybean plants treated with R1MY and R1+R3MY while under 70% FC, there was an increase in soluble sugars in soybean leaves amended with R1, R1MY, R3MY, R1+R3 compared to the well-watered treatment (Figure 5). These results agree with the findings of Zhang et al. (2010); Abbaspour et al. (2012); Yooyongwech et al. (2013); Ruiz-Lozano et al. (2012) and Talaat and Shawky (2011) who reported that soluble sugar accumulation in inoculated plants can serve as a defence strategy against drought and salt stresses. However, since will recorded the lowest content (6.02 mg equivalents of glucose) of soluble sugars in plants treated with MY under moderate or 70% FC (Figure 5), our results partially agree with the work of Zarik et al. (2016) who highlighted higher soluble sugar concentration in ‘non-mycorrhized plants’ than in ‘mycorrhized plants’ under drought conditions but disagree with this author’s conclusion at the level of 40% FC since there was an increase in soluble sugars from 6.02 to 21.2 mg equivalents of glucose which was higher than what was observed in the control and some of the mycorrhized and nom-mycorrhized treatments under this severe drought stress.

The amino acid proline is an essential ‘osmo-protectant osmolyte’ produced by plants to improve osmosis and prevent water loss (Ruiz-Lozano et al., 2012, Zarik et al., 2016). The proline (the osmo-regulator in addition to soluble sugars used in this investigation) concentration

in leaves was lower in non-inoculated soybean plants than in rhizobial and mycorrhizal inoculated soybean plants under severe drought (Figure 6). Under this drought condition, significant accumulation of proline was observed in the leaves of soybean plants treated with the dual inoculum R3MY (Figure 6). This finding corresponds to the result of Ortiz et al. (2015) who reported significant proline accumulation in some plant treatments dually inoculated with microbial species leading to a reduction in the osmotic potential of the plant cells and consequently enhanced uptake of water to 'maintain osmotic balance' (Dong et al., 2002). Also, the concentration of proline accumulated by soybean plants treated with MY under severe drought condition was approximately 4 $\mu\text{mol/g}$ dry weight which was comparatively higher than that of the non-mycorrhized treatments (control, R1, R3, R1+R3). This result disagrees with the conclusions of Zarik et al. (2016); Manoharan et al. (2010) and Wu and Xia (2006) who observed less proline content in the leaves of mycorrhized *Cupressus atlantica*, *vagiegata* and orange plants respectively than in non-mycorrhized plants grown under moderate and severe drought stresses. Furthermore, under moderate drought stress condition, soybean plants treated with R1+R3MY were shown to accumulate the highest level of proline while the lowest level was found in R1 treatment. Contrary results were found in the well-watered plant treatments where the highest and lowest proline concentrations were produced by plants inoculated with R3 and R3MY respectively.

Surprisingly, lower chlorophyll content was observed in virtually all soybean plants grown in 100% FC and increased with increasing drought stress (Figure 7). Overall, R3MY treatment and non-inoculated plants grown in 100% FC showed the highest (15.2 CCI) and lowest (10.9 CCI) chlorophyll content respectively. Chlorophyll is an important component that plays a vital role in the process of photosynthesis in plants (Tajini et al., 2012). Considering the

different water levels, chlorophyll content negatively correlated with below-ground-above-ground productivity, implying that as the drought stress increased, chlorophyll content increased and plant productivity decreased.

Furthermore, we found that the number of mycorrhizal spore (Figure 8) positively correlated with the % mycorrhization in this study as fungal spore (Fig. 8a) increased from 40% FC to 100% FC. The highest number of spore was observed in soybean plant rhizosphere inoculated with R1+R3MY in all the water treatments. Also, soybean root colonization or % mycorrhization (Figure 8) was positively correlated with RWC (Figure 3) and seed number of soybean plants inoculated with MY, R3MY and R1+R3MY (Table 3) indicating enhancement of plant water status and productivity. Perhaps, mycorrhizal fungi may have contributed to drought avoidance capacity of these treatments, thus resulting in water stress alleviation in soybean plants. The increase in seed number and other morphological components in soybean plants inoculated with MY, R1MY, R3MY, R1+R3MY may partly be ascribed to mycorrhizal colonization (Fig. 8b) and RWC (especially for MY, R1MY and R3MY treatments at 40% FC), signifying that plant water content was efficiently enhanced by this plant-microbial interactions resulting in enhanced productivity. This finding agrees with the results of Aliasgharzad et al. (2006) who reported that an increase in % mycorrhization led to an increase in RWC in soybean plants inoculated with both *Bradyrhizobium japonicum* and *Glomus etunicatum*.

One fascinating and unique result obtained in the current study under controlled environment is the inherent ability of the test *Rhizobium* spp. and mycorrhizal consortium to cope with and tolerate drought stress. In addition, not only single rhizobial and mycorrhizal inoculations, but dual inoculations of these bacteria and fungi proved very competent in aiding inoculated soybean plants to annul the deleterious effects of drought stress on not only the

aforementioned physiological and biochemical parameters but also the below-ground-above-ground/morphological parameters of soybean plants. It was reported in our previous work (Igiehon and Babalola, 2018a) that below-ground interactions between plants and rhizobacteria/AMF can trigger responses that may affect above-ground plant components. But in this present study, it became evident that below-ground interactions among rhizobial, mycorrhizal fungi and soybean plants under stressed condition improved the productivity of most of the above-ground components such as shoot height, shoot width, branch number, leaf number, shoot dry weight, pod number, pod fresh weight, seed number, seed fresh weight, highest seed number per pod and seed dry weight (Table 2 and 3).

At 40% FC, significant promotion of soybean plant growth under severe drought stress by R1+R3MY inoculation depicted by increased in shoot width and branch number (Table 2) could be as a result of the synergistic interaction between the microbial species and mutualistic interaction between the inoculum and soybean plant roots (Igiehon and Babalola, 2018a). This treatment in addition to the dual inoculum R1MY greatly affected root dry weight, although the effect was only significantly different ($P < 0.05$) from plants inoculated with R1. Similarly, under severe drought stress, taproot length was greatly affected by R3 inoculum and this result somewhat supported the outcome of our previous work (data not shown) in which R3 significantly affected soybean seedling root fresh and dry weight and number of lateral root in a growth chamber experiment where PEG was used as a drought factor. But in this study, under severe drought stressed condition, R3 treatments produced lateral roots with a mean value of 13.9 which was less than that produced by other microbial treatments but higher than that of non-inoculated plants (Table 2).

On the contrary, R3 treatments produced the highest (34.3) number of lateral roots under moderate drought stress (70% FC) which further supports our previous PEG experiment results (Table 2). Like in severe drought condition, R1+R3MY inoculum also enhanced soybean growth under moderate drought stress since it had the greatest effect on soybean shoot height under this condition (Table 2). Below-ground taproot length received the greatest impacts from both R1+R3MY and MY inocula compared to other treatments. Under this condition, we observed that below-ground synergistic interaction between *Rhizobium* sp. strain R1 and mycorrhizal consortium (RIMY) outstandingly impacted soybean shoot width, branch number, leaf number and shoot number.

For the well-watered treatments, it was found that MY glaringly enhanced soybean plant shoot height with a mean value of 291.3 mm (Table 2) and the non-inoculated plants showed the poorest shoot development as they presented the lowest (214 and 2.1 mm) shoot height and shoot width respectively. Thus, based on the shoot height and shoot width evaluation, the non-inoculated or control plants produced soybean plants with the lowest shoot biomass in all the water regimes (Table 2).

One of the indicators of sustainable agriculture and/or food security is increase in agricultural produce (Igiehon and Babalola, 2018c). Our present study has shown that combination of rhizobia and mycorrhizal fungal consortium resulted in increased yield (soybean seed number) under severe and moderate stressed conditions in a controlled environment (Table 3). Particularly, R1+R3MY treatments significantly ($P<0.05$) produced more seeds in 40% FC than other treatments (Table 3). The non-inoculated (control) plants and plants amended with R1 and R3 did not produced seeds under this condition probably because the absence of PGPR (in the control plants) and single inoculation (in R1 and R3 treated plants) could not ameliorate the

detrimental effects of drought stress to the extent of empowering the plants to produced seeds even though these treatments produced pods. It was reported in our previous studies (Igiehon and Babalola, 2017, Enebe and Babalola, 2018) that abiotic stresses such as drought, heat and salinity are part of the factors that militate against crop growth, development and yield. The impacts of these abiotic stresses may be abated by below-ground plant-microbial (AMF and rhizobacterial) interactions (Igiehon and Babalola, 2018a). Tripartite mutualistic interactions with AMF fungi and *Rhizobium* species (Igiehon and Babalola, 2018a) is therefore important for increased productivity in soybean (Igiehon and Babalola, 2018c) and the impacts of co-inoculation with mycorrhizal fungi and rhizobia on soybean plants need further research.

Also, co-inoculation with mycorrhizal consortium and rhizobia (R1MY) significantly enhanced soybean yield as indicated by pod number, pod fresh weight, seed number and seed fresh weight in the 70% FC but not in the 100% FC (Table 3). These outcomes further showed that there was a synergistic impact between mycorrhizal consortium and rhizobia on soybean yield in this investigation and that this impact ‘might’ be dependent on water status. However, previous study by Wang et al. (2011) indicated that such synergistic impact can be link to nutrient status. It is generally believed that mycorrhizal fungi principally benefit plants grown in soil environments where phosphorus is likely to hinder plant productivity by increasing the volume of soil penetrated by mycorrhizal hyphae compared to that of root hairs of non-mycorrhizal plants (Igiehon and Babalola, 2017, Wang et al., 2011) and in this current study, there was no addition of inorganic phosphorus and nitrogen to the soil and the natural soil total nitrogen was slightly low (0.095%-Table 1) compared to that (0.22%) reported by Ortiz et al. (2015). This low nutrient level of soil could be another reason (In addition to water status) for the synergistic impacts of dual inoculation with mycorrhizal consortium and rhizobia in this study.

The universal consensus is that mycorrhizal fungi enhance phosphorus nutrient uptake in legumes which eventually improves plant development and nitrogen fixation (Wang et al., 2011, Igiehon and Babalola, 2017, Igiehon and Babalola, 2018a, Barea and Azcon-Aguilar, 1983, Cluett and Boucher, 1983) and according to Xavier and Germida (2003), dual inoculation with rhizobial species and compatible AMF can intensely improve pea growth and nutrient uptake. On the basis of this, we observed that the rhizobia and mycorrhizal consortium used in this present study enhanced soybean plants tolerance to drought and improved their productivity. Therefore, the fungal consortium is compatible with our rhizobial species and soybean cultivar, which may have prospect for agronomic application.

9.4 Conclusions

The results of this study have shown that single and dual inoculations of rhizobia and mycorrhizal consortium had significant effects on the physiological and biochemical indicators of soybean plants grown under different water regimes. These effects culminated in improving the below-ground-above-ground and/or morphological components of soybean plants more than the non-inoculated treatments. Specifically, it was found that R1+R3MY enhanced soybean growth and yield as indicated for instance by shoot height and seed number under severe drought conditions. These results can help gain more insights into the impacts of mycorrhizal fungi and rhizobia on soybean growth and provide theoretical background for real-life application of these species in field-grown legumes. This biotechnological strategy can help us resolve or abate food insecurity problem endemic in most developing countries (especially the semi-arid and arid regions) of the world.

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9.6 Conflict of Interest

Author 1 declares that he has no conflict of interest. Author 2 declares that she has no conflict of interest.

9.7 Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

9.8 Authors Contribution

Igiehon NO wrote the article. Babalola OO provided academic input and thoroughly critiqued the article. Both authors approved the article for publication.

CHAPTER 10

Growth and Mineral Nutrient Enhancement of Soybean by New *Rhizobium* Species and Mycorrhizal Fungi under Drought Conditions

Abstract

Drought stress is one of the major abiotic components affecting the growth and nutrient status of crops such as soybean (*Glycine max* L.). Soybean can form tripartite mutualistic interaction with *Rhizobium* species and arbuscular mycorrhizal fungi (AMF), but much is not known about the impacts of such interaction on soybean growth and mineral nutrients under drought conditions. Therefore, in this study, the impact of new *Rhizobium* sp. strain R1 and *Rhizobium cellulosilyticum* strain R3 and mycorrhizal consortium on soybean was tested under drought conditions in a controlled environment and natural semi-arid environment. Single and dual inoculation of the *Rhizobium* spp. and AMF increased the relative water content (RWC), while dual inoculation of *Rhizobium* spp. and AMF decreased electrolyte leakage and increased soluble sugars and proline accumulation in soybean plants, suggesting their potential to alleviate drought stress. Single and dual inoculation of soybean with *Rhizobium* spp. and AMF also increased below-ground and above-ground plant components at different stages of growth of the plant as well as macro and micronutrient assimilation especially N, P, Mg, S, Ca, Co, Mo, Fe and B. However, co-inoculation with *Rhizobium* sp. strain R1 and mycorrhizal consortium (R1MY) resulted in a significant increase ($P < 0.05$) in almost all the macronutrients (N, P, Mg and Ca). Thus, the applications of these microorganisms can be considered as an effective biotechnological approach to improve soybean and other agricultural crops grown under drought conditions.

Keywords: biotechnology, drought, mineral nutrient, mycorrhizal fungi, *Rhizobium*, soybean

10.0 Introduction

Drought is the main factor militating against crop productivity worldwide (Sharp et al., 2004). It is also known to be the abiotic factor with the greatest impact on plant, since about 50% of global arable lands are constantly exposed to drought (Long and Ort, 2010). It has been forecasted by ‘the Intergovernmental Panel on Climate Change’ that there will be an increase in land area subjected to water stress, while water availability in the affected regions would reduce by 30% by mid-century (Christensen et al., 2007). Thus, there is a need for a sustainable strategy to abate the detrimental effects of drought on agricultural crops, especially in semi-arid and arid regions of the world. One of the ways to achieve this feat is through the use of drought tolerant microorganisms with plant growth promoting traits. In our previous study, it was reported that AMF can be harnessed in agriculture for dual purposes of improving nutrient uptake by plants and enhancing their tolerance to drought (Igiehon and Babalola, 2017). In another study, it was suggested that plant growth promoting rhizobacteria (PGPR) such as *Rhizobium* species, besides nitrogen fixation, have the ability to promote plant growth and increase macro and micronutrient of plants including legumes (Igiehon and Babalola, 2018c).

PGPR, therefore, have colossal prospect in agriculture since they are able to contribute to crop productivity by enhancing their tolerance to both abiotic and biotic stress (Vilchez and Manzanera, 2011, Igiehon and Babalola, 2018a). These PGPR are also termed rhizobacterial drought-tolerance enhancers (RDTE) (Timmusk et al., 2013). Over the last ten years, scientists have focused research on RDTE (Glick et al., 2007), and since it was reported by Mayak et al. (2004a) that *Achromobacter piechaudii* ARV8 provided drought-tolerant benefit to plants, numerous other microorganisms with similar potential have been revealed. In particular, these rhizobacteria are able to neutralize the deleterious effects of drought and promote plant growth

by helping plant to accumulate proline, soluble sugar, maintain water balance and cell membrane integrity (Ortiz et al., 2015, Zarik et al., 2016).

Furthermore, the accumulation of nutrient in plants under water stress might have a role to play in water stress tolerance (Samarah et al., 2004). According to Tanguilig et al. (1987), the uptake of potassium (K) and phosphorus (P) by soybean (*G. max* L) shoots and roots was not affected by water stress and they also reported that soybean grew better than rice (*Oryza sativa* L.) under drought conditions probably because soybean maintain its water status by reducing the transpiration rate and maintaining uptake of nutrients, specifically K, which is a key nutrient involved in the regulation of osmotic stress in plants. Indeed, the concentration of magnesium (Mg), calcium (Ca) and P was lower in leaf sap of drought-sensitive sorghum (CS3541) than ‘drought-tolerant sorghum’ (K866) (Premachandra et al., 1995). The content of K, P and chloride (the principal minerals that regulate osmotic stress) increased in the ‘drought-tolerant sorghum’ as leaf water content diminished but these mineral nutrients in the drought-sensitive sorghum remain constant (Premachandra et al., 1995). Mg and Ca concentrations in the ‘leaf cell sap of the two sorghum’ increased as the leaf water content decreased (Premachandra et al., 1995).

In addition, mineral nutrient content can contribute to plant seeds quality and seeds with high level of mineral nutrient would be expected to be high in quality. Food quality is one of the critical components of food security. According to Igiehon and Babalola (2018c), food security is reliant on the improvement of plant development all the way from the field. This can be accomplished ‘through inoculation of agricultural crops (such as soybean) with rhizospheric rhizobacteria (e.g., *Rhizobium* spp.)’.

Previous studies (Igiehon and Babalola, 2017, Tairo and Ndakidemi, 2013, Cely et al., 2016) have reported that *Rhizobium* and mycorrhizal fungal inoculation increased soybean shoot

height, leaf number, shoot width and pod number compared to the non-inoculated plant. Nevertheless, limited information is available on the effects of these microbial species on soybean growth at different stages of growth and mineral nutrient concentration in soybean seeds and whether the proliferation in the concentration of mineral nutrient in the seeds of soybean exposed to drought is dependent on microbial amendment or not. Although, a microbial-dependent increase of soybean growth and seed mineral nutrients under drought conditions may go a long way in sustaining global food security.

The aim of this research, therefore, was to determine the influence of *Rhizobium* species and AMF inoculation on soybean mineral nutrient content and growth parameters (at different growth stages) in a controlled and semi-arid environment.

10.1 Materials and Methods

10.1.1 Climatic Weather Time Series

Relative humidity (%) of the study area was monitored on an hourly basis (each day) and the maximum and minimum temperature was taken on a daily basis during the experimental period by the South Africa Weather Service and compared with previously collected data. The number of rainy days during the experimental period was also recorded by the South Africa Weather Service.

10.1.2 Sources of Microbial Isolates and Soybean

The two species of *Rhizobium* namely *Rhizobium* sp. strain R1 and *Rhizobium cellulosilyticum* strain R3 used for the greenhouse and field experiments in this study were isolated from Bambara groundnut rhizosphere and identified by molecular approach method in our previous study. The key characteristics of the Bambara groundnut rhizospheric soil used for the rhizobial isolation were pH 7.65, Fe 1.62 mg/kg, manganese 24.1 mg/kg, organic carbon

1.06%, organic matter 4.01%, potassium 285 mg/kg, magnesium 397 mg/kg and total nitrogen 0.066%. The two rhizobial species were proven to be drought tolerant in our previous experiment. Also, the mycorrhizal consortium was ascertained to be drought tolerant and comprised of *Gigaspora gigantea*, *Funneliformis mosseae* (previously *Glomus mosseae*), *Paraglomus occulum* (molecular determination), *Rhizophagus clarus* (previously *Glomus clarum*) and *Claroideoglomus etunicatum* (previously *Glomus etunicatum*)] and was obtained from the Department of Biochemistry and Microbiology, Rhodes University, Grahamstown, South Africa.

The soybean PAN 1532 R, which was classified as ‘moderate drought sensitive cultivar’ in our previous study (data not shown), was obtained from Agricultural Research Council, South Africa.

10.1.3 Greenhouse Experiment

Soil samples used for the greenhouse experiment were collected from the North-West University garden. Topsoil (10-25) from the soil was used and the major properties of the soil were Fe 1.950 mg/kg, pH 7.390, Mn 45.400 mg/kg, organic carbon 1.260%, organic matter 3.190%, potassium 406.0 mg/kg, magnesium 639.0 mg/kg, total nitrogen 0.095%, sand 70%, silt 8% and clay 22%. Soil samples were sieved and autoclaved three times at 121°C for 30 min. Plastic pots with a diameter of 0.3 m and height of 0.29 m were filled with 8 kg of sterilized soil. The experiment was set-up using a completely randomized designed (CRD). Three water regimes were used namely: 100, 70 and 40% field capacity (FC) and 24 treatments replicated 8 times with an overall total of 192 replicates were used. *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 used for the greenhouse experiment were grown in broth and harvested by centrifuging in autoclaved 0.85% NaCl. The OD of the *Rhizobium* spp. was

adjusted to 1.3 and *Rhizobium* sp. strain R1 colony count was 30×10^9 CFU/ml, while *R. cellulosilyticum* strain R3 was 31×10^9 CFU/ml.

Two hundred and forty (240) PAN 1532 R soybean seeds surface sterilized in 75% alcohol, 1% sodium hypochlorite and distilled water were singly inoculated with 0.2 L of *Rhizobium* sp. strain R1 suspension and 0.2 L of *R. cellulosilyticum* R3 suspension. Seeds (480) used for AMF and control treatments were suspended in 0.85% NaCl. Seeds were shaken in a rotary shaker at ambient temperature overnight, after which they were aseptically air-dried (seed coating). Before sowing, all pots containing the autoclaved soil were watered to the 100% FC with 0.36 L of water and 5 seeds were sowed in per pot. AMF inoculation was performed by adding 0.05 kg of mycorrhizal consortium in the soil before placing the seeds. Fourteen days after emergence, seedlings were thinned to two per pot. All the treatments were watered every three days to the FC for about 21 days after seed germination upon which drought stress was initiated in the 70 and 40% treatments. Precisely, water application to the 70 and 40% treatments were reduced to 0.252 and 0.144 L respectively, while water supply to the 100% treatments was maintained at 0.36 L. Water was applied to pots every two days for 21 days and drought stress was increased by watering every three days for the remaining 30 days before data were collected. Using a ruler, shoot height and shoot width were measured about 7 weeks after subjecting the plants to drought.

10.1.4 Field Experiment

Field experiment was performed at the North-West University, Mafikeng campus (25.82202S:025.61430E), South Africa. The climate of the study area is semi-arid with the highest average monthly rainfall of 3.88 mm recorded during the period of the experiment. The soil had the following physicochemical properties: pH (6.620), iron (3.870 mg/kg), Mn (43.50

mg/kg), organic carbon (1.060%), organic matter (3.230%), potassium (344 mg/kg), magnesium (858 mg/kg), total nitrogen (0.069%), sand (68%), silt (6%) and clay (26%). The experimental land was previously planted with maize crop, and to the best of our knowledge, there was no history of fertilizer application in the experimental site. In addition, chemical fertilization was not performed in this current study. The topography of the land was flat and thus was not prone to erosion. Each treatment was replicated eight times in a randomized complete block design (RCBD) and the area of each plot was 1.04 m² with a planting density of 50 x 13 cm. Soybean seeds were inoculated with approximately 5 g of mycorrhizal consortium with MPN values of 50 -100/g and 8-10 spore number/g. The fungal were applied at sowing time, just immediately below the seeds and four seeds were sowed per hole and seedlings were thinned to one 14 days after emergence. Manual removal of weeds was performed on weekly basis as reported by Sugiyama et al. (2014) including other 'regular agricultural practices' as described by Prakamhang et al. (2015).

Seeds were inoculated with *Rhizobium* spp. with counts of 520 x 10⁷ CFU (colony forming unit)/ml (for *Rhizobium* sp. strain R1) and 524 x 10⁷ CFU/ml (for *R. cellulosilyticum* strain R3). The *Rhizobium* species were harvested by spinning rhizobial spp. broths grown to early exponential phase at 10000 x g for 900 sec. The bacterial pellet was suspended in normal saline and bacterial OD adjusted to 1.301.

Before sowing, soybean seeds were surface sterilize in 1% Na hypochlorite and 75% alcohol as described by Ndeddy Aka and Babalola (2016). About 2000 seeds were inoculated with 100 ml of each bacterial formulation (single inoculation) and 50 ml of each bacterial formulation was used on the same number of seeds (co-inoculation) in round bottom flasks. Control seeds (2000) and seeds (2000) used as mycorrhizal treatments were also placed in two

separate flasks containing 700 ml normal saline solution. Seeds were incubated in a rotary shaker at 180 rpm at 28±2°C for 24 hr and air-dried (seed coating technique) prior to sowing in the field. The following were the microbial treatments used for the field experiment: CONTROL, R1, R3, MY, RIMY, R3MY, R1+R3, R1+R3MY corresponding to non-inoculated treatment, single inoculation with *Rhizobium* sp. strain R1, single inoculation with *R. cellulosilyticum* strain R3, single inoculation with mycorrhizal consortium, co-inoculation with *Rhizobium* sp. strain R1 and mycorrhizal consortium, co-inoculation with *R. cellulosilyticum* strain R3 and mycorrhizal consortium, co-inoculation with *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 as well as co-inoculation with *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 and mycorrhizal consortium.

In order to avoid border effects on data collection, samples were collected from the middle plants within the plot.

10.1.5 Leaf Relative Water Content

Leaf relative water content (RWC) was estimated according to the methods described by Ortiz et al. (2015) and Aroca et al. (2003) using the following formula

$$\text{Leaf relative water content (\%)} = \frac{(\text{FW}-\text{DW})}{(\text{TW}-\text{DW})} \times 100$$

10.1.6 Electrolyte Leakage

Electrolyte leakage was estimated according to the method described by Ortiz et al. (2015). A young leaf of almost the same size from the distal end of soybean shoot was collected from all the eight replicates per treatment and rinsed properly with deionized water to get rid of electrolytes that may have attached on the leaf surface. Leaf samples were placed in closed test-tubes and incubated at 28±2°C for 24 hr on a rotary shaker and thereafter, electrical conductivity

(L_t) of the sample solution was estimated using a PL-700AL conductivity meter made in Taiwan. Samples were subjected to autoclaving at 120 °C for 20 min and allowed to cool to 25 °C prior to obtaining the final electrical conductivity (L_0). The electrolyte leakage was obtained using the following formula:

$$\text{Electrolyte leakage (\%)} = \frac{L_t}{L_0} \times 100\%$$

10.1.7 Soluble Sugar Content in Soybean Leaves

Soluble sugars in soybean leaves were extracted from 100 mg of leaf samples in 80% ethanol and centrifuged according to the method described by Zarik et al. (2016) and Dubois et al. (1956). Soluble sugar content in the homogenate was estimated according to the method described by Porcel and Ruiz-Lozano (2004). In summary, 1 ml of the homogenate was mixed with 5 ml of concentrated H_2SO_4 and 1 ml of 5% phenol and agitated vigorously. Samples were allowed to cool for half an hour and analyzed spectrophotometrically at 485 nm. The concentrations of soluble sugars were extrapolated from a glucose standard curve.

10.1.8 Proline Content in Soybean Leaves

Proline in soybean leaves was extracted in 3% aqueous sulfo-salicyclic acid from 1 g of fresh leaves according to the method of Bates et al. (1973) with little modifications. The homogenate was subjected to centrifugation at 10000 x g for 10 min at 4 °C. Proline content was estimated using the supernatant. Proline content was quantified by spectrophotometric method at 520 nm using the acid-ninhydrin reaction (Ortiz et al., 2015) and the concentration of proline was calculated from a standard curve.

10.1.9 AMF Spore Counts and Symbiotic Development

AM fungal spore number was obtained by a wet sieving and decanting technique while root samples (for mycorrhizal colonization level) were collected from four replicates per treatment in the field. The percentage of mycorrhizal fungi root colonization was determined by visual examination under stereomicroscope of root colonized fungi after clearing in 10% KOH and staining with trypan blue solution which comprised of 0.82 g trypan blue, 480 ml glycerol, 640 ml distilled water and 520 ml lactic acid (Phillips and Hayman, 1970). Percentage mycorrhization was estimated as described by (GIOVANNETTI and Mosse, 1980).

10.1.10 Plant Sampling and Measurement of Plant Parameters

Soybean plants were sampled at various stages of soybean growth namely: beginning of blooming, beginning of pod development, beginning of seed development, full seed and full maturity. Plants were immediately transported to the laboratory to obtain the plant fresh weight. The shoots were separated from the roots as described by Masciarelli et al. (2014). Shoot height, shoot width and taproot/pod lengths were measured using a measuring tape. Lateral root, branch and leaf number were determined by counting. Pods and nodules were counted after detaching them from the shoots and roots respectively. Nodule, pod fresh and dry weights were obtained using a weighing machine (Wagi Elektroniczne, Poland).

10.1.11 Other Plant Parameters Determined

Pod, nodule, shoot and root dry weights were obtained according to the method described by Goicoechea et al. (1997b) with little modifications by oven-drying at 65°C for 48 hr. Oven-dried samples were weighed on a weighing balance (Wagi Elektroniczne, Poland) to obtain the dry weights.

10.1.12 Determination of Mineral Nutrients

Upon maturity, eight soybean plants per treatment (each plant represents a replicate) were harvested in the field and the pods were detached from the plants. The seeds were manually removed from the pods and oven-dried at 65°C for 48 hrs. The oven-dried seed samples were ground using a Grinder (Professional compressor, Cemotec 1090). For the determination of macro and micronutrients such as N, Mg, P, Ca, S and Mo, Cu, Mn, Zn, Na, B using ICP Mass Spectrometer (Perkin Elmer, NEXION™ 300Q), 1 g of ground and homogenized seeds were weighed, oven-dried at 160°C for 16 hrs in an oven (Protea Laboratory, South Africa) and ashed at 800°C in a micro-furnace (Merck Chemical Laboratory Supplies Division) for 16 hrs and processed further as described by Nouri et al. (2014) with little modifications. In summary, 5 and 2 ml of nitric acid (HNO₃) and hydrochloric acid (HCl) were added to the ashed samples in the crucibles and heated on a hot-plate for 30 min. The heated samples were digested in a round-bottom flask containing 100 ml of distilled water for 16 hrs. The digested samples were filtered using sterile and cleaned Whatman filter papers. Approximately 15 ml of the digested samples in falcon tubes were used to determine the macro and micronutrient contents using ICP Mass Spectrometer (Perkin Elmer, NEXION™ 300Q).

10.1.13 Statistical Analyses

Data obtained from greenhouse and field experiments were cleaned in a Microsoft excel sheet. To ascertain the impact of each treatment, two-way and one-way ANOVA tests were performed for greenhouse and field data respectively using SAS statistical package. The differences between means (post hoc test) were determined using Duncan's multiple range test and $P \leq 0.05$ was considered significant (Dytham, 2011, Duncan, 1955, Igiehon, 2015).

10.2 Results

10.2.1 Climatic Results of the Study Area

The relative humidity of the experimental area was lower at the early stage of soybean growth compared to the later stage. The relative humidity was 48% in December and 42% in January during the experimental period which were lower than the relative humidity recorded in the same months in the last ten years. However, the relative humidity became constant in February, March and April with a mean value of 65% (Fig. 1).

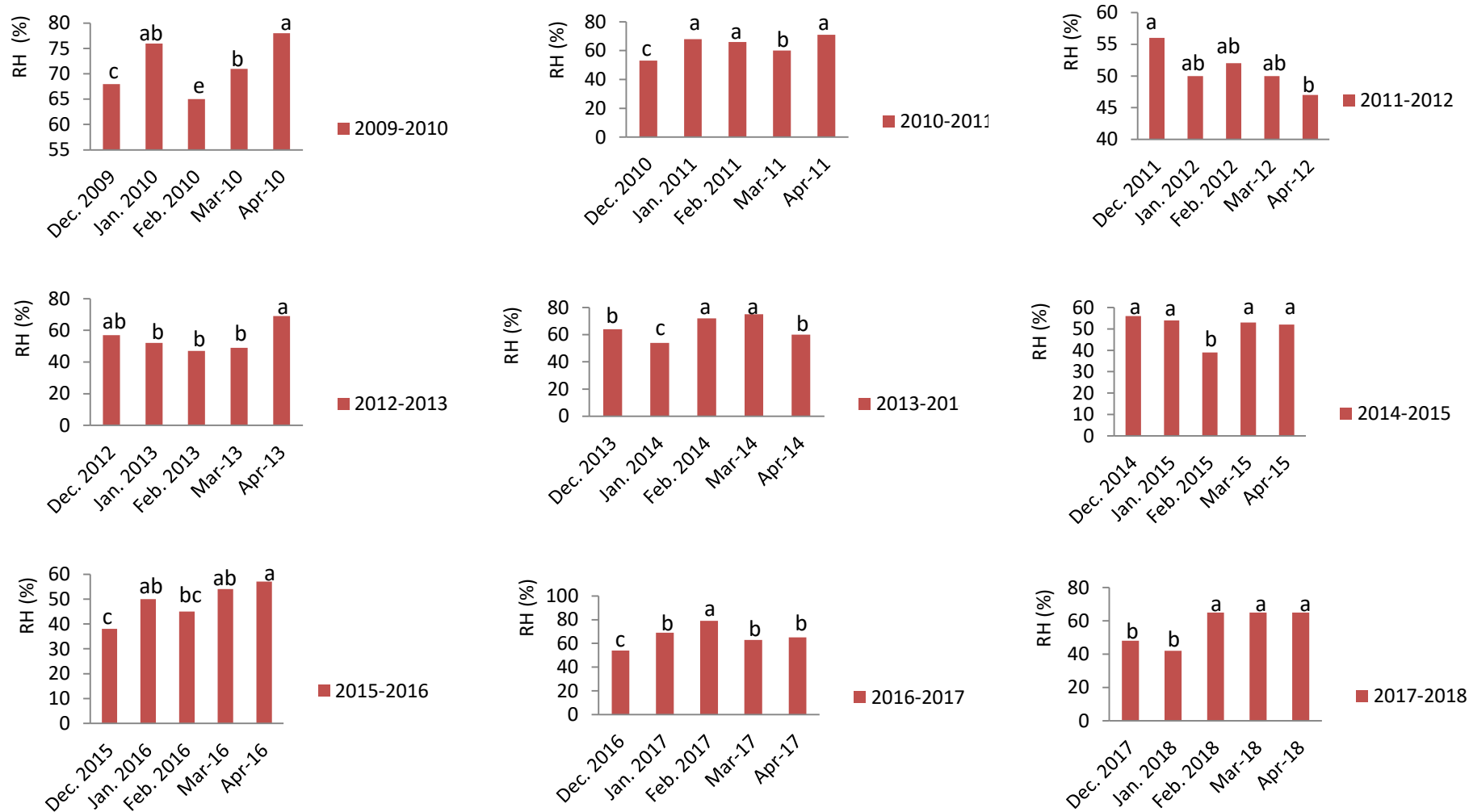


Fig. 10. 1 Climatic data (humidity) during the experimental period in comparison to similar periods in the past ten years. RH - relative humidity

The highest maximum temperature through-out the period of soybean growth (December 2017 – April 2018) was 33.8°C recorded in the second month (January) of the experiment. As a matter of fact, the temperature reading in January during this study was relatively higher than that recorded in the same month in previous years. The minimum temperature values in 2017-2018 increased from December till March and declined in April with a temperature value of 12.5°C which was similar to what was observed in April 2011 (Fig. 2).

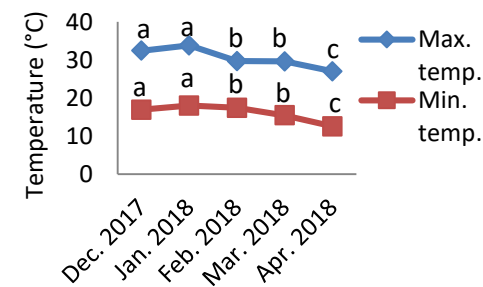
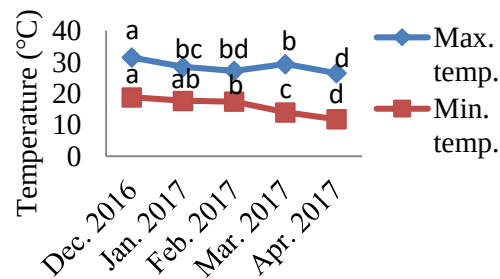
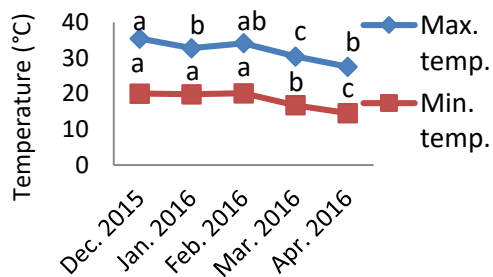
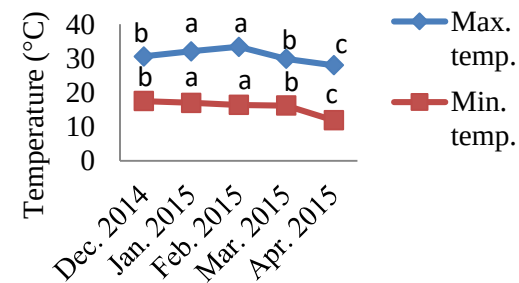
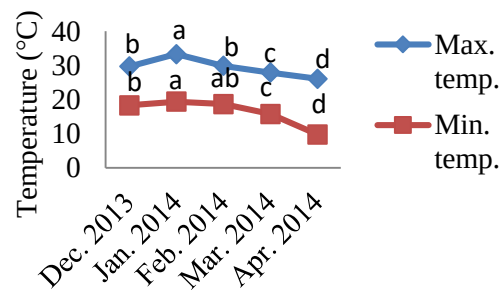
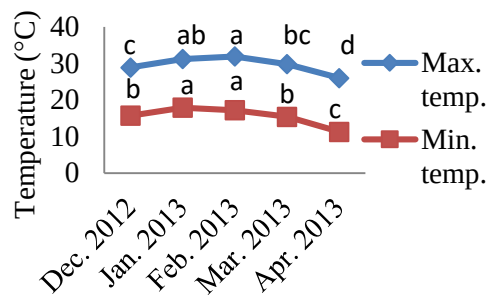
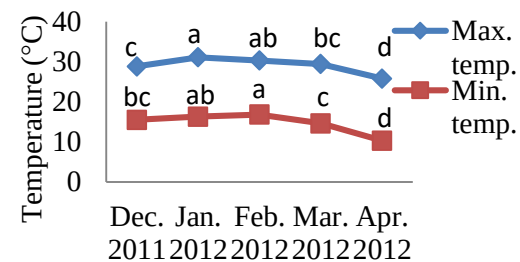
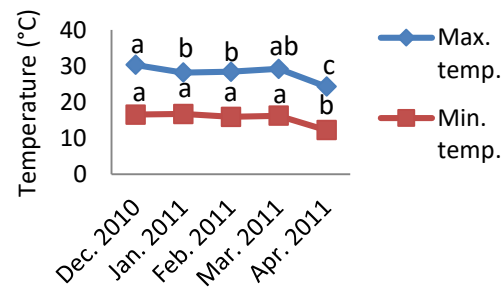
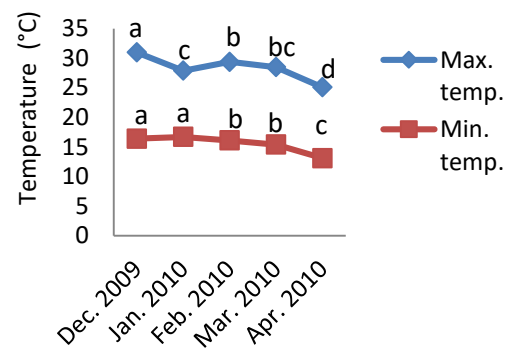


Fig. 10. 2 Climatic data (temperature) during the experimental period in comparison to similar periods in the past ten year

Also, during this study, it rained for 14 days in the first and fourth month, 13 days in the second and third month and 7 days in the last month (Fig. 3).

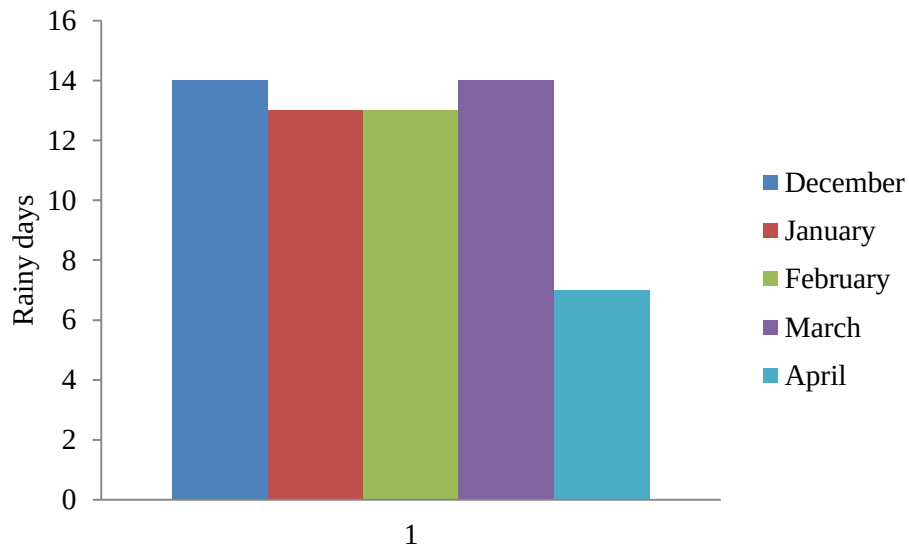


Fig. 10. 3 Number of rainy days during experimental period

10.2.2 Relative Water Content of Soybean Leaves

There was an increase in physiological values such as RWC in most of the soybean plants inoculated with rhizobial spp. and mycorrhizal fungal compared to the non-inoculated plants under this semi-arid condition. R1 and R3 strains were very efficient in increasing RWC (Fig 4). Nevertheless, the enhancement of RWC was principally pronounced in soybean plants treated with mycorrhizal consortium (MY), dual inoculation of *Rhizobium* sp. R1 and mycorrhizal consortium (R1MY) and *R. cellulosilyticum* strain R3 (R3). The interactions between rhizobial species and MY in the R1+R3MY treatment increased RWC by approximately 10% more than the non-inoculated plants.

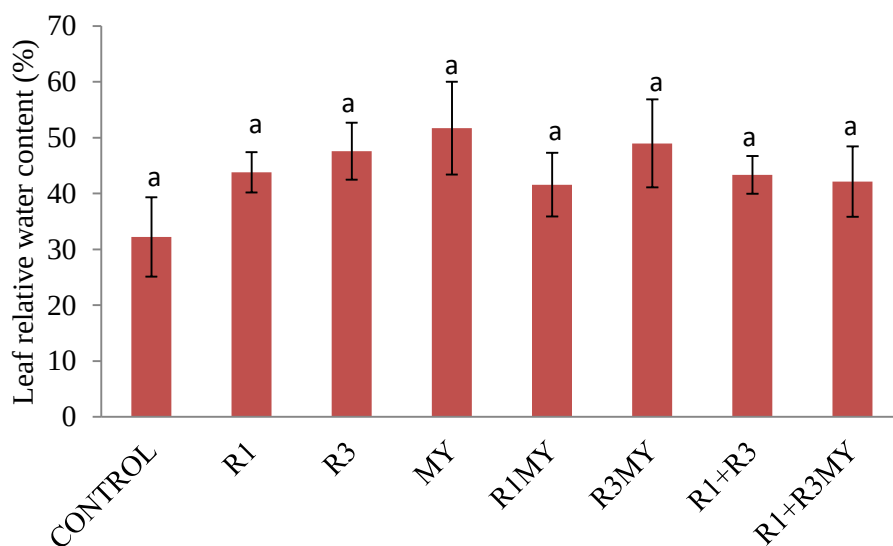


Fig. 10. 4 Leaf relative water content of non-inoculated (control) soybean plants and soybean plants inoculated with R1 (*Rhizobium* sp. strain R1), R3 (*Rhizobium cellulosilyticum* strain R3), MY (Mycorrhizal consortium), R1MY (*Rhizobium* sp. strain R1 and mycorrhizal consortium), R3MY (*R. cellulosilyticum* strain R3), R1+R3 (*Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3) and R1+R3MY (*Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium) in the field. Number of replicates (n) = 8. Data represent mean $\pm$ SE

10.2.3 Electrolyte Leakage of Soybean Leaves

R1+R3MY treatment was very efficient in decreasing electrolyte leakage but dual inoculation with *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 (R+R3) was the most competent treatment to reduce electrolyte leakage since it showed the lowest percentage electrolyte leakage of 29.5 (Fig. 5). The reduction in electrolyte leakage in R1+R3 and R3+R3MY treatments appears to be linked to a high soluble sugar accumulation in the leaves of the soybean plants (Fig. 6). In contrast, comparatively higher electrolyte leakage (40.9) was showed in the leaves of non-inoculated (control) plants but the highest electrolyte leakage (43.9) was revealed in R1 treatment.

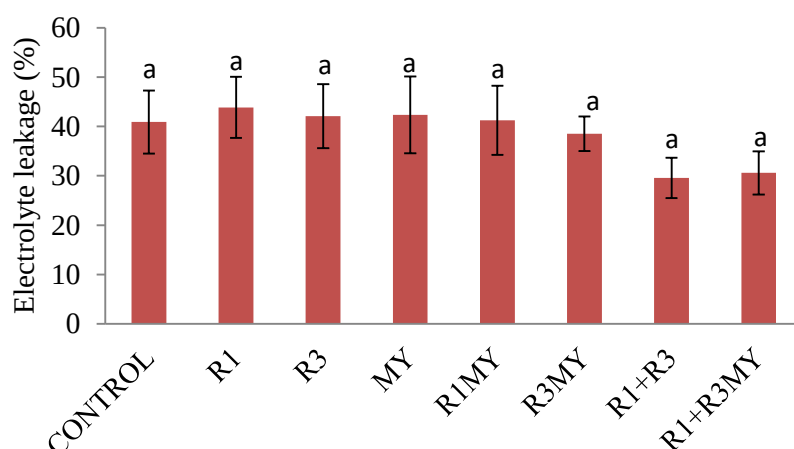


Fig. 10. 5 Leaf electrolyte leakage of non-inoculated (control) soybean plants and soybean plants inoculated with R1 (*Rhizobium* sp. strain R1), R3 (*Rhizobium cellulosilyticum* strain R3), MY (Mycorrhizal consortium), R1MY (*Rhizobium* sp. strain R1 and mycorrhizal consortium), R3MY (*R. cellulosilyticum* strain R3 and mycorrhizal consortium), R1+R3 (*Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3) and R1+R3MY (*Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium) in the field. Number of replicates (n) = 8. Data represent mean $\pm$ SE

10.2.4 Soluble Sugar and Proline Accumulation in Soybean Leaves

There is always an accumulation of soluble sugars and proline in the cells of plants grown under water stress conditions. A significant accumulation of soluble sugars was observed in the leaves of soybean inoculated with R3MY, R1+R3 and R+R3MY (Fig. 6). Indeed, this shows that amino acid may serve as ‘osmolyte and stabilizing protein’ (Ortiz et al., 2015). In this present study, the greatest proline content was observed in plants amended with R1MY (Fig. 7). In contrast, the lowest soluble sugar accumulation in the leaves of soybean plants was found under this treatment (Fig. 6). Nevertheless, the accumulated proline ($\mu\text{mol/g}$ dry weight) was considerably lower in the leaves of non-inoculated (control) plants than in inoculated plants (Fig. 7).

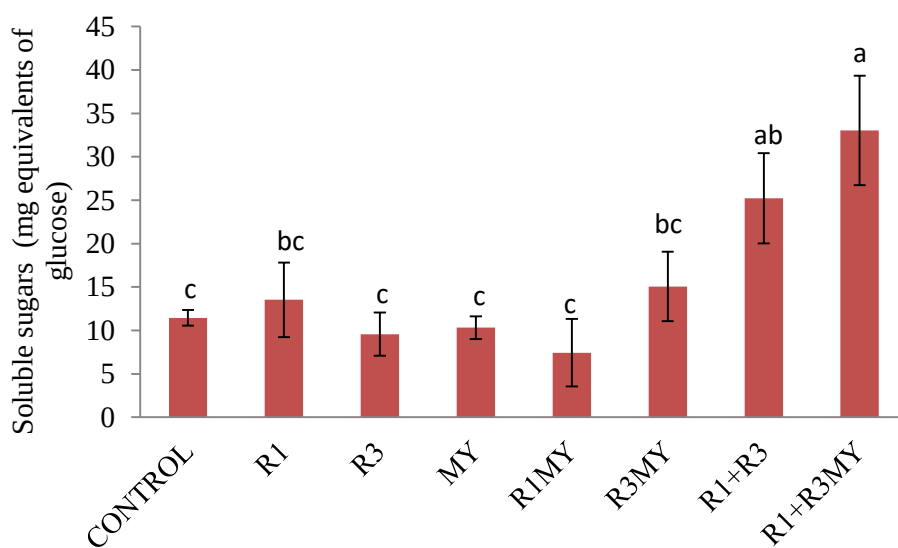


Fig. 10. 6 Leaf soluble sugar content of non-inoculated (control) soybean plants and soybean plants inoculated with R1 (*Rhizobium* sp. strain R1), R3 (*Rhizobium cellulosilyticum* strain R3), MY (Mycorrhizal consortium), R1MY (*Rhizobium* sp. strain R1 and mycorrhizal consortium), R3MY (*R. cellulosilyticum* strain R3), R1+R3 (*Rhizobium* sp. strain R1 and *R. cellulosilyticum*

strain R3) and R1+R3MY (*Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium) in the field. Number of replicates (n) = 8. Data represent mean $\pm$ SE

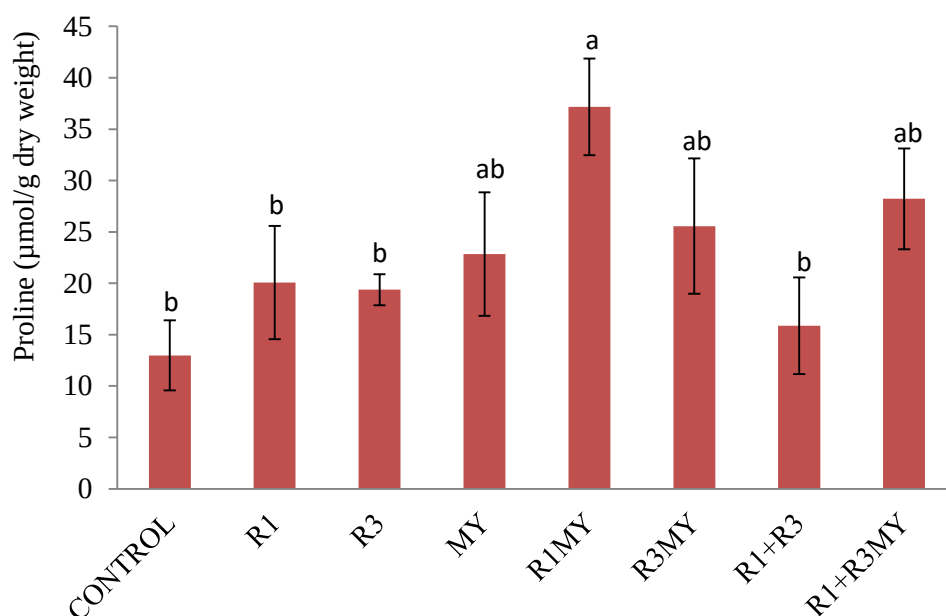


Fig. 10. 7 Leaf proline content of non-inoculated (control) soybean plants and soybean plants inoculated with R1 (*Rhizobium* sp. strain R1), R3 (*Rhizobium cellulosilyticum* strain R3), MY (Mycorrhizal consortium), R1MY (*Rhizobium* sp. strain R1 and mycorrhizal consortium), R3MY (*R. cellulosilyticum* strain R3 and mycorrhizal consortium), R1+R3 (*Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3) and R1+R3MY (*Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium) in the field. Number of replicates (n) = 8. Data represent mean $\pm$ SE

10.2.5 Fungal Spore Number and Symbiotic Development

Almost similar to the results observed for mycorrhizal root colonization level, mycorrhizal consortium (MY) was able to increase the number of fungal spores in soybean plant rhizosphere when associated with R1 and R1+R3. But co-inoculation of MY and R3 did not increase fungal spore number compared to single inoculation with MY. However, the lowest fungal spore number (29.25) was observed in non-inoculated (control) plants which significantly increased ($P < 0.05$) in the soybean plant rhizosphere treated with R1+R3MY (Fig. 8).

The inoculation of MY or R1+R3MY and particularly R1MY improved the symbiotic mycorrhizal establishment (Fig. 8). However, a low mycorrhizal infection level was revealed in the control plants while the mycorrhizal consortium (MY) which showed percentage mycorrhization of 83.5 increased this value upon co-inoculation with R1 and R1+R3 but colonization level was not enhanced when associated with R3 strain.

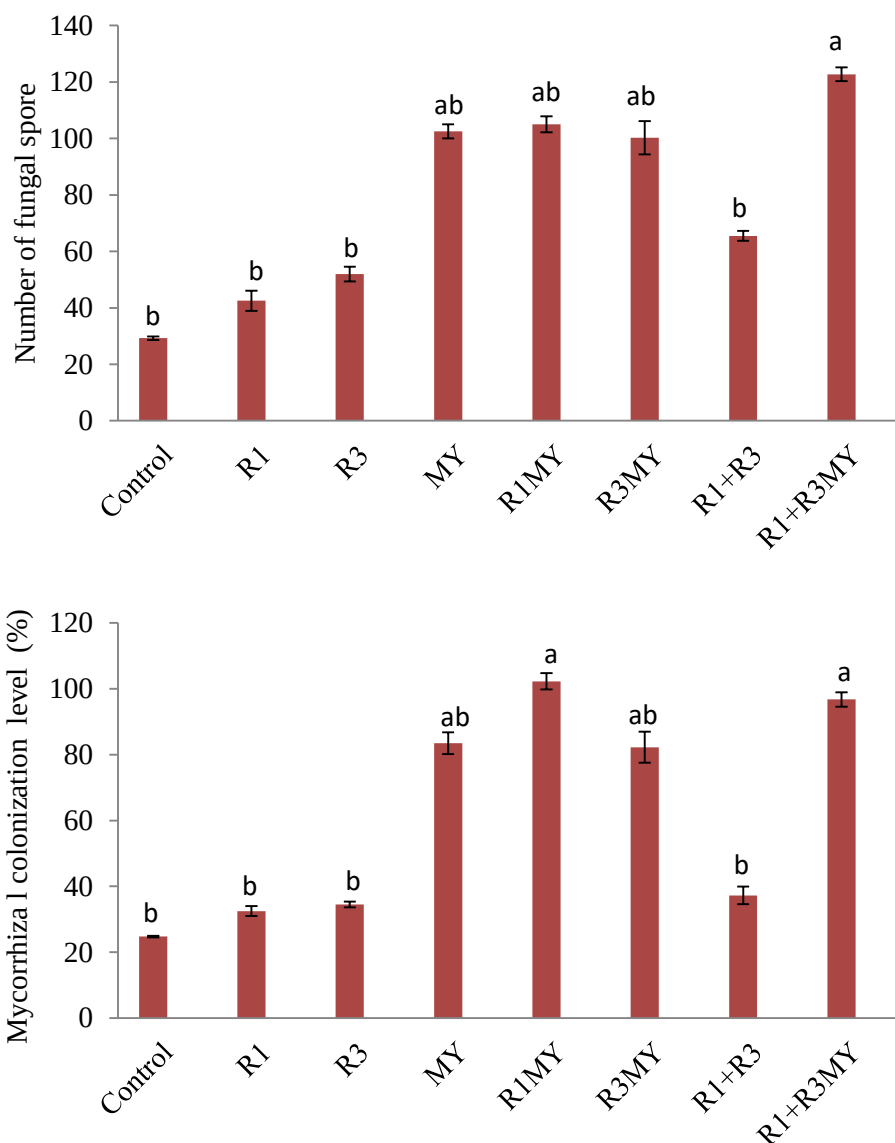


Fig. 10. 8 Fungal spore number and mycorrhizal colonization level of non-inoculated (control) soybean plants and soybean plants inoculated with R1 (*Rhizobium* sp. strain R1), R3 (*Rhizobium cellulosilyticum* strain R3), MY (Mycorrhizal consortium), R1MY (*Rhizobium* sp. strain R1 and mycorrhizal consortium), R3MY (*R. cellulosilyticum* strain R3), R1+R3 (*Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3) and R1+R3MY (*Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium) in the field. Number of replicates (n) = 4. Data represent mean $\pm$ SE

10.2.6 Effects of Rhizobia and AMF on Soybean Growth in the Greenhouse

Amazingly, single-inoculation with R1 had the greatest significant impact ($P < 0.05$) on soybean shoot width under controlled environment, particularly at 70% FC. This was followed by plants dually inoculated with R1+R3MY under the same FC (Fig. 8). On the other hand, the interaction between a bacterium and fungal consortium in the R3MY treatment produced soybean plants with the greatest width of 1.96 mm under severe drought stress (40% FC).

Overall, the shoot height of all the soybean plants grown in the greenhouse decreased as the field water capacity decreased (Fig. 9). At 100% FC, we observed the greatest shoot height (236.75 mm) in soybean plants inoculated with MY. In contrast, the greatest shoot height (223.3 mm) was observed in plants singly inoculated with R1 strain at 70% FC and in plants dually inoculated with R3MY at 40% FC. The decrease in shoot height with respect to decrease in percentage field capacity indicates that water had a negative effect on the growth of the soybean plants while relatively higher shoot height values recorded for most of the inoculated plants compared to the non-inoculated (control) plants show that the microbial treatments had positively affected soybean plants.

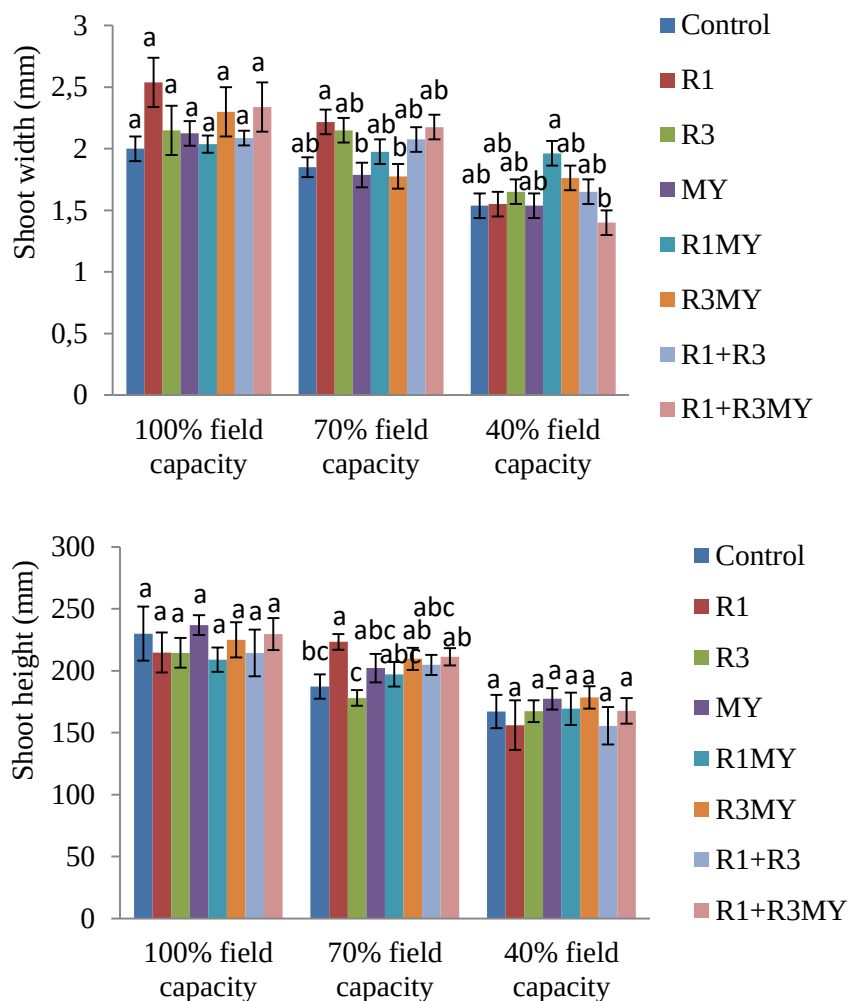


Fig. 10. 9 Shoot width and shoot height of non-inoculated (control) soybean plants and soybean plants inoculated with R1 (*Rhizobium* sp. strain R1), R3 (*Rhizobium cellulosilyticum* strain R3), MY (Mycorrhizal consortium), R1MY (*Rhizobium* sp. strain R1 and mycorrhizal consortium), R3MY (*R. cellulosilyticum* strain R3), R1+R3 (*Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3) and R1+R3MY (*Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium) exposed to a 49 day period of drought stress in the greenhouse. The different drought stress levels were 100, 70 and 40% field water capacity. Number of replicates (n) = 8. Data represent mean $\pm$ SE

10.2.7 Effects of Rhizobia and AMF on Soybean Growth at the Beginning of Blooming in the Field

At the beginning of blooming, R1+R3MY treatment exhibited the greatest shoot height (321.3 mm) which was significant at 0.05% confidence level compared to the non-inoculated plants. Single inoculation with R1 and R3 strains showed similar shoot height (>300 mm) which declined when both bacterial strains were co-inoculated (Table 1). A similar trend was observed in shoot width of soybean plants in which non-inoculated soybean plants were significantly lower in shoot width when compared with R3, MY, R3MY and R1+R3MY. Branch and leaf number were higher in R3 and R1+R3MY treatments respectively. Also, R1+R3MY treatment had the greatest positive impact on nodule number and taproot length including lateral root number and fresh plant biomass weight. In contrast, R1 showed the greatest impact on taproot width and nodule fresh and dry weight. Shoot (17.6 g) and root (5.6) dry weights were higher in plants inoculated with R3 bacterial strain and R1MY respectively (Table 1).

Table 10. 1 Effects of *Rhizobium* spp. and mycorrhizal fungi on soybean growth in the field at the beginning of blooming

Treatment	SH (mm)	SW (mm)	Branch number	Leaf number	Nodule number	Taproot length (mm)	Taproot width (mm)	Lateral root number	Fresh plant biomass (g)	Nodule fresh wt (g)	Nodule dry wt (g)	SH dry wt (g)	Root dry wt (g)
Control	268.3±32.6 ^a	6.2±1.2 ^b	5.2±0.7 ^a	41.5±9.8 ^b	2.3±2.1 ^a	152.5±19.1 ^{ab}	2.8±0.4 ^b	16.5±1.9 ^{bc}	26.9±9.7 ^{ab}	0.1±0.1 ^a	0.003±0.0 ^a	8.1±3.3 ^a	0.2±0.0 ^c
R1	308.0±8.9 ^a	8.5±1.9 ^{ab}	7.5±2.2 ^a	65.0±12.2 ^b	7.5±6.4 ^a	140.8±20.4 ^{ab}	5.7±0.9 ^a	9.5±1.1 ^c	45.4±12.2 ^{ab}	2.6±2.3 ^a	0.1±0.1 ^a	13.6±2.9 ^a	1.1±0.2 ^c
R3	305.5±31.1 ^a	7.5±1.2 ^{ab}	8.3±1.3 ^a	69.0±13.9 ^b	1.3±1.3 ^a	147.3±14.7 ^{ab}	4.8±0.6 ^{ab}	25.3±4.8 ^a	52.6±18.5 ^{ab}	0.0±0.0 ^a	0.0±0.0 ^a	17.6±2.4 ^a	1.9±0.3 ^b
MY	262.5±33.4 ^a	7.2±0.7 ^{ab}	4.7±0.7 ^a	51.3±4.9 ^b	3.5±2.2 ^a	161.2±16.1 ^{ab}	5.0±1.0 ^{ab}	15.0±1.3 ^{bc}	39.0±7.9 ^{ab}	0.01±0.01 ^a	0.01±0.0 ^a	10.2±1.9 ^a	1.1±0.2 ^a
R1MY	266.7±29.0 ^a	5.0±1.1 ^{ab}	8.2±1.9 ^a	39.5±9.7 ^b	0.0±0.0 ^a	150.5±34.1 ^{ab}	5.5±1.2 ^{ab}	17.2±4.4 ^{abc}	17.6±4.2 ^b	0.0±0.0 ^a	0.0±0.0 ^a	5.6±1.5 ^a	5.6±0.0 ^{bc}
R3MY	283.0±38.5 ^a	7.5±1.4 ^{ab}	6.2±1.6 ^a	72.0±16.2 ^b	7.7±6.5 ^a	164.5±16.4 ^{ab}	3.5±0.3 ^{ab}	18.8±0.8 ^{ab}	39.9±10.6 ^{ab}	0.01±0.01 ^a	0.01±0.0 ^a	14.9±5.3 ^a	1.3±0.4 ^{bc}
R1+R3	290.8±28.7 ^a	6.7±1.6 ^{ab}	5.2±0.7 ^a	52.5±12.6 ^b	4.8±3.6 ^a	130.5±27.6 ^b	4.67±0.4 ^{ab}	16.2±1.9 ^{bc}	40.1±16.7 ^{ab}	0.1±0.1 ^a	0.04±0.04 ^a	15.0±4.6 ^a	1.0±0.3 ^c
R1+R3MY	321.3±36.4 ^a	10.8±2.3 ^a	8.0±0.4 ^a	94.2±22.4 ^a	8.7±8.7 ^a	201.5±17.2 ^a	5.5±1.6 ^a	22.0±4.5 ^{ab}	66.8±23.8 ^a	0.03±0.03 ^a	0.02±0.02 ^a	15.1±7.7 ^a	1.5±0.4 ^c

Legend: Control – non - inoculated treatment, R - *Rhizobium* sp. strain R1, R3 - *R. cellulosilyticum* strain R3, MY - Mycorrhizal consortium, R1MY - *Rhizobium* sp. strain R1 and mycorrhizal fungal consortium, R3MY - *R. cellulosilyticum* strain R3 and mycorrhizal consortium, R1+R3 - *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3, R1+R3MY - *Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium, SH - shoot height, SW – shoot width, wt – weight, mm – millimeter, g – gram. Number of replicates (n) = 8. Data represent mean ± SE. Means with the same letters are not significantly different considering Duncan's multiple range test

10.2.8 Effects of Rhizobia and AMF on Soybean Growth at the Beginning of Pod Development in the Field

At the beginning of pod development, higher shoot height, shoot width, leaf number, nodule number, taproot length, taproot width, lateral root number, pod number nodule fresh weight, nodule dry weight and shoot dry weight were correspondingly observed in MY, R1MY/R3MY, R1+R3MY, R1MY, R1+R3MY, R3MY, R1MY, R1+R3, R3, R1MY, control and R1MY treatments (Table 2). In contrast, the shoot width, branch number, taproot length, taproot width and lateral root number of non-inoculated (control) plants were negatively impacted by drought as they were less productive, considering the afore-mentioned above-ground and below-ground parameters (Table 2).

Table 10. 2 Effects of *Rhizobium* spp. and mycorrhizal fungi on soybean growth in the field at the beginning of pod development

Treatment	SH (mm)	SW (mm)	Branch number	Leaf number	Nodule number	Taproot length (mm)	Taproot width (mm)	Lateral root number	Pod number	Nodule fresh wt (g)	Nodules dry wt (g)	SH dry wt (g)
Control	357.5±33.8 ^a	4.8±0.7 ^b	14.2±1.0 ^a	65.0±9.3 ^b	2.8±2.1 ^a	156.8±16.1 ^a	2.5±0.3 ^c	14.5±0.8 ^a	16.3±4.4 ^a	0.2±0.1 ^b	0.7±0.7 ^a	9.7±2.3 ^a
R1	324.0±20.9 ^a	5.3±0.4 ^b	15.3±1.3 ^a	63.5±13.4 ^b	2.7±2.7 ^a	173.8±14.5 ^a	4.8±0.3 ^{bc}	19.8±1.2 ^a	18.2±4.2 ^a	0.1±0.1 ^b	0.01±0.01 ^a	7.1±1.9 ^a
R3	370.0±31.8 ^a	6.7±0.9 ^{ab}	15.8±0.7 ^a	90.5±14.6 ^{ab}	1.7±1.1 ^a	191.0±20.2 ^a	7.2±0.4 ^{ab}	20.5±3.8 ^a	38.7±12.9 ^a	0.1±0.1 ^b	0.008±0.0 ^a	11.4±5.1 ^a
MY	383.0±22.5 ^a	6.8±0.4 ^{ab}	16.0±2.8 ^a	68.5±3.7 ^b	2.3±2.3 ^a	167.2±16.1 ^a	6.2±0.5 ^{ab}	16.8±2.4 ^a	24.0±1.7 ^a	0.1±0.1 ^b	0.014±0.01 ^a	8.6±2.6 ^a
R1MY	365.8±52.8 ^a	8.7±0.9 ^a	16.7±0.9 ^a	128.5±20.9 ^a	5.3±5.3 ^a	193.0±15.2 ^a	8.5±1.2 ^a	20.5±1.8 ^a	37.2±9.4 ^a	0.8±0.3 ^a	0.2±0.1 ^a	16.6±4.2 ^a
R3MY	361.7±23.8 ^a	8.7±1.5 ^a	15.3±0.7 ^a	95.2±14.7 ^{ab}	3.8±2.8 ^a	209.7±36.7 ^a	7.8±1.9 ^a	18.0±1.0 ^a	33.7±9.6 ^a	0.2±0.2 ^b	0.1±0.1 ^a	15.2±3.9 ^a
R1+R3	338.2±36.6 ^a	6.3±0.8 ^{ab}	16.2±1.4 ^a	85.5±18.9 ^{ab}	0.0±0.0 ^a	195.8±10.5 ^a	7.2±0.9 ^{ab}	20.7±2.9 ^a	14.3±7.4 ^a	0.0±0.0 ^b	0.0±0.0 ^a	9.8±2.5 ^a
R1+R3MY	370.8±16.2 ^a	7.2±0.9 ^{ab}	17.8±1.4 ^a	103.0±17.1 ^{ab}	5.7±5.7 ^a	176.3±17.4 ^a	7.3±0.6 ^{ab}	20.0±2.5 ^a	17.3±1.2 ^a	0.1±0.1 ^b	0.07±0.1 ^a	11.4±1.4 ^a

Legend: Control – non - inoculated treatment, R - *Rhizobium* sp. strain R1, R3 - *R. cellulosilyticum* strain R3, MY - Mycorrhizal consortium, R1MY - *Rhizobium* sp. strain R1 and mycorrhizal fungal consortium, R3MY - *R. cellulosilyticum* strain R3 and mycorrhizal consortium, R1+R3 - *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3, R1+R3MY - *Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium, SH - shoot height, SW – shoot width, wt – weight, mm – millimeter, g – gram. Number of replicates (n) = 8. Data represent mean ± SE. Means with the same letters are not significantly different considering Duncan's multiple range test

10.2.9 Effects of Rhizobia and AMF on Soybean Growth at the Beginning of Seed Development in the Field

At the beginning of seed development, R1MY significantly increased shoot height compared to other treatments, but co-inoculation of the two bacterial strains resulted in the production of soybean plants with wider shoots, more branches and leaves. Plants inoculated with mycorrhizal consortium did not only produce higher number of nodules, but they also had longer taproot compared to other treatments. Soybean inoculated with R1 strain had the lowest number of nodules and shortest taproot. Similar lateral root number (≥ 19) was observed in plants amended with R1, R3MY and R1+R3MY, but R3 had the greatest impact on lateral root number since it produced the highest root number of 23 (Table 3). The fresh plant biomass was tremendously affected by MY inoculation when compared to other treatments and soybean plants dually inoculated with both bacterial strains (R1+R3) were found to produce higher number of pod at this stage of soybean growth in this study. Although, MY treatment produced higher number of nodules, R1MY treatment produced nodules with greater fresh and dry weights. Pod fresh and dry weights as well as shoot dry weight were higher in R1+R3, while the lowest shoot dry weight was found in the control plants. Similar root dry weight (>2 g) was recorded in soybean plants inoculated with R3, R1MY, R3MY, R1+R3, R1+R3MY, but mycorrhizal treatment produced roots with greater dry weight (3.0 g) (Table 3).

Table 10. 3 Effects of *Rhizobium* spp. and mycorrhizal fungi on soybean growth in the field at the beginning of seed development

Treatment	SH (mm)	SW (mm)	Branch number	Leaf number	Nodule number	Taproot length (mm)	Taproot width (mm)	Lateral root number	Fresh plant biomass (g)	Pod number	Nodules fresh wt	Nodules dry wt	Pod fresh wt	Pod dry wt	SH dry wt	Root dry wt
Control	419.8±17.2 ^a	6.4±0.7 ^{ab}	15.6±0.9 ^a	93.6±11.2 ^{ab}	3.4±2.8 ^a	198.8±29.2 ^a	5.7±1.0 ^a	22.4±1.7 ^a	52.9±8.4 ^{ab}	45.6±6.6 ^{ab}	0.2±0.2 ^a	0.1±0.1 ^a	13.03±3.4 ^b	4.7±1.2 ^a	12.7±3.2 ^b	2.6±0.4 ^a
R1	380.3±20.8 ^a	5.5±0.6 ^b	14.5±0.7 ^a	69.0±17.9 ^b	0.2±0.2 ^a	193.8±16.1 ^a	4.2±0.4 ^a	19.1±3.3 ^a	37.9±5.8 ^b	36.0±5.1 ^b	0.1±0.1 ^a	0.0007±0.0 ^a	6.5±1.4 ^b	2.6±0.6 ^a	13.7±1.4 ^b	1.9±0.2 ^a
R3	391.2±27.7 ^a	5.8±0.7 ^b	15.2±0.6 ^a	94.2±10.8 ^{ab}	2.7±2.3 ^a	193.2±26.8 ^a	3.3±0.4 ^a	23.3±3.5 ^a	57.3±10.7 ^{ab}	38.7±8.2 ^b	0.4±0.2 ^a	0.09±0.1 ^a	6.3±1.9 ^b	3.4±0.9 ^a	15.8±2.7 ^b	2.5±0.4 ^a
MY	454.2±52.7 ^a	8.3±0.6 ^{ab}	16.8±1.6 ^a	118.0±12.3 ^{ab}	20.3±18.0 ^a	227.0±40.2 ^a	4.6±0.8 ^a	20.3±2.5 ^a	98.4±14.3 ^a	60.8±7.9 ^{ab}	0.3±0.2 ^a	0.1±0.1 ^a	15.9±2.8 ^{ab}	6.9±1.1 ^a	25.5±3.9 ^{ab}	3.0±0.5 ^a
R1MY	501.0±50.2 ^a	5.4±1.5 ^b	17.4±1.8 ^a	128.8±34.1 ^{ab}	10.8±5.4 ^a	159.6±28.9 ^a	5.2±0.9 ^a	15.0±3.2 ^a	90.2±23.4 ^a	60.2±14.9 ^{ab}	0.5±0.3 ^a	0.2±0.1 ^a	9.9±2.7 ^b	5.0±1.3 ^a	30.6±7.5 ^{ab}	2.6±0.6 ^a
R3MY	385.7±37.7 ^a	5.7±0.7 ^b	16.2±1.2 ^a	92.5±10.4 ^{ab}	6.2±3.8 ^a	200.5±33.4 ^a	3.3±0.5 ^a	19.0±2.3 ^a	53.1±6.8 ^{ab}	36.2±6.7 ^b	0.04±0.04 ^a	0.02±0.02 ^a	6.6±2.1 ^b	3.1±0.8 ^a	14.4±3.6 ^b	2.5±0.6 ^a
R1+R3	473.7±27.8 ^a	9.2±1.1 ^a	18.3±0.9 ^a	162.3±32.3 ^a	5.5±3.9 ^a	202.3±26.5 ^a	4.4±1.2 ^a	18.2±2.6 ^a	76.4±21.0 ^{ab}	89.7±31.6 ^a	0.4±0.2 ^a	0.1±0.1 ^a	27.9±10.9 ^a	7.3±3.2 ^a	33.8±10.2 ^a	2.2±0.4 ^a
R1+R3MY	414.7±56.0 ^a	7.3±1.1 ^{ab}	16.9±1.5 ^a	122.2±27.3 ^{ab}	6.6±4.1 ^a	217.5±24.4 ^a	3.5±0.4 ^a	19.9±3.8 ^a	88.0±21.7 ^a	54.9±12.8 ^{ab}	0.3±0.08 ^a	0.01±0.009 ^a	16.3±4.2 ^{ab}	5.7±1.4 ^a	21.7±5.8 ^{ab}	2.3±0.5 ^a

Legend: Control – non - inoculated treatment, R - *Rhizobium* sp. strain R1, R3 - *R. cellulosilyticum* strain R3, MY - Mycorrhizal consortium, R1MY - *Rhizobium* sp. strain R1 and mycorrhizal fungal consortium, R3MY - *R. cellulosilyticum* strain R3 and mycorrhizal consortium, R1+R3 - *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3, R1+R3MY - *Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium, SH - shoot height, SW – shoot width, wt – weight, mm – millimeter, g – gram.

Number of replicates (n) = 8. Data represent mean ± SE. Means with the same letters are not significantly different considering Duncan's multiple range test

10.2.10 Effects of Rhizobia and AMF on Soybean Growth at Full Seed in the Field

At full seed stage, the non-inoculated soybean plants were less productive as shown or indicated in all the below-ground and above-ground parameters measured (Table 4). With the exception of leaf number, pod fresh and dry weight, soybean plants dually inoculated with rhizobial spp. and mycorrhizal consortium (R1+R3MY) had the greatest effects on the measured above-ground parameters (such as shoot height, shoot width, branch number, pod number and shoot dry weight). R1MY and R3MY inoculations significantly increased ($P<0.05$) leaf and nodule numbers respectively while the greatest significant effects were correspondingly observed in taproot length and width of plants treated with R1+R3 and MY. Similarly, R1+R3 significantly increased plant fresh biomass, but soybean inoculated with R1MY produced nodules with comparative higher fresh and dry weights. The highest values for pod fresh/dry weight and root dry weight were observed in treatments amended with R3 and R1+R3 respectively (Table 4).

Table 10. 4 Effects of *Rhizobium* spp. and mycorrhizal fungi on soybean growth in the field at full seed

Treatment	SH (mm)	SW (mm)	Branch number	Leaf number	Nodule number	Taproot length (mm)	Taproot width (mm)	Lateral root number	Fresh plant biomass	Pod number	Nodule fresh wt (g)	Nodule dry wt (g)	Pod fresh wt (g)	Pod dry wt (g)	SH dry wt (g)	Root dry wt (g)
Control	319.9±16.5 ^b	3.8±0.4 ^b	12.5±0.6 ^b	45.0±5.9 ^b	5.0±5.0 ^a	180.4±16.1 ^a	3.0±0.3 ^a	32.8±4.7 ^{ab}	26.8±5.4 ^c	18.3±2.7 ^b	0.009±0.0 ^a	0.006±0.0 ^a	9.2±2.5 ^{ab}	2.9±0.9 ^b	4.4±0.9 ^b	0.5±0.1 ^b
R1	448.1±45.5 ^{ab}	6.4±0.9 ^{ab}	15.9±2.1 ^{ab}	118.3±34.7 ^{ab}	15.5±9.3 ^a	183.0±23.8 ^a	4.7±0.6 ^a	48.0±10.2 ^a	80.5±21.9 ^{bc}	57.6±22.2 ^{ab}	0.07±0.03 ^a	0.04±0.02 ^a	32.4±13.3 ^{ab}	11.7±4.8 ^{ab}	15.8±5.1 ^{ab}	2.0±0.5 ^{ab}
R3	479.4±61.4 ^a	7.9±1.3 ^a	17.6±2.5 ^{ab}	171.6±67.0 ^a	5.5±3.3 ^a	168.3±17.7 ^a	4.5±0.5 ^a	35.6±5.0 ^{ab}	139.2±42.8 ^{abc}	89.3±22.9 ^a	0.02±0.02 ^a	0.006±0.0 ^a	74.4±28.2 ^a	28.6±11.4 ^a	33.1±12.3 ^{ab}	1.8±0.5 ^{ab}
MY	448.8±39.9 ^{ab}	8.0±0.9 ^a	15.9±1.9 ^{ab}	109.1±32.2 ^{ab}	27.5±17.1 ^a	202.0±25.7 ^a	5.2±0.7 ^a	38.9±10.6 ^{ab}	103.5±22.6 ^{bc}	70.1±26.2 ^{ab}	0.4±0.3 ^a	0.1±0.1 ^a	48.7±21.9 ^{ab}	18.7±8.6 ^{ab}	25.4±11.8 ^{ab}	1.9±0.7 ^{ab}
R1MY	454.8±48.1 ^{ab}	6.1±0.9 ^{ab}	16.3±1.8 ^{ab}	105.8±35.0 ^{ab}	40.9±24.6 ^a	196.1±25.4 ^a	4.9±1.2 ^a	22.4±3.6 ^b	97.8±34.0 ^{bc}	63.1±29.6 ^{ab}	0.9±0.9 ^a	0.4±0.3 ^a	36.5±19.5 ^{ab}	12.9±6.8 ^{ab}	24.6±14.5 ^{ab}	2.2±0.6 ^a
R3MY	431.7±38.2 ^{ab}	6.7±0.6 ^a	18.4±1.6 ^{ab}	206.6±52.9 ^a	8.1±4.9 ^a	205.7±23.8 ^a	5.1±0.8 ^a	21.1±1.9 ^b	116.4±22.9 ^{bc}	65.6±12.7 ^{ab}	0.3±0.1 ^a	0.02±0.01 ^a	50.0±12.7 ^{ab}	18.9±4.8 ^{ab}	25.5±6.1 ^{ab}	2.9±0.5 ^a
R1+R3	487.7±49.5 ^a	7.9±1.3 ^a	17.9±1.9 ^{ab}	146.6±28.8 ^{ab}	10.9±6.9 ^a	223.7±25.8 ^a	4.7±0.9 ^a	35.0±8.5 ^{ab}	255.1±84.4 ^a	88.1±18.9 ^a	0.3±0.2 ^a	0.1±0.1 ^a	55.2±15.9 ^{ab}	19.7±5.7 ^{ab}	35.6±7.6 ^a	3.2±0.7 ^a
R1+R3MY	535.6±33.8 ^a	8.0±0.7 ^a	20.6±1.5 ^a	182.3±24.4 ^a	7.4±7.4 ^a	213.6±23.1 ^a	4.1±0.4 ^a	49.0±11.2 ^a	171.0±42.5 ^{ab}	90.9±11.5 ^a	0.01±0.01 ^a	0.002±0.0 ^a	73.4±19.9 ^a	26.8±7.4 ^a	39.8±8.7 ^a	2.9±0.9 ^a

Legend: Control – non - inoculated treatment, R - *Rhizobium* sp. strain R1, R3 - *R. cellulosilyticum* strain R3, MY - Mycorrhizal consortium, R1MY - *Rhizobium* sp. strain R1 and mycorrhizal fungal consortium, R3MY - *R. cellulosilyticum* strain R3 and mycorrhizal consortium, R1+R3 - *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3, R1+R3MY - *Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium, SH - shoot height, SW – shoot width, wt – weight, mm – millimeter, g – gram.

Number of replicates (n) = 8. Data represent mean ± SE. Means with the same letters are not significantly different considering Duncan's multiple range test

10.2.11 Effects of Rhizobia and AMF on Soybean Growth at Full Maturity in the Field

At full maturity, the control or non-inoculated soybean were the most negatively impacted plants considering the below-ground and above-ground components with the exception of lateral root number in which R3 treatment produced the lowest number of roots (Table 5) but significantly increased ($P < 0.05$) shoot dry weight. Dual inoculation of rhizobial spp. and mycorrhizal consortium (R1+R3MY) showed the greatest impact on soybean measured components such as shoot height, taproot length, pod length, and root dry weight. A similar shoot width (≥ 7 mm) was observed in soybean plants treated with R1, R3, R3MY, R1+R3 and R1+R3MY, but soybean dually inoculated with R1 and MY had the greatest positive effect on shoot width. The highest mean counts of 14.5 and 73.9 were recorded for branch and leaf number respectively in plants treated with R3MY while the highest taproot width (4.1 mm) was similar for soybean plants inoculated with MY, R3MY and R1+R3. R1+R3 inoculation significantly ($P < 0.05$) led to production of more pods and R3MY inoculation had the greatest significant impact on pod fresh weight (Table 5).

Table 10. 5 Effects of *Rhizobium* spp. and mycorrhizal fungi on soybean growth in the field at full maturity

Treatment	SH (mm)	SW (mm)	Branch number	Leaf number	Taproot length	Taproot width	Lateral root number	Fresh plant biomass (g)	Pod number	Pod fresh weight (g)	Pod length (mm)	SH dry weight (g)	Root dry weight (g)
Control	361.1±35.5 ^a	5.1±1.1 ^b	9.1±0.8 ^b	33.0±4.4 ^b	179.6±24.6 ^b	2.4±0.4 ^b	58.4±8.0 ^{ab}	45.2±15.9 ^a	33.1±13.4 ^b	26.3±9.2 ^a	45.0±1.5 ^b	6.6±1.9 ^a	0.7±0.3 ^b
R1	469.3±28.7 ^a	7.1±1.4 ^{ab}	12.3±1.2 ^{ab}	47.6±9.5 ^{ab}	193.4±12.4 ^b	3.8±0.5 ^{ab}	89.5±8.5 ^a	72.7±22.2 ^a	60.6±21.9 ^{ab}	45.9±15.5 ^a	46.3±0.5 ^{ab}	12.0±3.9 ^a	1.7±0.7 ^{ab}
R3	469.6±51.9 ^a	7.6±0.9 ^{ab}	13.5±1.8 ^{ab}	60.4±15.2 ^{ab}	256.4±33.9 ^{ab}	3.4±0.4 ^{ab}	35.0±2.9 ^b	95.2±23.5 ^a	67.9±19.8 ^{ab}	62.0±17.3 ^a	45.1±1.6 ^b	17.1±4.9 ^a	1.6±0.3 ^{ab}
MY	425.4±49.8 ^a	6.8±0.9 ^{ab}	14.0±1.7 ^{ab}	42.0±4.9 ^{ab}	187.0±25.8 ^b	4.1±0.7 ^a	71.8±7.7 ^{ab}	75.6±17.6 ^a	59.8±13.8 ^{ab}	48.1±12.9 ^a	46.5±0.8 ^{ab}	13.1±2.4 ^a	1.4±0.3 ^{ab}
R1MY	463.0±44.5 ^a	9.4±1.9 ^a	11.4±1.8 ^{ab}	47.3±11.9 ^{ab}	186.3±24.7 ^b	3.35±0.3 ^{ab}	97.0±25.6 ^a	58.6±8.8 ^a	49.5±12.9 ^{ab}	42.3±11.1 ^a	48.6±0.8 ^a	11.9±2.5 ^a	0.9±0.2 ^{ab}
R3MY	447.5±55.4 ^a	7.5±1.3 ^{ab}	14.5±1.7 ^a	73.9±10.6 ^a	217.2±21.3 ^{ab}	4.1±0.4 ^a	91.5±9.2 ^a	69.1±17.1 ^a	70.8±22.3 ^{ab}	62.8±19.0 ^a	47.4±0.9 ^{ab}	14.3±4.0 ^a	2.1±0.5 ^{ab}
R1+R3	475.0±38.9 ^a	7.5±1.0 ^{ab}	13.4±1.3 ^{ab}	62.6±17.3 ^{ab}	182.3±20.5 ^b	4.1±0.5 ^a	48.9±12.8 ^b	70.5±16.0 ^a	153.1±82.5 ^a	54.5±17.6 ^a	44.3±0.7 ^b	14.4±4.7 ^a	1.4±0.4 ^{ab}
R1+R3MY	490.0±37.5 ^a	7.0±0.7 ^{ab}	12.5±1.4 ^{ab}	59.8±7.2 ^{ab}	281.3±36.7 ^a	3.8±0.5 ^{ab}	64.3±13.9 ^{ab}	92.4±21.8 ^a	80.9±28.8 ^{ab}	57.3±16.1 ^a	49.5±1.3 ^a	10.4±2.5 ^a	2.3±0.6 ^a

Legend: Control – non - inoculated treatment, R - *Rhizobium* sp. strain R1, R3 - *R. cellulosilyticum* strain R3, MY - Mycorrhizal consortium, R1MY - *Rhizobium* sp. strain R1 and mycorrhizal fungal consortium, R3MY - *R. cellulosilyticum* strain R3 and mycorrhizal consortium, R1+R3 - *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3, R1+R3MY - *Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium, SH - shoot height, SW – shoot width, mm – millimeter, g – gram. Number of replicates (n) = 8. Data represent mean ± SE. Means with the same letters are not significantly different considering Duncan's multiple range test

10.2.12 Effects of Rhizobia and AMF on Macro and Micronutrients of Soybean Seeds

Grown in the field

Generally, soybean seeds inoculated with R1MY gave the best results for all the macronutrients (such as N, P, Mg and Ca) analyzed in this study (Table 6). Seed nitrogen (N) content under semi-arid condition was greatest in R1MY treatment followed by soybean inoculated with R1+R3MY (Table 6). A similar N content was observed in R3 and control treatments but N content of 2265.3 mg/L was observed in R3 treatment and 1725.1 mg/L in R3MY treatment. Seed phosphorus (P) content was similar in seeds of soybean inoculated with R1, R3 and R3MY while besides R1MY treatment, MY treatment showed the highest P content compared to other inoculated plants. Similar results were observed for magnesium (Mg), calcium (Ca) and sulphur (S) contents of soybean seeds.

With respect to the micronutrients, under semi-arid environment, seeds of R1MY had higher cobalt (Co), while R1 and R1MY seeds showed the highest concentrations of Mo. As regards zinc (Zn) content, MY, R1MY, R1+R3, R1+R3MY and control plants showed similar results. Also, similar manganese (Mn) concentration (0.1 mg/L) were observed in plants inoculated with R1, R3, and R1+R3 while MY, R1MY, R1+R3MY and control plants displayed similar Mn content of 0.2 mg/L. R1, R3, MY, R1MY and R3MY had higher copper (Cu) content, but the non-inoculated plants showed the highest concentration of sodium (Na) (Table 6).

Table 10. 6 Effects of *Rhizobium* spp. and mycorrhizal fungi on macro and micro nutrient contents of soybean seeds grown in the field

Treatment	Macronutrients (mg/L)						Micronutrients (mg/L)							
	N	P	K	Mg	Ca	S	Co	Mo	Zn	Mn	Cu	Na	Fe	B
Control	1504.4± 915.9 ^{bc}	25.6±1.2 ^a	ND	23.8±1.2 ^a	65.9±6.9 ^{ab}	28.4±4.4 ^a	0.001±0.0 ^a	0.015±0.0 ^{bc}	0.1±0.01 ^b	0.2±0.03 ^a	0.1±0.0 ^{bc}	23.9±3.3 ^a	1.8±1.2 ^a	0.2±0.04 ^b
R1	2265.3 ±244.7 ^c	11.9±0.5 ^d	ND	8.3±0.4 ^c	40.8±3.2 ^d	29.3±0.6 ^a	0.001±0.0 ^a	0.032±0.0 ^a	0.05±0.0 ^c	0.1±0.02 ^{ab}	0.044±0.01 ^{bc}	5.5±0.5 ^{de}	0.6±0.1 ^a	0.02±0.0 ^d
R3	1536.6±193.7 ^c	11.4±0.9 ^d	ND	8.2±0.7 ^c	35.7±2.8 ^d	30.4±0.9 ^a	0.0009±0.0 ^a	0.026±0.0 ^{ab}	0.07±0.0 ^{bc}	0.1±0.0 ^b	0.037±0.0 ^{bcd}	3.0±0.4 ^e	0.4±0.1 ^a	0.03±0.0 ^d
MY	633.6±480.0 ^c	19.2±1.6 ^b	ND	16.9±1.4 ^b	55.4±6.6 ^{bc}	9.8±0.8 ^c	0.001±0.0 ^a	0.01±0.0 ^{abc}	0.1±0.0 ^b	0.2±0.03 ^a	0.04±0.0 ^{bc}	11.1±1.3 ^{bc}	0.9±0.3 ^a	0.2±0.02 ^c
R1MY	5902.1±823.5 ^a	26.9±0.9 ^a	ND	24.1±1.5 ^a	69.8±5.1 ^a	24.9±6.5 ^{ab}	0.01±0.0 ^a	0.03±0.0 ^a	0.15±0.01 ^a	0.2±0.01 ^a	0.06±0.0 ^b	13.5±0.9 ^b	1.14±0.11 ^a	0.4±0.03 ^a
R3MY	1725.1±164.2 ^c	11.7±0.6 ^d	ND	7.7±0.3 ^c	39.4±1.8 ^d	33.8±1.0 ^a	0.001±0.0 ^a	0.02±0.0 ^{abc}	0.08±0.0 ^{bc}	0.9±0.01 ^b	0.03±0.0 ^{cd}	4.5±0.4 ^{de}	0.7±0.2 ^a	0.04±0.0 ^d
R1+R3	675.1±675.1 ^c	15.1±1.8 ^{cd}	ND	16.7±1.9 ^b	50.0±5.7 ^{cd}	17.4±2.4 ^{bc}	0.001±0.0 ^a	0.008±0.0 ^c	0.1±0.0 ^{bc}	0.1±0.02 ^b	0.021±0.0 ^d	11.1±1.7 ^{bc}	0.4±0.2 ^a	0.1±0.02 ^c
R1+R3MY	3713.3± 962.3 ^b	16.0±1.9 ^{bc}	ND	14.9±2.7 ^b	46.9±2.6 ^{cd}	28.0±2.6 ^a	0.001±0.0 ^a	0.02±0.0 ^{abc}	0.1±0.01 ^a	0.2±0.01 ^a	0.1±0.01 ^a	8.3±1.4 ^{cd}	0.5±0.1 ^a	0.1±0.04 ^c

Legend: Control – non - inoculated treatment, R - *Rhizobium* sp. strain R1, R3 - *R. cellulosilyticum* strain R3, MY - Mycorrhizal consortium, R1MY - *Rhizobium* sp. strain R1 and mycorrhizal fungal consortium, R3MY - *R. cellulosilyticum* strain R3 and mycorrhizal consortium, R1+R3 - *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3, R1+R3MY - *Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium, ND - no detected level, mg/L – milligram per litre, N – nitrogen, P – phosphorus, K – potassium, Mg – magnesium, Ca – calcium, S – sulphur, Co – cobalt, Mo – molybdenum, Zn – zinc, Mn – Manganese, Cu – copper, Na – sodium, Fe – iron, B – boron. Number of replicates (n) = 8. Data represent mean ± SE. Means with the same letters are not significantly different considering Duncan's multiple range test

10.3 Discussion

Relative humidity is the amount of moisture in the atmosphere at a particular temperature. In this study, the mean relative humidity during the period of the experiment ranged from 42 – 65% (Fig. 1), which is slightly different from the range (63.1 – 69.2%) reported by Prakamhang et al. (2015). In addition, during the period of this experiment, the temperature range was 12.5 – 33.8°C (Fig. 2) which partly agrees with a temperature of 19.2°C reported by Ortiz et al. (2015) in a study performed in an arid soil and almost similar climatic trends were observed in preceding years (Fig. 1 and 2). Also, it only rained for 61 days during the period of this experiment (Fig. 3). Hence, the climatic data obtained in this study indicate that the study area is exposed to drought stress.

Furthermore, plants are continually subjected to abiotic stress (e.g. drought) which is among the major challenges connected to plant development impacting agronomic demands. Inoculation with rhizobacteria and mycorrhizal fungi has been reported to efficiently enhance productivity under semi-arid and arid environments (Enebe and Babalola, 2018, Igiehon and Babalola, 2017). Consequently, knowledge of the influence of interactions between microorganisms and plants on crop growth, nutritional level and tolerance to stress is essential. Indeed, inoculation with two drought tolerant *Rhizobium* spp. strains R1 and R3 as well as mycorrhizal consortium for growth and nutrient enhancement under water-stress environments resulted in increased RWC, soluble sugars, proline, above-ground and below-ground biomass, nutrient content and decreased electrolyte leakage.

Accordingly, the results of this study showed that rhizobial and mycorrhizal inoculations enhanced RWC of soybean leaves compared to the non-inoculated plants (Fig. 4). Mycorrhizal consortium (MY) treatment gave the highest RWC followed by the rhizobial and mycorrhizal

combination (R3MY) treatment. This increase in RWC can be attributed to the capacity of mycorrhizal and nodulated root to enhance water uptake in soil deficient in water. Similar findings were also reported in plants grown under water stress by Baslam and Goicoechea (2012) and Jie et al. (2000). According to Beltrano and Ronco (2008), mycorrhizal fungi positively impacted wheat water status under drought stress conditions. In short, maintaining higher RWC in mycorrhizal and nodulated plants indicates a tolerance tactic by minimizing water loss and as such assisting plant to evade drought stress pressure.

Conversely, electrolyte leakage was in soybean plants co-inoculated with both rhizobial species (R1+R3) and plants dually inoculated with both rhizobia and mycorrhizal consortium (R1+R3MY). We had similar finding in our previous study in which R1+R3MY significantly reduced ($P < 0.05$) electrolyte leakage under different water-stressed conditions. Data based on electrolyte leakage give information on cell membrane integrity. An intact cell would have stable and less permeable membrane and, therefore, less electrolyte diffusion out of the cell. Thus, the relatively low electrolyte values observed in soybean plants inoculated with R1+R3 and R1+R3MY (Fig. 4) showed that the plant cells or tissues were not injured by drought stress. This result is in agreement with the findings of Ortiz et al. (2015) who reported reduction in cell membrane damage in stressed plants by plant growth promoting bacteria and mycorrhizal fungi.

Admittedly, microbial inoculation also enhanced soluble sugar concentration more than the non-inoculated plants, suggesting that microbial species help in catabolic breakdown of starch to sugars for osmotic adjustment to minimize the effects of stress since it was reported by Enebak et al. (1998) that starch degradation may be the cause of increase in soluble sugars in cells. Accumulation of soluble sugars was more pronounced in soybean leaves treated with R3MY, R1+R3 and R1+R3MY, but accumulation was highest in R1+R3MY inoculated plants.

The absence of rhizobia and mycorrhizae in the non-inoculated (control) plants perhaps led to a decrease in starch catabolism and soluble sugar content and it consequently affected the plant growth as depicted in Table 1, 2, 3, 4 and 5. Similarly, previous studies have shown starch reduction in grapevine leaves (Patakas and Noitsakis, 2001) and soluble sugar accumulation in stressed plants (Dekánková et al., 2004) in response to drought stresses.

Indeed, in this study, rhizobial and mycorrhizal fungal inoculations enhanced proline accumulation under semi-arid condition, which might be as a result of up-regulation of proline biosynthetic pathway. High level of proline helps to maintain cell water balance, protect protein and cell membranes from stress (Yoshiba et al., 1997). Accumulation of proline was higher in mycorrhizal treatments particularly in MY, R1MY, R3MY and R1+R3MY treatments (Fig. 7). Proline accumulation may be related to the high level of nutrients (Table 6) observed in soybean seeds amended with R1MY. Soluble sugars and proline are essential osmolyte involved in osmotic adjustment (Zarik et al., 2016) and accumulation of proline under water stressed conditions has been reported in sorghum, wheat, pepper, *Cupressus atlantica* and *Trifolium repens* (Zarik et al., 2016, Ortiz et al., 2015, Sandhya et al., 2010) and therefore, an increase in proline and soluble sugars is an indication of drought stress tolerance. Like the soluble sugars, the accumulation of proline may be as a result of protein degradation in response to drought stress.

In reality, the positive effects observed in most of the mycorrhizal soybean plants with regard to most of the physiological parameters (RWC and electrolyte leakage), biochemical parameters (soluble sugars and proline content), morphological parameters (at different growth stages) and mineral nutrients of seeds can be linked to increase in spore number and mycorrhizal root colonization (Fig. 8), since the use of mycorrhizal fungi as biofertilizer for sustainable

agriculture as reported in our previous studies (Igiehon and Babalola, 2017, Igiehon and Babalola, 2018a) is dependent on their inherent ability to penetrate the roots of their host plants.

Furthermore, the results of our previous work (data not shown) indicated that achievement of sustainable agriculture/food security (Igiehon and Babalola, 2018c) starts in a controlled environment. Thus, in this present study, single and dual inoculations of soybean plants were performed in a greenhouse under different watering levels spanning from 100 % (watering control) to 40% FC. A significant increase was observed in the shoot height of most of the inoculated plants with soybean plants dually inoculated with R3MY showing the greatest shoot height under severe drought condition (40% FC) (Fig. 9). But under severe drought condition, R1MY treatment had the greatest impact on shoot width followed by R3MY. Similarly, in this study, R1MY inoculation gave the best result as regards shoot width of soybean plants harvested at full maturity (Table 5) under semi-arid condition in the field, while R1MY and R3MY treatments had the highest significant impact on soybean width at the beginning of pod development (Table 2). However, under moderate drought stress (70% FC), R1 inoculated soybean plants had the greatest impact on shoot height and shoot width/diameter since the treatment showed a mean height of 223.3 mm and width of 2.21 mm (Fig. 9). This is in agreement with our previous study where it was reported that *Rhizobium* species inoculation enhanced the shoot biomass of a legume (Igiehon and Babalola, 2018c). Also, under this drought condition, R1+R3MY inoculation also proved to enhance shoot height, while the mycorrhizal consortium (MY) inoculation resulted in soybean plants with higher shoot height under well-watered condition (100% FC).

Obviously, *Rhizobium* is a group of bacteria that have the ability to fix atmospheric N for their host via the development of nodules on the roots of plants such as legumes (Mfilinge et al.,

2014, Igiehon and Babalola, 2018c). In this study, at the beginning of blooming, we observed that R1 inoculation was effective to increase nodule formation of soybean plants and this effect increased when it was co-inoculated with R3 and MY in the treatment R1+R3MY (Table 1). The increase in nodule number observed in R1+R3MY treatment can be linked to the significant increase in most of the growth parameters observed in the treatment, since more nodule formation may result in increase in nitrogen fixation which is needed for growth. To be specific, soybean plants inoculated with R1+R3MY were significantly higher ($P < 0.05$) in shoot height, shoot width, leaf number, nodule number, taproot length, lateral root number and fresh plant biomass (Table 1) which conformed with the result of Tairo and Ndakidemi (2013) who reported increase in shoot height, width and other growth parameters of soybean plants grown in the field. On the other hand, R3 inoculated plants gave the best result with regard to branch number, but highly significant increase was observed in soybean nodule fresh and dry weight amended with R1 (Table 1).

Compared to the non-inoculated plants, significant effects were observed at the beginning of pod formation in soybean plants inoculated with rhizobia and mycorrhizal consortium. At this stage of soybean growth, mycorrhizal consortium (MY) had the greatest effect on soybean shoot height, but synergistic interaction between R1 and MY (R1MY) showed high significant impacts on shoot width, leaf number, nodule number, taproot width, nodule fresh weight and shoot dry weight. The increase in the productivity of soybean that was dually inoculated could be linked to the fact that one of the microorganisms serves as a helper species to the other microorganism for effective growth promotion, since it was reported by Igiehon and Babalola (2018c) that ‘in co-inoculation, certain microorganisms function as helper microorganisms to enhance the effectiveness of the other microorganisms’. Amazingly, the non-inoculated soybean plants

produced nodules with the highest dry weight, and the highest pod number with a mean of 38.7 was produced by R3 inoculated soybean (Table 2) which contradicts the report of Mfilinge et al. (2014) who asserted that amendment of soybean plants with *Rhizobium* species resulted in increase in the number of pod produced.

At the beginning of seed development, co-inoculation with R1 and R3 appears to have the greatest effects on seed development since R1+R3 had the greatest impact on pod number, pod fresh weight and pod dry weight (Table 3). Similarly, R1+R3 co-inoculation considerably impacted shoot width, branch number, leaf number and shoot dry weight. These results further buttress the synergistic effects of microbial co-inoculations on plant growth and yield as reported by Mfilinge et al. (2014); Ortiz et al. (2015); Zarik et al. (2016); Igiehon and Babalola (2018c).

At full seed, tripartite interaction with R1, R3 and MY (R1+R3MY) significantly affected seed biomass since this treatment was able to increase pod fresh and dry weight. Also, R1+R3MY inoculation significantly increased pod number, but single inoculation with R3 gave the best results for pod fresh and pod dry weight (Table 4). R1+R3MY also greatly impacted above-ground biomass such as shoot height, shoot width, branch number and shoot dry weight as well as below-ground biomass such as lateral root number. A significant increase ($P < 0.05$) was observed in shoot and taproot width in plants inoculated with MY while co-inoculation of soybean with MY and R1 (R1MY) resulted in higher nodule number, nodule fresh weight and nodule dry weight. Similar effects of co-inoculation on nodulation of agricultural crops have previously been reported. For instance, it was revealed that co-inoculation of *Pseudomonas* and *Mesorhizobium* sp. significantly improved nodulation as well as shoot and root dry weight of chickpea (Verma et al., 2013). In contrast, dual inoculation of soybean with R3 and MY (R3MY) significantly increased leaf number (Table 4).

At maturity, R1+R3MY showed the greatest effect on shoot height (490 mm) and pod length (49.5 mm) (Table 5). Unlike at the beginning of pod development (Table 2), R1+R3 and R3MY had the greatest impact on pod number and pod fresh weight respectively at full maturity (Table 5). Similarly, R3MY significantly increased branch number, leaf number, taproot width and lateral root number, but R1+R3MY had the greatest effects on taproot length and root dry weight. In this present study, with the exception of pod length and lateral root number, drought stress decreased other above-ground and below-ground biomass measured at full maturity (Table 5) in the non-inoculated (control) plants which agrees with the reports of our previous study (Igiehon and Babalola, 2018c).

In the same way, the nutritional components in soybean were increased by most of the rhizobial and mycorrhizal species inoculated under semi-arid environment in the field (Table 6). Similar findings were reported by Ortiz et al. (2015); Zarik et al. (2016); Al-Karaki and Clark (1999); Subramanian and Charest (1999) and Meddich et al. (2000). Most of the macro and micronutrients in this study were improved in soybean seeds. Specific enhancement on soybean seeds nutritional status (principally macronutrients) was significantly pronounced ($P < 0.05$) in plants dually inoculated with the new rhizobial strain R1 and mycorrhizal consortium (R1MY). In relation to the non-inoculated (control) plants, soybean plants dually treated with R1MY augmented N content by 292.4%, P by 5.07%, Mg by 1.26% and Ca by 5.9%. In contrast, the Na content was significantly increased in non-inoculated (control) plants (Table 6) while Mo content in R1 inoculated soybean was increased by 113.3%. The improved nutrient content observed in soybean seeds amended with microbial inoculants, particularly R1MY, could be due to increased absorption space created by extensive mycorrhizal fungal mycelia (Zarik et al., 2016, Igiehon and Babalola, 2017, Igiehon and Babalola, 2018a). Indeed, it was reported in our previous study

(Igiehon and Babalola, 2018c) that a ‘study carried out by Makoi et al. (2013) on the impact of *Rhizobium* strains on mineral nutrient absorption by *Phaseolus vulgaris* showed significant increase in the uptake of P, K, Mg, Ca and S in the entire plant parts. It was reported by the author that even though the concentration of P and K skyrocketed in the root region due to *Rhizobium* introduction, the increase was only significant in the greenhouse experiment and not in the field condition. *Rhizobium* inoculation enhancement of micronutrient (such as Mn, Fe, Cu, Zn, B and Mo) uptake in the shoots, roots, pods and the entire plant with the exception of Mo intake in the roots has been reported’.

It is commonly believed that mycorrhizal fungi hyphae are endowed with the ability to convey P and other mineral nutrients into their host plant (Goicoechea et al., 1997a), while *Rhizobium* species are notable for increasing the N content of their host plants through the process of nitrogen fixation (Igiehon and Babalola, 2018c). Hence, in this study, the increase in N and P nutrients by R1MY treatment could be linked to the effect of the synergistic interaction between the rhizobial sp. and mycorrhizal fungi even though such effect was not glaring in other rhizobial-mycorrhizal combinations.

Furthermore, the accumulation of nutrient in seeds under water stress might have a role in water stress tolerance. In a study carried out by Tanguilig et al. (1987), it was highlighted that soybean grew better than rice under water stress, probably because the legume ‘maintain their turgidity’ under water stress by reducing the rate of transpiration and maintaining nutrient uptake. Therefore, in this study performed under semi-arid condition, the increase in macronutrients (Table 6) and RWC (Fig. 4) observed in R1MY could partly be responsible for the enhancement of most of the measured components of the R1MY inoculated plants compared to the non-inoculated plants, particularly at the beginning of pod and seed development as well

as full seed and maturity stages (Table 2, 3, 4, 5). Correspondingly, Samarah et al. (2004) and Premachandra et al. (1995) reported that P, K, Cl were responsible for osmotic adjustment of water-stress tolerant sorghum line (K866), while Salisbury and Ross (1992) reported that molybdenum was a cofactor in enzymes involved in nitrogen fixation (nitrogenase enzyme) and nitrogen assimilation (nitrate reductase) in plants, thus suggesting that Mo was a key nutrient in nitrogen metabolism and assimilation. Also, drought has been reported to repress nitrogen fixation and reduce accumulation of N in the shoot (Purcell and King, 1996, Serraj et al., 1999). However, in this current study, we observed N accumulation in the seeds of most inoculated soybean plants specifically in R1, R3, R1MY, R3MY and R1+R3MY treatments indicating that N concentrations in seeds was not negatively affected by water stress. This finding is in agreement with the results of Samarah et al. (2004) who similarly reported high N concentration in soybean seeds grown under drought stress. Since Mo is an important nutrient in N metabolism and assimilation, the intake and transport of this micronutrient from water stressed plants may play a role in the uptake and transport of N to the seeds since it was reported by Samarah et al. (2004) that previous studies have shown that Mo application enhanced shoot N concentration and N translocation to the seed.

10.4 Conclusions

Evidence indicated that water stress could have negative effects on plant growth and productivity by affecting nutritional contents and water balance. Nevertheless, the effects of such stress can be mitigated by naturally occurring soil microorganisms such as *Rhizobium* spp. and arbuscular mycorrhizal fungi. As indicated in this study, *Rhizobium* spp. and mycorrhizal fungal symbioses can confer tolerance against water stress in soybean plants. The results of this study showed that the inoculation of soybean with mycorrhizal fungi and *Rhizobium* spp. enhanced

drought tolerance as demonstrated by improved water uptake, cell membrane stability as a result of decreased electrolyte leakage, osmotic adjustment thanks to accumulation of soluble sugars and proline, below-ground and above-ground plant biomass, macro as well as micronutrient assimilation especially N, P, Mg, S, Ca, Co, Mo, Fe and B. In brevity, this can be considered as an effective biotechnological approach to improve soybean and other agricultural crops grown under drought conditions.

10.5 Acknowledgements

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10.6 Conflict of Interest

Both authors have no conflict of interest.

10.7 Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

10.8 Authors Contribution

Igiehon NO performed the experiment, analyzed the data and wrote the article while Babalola OO secured funding, provided academic input in writing the manuscript and thoroughly critiqued the article. Both authors approved the article for publication.

CHAPTER 11

Effects of Rhizobia and Arbuscular Mycorrhizal Fungi on Yield, size Distribution and Percentage Crude Fatty acid of Soybean Seeds Grown under Drought Stress

Abstract

The aim of this study was to assess the ability of *Rhizobium* spp. and mycorrhizal fungi to enhance the yield, seed size and fatty acid content of soybean grown under semi-arid environment. Soybean (*Glycine max* L) seeds inoculated with *Rhizobium* spp. and mycorrhizal fungi were cultivated in soil exposed to drought stress. *Rhizobium* spp. inoculation and mycorrhization alleviated drought stress and increased yield, size and percentage (%) crude fat of soybean seeds but more increase was observed in soybean plants dual inoculated with *Rhizobium* sp. strain R1/*Rhizobium cellulosilyticum* strain R3 and mycorrhizal consortium (R1+R3MY). This increase in the aboveground parameters was accompanied with increase in belowground mycorrhizal spore number, percentage root mycorrhization and aboveground shoot relative water content (RWC) in the dually inoculated (R1+R3MY) soybean plants. In particular, the dually inoculated (R1+R3MY) soybean plants revealed 34.3 g fresh weight, 15.1 g dry weight and 23% crude fat and soybean plants singly inoculated with *Rhizobium* sp. strain R1 (R1) produced more large seeds with 12.03 g dry weight. The non-inoculated (control) seeds contained higher percentage of moisture content compared to the microbially amended seeds suggesting that the rhizobial and fungi used in this study can also produce soybean seeds with long storage capacity.

Keywords: crude fat, drought, mycorrhizal fungi, *Rhizobium* spp., soybean, yield

11.0 Introduction

Soybean (*Glycine max* L.) is among the most economically important legumes that provide more than 1/4 of food (for man) and animal feed (Graham and Vance, 2003), however its

yield is comparatively low (Egli, 2008) most especially under drought stress. Drought is one of the most crucial abiotic factors affecting soybean yield (Pathan et al., 2014, Purcell and Specht, 2004). It has been acknowledged that drought tolerance is an important target area for crop development and enhancement (Pennisi, 2008). Soybean cultivation mainly relies on natural rainfall, but irregular delivery of rainfall can cause yield difference in the same field and soybean is exceedingly sensitive to drought especially in the reproductive growth phase (Oya et al., 2004). Irrigation is not a possible alternative for the cultivation of soybean in most parts of the world because of its high cost. There is therefore the need for cost-effective means to alleviate drought stress and improve seed yield particularly in semi-arid environment.

“Seed yield per unit area is the product of number of plants per unit area, number of seeds per plant and 100-seed weight. The 100-seed weight is affected by seed size, measured by length, width and thickness (Niu et al., 2013)”. According to Boyer and Wisniewski-Dyé (2009), seed yield reduced maximally during blooming and early pod formation phase. The decrease in yield is mainly due to a reduction in pod number per plant (Kokubun et al., 2001). Previous researches have indicated that the decrease in the number of pod per plant under water stress is as a result of increased rate of flower loss (Momen et al., 1979).

In addition, variations in rainfall and temperature which can be caused by differences in atmospheric weather conditions can eventually affect plant yield (Egli, 2008). Thus, crop models were used by Anderson et al (2001) to assess the effects of weather on soybean and corn yields and found that improved weather was responsible for some of the yield increase. Lobell and Asner (2003); Egli (2008) reported that the yield of soybean and corn cultivated within a certain period at some locations was improved by decreasing temperatures, ‘but the effects was the same for both crops’. However, their outcomes were queried on technical grounds (Gu, 2003). Low

temperature and/or drought are also known to affect soybean yield components such as seed weight and size.

Drought stress may decrease seed weight and size (during seed fill) depending on the time, duration and severity of the stress. Soybean plants subjected to severe drought at a particular stage of their life cycle diminished individual seed weight from 0.21 - 0.182 g in one greenhouse trial and from 0.2 - 0.172 g in another trial experiment (Dornbos et al., 1989). Vieira et al. (1998) reported that soybean plants that were exposed to drought at the beginning of seed fill reduced individual seed weight from 0.144 - 0.096 g per seed. However, Smiciklas et al. (1992) reported that soybean plants exposed to water stress during reproductive phase marginally reduced seed weight from 0.164 - 0.155 g, which was not significantly different from seeds harvested from well watered plants. Soybean plants exposed to drought stress prior to the linear phase of seed fill reduced seed number and also “seed number was maintained by remobilization of reserves” when plants were subjected to drought stress after linear filling stage (New et al., 1994, Samarah et al., 2004). But when reserves were depleted under prolonged stress, seed size reduced due to the fact that the seed filling period was abridged (New et al., 1994).

Moreover, fatty acid content is a quality indicator use to classify soybean cultivars and soy-based products and such classification can influence consumers choice for this crop (Lee et al., 2013). Soybean contains 40 - 42 % protein and 18 - 22 % oil in which 85 % is unsaturated fatty acid (Anwar et al., 2016) and so to produce and sustain soybean with high quality particularly in semi-arid environments, the use of *Rhizobium* spp. and mycorrhizal fungal fungi should be encouraged. The genus *Rhizobium* is one of the plant growths promoting rhizobacteria (PGPR) well-known for its plant growth promoting traits (Igiehon and Babalola, 2018c). “*Rhizobium*, *Pseudomonas*, *Azospirillum* and *Bacillus*, have been found to have positive impacts

on crops by enhancing both above and belowground biomass and could therefore play positive roles in achieving sustainable agriculture (Igiehon and Babalola, 2018c)”. Although, increase in nutrient uptake and yield in soybean inoculated with mycorrhizal fungi and *Rhizobium* spp. has previously been reported (Igiehon and Babalola, 2017, Liu et al., 2012a, Tian et al., 2013, Igiehon and Babalola, 2018c), limited information is available on rhizobial and mycorrhizal fungal enhancement of soybean aboveground components particularly its nutritional contents under drought or semi - arid environments. But, it has been reported that the contribution of mycorrhizal fungal symbiosis to plant drought tolerance is connected to nutrient accumulation (Aliasgharzad et al., 2006, Augé, 2001). Microbial improvement of yield and organic nutrient(s) in seeds from soybean plants exposed to drought stress can be a vital response in water stress tolerance. The aim of this study therefore was to investigate the effects of microbial inoculants on yield, seed size and percentage crude fat of soybean plants exposed to drought stress in semi-arid environment.

11.1 Materials and Methods

11.1.1 Soil Samples for Physicochemical Parameters

Prior to planting, soil samples were collected 10 cm below the top-soil from five (05) different spots in the field, homogenized and kept at 4°C before analyses. Homogenized soil sample was sieved using 2 mm sieve. Iron, manganese, organic carbon, organic matter, potassium, magnesium content, pH, total nitrogen, percentage sand, silt and clay of soil sample were determined according to the methods of Rascovan et al. (2016), Tajini et al. (2012) with little modifications.

11.1.2 Climatic Weather Time Series

Amount of rainfall was the drought index used in this study. The amount of rainfall for the period of soybean growth in the experimental area was monitored on a daily basis by South Africa Weather Service and compared with previous rainfall data for the past 10 years.

11.1.3 Soybean Seeds

In this study, we used soybean seeds (PAN 1532 R cultivar) obtained from Agricultural Research Council (ARC) located at Potchefstroom, South Africa and classified as “moderately drought-sensitive cultivar” in our previous experiment. The viability of the seeds was confirmed by the ARC representative.

11.1.4 Bacterial Growth and Preparation

Rhizobium spp. growth and processing was carried out as described by Prakamhang et al. (2015) with little modifications. Briefly, *R. cellulosilyticum* strain R3 was grown in two bottles containing 500 ml Luria Bertani (LB) broth and *Rhizobium* sp. strain R1 was grown in 800 ml LB broth and both were incubated in a shaker incubator at ambient temperature ($28 \pm 2^\circ\text{C}$). Bacteria were grown to early exponential phase within 5 days and bacterial broth was harvested by centrifuging at 10000 g for 15 min. Bacterial pellet was suspended in 0.85% (w/v) sterile NaCl. Bacterial inocula were quantified by spectrophotometric and plate count methods. The suspended bacterial pellet optical density (OD) were obtained and adjusted at 600 nm to 1.301. One ml (1 ml) of bacterial suspension was serially diluted and samples from 10^{-6} dilution were cultured by pour plate method and incubated at 37°C for 48 hrs. *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 colonies were counted and recorded as 520×10^7 and 524×10^7 CFU (colony forming unit) ml^{-1} respectively.

11.1.5 Mycorrhizal Consortium

A combination of arbuscular mycorrhizal fungi (AMF) which include *Rhizophagus clarus* (previously *Glomus clarum*), *Gigaspora gigantea*, *Funneliformis mosseae* (previously *Glomus mosseae*), *Claroideoglomus etunicatum* (previously *Glomus etunicatum*) and *Paraglomus occulum* (molecular determination) were used for soybean inoculation in this study. The infectivity potential of the fungi was evaluated by culturing them in pot cultures of sorghum plants for three (3) months. The AMF spore number was 8 - 10 per gram with most probable number (MPN) of propagules between 50 -100 per gram.

11.1.6 Soybean Sterilization and Inoculation

Soybean seeds (PAN 1532 R) were prepared for inoculation as described by Ndeddy Aka and Babalola (2016) with little modifications. Seeds which were surface sterilized in 75% ethanol and 1% sodium hypochlorite were rinsed severally in sterile distilled water. For the single bacterial inoculation, 100 ml of *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 suspension was used to inoculate approximately 2000 soybean seeds. For the co-inoculation, 50 ml each of *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 were used to inoculate approximately 2000 soybean seeds in sterile flasks. The flasks were top-up by adding 600 ml of 0.85% (w/v) NaCl. Seven hundred ml (700 ml) of 0.85% (w/v) NaCl was added to approximately 2000 seeds (for the arbuscular mycorrhizal treatment) and approximately 2000 seeds (for the control). Seeds were subjected to shaking at 180 rpm in a shaker incubator at room temperature for 24 h and air-dried in a sterilized laminar flow cabinet before sowing.

11.1.7 Field Experiment

Field experiment was conducted as described by Prakamhang et al. (2015) with little modifications. Eight (8) different treatments were used as follow: (1) Non-inoculated (control)

soybean seeds, (2) R1; single inoculation of soybean seeds with *Rhizobium* sp. strain R1, (3) R3; single inoculation of soybean seeds with *R. cellulosilyticum* strain R3 (4) R1+R3; co-inoculation with *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 (5) MY; inoculation of soybean seeds with arbuscular mycorrhizal fungal (AMF) consortium, (6) R1MY; co-inoculation of soybean seeds with *Rhizobium* sp. strain R1 and AMF consortium, (7) R3MY; co-inoculation of soybean seeds with *R. cellulosilyticum* strain R3 and AMF consortium and (8) R+R3MY; co-inoculation of soybean seeds with *Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and AMF consortium. The field site was located in North-West University school farm, Mafikeng campus (GPS coordinates 25.82202S:025.61430E), North-West Province, South Africa which has history of maize cultivations but no history of fertilizer application. The experiment was conducted within December 2017 – April 2018. The experiment was set-up using 4 X 2 factorial randomized complete block design (RCBD) by dividing into eight (8) treatments (as described above), and each treatment was replicated eight (8) times making a total of sixty four (64) plots. Five-row plots were used with 0.5 m inter-row spacing, 0.13 m intra-row spacing, 1 m inter-plot spacing and 1 m inter-block spacing. Each plot size was 2 x 0.52 m (1.04 m²). Bacterial inoculated seeds and control seeds were placed 10 cm below top-soil and for treatments involving AMF consortium inoculation, approximately 5 g of AMF consortium was poured 12 cm into the soil and covered sparingly with soil before seeds were added. Four (4) seeds were sowed per hole. Two weeks after emergence, soybean seedlings were thinned to one. “The regular agricultural practices were done except chemical fertilizer application and pesticide spraying (Prakamhang et al., 2015)”. Plants were harvested at maturity and data were collected from the soybean plants at the middle of the plots and not from the edge to avoid border effects. Number of pod, seed number per pod, seed area were determined according to previous methods

(Tiquia et al., 1996, Samarah et al., 2004). Seed sizes were determined according the method of Samarah et al. (2004) with little modifications. Briefly, Soybean seeds were classified into small and large sizes by passing the seeds through a 5.6 mm diameter sieve. Large seeds represented seeds retained above a 5.6 mm diameter round sieve and small seeds represented seeds that passed through the 5.6 mm diameter round sieve. Pods were separated by hand and seed yield (fresh and dry weight) and weight per seed (fresh and dry) were determined by weighing on a weighing machine (Radwag weighing machine by Lasec, made in Poland).

11.1.8 Percentage (%) Crude Fat Determination

Oven dried soybean seeds were ground in a Grinder (Professional compressor, Cemotec 1090) and approximately 0.5 g (W_1) was weighed on a weighing machine (Ohaus, Model: A X 224, Assemble in China) and poured into filter bags. Heat sealer [automatic table type vacuum packager (Valve CE)] was used to heat seal the filter bag and oven-dried for 60 min at 105 °C, cooled in a Desiccant Pouch and weighed (W_2). Filter bags with sample were placed into the bag holder and placed in the Extraction System or extractor (ANKOM EXTRACTOM, made in USA) and extracted using petroleum ether (B.P. 35 – 65°C) at 90°C for 60 min. Filter bags with sample were oven-dried at 105°C for 60 min, cooled in a Desiccant Pouch and weighed (W_3). % crude fat was calculated using the formula below:

$$\% \text{ Crude fat} = \frac{100 \times (W_2 - W_3)}{W_1}$$

$$W_1$$

Where: W_1 = Original weight of sample

W_2 = Weight of pre-extracted dried sample with filter bag

W_3 = Weight of dry sample with filter bag after extraction

11.1.9 Percentage (%) Moisture

Percentage (%) moisture content of soybean samples used for the determination of % crude fat was calculated using the formula:

$$\% \text{ moisture} = \frac{100 \times [(\text{filter bag weight} + \text{sample weight}) - \text{Weight after drying}]}{\text{Sample weight}}$$

11.1.10 Percentage Mycorrhizal Colonization

The percentage of AMF root colonization was calculated by the methods of Ortiz et al. (2015); Phillips and Hayman (1970) with little modifications. Briefly, soybean root samples (4 replicates per treatment) were treated with 10% KOH and stained with trypan blue solution prepared by dissolving 0.82 g of trypan blue powder in a mixture of 640 ml of distilled water, 480 ml glycerol and 520 ml lactic acid. Stained root samples were examined under a stereomicroscope at X4 and X3 magnification.

11.1.11 AMF Spore Count

AMF spore counts were performed by wet sieving and decanting techniques according to the methods of Pacioni (1992) with little modifications. One hundred g (100 g) of soil samples were collected from soybean rhizosphere and mixed with 1000 ml of sterile distilled water and poured through 212, 106, 63 and 53 μm sieves stacked together from the top to the base in a descending order. The content of the 53 μm was backwashed into vials and centrifuged at 1800 rpm for 5 min. One ml (1 ml) of the supernatant was mixed with 0.5 ml of 60% sucrose in a fresh vial and centrifuged at 1800 rpm for 5 min. With a syringe, AMF spore suspension was removed from the supernatant and poured in a sterile Petri-dish and examined under a stereomicroscope at X4 and X3 magnification.

11.1.12 Shoot Relative Water Content

Fifty mm (50 mm) of shoot relative water content was estimated in soybean plants as follows: $(FW-DW)/TW-DW) \times 100$ (Ortiz et al., 2015, Aroca et al., 2003).

11.1.13 Statistical Analyses

Data were submitted to normality and homogeneity of variances. Data that were not normally distributed were transformed to satisfy the assumptions of analysis of variance (ANOVA). Differences between means were determined using Duncan test and differences at $P < 0.05$ were considered significant (Igiehon, 2015, Dytham, 2011)

11.2 Results

The physical properties of the experimental field soil that were analyzed are percentage sand, silt and clay. The soil had the highest percentage of sand (68%) followed by 26% clay and 6% silt. Chemical analyses of the soil showed that the field soil had 858, 344, 43.5 and 3.87 mg/kg of magnesium (mg), potassium (K), manganese (Mn) and iron (Fe) respectively. The pH of the soil was closed to neutral (pH 7.62) with 0.069% of total nitrogen. However, 3.23 % of the soil represents organic matter and 1.06% organic carbon (Table 1).

Table 11. 1 Physicochemical parameters of the field soil sample

Parameter	Result
pH (H ₂ O)	7.620
Fe (mg/kg)	3.870
Mn (mg/kg)	43.50
Organic carbon (%)	1.060
Organic matter (%)	3.230
Phosphorus	N/D
Potassium (mg/kg)	344.00
Magnesium (mg/kg)	858.00
Total nitrogen (%)	0.069
Sand (%)	68.000
Silt (%)	6.000
Clay (%)	26.000

The location of the experimental field was North-West University, Mafikeng campus in North-West Province which is semi-arid due to the low amount of rainfall the Province experiences annually. Between December and April, this region had recorded less than 5 mm of rainfall for the past 10 years except February, 2017 in which approximately 9 mm of rainfall was recorded (Fig. 1). Soybean is a summer crop since it grows well during this season and summer season is usually between October and April in South Africa. During the period of this current study, the highest amount of rainfall was 3.88 mm recorded in February and the least was 0.95 mm in April (Fig. 1). These data indicate that this region does experience low amount of rainfall and therefore crops grown therein are exposed to drought stress.

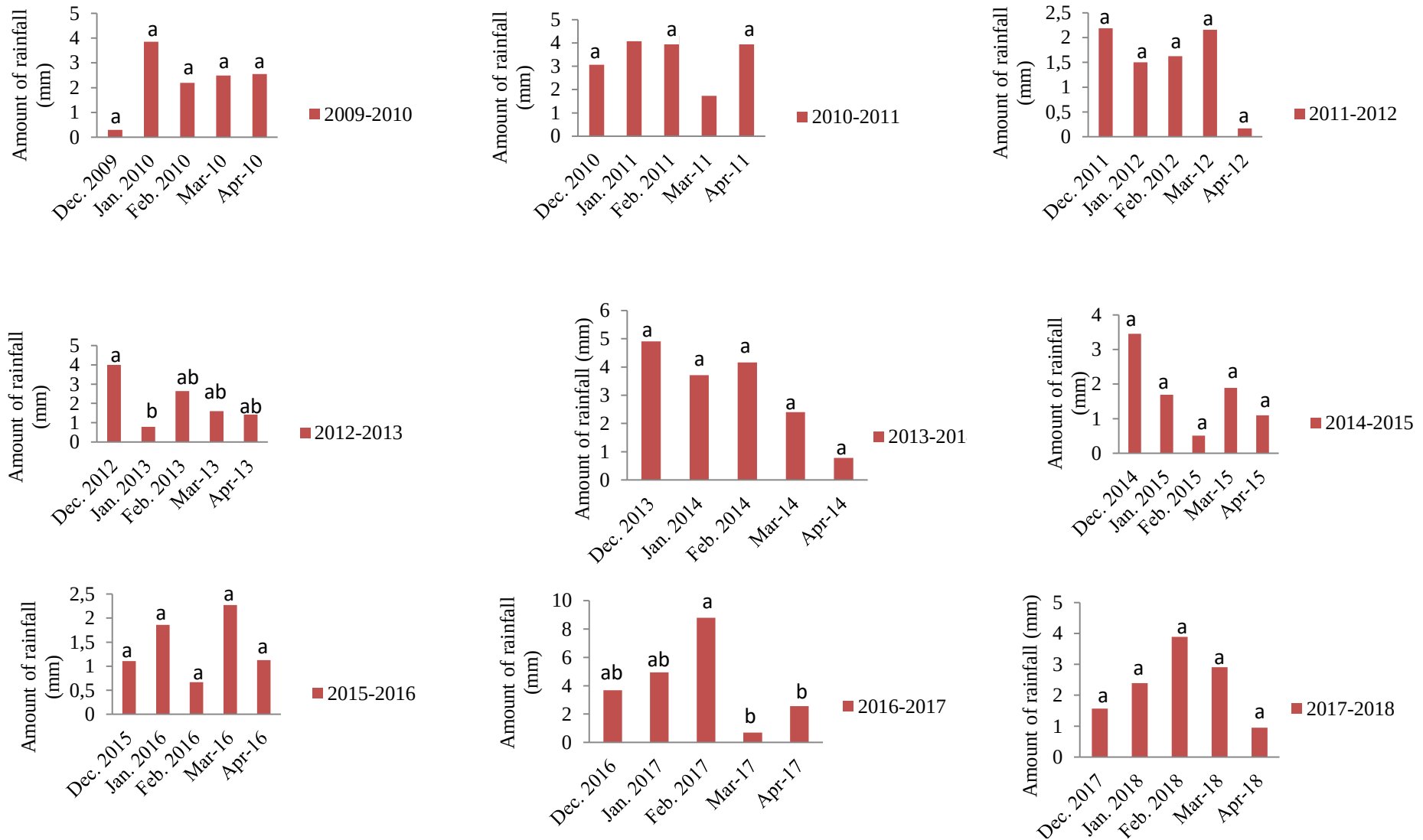


Fig. 11. 1 Amount of rainfall recorded in the study area between 2009 and 2018 for the months of December to April

Furthermore, in this study, seed yield was evaluated by weighing the fresh and dry weights of soybean seeds. The highest fresh weight (34.3 g) was recorded for soybean plants co-inoculated with *Rhizobium* sp. strain R1/*R. cellulosilyticum* strain R3 and mycorrhizal fungi (R1+R3MY) and the least fresh weight (15.1 g) was recorded for the non-inoculated control plants. *Rhizobium* sp. strain R1 (R1), *R. cellulosilyticum* strain R3 (R3), *Rhizobium* sp. strain R1/*R. cellulosilyticum* strain R3 (R1+R3), mycorrhizal consortium (MY), *Rhizobium* sp. strain R1 and mycorrhizal consortium (R1MY) and *R. cellulosilyticum* strain R3 and mycorrhizal consortium (R3MY) had fresh weights of 19.9, 21.2, 31.9, 27.3, 20.6 and 32.6 g respectively (Fig. 2). Seed dry weight was also significantly increased by fungal and rhizobial inoculation compared to the non-inoculated plants (Fig. 2). Soybean seeds amended with R1+R3MY had the highest dry weight followed by seeds treated with R3MY (Fig.2).

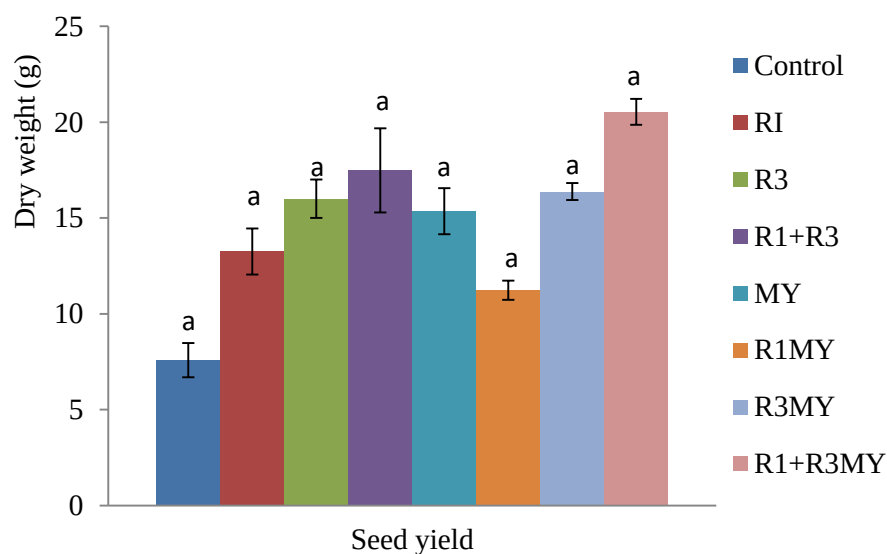
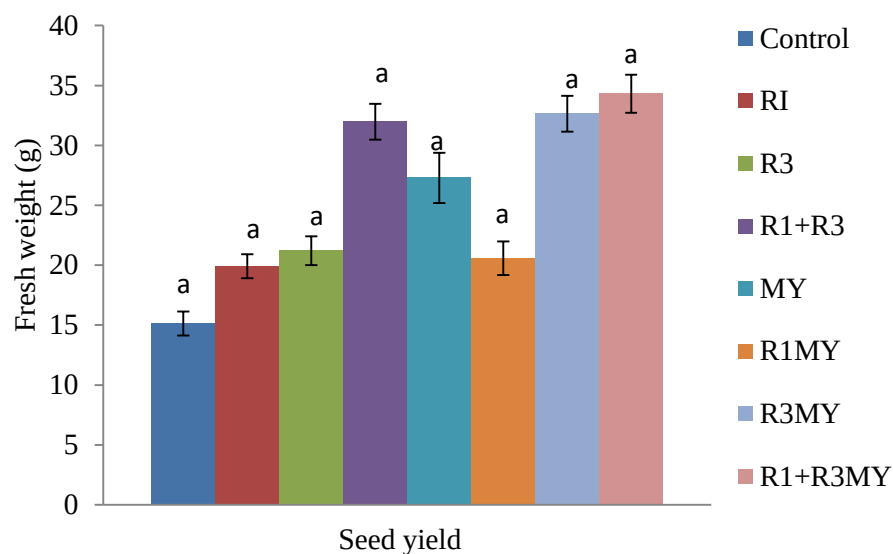


Fig. 11. 2 Fresh and dry weight of entire seed of non-inoculated control soybean plants (control), soybean singly inoculated with *Rhizobium* sp. (R1), *Rhizobium cellulosilyticum* (R3), dually inoculated with *Rhizobium* sp. and *Rhizobium cellulosilyticum* (R1+R3), mycorrhizal consortium (MY), *Rhizobium* sp. and mycorrhizal consortium (R1MY), *Rhizobium cellulosilyticum* and mycorrhizal consortium (R3MY), *Rhizobium* sp./*Rhizobium cellulosilyticum* and mycorrhizal consortium (R1+R3MY) under semi-arid environment. Number of replicates (n) = 8. Data represent mean $\pm$ SE

Similarly, the weight of individual soybean seeds similarly showed that R1+R3MY effectively increased the fresh and dry weight of soybean seed (Fig. 3). This indicates that the combined interaction among the microbial inocula used in this study had a positive effect on soybean seeds. However, there was no significant difference between the individual seed fresh weight of non-inoculated soybean plant and soybean plants inoculated with the different microbial inocula. Similarly, the dry weight per seed for all the treatment was approximately 0.1 g.

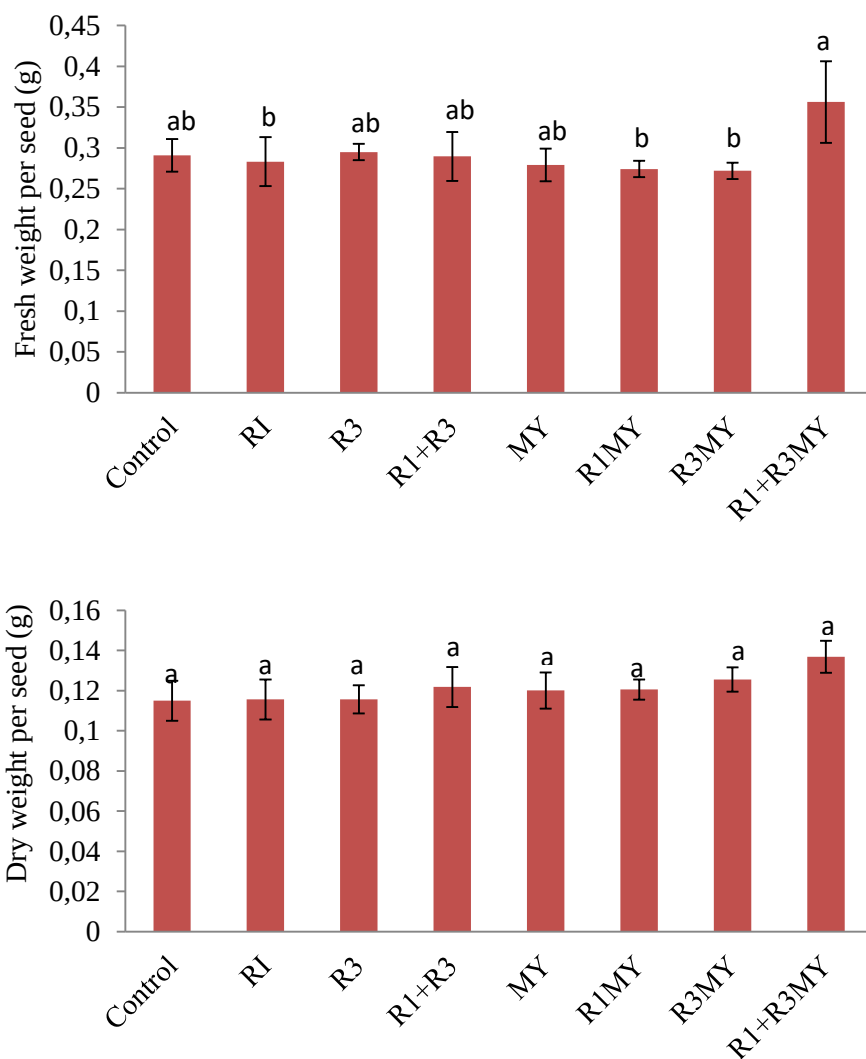


Fig. 11. 3 Fresh and dry weight of individual seed of non-inoculated control soybean plants (control), soybean singly inoculated with *Rhizobium* sp. (R1), *Rhizobium cellulosilyticum* (R3), dually inoculated with *Rhizobium* sp. and *Rhizobium cellulosilyticum* (R1+R3), mycorrhizal consortium (MY), *Rhizobium* sp. and mycorrhizal consortium (R1MY), *Rhizobium cellulosilyticum* and mycorrhizal consortium (R3MY), *Rhizobium* sp./*Rhizobium cellulosilyticum* and mycorrhizal consortium (R1+R3MY) under semi-arid environment. Number of replicates (n) = 8. Data represent mean $\pm$ SE

Additionally, the highest (average of 202.18) seed area which is a product of seed length, width and 2.325 was observed in soybean plants amended with MY. On the contrary, the least seed area was recorded for the control treatment (Fig. 4). Soybean plants co-inoculated with R1 and R3 had the highest number of seed per pod with an average of 18.3 followed by the non-inoculated plants with a mean of 17.3. MY, R1MY, R3MY and R1+R3MY treatments had similar seed number per pod of approximately 16.2 while the least number was observed in soybean inoculated with R1 (Fig. 5).

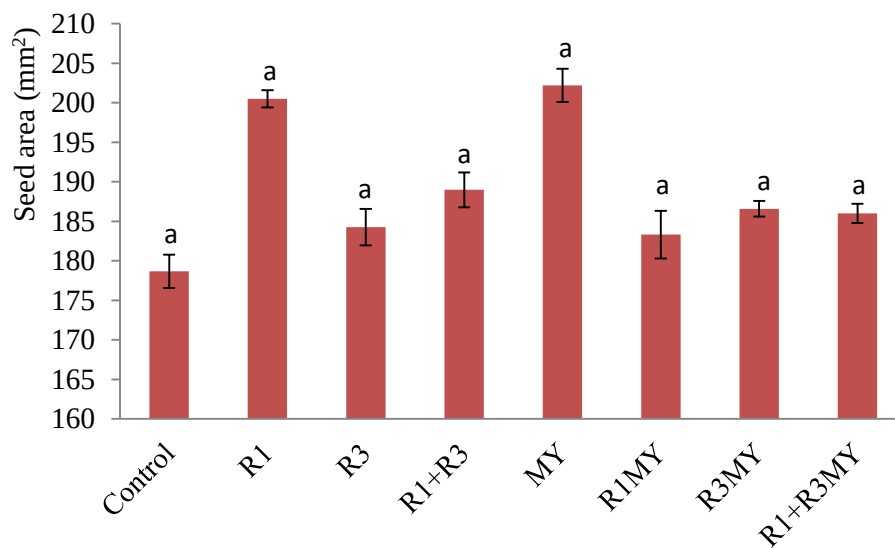


Fig. 11. 4 Seed area of non-inoculated control soybean plants (control), soybean singly inoculated with *Rhizobium* sp. (R1), *Rhizobium cellulosilyticum* (R3), dually inoculated with *Rhizobium* sp. and *Rhizobium cellulosilyticum* (R1+R3), mycorrhizal consortium (MY), *Rhizobium* sp. and mycorrhizal consortium (R1MY), *Rhizobium cellulosilyticum* and mycorrhizal consortium (R3MY), *Rhizobium* sp./*Rhizobium cellulosilyticum* and mycorrhizal consortium (R1+R3MY) under semi-arid environment. Number of replicates (n) = 8. Data represent mean $\pm$ SE

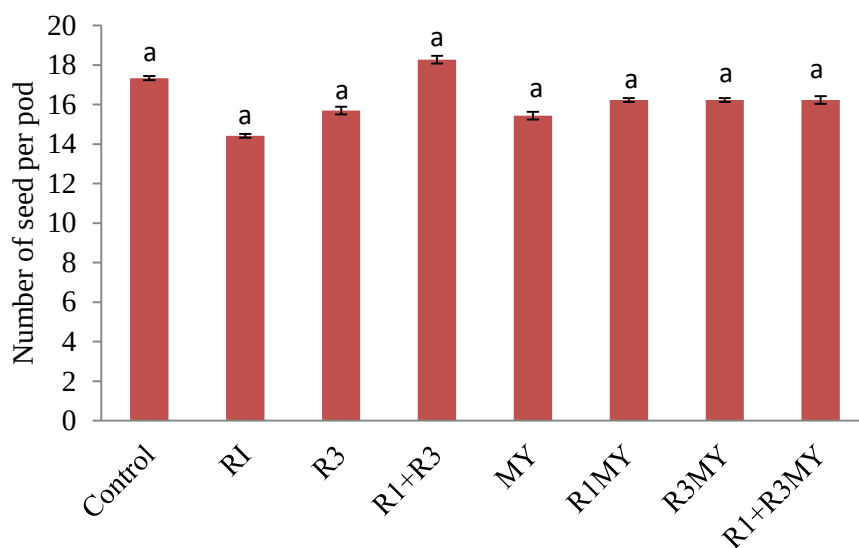


Fig. 11. 5 Number of seed per pod of non-inoculated control soybean plants (control), soybean singly inoculated with *Rhizobium* sp. (R1), *Rhizobium cellulosilyticum* (R3), dually inoculated with *Rhizobium* sp. and *Rhizobium cellulosilyticum* (R1+R3), mycorrhizal consortium (MY), *Rhizobium* sp. and mycorrhizal consortium (R1MY), *Rhizobium cellulosilyticum* and mycorrhizal consortium (R3MY), *Rhizobium* sp./*Rhizobium cellulosilyticum* and mycorrhizal consortium (R1+R3MY) under semi-arid environment. Number of replicates (n) = 8. Data represent mean $\pm$ SE

Soybean seeds classified into small and large sizes showed that soybean plants inoculated with R1 produced more seeds with large sizes seconded by plants treated with R1+R2MY. The non-inoculated soybean plants produced large seeds with a mean dry weight of approximately 2.4 g which was significantly different from that of R1 treatment. The outcome of this field work showed that R1+R3, MY, R1MY, R3MY and non-inoculated treatments produced more of large seeds than small seeds.

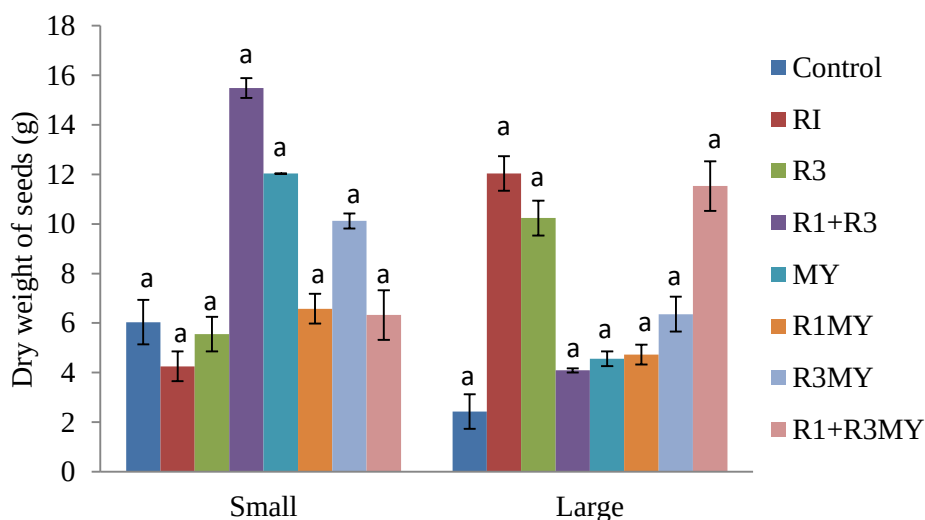


Fig. 11. 6 Dry weight of small and large soybean seeds harvested from non-inoculated control soybean plants (control), soybean singly inoculated with *Rhizobium* sp. (R1), *Rhizobium cellulosilyticum* (R3), dually inoculated with *Rhizobium* sp. and *Rhizobium cellulosilyticum* (R1+R3), mycorrhizal consortium (MY), *Rhizobium* sp. and mycorrhizal consortium (R1MY), *Rhizobium cellulosilyticum* and mycorrhizal consortium (R3MY), *Rhizobium* sp./*Rhizobium cellulosilyticum* and mycorrhizal consortium (R1+R3MY) under semi-arid environment. Number of replicates (n) = 8. Data represent mean $\pm$ SE. Large seeds represented seeds retained above a 5.6 mm diameter round sieve; small seeds represented seeds that passed through the 5.6 mm diameter round sieve

Furthermore, as shown in Fig. 7, % crude fat increased ($P>0.05$) in soybean plants inoculated with R1+R3MY compared to the control. As expected, all the microbial amended plants showed relatively higher amount of % crude fat than the control. The single inoculation with R1 and R2 effectively increased % crude fat compared to the soybean plants that were co-inoculated. However, % crude fat of 21.3, 21.9, 22.3, 22.5, 22.6, 22.8 and 23.0 were observed in soybean plants treated with R1, R3, R1+R3M0, MY, R1MY, R3MY and R1+R3MY respectively while the control had 20.9 % crude fat.

% moisture content of the homogenized seed samples used to determine % crude fat ranged from 7.2 - 3.2% (Fig. 7). The least (3.2 %) percentage moisture content was observed in soybean amended with R1 and the highest (7.2) was observed in the control treatment. There were no significant differences ($P > 0.05$) in the % moisture content of soybean seeds treated with the different microbial inocula. The % moisture content of soybean samples inoculated with R3, R1+R3, MY, R1MY, R3MY and R1+R3MY were 4.0, 4.4, 3.6, 3.8, 3.4 and 3.5 respectively.

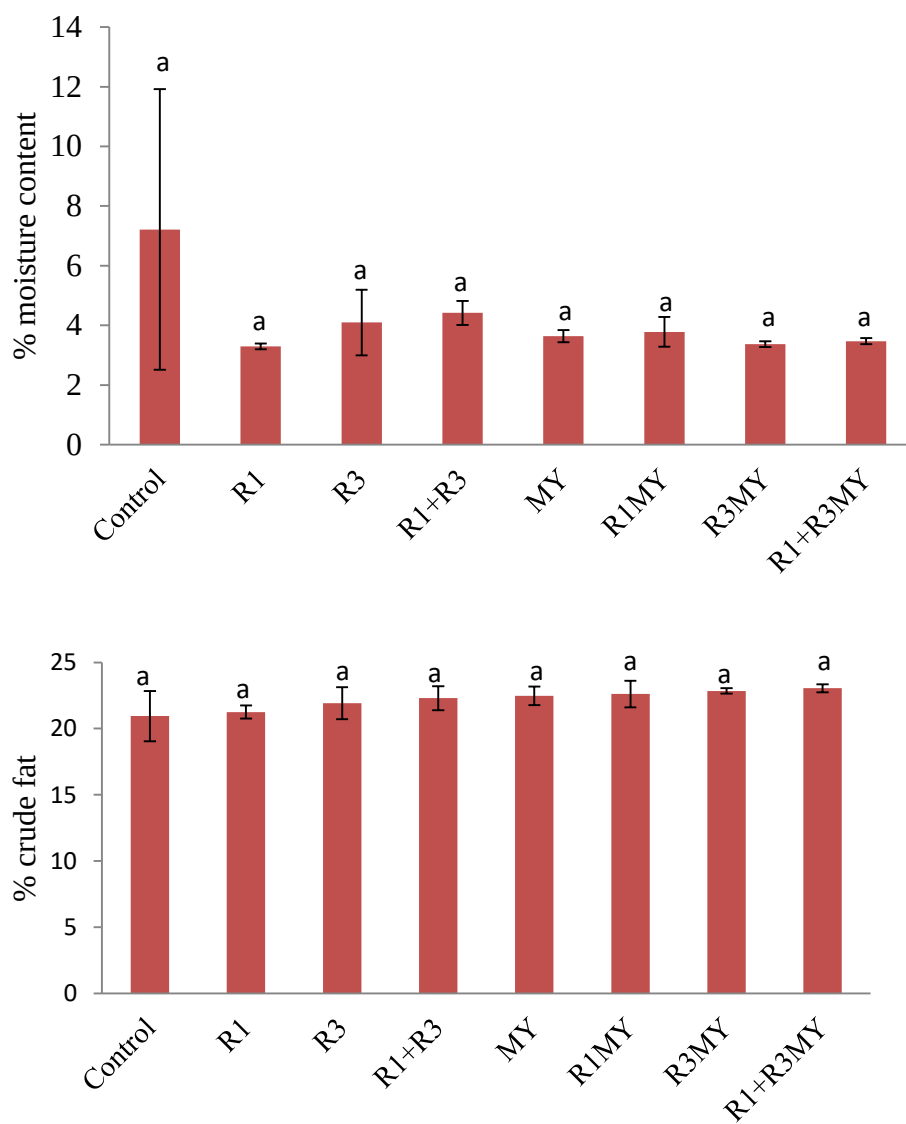


Fig. 11. 7 Percentage crude fat and moisture content in soybean seeds of non-inoculated control soybean plants (control), soybean singly inoculated with *Rhizobium* sp. (R1), *Rhizobium cellulosilyticum* (R3), dually inoculated with *Rhizobium* sp. and *Rhizobium cellulosilyticum* (R1+R3), mycorrhizal consortium (MY), *Rhizobium* sp. and mycorrhizal consortium (R1MY), *Rhizobium cellulosilyticum* and mycorrhizal consortium (R3MY), *Rhizobium* sp./*Rhizobium cellulosilyticum* and mycorrhizal consortium (R1+R3MY) under semi-arid environment. Number of replicates (n) = 8. Data represent mean $\pm$ SE

On the other hand, Non-inoculated (control) treatments showed the least colonization with AMF in soybean roots. Colonization rate increased significantly especially in plants inoculated with R1+R3MY. The colonization rate of R1+R3MY was 97.75% and control was 19.25% (Fig. 8). However, compared to the control, increased colonization rate was observed in soybean plants inoculated with R1, R3, R1+R3M0, MY, R1MY and R3MY. Similar results were also obtained for number of fungal spore in soybean rhizosphere with soybean plants amended with R1+R3MY showing the highest number of spore and non-inoculated plants had the least spore number (Fig. 8).

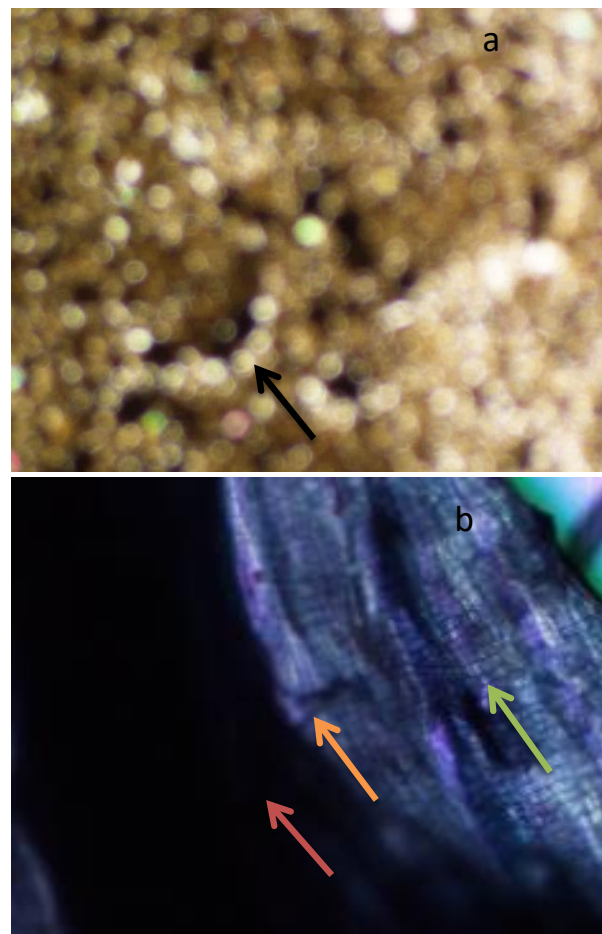
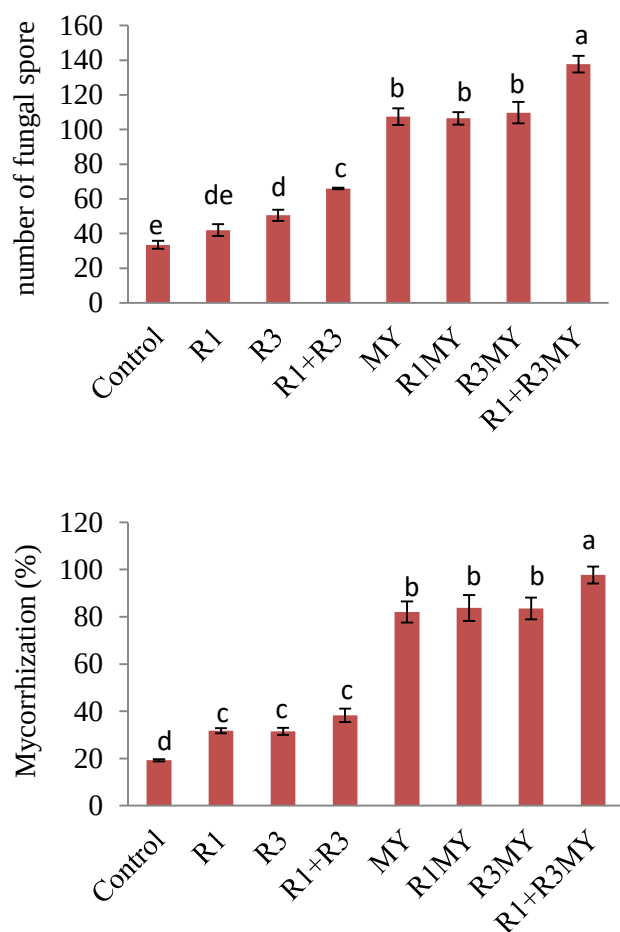


Fig. 11. 8 Number of fungal spore and percentage mycorrhizal colonization in non-inoculated control soybean plants (control), soybean singly inoculated with *Rhizobium* sp. (R1), *Rhizobium cellulosilyticum* (R3), dually inoculated with *Rhizobium* sp. and *Rhizobium cellulosilyticum* (R1+R3), mycorrhizal consortium (MY), *Rhizobium* sp. and mycorrhizal consortium (R1MY), *Rhizobium cellulosilyticum* and mycorrhizal consortium (R3MY), *Rhizobium* sp./*Rhizobium cellulosilyticum* and mycorrhizal consortium (R1+R3MY) under semi-arid environment. Number of replicates (n) = 4. Data represent mean $\pm$ SE. (a) represents AMF spores (b) represents root colonized with AMF. Black, green, orange and red arrows point towards fungal spores, arbuscule, hyphal and soybean plant root respectively

Percentage (%) relative water content (RWC) is one of the parameters used to determine the response of plants to environmental stress. As depicted in Fig. 9, RWC increased in the inoculated soybean plants under this semi-arid condition. How RWC increase was particularly glaring in soybean plants co-inoculated with R1+R3MY. The synergistic interaction of the two rhizobial species and mycorrhizal consortium increased RWC at 63.3%, was higher than that of the non-inoculated control at 43.6%. Co-inoculation of R3 and mycorrhizal consortium also resulted in a significant increase of RWC by 58.8% compared to the control.

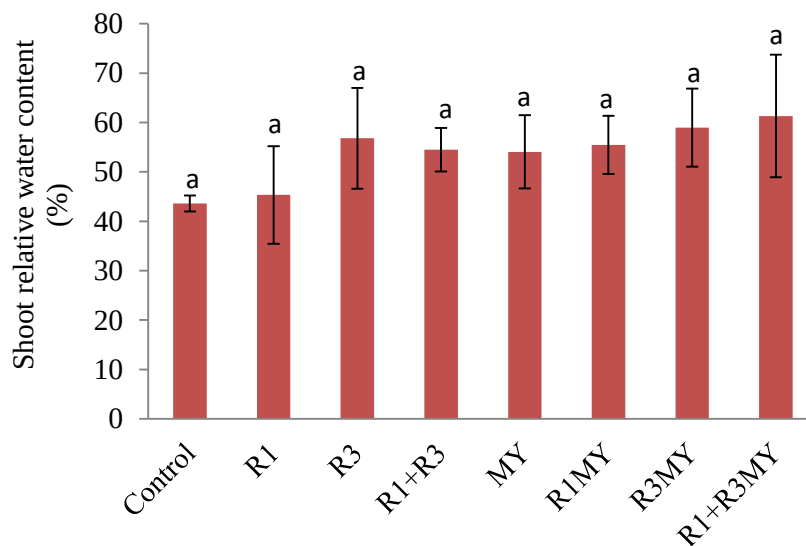


Fig. 11. 9 Relative water content in the shoot of non-inoculated control soybean plants (control), soybean singly inoculated with *Rhizobium* sp. (R1), *Rhizobium cellulosilyticum* (R3), dually inoculated with *Rhizobium* sp. and *Rhizobium cellulosilyticum* (R1+R3), mycorrhizal consortium (MY), *Rhizobium* sp. and mycorrhizal consortium (R1MY), *Rhizobium cellulosilyticum* and mycorrhizal consortium (R3MY), *Rhizobium* sp./*Rhizobium cellulosilyticum* and mycorrhizal consortium (R1+R3MY) under semi-arid environment. Number of replicates (n) = 5. Data represent mean $\pm$ SE. (a) represents AMF spores (b) represents root colonized with AMF

11.3 Discussion

Table 1 shows that the pH of the soil in the study site was 7.620 suggesting that the soil is slightly above the optimum pH range of 5.5 – 7 (Gurmu, 2007, Zerihun et al., 2015) needed for soybean growth. However, this pH will support the growth of bacteria but may not favour the growth of fungi since the latter grow best in acidic environments.

From the rainfall data (Fig. 1), low rainfall was recorded during the experimental period (December-April) which also corresponds with the amount of rainfall recorded for the same period for the past 10 years in the study area. In addition, in Thailand, Prakamhang et al. (2015) reported mean rainfall of 31.07 mm in a well-watered field study involving *Bradyrhizobium* spp. and soybean which is above the highest mean rainfall of 3.88 mm observed in February in this present study (Fig. 1). These data suggest that the study area for the present study (which may not be able to support the growth of most agricultural crops such as soybean) is not well-watered and therefore is semi-arid since it was also reported that soybean grow best with an annual rainfall of 900 -1300 mm (Gurmu, 2007) which is far above the amount of rainfall record in the study area; as results shown in this study represent significant data for the yearly rainy period in this region.

Therefore, in this study, the effects of rhizobia and mycorrhizal fungi on the yield, size and fatty acid content of soybean grown under the semi-arid environment of South Africa were evaluated. It was observed that co-inoculation of soybean plants with *Rhizobium* sp./*Rhizobium cellulosilyticum* and mycorrhizal consortium (R1+R3MY) resulted in a significant increase in the fresh and dry weights of soybean seeds compared to the non-inoculated control plants. Similarly, other microbial treatments were effective in enhancing the fresh and dry weight (parameters that were used to determine seed yield in this study), but the least fresh (19.9 g) and dry (13.24 g)

weights were obtained for soybean treated with *Rhizobium* sp. strain R1 (R1) unlike *R. cellulosilyticum* strain R3 treatment (R3) which was more effective in improving soybean yield (Fig. 2). These results are in agreement with our previous *in vitro* soybean seed germination experiment (data not shown) in which soybean seedlings singly inoculated with R3 showed a better performance than those amended with R1 or *Sinorhizobium meliloti* strain R5. Amazingly, R3 treatment produced seeds with dry weight (16 g) greater than soybean inoculated with mycorrhizal consortium even though this consortium contains five (5) different drought tolerant AMF namely: *Rhizophagus clarus*, *Gigaspora gigantea*, *Funneliformis mosseae*, *Claroideoglomus etunicatum* and *Paraglomus occulum*. An increase ($P>0.05$) was however observed in the yield of soybean dually inoculated with *R. cellulosilyticum* strain R3 and mycorrhizal consortium (R3MY).

There significant differences ($P<0.05$) among the treatments as regards fresh weight per seed (Fig. 3). However, the highest fresh (0.35 g) and dry (0.13 g) weights were recorded in soybean seed co-inoculated with R1+R3MY. This increase may be due to synergistic interaction between the rhizobia and mycorrhizal fungi since many researchers have reported that yield of legumes are affected by AMF and rhizobia interactions (Ahmad, 1995, Redecker et al., 1997, Xavier and Germida, 2003). In comparing the impacts of associations between *Glomus aggregatum*, *Sclerocystis microcarp* and *Glomus pallidum* and four *Rhizobium phaseoli* on the yield of different kidney bean genotypes, Ahmad (1995) observed that the symbiotic efficacy was reliant on the actual combination of the mycorrhizal species, bacterial strain and the host genotype. However, contrary to other studies, both effective and ineffective *Rhizobium* species were included in their study so as to determine the role of *Rhizobium* spp. in the tripartite association. They found that co-inoculation of an effective *Rhizobium* strain LX43 with an AM

fungus enhanced the yield, nitrogen and phosphorus content of the plant. Similarly, co-inoculation of less effective *Rhizobium* isolate with another AM fungus increased the yield of the legume. However, this significantly enhanced productivity than that of an effective *Rhizobium*-AMF combination.

The area of seed randomly picked from each treatment revealed that soybean treated with mycorrhizal consortium (MY) had the greatest area of 202.18 mm². In addition, R1 treatment seed area (200.48 mm²) was relatively higher than other treatments. This result indicates that MY and R1 relatively affect the length and width of soybean seeds, although the effect was not significant compared to the non-inoculated (control) soybean plants (Fig. 4). The effect of seed area was very pronounced in the seed size distribution of R1 since this treatment produced large seeds with the greatest weight (Fig. 6). However, seed area effect did not significantly affect yield (fresh and dry weight of entire seed produced) since much yield increase was observed in soybean dually inoculated with *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 (R1+R3), R1MY and R1+R3MY.

Number of seed per pod is also a component that can affect soybean seed yield. *Rhizobia* and fungi did not significantly ($P>0.05$) affect the number of seed per pod; soybean dual inoculated with R1+R3 had the highest number of seed per pod with an average number of 18.27 however (Fig. 5). Single inoculation with either R1 or R3 resulted in either 14.4 or 15.69 seed number per pod respectively. But co-inoculation with only the *rhizobia* strains slightly affected pod seed number. This effect slightly decreased when each of the *rhizobial* strains was co-inoculated with mycorrhizal consortium. Interestingly, such slight decrease was also observed in soybean dually inoculated with R1+R3MY. Perhaps, the significant increase in the yield of soybean (Fig. 2) dually inoculated with R1+R3MY can be related to production of more pods per

plant than other treatments which confirm the conclusions drawn by Liu et al. (2003); Westgate and Peterson (1993) that yield increase under drought stress is caused by increase in the number of pod.

R1, R3 and R1+R3MY were more effective in producing large soybean seeds since these treatments produced large seeds with greater weight (Fig 6). Comparatively, R1+R3 and MY treatments did not favour the production of large seeds suggesting that the effects seen in soybean seeds amended with R1+R3MY treatment could largely be attributed to the synergistic interaction between the rhizobial species and mycorrhizal consortium. Naturally, leguminous crops such as soybean are able to form symbiotic interactions with both AMF and rhizobia (Xavier and Germida, 2003, Wang et al., 2011). Tripartite mutualistic symbiotic interactions with AMF and rhizobia should be very crucial for soybean productivity (Igiehon and Babalola, 2018) and the impacts of soybean inoculation with rhizobia and AMF need further research.

Furthermore, the interactions between rhizobial species and mycorrhizal fungi enhanced the percentage (%) crude fat of soybean seeds. The highest percentage (23.04 %) was observed in soybean seeds treated with R1+R3MY and the least (20.9 %) crude fat was recorded for the non-inoculated control soybean plants (Fig. 7). These results showed that drought stress reduced the fatty acid content in seeds of non-inoculated plants which is in agreement with the work of Hou et al. (2006) who reported that drought stress is among the environmental factors that affect the content and/or composition of fat in soybean seeds. However, there was no significant difference in the % crude fat of inoculated and non-inoculated (control) soybean seeds. The quality of soybean is evaluated based on the concentration and composition of fat, oil and protein. Soybean seeds have been reported to contain 24% oleic acid, 8% linolenic acid and 54% linoleic acid (Schnebly and Fehr, 1993). Higher concentration of oleic acid is a desirable

quality for long shelf-life and stability of oil for industrial purposes, but higher concentrations of polyunsaturated fatty acids such as linolenic acids are needed for human nutrition (Bellaloui, 2011). However, fatty acids (such as oleic acid) are less vulnerable to oxidative modifications during processing, storage cum frying. Therefore, the food industry is currently concerned in producing soybean seed that contain high concentration of oleic acid (Rahman et al., 2001). According to Opperman and Varia (2011), in 2010, South Africans demand for soybean oil was at 1.3 million tons followed by other countries such as Mozambique and Zambia with 0.2 million tons demand each for soybean oil in the same year. Thus, the demand for soybean seeds can greatly be influenced by its fatty acid content and production of soybean rich in fatty acid will therefore contribute positively to human nutrition and economy growth since it has also been reported that availability of high quality seed plays a significant role in the growth and development of any crop-producing enterprise (Dlamini et al., 2014). But, the % moisture content of non-inoculated (control) soybean seeds was relatively higher than the inoculated soybean seeds. The seeds of the non-inoculated plants showed 7.2% of moisture unlike the seeds of soybean plants amended with rhizobial species and mycorrhizal fungi which showed relatively low moisture content. This indicates that the microbial candidates used in this study may not only improve the nutrient status of soybean seeds but could also be harnessed to produce soybean with long shelf-life.

In addition, soybean root dually inoculated with R1+R3MY was most heavily colonized by AMF (Fig. 8, b). The % mycorrhization of roots of soybean inoculated with MY was 82 which was slightly less than plants dually inoculated with *Rhizobium* sp. strain R1/mycorrhizal consortium (R1MY), R3MY and R1+R3MY. The increase in mycorrhizal colonization level observed in the R1MY, R3MY and R1+R3MY treatments might be traced to the fact that the

rhizobial species aided the mycorrhizal fungi to penetrate the soybean roots. Also, the efficacy of dual inocula, particularly R1+R3MY, could be linked to % mycorrhizal colonization as previously reported (Marulanda et al., 2009). In contrast, Ortiz et al. (2015) highlighted that the efficiency of dual inoculation in a study carried out under drought conditions in the field was not related to colonization level. However, in this study, mycorrhizal vesicles, arbuscules and hyphae were also seen in soybean plant roots inoculated singly with R1, R3 and the non-inoculated (control) plant roots but at a relatively low colonization level. These soybean roots may have been colonized by indigenous mycorrhizal fungi present in the soil (Igiehon and Babalola, 2017). Similar results were also obtained for spore number (Fig. 8a) in which soybean plant rhizosphere co-inoculated with R1+R3MY showed the highest number of fungal spore (average of 137.75) and the non-inoculated plant rhizosphere showed the lowest fungal spore number with an average number of 33.5.

One new and remarkable result obtained in the current research under semi-arid environmental conditions is the inherent ability of the microorganisms to cope and tolerate drought conditions. Additionally, not only rhizobial, but also mycorrhizal inoculants proved competent in assisting inoculated plants to reduce the harmful drought effects on yield and fatty acid content of soybean seeds.

In this present study, most of the inocula applied were able to increase water content that reduced yield and nutrient content caused by drought. Rhizobial and fungal inocula applied induced different natural processes that helped soybean plants to grow and develop under drought.

Relative water content (RWC) is 'involved in plant response to drought' (Aliasghar zad et al., 2006, Ortiz et al., 2015). The lowest RWC observed in control soybean plants showed that

they were relatively less effective in coping with drought. In this study, plants colonized by rhizobial species and mycorrhizal consortium increased plants RWC content by 45.3% (R1), 56.8% (R3), 54.5% (R1+R3), 54.1% (MY), 55.5% (R1MY), 58.9% (R3MY), 61.3% (R1+R3MY). However, the increase in RWC was not significantly different ($P>0.05$).

In conclusion, although, most of the soybean plants singly and dually inoculated with *Rhizobium* spp. and mycorrhizal consortium enhanced yield, seed fresh weight and dry weight, seed size, seed fatty acid content and shoot water content, the results of this present study largely suggest that synergistic interactions are involved in the enhancement of yield, seed size and fatty acid content of soybean plants. This interaction between rhizobia and mycorrhizal fungi is an effective biotechnological strategy to improve plant yield and nutrition in semi-arid environments. However, insights into the adaptation cum functioning of microorganisms and plants to extreme environmental conditions need further studies.

11.4 Acknowledgements

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11.5 Conflict of Interest

Author 1 declares that he has no conflict of interest. Author 2 declares that she has no conflict of interest.

11.6 Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the author.

CHAPTER 12

Microbial Dynamics of Soybean Rhizosphere at different Growth Stages in the Field

Abstract

Greatly diverse bacteria populating soybean rhizosphere play fundamental roles in the growth of plants as well as crop production; nevertheless, limited information is known about the alterations that arise in these regions at different growth stages. In this study Next Generation Sequencing technique (NGS) was used to sequence bacterial communities of rhizospheric soil at different stages of growth of soybean inoculated with drought tolerant *Rhizobium* sp. strain R1 and *Rhizobium cellulosilyticum* strain R3 in a semi-arid environment. Stack bar/area plot and Heatmap clustering analysis unveiled variations among the rhizospheric bacterial communities at the class and order levels respectively. Particularly, *Actinobacteria* was the most abundant bacterial group with the highest reads counts observed at full seed (FS) stage followed by *Proteobacteria* which contained bacterial species such as *Streptomyces* and *Rhizobium* species respectively with plant growth promoting traits. Alpha-diversity and Bray Curtis Index analyses at the family, genus, species and operational taxonomic units (OTUs) levels similarly showed that bacterial composition and abundance of the rhizosphere changed significantly during soybean growth. These results revealed that rhizosphere bacterial community structured varied at different growth stages of soybean in the field.

Keywords: alpha-diversity, bacterial community structure, Bray Curtis Index, rhizosphere microbial dynamics, next generation sequencing, soybean growth stage

12.0 Introduction

The rhizosphere is the thin layer of soil surrounding the root region (Darrah, 1993, Hartmann et al., 2008) where millions of microorganisms interact with one another and with

plant roots (Mendes et al., 2013a). Rhizosphere microorganisms have been indicated to have great activity and to be essential for plant growth and health (Pieterse et al., 2014). For instance, rhizobial species and arbuscular mycorrhizal fungi (AMF) supply nitrogen and phosphorous respectively and microorganisms termed plant growth promoting rhizobacteria (PGPR) directly and indirectly affect plant growth by modulating plant immunity, enhancing plant tolerance to stress and preventing pathogen colonization (Bloemberg and Lugtenberg, 2001, Igiehon and Babalola, 2018a, Van der Ent et al., 2009, Lugtenberg and Kamilova, 2009). The rhizosphere microorganisms are considered as prominent constituents of sustainable agriculture that can decrease the use of chemical pesticides and fertilizers (Beneduzi et al., 2012). It has been reported that plant harbor rhizosphere microorganisms by providing an enabling environment and nutrients majorly as root exudates, which are obtained from photosynthetic products (Oger et al., 2004, Bais et al., 2006, el Zahar Haichar et al., 2008, Badri and Vivanco, 2009). Besides climatic effects, resident plants and alien microorganisms exert effect on rhizosphere microbial community structure. Microbial community structure have been reported to depend on plant cultivar ‘grown in the same type of soil’ (Innes et al., 2004, Batten et al., 2006, Garbeva et al., 2008, İnceoğlu et al., 2011), signifying an intimate interaction between plants cum rhizosphere microorganisms (Bakker et al., 2012).

Due to the fact that rhizosphere microorganisms are essential in plant performance and growth, microbial community of plant rhizosphere has been broadly studied using culture-based and culture-independent techniques (Igiehon and Babalola, 2018c, Berendsen et al., 2012a, Bakker et al., 2012, DeAngelis et al., 2009). Latest developments in next generation sequencing techniques have allowed in-depth investigation of rhizosphere microbiome (Igiehon and Babalola, 2018c). Indeed, *Arabidopsis* rhizosphere microbial communities have been analyzed

extensively (Bulgarelli et al., 2012b, Lundberg et al., 2012, Bodenhausen et al., 2013) and the investigation of soils sampled from *Arabidopsis* rhizosphere in their natural environments showed plausible interactions between the plant growth and the soil microbiome (Sugiyama et al., 2012). Nevertheless, despite increases in community based investigations of rhizosphere bacterial diversities (Berendsen et al., 2012b, Bakker et al., 2012), it is still not clear how plant cum bacteria interact to create ‘rhizosphere bacterial communities’ from surrounding or bulk soil. Also, few studies have extensively investigated ‘rhizosphere bacterial communities’ at different stages of plant growth (such as leguminous plant) using next generation sequencing technique (İnceoğlu et al., 2011, Chaparro et al., 2014a, Chaparro et al., 2013), even though ‘growth-dependent’ investigation of rhizosphere microbiome have been done using techniques such as denaturing gradient gel electrophoresis (DGGE) and automated ribosomal interspacer analysis (ARISA) (Mougel et al., 2006, Houlden et al., 2008, Xu et al., 2009, Micallef et al., 2009).

In summary, leguminous plants include essential crops such as soybean (*Glycine max* L) which provide nutrients that are rich in oil and protein for ‘human consumption’. Legumes have been utilized to study plant-microbial interactions in the root regions, as a result of their agronomic significance and capacity to establish symbiotic associations with AMF and *Rhizobium* species (Denison and Kiers, 2011, Yokota and Hayashi, 2011, Jie et al., 2013). Molecular analyses of model leguminous plants such as *Medicago truncatula* and *Lotus japonicus* unveiled pathways resulting in symbiosis. Despite the myriad of reports on investigation of the components of legume-AMF and legume-*Rhizobium* species symbiosis, the wide-range of rhizosphere bacterial species which may exert impacts on leguminous plant interactions with AMF and *Rhizobium* species, are yet to be characterized in-depth. Thus, the

mechanisms supporting legume interactions with different soil microorganisms during growth especially in semi-arid and arid environments remain obscure. Knowledge of rhizosphere bacterial community composition at different stages of growth in the field can ‘provide a basis for optimizing agricultural utilization of rhizosphere microbes’. For instance, DGGE revealed that soybean rhizosphere microbial composition altered during growth phase with changes in the comparative contributions of different phyla including *Acidobacteria*, *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Verrucomicrobia* and *Nitrospirae* (Xu et al., 2009). To give more insights into legume – microbial interactions in a natural soil and to acquire fundamental data on soybean rhizosphere bacterial community structures for subsequent research, this study was therefore designed to investigate how rhizosphere bacterial community structures change at different growth stages of soybean amended with alien rhizobia and mycorrhizal fungi in a field located in a semi-arid environment of North-West Province, South Africa using next generation sequencing technique.

12.1 Materials and Methods

12.1.1 Description of Study Site and Sample Collection

Field experiment was performed in an experimental plot of North-West University (25.82202S 025.61430E), Mahikeng campus at Ngaka Modiri Molema district in Mahikeng town, North-West Province, South Africa (Fig. 1). The field has been grown with crops for a couple of years and maize was grown there the preceding year. The highest average amount of rainfall of 3.88 mm was recorded in February and 48% (December), 42% (January) and 65% (February, March and April) relative humidity with a maximum temperature of 33.8°C January (data obtained by South Africa Weather Service) were observed during the experimental period. The field had the following soil properties: 6.620 pH, 3.870 mg/kg Fe, 43.50 mg/kg Mn, 1.060%

organic C, 3.230 organic matters, 344 mg/kg K, 858 mg/kg Mg, 0.069% total N, 68% sand, 6% silt and 26% clay.

According to the method of Prakamhang et al. (2015), soybean seeds (*G. max* L cv. PAN 1532 R) obtained from Agricultural Research Council (Pretoria, South Africa) were sown in December, 2012 upon dual inoculated with *Rhizobium* sp. strain R1, *Rhizobium cellulosilyticum* strain R3 and mycorrhizal consortium which comprised of *Funneliformis mosseae* (previously *Glomus mosseae*), *Paraglomus oculum* (molecular determination), *Gigaspora gigantea*, *Claroideoglomus etunicatum* (previously *Glomus etunicatum*) and *Rhizophagus clarus* (previously *Glomus clarum*). The mycorrhizal consortium was acquired from Department of Biochemistry and Microbiology, Rhodes University, Grahamstown, South Africa. Mycorrhizal consortium was cultured for ninety days (90 days) in pot cultures using sorghum as a host to check the colonization potential of the fungi. The fungi had 8-10 per gram spore number and 50-100 per gram most probable number (MPN). The two rhizobial species were isolated from Bambara groundnut rhizosphere and identified by molecular method in our previous study. The two *Rhizobium* spp. and fungal consortium used for soybean inoculation in this study were not in any way altered genetically. The experimental area is semi-arid and plants were irrigated sparingly and weeds were manually eliminated throughout the experimental period on a weekly basis. Rhizosphere soil samples were collected by destructive sampling on 10th February, 2018 (at the beginning of blooming); 7th March, 2018 (Beginning of pod development); 14th March, 2018 (Beginning of seed development); 31st March, 2018 (Full seed) and 27th April, 2018 (Maturity). Soil samples were collected from rhizosphere of eight soybean plants, homogenized and pooled into one sample. Rhizosphere soil was defined as soil that adhered to soybean plant

roots after mild shaking (İnceoğlu et al., 2010). Soil samples were instantly transferred to the laboratory in an ice box and kept at -20°C prior to DNA extraction.

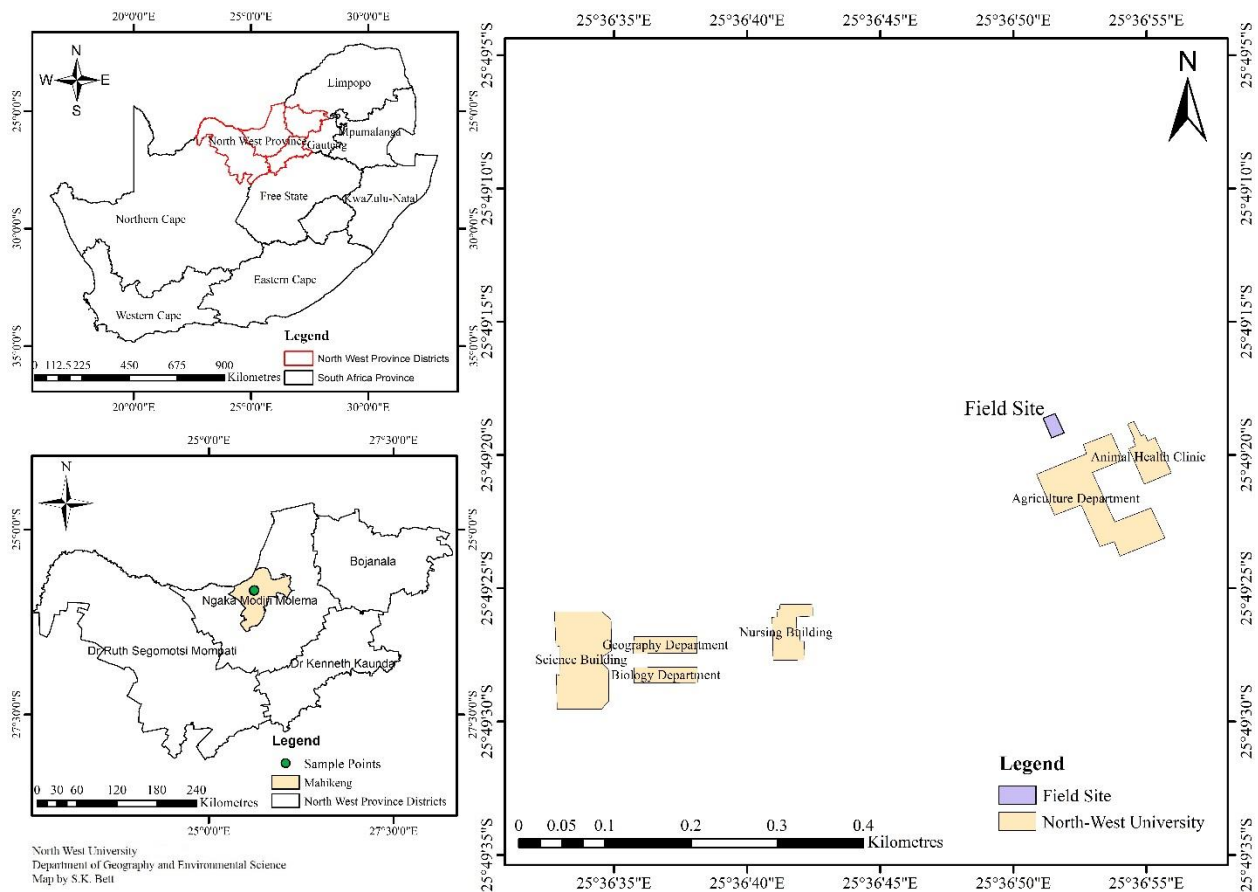


Fig. 12. 1 Rhizosphere soil collection site in South Africa. The top left sketch represents a map of South Africa encompassing North-West Province (the region in red sketch) and at the bottom left is a map of North-West Province encompassing a map of Mahikeng (light yellow area) which encompasses Ngaka Modiri Molema district (the green spot) where North-West University Mahikeng campus is located. To the right, is a sketch representing the field site where sample were collected

12.1.2 DNA Extraction

DNA was extracted from 0.25 g of soil using DNeasy Power Soil Kit (QIAGEN GmbH, Hilden, GERMANY) following manufacturer's instructions. Extracted DNA was checked in 1% agarose to confirm the success of extraction process cum relative band thickness. The concentrations of extracted DNA were determined using a NanoDrop spectrophotometer (Thermo Scientific, NANODROP LITE Spectrophotometer). The DNA concentrations were found to be 12.2 ng/μl, 9.2 ng/μl, 25.9 ng/μl, 20.3 ng/μl and 18.5 ng/μl corresponding to rhizosphere soil obtained at the beginning of blooming (BB), beginning of pod development (BP), beginning of seed development (BS), full seed (FS) and full maturity (FM). DNA samples were stored at -20°C until use. Upon which, 40 μl of DNA of each DNA sample was sent to the next generation sequencing laboratory of Molecular Research (Mr. DNA), Texas, USA.

12.1.3 Miseq Sequencing of DNA Samples

Polymerase chain reaction (PCR) primers 515/806 that target V4 variable region of 16S rRNA with a forward primer having a barcode were used to amplify the DNA extract using a HotStarTaq Plus Master Mix Kit (Qiagen, USA) in a 28 cycle PCR according to the following conditions: 94°C for 3 min, 94°C for 30 sec (28 cycles but 5 cycles were used on PCR amplified products), 72°C for 1 min and 72°C for 5 min (elongation step).

To determine the success of PCR amplification and bands intensity, PCR products were checked in 2% agarose gel. Multiple samples were mixed together in the same proportion on the basis of their DNA concentrations cum molecular weight. Calibrated Ampure XP beads were used to purify the mixed samples and thereafter illumina DNA library was prepared from the purified samples. Following manufacturer's instructions, sequencing of purified samples was done on Miseq at MR DNA (www.mrdnalab.com, Shallowater, TX, USA). MR DNA analysis

pipeline (MR DNA, Shallowater, TX, USA) was used to process the sequenced data. In brevity, sequences were fused, ‘depleted of barcodes’ and sequences less than 150bp and ‘ambiguous base calls’ were removed. Sequences were subsequently denoised, operational taxonomic units (OTUs) generated and chimeras were removed. ‘OTUs were defined by clustering at 3% divergence (97% similarity)’. OTUs were finally classified taxonomically using ‘BLASTn against curated database’ obtained from NCBI and RDP II (www.ncbi.nlm.nih.gov, <http://rdp.cme.msu.edu>).

12.1.4 Bioinformatics

Data were uploaded online and analyzed through MicrobiomeAnalyst pipeline (Dhariwal et al., 2017).

12.2 Results

12.2.1 Rhizosphere Bacterial Community Read Counts

Soybean plants were grown from December 2017 to April 2018 in a field in North-West Province, South Africa. Rhizosphere soil samples were collected at the beginning of blooming (BB), beginning of pod development (BP), beginning of seed development (BS), full seed (FS) and full maturity (FM) as described in the material and methods section. DNA samples extracted from rhizosphere soil samples were stored at -20°C. 16S amplicon (V4) of PCR products were sequenced on Miseq at MR DNA (www.mrdnalab.com, Shallowater, TX, USA). In this current study, the total reads count for the five DNA samples analyzed was 251406 (Fig. 2). The maximum read count was observed in FS sample and the minimum read count was found in BP sample (Fig. 2). The operational taxonomic unit (OTU) number generated was 3193 and OTU with ≥ 2 counts was 3064.

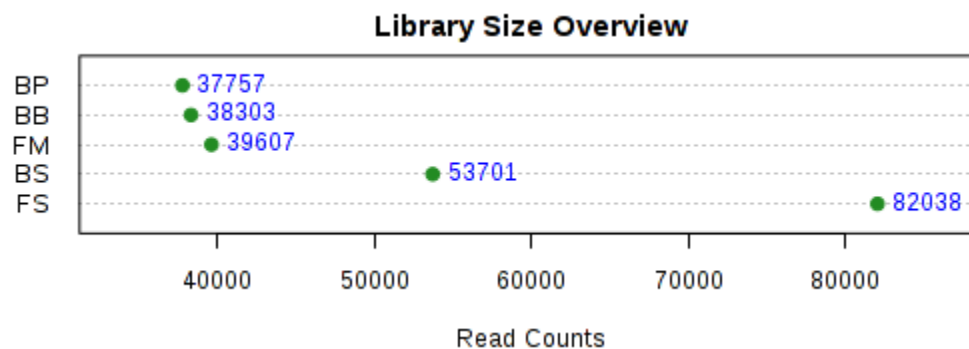


Fig. 12. 2 Read counts for different rhizosphere soil samples. The vertical line shows the sample type (BB - beginning of blooming, BP – beginning of pod development, BS – beginning of seed, FS – full seed, FM – full maturity) and the horizontal shows the read counts

12.2.2 Class-based Classification of Rhizosphere Bacterial Communities during Soybean Growth

Upon analysis using MicrobiomeAnalyst platform, all sequences were classified into 17 classes (Fig. 3). We chose to classify bacterial community into classes and not phyla because class-based classification is more precise than phyla-based classification. *Proteobacteria* was more dominant followed by *Actinobacteria* in BB and BP soil samples. In contrary, *Actinobacteria* was more abundant in BS, FM and FS soils but FS sample had the highest abundance of *Actinobacteria* with a read count of 47382 (Fig. 3). We estimated that rhizosphere soils showed distinctive changes during soybean growth, with *Actinobacteria* and *Proteobacteria* decreasing by 1.24% and 1.80% respectively from beginning of blooming to beginning of pod development while *Actinobacteria* increased by 39.67% from beginning of seed to full seed stage and further decreased by 65.87% from full seed stage to full maturity. *Firmicutes* increased from 6.93% at the beginning of blooming to 9.82% at full seed stage and decreased to 7.95% at FM stage (Fig. 3). Other classes of bacterial community identified followed almost the same pattern of microbial dynamics during soybean growth (Fig. 3). *Spirochaetes* were absent at BB and BS stages but was present in BP, FS and FM with 1, 6 and 11 read counts respectively during soybean growth.

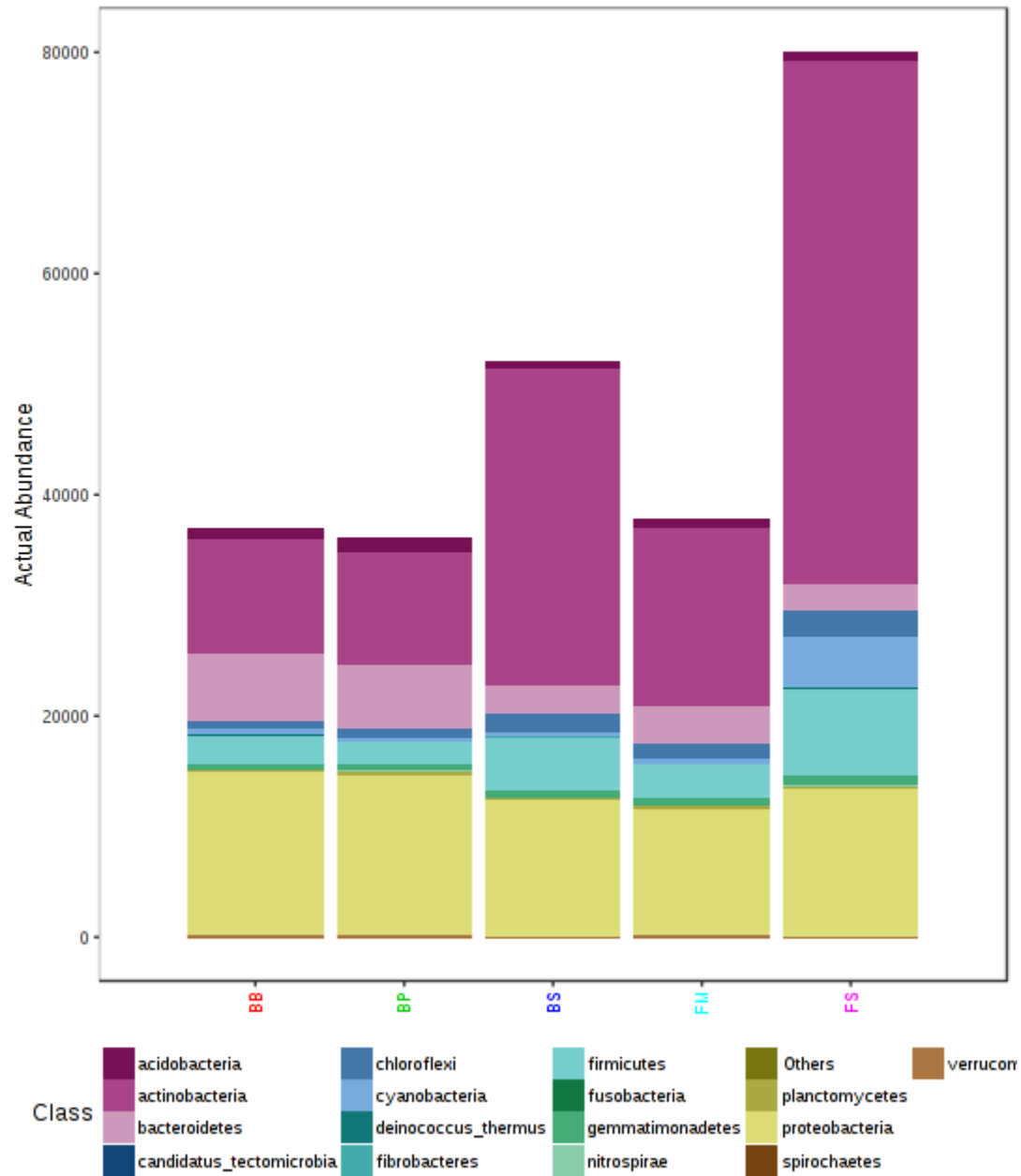


Fig. 12. 3 Actual Abundance of bacteria at the class level on a 16S Amplicon data reads from rhizosphere soil at different growth stages of soybean plants. Y-axis represents bar plot of the total reads (the actual abundance) and X-axis represents the sample type (BB – beginning of blooming, BP – beginning of pod development, BS – beginning of seed development, FS – full seed, FM – full maturity). The colour code for each class is shown at the base of the figure

12.2.3 Heatmap Analysis of Rhizosphere Bacterial Communities at OTU Level

In addition, to gain knowledge into the impact of soybean growth stages on bacterial community structure, Heatmap analysis (which gives information on relative distribution and abundance) was applied on the entire OTUs at order taxonomic level (Fig. 4). The results showed that growth stages had a significant impact on most of taxonomic units (order). In particular, growth stage significantly affected the order *Dehalococcoida*, *Candidatus_tectomicrobia* and *Bacteroidia* at the BB. However, growth stages had a greater effect at FS stage with highly significant effects observed in the order *Cytophagia*, *Spartobacteria*, *Alphaproteobacteria*, *Deinococci*, *Plantomycetia*, *Deltaproteobacteria*, *Erysipelotrichia*, *Ardenticatenia*, *Cyanobacteria* and *Mollicutes*.

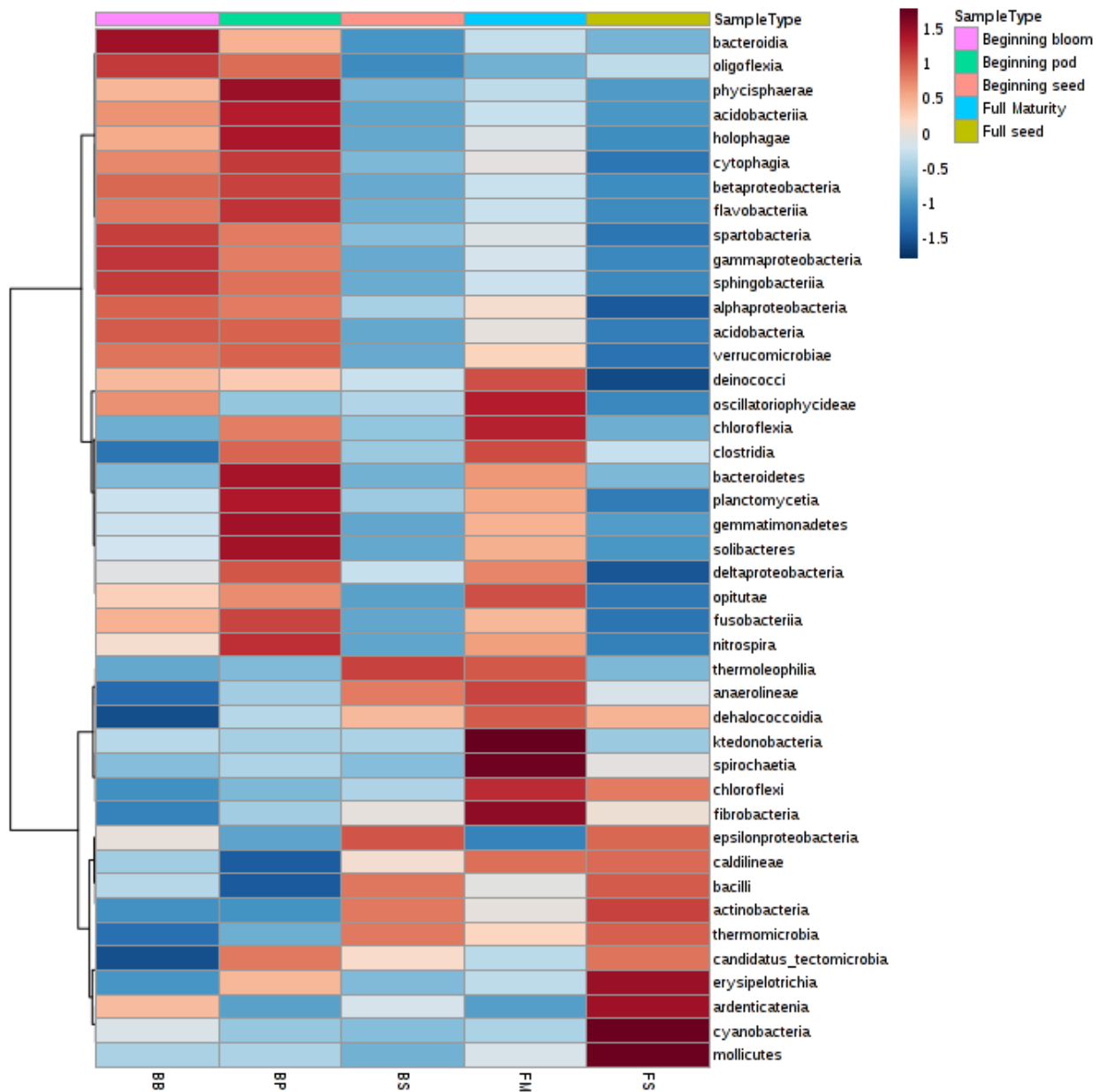


Fig. 12. 4 Hierarchical clustering and Heatmap visualization at the order level. The intensity of the coloured boxes is directly proportional to the taxon abundance

12.2.4 Variations within Rhizosphere Bacterial Communities during Soybean Growth

Alpha diversity, which is the richness and evenness of taxonomic units within a community, was extremely variable across the five soil samples. Alpha diversity graphical visualizations at generic (p-value: 0.40601; Kruskal-Wallis statistics: 4) and specific (p-value: 0.40601; Kruskal-Wallis statistics: 4) taxonomic levels are shown in Fig. 5. Mann-Whitney/Kruskal-Wallis statistical method was executed to compare our samples. The diversity measure used was the non-parametric Chao1, which is well-known to be a precise evaluator of richness (Fig. 5) was highest in BP (diversity value: 186; se: 8.877581) and lowest in FS samples (diversity value: 185; se: 3.890475) at the generic taxonomic level. But, at the species level, the variations at the BB and BP samples were almost at par but BB (diversity value: 185 and se: 3.890475) was slightly higher than BP (diversity value: 185; se: 3.890475) and higher species richness was observed at FS and FM growth stages.

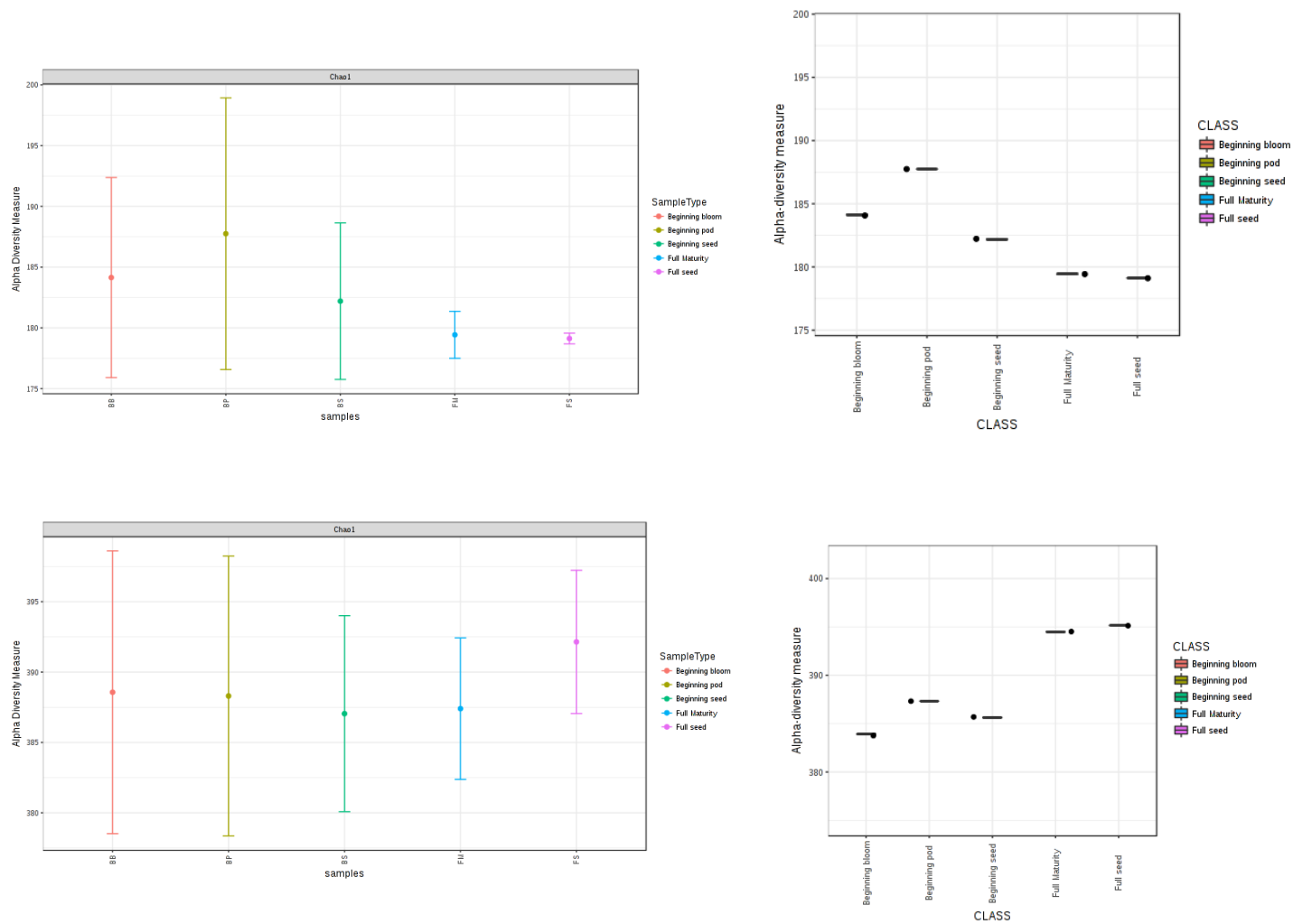


Fig. 12. 5 Alpha-diversity of samples at the genus (top) and species (bottom) levels. Y-axis indicates the taxonomic richness of the rhizospheric soil bacterial communities. X-axis indicates the sample type

12.2.5 Variations between Rhizosphere Bacterial Communities during Soybean Growth

In order to examine the variations in bacterial community structure between sample types (beta-diversity), the Bray-Curtis Index was computed at the family, genus, species and strain/OTU levels using permutational MANOVA and principal coordinate analysis (PCoA) statistical methods (Fig. 6). Samples were clustered according to growth stages at every taxonomic level (Family level: F-value = 0, R-squared = 1, p-value < 1; Genus level: F-value = 0, R-squared = 1, p-value < 1; Species level: F-value = 0, R-squared = 1, p-value < 1; strain/OTU level: F-value = 0, R-squared = 1, p-value < 1). At the family level, BS and FM rhizosphere soils were more similar to each other than BB, BP and FS rhizosphere soils. At the generic level, BB and BP rhizosphere soils were more similar than other rhizosphere sample types and the same trend of close similarity between BB and BP was observed at the species level. Similarly, at the strain/OTU level, BB and BP rhizosphere soil samples were more similar followed by somewhat similarity between BS and FS but FS rhizosphere soil sample was relatively far apart at this level. This analysis disclosed clear distinctions in the bacterial community structure between rhizosphere soil types at different soybean growth stages.

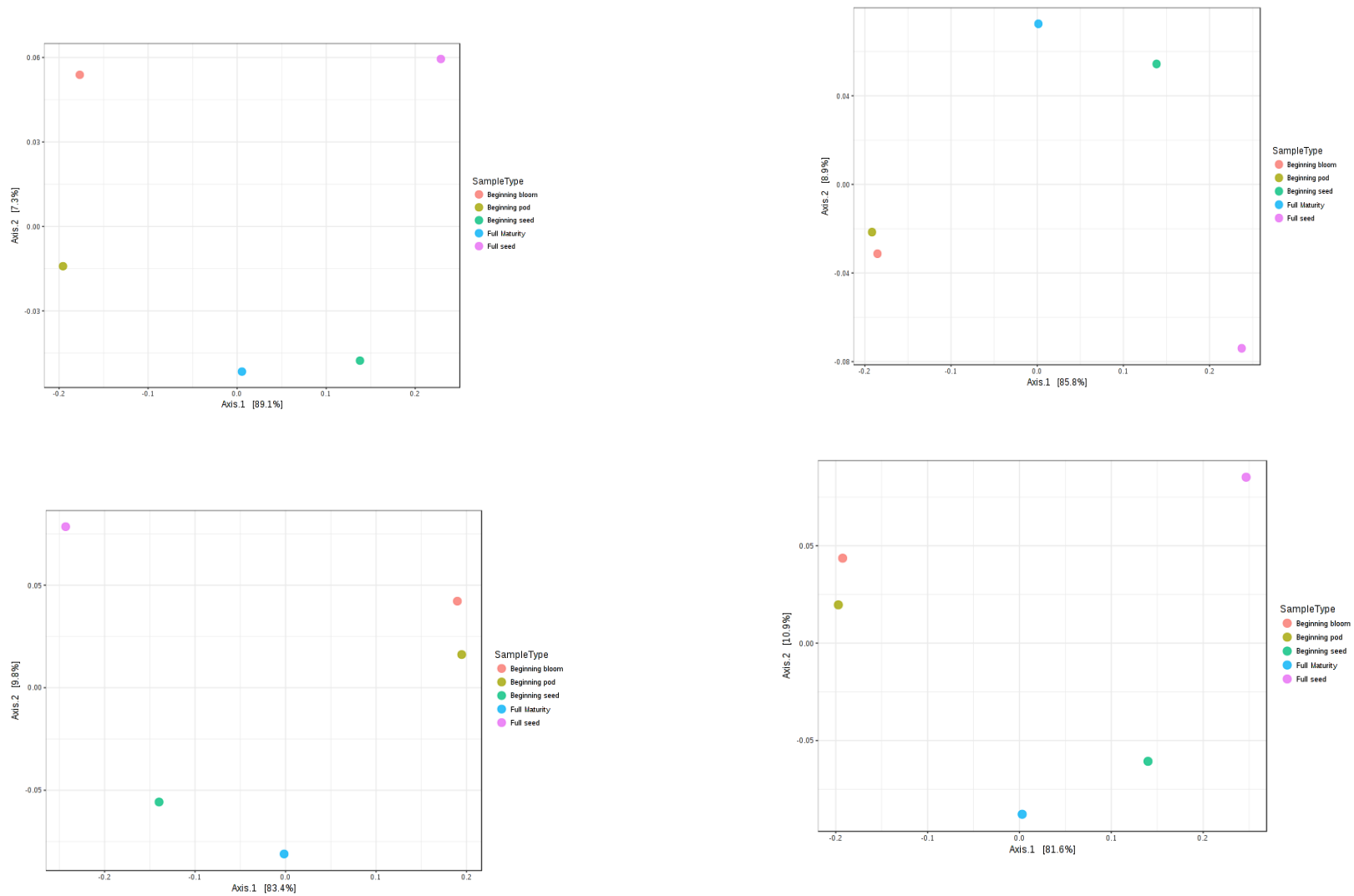


Fig. 12. 6 Beta-diversity of samples at the family (top left), genus (top right), species (bottom left) and strain/OTU (bottom right) level

12.2.6 Bray-Curtis Dendrogram Community Profiling of Rhizosphere Bacterial

Communities

In this study, we used dendrogram hierarchical clustering method with Bray-Curtis Index to validate the phylogenetic β -diversity of bacterial communities. The two major clusters were comparatively consistent with respect to soybean growth stages marked with different colors (Fig. 7). The dendrogram tree revealed that bacterial communities from BB clustered with those from BP and soil samples collected at the later stages of soybean growth (BS, FS and FM) exhibited clustering that crossed over (Fig. 7).

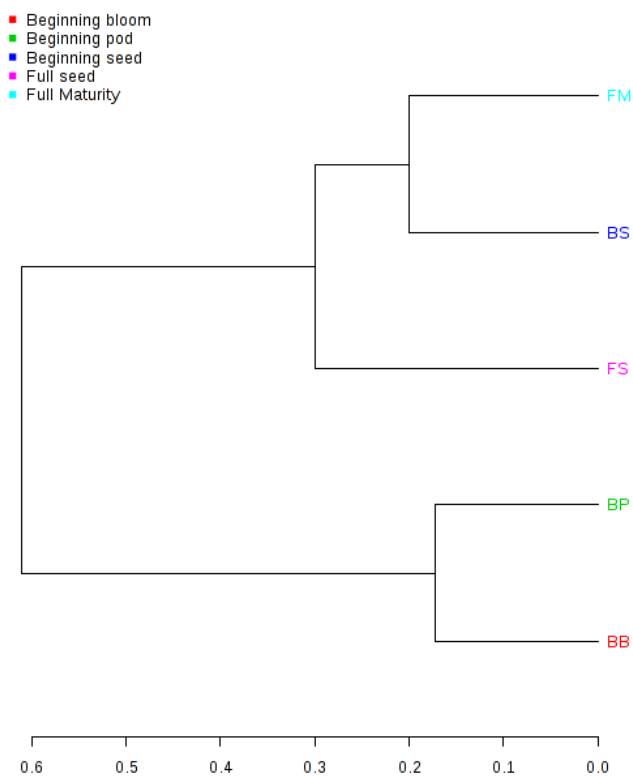


Fig. 12. 7 Dendrogram drawn from a Bray-Curtis Index of bacterial 16S (V4) region. The two major clusters showed the relationships among the bacterial communities at different soybean

growth stages. The first cluster (top) showed FS (pink), BS (blue) and FM (turquoise), while the second cluster (bottom) showed BB (red) and BP (green)

Online data availability

BB with the online name B_L001: Bioproject number PRJNA496421, Biosample number SAMN10341378, SRA Accession number SRR8127646

<https://www.ncbi.nlm.nih.gov/sra/SRX4948854>[accn]

BP with the online name P_L001: Bioproject number PRJNA496421, Biosample number SAMN10341908, SRA Accession number SRR8127966

<https://www.ncbi.nlm.nih.gov/sra/SRX4949174>[accn]

BS with the online name AMPLICON: Bioproject number PRJNA496421, Biosample number SAMN10341954, SRA Accession number SRR8128850

<https://www.ncbi.nlm.nih.gov/sra/SRX4949972>[accn]

FS with the online name FS_L001: Bioproject number PRJNA496421, Biosample number SAMN10341927, SRA Accession number SRR8128629

<https://www.ncbi.nlm.nih.gov/sra/SRX4949836>[accn]

FM with the online name H_L001: Bioproject number PRJNA496421, Biosample number SAMN10342630, SRA Accession number SRR8129599

<https://www.ncbi.nlm.nih.gov/sra/SRX4950721>[accn]

12.3 Discussion

Plant growth stages have been reported to affect bacterial communities of the rhizosphere which are essential for plant growth and yield (Berendsen et al., 2012b, Uroz et al., 2010). In this present study, bacterial communities of soybean rhizosphere in semi-arid field soil were analyzed using cultured independent 16S amplicon (V4) sequencing through NGS platform. Large numbers of bacteria have been found to thrive in the root region, mostly due to nutrient supply and platform provided by indigenous plants (Mendes et al., 2013b, Bakker et al., 2012, Sugiyama et al., 2014). In our results, *Proteobacteria* abundance modulates across soybean growth stages with the highest abundance recorded at BB stage which further decreased during soybean growth until the BS stage and increased at BS stage and later decreased at FM stage. These results indicate that soybean growth stages influenced bacterial communities of the rhizosphere with changes possibly taking place during subsequent growth stages to sustain ‘physiologically active’ rhizospheric bacterial compositions. However, the most abundant class of bacteria was *Actinobacteria* with the highest count of 47382 observed at FS growth stage. This result is contrary to what was observed by Sugiyama et al. (2014) who reported *Proteobacteria* as the most abundant bacterial group in the rhizosphere of soybean.

Heatmap analysis based on order-level classification grouped bacterial into 43 orders. Several orders were recognized as components of each sample including *Betaproteobacteria*, *Gammaproteobacteria*, *Alphaproteobacteria*, *Acidobacteria*, *Bacilli*, *Clostridia*, *Actinobacteria* and *Mollicutes* (Fig. 4) which is in agreement with the results of Mendes et al. (2014b) who also found in soybean rhizosphere most of the microbial groups observed in this study.

Genus and species-based analyses demonstrated that bacterial communities of the rhizosphere changed as the growth stage of soybean changed, indicating ‘stage-specific

formation' of peculiar rhizospheric bacterial communities (Fig. 5). This observation is in agreement with the changes that occurred in the bacterial communities of soybean rhizosphere bacterial reported by Sugiyama et al. (2014).

In order to evaluate the impacts of growth stages on the bacterial diversity, we compared the five datasets using two standard comparison methods utilized in community ecological studies, focusing on taxonomic abundance, richness and community composition. Our comparison platforms- PCoA (at the generic and specific levels) and Bray-Curtis Index (at the family, generic, specific and OTU levels) – showed distinct differences (Fig. 5 and 6 respectively). At the generic level, PCoA showed relatively lower taxonomic richness at BS, FS and FM and higher richness at BB and BP soybean growth stages while at the specific level, lower richness was revealed at BB, BP and BS and higher richness at FS and FM growth stages (Fig. 5). These results are in agreement with several other studies that revealed that plant species shaped the microbial composition and abundance in the rhizosphere. Some of these studies include influence on microbial dynamics by *Arabidopsis* (Bulgarelli et al., 2012b, Lundberg et al., 2012), soybean ((Xu et al., 2009), rice (Knief et al., 2012), wild oats (DeAngelis et al., 2009), oak (Uroz et al., 2010), tobacco (Robin et al., 2006), Norway spruce (Calvaruso et al., 2009) and potato (Rasche et al., 2006).

Moreover, analyses of the richness and evenness of bacterial community among samples showed similarity between BB and BP samples at the genus, species and strain/OTU levels while BS, FS and FM samples were more similar (Fig. 6) which also conforms to Bray-Curtis Index dendrogram bacterial community profiling (Fig. 7). In contrast, at the family level, BS and FM samples showed closer similarity while BB, BP and FS bacterial community were similarly

clustered together (Fig. 6). These further buttressed that there were changes in the rhizosphere bacterial community at different growth stages of soybean grown in this semi-arid environment.

Thus, the growth and yield of agricultural crops in semi-arid and arid environments can be hindered by drought stress and soybean crop is not an exception. In particular, it was reported by Igiehon and Babalola (2018a); Igiehon and Babalola (2018c); Igiehon and Babalola (2017) that drought stress caused by climate change is one of the main factors that can affect rhizosphere soil microbial diversity and plant growth and it was reported by Hungria et al. (2013) that the contribution of soybean to soil fertility enhancement via nitrogen fixation process is being threatened by global climate change as a result of the ever increasing drought stress (Zullo Junior et al., 2008).

In addition, drought stress increases plant vulnerability to pathogens leading to seed amendment with different insecticides and fungicides that are presently used on more than 90% of soybean plants in some parts of the globe and this can be detrimental to the entire ecosystem (Igiehon and Babalola, 2018b) including PGPR (Campo et al., 2009) with drought tolerant and antimicrobial potentials. Furthermore, it has been reported that indigenous plant species contain a definite assemblage of rhizospheric bacterial communities that can enhance plant growth and thwart infection by pathogenic organisms (Berendsen et al., 2012a). *Actinobacteria* and *Proteobacteria* are known to accommodate bacterial species with plant growth promoting traits. The abundance of these groups in soybean rhizosphere in this study could be due to chemotaxis of bacteria in these groups to the soybean root exudates resulting in their proliferation in the rhizosphere (Barbour et al., 1991). *Proteobacteria* such as *Rhizobium* and *Pseudomonas* have been implicated in plant tolerance to drought stress (Igiehon and Babalola, 2018a, Igiehon and Babalola, 2018c). Indeed, *Enterobacter cloacae* was reported to be involved in phosphate

solubilization and production of indole-3-acetic acid (Richardson et al., 2009, de Souza et al., 2013) and *Stenotrophomonas maltophilia* under *Gammaproteobacteria* group has been found to reduce the population of nematode in the soils (Insunza et al., 2002). Added to this, *Sphingomonas* sp. was shown to produce indole-3-acetic acid (Tsavkelova et al., 2007) and *Streptomyces* sp. (*Actinobacteria*) to abate pine root infection by *Armillaria* and *Fusarium* (de Vasconcellos and Cardoso, 2009), although both genera similarly contain pathogens of plants such as *Streptomyces scabies* and *Sphingomonas melonas* (Buonaurio et al., 2002, Johnson et al., 2008, Seipke et al., 2011). Other classes and orders present in the rhizospheric soil (Fig. 3 and 4 respectively) also accommodate potential PGPRs. For instance, *Firmicutes* such as *Bacillus* species have been reported to be connected with antibiotics production, phosphate solubilization and ‘systemic resistance to pathogens’ (Choudhary and Johri, 2009, Govindasamy et al., 2010, Kumar et al., 2011). We observed that many genera and species (Fig. 5) of these likely PGPRs were increased in the soybean rhizosphere at ‘growth stage-specific’ pattern. This might be due to fluctuations in root exudates during soybean growth since it has been reported that rhizosphere microbial richness and root exudate profile correlated during *Arabidopsis* growth (Chaparro et al., 2014b, Chaparro et al., 2013).

12.4 Conclusions

Conclusively, this current study unveiled the changes that occurred in bacterial communities of field grown soybean rhizosphere exposed to drought. Bacterial communities of the rhizosphere were altered during growth of soybean plants and rhizospheric bacterial communities contained classes and orders with likely PGPR genera and species with variations in the level of abundance at different growth stages. Bacterial fluctuations observed in soybean rhizosphere are partly due to the semi-aridity of the soil, but considerable changes detected for

rhizosphere microbial composition and abundance suggest the effect(s) from soybean growth. Further studies on soybean root exudation and effects of bacterial communities on soybean growth are necessary to elucidate the mutual interactions between soybean and rhizosphere microorganisms in the field for better use of these bacteria for agricultural sustainability.

12.5 Acknowledgements

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12.6 Conflict of Interest

Author 1 declares that he has no conflict of interest. Author 2 and 3 declare that they have no conflict of interest

12.7 Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

12.8 Authors Contribution

Igiehon NO performed the experiment and wrote the article; Babalola OO secured funding, provided academic input in writing the manuscript and thoroughly critiqued the article while Igiehon NO and Aremu BR did bioinformatics analyses of the data. All authors approved the article for publication.

CHAPTER 13

13.0 General Discussion

Drought stress is one of the most detrimental abiotic stresses that has considerably increased ‘over past decades’ affecting global food security. Drought stress might range from short and moderate to prolonged and very severe, limiting agricultural yields (Austin, 1989, Pereira and Chaves, 1995, Bottner et al., 1995, Vurukonda et al., 2016). Also, it is expected that drought stress will cause severe plant growth challenges for more than 50% of agricultural lands by 2050 (Ashraf and Wu, 1994, Vinocur and Altman, 2005, Kasim et al., 2013). Thus, the present study explored the ability of new *Rhizobium* species isolated from Bambara groundnut (*V. subterranea*) biome and AMF to enhance the growth of soybean (*G. max* L) under drought conditions.

The new *Rhizobium* species that were isolated were identified through 16S rRNA amplicon sequence analyses as *Rhizobium* sp. strain R1 (accession no. MG309875), *Rhizobium tropici* strain R2 (accession no. MG851722), *Rhizobium cellulosilyticum* strain R3 (accession no. MG309874), *Rhizobium taibaishanense* strain R4 (accession no. MG851723) and *Sinorhizobium meliloti* strain R5 (accession no. MG851724). The evolutionary relationships of these species with other species in database showed that R2 strain was closely related to *R. tropici* but R1 and R3 strains were grouped under same clade in the phylogenetic tree. This might be due to nucleotide similarity observed in these two strains.

Furthermore, our findings showed that these rhizobial species possessed PGP traits that enable soil microorganisms to support plant growth and development (Igiehon and Babalola, 2018c). In particular, all the rhizobial species produced siderophore and NH₃ including IAA. IAA production by rhizobial species such as *B. japonicum* has previously been reported by

Masciarelli et al. (2014); Boiero et al. (2007). In addition, these species were also positive to ACC deaminase test but all the species except R1 and R4 strains were positive to HCN.

Also, the growth of the rhizobial species in NB broth amended with different concentrations of PEG indicated that these species are able to survive and grow under stress conditions. PEG is an osmotic-stress stimulant that can be used to induce stress on plants in drought tolerance experiments (Ortiz et al., 2015). In this study, it was found that R1 and R3 strains demonstrated the highest level of tolerance to osmotic stress while, strain R5 showed the lowest level of tolerance in the PEG experiment. Hence, R1 and R3 (the most tolerant strains) and R5 (the least tolerant strain) were selected for soybean inoculation and growth under stress condition in a growth chamber under in the laboratory. Thus, PAN 1532 R soybean seeds which were noticed to be moderately sensitive to drought stress were inoculated with R1, R3 and R5 strains in 4% PEG solution. In particular, soybean seedlings colonized with specific bacterial species inocula enhanced the seedling fresh weight. Moreover, R1 and R3 strains were able to more effectively enhance soybean seed germination than R5. These findings are in agreement with the results of Jalili et al. (2009) who reported that ‘ACC deaminase – producing fluorescent pseudomonads’ improved canola (*Brassica napus* L) seed germination under osmotic stress. Thus, whole genome sequencing was performed for R1 and R3 strains in order to gain genomic insights into some of the functional genes that may be involved in symbiotic establishment as well as plant survival and growth promotion under stress and were further used to inoculate soybean seeds (PAN 1532 R) in the greenhouse and field under drought conditions.

Upon trimming and *de novo* assembly using Kbase pipeline of sequenced R1 and R3 strain data, the Prokka (V1.12) and RAST online pipeline annotation was performed for assembled sequence without using a known reference strain (Seemann, 2014, Tejerizo et al.,

2017, Aziz et al., 2008). Annotation of R1 and R3 sequenced genome revealed the presence of *exoX*, *htrA*, *Nif*, *nodA*, *eptA*, *IAA* and siderophore-producing genes in both rhizobial strains. Most of these genes are involved in stress (such as drought) tolerance (Vurukonda et al., 2016), plant growth promotion (Igiehon and Babalola, 2018c), establishment of symbiotic interactions and antibiosis.

For instance, *exoX* genes are responsible for the production of EPS by rhizobial (Zhan and Leigh, 1990). Although, it has been reported that EPS production is a survival strategy needed by microorganisms under drought stress condition, Ormeño-Orrillo et al. (2012) reported that production of EPS is connected with acid tolerance. In reality, EPS has been produced by *Agrobacterium tumefaciens* and *S. meliloti* in acidic environments (Yuan et al., 2008, Hellweg et al., 2009, Cunningham and MuNNS, 1984). R1 and R3 strains also possess high-temperature tolerant genes called *htrA* which may have been responsible for the ability of these species to grow at a temperature of 45°C. Five (5) *htrA* and 14 *htrA* genes were found in R1 and R3 strain respectively and among the *htrA* found in the rhizobial strains in this present study are the R1 strain extracellular serine protease and microbial serine proteinase *htrA* as well as the R3 strain serine protease Do-like *htrA* and serine protease Do-like *htrA* genes. Both *htrA* genes for R1 strain and R3 strain (Fig. 12 c, d) have their unique contigs locations within the genome.

Furthermore, R1 and R3 strains harbored nitrogen fixing (*nif*) genes with 6 different biological functions. Nitrogen fixing (*nif*) genes are involved in the conversion of atmospheric N to the form that can be utilized by plants. One of the *nif* genes reported for R3 strain was located between contigs 38, 300 – 39,443 (IscS gene) while *nifS* was located between contigs 72 – 336 but the *nif* genes in R1 were found at contigs locations different from that of R3 strain. IscS whose product function provides sulphur for the synthesis of iron-sulphur cluster *in-vitro*,

nevertheless *in-vivo* role of IscS in iron-sulphur formation is yet to be established (Schwartz et al., 2000). Studies of the *Azotobacter vinelandii* nitrogen fixation gene cluster revealed that there are activities that enhance the effectiveness of iron-sulphur cluster assembly (Dean et al., 1993). To be specific, study of *nifS* led to the detection that the protein produced by IscS gene is a pyridoxal 59-phosphate-haboring cysteine desulfurase that helps to transfer the sulfur moiety from cysteine to cysteinyl active site of *nifS* leading to the formation of enzyme-bound per-sulfide (Zheng et al., 1994). After reduction and incorporation of an Fe source, the sulphur can be released and effectively integrated into the iron-sulphur protein cluster of nitrogenase (Zheng et al., 1994).

We also found 23297 and 12242 *nodA* genes in R1 and R3 strains respectively but only two are reported for each strain in this study. Particularly, we observed *NodA* genes encoding putative MFS-type transporter YcaD and riboflavin transporter in R1 strain and Beta-N acetylglucosaminidase and Teichoic acid poly (ribitol-phosphate) polymerase in R3 strain. The location of putative MFS-type transporter YcaD was between contigs 93 – 735, riboflavin transporter was between contigs 734 – 1091, Beta-N acetylglucosaminidase was between contigs 4582 – 7225 and Teichoic acid poly (ribitol-phosphate) polymerase was between contigs 7268 – 9128. It is a common knowledge that *Nod* genes help in the formation of nodules, the site of nitrogen fixation by nitrogen fixing bacteria such as *Rhizobium* species. Moreover, it was suggested that the kind of ‘Nod factor acyl group attached by *NodA* can contribute to the determination of host range’ (Debellé et al., 1996, Ormeño-Orrillo et al., 2012). *NodA* gene was also reported by Ormeño-Orrillo et al. (2012) as one of the nodulation genes found in *Rhizobium* species.

Moreover, 13 and 33 siderophore producing genes were found in R1 and R3 respectively. Siderophore production by these species may have been due to the presence of these genes and the role of siderophore producing-rhizobia under drought conditions is highly appreciated since it was reported by Hungria et al. (2013) that plants are highly prone to pathogenic attack under water stress conditions. Siderophore is obviously antimicrobial in nature (Igiehon and Babalola, 2018c) and thus application of siderophore-producing rhizobia will certainly help to improve plant health under drought stress and consequently increase agricultural productivity (Igiehon and Babalola, 2018a). All the same, IAA genes involved in the production of Isoaspartyl peptidase protein were detected in R1 strain. Similarly, 2 of the 6 IAA genes found in R3 strain are responsible for the production of inner membrane protein YiaA and tRNA dimethylallyltransferase and the locations of the respective genes were between 536 and 704 for the R1 strain and between contigs 11,701 and 11,965 as well as 10,955 and 11,900 for the R3 strain. IAA helps in root elongation and production of lateral roots and root hairs involved in nutrient absorption (Mohite, 2013). Elongation and increase in the number of roots produced by plants as a result of IAA production can serve as a survival strategy to plants under drought stress condition and may even contribute in some other ways to plant development since it was reported by Masciarelli et al. (2014) that the increased production of IAA by *B. japonicum* shows that, in addition to plant promotion, the bacterium could have other beneficial traits needed for plants (such as soybean) survival. In short, considering these benefits, the interaction between plants especially legumes and rhizobia is key to plant productivity.

Based on the results of the growth response to different environmental pH, R1 and R3 strains can be considered acid-tolerant strains since they were able to survive and grow at a low pH of 4. Again, upon application in the field, these strains may be confronted with acid stress in

acidic soils and within the symbiosome. According to Ormeño-Orrillo et al. (2012), the mechanisms involved in rhizobial survival and growth in acidic environments are yet to be understood. *EptA* genes coding for UDP-N-acetylmuramate-L-alanyl-gamma-D-glutamyl-meso-2, 6-diaminoheptandioate ligase and Phosphoethanolamine transferase were found in R1 strain while those coding for Heptaprenyl diphosphate synthase component-1 and septation ring formation regulator EzrA were found in R3 strain. Ormeño-Orrillo et al. (2012) also found genes in *R. tropici* CIAT 899 and *Rhizobium* sp. PRF 81 coding for ‘putative lipid A Phosphoethanolamine transferase’ similar to that found in R1 strain in this study. In *Salmonella typhimurium* and *Escherichia coli*, *eptA* gene is activated under mildly acidic environments and it was reported that *eptA* imposed acid tolerance in *Shigella flexneri* 2a (Martinić et al., 2011). *EptA* genes were also reported in *R. rhizogenes* K84, sinorhizobia, agrobacteria, but not in other *Rhizobium* spp.

With respect to the greenhouse experiment, physiological, biochemical and below-ground-ground or morphological parameters connected to drought tolerance in soybean plants amended with rhizobia and mycorrhizal consortium (MY) under water stress conditions were investigated. It was observed that water levels (40 and 70% FC) had impacts on virtually all the physiological (% RWC and % electrolyte leakage), biochemical (soluble sugars and proline content) and below-ground-above-ground or morphological (shoot height, shoot width, branch number, leaf number, taproot length, taproot width, lateral root number, plant fresh weight, shoot dry weight, root dry weight, pod number, pod fresh weight, seed number, seed fresh weight, highest seed number per pod, seed dry weight, number of fungal spore and % mycorrhizal) parameters of soybean plants grown.

RWC is used to evaluate water level balance in plants and it is a dependable strategy by which the level of osmotic stress is determined (Zarik et al., 2016). Thus, in this study, MY treatment was able to increase RWC under 40% FC. This confirms the reports that mycorrhizal fungi increased leaf RWC (Baslam and Goicoechea, 2012, Aroca et al., 2008). In addition, some other single and dual inoculations similarly led to increase in RWC and this increase was pronounced in plants treated with R1+R3.

In addition to the physiological approach, plants also use biochemical strategy to endure or tolerate water stress by accumulating high level of soluble sugars and proline which help to regulate cell osmotic potential under water deficit conditions (Zhang et al., 2010). According to the results obtained in this study, under 40% FC, leaf soluble sugars increased in soybean plants treated with R1MY and R1+R3MY while under 70% FC, there was an increase in soluble sugars in soybean leaves amended with R1, R1MY, R3MY, R1+R3 compared to the well-watered treatment . These results agree with the findings of Zhang et al. (2010); Abbaspour et al. (2012); Yooyongwech et al. (2013); Ruiz-Lozano et al. (2012) and Talaat and Shawky (2011) who reported that soluble sugar accumulation in inoculated plants can serve as a defence strategy against drought and salt stresses. However, since will recorded the lowest content of soluble sugars in plants treated with MY under moderate or 70% FC, our results partially agree with the work of Zarik et al. (2016) who highlighted higher soluble sugar concentration in ‘non-mycorrhized plants’ than in ‘mycorrhized plants’ under drought conditions but disagree with this author’s conclusion at the level of 40% FC since there was an increase in soluble sugars which was higher than what was observed in the control and some of the mycorrhized and non-mycorrhized treatments under this severe drought stress.

The amino acid proline is an essential ‘osmo-protectant osmolyte’ produced by plants to improve osmosis and prevent water loss (Ruiz-Lozano et al., 2012, Zarik et al., 2016). The proline (the osmo-regulator in addition to soluble sugars used in this investigation) concentration in leaves was lower in non-inoculated soybean plants than in rhizobial and mycorrhizal inoculated soybean plants under severe drought. Under this drought condition, significant accumulation of proline was observed in the leaves of soybean plants treated with the dual inoculum R3MY. This finding corresponds to the result of Ortiz et al. (2015) who reported significant proline accumulation in some plant treatments dually inoculated with microbial species leading to a reduction in the osmotic potential of the plant cells and consequently enhanced uptake of water to ‘maintain osmotic balance’ (Dong et al., 2002). Also, the concentration of proline accumulated by soybean plants treated with MY under severe drought condition was comparatively higher than that of the non-mycorrhized treatments (control, R1, R3, R1+R3). This result disagrees with the conclusions of Zarik et al. (2016); Manoharan et al. (2010) and Wu and Xia (2006) who observed less proline content in the leaves of mycorrhized *Cupressus atlantica*, *vagiegata* and orange plants respectively than in non-mycorrhized plants grown under moderate and severe drought stresses. Furthermore, under moderate drought stress condition, soybean plants treated with R1+R3MY were shown to accumulate the highest level of proline while the lowest level was found in R1 treatment. Contrary results were found in the well-watered plant treatments where the highest and lowest proline concentrations were produced by plants inoculated with R3 and R3MY respectively.

Surprisingly, lower chlorophyll content was observed in virtually all soybean plants grown in 100% FC and increased with increasing drought stress. Overall, R3MY treatment and non-inoculated plants grown in 100% FC showed the highest and lowest chlorophyll content

respectively. Chlorophyll is an important component that plays a vital role in the process of photosynthesis in plants (Tajini et al., 2012). Considering the different water levels, chlorophyll content negatively correlated with below-ground-above-ground productivity, implying that as the drought stress increased, chlorophyll content increased and plant productivity decreased.

Furthermore, we found that the number of mycorrhizal spore positively correlated with the % mycorrhization in this study as fungal spore increased from 40% FC to 100% FC. The highest number of spore was observed in soybean plant rhizosphere inoculated with R1+R3MY in all the water treatments. Also, soybean root colonization or % mycorrhization was positively correlated with RWC and seed number of soybean plants inoculated with MY, R3MY and R1+R3MY indicating enhancement of plant water status and productivity. Perhaps, mycorrhizal fungi may have contributed to drought avoidance capacity of these treatments, thus resulting in water stress alleviation in soybean plants. The increase in seed number and other morphological components in soybean plants inoculated with MY, R1MY, R3MY, R1+R3MY may partly be ascribed to mycorrhizal colonization and RWC (especially for MY, R1MY and R3MY treatments at 40% FC), signifying that plant water content was efficiently enhanced by this plant-microbial interactions resulting in enhanced productivity. This finding agrees with the results of Aliasgharzad et al. (2006) who reported that an increase in % mycorrhization led to an increase in RWC in soybean plants inoculated with both *B. japonicum* and *Glomus etunicatum*.

Indeed, at 40% FC, significant promotion of soybean plant growth under severe drought stress by R1+R3MY inoculation depicted by increased in shoot width and branch number could be as a result of the synergistic interaction between the microbial species and mutualistic interaction between the inoculum and soybean plant roots (Igiehon and Babalola, 2018a). This treatment in addition to the dual inoculum R1MY greatly affected root dry weight, although the

effect was only significantly different ($P < 0.05$) from plants inoculated with R1 strain. Similarly, under severe drought stress, taproot length was greatly affected by R3 inoculum and this result somewhat supported the previous outcome in which R3 strain significantly affected soybean seedling root fresh and dry weight and number of lateral root in a growth chamber experiment where PEG was used as a drought factor. But in the greenhouse, under severe drought stressed condition, R3 treatments produced lateral roots which were less than that produced by other microbial treatments but higher than that of non-inoculated plants.

One of the indicators of sustainable agriculture and/or food security is increase in agricultural produce (Igiehon and Babalola, 2018c). Our present study has shown that combinations of rhizobia and mycorrhizal fungal consortium resulted in increased yield (soybean seed number) under severe and moderate stressed conditions in a controlled environment. Particularly, R1+R3MY treatments significantly ($P < 0.05$) produced more seeds in 40% FC than other treatments. The non-inoculated (control) plants and plants amended with R1 and R3 did not produced seeds under this condition probably because the absence of PGPR (in the control plants) and single inoculation (in R1 and R3 treated plants) could not ameliorate the detrimental effects of drought stress to the extent of empowering the plants to produced seeds even though these treatments produced pods. It was reported in our previous studies (Igiehon and Babalola, 2017, Enebe and Babalola, 2018) that abiotic stresses such as drought, heat and salinity are part of the factors that militate against crop growth, development and yield. The impacts of these abiotic stresses may be abated by below-ground plant-microbial (AMF and rhizobacterial) interactions (Igiehon and Babalola, 2018a). Tripartite mutualistic interactions with AMF fungi and *Rhizobium* species (Igiehon and Babalola, 2018a) is therefore important to increase

productivity in soybean (Igiehon and Babalola, 2018c) and the impacts of co-inoculation with mycorrhizal fungi and rhizobia on soybean plants need further research.

Also, co-inoculation with mycorrhizal consortium and rhizobia (R1MY) significantly enhanced soybean yield as indicated by pod number, pod fresh weight, seed number and seed fresh weight in the 70% FC but not in the 100% FC. These outcomes further showed that there was a synergistic impact between mycorrhizal consortium and rhizobia on soybean yield in this investigation and that this impact ‘might’ be dependent on water status. However, previous study by Wang et al. (2011) indicated that such synergistic impact can be link to nutrient status. It is generally believed that mycorrhizal fungi principally benefit plants grown in soil environments where phosphorus is likely to hinder plant productivity by increasing the volume of soil penetrated by mycorrhizal hyphae compared to that of root hairs of non-mycorrhizal plants (Igiehon and Babalola, 2017, Wang et al., 2011) and in this current study, there was no addition of inorganic phosphorus and nitrogen to the soil and the natural soil total nitrogen was slightly low compared to that reported by Ortiz et al. (2015). This low nutrient level of soil could be another reason (in addition to water status) for the synergistic impacts of dual inoculation with mycorrhizal consortium and rhizobia in the greenhouse study.

The effects of rhizobia and mycorrhizal fungi on soybean in the field under semiarid environment of South Africa were further evaluated. During the period of field the experiment, climatic data such as relative humidity, temperature and number of days of rainfall were collected. the mean relative humidity during the period of the experiment was slightly different from that reported by Prakamhang et al. (2015). In addition, during the period of this experiment, the temperature range was in partial agreement with a temperature reported by Ortiz et al. (2015) in a study performed in an arid soil and almost the similar climatic trends were

observed in preceding years. Also, it only rained for 61 days during the period of the field experiment. Hence, the climatic data obtained in this study indicate that the study area is exposed to drought stress.

Furthermore, plants are continually subjected to abiotic stress (e.g. drought) which is among the major challenges connected to plant development impacting agronomic demands. Inoculation with rhizobacteria and mycorrhizal fungi has been reported efficient to enhance productivity under semi-arid and arid environments (Enebe and Babalola, 2018, Igiehon and Babalola, 2017). Consequently, knowledge of the influence of interactions between microorganisms and plants on crop growth, yield nutritional level and tolerance to stress is essential. Indeed, inoculation with two drought tolerant *Rhizobium* spp. strains R1 and R3 as well as mycorrhizal consortium for growth and nutrient enhancement under water-stress environments resulted in increased RWC, soluble sugars, proline, above-ground and below-ground biomass, yield, nutrient content and decreased electrolyte leakage.

Accordingly, the results of this study in the field showed that rhizobial and mycorrhizal inoculations enhanced RWC of soybean leaves compared to the non-inoculated plants. Mycorrhizal consortium (MY) treatment gave the highest leaf RWC followed by the rhizobial and mycorrhizal combination (R3MY) treatment. In contrast, R1+R3MY gave the highest shoot RWC. This increase in RWC can be attributed to the capacity of mycorrhizal and nodulated root to enhanced water uptake in soil deficient in water. Similar findings were also reported in plants grown under water stress by Baslam and Goicoechea (2012) and Jie et al. (2000). According to Beltrano and Ronco (2008), mycorrhizal fungi positively impacted wheat water status under drought stress conditions.

Conversely, electrolyte leakage was decreased in soybean plants co-inoculated with both rhizobia species (R1+R3) and plants dually inoculated with both rhizobia and mycorrhizal consortium (R1+R3MY). Similar findings were observed in the greenhouse experiment in which R1+R3MY reduced electrolyte leakage under different water-stressed conditions. Thus, the relatively low electrolyte values observed in soybean plants inoculated with R1+R3 and R1+R3MY showed that the plant cells or tissues were not injured by drought stress. This result is in agreement with the findings of Ortiz et al. (2015) who reported reduction in cell membrane damage in stressed plants by plant growth promoting bacteria and mycorrhizal fungi.

Admittedly, microbial inoculation also enhanced soluble sugar concentration more than the non-inoculated plants suggesting that microbial species help in catabolic breakdown of starch to sugars for osmotic adjustment to minimize the effects of stress since it was reported by Enebak et al. (1998) that starch degradation may be the cause of increase in soluble sugars in cells. Accumulation of soluble sugars was more pronounced in soybean leaves treated with R3MY, R1+R3 and R1+R3MY but accumulation was highest in R1+R3MY inoculated plants. The absence of rhizobia and mycorrhizae in the non-inoculated (control) plants perhaps led to a decrease in starch catabolism and soluble sugar content and consequently affected the plant growth. Similarly, previous studies have shown starch reduction in grapevine leaves (Patakas and Noitsakis, 2001) and soluble sugar accumulation in stressed plants (Dekánková et al., 2004) in response to drought stresses.

Indeed, in the study, rhizobial and mycorrhizal fungal inoculations enhanced proline accumulation under semi-arid condition, which might be as a result of up-regulation of proline biosynthetic pathway. High level of proline helps to maintain cell water balance, protect protein and cell membranes from stress (Yoshida et al., 1997). Accumulation of proline was higher in

mycorrhizal treatments particularly in MY, R1MY, R3MY and R1+R3MY treatments. Proline accumulation may be related to the high level of nutrients observed in soybean seeds amended with R1MY.

In reality, the positive effects observed in most of the mycorrhizal soybean plants with regard to most of the physiological parameters (RWC and electrolyte leakage), biochemical parameters (soluble sugars and proline content), morphological parameters (at different growth stages) and mineral nutrients of seeds can be linked to increase in spore number and mycorrhizal root colonization since the use of mycorrhizal fungi as biofertilizer for sustainable agriculture as reported in our previous studies (Igiehon and Babalola, 2017, Igiehon and Babalola, 2018a) is dependent on their inherent ability to penetrate the roots of their host plants.

Obviously, *Rhizobium* is a group of bacteria that have the ability to fix atmospheric N for their host via the development of nodules on the roots of plants such as legumes (Mfilinge et al., 2014, Igiehon and Babalola, 2018c). Thus in the field study, at the beginning of blooming, we observed that R1 inoculation was effective to increase nodule formation of soybean plants and this effect increased when it was co-inoculated with R3 and MY in the R1+R3MY treatment. The ability of this rhizobial strain to enhance nodule formation can be linked to its inherent nodulation genes. The increase in nodule number observed in R1+R3MY treatment could also be the cause for the significant increase in most of the growth parameters observed in the treatment since more nodule formation may result in increase in nitrogen fixation which is needed for growth. To be specific, soybean plants inoculated with R1+R3MY were significantly higher ($P < 0.05$) in shoot height, shoot width, leaf number, nodule number, taproot length, lateral root number and fresh plant biomass which conformed with the result of Tairo and Ndakidemi (2013) who reported increase in shoot height, width and other growth parameters of soybean plants

grown in the field. On the other hand, R3 inoculated plants gave the best result with regard to branch number but highly significant increase was observed in soybean nodule fresh and dry weight amended with R1.

Compared to the non-inoculated plants, significant effects were observed at the beginning of pod formation in soybean plants inoculated with rhizobia and mycorrhizal consortium. At this stage of soybean growth, mycorrhizal consortium (MY) had the greatest effect on soybean shoot height but synergistic interaction between R1 and MY (R1MY) showed high significant impacts on shoot width, leaf number, nodule number, taproot width, nodule fresh weight and shoot dry weight. The increase in the productivity of soybean that was dually inoculated could be linked to the fact that one of the microorganisms served as a helper species to the other microorganism for effective growth promotion since it was reported by Igiehon and Babalola (2018c) that ‘in co-inoculation, certain microorganisms function as helper microorganisms to enhance the effectiveness of the other microorganisms’. Amazingly, the non-inoculated soybean plants produced nodules with the highest dry weight and the highest pod number was produced by R3 inoculated soybean which contradicts the report of Mfilinge et al. (2014) who asserted that amendment of soybean plants with *Rhizobium* species resulted in increase in number of pod produced.

At the beginning of seed development, co-inoculation with R1 and R3 strains appears to have the greatest effects on seed development since R1+R3 had the greatest impact on pod number, pod fresh weight and pod dry weight. Similarly, R1+R3 co-inoculation considerably impacted shoot width, branch number, leaf number and shoot dry weight. These results further buttress the synergistic effects of microbial co-inoculations on plant growth and yield as reported by Mfilinge et al. (2014); Ortiz et al. (2015); Zarik et al. (2016); Igiehon and Babalola (2018c).

At full seed, tripartite interaction with R1, R3 and MY (R1+R3MY) significantly affected seed biomass since this treatment was able to increase pod fresh and dry weight. Also, R1+R3MY inoculation significantly increased pod number but single inoculation with R3 gave the best results for pod fresh and pod dry weight. R1+R3MY also greatly impacted above-ground biomass such as shoot height, shoot width, branch number and shoot dry weight as well as below-ground biomass such as lateral root number. Significant increase ($P < 0.05$) was observed in shoot and taproot width in plants inoculated with MY while co-inoculation of soybean with MY and R1 (R1MY) resulted in higher nodule number, nodule fresh weight and nodule dry weight. Similar effects of co-inoculation on nodulation of agricultural crops has previously been reported, for instance it was revealed that co-inoculation of *Pseudomonas* and *Mesorhizobium* sp. significantly improved nodulation as well as shoot and root dry weight of chickpea (Verma et al., 2013). In contrast, dual inoculation of soybean with R3 and MY (R3MY) increased significantly leaf number.

At maturity, R1+R3MY showed the greatest effect on shoot height and pod length. Unlike at the beginning of pod development, R1+R3 and R3MY had the greatest impact on pod number and pod fresh weight respectively at full maturity. Similarly, R3MY significantly increased branch number, leaf number, taproot width and lateral root number but R1+R3MY had the greatest effect on taproot length and root dry weight. In this present field study, with the exception of pod length and lateral root number, drought stress decreased other above-ground and below-ground biomass measured at full maturity in the non-inoculated (control) plants which agree with the reports of our previous study (Igiehon and Babalola, 2018c).

Additionally, upon harvest in the field, it was observed that co-inoculation of soybean plants with R1+R3MY resulted in a significant increase in the fresh and dry weights of soybean

seeds compared to the non-inoculated control plants in the field. Similarly, other microbial treatments were effective in enhancing the fresh and dry weight (parameters that were used to determine seed yield in this study), but the lowest fresh and dry weights were obtained for soybean treated with R1 strain unlike R3 strain which was more effective in improving soybean yield. These results are in agreement with the growth chamber experiment in which soybean seedlings singly inoculated with R3 showed a better performance than those amended with R1 or R5 strain. Amazingly, R3 treatment produced seeds with dry weight greater than soybean inoculated with mycorrhizal consortium even though this consortium contains 5 different drought tolerant AMF namely: *Rhizophagus clarus*, *Gigaspora gigantea*, *Funneliformis mosseae*, *Claroideoglomus etunicatum* and *Paraglomus occulum*. There was an increase in the yield of soybean dually inoculated with *R. cellulosilyticum* strain R3 and mycorrhizal consortium (R3MY).

In addition, there were no significant differences among the treatments as regards fresh weight per seed. However, the highest fresh and dry weights were recorded in soybean seed co-inoculated with R1+R3MY. This increase may be due to synergistic interaction between the rhizobia and mycorrhizal fungi since many researchers have reported that yield of legumes are affected by AMF and rhizobia interactions (Ahmad, 1995, Redecker et al., 1997, Xavier and Germida, 2003). In comparing the impacts of associations between *Glomus aggregatum*, *Sclerocystis microcarp* and *Glomus pallidum* and four *Rhizobium phaseoli* on the yield of different kidney bean genotypes, Ahmad (1995) observed that the symbiotic efficacy was reliant on the actual combination of the mycorrhizal species, bacterial strain and the host genotype. However, contrary to other studies, both effective and ineffective *Rhizobium* species were included their study so as to determine the role of *Rhizobium* spp. in the tripartite association.

They found that co-inoculation of an effective *Rhizobium* strain LX43 with an AM fungus enhanced the yield, nitrogen and phosphorus content of the plant. Similarly, co-inoculation of less effective *Rhizobium* isolate with another AM fungus increased the yield of the legume. However, this significantly enhanced productivity than that of an effective *Rhizobium*-AMF combination.

The area of seed randomly picked from each treatment revealed that soybean treated with mycorrhizal consortium (MY) had the greatest area. In addition, R1 treatment seed area was relatively higher than other treatments. This result indicates that MY and R1 relatively affect the length and width of soybean seeds, although the effect was not significant compared to the non-inoculated (control) soybean plants. The effect of seed area was very pronounced in the seed size distribution of R1 since this treatment produced large seeds with the greatest weight. However, seed area effect did not significantly affect yield (fresh and dry weight of entire seed produced) since much yield increase was observed in soybean dually inoculated with *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 (R1+R3), R1MY and R1+R3MY.

Number of seed per pod is also a component that can affect soybean seed yield. Rhizobia and fungi did not significantly ($P>0.05$) affect the number of seed per pod; soybean dual inoculated with R1+R3 had the highest number of seed per pod. Single inoculation with either R1 or R3 strain resulted in either 14.4 or 15.69 seed number per pod respectively. But co-inoculation with only the rhizobial strains slightly affected pod seed number. This effect slightly decreased when each of the rhizobial strains was co-inoculated with mycorrhizal consortium. Interestingly, such slight decrease was also observed in soybean dually inoculated with R1+R3MY. Perhaps, the significant increase in the yield of soybean dually inoculated with R1+R3MY can be related to production of more pods per plant than other treatments which

confirm the conclusions drawn by Liu et al. (2003); Westgate and Peterson (1993) that yield increase under drought stress is caused by increase in the number of pod.

R1, R3 and R1+R3MY were more effective in producing large soybean seeds since these treatments produced large seeds with greater weight. Comparatively, R1+R3 and MY treatments did not favour the production of large seeds suggesting that the effects seen in soybean seeds amended with R1+R3MY treatment could largely be attributed to the synergistic interaction between the rhizobial species and mycorrhizal consortium. Naturally, leguminous crops such as soybean are able to form symbiotic interactions with both AMF and rhizobia (Xavier and Germida, 2003, Wang et al., 2011). Tripartite mutualistic symbiotic interactions with AMF and rhizobia should be very crucial for soybean productivity (Igiehon and Babalola, 2018) and the impacts of soybean inoculation with rhizobia and AMF need further research.

In the same way, the nutritional components in soybean were increased by most of the rhizobial and mycorrhizal species inoculated under semi-arid environment in the field. Similar findings were reported by Ortiz et al. (2015); Zarik et al. (2016); Al-Karaki and Clark (1999); Subramanian and Charest (1999) and Meddich et al. (2000). Actually, most of the macro and micronutrients in this study were improved in soybean seeds. Specific enhancement on soybean seeds nutritional status (principally macronutrients) was significantly pronounced ($P < 0.05$) in plants dually inoculated with the new rhizobial strain R1 and mycorrhizal consortium (R1MY). In relation to the non-inoculated (control) plants, soybean plants dually treated with R1MY augmented N content. In contrast, the Na content was significantly increased in non-inoculated (control) plants while Mo content in R1 inoculated *G. max* L was increased. The improved nutrient content observed in soybean seeds amended with microbial inoculants particularly R1MY could be due to increased absorption space created by extensive mycorrhizal fungal

mycelia (Zarik et al., 2016, Igiehon and Babalola, 2017, Igiehon and Babalola, 2018a). Indeed, it was reported in our previous study (Igiehon and Babalola, 2018c) that a ‘study carried out by Makoi et al. (2013) on the impact of *Rhizobium* strains on mineral nutrient absorption by *Phaseolus vulgaris* showed significant increase in the uptake of P, K, Mg, Ca and S in the entire plant parts. It was reported by the author that even though the concentration of P and K skyrocketed in the root region due to *Rhizobium* introduction, the increase was only significant in the greenhouse experiment and not in the field condition. *Rhizobium* inoculation enhancement of micronutrient (such as Mn, Fe, Cu, Zn, B and Mo) uptake in the shoots, roots, pods and the entire plant with the exception of Mo intake in the roots has been reported’.

It is commonly believed that mycorrhizal fungi hyphae are endowed with the ability to convey P and other mineral nutrients into their host plant (Goicoechea et al., 1997a) while *Rhizobium* species are notable for increasing the N nutrient of their host plants through the process of nitrogen fixation (Igiehon and Babalola, 2018c). Hence, in this study, the increase in N and P nutrients by R1MY treatment could be linked to the effect of the synergistic interaction between the rhizobial sp. and mycorrhizal fungi even though such effect was not glaring in other rhizobial-mycorrhizal combinations.

Furthermore, accumulation of nutrient in seeds under water stress might have a role in water stress tolerance. In a study carried out by Tanguilig et al. (1987), it was highlighted that soybean grew better than rice under water stress probably because the legume ‘maintain their turgidity’ under water stress by reducing the rate of transpiration and maintaining nutrient uptake. Therefore, in this study performed under semi-arid condition, the increase in macronutrients and RWC observed in R1MY could partly be responsible for the enhancement of most of the measured components of the R1MY inoculated plants compared to the non-

inoculated plants particularly at the beginning of pod and seed development as well as at full seed and maturity stages. Samarah et al. (2004) and Premachandra et al. (1995) reported that P, K, Cl were responsible for osmotic adjustment of water-stress tolerant sorghum line (K866) while Salisbury and Ross (1992) reported that Mo was a cofactor in enzymes involved in nitrogen fixation (nitrogenase enzyme) and nitrogen assimilation (nitrate reductase) in plants, suggesting that Mo was a key nutrient in nitrogen metabolism and assimilation. Also, drought has been reported to repress nitrogen fixation and reduce accumulation of N in the shoot (Purcell and King, 1996, Serraj et al., 1999). However, in this current study, we observed N accumulation in seeds of most inoculated soybean plants specifically in R1, R3, R1MY, R3MY and R1+R3MY treatments indicating that N concentrations in seeds was not negatively affected by water stress. This finding is in agreement with the results of Samarah et al. (2004) who similarly reported high N concentration in soybean seeds grown under drought stress. Since Mo is an important nutrient in N metabolism and assimilation, the intake and transport of this micronutrient from water stressed plants may play a role in uptake and transport of N to the seeds since it was reported by Samarah et al. (2004) that previous studies have shown that Mo application enhanced shoot N concentration and N translocation to the seed.

Furthermore, the interactions between rhizobial species and mycorrhizal fungi enhanced the percentage (%) crude fat of soybean seeds. The highest percentage was observed in soybean seeds treated with R1+R3MY and the lowest crude fat was recorded for the non-inoculated control soybean plants. These results showed that drought stress reduced the fatty acid content in seeds of non-inoculated plants which is in agreement with the work of Hou et al. (2006) who reported that drought stress is among the environmental factors that affect the content and/or composition of fat in soybean seeds. However, there was no significant difference in the % crude

fat of inoculated and non-inoculated (control) soybean seeds. The quality of soybean is evaluated based on the concentration and composition of fat, oil and protein. Soybean seeds have been reported to contain oleic acid, linolenic acid and linoleic acid (Schnebly and Fehr, 1993). Higher concentration of oleic acid is a desirable quality for long shelf-life and stability of oil for industrial purposes, but higher concentrations of polyunsaturated fatty acids such as linolenic acids are needed for human nutrition (Bellaloui, 2011). However, fatty acids (such as oleic acid) are less vulnerable to oxidative modifications during processing, storage cum frying. Therefore, the food industry is currently concerned in producing soybean seed that contain high concentration of oleic acid (Rahman et al., 2001). According to Opperman and Varia (2011), in 2010, South Africans demand for soybean oil was at 1.3 million tons followed by other countries such as Mozambique and Zambia with 0.2 million tons demand each for soybean oil in the same year. Thus, the demand for soybean seeds can greatly be influenced by its fatty acid content and production of soybean rich in fatty acid will therefore contribute positively to human nutrition and economy growth since it has also been reported that availability of high quality seed plays a significant role in the growth and development of any crop-producing enterprise (Dlamini et al., 2014). But, the % moisture content of non-inoculated (control) soybean seeds was relatively higher than the inoculated soybean seeds. The seeds of the non-inoculated plants showed higher percentage of moisture content than the seeds of soybean plants amended with rhizobial species and mycorrhizal fungi. This indicates that the microbial candidates used in this study may not only improve the nutrient status of soybean seeds but could also be harnessed to produce soybean with long shelf-life.

Plant growth stages have been reported to affect bacterial communities of the rhizosphere which may be essential for plant growth and yield (Berendsen et al., 2012b, Uroz et al., 2010).

Also, introduction of alien microorganisms could affect the richness and abundance of indigenous bacterial communities in a given ecosystem. Therefore, in this study, the richness and abundance of bacterial communities at different stages of soybean growth in the field was investigated by using cultured independent 16S amplicon (V4) sequencing through NGS platform. Large numbers of bacteria have been found to thrive in the root region, mostly due to nutrient supply and platform provided by indigenous plants (Mendes et al., 2013b, Bakker et al., 2012, Sugiyama et al., 2014). In our results, *Proteobacteria* abundance modulates across soybean growth stages with the highest abundance recorded at the beginning of blooming (BB) stage which further decreased during soybean growth until the beginning of seed (BS) stage and increased at BS stage and later decreased at full maturity (FM) stage. These results indicate that soybean growth stages influenced bacterial communities of the rhizosphere with changes possibly taking place during subsequent growth stages to sustain ‘physiologically active’ rhizospheric bacterial compositions. However, the most abundant class of bacteria was *Actinobacteria* with the highest count was observed at full seed (FS) stage. This result is contrary to what was observed by Sugiyama et al. (2014) who reported *Proteobacteria* as the most abundant bacterial group in the rhizosphere of soybean.

Heatmap analysis based on order-level classification grouped bacterial into 43 orders. Several orders were recognized as components of each sample including *Betaproteobacteria*, *Gammaproteobacteria*, *Alphaproteobacteria*, *Acidobacteria*, *Bacilli*, *Clostridia*, *Actinobacteria* and *Mollicutes* which are in agreement with the results of Mendes et al. (2014b) who also found in soybean rhizosphere most of the microbial groups observed in this study.

Genus and species-based analyses demonstrated that bacterial communities of the rhizosphere changed as the growth stage of soybean changed indicating ‘stage-specific

formation' of peculiar rhizospheric bacterial communities. This observation is in agreement with the changes that occurred in the bacterial communities of soybean rhizosphere bacterial reported by Sugiyama et al. (2014).

In order to evaluate the impacts of growth stages on the bacterial diversity, we compared the 5 datasets using two standard comparison methods utilized in community ecological studies, focusing on taxonomic abundance, richness and community composition. Our comparison platforms- PCoA (at the generic and specific levels) and Bray-Curtis Index (at the family, generic, specific and OTU levels) – showed distinct differences. At the generic level, PCoA showed relatively lower taxonomic richness at BS, FS and FM and higher richness at BB and BP soybean growth stages while at the specific level, lower richness was revealed at BB, BP and BS and higher richness at FS and FM growth stages. These results are in agreement with several other studies that revealed that plant species shaped the microbial composition and abundance in the rhizosphere. Some of these studies include influence on microbial dynamics by *Arabidopsis* (Bulgarelli et al., 2012b, Lundberg et al., 2012), soybean ((Xu et al., 2009), rice (Knief et al., 2012), wild oats (DeAngelis et al., 2009), oak (Uroz et al., 2010), tobacco (Robin et al., 2006), Norway spruce (Calvaruso et al., 2009) and potato (Rasche et al., 2006).

Moreover, analyses of the richness and evenness of bacterial community among samples showed similarity between BB and BP samples at the genus, species and strain/OTU levels while BS, FS and FM samples were more similar which also conforms to Bray-Curtis Index dendrogram bacterial community profiling. In contrast, at the family level, BS and FM samples showed closer similarity while BB, BP and FS bacterial communities were similarly clustered together. These further buttressed that there were changes in the rhizosphere bacterial communities at different growth stages of soybean grown in this semi-arid environment.

Thus, the growth and yield of agricultural crops in semi-arid and arid environments can be hindered by drought stress and soybean crop is not an exception. In particular, it was reported by Igiehon and Babalola (2018a); Igiehon and Babalola (2018c); Igiehon and Babalola (2017) that drought stress caused by climate change is one of the main factors that can affect rhizosphere soil microbial diversity and plant growth and it was reported by Hungria et al. (2013) that the contribution of soybean to soil fertility enhancement via nitrogen fixation process is being threatened by global climate change as a result of the ever increasing drought stress (Zullo Junior et al., 2008).

In addition, drought stress increases plant vulnerability to pathogens leading to seed amendment with different insecticides and fungicides that are presently used on more than 90% of soybean plants in some parts of the globe and this can be detrimental to the entire ecosystem (Igiehon and Babalola, 2018b) including PGPR (Campo et al., 2009) with drought tolerant and antimicrobial potentials. Furthermore, it has been reported that indigenous plant species contain a definite assemblage of rhizospheric bacterial communities that can enhance plant growth and thwart infection by pathogenic organisms (Berendsen et al., 2012a). *Actinobacteria* and *Proteobacteria* are known to accommodate bacterial species with plant growth promoting traits. The abundance of these groups in soybean rhizosphere in this study could be due to chemotaxis of bacteria in these groups to the soybean root exudates resulting in their proliferation in the rhizosphere (Barbour et al., 1991). *Proteobacteria* such as *Rhizobium* and *Pseudomonas* have been implicated in plant tolerance to drought stress (Igiehon and Babalola, 2018a, Igiehon and Babalola, 2018c). Indeed, *Enterobacter cloacae* was reported to be involved in phosphate solubilization and production of indole-3-acetic acid (Richardson et al., 2009, de Souza et al., 2013) and *Stenotrophomonas maltophilia* under *Gammaproteobacteria* group has been found to

reduce the population of nematode in the soil (Insunza et al., 2002). Added to this, *Sphingomonas* sp. was shown to produce indole-3-acetic acid (Tsavkelova et al., 2007) and *Streptomyces* sp. (*Actinobacteria*) to abate pine root infection by *Armillaria* and *Fusarium* (de Vasconcellos and Cardoso, 2009), although both genera similarly contain pathogens of plants such as *Streptomyces scabies* and *Sphingomonas melonas* (Buonaurio et al., 2002, Johnson et al., 2008, Seipke et al., 2011). Other classes and orders of bacteria present in the rhizospheric soils also accommodate potential PGPRs. For instance, *Firmicutes* such as *Bacillus* species have been reported to be connected with antibiotics production, phosphate solubilization and ‘systemic resistance to pathogens’ (Choudhary and Johri, 2009, Govindasamy et al., 2010, Kumar et al., 2011). We observed that many genera and species of these likely PGPRs were increased in the soybean rhizosphere at ‘growth stage-specific’ pattern. This might be due to fluctuations in root exudates during soybean growth since it has been reported that rhizosphere microbial richness and root exudate profile correlated during *Arabidopsis* growth (Chaparro et al., 2014b, Chaparro et al., 2013).

13.1 General Conclusions

The new *Rhizobium* spp. isolated from Bambara groundnut rhizosphere in this study were identified as *Rhizobium* sp. strain R1 (accession no. MG309875), *Rhizobium tropici* strain R2 (accession no. MG851722), *Rhizobium cellulosilyticum* strain R3 (accession no. MG309874), *Rhizobium taibaishanense* strain R4 (accession no. MG851723) and *Sinorhizobium meliloti* strain R5 (accession no. MG851724) and they were found to possess plant growth promoting traits.

In particular, it was found that *Rhizobium* sp. strain R1, *Rhizobium tropici* strain R2, *Rhizobium cellulosilyticum* strain R3, *Rhizobium taibaishanense* strain R4 and *Sinorhizobium meliloti* strain R5 produced EPS, ACC and in addition were able to survive and grow at a temperature of 45°C and in an acidic condition. Consequently, *Rhizobium* sp. strain R1, *R. cellulosilyticum* R3 and *S. meliloti* R5 strains enhanced the germination of soybean seeds (PAN 1532 R) including soybean seedling fresh weight, shoot and root parameters under drought condition imposed by 4% PEG. Nevertheless, *Rhizobium* sp. R1 and *R. cellulosilyticum* strain R3 inoculations were able to improve seeds germination more than other strains. Thus, genomic insights into *Rhizobium* sp. R1 and *R. cellulosilyticum* strain R3 revealed the presence of some genes with their respective proteins involved in symbiotic establishment, drought tolerance and plant growth promotion. To be specific, *exoX*, *htrA*, *Nif*, *nodA*, *eptA*, *IAA* and siderophore-producing genes were found in the two rhizobial strains.

The results of the greenhouse experiment similarly showed that single and dual inoculations of rhizobia and mycorrhizal consortium had significant effects on the physiological and biochemical indicators of soybean plants grown under different water regimes. These effects culminated in improving the below-ground-above-ground and/or morphological components of

soybean plants more than the non-inoculated treatments. Specifically, it was found that R1+R3MY enhanced soybean growth and yield as indicated for instance by shoot height and seed number under severe drought conditions.

Furthermore, the results of the field experiment showed that *Rhizobium* spp. and mycorrhizal fungal inoculation enhanced soybean tolerance to drought as demonstrated by improved water uptake, cell membrane stability as a result of decreased electrolyte leakage, osmotic adjustment thanks to accumulation of soluble sugars and proline, below-ground and above-ground plant biomass such as yield, seed fresh weight and dry weight, seed size, seed fatty acid content, macro and micronutrient assimilation especially N, P, Mg, S, Ca, Co, Mo, Fe and B. In addition, one of the new and remarkable results obtained in the current research under semi-arid environmental conditions is the inherent ability of the microorganisms to cope and tolerate drought conditions.

It was also found in the field experiment that bacterial communities of soybean rhizosphere were altered during growth of soybean plants and rhizospheric bacterial communities contained classes and orders with likely PGPR genera and species with variations in the level of abundance at different growth stages. Bacterial fluctuations observed in soybean rhizosphere are partly due to the semi-aridity of the soil, but considerable changes detected for rhizosphere microbial composition and abundance suggest the effect(s) from soybean growth.

13.2 Recommendations

- The availability of the whole genome sequences of R1 and R3 strains in public databases may further be exploited to comprehend the interaction of drought tolerant rhizobia with soybean and other legumes and the PGP ability of these rhizobial strains can also be

harnessed for biotechnological application in the field especially in semiarid and arid regions of the globe.

- Results of this study can also help to gain more insights into the impacts of mycorrhizal fungi and rhizobia on soybean growth and provide theoretical background for real-life application of these species in field-grown legumes. This biotechnological strategy can help us resolve or abate food insecurity problem endemic in most developing countries of the world.
- The results of this present study largely suggest that synergistic interactions are involved in the enhancement of yield, seed size and fatty acid content of soybean plants. This interaction between rhizobia and mycorrhizal fungi is an effective biotechnological strategy to improve plant yield and nutrition in semi-arid environments. However, insights into the adaptation and functioning of microorganisms and plants to extreme environmental conditions need further studies.
- Further studies on soybean root exudation and effects of bacterial communities on soybean growth are necessary to elucidate the mutual interactions between soybean and rhizosphere microorganisms in the field for better use in agricultural sustainability development.

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