Improving cowpea production in North West Province using endophytic bacteria and fungi

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DECLARATION

I, the undersigned, Israel Olawale OMOMOWO 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

I heartily give Almighty Jehovah the deserved foremost position and place in making a success of this research study. I also dedicate this study to my late father Prince Omotayo Omomowo and my wonderful family for their unflinching support.

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GENERAL ABSTRACT

Providing adequate, nutritious and safe food for human race is a huge challenge in the light of the rising cost of living, corona virus pandemic, climatic changes, high energy cost and war. The means of taken care of the global food supply is through the application of agrochemicals that are costly, non-renewable and destructive to the ecological balance. However, this highly intensive agrochemicals production system is not agro-ecologically sustainable. Therefore, there is ongoing efforts by various stakeholders in the agricultural sector to search for substitutes/alternatives to synthetic agrochemicals to achieve sustainable agro-ecological production system. A veritable means of achieving sustainable agroecological production is through the use of microbial resources that are renewable and eco-friendly. Endophytic microbiota research is on the upward trend as important partner in the plant-microbiome production system. Endophytic bacterial and fungal inoculants have shown good potentials in improving plant growth as biofertilizing, bio-stimulating, and bio-pesticidal renewable microbial resources with prospects in attaining agro-ecological balanced and sustainable crop improvement. Cowpea known scientifically as *Vigna unguiculata* L. Walps is multi-beneficial, highly valuable leguminous crop. It is a crop with high value-addition to human nutritional, animal husbandry sector and also agro-ecological benefits/soil health improvement. However, it is still an under-developed/neglected leguminous crop despite its numerous benefits. Although the African continent remains the major producer of this leguminous crop, a lot of challenges still persists in limiting cowpea optimum productivity and includes abiotic and biotic constraints, such as pest infestations, climatic weather variations among others. A veritable means of changing this narrative of low productivity of cowpea in Africa is by adopting sustainable agro-ecological microbial biotechnology approaches. Hence, the aim of this study is to assess the prospect of using endophytic bacteria and fungi to improve cowpea production yield in North West Province: a semi-arid zone of South Africa.

Methodology: Healthy looking and disease-free cowpea plant aerial parts and root zone tissues were collected from farms in North west province, South Africa. Also, cowpea seeds cultivars PAN311 and Bechuana white were collected from Agricultural Research Council, South Africa for the purpose of this study. Standard approaches were deployed in the storage, processing, isolation and authentication of endophytic bacteria and fungi from the cowpea seeds and tissues through morphological and molecular techniques.

The morphometrics methodology that was deployed in characterizing the endophytic microbiota include Gram staining techniques, sugar utilization, varied biochemical test and growth at different temperature for the endophytic bacteria. For the fungi isolates, macroscopic growth characteristics on different mycological growth media as well as microscopic features were deployed for morphological identification. For the molecular authentication, genomic extraction kits were used for extracting the genomic DNA, polymerase chain reaction (PCR) amplification protocol using both bacteria and fungi primers, was used to amplify 16S rRNA gene for the bacteria isolates, and ITS rRNA gene was used to amplify the fungi isolates. Agarose gel electrophoresis techniques was used in checking the quality of amplicons for both bacteria and fungi isolated. The amplicons were sequenced in both the forward and reverse directions. Different bioinformatics software were used to trim, clean and edit the sequence chromatograms before comparing it with existing sequences through BLAST search of the national center for biotechnology information (NCBI) database platform. Phylogenetic tree was then constructed using MEGA 7 software. Thereafter, plant growth promoting assay procedures were deployed in assessing the plant growth-improving attributes of both bacteria and fungi isolates. The various plant growth promoting attributes assay determined include: exopolysaccharide production, ammonia production, siderophore production, biofilm forming potential, hydrogen cyanide producing traits, solubilization of phosphate traits, as well as 1-aminocyclopropane-1-carboxylic acid deaminase capability. In addition to their invitro growth promoting potential experiment, growth chamber studies were carried out to assess the in-planta growth promoting capability of the endophytic isolates. To further elucidate the mechanistic approaches used by the highly effective endophytic microbiota isolated, selected isolates that included 2 bacterial isolates and a fungal isolate were subjected to in-depth genomic exploration using next generation whole genome sequencing techniques. More so, field experimental planting of two cultivars of cowpea was done over 2 planting seasons in Mafikeng, South Africa. A 2 X 5 factorial experimental design were randomized in completely block layout with three replications. The treatments deployed through seed biopriming with endophytic bacteria and fungi culture were applied as a single bioinoculant and as mixed bioinoculants. The control treatments were cowpea cultivars planted without inoculation with endophytic microbiota. Agronomic yield and growth parameters data were monitored and subjected to statistical analysis using SPSS version 20 was deployed for analysis of data. Results: The results indicated that 33 endophytic bacteria isolates were morpho-genetically authenticated, while 26 fungi isolates were also characterized. The 33 endophytic bacteria strains are diverse in terms of taxonomy, molecular and metabolic characteristics and falls into 11 genera that include: *Bacillus*, *Lynsinibacillus*, *Pseudomonas*, *Staphylococcus*, *Paenibacillus*, *Mammalicoccus*, *Brevundimonas*, *Stenotrophomonas*, *Myroides*, *Micrococcus* and *Ignatzscineria*. All the endophytic bacteria isolates possess multifarious invitro plant growth improving attributes and improves cowpea seeds and seedling germination. Likewise, the 26 fungal isolated strains possess multiple plant-growth improving attributes. Three endophytic microbiota showing high-level of bioactivity were selected for further genomic exploration and then deployed in cowpea field experimentation. They are *Stenotrophomonas geniculata* NWUBe21 and *Pseudomonas carnis* NWUBe30, as well as *Aspergillus minisclerotigenes* NWUFe10. The whole genome exploration studies indicated that these cowpea endophytic isolates possess plant growth improving genes and also vital secondary metabolites gene clusters. The field studies data showed that both single and mixed culture endophytic inoculants enhanced cowpea agronomic yield and growth parameters significantly compared to the un-inoculated control. Conclusion: The results of this findings have shown that endophytic microbiota from cowpea plants are potential renewable microbial resources that could be deployed in cowpea production for productivity enhancement in order to achieve sustainable agroecological production of this vital leguminous crop.

Keywords: Agro-food system crop enhancement; endophytic microbial resources; sustainable agricultural intensification; whole genome sequencing; next generation sequencing; cowpea;

LIST OF PUBLICATIONS

Chapter Two: Bacterial and fungal Endophytes: tiny-giant with immense beneficial potentials on plant growth and sustainable agricultural productivity. Published in Microorganisms (2019).

Authors: Olawale Israel Omomowo, Olubukola Oluranti Babalola

Candidate's Contributions: OIO suggested the review topic; OIO conducted the literature search; OIO wrote the first draft of the manuscript and the final article for publication.

Chapter Three: Constraints and prospects of improving cowpea productivity to ensure food, nutritional security and environmental sustainability. Published in Frontiers in Plant Science (2021)

Authors: Olawale Israel Omomowo, Olubukola Oluranti Babalola

Candidate's Contributions: OIO conducted the literature search and undertook the drafting and writing of the manuscript, and the final revision based on the reviewer's comments

Chapter Four: Draft genome sequence of endophytic *Aspergillus minisclerotigenes* strain NWUFe10 and its multifaceted-attributes as plant growth promoter. This manuscript is formatted for submission as short communication in Mycobiology.

Candidate's Contributions: OIO conceptualized this project and also carried out the wet lab and bioinformatics analyses of the genomes.

Chapter Five: Genomic insights into endophytic strains *Stenotrophomonas geniculata* NWUBe21 and *Pseudomonas carnis* NWUBe30 from cowpea with plant growth-stimulating attributes.

This manuscript is under review in Applied sciences -MDPI.

Candidate contributions: OIO conceptualized this project, carried out the wet lab and bioinformatics analyses of the genomes. OIO wrote the first and final draft of the manuscript for publication.

Chapter Six: Bioassessment of phylogenetic relatedness and plant growth enhancement of endophytic bacterial isolates from cowpea (*Vigna unguiculata*) seeds and plant tissues. This manuscript is formatted for submission to Journal of Microbiology and Biotechnology. OIO conceptualized this project, carried out all the wet-lab analyses of the study, including the isolation, characterization, and bioinformatics analysis. The first and final draft of the manuscript for publication was written by the candidate

Chapter Seven: Molecular diversity and growth promoting potentials of culturable endophytic fungal isolates from the seeds and root zone tissues of cowpea. This manuscript is formatted for submission to Mycology. OIO carried out the isolation, characterization, and all other wet-lab analyses of the project. OIO wrote the first draft of the manuscript as well as the final draft of the manuscript for publication.

Chapter Eight: Agronomic growth and yield enhancement potential of Cowpea plant by culturable bacterial and fungal endophytes in field experiment in Mafikeng, South Africa. This manuscript is formatted for submission to Annals of Agricultural Science. OIO is responsible for conceptualization of this project and carried out the wet-lab protocol, the field experimental planting and data analysis of the project. The first and second draft of the manuscript for publication was drafted by the candidate for publication.

Chapter Nine: Conclusion and recommendations

LIST OF ABBREVIATIONS

ACCD	1-aminocyclopropane-1-carboxylic acid deaminase
ALP	Alkaline phosphate
AM	Arbuscular mycorrhizal
AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of Variance
ARC	Agricultural Research Council
Bp	Base pair
BLAST	Basic Local Alignment Search Tool
BLASTn	Basic Local Alignment Search Tool n
°C	Degree centigrade
CAS	Chrome azurol S
CDS	Coding sequences
CFU	Colony forming unit
Cl	Chlorine
Cm	Centimeter
CO_2	Carbon (IV) Oxide
CRBD	Completely randomized block design
DAS	Days after sowing
DF	Dworkin and Foster
DNA	Deoxyribonucleic acid
DW	Dry weight
E	East
EPS	Exopolysaccharide
F	Forward

FAO	Food Agriculture Organization
G	Gram
GC	Guanine cytosine
g/l	Gram per liter
H	Hour
H_2O	Water
hr.	Hour
HCl	Hydrochloric acid
HCN	Hydrogen cyanide
HNO_3	Nitric acid
HSPs	heat shock proteins
H_2S	Hydrogen sulphide
H_2SO_4	Sulphuric acid
HTS	High throughput sequencing
IAA	Indole-3-acetic acid
IND	Indole production
INO	Inositol
ISR	Induced systemic resistance
K	Kilogram
K	Potassium
Kb	Kilo-base
KEGG	Kyoto Encyclopedia of Genes and Genomes
KOH	Potassium hydroxide
LB	Luria Bertani agar
LDC	Lysine decarboxylase

LNF	Level of nitrogen fertilizer
LPS	β-1, 2-glucans and lipopolysaccharides
M	Molarity
m^2	Metre square
mm^2	Millimetre square
MAN	Mannitol
MEGA	Molecular Evolutionary Genetics Analysis
Mg	Milligram
Min.	Minimum
Ml	Milliliter
Mm	Millimeter
Mn	Manganese
N	Nitrogen
Na	Sodium
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NB	Nutrient broth
NCBI	National Center for Biotechnology Information
ND	Not determined
NGS	Next Generation Sequencing

NH_3	Ammonia
Nif	Nitrogen fixing
Nm	Nanometer
No	Number
NRA	Nitrate reductase activity
NS	Non significant
OD	Optical density
OTU	Operation taxonomic unit
P	Probability
P	Phosphorus
PAN	Pannar
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGP	Plant growth promoting
PGPR	Plant growth promoting rhizobacteria

PGPT	Plant growth promoting traits
R	Reverse
RAST	Rapid Annotation using Subsystem Technology
RCBD	Randomize Complete Block Design
Rev/min	Revolution per minute
Rpm	Revolution per minute
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
ROS	Reactive oxygen species
S	South
SDA	Sabouraud dextrose agar
SE	Standard error
Sec	Second
SH	Shoot height
Sp.	Species
Spp.	Species
SPSS	Statistical Package for the Social Sciences
SRA	Sequence read archive
SYM	Symbiotic
TAE	Tris-Acetate-EDTA

Temp.	Temperature
tRNA	Transfer ribonucleic acid
VP	Voges Proskauer
UV	Ultra violet
v/v	Volume per volume
W	Weight
WGS	Whole genome sequencing
Wt	Weight
w/v	Weight per volume
YEM	Yeast mannitol agar
%	Percentage
µl	Microliter
<	Less than
≤	Less than or equal to
>	Greater than
≥	Greater than or equal to
µg/ml	Microgram per millilitre

CHAPTER ONE

GENERAL INTRODUCTION

1.0 BACKGROUND

Cowpea (*Vigna unguiculata* L. Walp) remains the most economically important and extensively produced indigenous African legume crop in sub-Saharan Africa (Walker et al. 2016). It is an annual leguminous crop that is indigenous to Africa. Cowpea can contribute significantly to increased food security and sustainability of crop-livestock systems. It is considered the most economically important traditional legume crop in Africa (Langyintuo et al., 2003). It is a major component in farming systems in diverse ecological zones and serve as a source of nutritious human food, livestock feed, green manure and income source for smallholder farmers (Timko and Singh, 2008). Cowpea production occurs under low input agricultural production on small-scale farms in developing countries like Nigeria and South Africa, and under such conditions, yield is mostly below its potential for the crop (Singh, 2014).

The main constraints militating against improved yield of cowpea production in most cowpea growing zones include; inadequate rainfall (due to frequent and erratic droughts), declining soil nutrients, pests and microbial pathogens, use of poor yielding varieties among others. Compounding these constraints and worsening this issue is climate change and problem of exponential human population growth rate, which necessitate expanding areas of production to obtain higher yields to meet food and nutritional security needs (Afutu et al. 2017; Springmann et al. 2016). Also, inorganic fertilizers are a challenge due to high cost and its attendant environmental polluting potentials (Rascovan et al., 2016). In addition to these, semi-arid region like NorthWest Province is characterized by little rainfall, declining soil nutrients, pests and plant pathogens infestation among others (Afutu et al., 2017).

More so, there is insufficient data on plant probiotics beneficial and abiotic stress tolerant functional genes associated with cowpea crops grown in North West Province of South Africa. This is a problem that needs to be solved. Therefore, there is the need to search for morphological and biochemical functional traits and molecular genes that are associated with beneficial endophytic bacteria and fungi that can be used to boost cowpea production in this semi-arid region.

To effectively combat these highlighted challenges, there is the need to formulate simple biological alternatives to take care of these highlighted abiotic and biotic stresses. This is imperative because plant infestation by pests and disease causing pathogens and its susceptibility to different environmental stress conditions is often due to the reduction in beneficial microbial diversity and imbalance in microbiome of the plant-host relationship. The plant microbiome is a fundamental partner in protecting plant from stresses by synthesizing enzymes or metabolites that can negatively affect plant pathogens, it produces important phytohormones and ensure / enable plant tolerance to environmental stress (Braga et al., 2016).

Endophytes represent promising alternatives to synthetic chemicals in crop production and protection against abiotic and biotic stresses, thereby fitting well into sustainable agriculture criteria strategies (Gundel et al., 2013).

Therefore, the biodiversity of the cowpea endophytic bacteria and fungi needs to be morpho-genetically characterized in-order for them to be taxonomically identified and to identify symbiotic strains that can be used as bio-inoculants for improving cowpea yield.

1.1 Problem statement

Cowpea is a versatile, resilient and multi-beneficial grain legume of enormous importance to man and its environment in most part of the developing world (Jayathilake et al. 2018; Olorunwa et al. 2022). The production of cowpea occurs in marginal fields by subsistence/rural farmers on a small-scale, deploying low-input agricultural production in developing countries like Nigeria and South Africa. The production output under such conditions is low yield that is below its optimum potential for the crop (Singh, 2014). Cowpea still remain an under-explored and under-utilized legume in most production areas in Africa. There are constraints/challenges that hinders productivity enhancement of cowpea yield in most producing countries in Africa. The main militating factors against improvement in yield of cowpea production in most cowpea growing zones include; inadequate rainfall (due to frequent and erratic droughts), declining soil nutrients, pests and microbial pathogens, use of poor yielding varieties among others. In addition to these challenges, the issue of climate vulnerabilities and astronomical global human population growth rate that is projected to reach 9 billion in a few decade is also a huge problem in achieving food and nutritional security (Fasolin et al. 2019; FAO, 2017). (Afutu et al., 2017; Springmann et al., 2016; Wiebe et al., 2015). Also, inorganic fertilizers are import dependent and non-renewable, this is a challenge due to high cost and its attendant public health and environmental polluting potentials (Rascovan et al., 2016).

The plant microbiome is a fundamental partner in protecting plant from stresses by synthesizing enzymes or metabolites that can negatively affect plant pathogens, it produces important phytohormones and also ensure / enable plant tolerance to environmental stress (Berg et al., 2015). The reduction / imbalance in beneficial microbiota allows for plant infestation by pests and disease causing pathogens in a microbe-plant host relationship.

Drought tolerance is a complex trait defined as the ability of plants to live, grow and reasonably produce with limited soil water supply or periodic water deficiencies. One of the most important limiting factors of cowpea production in the North West Province is the issue of low and erratic rainfall. This is a semi-arid region and drought is a major constraint for crop yield and productivity. There is insufficient data on drought tolerant functional traits of cowpea crops grown in NorthWest Province of South Africa. This problem requires a solution. Therefore, there is the need to search for morphological and biochemical functional traits and molecular genes that are associated with drought tolerant mechanisms in endophytic bacteria and fungi that can be used to boost cowpea production in this semi-arid region.

Therefore, the biodiversity of the cowpea endophytic bacteria and fungi needs to be morpho-genetically characterized in-order for them to be taxonomically identified and to identify symbiotic strains that can be used as bio-inoculants for improving cowpea yield.

1.2 Aim of the study

The aim of this study is to assess the prospect of using endophytic bacteria and fungi to improve cowpea production yield in North West Province: a semi-arid zone of South Africa.

1.2.1. Objectives of the research study

1. Isolation and identification of putative bacteria and fungi endophytes from the seeds, and cowpea plant and screening of the endophytes for beneficial traits in vitro.
2. Phenotypic and molecular characterization of bacteria and fungi endophytes and assessment of their phylogenetic relatedness.
3. Assessment of beneficial traits of the bacteria and fungi endophytes in growth chamber and greenhouse experiment.
4. Deploy high throughput sequencing to detect probiotics and stress tolerance genes in bacterial and fungal endophytes.
5. To assess the cowpea improvement yield in field experiment due to inoculation with bacterial and fungal endophytes.

CHAPTER TWO

2.0 BACTERIAL AND FUNGAL ENDOPHYTES: TINY-GIANT WITH IMMENSE BENEFICIAL POTENTIALS ON PLANT GROWTH AND SUSTAINABLE AGRICULTURAL PRODUCTIVITY. PUBLISHED IN MICROORGANISMS (2019).

Abstract

The conventional means of achieving enhanced agricultural productivity is not ecologically balanced and sustainable. The excessive use of synthetic agrochemicals, declining soil nutrients, water use issues among others is a threat to the ecosystem. To add to these environmental destructions is increasing global populace that will get to 9 billion by 2030. This means a decline in the volume of food resources to feed the world. Therefore, sustainably increasing agricultural productivity is a must to restore soil fertility, feed the populace and improve ecological balance. A way to achieve this is by the use of eco-friendly microbial inoculants. Endophytes inhabit the tissues of a plant without causing any adverse effects in their host plant. Bacteria and fungi endophytes benefit plants through growth promotion, pathogen suppression, improving stress tolerance and plant immunity. Despite this vital role played by endophytes in their interactions with the host plant, there is still a paucity of review data. More importantly, their prospects as an alternative to synthetic agrochemicals in ensuring sustainable agroecological crop productivity are not well review in literature. Therefore, this review seeks to highlight the potential of using endophytic microbial resources to achieve agro-food system crop enhancement in a sustainable and eco-friendly manner.

Keywords: Agro-food system crop enhancement; endophytic microbial resources; sustainable agricultural intensification; soil fertility; agroecological crop productivity; bio-fertilizers

2.1. Introduction

The global community is faced with constraints that adversely affect agricultural productivity in a sustainable and eco-friendly manner. Improving crop yield and productivity are hampered by a lot of limiting factors that include poor/erratic rainfall and inadequate water supply/water use efficiencies, diminishing soil fertility/declining soil nutrients. These militating constraints are also due to over-use of agricultural soil, urbanization, land degradation, excessive use of synthetic agrochemicals, the challenges of destructive phytopathogens and pests, abiotic stresses among others (Doni *et al.*, 2019). These constraints become exacerbated with the increasing number of people to feed: the human population will be more than 9 billion by the year 2030 (Hannah *et al.*, 2017). Also, the issue of climatic change and its adverse effects on crop productivity add to this herculean task. Changes in climatic conditions do affect plant growth and productivity. Although these effects are reliant on variation in climatic peculiarities like radiation, precipitation, humidity, as well as temperature changes. Anthropogenic activities of humans are affecting the global climate and, thus, leads to negative consequences for the sustainable productivity of crops in the agricultural sector and the ecological system (Springmann *et al.*, 2016).

In a bid to overcome these limitations and improve agricultural productivity, modern agricultural production is strongly intensified with the use of huge quantities of agro-chemicals in the form of synthetic fertilizers and pesticides (Duan *et al.*, 2016). Indeed, the conventional means of achieving agri-food security in this era is through the intensification of agricultural production in-puts by deploying excessive use of agrochemicals that are expensive and pollute the environment, thereby posing threat to man and the ecosystem (Vanlauwe *et al.*, 2014).

However, despite the successes recorded with the use of these synthetic agrochemicals, they do have their drawbacks. The excessive use of these synthetic agrochemicals have negative impacts on the well-being of humans and the proper ecological system functionality, as well as it reduces crop production sustainability (Smith & Siciliano, 2015). Agricultural intensification thus adversely affects ecological balance, it reduces fertility of the soil, contaminates the food chain, pollutes the groundwater, reduces microbial diversity, reduces soil pH and leads to increases in microbial resistance (Uphoff & Dazzo, 2016).

Yes, to keep up with the pace of increasing population and the attendant challenge of feeding a huge populace, agricultural production needs to be intensified. However, this must be done sustainably. Other alternatives are urgently needed to achieve environmental balance and sustainability. Globally, scientists that are in the field of agriculture and allied sciences are researching on an innovative means of improving agricultural productivity and its sustainability. Achieving sustainable agricultural intensification is necessary to achieve an increase in productivity and maintain the ecological balance. This will translate to higher crop yield due to improvement in plants resilience and adaptation to change in climatic conditions, biotic and abiotic stress shocks (Pretty *et al.*, 2011).

Improving crop production out-put in this era of enormous constraints that limit agricultural productivity, requires maximizing productivity sustainably without causing damage to the ecological balance (Busby *et al.*, 2017; Timmusk *et al.*, 2017). A cheaper, eco-friendly and sustainable means of achieving agricultural intensification and improving productivity is by adopting the use of microbial inoculants in enhancing vital soil nutrient availability and use, as well as their usefulness in conferring both abiotic and biotic stresses mitigating functional traits in planted crops (Jambon *et al.*, 2018).

There has been an upsurge in the use of microbial inoculants in contemporary agricultural production to boost soil fertility, improve cycling of nutrients, enhance growth, vitality and also improve the productivity of crops (Ajilogba & Babalola, 2019; Enebe & Babalola, 2018; Igiehon & Babalola, 2018). The use of microbial inoculants that include, bio-fertilizers, bio-pesticides, bio-flocculant, bio-remediation agents and bio-stimulants in a sustainable way to improve the growth, disease-resistant capability of plants and fitness vitality is the right way forward. A lot of studies on beneficial roles of microorganisms that are intimately associated with plants in the rhizosphere, phylloplane and rhizoplane as rhizobacteria that promote plant growth (PGPR) or as microorganisms that promote plant growth (PGPM) are well-documented (Aremu *et al.*, 2017; Igiehon *et al.*, 2019; Olanrewaju *et al.*, 2019; Uzoh *et al.*, 2019).

Reports, highlighting the importance/beneficial properties of microbial-based formulations in enhancing crop development and productivity have been in the news as the basis of the revitalized "Greener Revolution in agricultural production" (Baez-Rogelio *et al.*, 2017; Singh *et al.*, 2016). Other alternatives are urgently needed to achieve environmental balance and sustainability. The focus, therefore, must be to bring to the forefront, simple biological alternatives to take care of these highlighted abiotic and biotic stresses. To effectively combat these highlighted challenges, there is a need for novel, trait-specific microbial strains for crop improvement. The application of a single strain does not always lead to positive results. More so, when consortia or co-inoculation of beneficial microorganisms are used as inoculants on plants, they do confer better results. Moreover, moving ahead in this direction is imperative because plant infestation by pests and disease-causing pathogens and its susceptibility to different environmental stress condition is often due to the reduction in beneficial microbial diversity and imbalance in microbiome of the plant-host relationship. The plant microbiome is a fundamental partner in protecting the plant from stresses by synthesizing enzymes or metabolites that can negatively affect plant pathogens, it produces important phytohormones and ensures /enable plant tolerance to environmental stress (Le Cocq *et al.*, 2017).

However, lately, the search is now on the other important partner of the plant microbiome that colonizes internal tissues of their host plant without causing any disease symptom endophytes. Microbial endophytes are microorganisms that live in the tissues of a plant without causing any adverse effects in their host plant. These endophytic bacterial and fungi do interact with their plant host and elicit positive response to plant pathogens, herbivore pests, and even respond positively to abiotic environmental stresses; also, they synthesize important bioactive metabolites. They are ascribed with many beneficial traits/functions in their plant host that include plant growth promotion, plant pathogens suppressive ability, improving plant stress tolerance, as well as improving plant immunity. Also, reports that indicated *in vitro* and in planta growth-promoting and disease suppressive potentials of bacterial and fungi endophytes are confirmed (Mehmood *et al.*, 2019b; Soldan *et al.*, 2019).

There is no plant without intimately linked endophytes colonizing its tissues (Zabalgogeazcoa, 2008). Despite this vital link/symbiotic role played by endophytes in their host plant/environment/pathogen interactions, there is still a paucity of review data. More importantly, their prospects as alternative to synthetic agrochemicals in ensuring sustainable agroecological crop productivity is not well review in the literature, unlike plant growth-promoting rhizospheric microorganisms.

The focus of this review is to highlight selected research published in the web of science that delves on endophytic bacterial and fungal as potential plant growth-promoting, as well as phytopathogens inhibitory bio-inoculants. The mechanisms of this important potential bio-inoculants used in achieving this will be elucidated. Also, the present state of knowledge/potentials of endophytic bacteria and fungi, as well as the prospects of utilizing this important partner of the plant microbiome as biological inoculants in crop management in a sustainable way using smart biotechnological approaches will be highlighted. Also, the need to bioprospect for novel endophytes in different ecological niches using high throughput molecular techniques, characterizing their functions and screening and optimizing their environmental stress fitness for effective competition/survival in the field will be review. Therefore, this review seeks to highlight the important roles and potential of using endophytic microbial resources to achieve agro-food system crop enhancement in a sustainable and eco-friendly manner.

2.2. Endophytes

The inter-relationship of all living things on earth is much more apparent in the plant world. The plant is in close relationship with micro-organisms in their various ecological niche. The relationship that exists between the plant host and a diverse group of microorganisms is often symbiotic. We have epiphytic, rhizospheric and endophytic microorganisms that inhabit different environmental niche of their plant host. Among these different groups of microorganisms that are in symbiotic association with their plant host, the endophytes are the closest and most intimately linked with their plant host. Endophytes can simply be defined as microbial forms that aids in colonizing plant tissues without causing any adverse impacts (Bamisile *et al.*, 2018; Kandel *et al.*, 2017). All living plants are colonized by different endophytes without an exception, and indeed plants are host to a diverse group of endophytic microbiota in a mutualistic beneficial way, that are vital to plant growth and health (Verma & White, 2018).

Endophytic microbiota can enter and colonize plants through vertical seeds method or by horizontal transmission from soil to the plants. In either way, the mutualistic benefits are still conferred in the plant-microbe interactions (Huang *et al.*, 2016; Verma & White, 2018). This highly beneficial mutualistic interaction makes it possible for the endophytic microbiota to confer the following benefits on their plant host increasing the nutrients made available to the plant host, acting as a protective defender against pathogens and destructive pests, improving capability of the plant to withstand/tolerate environmental/abiotic stresses, help in modulating development in their plant host and also help in tackling the issues that relate to the growth of unwanted weeds (Irizarry & White, 2018; Prieto *et al.*, 2017; Verma *et al.*, 2018; Verma *et al.*, 2017). The beneficial traits are achieved using different mechanisms by the endophytic microbiota to confer these important functional roles on their host plant.

2.3. Bacterial endophytes

For plants to effectively thrive in their ecological niche, they do form mutualistic interrelationships that are of benefits with different living things in the ecological systems. One such positive association is the microorganisms-plant interactions (Santoyo *et al.*, 2016a). Some tissue colonizing bacteria form closely linked association with their host plant and confer a benefit to the plants under both ideal and challenging situations.

These endophytic bacteria confer advantages to their host plants, which include helping in abating growth limiting biotic and abiotic parameters (Miliute *et al.*, 2015). Endophytic bacteria confers stress tolerance capabilities on their host plant, they also induce allelopathic effects in their host plant in addition to improving the growth of their host plant (Mishra *et al.*, 2013). The highlighted functional traits of endophytic bacteria in Table 2.1 have been linked or associated with the capability of their host plant to thrive and survive better in their different ecological niche.

Table 2.1: Plant productivity improvement using bacterial endophytes.

Bacteria endophyte	Host plant	Bioactive influence	References
Bradyrhizobium sp SUTNa-2	*Oryza sativa*	Plant growth-promoting	Greetatorn *et al.* (2019)
Pantoea dispersa IAC-BECa-132; *Pseudomonas sp*; *Enterobacter sp*	*Saccharum officinarum*	Plant growth-promoting	De Silva *et al.* (2019)
Enterobacter cloacae RCA25; *Herbaspirillum huttiense* RCA24	*Oryza sativa*	Plant growth-promoting	Andreozzi *et al.* (2019)
Pseudomonas granadensis T6; *Rhizobium larrymoorei* E2	*Oryza sativa*	Plant growth-promoting and Pesticide tolerance	Shen *et al.* (2019)
Bacillus amyloliquefaciens EPP90; *Bacillus subtilis*; *Bacillus pumilus*	*Pennicetum glaucum*	PGP and abiotic stress tolerance	Kushwaha *et al.* (2019)
Gordonea terrae	*Avicena marina*	Plant growth-promoting	Soldan *et al.* (2019)
Pantoea, *Pseudomonas*, *Enterobacter*	*Eleusine coracana*	Plant growth-promoting	Misganaw *et al.* (2019)
Bacillus subtilis LE24, *Bacillus amyloliquefaciens* LE109, *Bacillus tequilensis* PO80	*Citrus spp*	Biocontrol of pathogens	Daungfu *et al.* (2019)

Curtobacterium sp SAK 1	*Glycine max*	PGP and salinity stress tolerance	Khan *et al.* (2019)
Bacillus tequilensis (PBE1)	*Solanum lycopersicum*	PGP and biocontrol of pathogens	Bhattacharya *et al.* (2019)

Bacteria endophytes have been isolated and characterized from diverse plants host, environment and different plant parts; above and below plant part that includes root tissues, stems, leaves, seeds, fruits, tubers, ovules, and nodules (Nair & Padmavathy, 2014). However, the frequency of occurrence of bacterial endophytes is more in the root tissues, when compared with their frequency in aerial plants tissues (Rosenblueth & Martínez-Romero, 2006). There are a lot of studies that have reported growth-promoting potency of bacteria endophytes on different crops that include rice, wheat, potato, canola, tomato and other crops (Ma *et al.*, 2011; Mei & Flinn, 2010). In their various investigative studies, they all alluded to the huge agro-biotechnological potential of using endophytic bacteria as bioinoculants to achieve a sustainable, eco-friendly and enduring agricultural production system.

2.4. Fungal endophytes

Fungal endophytes are in close association with their plant host in a mutually beneficial way, providing ecological support to their host plant in terms of conferring ability to survive adverse biotic and abiotic stresses, while the endophytes derived nutrients and protection from the plant too (Suryanarayanan, 2017).

Fungal endophytes colonize their plant host tissues such as stems, fruits, flowers, roots, leaves and branches, and this is done asymptomatically without any adverse effects (Saikkonen *et al.*, 2006). They form a key part of the huge biodiversity of the fungi kingdom. Fungi endophytes are known to have conferred beneficial effects on their plant hosts. These beneficial functional traits they induce on their plant host include the containment or reduction of damage caused by pests or destructive insects (Resquín-Romero *et al.*, 2016; Sánchez-Rodríguez *et al.*, 2018). More so, reports indicated fungal endophyte colonized plants as being less susceptible to the destructive effects of pests. They confer these benefits to their host plant by interrupting the pest growth development, affecting also the feeding pattern and reproductive stages of the pest, thereby affecting their overall survival (Vega, 2018). The containment of pest damages on different crops attributed to fungal endophytes has been highlighted in maize (Cherry *et al.*, 2004). The amelioration of pests' damage was also reported in a tomato plant, cotton, coffee plant, banana, faba bean, common bean (Klieber & Reineke, 2016; Qayyum *et al.*, 2015). Also, other investigators reported suppression/containment of pest damage in plants due to the effective action of fungal endophytes (Jaber & Araj, 2018; Rondot & Reineke, 2018). These reductions in damage or containment of pest by the fungal endophytes is attributed to their production of secondary mycotoxigenic metabolites in their host plants that are toxic to the pests (Gurulingappa *et al.*, 2010).

Another important mutualistic benefits of endophytic fungal inter-relationship with their plant host is their ability to confer both abiotic and biotic stress tolerant traits on their plant host, thereby helping their host to improve growth and to be less susceptible to diseases (Busby *et al.*, 2016; Khan *et al.*, 2016). Fungal endophytes have also proven that they are important sources of metabolically active compounds (Jia *et al.*, 2016; Lahrmann *et al.*, 2013). They have been attributed with the ability to produce vital plant hormonal compounds like piperine, gibberellic acid, indole-3- acetic acid that are required for growth promotion in a plant (Chitra et al2017; Jia *et al.*, 2016). They are capable of inhibiting plant diseases causing pathogens, and enable plants to tolerate salinity stress among others (Leitão & Enguita, 2016; Yao *et al.*, 2017).

It can be asserted from studies in the last few years that endophytic fungi play key functional roles in the ecosystem. Their main effect or beneficial interactions with plants include their ability to make vital growth-promoting nutrients available to plant, they help suppress noxious plant pests, pathogens, nematodes, and other destructive insects, they help in mitigating environmental stress, and they are also useful in bioremediation of environmental contaminants. This much they achieved by using different mechanisms (Ali *et al.*, 2018; Kumar *et al.*, 2019b).

They have also been reported as potential sources of bioactive inoculant that could be useful in achieving agricultural sustainability (Yadav, 2019). Chhipa and Deshmukh (2019), reported that endophytic fungi positively influences the exudation in the root of plants, thereby attracting beneficial rhizosphere microbiota that facilitates the transportation of minerals needed by plants from the soil. They are also beneficial partners in plant immune-fitness or ecological and physiological adaptations against pathogens and environmental stress adaptations (Ali *et al.*, 2018; Jain & Pundir, 2017).

With all the highlighted agro-biotechnological beneficial potentials of endophytic bacteria and fungi, it is not surprising, therefore, that the recent interest in finding safe, environmentally friendly and sustainable means of improving agricultural out-put is focused on endophytic microbial formulation used as an attractive alternative. Fungal endophytes have proven to be a potential option for use in attaining sustainable crop intensification due to their ability in producing vital compounds that promote the growth of plants, inhibit plant damaging pest and pathogens, as well as their ability to confer immunity fitness and abiotic stress tolerant traits in the plant. They can, therefore, be biotechnologically manipulated to improve agricultural productivity yield sustainably (Table 2.2).

Table 2.2: Plant productivity enhancement using fungal endophytes.

Fungal endophyte	Host plant	Bioactive influence	References
Penicillium aurantiogriseum 581PDA3; *Alternaria alternate* 581PDA5; *Trichoderma harzianum* 582PDA7	*Triticum aestivum*	Plant growth-promoting and abiotic stress tolerance	Ripa *et al.* (2019)
Mucor sp	*Arabidopsis arenosa*	Metal toxicity tolerance	Domka *et al.* (2019)
Fusarium sp.	*Dendrobium moniliforme*	Plant growth-promoting	Shah *et al.* (2019a)
Piriformospora indica	*Cymbidium aloifolium*	Plant growth-promoting and abiotic stress tolerance	Shah *et al.* (2019b)
Porostereum spadiceum AGH786	*Glycine max*	Plant growth-promoting and salinity stress tolerance	Hamayun *et al.* (2017)
Aspergillus awamori W11	*Withania somnifera*	Plant growth-promoting	Mehmood *et al.* (2019b)
Aspergillus fumigatus TS1; *Fusarium proliferatum* BRL1	*Oxalis corniculata*	Plant growth-promoting	Bilal *et al.* (2018)
Yarrowia lipolytica	*Euphorbia milii* L.	Plant growth-promoting and salinity stress tolerance	Jan *et al.* (2019)

Aspergillus oryzae	*Raphanus sativus*	Plant growth-promoting and biocontrol trait	Sun *et al.* (2018)
Paecilomyces variotii, *Penicillium purpurogenum*	*Caralluma acutangula*	Plant growth-promoting	Ali *et al.* (2019)

2.5. The entry of endophytes into the plant for colonization and the mechanisms that are involved in plant tissues colonization

In recent times, attention has been focused on endophytic microbiota that inhabits inner tissues of the plant without any adverse effects on their host plant. A lot of report on the potential of using endophytic microbiota as bio-inoculating agents to control plant pathogens, improve their immunological fitness, improve their growth parameters and also induce functional traits that enable plant tolerance to abiotic stresses to abound (Ripa *et al.*, 2019; Yadav, 2019). The question that then readily begs for answers will be how do endophytic microbiota enters their plant's host and colonizes their tissues?

There are many complexities involved in the entry and eventual colonization of microorganisms in their plant host. Usually, the colonization process begins with an initial preliminary entry communication/signaling of associated microorganisms and the plant root exudates (Rosenblueth & Martínez-Romero, 2006). The rhizospheric zones of plant and the root exudates produced vital organic compounds that act as chemo-attractants, hence it helps in facilitating signals or communication between microbes and plant roots, then recruiting microbes and this begins endophyte entry for colonization of tissues of plant host (Lopez-Raez *et al.*, 2017; Rozpądek *et al.*, 2018).

2.5.1. Post preliminary entry (adhesion, attachment) of endophytes for plant tissues colonization

As earlier stated, signaling/communication takes place between endophytic microbiota and plant rhizospheric root exudates as the preliminary entry/first step in endophytes colonization of plant tissues. This step is then followed by the adhesion of the endophytic organism to the plant host surface as the next step in colonization. The next stage is endophytes migration to the plant surfaces as a response to chemo-tactic exchanges between the endophyte and plant root exudates; this is finally followed by attachment (Begonia & Kremer, 1994). This attachment is made possible with the aid of structural secretory biomolecules/organelles that include flagella, pili, fimbriae, lipopolysaccharide and exopolysaccharide among others (Berne *et al.*, 2015; Zheng *et al.*, 2015). There are reported initial attachment/colonization of plant by endophyte facilitated by exopolysaccharide metabolites (Janczarek *et al.*, 2015; Marczak *et al.*, 2017). Also reported was initial attachment/colonization that was facilitated or aided by lipopolysaccharide (Balsanelli *et al.*, 2013).

The next stage in the complex steps that are involved in colonization of plant tissues by endophytes entails looking at ways of entering the host tissues after establishing their presence on the plant epiphytic surfaces. They mainly use passive/active penetration processes to enter host tissues where cracks open in the root zones/other aerial parts of plants; stems, flowers, cotyledons, leaves (Hardoim *et al.*, 2008). Penetration proliferation and attachment are reported as being mediated by deploying bioactive secretory components like quorum sensing, exopolysaccharide, lipopolysaccharide, production of a lytic enzyme that includes cellulases, cell wall degrading enzymes, lysozymes among others (Naveed *et al.*, 2014). There must be compatibility between the plant host and microbial endophyte for colonization of plant tissues to be successful. Also, there must be effective communication/signaling of the endophytes with the plant bioactive metabolites (Brader *et al.*, 2014; Compant *et al.*, 2010). Although colonization success depends on diverse factors like host plant genotype, biotic/abiotic factors, the extent of nutrient limitations among others, endophytic microbial strains that are efficiently adapted to these factors succeed better (Hardoim *et al.*, 2015).

2.5.2. Transmission of endophytes (Vertical or horizontal) for plant tissue colonization

Endophytic bacteria and fungi that are associated with the colonization of plant tissues are transmitted horizontally (plant or soil to plant) vertically (parent plant to seed) or in a mixed way (Bright & Bulgheresi, 2010). Most fungi endophytes are seed/vertically transmitted (Foster & Wenseleers, 2006). Endophytic bacteria, on the other hand, prefers horizontal transmission (Khan *et al.*, 2012). Bacteria endophytes are optimally adapted to the horizontal transmission route (Edwards *et al.*, 2015; Verma *et al.*, 2017). Although there are reports of vertically transmitted seed-borne bacterial endophytes of biotechnological importance (Verma & White, 2019).

Different seed-borne endophytic microbial isolates reports were highlighted by different investigators (Glassner *et al.*, 2018; Mitter *et al.*, 2017; Pitzschke, 2016). The best-supporting proof/evidence of endophytic microbiota colonization of plant host through seed-borne/vertical transmission are research reports that indicated exchange/overlap in the taxonomy of endophytes in seed and their accompanying seedling (Gagne-Bourgue *et al.*, 2013; Verma & White, 2019).

2.6. Potentials of using endophytic bacteria and fungi to improve agricultural productivity

In an era of multi-fold constraints that are adversely affecting improvement in agri-food system productivity, there is an urgent need to look beyond these challenges in a modernized agriculturally sustainable manner, to maintain an environmental balance and achieve enduring crops intensified productivity. The potential use of endophytic bacteria and fungi to improve agricultural productivity is a sustainable alternative that is gaining attention in recent times. Endophytic bacteria and fungi have shown great potentials in promoting different plant growth (Fig. 1), in the biological control of phytopathogens, destructive pests, and insects, in inducing tolerant traits to abiotic stresses, as well as inducing greater immune-fitness in different plants (Arora & Ramawat, 2017; Chhipa & Deshmukh, 2019; Khare *et al.*, 2018).

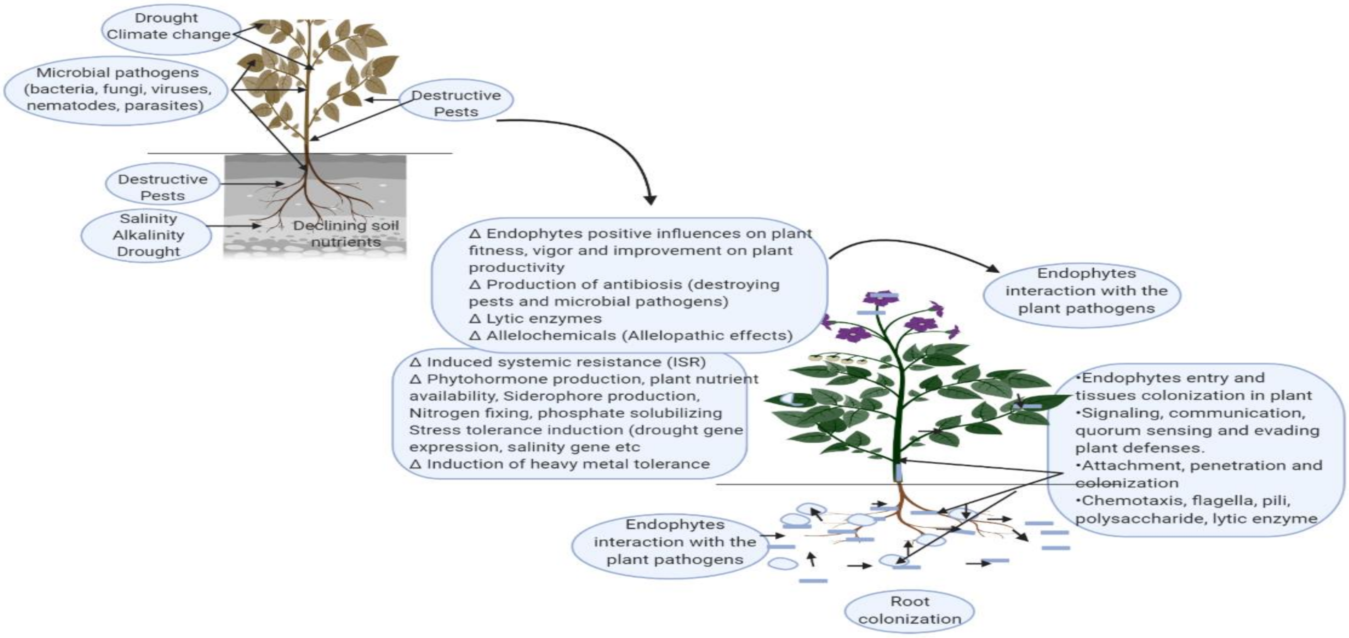


Figure 2.1. Diagrammatic representation of endophytic bacteria and fungi functional traits that are of benefits to plants.

The ability of endophytic microorganisms to enter, establish and firmly colonized plant tissues, makes them show multi-faceted functional traits that positively influence plant productivity. The question that automatically will be raised is how endophytes do achieve these highlighted beneficial functional traits in plants. Endophytes are a group of ubiquitous and diverse microbiota that are found in different tissues of a plant in diverse ecological niches. They inhabit all plant asymptomatically and serves as a goldmine of biologically important metabolites that could be used for potential plant growth-promoting, biocontrol potency against pathogens and pests, induction of plant immune defense/fitness, as well as conferring functional traits in plant to be able to withstand/tolerate external stresses (Doilom *et al.*, 2017; Potshangbam *et al.*, 2017).

A unique ability/resourcefulness of endophytes is that they are multi-functional, being able to simultaneously act as a biocontrol agent, as well as agents of improving yield/growth of plants (Larran *et al.*, 2016; Potshangbam *et al.*, 2017; Rojas-Solís *et al.*, 2018). Endophytic microbiota are key players in inter-relationship with plant hosts in the ecological environment, that confers functional traits benefits in the plant in tolerating abiotic stresses, combating/suppressing disease-causing pathogens, as well as promoting growth and nutrient acquisitions in a plant (Rodriguez & Redman, 2008). In their research, Redman *et al.* (2011) highlighted that endophytes play consequential roles in a plant, as well as confers the ability to grow on marginal fields to plants.

Research have highlighted/implicated endophytes in having functional traits. This confers the ability to increase nutrients available to plants, suppress plant pathogens; pests, insects and nematodes, abilities to produce phytohormones in a plant, abilities to tolerate abiotic stresses in plants, abilities to fix, solubilize and mobilize essential elements for the plant to utilize (Khare *et al.*, 2018; Kumar *et al.*, 2019b). Endophytic microorganisms facilitate the improvement in plant productivity by using different mechanisms. For endophytic microorganisms mediated plant growth promotion, the different mechanisms they use include; breaking down of inorganic nutrient substances from the soil into host roots, the production of enzymes and other essential bioactive metabolites (Khan *et al.*, 2016). Besides, endophytes also improve productivity by protecting their plant host from pathogens by regulating important phytohormones production and thereby influencing the physiological response of the host plant.

Another key role played by endophytes in improving agricultural productivity is their beneficial interactions with the plant on abiotic stress tolerance. Also, with the associated adverse effects of climate change and vulnerability on plant growth, research reports from the literature support the fact that endophytes can help the plant to mitigate environmentally induce stresses that include drought, higher temperatures, excess salinity, frequent flooding among others (Rho *et al.*, 2018). Endophytes use different mechanisms to confer abiotic stress-tolerant capability on their plant host, these they accomplish through inducing and expressing genes that are responsive to stress, they synthesize metabolites that act against stress, and also produce scavengers like reactive oxygen species to take care of free radicals (Lata *et al.*, 2018).

Reports in literature supporting endophyte utility in abating abiotic stresses using an array of mechanisms are highlighted in different studies (de Zélicourt *et al.*, 2018; Ilangumaran & Smith, 2017; Ma *et al.*, 2016; Pandey *et al.*, 2016). Another important functional role of endophytes in improving agricultural productivity is their ability to resist biotic stresses by suppressing phytopathogens via antagonistic actions (Gunatilaka, 2006). Endophytes have also been reported to inhibit phytopathogens through the expression of genetically linked physiological and defensive pathways in their plant host against disease-causing pathogens and pests' (Khare *et al.*, 2016; Salam *et al.*, 2017).

To achieve effective inhibition of phytopathogens and pests, endophytes produces and secretes bioactive metabolites like salicylic acid, jasmonic acid, phytoalexins, siderophores, volatile organic carbon. All these bioactive metabolites are known for their significant inhibitory action against phytopathogens and pests of the plant (Etminani & Harighi, 2018).

2.7. Conclusion and prospects of endophytes in sustainable agricultural intensification

Bacteria and fungi endophytes have shown that they have great potentials as an eco-friendly, natural resource that needs to be applied in agri-food system applications to achieve sustainable agricultural intensification, thereby achieving both environmental sustainability and improving agricultural productivity using cheaper, readily available natural bioresources sustainably. The present knowledge of research on endophytic organisms in terms of their bio-effector potentials as bio-fertilizers, biocontrol agents/bio-pesticidal and bio-stimulant activity is good enough for their applications in the field of agricultural crop production.

Therefore, endophytic bioformulated inoculants (encompassing synergetic consortia of both bacteria and fungi endophytes) should be developed for use as an agricultural production input, to minimize the use of conventional agrochemicals, thereby ensuring the sustainability of the ecological balance. The time to act is now to begin the salvation of the ecosystem by engaging in sustainable agricultural intensification. Endophytic microorganisms-based inoculant bioformulation have a great role to play in the future next green revolution to salvage the ecosystem. As more enlightenment on genetics, metabolism and physiological processes in endophytic microorganisms and plant symbiotic inter-relationships are made manifest through advanced biotechnological screening/investigative processes, better exploitation/utilization in the field application as bio-inoculants for sustainable agricultural intensification will be achievable in no distance future. However, there are still gaps to be filled. Important gaps that need to be filled in the future are.

1. The use of advanced biotechnological omics tools to investigate both the community and functionalities of endophytic microorganisms. A further exploratory investigation of the entire plant tissue endomicrobiome can lead to the unraveling of novel endophytic microorganisms with significant functional traits that could be exploited further in the quest for enhancing crop improvement in a sustainable way. This must entail using next-generation molecular techniques to obtain an optimum result. There is a need to use newer biotechnological tools to study endomicrobiome in terms of genomics, proteomics, and transcriptomic functional traits.
2. There is the need to do an extensive bioprospecting study of endophytic microorganisms from diverse ecological niches, for example, extreme environments, marine environment, etc., to isolate and characterize novel endophytes with specific traits that will be beneficial for agricultural input
3. There is also a need for effective screening for essential/important metabolites that could be deployed in the field directly to circumvent known field environmental/competitive challenges.

CHAPTER THREE

3.0. CONSTRAINTS AND PROSPECTS OF IMPROVING COWPEA PRODUCTIVITY TO ENSURE FOOD, NUTRITIONAL SECURITY AND ENVIRONMENTAL SUSTAINABILITY. PUBLISHED IN FRONTIERS IN PLANT SCIENCE (2021).

Abstract

Providing safe and secure food for an increasing number of people globally is challenging. Coping with such a human population by merely applying the conventional agricultural production system has not proved to be agro-ecologically friendly; nor is it sustainable. Cowpea (*Vigna unguiculata* (L) Walp) is a multi-purpose legume. It consists of high-quality protein for human consumption and it is rich in protein for livestock fodder. It enriches the soil in that it recycles nutrients through the fixation of nitrogen in association with nodulating bacteria. However, the productivity of this multi-functional, indigenous legume that is of great value to African smallholder farmers and the rural populace, and also to urban consumers and entrepreneurs, is limited. Because cowpea is of strategic importance in Africa, there is a need to improve on its productivity. Such endeavors in Africa are wrought with challenges that include drought, salinity, the excessive demand amongst farmers for synthetic chemicals, the repercussions of climate change, declining soil nutrients, microbial infestations, pest issues, and so forth. Nevertheless, giant strides have already been made and there have already been improvements in adopting sustainable and smart biotechnological approaches that are favorably influencing the production costs of cowpea and its availability. As such, the prospects for a leap in cowpea productivity in Africa and in the enhancement of its genetic gain are good. Potential and viable means for overcoming some of the above-mentioned production constraints would be to focus on the key cowpea producer nations in Africa and to encourage them to embrace biotechnological techniques in an integrated approach to enhance for sustainable productivity. This review highlights the spectrum of constraints that limit the cowpea yield, but looks ahead of the constraints and seeks a way forward to improve cowpea productivity in Africa. More importantly, this review investigates applications and insights concerning mechanisms of action for implementing eco-friendly biotechnological techniques, such as the deployment of bio inoculants, applying climate-smart agricultural practices, agricultural conservation techniques, and multi-omics smart technology in the spheres of genomics, transcriptomics, proteomics, and metabolomics, for improving cowpea yields and productivity to achieve sustainable agro-ecosystems, and ensuring their stability.

Keywords: cowpea productivity enhancement, indigenous legume, *Vigna unguiculata*, nutritious human food, the largest producer status, smart biotechnological approaches, protein-rich fodder-for livestock, climate-smart agricultural practices.

3.1 Introduction

With the world population expected to increase by up to 70 % by 2050, the global community is faced with the constraint of providing safe and secure food supplies to an increasing number of people. The human population is projected to reach the 9.8 billion mark by 2030, bringing immense challenges in feeding the global populace (Pais *et al.*, 2020; Tian *et al.*, 2016). This will be a huge task, especially for the African continent, to handle in an era of climatic change and a growing population that will double by the year 2050 (Adedeji *et al.*, 2020). Not only is the task of coping with this high human population growth rate in terms of the conventional agricultural production system daunting; it is also not environmentally/ecologically sustainable (Roell & Zurbriggen, 2020). In addition to the burgeoning human population, other factors that are posing threats to improvement in agricultural productivity include amongst others, climatic change, the loss of fertile agricultural land to urbanization, the challenges of phytopathogens and pests, abiotic challenges; high levels of salinity, and drought. Therefore, there is an urgent need to devise novel and workable solutions to achieve sustainable means of enhancing productivity in terms of agro-products and their nutritional composition.

Cowpea (*Vigna unguiculata* (L.) Walp.) is an annual leguminous crop that is grown throughout the world, but it is grown mainly in semi-arid regions. Cowpea is a diploid, having 2n=2x=22, with the size of its genome consisting of approximately 620 million base pairs (Lonardi *et al.*, 2019). In terms of its importance, this indigenous African legume is economically, nutritionally, and environmentally the foremost crop that serves as a source of essential human dietary nutrients and as, a means of providing fodder for livestock. It also presents with other multi-functional traits, including the maintenance of the soil-ecological balance through nitrogen fixation in that it facilitates a symbiosis with nodulating bacteria (Ravelombola *et al.*, 2017). Cowpea is of strategic importance to Africa in terms of the large quantities that can be produced and is, therefore, an important component in the economy (Walker *et al.*, 2016). Having originated in Africa, cowpea is now grown worldwide in 100 countries (Gonçalves *et al.*, 2016; Singh, 2014). The cowpea yield in 2020 was estimated to be in the region of 9.8 million, while by 2030, the projected yield is expected to rise to 12.3millon tons (Boukar *et al.*, 2016). Cowpea is indeed a multi-faceted crop, providing revenue for millions of smallholder farmers, as well as for traders who sell the nutritious grain. By providing essential protein, minerals, and vitamins, it serves in most African countries, as a means of balancing the diet, thereby providing a cheaper means for accessing the necessary dietary nutrients and for positively influencing the well-being and health of the populace. In addition, all of its components are valuable as nutrients (Gonçalves *et al.*, 2016);- the leaves, pods, and seeds are nutritionally high in protein, with less fat, and are used extensively as the vegetable component in diets. In both the urban and rural settlements in most African countries, women generate income by trading in processed cowpea food and snacks. Cowpea is also important in livestock production, where the plant's leaves and vines are dried and used as fodder/feed supplements in livestock husbandry. Cowpea is a key resource for a large number of people in the developing world, mainly in the arid/semi-arid tropical regions of the world (Muñoz-Amatriaín *et al.*, 2017). Cowpea dry grain contains 23 to 32% protein and essential amino acids (Carvalho *et al.*, 2017). Also, the green cowpea seeds, fresh and immature pods, and leaves contribute vegetable sources for human consumption (Gerrano *et al.*, 2019; Gerrano *et al.*, 2017). Its fresh leaves are used as vegetables, the haulms (cowpea pod walls, stems, and leaves) are used as livestock fodder, providing dietary nutrients for animals, and as income for the farmers (Kebede & Bekeko, 2020). Cowpea is highly prized as a source of food, for fodder in livestock feeds, and an important but cheaper means of improving and boosting soil fertility through biological nitrogen fixation. As important as it is in human nutrition, cowpea is equally useful in providing the necessary energy and protein in livestock production. More so, owing to its adaptation to different climatic conditions and its ability to grow in a less-fertile soil environment, it is highly appreciated as forage and a potential fodder crop for the future (Alemu *et al.*, 2016a). It is a key leguminous crop in the arid and tropical regions of Africa, Asia, and Latin America (Xiong *et al.*, 2016). Cowpea is relished as a source of nutritious food and a variety of snacks that provide humans with cheaper proteins, thereby enhancing food security (Agbogidi & Egho, 2012; Muranaka *et al.*, 2016). Cowpea is a vital source of beneficial micronutrients, proteins, amino acids, antioxidants, vitamins, and minerals, with immense therapeutic and nutritional security benefits (Gondwe *et al.*, 2019; Irondi *et al.*, 2019; Jayathilake *et al.*, 2018; Olabanji *et al.*, 2018; Owade *et al.*, 2020). It is often used in mixed cropping systems to offer the multi-functional benefits of a nutritious grain, as a fodder crop, and as a means to improve soil fertility (Agza *et al.*, 2012; Belay *et al.*, 2017). Importantly, it is useful in agro-ecological conservation. It is used mainly as an inter-crop with other food crops to boost soil fertility and add nutrients to degraded soil through its nitrogen-fixing property (REGO *et al.*, 2015). It is postulated that cowpea can fix about 337 kg N. ha-1 (Yahaya, 2019). The average nitrogen addition/contribution to the soil during the cowpea growth and development phase is in the range of 40 to 80 kg N. ha-1 and sometimes up to 200 kg N. ha-1 (Meena *et al.*, 2015). Also, it is useful as a cover crop or an erosion-preventing crop; it helps in suppressing weeds; and also aids in the retention of moisture (Das *et al.*, 2018). Another key advantage of cowpea production is that when used as an inter-crop with other crops, it induces the growth of beneficial soil microorganisms and reduces the use of synthetic agrochemicals (Bukovsky-Reyes *et al.,* 2019; Sun *et al.*, 2019). In terms of importance, cowpea production contributes significantly to economic productivity and environmental sustainability in Africa (Cardona-Ayala et al., 2020; (Martins *et al.*, 2003; Olajide & Ilori, 2017; Ovalesha *et al.*, 2017).

The main cowpea-producing countries of the world are in sub-Saharan Africa, that is the Sudano-Sahelian vegetation region (Boukar *et al.*, 2019). Nigeria has the highest production output, followed by Niger and Burkina Faso - in that order. In terms of the metric - ton production levels of cowpea grain, Nigeria is the largest producer in the world (FAO, 2020).

The productivity of cowpea in different countries differs in terms of the production output per area cultivated as highlighted in Table 3.1. However, despite all of the mentioned benefits of cowpea production in Africa in terms of the economies of scale, agri-food / nutritional benefits, and environmental stability influences, its productivity output is limited, and its status as an under-utilized leguminous crop persists. The challenges militating against improved cowpea productivity in Africa include the following: Climatic change and its adverse consequences on crop productivity include the issue of infrequent and erratic rainfall arising from, amongst others, drought and aridity issues, the decline in soil nutrients, the excessive use of synthetic chemicals, low-yielding seed cultivars, and infestations of pests and microbial pathogens (Afutu *et al.*, 2017; Rascovan *et al.*, 2016). Diverse strategies have been deployed by researchers in an attempt to breed cowpea for productivity enhancement. These strategies span through the initial selected germplasm collection from cowpea wild relatives and its' natural population for desired genetic traits in order to create an improve cowpea genotypic varieties with agronomic traits and morphology through conventional hybridization and progeny crossbreeding techniques. These earlier breeding research techniques contributed to the development of many improved cowpea accession lines in the germplasm. However, significant barriers of improving cowpea varieties through the conventional breeding techniques like the challenges of intraspecific and interspecific crossing, genetic variation, genotype-by environment interaction, among others still persist. The advent of molecular tools such as RAPD, AFLP, ISSR, and other assisted marker selection genotypic breeding, was a milestone that led to genetic gains in cowpea productivity improvement. The advantages that are associated with this DNA molecular tools include: they are highly reproducible, cost effective and also it can deploy in the analysis of a large number of samples having genetic differences. Moving ahead, the advancement in molecular biology techniques that span genomics, proteomics, transcriptomics and metabolomics, means cowpea breeding research could now encompass assessing gene regulation and expression patterns for both abiotic and biotic resilient cultivars. These advance molecular technologies have been deployed to discern genotypic diversity existing in cowpea genome globally. Also, these advanced techniques have help cowpea breeders through genetic engineering to select desired gene traits and transfer across genetic barriers for cowpea improvement.

In summary, diverse technological tools have been deployed by researchers for cowpea breeding enhancement, spanning the past, the present and future prospects that include (markers systems, genetics maps, high-throughput genotyping, and quantitative trait loci (QTL) analysis). In addition, mutation breeding, tissue culture, reverse genetics, clustered regularly interspaced short palindromic repeats (CRISPR) technologies are being apply for genetic gain in cowpea. In spite of this progress, major efforts are still needed for cowpea productivity improvement because cowpea plant is a diploid with a very narrow genetic make-up and also, it reproduces through means of self-pollination. Therefore, to overcome this gap, innovative research efforts that transverse different continents are still required towards breeding cowpea for enhanced productivity. For Africa to leverage its position as the foremost producer of this vital indigenous legume, the continent must look ahead at ways of improving productivity by closing the gaps in yield and by limiting the constraints to cowpea productivity in an agro-ecologically sustainable way. Therefore, this review highlights the constraints of cowpea production in Africa, and also gives an overview into the way these challenges can be circumvented through the deployment of smart biotechnological techniques/applications and insights concerning mechanisms of action for implementing eco-friendly biotechnological techniques, such as the deployment of bio inoculants, applying climate-smart agricultural practices, agricultural conservation techniques, and multi-omics smart technology in the spheres of genomics, transcriptomics, proteomics, and metabolomics, for improving cowpea yields and productivity to achieve sustainable agro-ecosystems, and ensuring their stability.

3.2. Production constraints

The production of cowpea in Africa, the epicentre of this foremost indigenous legume, is carried out mainly by subsistence farmers. The production output of these smallholder farmers is limited by diverse constraints that lead to low agronomic yields/productivity. The average yield of cowpea in Africa is about 600 kg / ha, which is still below its estimated optimum potential yield of 1500 to 2500 kg /ha (Kamara *et al.*, 2018). Numerous constraints limit the improvement of cowpea yield and productivity in Africa. These limiting factors can broadly be termed as abiotic/biotic stresses and climatic variations, and they have had a huge influence on the overall productivity of cowpea grains and fodder vegetables that are produced in the different cowpea-producing nations of the world, and particularly in Africa.

3.2.1 Abiotic stresses

3.2.1.1 Drought

Drought is a major challenge/constraint to achieving worldwide food security and production enhancement. Drought adversely affects plant growth at all developmental stages, impairing the morphology of the plant and the biochemical and physiological processes operating in the planted crops. These aspects subsequently affects, amongst others the uptake of vital nutrients for plant growth and the ability of the seeds to germinate and of the plant to photosynthesize (Fahad *et al.*, 2017; Lamaoui *et al.*, 2018). Drought stress has negative consequences on the vitality and vigor of seeds and impairs seedling growth (Hatzig *et al.*, 2018). The optimum growth/developmental stages in planted crops are adversely affected by drought, as observed in a decline in the rate of germination, seedling emergence and growth; impairments in vegetative growth, cell division and elongation; with mitotic processes also being affected (Farooq *et al.*, 2009).

Drought stress can adversely affect the functioning of vital enzymes. Among other influences, the flowering stage of the plant could be negatively affected, as also the photosynthetic rate and the assimilate partitioning process. All these conditions eventually reduce the planted crop yields (Anjum *et al.*, 2011).

Drought also impairs the proper functioning of the plant cell by producing oxidative damaging reactive species (ROS), which destroy plant lipids and proteins (You & Chan, 2015). Drought leads to adverse influences on the growth, development, and reproduction ability of planted cowpea, which limits the yield and productivity of the planted crops (Daryanto *et al.*, 2017; Ravelombola *et al.*, 2018a; Verbree *et al.*, 2015).

Numerous studies have been done and are also on-going due to the enormity of drought stress challenges on cowpea productivity enhancement. In a study by Cui *et al.* (2020), they evaluated cowpea drought tolerance potentials at seedling stage. The experiment was done using a total of 36 cowpea breeding lines in a completely randomized manner under drought stress conditions. Their results revealed that four (4) Arkansas cowpea breed lines are drought tolerant and they ranked better in terms of chlorophyll, healthiness and lodging score when compared to the other 32 genotypes. Therefore, these 4 cowpea breed lines could be further exploited in cowpea breeding improvement. Also, in a study to highlight the constraining effects of drought stress on above ground traits in cowpea plant (Ravelombola *et al.*, 2018a) assessed drought stress induced changes in 17 above ground traits in 30 cowpea genotypes at the seedling stage of growth for 28 days. Their findings showed that cowpea genotypes PI293568, PI349674, and PI293469 are slow to wilting, better adapted to drought, while the other susceptible genotypes are fast to wilt, the chlorophyll content is lower and they undergo senescence faster too. The three (3) cowpea drought tolerant genotype could be exploited further for advanced breeding.

More so, in a comprehensive study of drought tolerance response in cowpea plant, Carvalho *et al.* (2019) used four (4) cowpea genotypes to determine their physiological, biochemical and molecular response under water limiting stress conditions. The output from this study highlighted the importance of stomata conductance, photosynthetic parameters, compatible solutes like anthocyanin and proline, as well as increase in enzymatic activity of reactive oxygen species scavenging enzymes like catalase, superoxide dismutase, glutathione reductase, guaiacol peroxidase. This study, also characterized the drought gene expression profile of the four cowpea genotypes. Thirteen drought related genes were profile, and some of the genes were expressed higher than others under drought stress. The hallmark of the study was that cowpea genotype Cp5051 was the most drought tolerant due to a higher expression of drought tolerant marker genes *VuHsp17.7* and *VuCPRD14*

3.2.1.2 Salinity

Soil salinity is a major abiotic constraint to plant productivity. Salinity adversely impacts the metabolic and physiological processes in plants. Statistical report stipulated that upward of over 45 million hectares of agricultural soil are affected by this problem and that climatic change, as well as current irrigation practices, will exacerbate this situation (Munns & Gilliham, 2015; Parihar *et al.*, 2015).

In addition, salinity stress negatively influences the rate of plant growth. The adverse influence of salinity reduces the fresh and dry weight of plants, while other vegetative growth traits are also adversely impacted (El-Beltagi *et al.*, 2013; Mohamed *et al.*, 2018).

Salinity stress leads to extensive damage in the adductive capacity of planted crops. It reduces lipid peroxidation and leads to the production of destructive oxidation species (ROS), that in turn cause damage to the key plant biomolecules (Ghonaim *et al.*, 2021).

Salinity stress ultimately reduces yields and the productivity of planted cowpea crops, thereby affecting the goal of achieving enhanced global food security (da Silva Sá *et al.*, 2017; Ravelombola *et al.*, 2018b).

Research into the impact of salinity on cowpea has indicated that it impairs cowpea seed germination, its vigor and growth (Mini *et al.*, 2015; Zahedi *et al.*, 2012).

In order to evaluate salinity stress impact on cowpea cultivars, Ravelombola *et al.* (2019), investigated a simple protocol that could be deployed to assess the response of 30 cowpea genotypes to salinity constraint at seedling growth stage in a greenhouse experiment that profile 14 above ground traits response to sodium chloride (NaCl) induced salinity. The findings indicated that relative salinity tolerance (RST) of cowpea genotype PI255774, all the plants were completely dead, while PI582438 performed best and the leaves were all green and had higher chlorophyll content. The outcome of this study validated simple protocol of assessing chlorophyll content and leaf injury for assessing salinity at seedling stage in cowpea. Also, in a study to investigate further the utility of chlorophyll content as a means of assessing salinity tolerance in cowpea seedling over time (Dong *et al.*, 2019), investigated how 24 different cowpea genotypes responded to salinity induced stress by monitoring the chlorophyll changes over a period of 24 days using a split-plot design. The results indicated the importance of genotype and the timing in relation to cowpea seedling response to salinity stress. Also, the chlorophyll content of the cowpea salt tolerant cultivar was higher at day 24 of the experiment, while all the cowpea salt sensitive plant were dead at the end of the 24 days. In addition, salinity induce stress could further predispose cowpea cultivars to viral infestation. In their study (Varela *et al.*, 2019), assessed the consequences of exposing a cowpea severe mosaic virus (CPSMV) resistant genotype to salinity induced stress. The results signify that vital protein pathways were altered, and there was proliferation of the (CPSMV), leading to the cowpea genotype changing from resistant to susceptible.

3.2.1.3 Heavy Metals

Heavy metals pose serious environmental constraints for and can adversely impact on plants and humans when the former bioaccumulate in plants and ultimately reach human beings via the food chain (Sidhu, 2016).

Heavy metals pose environmental and public health threats when they are discharged as by-products of industrial processes in the form of effluents (Wuana & Okieimen, 2011). Heavy metals such as, amongst others, Cadmium (Cd), Lead (Pb), Arsenic (As), Mercury (Hg), Chromium (Cr), and Antimony (Sb), affect plant productivity and plant yields.

Heavy metals adversely affect the metabolic processes of the plant during the course of its growth and development. Heavy metals negatively influence the germination of seeds, while the vegetative growth rate (leaf, shoot, root) is also impaired. Plants are adversely affected by heavy metals, as in the case of various physiological and biochemical processes such as the rate of photosynthesis, the uptake of nutrients, vital enzymatic reactions, as well as in the case of emergence of destructive oxidation species (ROS) (Azevedo & Rodriguez, 2012; Tiwari & Lata, 2018).

Research reported by (Asagba *et al.*, 2019) detailed the impact of Nickel toxicity on cowpea germination and other biochemical parameters. The investigation on phytotoxicity of Nickel at varying concentration on cowpea seedling growth rate, length, fresh weight, as well as Ca2+ ATPase activity was assessed. The results indicated toxic impact of this heavy metal on cowpea seedling agronomic and biochemical parameters.

Also, in a study by Ogunkunle *et al.* (2020) that applied co-inoculation of arbuscular mycorrhizal fungi (AMF) and nano-TiO2 to reduced oxidative stress and bioaccumulation of Cd in cowpea, it was reported that the total chlorophyll of the cowpea plant, as well as different reactive oxygen species enzymes were impacted negatively due to Cd induced toxicity.

3.2.1.4 Temperature Stress

As an abiotic stress factor, temperature in the case of low temperatures (chill stress) and high temperatures (heating stress), is a potential constraint in limiting the productivity and yield of planted crops globally. Temperature is a key abiotic parameter that influences the growth and development of plants (Hatfield & Prueger, 2015).

High temperatures limit the photosynthetic rate of the plant. The vegetative growth parameters and the metabolic activities of the plant are also adversely affected. Also, emergence, maturity/ripening, harvesting time (length of period/stage), and plant yield are affected (Prasad *et al.*, 2008; Shah *et al.*, 2011). Likewise, low-temperatures (chilling stress) adversely influence plant metabolic activity and negatively impact the growth/development of plants (Tian *et al.*, 2011). Low temperatures (chilling stress) also negatively affect the germination rate, seedling emergence, and the vigour of the plant, so that the productivity of the plant is ultimately reduced (Abbas, 2012). In a study on the impact of elevated temperature on the agronomic growth parameters and the nutritional status of cowpea at different growth phase (Nevhulaudzi *et al.*, 2020b), reported that there were differences in both the agronomic growth and nutritional parameters, and this is more pronounced at the flowering and pre-flowering stage.

3.2.1.5 Waterlogging stress

Waterlogging stress affects the gaseous exchange in agricultural soil and negatively impacts crop productivity globally. It leads to an insufficient supply of oxygen to the plant roots and this in turn reduces the growth and development of the plant roots. It also leads to the inability of the plant to take up the necessary nutrients and nitrogen. Waterlogging affects the photosynthetic rate, reduces the vegetative agronomic growth rate of plants, leads to the senescence of leaves, and ultimately, negatively affects crop yield and productivity (Ren *et al.*, 2014).

Higher or excessive soil water availability do not always favor cowpea growth. In a field study done in the Sudan Savanna zone taking genotype environment interaction into focus (Iseki *et al.*, 2021) reported that excess water can inhibit the nitrogen fixing capability of cowpea and lower its productivity.

3.2.1.6 Climatic change stress

Climatic changes in weather, as denoted by, amongst others variability or fluctuations in the prevailing temperatures, rainfall, and the volume of greenhouse gases are potentially limiting factors on various agro-input variables and ultimately affect the productivity of planted crops on a global scale (Awoye *et al.*, 2017; Hounnou *et al.*, 2019; Kukal & Irmak, 2018).

Climatic changes also adversely threaten the agri-food system at all scales; globally, nationally, regionally, and locally (Ajadi *et al.*, 2011).

Climatic change negatively impacts agri-food input and output production systems because it influences the biotic and abiotic parameters of agricultural production. Hence, it affects planted crop yields (Challinor *et al.*, 2014).

Changes in climatic conditions affect the biochemical, physiological and metabolic activities of plants; the photosynthetic rate is affected, as are factors such as plant growth and development, and the rate of transpiration; there is also an imbalance in the elimination of CO_2, and a reduction in enzyme reactions; flowering may be affected, which could also lead to senescence (Srivastava *et al.*, 2019). Predictive studies have forecast a reduction in food grain yields toward the later years of this current century (Pachauri *et al.*, 2014), this hinging on expected extremes in global temperatures. Furthermore, most, if not all of the major food crops are adversely impacted by stress arising from heat at the different growth and developmental stages (Atlin *et al.*, 2017; Kaushal *et al.*, 2016). Global changes in climatic conditions have been found to adversely affect the health of humans, animal/livestock production, as well as planted crop productivity (Lesk *et al.*, 2016; Mora *et al.*, 2017).

In summary, many huge tasking constraints are militating against and slowing down the optimum yield production of cowpea in Africa. Some of these limiting challenges are highlighted in Table 3. 2.

3.2.2 Biotic stress

Worldwide, biotic stressors (roots and membrane pathogens) in large numbers lead to low productivity and low-quality agricultural products. Destructive pests and pathogens result in food insecurity on every scale – from the smallest to the largest thus leading to massive monetary losses on a global scale in terms of crop yield (Savary *et al.*, 2019).

The main production constraints concerning biotic stress factors limiting cowpea productivity are exemplified by a wide range of organisms, including destructive pests; parasitic weeds, viral pathogens, bacterial pathogens, as well as fungal pathogens (Boukar *et al.*, 2019)

3.2.2.1 Bacterial diseases/ pathogens affecting cowpea seeds, plants and pods

A major constraint in limiting cowpea yields can be attributed to bacterial pathogens, which lead to massive crop losses of upward of 70 % in the form of seed grain, pod, and fodder reduction (Agbicodo *et al.*, 2010). Some of these destructive pathogens are transmitted via the seed (de Lima-Primo *et al.*, 2015), while some are transmitted via the soil-borne route (Constantin *et al.*, 2016). Some of the damaging symptoms of bacterial pathogen infestation in cowpea are brownish leaf spots, necrotizing and yellow halo leaf shapes, cracks noticeable on the stem, and pods filled with water, and blotch (Claudius-Cole *et al.*, 2014). Among the most destructive bacterial pathogens of cowpea are members of the *Xanthomonas* genus (Durojaye *et al.*, 2019; Shi *et al.*, 2016).

3.2.2.2 Root-knot nematodes

Nematodes are responsible for huge losses in cowpea production and are also one of the constraints limiting improvements in cowpea production (Dareus *et al.*, 2021; Haegeman *et al.*, 2012). This they accomplish by impeding the uptake of water and nutrients. Also, nematodes limit cowpea growth and development by interfering in the pathways towards cell differentiation and in the transportation of auxin (Gheysen & Mitchum, 2011). *Meloidogyne javanica* and *Meloidogyne incognita* are the two major nematodes destroying cowpea (Oliveira *et al.*, 2012)

3.2.2.3 Fungal diseases/pathogens associated with cowpea

Fungal pathogens are the topmost destructive agents/phytopathogens of planted crops globally (Fisher *et al.*, 2012). Very many species of different genera of fungi destroy cowpea in the field and during the post-harvest stage. Furthermore, seed and soil-borne fungal pathogens have been implicated in the loss of cowpea production that sometimes rises to 100% (Mbeyagala *et al.*, 2014). Notable fungal pathogens of cowpea include *Rhizoctonia solani*, *Colletotrichum spp*, *Fusarium oxysporum*, *Macrophomina phaseolina*, *Sclerotium rolfsii*(Adegbite & Amusa, 2010; Pottorff *et al.*, 2014).

3.2.2.4 Viral diseases/ pathogens associated with cowpea

Viral pathogens can adversely impact cowpea productivity; some of these have been linked in some cases to cowpea losses of up to 100% (Nsa & Kareem, 2015). Their destructive mechanisms that negatively affect cowpea include the reduction they cause in the population/growth and development of Rhizobium, thereby reducing the necessary root nodulation in cowpea (Taiwo *et al.*, 2014). Up to 40 viruses adversely affect cowpea yields globally. Some of the most devastating viral pathogens of cowpea are the cowpea aphid-borne mosaic virus (CABMV), cowpea wild mottle virus (CPMMV), and, cowpea severe mosaic virus (CPSMV) (Boukar *et al.*, 2013; Odedara & Kumar, 2017).

3.2.2.5 Parasitic weeds

Parasitic weeds cause serious losses in cowpea production/yields (Horn *et al.*, 2015; Li & Timko, 2009; Omoigui *et al.*, 2017). Eliminating these weeds in the course of cowpea production is difficult because they could be dormant in the soil for upward of 20 years (Kamara *et al.*, 2014). The major parasitic weeds that adversely affect the enhancement of cowpea production in Africa are *Striga gesnerioides* and *Alectra vogelii*.

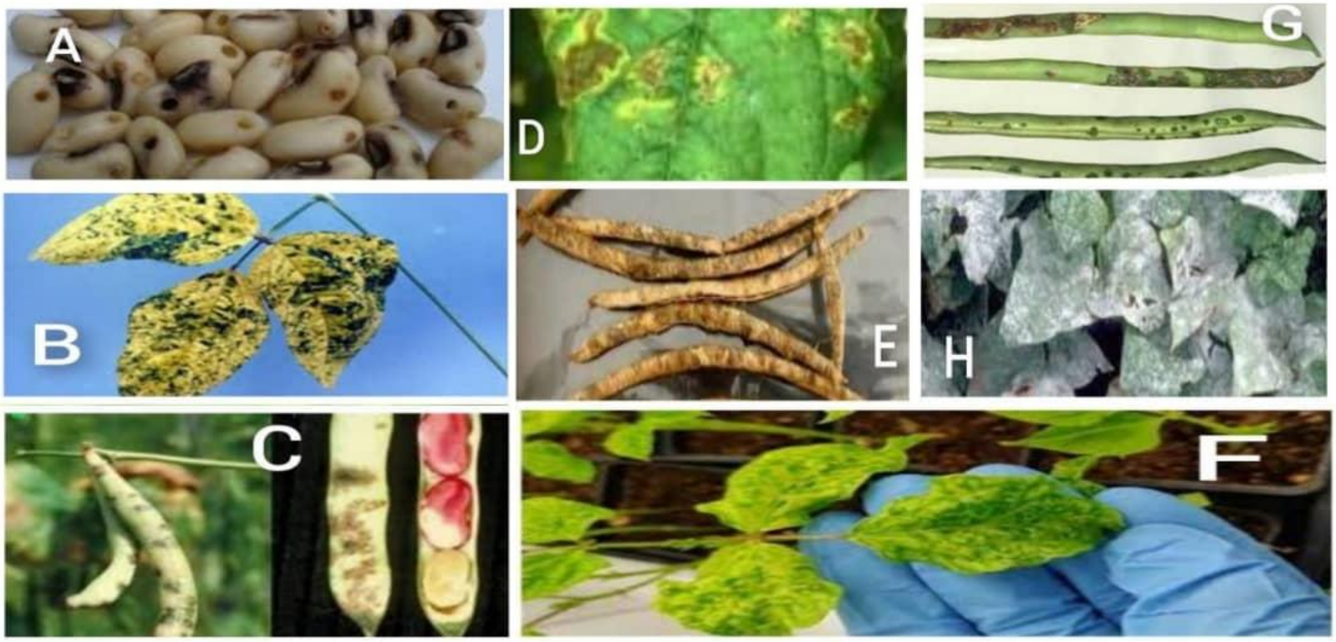


Figure 3.1: Microbial diseases of cowpea: A – cowpea seed beetle, B – Yellow mosaic virus infected cowpea, C – cowpea halo blight, D – bacterial blight, E – anthracnose, F – cowpea mosaic diseased leaf, G – bacterial bean blight, H – powdery mildew

3.3 Looking ahead beyond the constraints for cowpea productivity enhancement with sustainable bioinoculants and smart biotechnological techniques

It is worth re-emphasizing that the challenge of attaining enhanced cowpea productivity on a sustainable level is not merely a single limitation. Rather, it is a diversity of limitations requiring a high level of multi-tasking.

However, there are also multiple smart, and sustainable agro-biotechnological techniques that could be deployed in a sustainable manner to achieve improvements in cowpea productivity and production outputs. Elements of this technology, which is geared towards maximizing eco-friendliness and guaranteeing an improvement in safer agro-biotechnological productivity, are briefly listed, and their associated mechanisms of action are also explained.

1. The sustainable deployment of bio-inoculants (biofertilizers and biostimulants) to serve as an alternative to synthetic chemicals
2. The sustainable deployment of biological antagonists in the form of biopesticides to tackle pests in the field and during the post-harvest storage stage
3. The deployment of climate-smart agricultural (CSA) practices as an adaptive technology option to mitigate the effects of climate change on the vulnerabilities of crop production
4. The deployment of smart and advanced biotechnological applications, such as metabolomics, transcriptomics, proteomics and genomic breeding tools for the improvement of cowpea varieties, which would possess the desired traits, such as drought tolerance, favourable salinity stress tolerant levels, high yields, resistance to high temperatures and thermotolerance, resistance to disease, and a high potential for nodulation.
5. The application of conservation practices in agriculture

3.3.1. Sustainable deployment of bio-based/microbial resources as alternative to synthetic agrochemicals

Microbial-based formulations have proved to be an effective alternative to the use of synthetic agrochemicals in crop production. These natural, eco-friendly and sustainable bioformulants are categorized as biopesticides, biostimulants, and biofertilizers.

To minimize crop losses and improve productivity, natural microbial-based formulations have been successfully deployed in agro-ecological crop production. The salient features of these resources are that they are cheaper, renewable, easy to handle, and more importantly, safe for human beings and the living environment (Castaldi *et al.*, 2021; Kour *et al.*, 2020).

To meet up with the challenge of feeding the rapidly increasing global population, there is a need to increase crop productivity. One popular means of solving the problem of global food insecurity is by boosting agricultural outputs /productivity through the application of synthetic agro-fertilizers.

Conventionally, synthetic agrochemicals are applied as inputs to intensify agricultural production systems. Various fertilizers, fungicides, herbicides, and pesticides are thus used in large-scale crop production systems. Initially, the advent of the chemical fertilizer was widely accepted because it helps to increase agricultural productivity and to solve global food consumption issues (Duan *et al.*, 2016; Liu *et al.*, 2015). However, the indiscriminate use of chemical fertilizers has led to air and groundwater pollution, which, mainly in the case of the latter has led to the eutrophication of water bodies (Vanlauwe *et al.*, 2014). Also, the long-term effect of using chemical fertilizers results in bio-magnification and bio-accumulation in living organisms which have in their turn had negative impacts on the soil environment and ultimately on human and animal health (Calderón *et al.*, 2017).

Therefore, the increasing concern of consumers and governments for food safety issues, has led stakeholders to explore newer ecologically and environmentally-friendly methods to replace or supplement the current chemical-based practices in agriculture. In fact, the use of bio-pesticides, bio-herbicides, and bio-insecticides has emerged as a promising alternative to chemical pesticidal products (Ahirwar *et al.*, 2020).

Also, Nicolopoulou-Stamati *et al.* (2016) reported that the use of chemicals in the form of pesticides, insecticides, and herbicides could affect the quality of the plant products and thus adversely affect human and animal health.

However, the search for environmentally and agro-ecologically sustainable alternatives to these synthetic agrochemicals has led to the deployment of quite an array of diverse forms of microorganisms being applied to function as biofertilizers, biostimulants, biopesticides, and plant growth promoters. Hence, they are being used to enhance a diversity of crop growth in numerous countries around the world, especially in the developing and emerging world (Alori & Babalola, 2018; Igiehon & Babalola, 2017; Omomowo & Babalola, 2019).

Different groups of microorganisms constitute different types of association with different host plants in the form of endophytic, epiphytic, and rhizospheric associations (Yadav, 2021). Thus, based on these associations, scientists have formulated bio-inoculants to solve the food security problem in an eco-friendly way.

Diverse terminologies have been used to qualify these metabolically and physiologically - important microbial forms. They are known under terms such as biocontrol agents (BCAs), and are referred to as agriculturally - beneficial microorganisms, e.g., arbuscular mycorrhizal fungi (AMFs), which are sometimes referred to as, amongst others, plant growth-promoting rhizobacteria, plant growth-promoting fungi (PGPFs), and plant growth-promoting bacteria (PGPBs). A lot of research in the field of applying microbial inoculants to different planted crops has been conducted by scientists and is still ongoing (Chaudhary *et al.*, 2021; Chen *et al.*, 2021; Igiehon *et al.*, 2019). These beneficial species help to control or suppress plant diseases caused by pathogenic bacteria and fungi through different antagonistic mechanisms in that they produce antifungal and antibacterial compounds or feed as parasites on them (El-Sharkawy *et al.*, 2018).

To solve the problem of food safety and the increasing concerns in respect of the environment in an eco-friendly manner, the use of biofertilizers, biopesticides, and biostimulants is gaining the necessary attention in the agricultural sector (Oleńska *et al.*, 2020). Based on plant-microbial associations, the utilization of viable and sustainable microbiota or their groupings has long been established as a means to improve agricultural productivity, and is in fact on an upward rise (Adeleke & Babalola, 2021b; Chukwuneme *et al.*, 2020; Fasusi *et al.*, 2021).

More importantly, with the advent of next generation sequencing technological availability and cheaper cost, research efforts in the field of metagenomics, metabolomics, proteomics, transcriptomics and genomics have revolutionize the prospects of applying plant growth promoting microbiota as bioinoculants that are deployed as biofertilizer, biopesticides and biostimulants for the improvement of planted crops. With the advent of these advanced biotechnological techniques, researchers have elucidated studies on the root microbiome as the hidden treasure that possesses immense potential to revolutionize the strategies for improving plant growth, as well as abating biotic and abiotic constraints in plants (Mathur & Roy, 2021).

These roots associated microbiome are known as prolific producers of phytohormones, mainly auxins, cytokinin, and ethylene as well as enzymes like the 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase) and exopolysaccharides that help plants in inducing systemic resistance to both biotic and abiotic stressors. Newer and effective techniques have been deployed in isolating and characterizing root associated microbiome, and applying them as bioinoculants in improving the growth and development of planted crops (Liu *et al.*, 2020; Romano *et al.*, 2020).

The root microbiome consists of an enormous number of beneficial microbes such as plant growth-promoting rhizobacteria (PGPRs), fungal and bacterial endophytes and mycorrhizal fungi (Yu *et al.*, 2019).

Metabolites that are secreted by this microbiota are associated with marked influences on plant growth promotion, response and mitigation to biotic and abiotic stressors. These bioactive metabolites include ACC deaminase, gibberellic acid (GAs), indole acetic acid (IAA), exopolysaccharides, melatonin, volatiles and cytokinins (Jones *et al.*, 2019; Qu *et al.*, 2020).

It is anticipated that root exudates influence the rhizospheric microbial community and that analysis of the root microbiome signifies ecosystem functioning (Williams & de Vries, 2020). Therefore, a lot of research effort abound on exploration of the root microbiome as reservoir of novel microbial isolates and genes that may be beneficial as biofertilizers, biopesticides and biostimulants in an era of climate change.

Plant growth promoting rhizobacteria (PGPR) strains are able to produce IAA, solubilize phosphate, induce ACC deaminase, and chelate iron by producing siderophore. Therefore, their application is an effective means of alleviating stress in planted crops (Etesami & Jeong, 2018).

The PGPR strains achieve improvement in the growth and tolerance of planted crops through the accumulation of compatible solutes like proline or glycine betaine, by enhancing the secretion of bioactive metabolites, as well as through inducing the expression of plant growth beneficial genes.

Recently, the Metabolomics profiling of *Sorghum bicolor* that was primed with PGPR isolates (*Bacillus* and *Pseudomonas*) and exposed to drought stress, induced systemic tolerance in the plants (Carlson *et al.*, 2020).

Also, proteomic analyses of *Medicago truncatula* that was inoculated with *Sinorhizobium sp*. and exposed to drought stress, led to the upregulation of JA pathway and downregulation of ethylene biosynthesis which are vital for improved drought tolerance (Staudinger *et al.*, 2016).

In addition, the inoculation of *Trichoderma* and *Pseudomonas* in rice plants that was subjected to drought stress induced the overexpression of antioxidative enzymes and the phenylpropanoid biosynthesis pathway, as well as other key drought responsive genes (Singh *et al.*, 2020).

PGPR remains a promising option for improving crop drought resistance, as reveal in a transcriptomics study by Morcillo *et al.* (2021) applying the bioinoculant B. megaterium TG1-E1 on different tomato cultivars under drought conditions. The findings reveal several key mediators of TG1-E1-induced transcriptional regulation in tomato plants, including transcription factors, stress signaling components and regulators, and putative regulators of cell wall organization. Also, analysis of the metabolites indicated the presence of important compounds that include ethanolamine, amino acid, sugars, and pinitol, which aided in TG1-E1-triggered plant drought resistance.

By using high-throughput RNA-sequencing techniques (Thomas *et al.*, 2019), characterized differentially expressed genes (DEGs) in rice roots upon inoculation with *A. brasilense*. The findings reveal plant growth promotion impacts, pathways and genes that are involved in the plant-microbe interactions.

Furthermore, in a study by Zhang *et al.* (2020) using culture independent 16S rRNA gene amplicon sequencing and culture-dependent functional analyses of *Alhagi sparsifolia* rhizosphere and root endospheric microbiome, identify key endophytic bacterial taxa and their genes facilitating drought resistance in wheat. Through comparative genomics analysis, a drought resistance-promoting strain was characterized, as well as the mechanisms deployed in colonization and enhancement of drought resistance in wheat was elucidated.

3.3.2 Deployment of climate-smart agricultural practices for improving productivity

One of the major challenges faced by humanity over the ages has been the task of tackling in a sustainable way environmental degradation and the consequences of climate change which are more pronounced in the case of natural ecosystems (Sarkar *et al.*, 2020). The effects of climate change are more pronounced in agro-ecosystems because the sum total of all agricultural activities takes place on them and that is why they are the most vulnerable of all of the natural ecosystems (Dubey *et al.*, 2020).

The deployment of ecologically and environmentally - unfriendly practices such as the excessive intensification of agricultural practices on the land, the indiscriminate use of agrochemicals, such as pesticides, herbicides and fertilizers, as well as the consequences of anthropogenic activities, such as like urbanization, deforestation, industrialization, and the burning of fossil fuels, collectively result in greenhouse gas (GHG) emissions and the ultimate disruption of the agro-ecological balance (Dubey *et al.*, 2016; Lawrence & Vandecar, 2015).To meet up to the challenges posed by the high consumption levels of a rapidly growing population has proved to be a huge task. This is especially true for the developing world where, under the changing climatic conditions, there is a need to adopt strategies and practices that are socially, economically, and ecologically acceptable in the management of our natural resources (Abhilash *et al.*, 2016; Sarkar *et al.*, 2017). Climate-smart agriculture (CSA) presents various innovative practices that can be adopted to meet the global food demand while concomitantly mitigating the effects of unfavorable climatic conditions on the production of climatically vulnerable crops. CSA is based on existing knowledge, technologies, and sustainable agriculture (FAO, 2015) and presents an integrated approach to managing cropland, livestock, forests, and fisheries in order to achieve food security, reduced greenhouse gas emissions and to contribute to other development goals in the face of climatic changes (Palombi & Sessa, 2013).

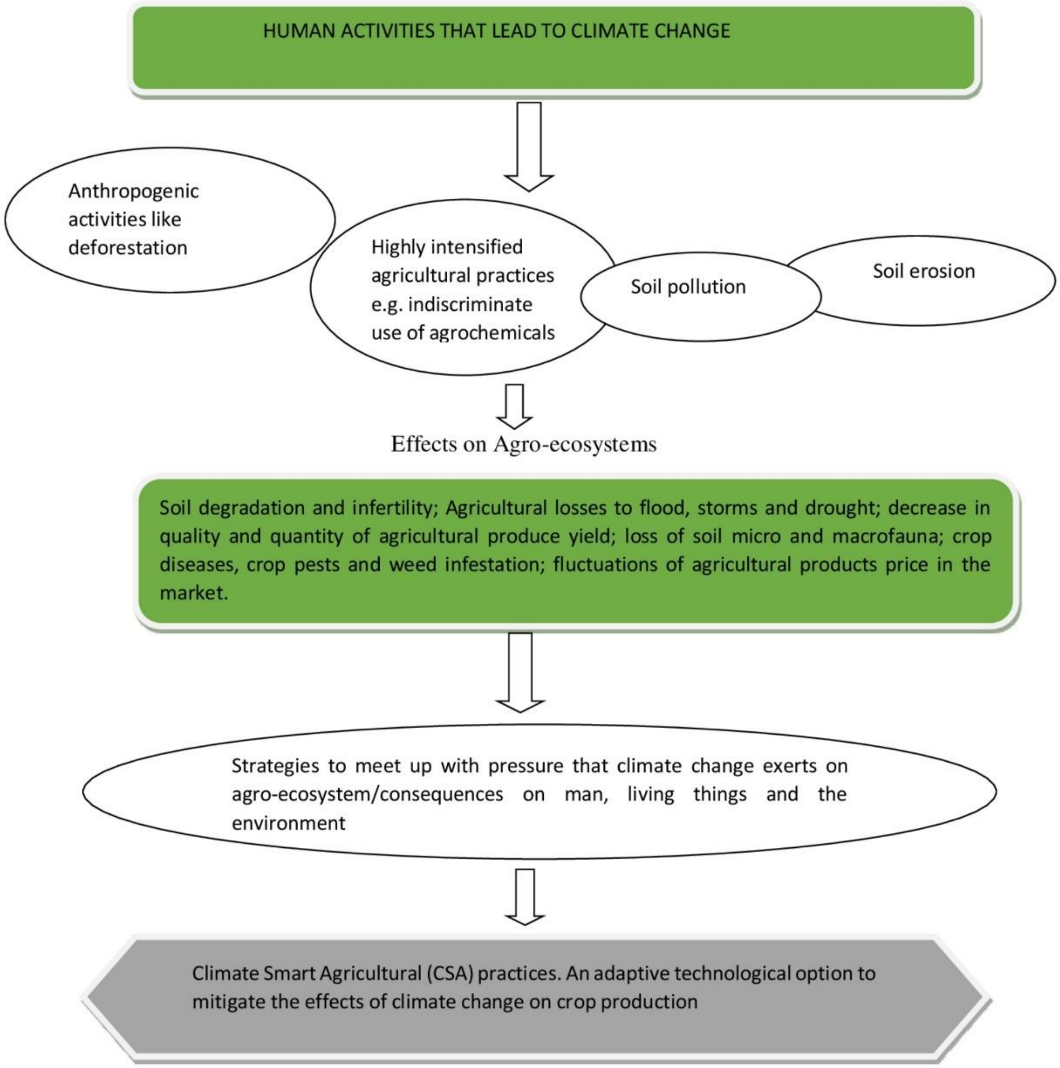


Figure 3.2: Schematic highlighting of the different pressures exerted by climatic change and CSA as a mitigating practice to improve agricultural production

According to Kumar *et al.* (2019a), some of the CSA practices and technologies are able to mitigate the effects of climate change on the agro-ecosystem, to boost agricultural production and to reduce the effects of GHGs. They include the use of quality seeds and the planting of well-adapted crops, effective biodiversity management, and integrated pest management systems, efficient water management, sustainable land and soil management to ensure increased crop production, and sustainable and efficient mechanization.

Other CSA mitigation practices include low-input sustainable agriculture (LISA) practices, which focus on safe farming and that incorporate local knowledge of the farming system, and in so doing, produce abundant, nutritious, profitable food products without causing negative effects to both the natural agro-ecosystem and human health (Najafabadi *et al.*, 2012). According to Sarkar *et al.* (2015) indigenous technical knowledge (ITR) concerns the knowledge that local people have gathered through their interactions with nature and that has allowed them to adopt mitigating measures to counter the effects of climate change and thus to boost their crop production.

Also, simulation model studies are vital tools that can be used to conduct studies of different agro-ecological regions in order to implement sustainable agricultural measures, to achieve effective and maximum production levels (Sarkar *et al.*, 2020). Organic farming also goes a long way to reducing the effect of GHG emissions (Rakshit *et al.*, 2010).

Importantly, (Cammarano *et al.*, 2020) used the Agricultural Model Intercomparison and Improvement Project (AgMIP) as a tool that, in the face of the prevailing drought problem in the north-eastern area of Free-state, South Africa, incorporated data about climate change, crops and the economy to provides and implement adaptation strategies to improve and increase the production of maize in this region. Likewise, (Ishikawa *et al.*, 2020) used the farmers' participatory varietal selection (FPVS) method to collect information from local farmers in the southern regions of Burkina Faso, in West Africa. They used the collected data to gather information on how to breed and select newly improved drought-resistant cowpea seeds for maximum production, which would prove to be economically and socially beneficial.

3.3.3 Prospects of advanced multi-omics biotechnological techniques for improving cowpea productivity

In this modern era, where there is a notion of smart biotechnological techniques that can turn around the immense challenges of optimizing agricultural system outputs productivity, the multi-omics biotechnological tools are usually the game-changer. These multifaceted biotechnological techniques encompassing genomics, transcriptomics, proteomics, and metabolomics offer great prospects for improving crop protection, crop yields/productivity, and for ensuring nutritional food sources that are safe and secured for human consumption.

Through the application of the techniques of genomics, transcriptomics, proteomics and metabolomics, plant breeding has improved, and biotic and abiotic stress-resistant and resilient crop cultivars have been developed, thus leading to the production of better-quality crops.

Multi-omics biotechnological tools encompass a knowledge of analytical chemistry, computational biology, and bioinformatics analysis, as well as other thematic areas of biology, to facilitate a systematic approach to research studies, which would then lead to crop production and productivity enhancement.

Metabolites, proteins, and genes are specific components that are targeted and researched to improve crop cultivars and to better understand their growth characteristics.

These smart biotechnological techniques are advanced, concise, precise, and valuable tools that can be specifically targeted for improving crops. In fact, they are vital tools for sparking the latest green revolution in agricultural productivity. They can be used to introduce genes, proteins, or metabolites of interest with good traits to improve and intensify the productivity of planted crops. Thus, fewer agro-resource inputs would then be necessary in agricultural systems to attain better agro-product outputs.

Multi-omics biotechnological tools can be deployed to reveal key information on (plants and microbes). Furthermore, these tools could be applied to orchestrate metabolic and physiological changes, and also in genetic engineering for crop improvement (Chassy, 2010). Multi-omics techniques can also be used in breeding transgenic crops with specific key agronomic traits (Ahmad *et al.*, 2012).

The multi-omics biotechnological tools, namely genomics, transcriptomics, proteomics, and metabolomics, are inter-woven techniques, that are closely linked and that can be applied to overcome the daunting challenges of feeding the burgeoning global population in this era of climatic vulnerabilities. They can also be deployed to consolidate the foremost producer status of the African continent in that they are able to enhance cowpea productivity and production.

3.3.3.1 Genomics

Genomics is the foremost pioneer omics that is presented as an advanced biotechnological technique and that uses genes and the genome transformation of plants and microbes for molecular breeding in order to establish improved crop cultivars. Genomics techniques are fast and precise, and can be selectively used to highlight the functional genes of desired traits for the improvement of a plant. Specifically, genomics techniques can be applied in the modification of genes in that they add genes to a plant, or by using RNAi they knock down genes from a plant, in so doing, accomplish phenotypic traits of interest faster than the conventional plant breeding method does. In the quest to enhance cowpea productivity, genomics-based smart biotechnology techniques have been deployed to breed improved cowpea cultivars. In such cases, the focus is on looking at the whole genome in terms of genotypic diversity and fingerprinting for cultivar improvement traits (Seo *et al.*, 2020; Wu *et al.*, 2018; Xu *et al.*, 2017).

Molecular based approach has been deployed towards improving cowpea cultivars using molecular markers and genomic breeding techniques. An authenticated cowpea genetic resource is the foundation for efficient breeding and conservation. Genotypic diversity assessment is done by using both phenotypic and molecular traits characterization.

Research efforts at genetic breeding of cowpea cultivars using these DNA markers have been investigated by (Chen *et al.*, 2017b; Kolade *et al.*, 2016).

SNPs are the preferred markers in genotypic assessment studies due to their wide distribution in the genome and they are highly efficient (Nkhoma *et al.*, 2020).

Following advancement in plants genome resources, molecular markers are now widely deployed in genetic variability assessment, molecular breeding and DNA fingerprinting (Su *et al.*, 2018).

Among the genomic breeding research effort, the Illumina Cowpea iSelect Consortium Array (Muñoz-Amatriaín *et al.*, 2017) was an important landmark. This great research effort led to the development of a minicore (referred to as the "UCR Minicore") which composed of 368 domesticated cowpeas selected from a larger set of _5000 accessions comprising the UC Riverside cowpea collection.

This array contained 51,128 SNPs derived from whole genome sequences (WGS) of 37 diverse cowpea accessions. Single nucleotide polymorphism (SNPs) is distributed uniformly in cowpea genome and indicates variation in genes of cowpea. Thus, they provide an ideal resource for cowpea molecular breeding and new variety protection. SNPs are vital genomics techniques for assessment of key traits in cowpea like constructing genomic linkage map, for quantitative trait loci (QTL), for the detection as well as assessing germplasm genetic diversity (Paudel *et al.*, 2021).

Also, the majority of the international institute of tropical agriculture (IITA) minicore collection (298 accessions) was genotyped using genotypic base sequencing (GBS) with 2276 SNPs, this identified three major subpopulations (Fatokun *et al.*, 2018), but showed dispersion of West and Central African accessions across the three sub-populations.

Another giant stride in the progress of cowpea genomics study was achieved by using next generation sequencing advancement (Lonardi *et al.*, 2019) to authenticate the whole genome of an improved cowpea genotypes, thus providing a key resource that is crucial to deciphering the morpho-physiological response of cowpeas.

Building on this developments and report of full SNP data release for the UCR Minicore, numerous follow up studies has been investigated for more focus cowpea research, that include studies on pattern of seed coat (Herniter *et al.*, 2019), color of seed coat (Herniter *et al.*, 2018), size of seeds (Lo *et al.*, 2019a), resistance to bruchid infestation (Miesho *et al.*, 2019), plant herbivore resistance (Steinbrenner *et al.*, 2020) and pod shattering (Lo *et al.*, 2021).

With better comprehension of genomic basis of variation, genome-wide association studies (GWAS) studies have been highlighted in cowpea for pod length (Xu *et al.*, 2017), root architecture (Burridge *et al.*, 2017), cowpea plant improvement traits, as well as the flowering period (Muñoz-Amatriaín *et al.*, 2021). All these findings are appreciated because cowpea genetic diversity assessment is necessary for strengthening breeding programs in order to develop high yielding dual-purpose cultivars with good grain and fodder yields.

3.3.3.2 Transcriptomics

Transcriptomics is a vital biotechnological technique that makes for a comprehensive understanding of genomics functionality (Valdés *et al.*, 2013). Transcriptomics regulates the expression of genes in the context of biotic and abiotic stresses. Transcriptomics is a dynamic technique that expresses genes at any given time and under different circumstances.

With the advancement of functional genomics, the identification of novel genes having vital functions in plant growth/development and adaptation to stressful conditions have been characterized for crop cultivars (Zhang *et al.*, 2017). Also, RNA expression profiling is important in understanding plant functionality.

Transcriptomics as a part of multi-omics biotechnological techniques have led to the detection of novel genes useful in response to both biotic and abiotic stresses in plants.

Transcriptomics approaches utilizes high throughput sequencing platforms to generate enormous useful transcript data through techniques such as RNA sequencing, microarray and serial analysis of gene expression (SAGE) to elucidate non-coding and coding RNAs expression profiles to plant biotic and abiotic stresses (Cramer *et al.*, 2011; Santos *et al.*, 2018).

Several factors like the duration and extent of stress conditions, determines the adaptability and tolerance of a plant to stresses. However, experimental design, handling of tissue samples, isolation of RNA and stability of RNA also play major role in any transcriptomic analysis (Gokce et al. 2020).

The characterization of different parts of the cowpea plant through transcriptomics has been carried out in studies that express the diverse genes essential for cowpea growth and development. The stress-resilience genes have also been characterized and their role in the overall improvement of cowpea has also been highlighted (Amorim *et al.*, 2018; Chen *et al.*, 2017c; Spriggs *et al.*, 2018; Yao *et al.*, 2016).

3.3.3.3 Proteomics

Proteins are a vital constituent of plants. Large quantities of protein are responsible for the key functional roles that plants perform. As a smart biotechnological technique, proteomics entails the expression of functional characteristics, structural features, and the translation/manifestation of beneficial traits in plants. Another important attribute of the proteomics technique is that it can be used to better elucidate a pesticide's mode of action, its mechanisms, and its biodegradation. The outputs/benefits that can be derived by applying proteomics include the authenticity of the food product, the assurance of food security that it represents and the sustainability of energy that the food product offers to consumer, as well as the maintenance of an environmental balance (Agrawal *et al.*, 2012; Landim *et al.*, 2017).

Proteomics as a key branch of ‘omics’ technology aims at investigating protein’s structure, function, as well as their interactions with other proteins and other components, including the modifications arising from these interactions through the use of analytical techniques.

Proteomics approach involves analysis and the elucidation of functional expression of proteins in order to understand biological processes (Chen & Weckwerth, 2020; Iwamoto & Shimada, 2018).

Proteins are vital components of all biological process. To fully comprehend the response of plants to biotic and abiotic stresses, proteomics studies must be assessed, along with other multi-omics technology (Gokce *et al.*, 2020). Changes in gene expression influences appropriate response in protein composition/abundance and affect cellular functions.

Proteomics studies are assessed using spectroscopic method usually by mass spectroscopy (MS)-based technology. This is done by MALDI-TOF MS or with liquid chromatography mass spectrometry (LC–MS) techniques. Proteomics studies have led to the characterization of different stress response proteins in planted crops under stress conditions (Kosová *et al.*, 2018; Matamoros & Becana, 2021; Rathi *et al.*, 2016).

3.3.3.4 Metabolomics

Metabolomics is an advanced and powerful smart biotechnique that identifies functionally - active metabolites, their roles, and the diverse biochemical processes that the metabolites play in plant genotypes and phenotypic expressions (Aliferis & Chrysayi-Tokousbalides, 2011; Führs *et al.*, 2009). Metabolomics tools can be deployed in identifying and monitoring physiological responses in plants and the metabolic pathways or linkages arising from the biotic and abiotic stress exerted upon plants. In fact, these tools are able to enhance crop development and improve plant health (Dixon *et al.*, 2006; Goufo *et al.*, 2017).

In a study on drought response of 3 cowpea landraces using leaf physiological and metabolites profiling assessment, (Gomes *et al.*, 2020), used gas chromatography time of flight mass spectrometry (GC-TOF-MS) and reported that cowpea landrace A116 genotype drought response was best with the accumulation of 14 bioactive metabolites that included proline, valine, and rhamnose and raffinose, isoleucine, fucose, urea, alanine, sucrose and putrescine.

Also, in a study on metabolites (polyphenols and carotenoids) in *V. unguiculata* sprouts by (Yeo *et al.*, 2018), investigated using high-performance liquid chromatography (HPLC), electrospray ionization-mass spectrometry (ESI-MS), gas chromatography-mass spectrometry (GC-MS), and gas chromatography, 39 hydrophilic compounds were identified and quantitated. Thus, the study provides a new approach for enhancing the carotenoid and phenylpropanoid production of *V. unguiculata*.

Metabolomics as a powerful omics-based approach can be applied as a tool to explore different aspects in plant breeding, the regulatory mechanisms related to plant growth and development (including those related to crop productivity and performance), adaptation to biotic and abiotic stresses, nutritional improvement, and selection of cultivars for agriculture. Metabolomes are simply metabolites (both secondary and primary) having low molecular-weight (usually <1500 Da), including their precursors and intermediates of the corresponding biosynthetic pathways. Such compounds are considered the end products of gene expression and protein activity, modulating processes between the genome and environment and indicating the functional status of the organism. Moreover, they are an indispensable part of the plant metabolism, influencing all biological processes, such as plant biomass and architecture, and those involved in plant defense or adaptation to biotic and abiotic stresses (Sharma *et al.*, 2021).

In a comprehensive study on cowpea osmoregulation response under drought stress, Goufo *et al.* (2017) investigated and provided a detailed metabolic profile of a broad range of primary and secondary metabolites in cowpea, including elemental solutes using (leaves and roots). Their findings revealed that the mechanisms deploy in modifying cowpea metabolism response to water deficit is through interplay between the shikimate and arginine/proline pathways, leading to three drought-responsive metabolites, namely galactinol, proline and quercetin 3-O-6″-malonylglycoside.

In a study aimed at identifying metabolic responses and key factors associated with Mn tolerance using Mn-tolerant and Mn-sensitive genotypic cultivars, Führs *et al.* (2012) reported that manganese tolerance is a consequence of genotypic/constitutive higher concentrations of metabolites detoxifying manganese and reactive oxygen species.

3.4 Agricultural conservation practices for crop productivity enhancement

Agricultural conservation practices are simple and cost-effective techniques for achieving sustainable productivity enhancement in planted crops. This technique is based on the use of a limited number of natural resources as inputs. Crop rotation, mixed farming methods, intercropping, the manual tillage of the soil, and the use of crop residues to reduce soil moisture loss through mulching are some of the methods employed. These simple, cost-effective techniques, using a limited number of resources as inputs, ultimately lead to crop productivity enhancement.

However, in order to effectively enhance crop productivity, it is necessary to find effective ways of adapting to climate change and the vulnerability it imposes on crops and farmers. The objective should always be to mitigate the adverse impacts of climate change on the environment (Lipper *et al.*, 2014).

Conservation agriculture improves the quality of the soil - biologically, physically, and chemically, and thus ultimately makes an impact on the crop production outputs, with both positive and sustainable effects (Basavanneppa *et al.*, 2017). In addition to improving crop yields and achieving sustainability, conservation agriculture also augments microbial diversity and enhances microbial functionality (Yadav *et al.*, 2017).

Conservation agriculture is increasingly being promoted as an adaptive climate-smart agricultural technique that can minimize the adverse effects of synthetic agrochemical usage in agricultural systems that generally lead to poor and depleted soil fertility (Pretty & Bharucha, 2014).

As an agroecological system tool, conservation agriculture can lead to enhanced crop productivity, the diminished use of agro-resource inputs, environmental sustainability, and advance the income generation potential of farmers (Pariyar *et al.*, 2019; Prasai *et al.*, 2018).

Conservation agriculture helps in enhancing soil fertility and in reducing the cost of the associated inputs. The application of conservation practices improves soil water conservation and soil moisture, minimizes runoff, reduces moisture losses through evaporation, boosts the biological properties of the soil, and enhances crop productivity (Hossain *et al.*, 2015).

The beneficial effects of conservation agriculture on crop productivity can be classified into three main categories:

1. Conservation agriculture provides agronomic growth benefits and enhances soil health.
2. The sustainability of the environment and the soil and the sociological benefits of the agricultural production system are enshrined.
3. Conservation agriculture can lead to enhanced economic benefits and also improve efficiency in the agricultural sphere.

In a nutshell, conservation agricultural practices enhance the quality of planted crops, improve the fertility of the soil, and ultimately provide both socio-economic and environmental benefits in a sustainable manner (Bell *et al.*, 2019; Calcante & Oberti, 2019).

The applications of bio-based, renewable, agro-ecologically - balanced, and advanced smart biotechnological techniques in achieving improvements in the productivity of cowpea and a few selected crops of economic importance are presented in (Table 3) as effective sustainable alternatives for crop improvement.

3.5 Mechanistic physiological processes/actions involved in the deployment of smart biotechnological techniques to improve cowpea productivity

Owing to the application of smart biotechnological techniques, diverse physiological and metabolic modes of activities are involved in improving the productivity outputs of cowpea. This can be achieved through direct and indirect modes of action such as those involved in, amongst others, directly supplying nutrients to plants, suppressing phytopathogens through the production of plant growth effectors, regulating the hormonal balance of plants, triggering various immune responses, and through the secretion of vital proteins (Santos Villalobos *et al.*, 2018; Villarreal-Delgado *et al.*, 2018). An overview is presented in (Figure 3)

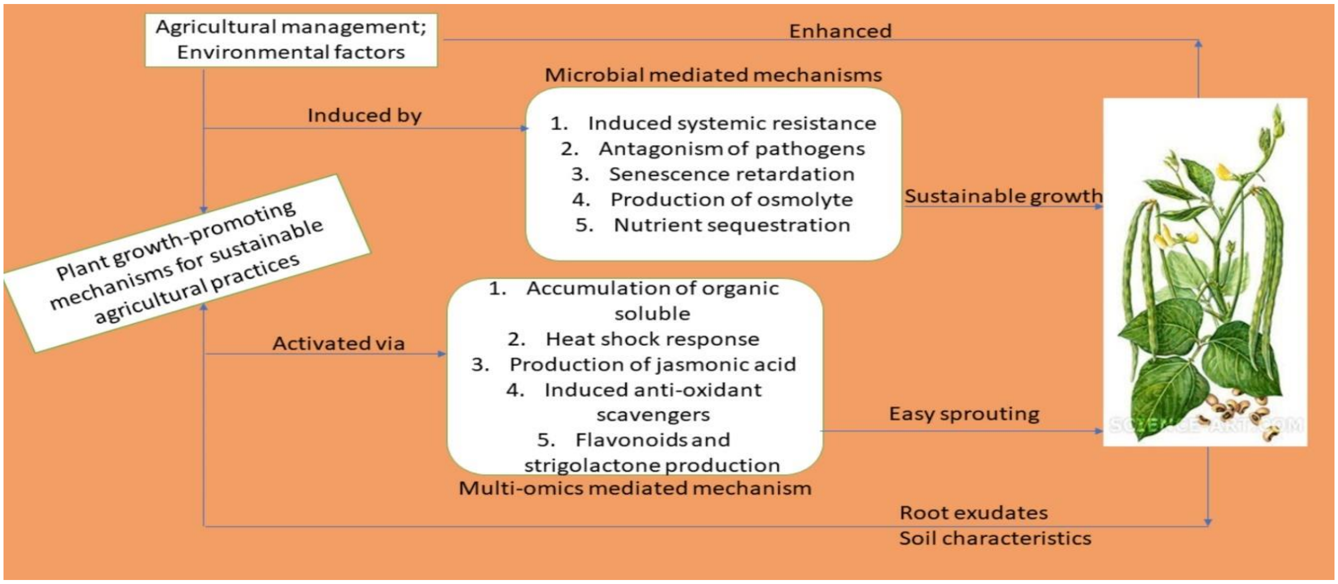


Figure 3.3: Mechanisms of action of smart biotechnological techniques deployed in cowpea productivity enhancement

3.6 Concluding remarks and future perspectives

. Yes! Daunting and herculean are the constraints that almost all African nations face in terms of improving their crop yields and productivity in the light of the current global challenges. These are aggravated by the global pandemic, climatic change, and a burgeoning population growth rate. However, a concerted effort directed at achieving the sustainable development goals of reducing poverty and eliminating hunger and malnutrition is what is called for. The first priority is to strive for an improvement in the agricultural system. The use of agro-ecologically balanced improvement techniques remains the surest way to achieve this. The constraints of low yields and the limited productivity of the cowpea - a valuable indigenous African legume at the forefront on the continent in terms of its potential as a food product were highlighted in this review. The prospect of circumventing and overcoming these constraints is in fact a very real possibility. An essential requirement would be the use of viable tools. These would include the deployment of sustainable, ecosystem-friendly smart biotechnological tools: the application of bioinoculants, climate-smart agricultural practices, agricultural conservation techniques, as well as advanced multi-omics biotechnological tools for the improvement in cowpea yields and productivity enhancement. However, there are research gaps that still need to be worked upon to ensure success. Several collaborative efforts should be directed at building the capacity of plant breeders, agronomists, biotechnologists, and other allied stakeholders in the agri-food value chain in Africa to embrace these sustainable biotechnological techniques. Further research efforts should be directed at attaining specific functional traits in cowpea plants, in order to develop locality adaptive and climate-specific traits - the latter in response to climatic vulnerabilities and other external stressors – all for the benefit of the planted cowpea crop. Furthermore, efforts should also be directed at exploring an integrative and holistic approach to systematic biology that would combine systemic knowledge in the field of multi-omics biotechniques, genetic engineering tools, precision agricultural practices, techniques in genome editing technology (CRISPR/Cas), synthetic biology, bio-computational technology, as well as the emerging field of agro-nanobiotechnology for the improvement of the cowpea crop. The use of a synthetic microbial consortium (SYNCOMs) should be deployed to the field to vigorously phenotype cowpea cultivars that are trait-specific and can be grown as a crop adapted to a niche environment, and favoured by most cowpea producing marginal communities in Africa.

There is, however, a need to integrate socio-economic policy into this sound biotechnological know-how system in order, to reach a balance, as well as a guaranteed and steady flow of the necessary financial support for the associated research efforts. Attention should also be directed to developing a policy of backward integration to achieve positive and sustainable results in the context of improving and enhancing the productivity and yields of cowpea, a key leguminous crop that is considered to be of great importance in Africa.

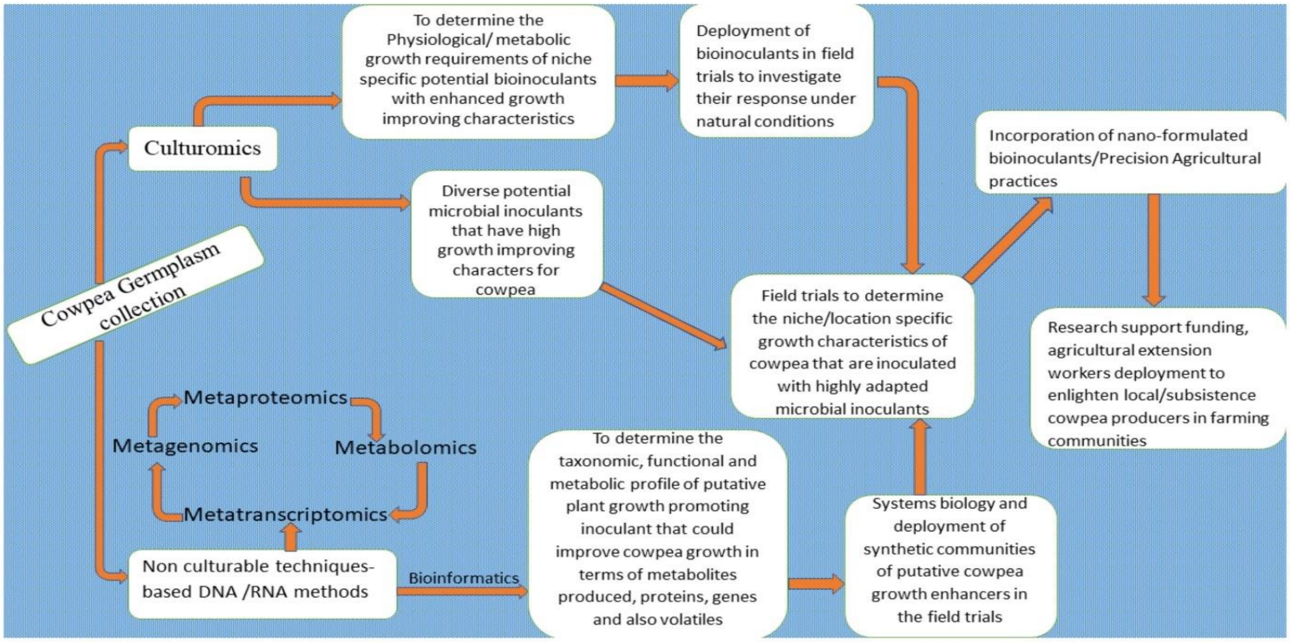


Figure 3.4: Holistic approach and futuristic perspectives for improving cowpea productivity enhancement in Africa and consolidating the continent's foremost producer status

Table 3.1: Production output and productivity of cowpea by some selected countries in the world, excluding Brazil as (adapted from FAOSTAT 2020).

S/N	Country	Production in tons	Yield per hectare	Area harvested	Inference on Production	Inference on Productivity
1	Nigeria	2606912	9137	2853097	1st	7th
2	Niger	2376727	4035	5889677	2nd	18th

3	Burkina Faso	630965	4826	1307336	3rd	12th
4	Ghana	215350	19862	11898	4th	2nd
5	Tanzania	202865	4096	30366	5th	6th
6	Cameroon	185832	4043	258898	6th	9th
7	Kenya	179399	4367	11154	7th	10th
8	Mali	157739	3767	160412	8th	11th
9	Myanmar	136411	11425	119398	9th	4th
10	Sudan	104667	2678	333638	10th	17th
11	Mozambique	89356	5545	284451	11th	20th
12	Democratic Republic of Congo	72726	4432	95803	12th	15th
13	Senegal	60422	6889	260408	13th	19th
14	Malawi	42456	13515	159345	14th	13th
15	United States	23632	4296	169279	15th	1st
16	China	15652	8876	209371	16th	5th

17	Madagascar	13000	8907	14596	17th	8th
18	Uganda	12439	9750	208059	18th	16th
19	Sri Lanka	11180	11770	9499	19th	3rd
20	South Africa	4871	10360	15108	20th	14th

Table 3.2: Highlight of constraints limiting productivity enhancement of cowpea plant in major producing nations of the world

Productivity constraint	Crop of interest	Bioactive roles of stressors	References
Biotic limitation involving Cowpea Severe Mosaic Virus	*Vigna unguiculata*	The chlorotic lesion, mosaic formation, and necrosis	Oliveira *et al.* (2020)
Combine abiotic stressors of CO_2, High temperature and UVB irradiation	*Vigna unguiculata*	Vegetative and reproductive growth stage impaired adversely	Singh *et al.* (2010)
Drought stress	*Vigna unguiculata*	Reduction in vegetative biomass Photosynthesis, transpiration, and stomatal conductance	Cardona-Ayala *et al.* (2020)

Abiotic limitation involving heavy metals (Chromium)	*Vigna unguiculata*	Adverse impact on nodulation and biological nitrogen fixation	Miranda *et al.* (2014)
Biotic constraint caused by Legume Pod Borer (*Marucavitrata* Fabricius) (LPB)	*Vigna unguiculata*	Complete crop failure due to feeding on all parts of cowpea	Sodedji *et al.* (2020)
Biotic constraint caused by *Aplosporella hesperidica*	*Vigna unguiculata*	Adverse impact on cowpea leading to collar rot symptoms	Deepika *et al.* (2020a)
Biotic constraint caused by *Fusarium equiseti*	Vigna unguiculata	Negative impact on cowpea resulting in root rot symptoms	Li *et al.* (2017)
Biotic constraint caused by *Fusarium oxysporum*	*Vigna unguiculata*	Negative impact on cowpearesulting in stem and root rot symptoms	(Shrestha *et al.*, 2016b)
Biotic constraint caused by *Fusarium proliferatum*	*Vigna unguiculata*	Negative impact on cowpea resulting in stem and dry root rot symptoms	Shrestha *et al.* (2016a)
Biotic constraint caused by Singly and Interactive effects of cowpea mosaic viruses	Vigna unguiculata	Negative impact on Rhizobium nodulating ability	Taiwo *et al.* (2014)
Biotic constraint caused by *Rhizoctonia solani*	*Vigna unguiculata*	Negative impact on cowpea resulting in collar rot and web blight symptoms	Vavilappalli and Celine (2014)
Biotic constraint caused by *Helminthosporium vignicola*	*Vigna unguiculata*	Negative impact on cowpea resulting in leaf spot disease symptoms	Sahoo and Beura (2019)
Biotic constraint caused by *Epicoccum nigrum*	*Vigna unguiculata*	Negative impact on cowpea resulting in leaf spot disease symptoms	Deepika *et al.* (2020c)
The abiotic constraint of Drought on cowpea Landrace (A55)	*Vigna unguiculata*	Reduction in net productivity and photosynthetic ability	Gomes *et al.* (2020)

The abiotic constraint of high temperature	*Vigna unguiculata*	Adverse impacts on physiology biochemistry and breeding traits in cowpea plant	Jha *et al.* (2020)
Biotic constraint caused by *Dactuliophora mysorensis* sp. nov	*Vigna unguiculata*	Zonate leaf spot disease	Deepika *et al.* (2020b)
Biotic constraint caused by *Nigrospora sphaerica*	*Vigna unguiculata*	Leaf spot disease	Deepika *et al.* (2021)
The abiotic constraint of high salinity	*Vigna unguiculata*	Adverse impacts on chlorophyl content and eventual death	Dong *et al.* (2019)
The abiotic constraint of high-temperature stress	*Vigna unguiculata*	Adverse impacts on plant development, with severe damage to vegetative and reproductive growth stages of cowpea	Barros *et al.* (2021)
The abiotic constraint of combined high salinity and temperature stress	*Vigna unguiculata*	Adverse impacts on plant development, with the germination and vigor of cowpea plant, impaired	Nunes *et al.* (2019)
Climate change limitation involving temperature and Relative humidity	*Vigna unguiculata*	Adverse impacts on the yield and development of cowpea plant as well as reduction in evapotranspiration	Cavalcante Junior *et al.* (2016)
Biotic constraints caused by *Diplodia seratia*	*Vigna unguiculata*	Wilt and necrosis adverse effects on cowpea	Swilling *et al.* (2020)

Table 3.3: Sustainable deployment of smart biotechnological techniques for the productivity enhancement of cowpea and some selected food crops

Beneficial microbial inoculants	Crop of interest	Bioactive roles of inoculants	References
Inoculation with Rhizobia strains and AMF species	*Glycine max*	Yield and nutrient improvement of soybean	Igiehon *et al.* (2021)
Inoculation with *Rhizobium* and Mycorrhizal Fungi species	*Glycine max*	Yield improvement of soybean Under drought stress	Igiehon and Babalola (2021)
Inoculation with *Trichoderma* Isolates	*Glycine max*	Biocontrol of destructive nematode of soybean	de Oliveira *et al.* (2021)
Bacillus sp. PS2 and PS10	*Zea mays*	Plant growth and Yield enhancement of Maize	Chaudhary *et al.* (2021)
Mixed inoculation of *Bacillus cereus* BI-8 and *Bacillus subtilis* BI-10	*Zea mays*	Plant growth and nutrient Yield enhancement of Maize	Fouda *et al.* (2021)
Azotobacter chroococcum	*Zea mays*	Soil health improvement and nutrient Yield enhancement of Maize	Song *et al.* (2021)
Co-inoculation of *Bradyrhizobia* strains	*Vigna unguiculata*	Growth improvement of cowpea	do Nascimento *et al.* (2021)
Application of different Microbial inoculants	*Wheat*	Improvement in wheat growth and soil microbiome diversity	Chen *et al.* (2021)
Inoculation with endophytic fungi *Nectria haematococca*	*Green gram*	Growth and nutritional improvement of Green gram	Muthukumar and Sulaiman (2021)
Inoculation with Potassium solubilizing B*acillus cereus*	*Potato*	Growth and yield improvement of potato	Ali *et al.* (2021)
Application of different Arbuscular Mycorrhizal fungi	*Cicer arietinum L*	Improving Arsenic metalloid tolerant and yield of chickpea	Garg and Cheema (2021)
Rhizobium leguminosarum RPN5	*Vigna unguiculata*	Used for cowpea yield and soil fertility improvement	Kumar et al., 2016
Pseudomonas sp.MX6	*Vigna unguiculata*	Used for cowpea growth promotion in metal contaminated soils	Shafique et al., 2017

Mutant of *Glomus sp* and *Trichoderma harzianum* (AMF60+TH)	*Vigna unguiculata*	Used for growth promotion and biocontrol of powdery mildew disease of cowpea	Omomowo *et al.* (2018)
Mutant strains of *Glomus versiforme* and *Trichoderma harzianum*	*Vigna unguiculata*	Used for growth promotion and biocontrol of powdery mildew disease of cowpea	Omomowo *et al.* (2020)
Bacillus spp	*Pearl Millet*	Used as biocontrol agent for fungal pathogens affecting Pearl millet	Kushwaha *et al.* (2020b)
Bacillus subtilis	*Oryza sativa*	Biocontrol agent for control of fungal disease of rice	Kumar et al., 2020
Bacillus pumilus strain JPVS 11	*Oryza sativa*	Improving growth/yield and salinity tolerance in rice	Kumar *et al.* (2021b)
Inoculation with *Piriformospora indica*	*Oryza sativa*	Improving yield and arsenic tolerance in rice	Ghorbani *et al.* (2021)
Single and co-inoculation with mycorrhiza	*Phaseolus vulgaris*	Improving yield and nutrition of snap bean	Beltayef *et al.* (2021)
Inoculation with single and co-inoculation with AMF and PSB	*Zea mays*	Improvement in productivity of maize	Pacheco et al., 2021
Inoculation with *Funneliformis mosseae*	*Triticum aestivum L*	Improving wheat productivity and enhancing soil health	Duan *et al.* (2021)
Single and co-inoculation with *Piriformospora indica* and *Pseudomonas putida*	*Triticum aestivum L*	Enhancement in growth and nutritional status of wheat	Abadi *et al.* (2021)
Genome wide association studies (GWAS)	*Vigna unguiculata*	Enhancement of drought tolerance of cowpea	Ravelombola *et al.* (2021)
Chitin-binding protein studies (CBV)	*Vigna unguiculata*	Toxic influence and reduction in larval mass and length of	Ferreira *et al.* (2021)

		Callosobruchus maculatus (Cowpea weevil)	
Genome wide association studies (GWAS), metal-analysis and Sequence homology combination	*Vigna unguiculata*	Identification of candidate genes for cowpea seed size enhancement	Lo *et al.* (2019b)
Synergistic effects of co-inoculation with different AMF isolates and *Sinorhizobium meliloti*	*Vigna unguiculata*	Enhancement of above ground biomass production and nitrogen content	Kavadia *et al.* (2021)
SSR typing for diversity assessment and nitrogen fixation potentials	*Vigna unguiculata*	Identification of SSR marker for nitrogen fixation and other Symbiosis-related traits	Mohammed *et al.* (2020)
Synergistic influence of *Trichoderma* and *Bradyrhizobia* on cowpea growth improvement	*Vigna unguiculata*	Enhancement of cowpea growth biomass and photosynthetic pigments	Mendes *et al.* (2020)
Proteomic approaches using miRNAs and Argonaute genes in response to CPSMV stress	*Vigna unguiculata*	Detection of miRNAs and genes that elicits response to CPSMV	Martins *et al.* (2020)
Transgenic cowpea plants response to *Maruca vitrata* legume pod borer	*Vigna unguiculata*	Improvement in prevention of damage cause by pod borer due to genetically engineered cowpea	Kumar *et al.* (2021a)
Deployment of Entomopathogenic fungi together with intercropping in managing *Aphis craccivora* infestation of cowpea	*Vigna unguiculata*	Reduction in damage cause by aphid infestation of cowpea	Mweke *et al.* (2020)
Deployment of conservation agricultural practices of no tillage and planting of cover crops	*Vigna unguiculata*	Improvement in soil carbon and nitrogen nutrient concentration, as well as good adaptation to water stress	Guzzetti *et al.* (2020)

Deployment of yeast isolates in controlling *Rhizoctonia solani* infestation in cowpea	*Vigna unguiculata*	Effective in the biocontrol of damping off and stem rot of cowpea plant caused by *R. solani*	de Tenório *et al.* (2019)
Deploying encapsulated *Pseudomonas libanensis* in alleviating cowpea drought stress	*Vigna unguiculata*	Encapsulation of the beneficial microbe, highlighted its positive impact for managing drought stress in cowpea	Souza-Alonso *et al.* (2021)
Application of embryonic axis explants for efficient regeneration, transformation and genome editing of cowpea	*Vigna unguiculata*	CRISPR/cas was used successfully to develop transgenic cowpea plantlet	Che *et al.* (2021)
Application of *Bacillus subtilis* Dcl1in cowpea plant as growth enhancer, biocontrol and abiotic stress abatement agent	*Vigna unguiculata*	Improvement in cowpea growth, biotic and abiotic stress effectors	Jayakumar *et al.* (2021)
Deployment of MgO nanoparticles in enhancing cowpea growth and controlling nematode infestation	*Vigna unguiculata*	Improvement in cowpea growth and control of root-knot nematode infestation	Tauseef *et al.* (2021)
Deploying *Bacillus sp* Fcl1as pesticide toxicity alleviating and growth promoting impact on cowpea plant	*Vigna unguiculata*	Improvement in cowpea growth and also toxicity alleviating effects of pesticide	Juby *et al.* (2021)
Application of *Bacillus cereus* NDRMN001 and *Kosakonia sp* MGR1 to improve cowpea growth and remediate heavy metal toxicity	*Vigna unguiculata*	Enhancement in the growth characteristics of cowpea plant and also remediation of heavy metal toxicity	Narayanan *et al.* (2021)
Inoculation of *Bradyrhizobium* and salicylic acid effects in mitigating water stress deficit in cowpea plant	*Vigna unguiculata*	Effective in the improvement of cowpea growth, proline content, superoxide dismutase and ascorbate peroxidase	de Andrade *et al.* (2021)
Inoculation using *Bradyrhizobium* BR3267 with phosphorus and potassium fertilizer improves cowpea growth	*Vigna unguiculata*	The combine inoculant treatment was effective in increasing cowpea yield and growth parameters	Emmanuel *et al.* (2021)
Seed inoculation using *Trichoderma harzianum* improves physiological quality of cowpea seeds	*Vigna unguiculata*	Trichoderma harzianum seed treatment was effective in improving cowpea seed germination speed index and percentage	Pimentel et al., 2021
Interactive influence of Bacillus subtilis that was co-inoculated with mine water on the physiological and	*Vigna unguiculata*	Bacillus subtilis co-inoculated with mine water, sequester heavy metals and improve nutritional content and growth of cowpea	Nevhulaudzi *et al.* (2020a)

nutritional growth enhancement of cowpea			
Influence of inoculation using dark septate endophytic fungi on cowpea productivity under salinity stress	*Vigna unguiculata*	Improvement in nutritional content and photosynthetic rate of cowpea plant	Farias *et al.* (2020)
Application of indigenous mycorrhizal and nano-Ti0_2 in reducing cowpea oxidative stress and Cd uptake	*Vigna unguiculata*	There was reduction in both the Cd metal uptake and oxidative stress of cowpea due to the co-inoculation treatment	Ogunkunle et al., 2020
Response of field grown cowpea to inoculation with *Bradyrhizobium*	*Vigna unguiculata*	Improvement in agronomic growth parameters of cowpea plant due to bioinoculant treatment	Ayalew et al., 2021
Seed inoculant treatments using rhizobacteria and mycorrhizal, improves the growth and nutrition of cowpea under water stress	*Vigna unguiculata*	Improvement in growth and nutritional content of cowpea due to mycorrhizal and rhizobacteria application via seed coating	Rocha et al., 2019

CHAPTER FOUR

4.0 DRAFT GENOME SEQUENCE OF ENDOPHYTIC *ASPERGILLUS MINISCLEROTIGENES* STRAIN NWUFE10 AND ITS MULTIFACETED ATTRIBUTES AS PLANT GROWTH PROMOTER

Abstract

A promising endophytic fungi strain (*Aspergillus minisclerotigenes* NWUFe10) with multiple attributes of plant growth enhancing potential was isolated from tissues of cowpea (*Vigna unguiculata* L. Walps). Morphological, molecular and biochemical characteristics of this potential bioinoculant confirmed its multifaceted potentials as a plant growth enhancing microorganism. The genomic insight of strain NWUFe10 revealed assembly size of 31.5Mb, the G+C content percentage was 49.04. The complete genomic features of *Aspergillus minisclerotigenes* strain NWUFe10 showed the presence of important gene clusters responsible for plant growth promotion like Siderophore, phosphate, indole, and other non-ribosomal peptides (NRPs).

4.1 Introduction

In recent times, the focus of most agro-products research theme is how to ensure agroecological crop productivity enhancement by looking at readily available, cheaper and natural resource based alternatives to synthetic agrochemicals (Oleńska *et al.*, 2020; Omomowo & Babalola, 2019). Endophytes are microbial colonizers of inner tissues and seeds of all living plants worldwide without causing any adverse effects on their plant host (Compant *et al.*, 2021; Rodriguez & Redman, 2008). Research studies have highlighted endophytic microorganisms as vital resources that have shown remarkable success in achieving plant growth promotion, phytopathogen biocontrol ability and also promoting environmental sustainability (del Carmen Orozco-Mosqueda & Santoyo, 2021). Endophytes have shown unique agro-biotechnological attributes that indicates they can be deploy as plant probiotics natural microbial resources for achieving sustainable agroecological crop improvement (Adeleke & Babalola, 2021a).

Aspergillus genera are filamentous fungi that are known as prolific producers of important secondary metabolites that include both beneficial and deleterious substances (Hung & Rutgers, 2016; Ortega *et al.*, 2020). Moreover, endophytic *Aspergillus* species capable of producing important secondary metabolites have been reported in literature (Ismail *et al.*, 2020; Lubna *et al.*, 2018). Although a lot of studies detailing endophytic *Aspergillus* species with plant growth promoting abilities have been reported, there is little information on the genes that are responsible for the growth promoting traits in this fungi, hence the need for the genomic analysis of this potential plant growth enhancing bioinoculant strain.

4.2 Materials and Methods

Aspergillus minisclerotigenes strain NWUFe10 was isolated following standard mycological protocol from the root tissues of healthy cowpea plant from farms in Mafikeng, North West province, South Africa. Morphological characteristics of *A. minisclerotigenes* showed whitish mycelium, floccose and velvety, dark sclerotia at the center with a reverse brownish-orange colour on PDA Petri-plate. The conidial heads are biseriate, and the conidiophore stipes are hyaline, coarsely roughened as visualized with microscopes. Standard invitro assay protocols was deployed in confirming the plant growth promoting characteristics of this fungi isolate, and these assay include phosphate solubilization, ammonia production, indole acetic acid production, siderophore production, exopolysaccharide production and hydrogen cyanide production, as well as Growth chamber assay to confirmed the growth promoting properties of the endophytic isolate (data unpublished).

4.2.1 Genotypical authentication and whole genome sequencing

For the genomic studies, actively growing mycelia in mycological broth was used in extracting the genomic DNA using zymoclean bacteria and fungi extraction kit following the directives from the manufacturer.The internal transcribe spacer (ITS) region, containing 18S rRNA gene, for fungi strain identification as well as key plant growth promoting genes like Gamma glutamyl phosphate reductase gene, Indole 3-glycerol phosphate synthetase gene, Siderophore synthetase acetyl transferase gene, Glucose -1- dehydrogenase gene and Diphthine-ammonia gene were amplified by polymerase chain reaction (PCR). The phylogeny inference was determined in relation to other fungal strains in GenBank repository through NCBI blast and Molecular Evolutionary Genetics Analysis (MEGA7) software (Kumar *et al.*, 2016). Overview of the complete genomic features of *Aspergillus minisclerotigenes* strain NWUFe10 was sequenced at Mr DNA laboratory, Shallowaters, Texas, U.S.A. by deploying Illumina NovaSeq 6000 platform. The libraries were prepared using Illumina DNA Prep, (M) Tagmentation library preparation kit (Illumina) following the manufacturer's user guide. 50 ng DNA was used to prepare the libraries. The samples underwent the simultaneous fragmentation and addition of adapter sequences. These adapters are utilized during a limited-cycle PCR in which unique indices were added to the sample. The sequences processing, reads quality check and trimming was done by deploying FastQC v.1.0.1 , Trimmomatic v0.36 , (Arkin *et al.*, 2016; Bolger *et al.*, 2014). The genomics standard features were determined using varied bioinformatics tools like glimmers 3, Prodigal, kmers V2, Kmers V1, Protein similarity, while genome annotation was done by applying RAST algorithm (Brettin *et al.*, 2015). Subsequently, genome assembling and annotation was done using SPAdes v.3.12.0, and Prokaryotic Genome Annotation Pipeline (PGAP) version (v4.7) (Aziz *et al.*, 2008; Nurk *et al.*, 2013). For detecting gene clusters, antiSMASH v6.0 (Blin *et al.*, 2021) was deployed. The circular view of the genome was created using PATRIC v3.6.12 (Wattam *et al.*, 2017).

4.3. Results

A total of 54 773634 reads was obtained, and the assembling resulted in 236 contigs that had base length of 2224084 and N50 value of 256596. The GC value of the genome was 49.04 %. Overall, the genes have 1633 distinct functions. The genes include 7137 genes with a seed annotation ontology. The coding gene was 63971, non coding repeat was 1030 while the number of rRNA was 172.

Strain identification and plant growth enhancing characteristics

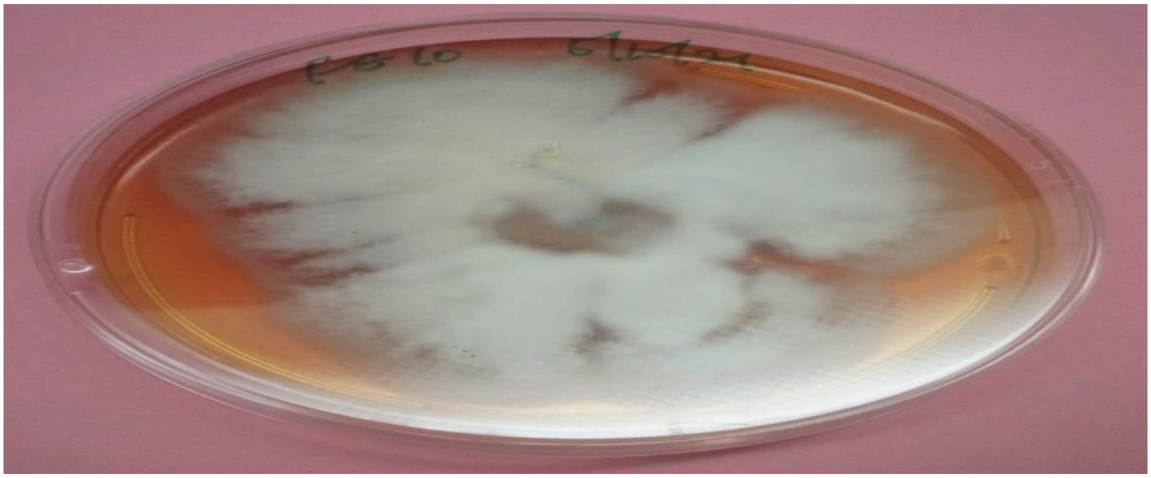


Figure 4.1: Growth of *Aspergillus minisclerotigenes* strain NWUFe10 on PDA Petri-plate

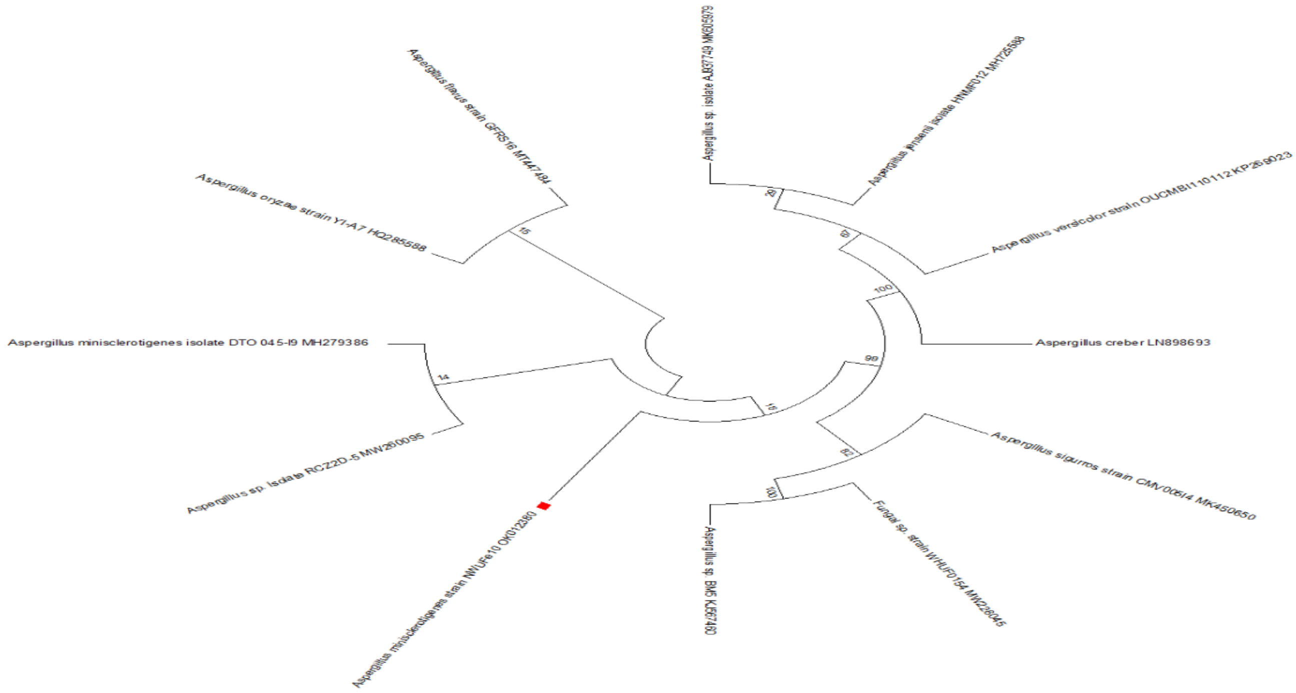
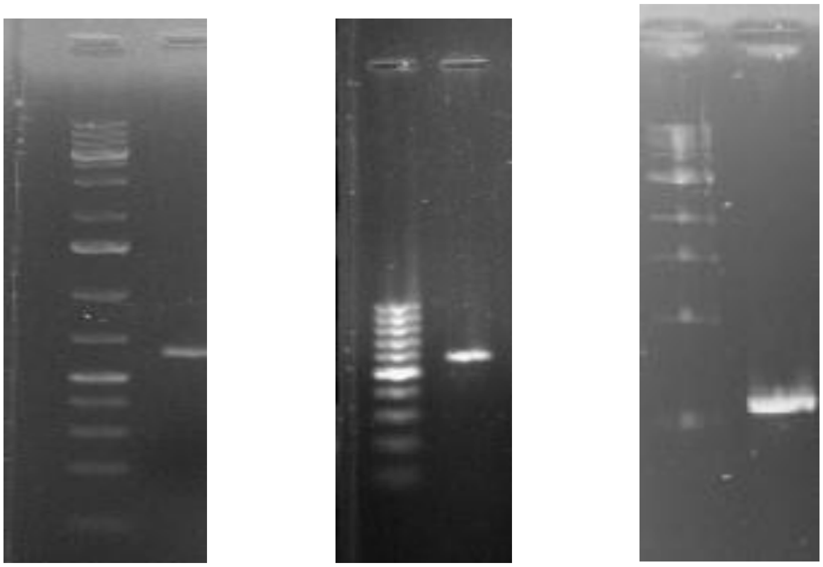


Figure 4.2: Phylogenetic relatedness of endophytic *Aspergillus minisclerotigenes* strain NWUFe10 with similar strains in GenBank deduced using maximum likelihood method



(A)18SrRNA (B) Asb (C) ACPHO

Figure 4.3: The 18S rRNA and plant growth promoting genes amplification of deoxyribonucleic acid (DNA) sequence of *A. minisclerotigenes* strain NWUFe10

Table 4.1: Genomic features of endophytic *Aspergillus minisclerotigenes* strains NWUFe10

Genome	Remarks
Size	31565265
GC value	49.04
N50	256596
L50	8
Number of contigs	236
Non coding rna	172
Number of coding sequences	63971
Protein coding genes	63971
Non coding repeat	1030
Genes with SEED annotation	7137

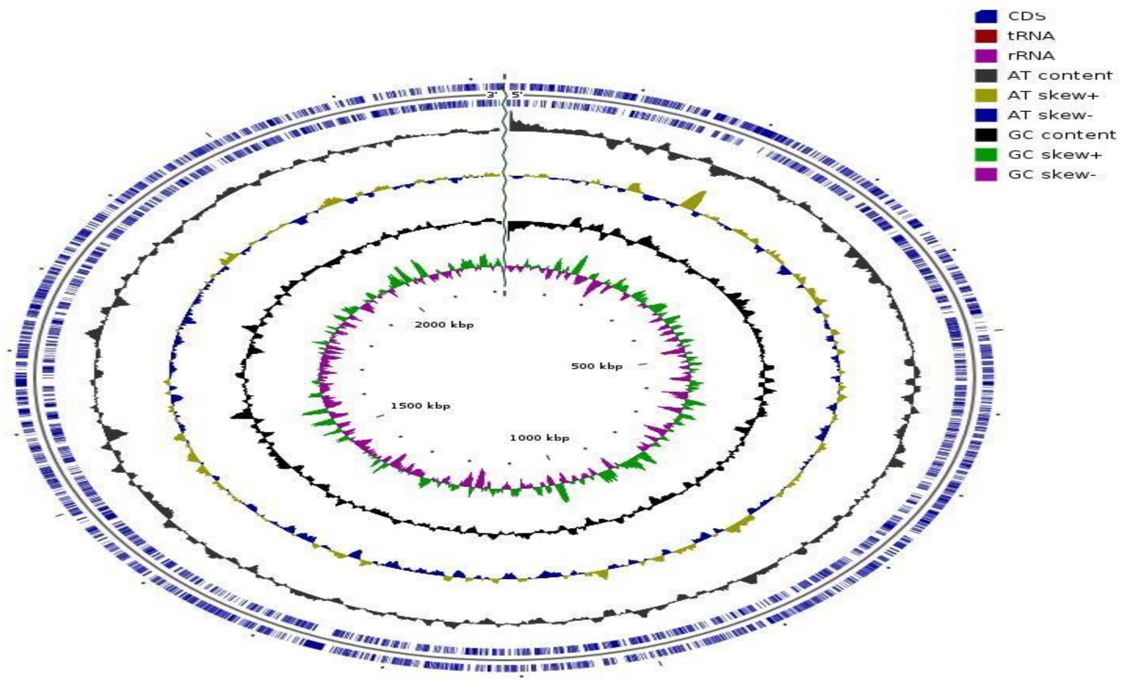


Figure 4.4 : The circular view of the genome of *Aspergillus minisclerotigenes* strain NWUFe10

Table 4.2: Growth enhancing attributes of endophytic *A. minisclerotigenes* strain NWUFe10

Plant growth promoting assay	Qualitative screening output	Quantitative screening output
Exopolysaccharide production	+++	
Ammonia production	+++	
Indole acetic acid (IAA) production	+++	16.27±1.22
Phosphate solubilization	++	33.65±0.43
Siderophore production	++	85.45±0.64
Biofilm formation	+	12.34±0.17
Acds production	+	

Key: Highly active = +++; High activity = ++; Slightly high = +; None active = -

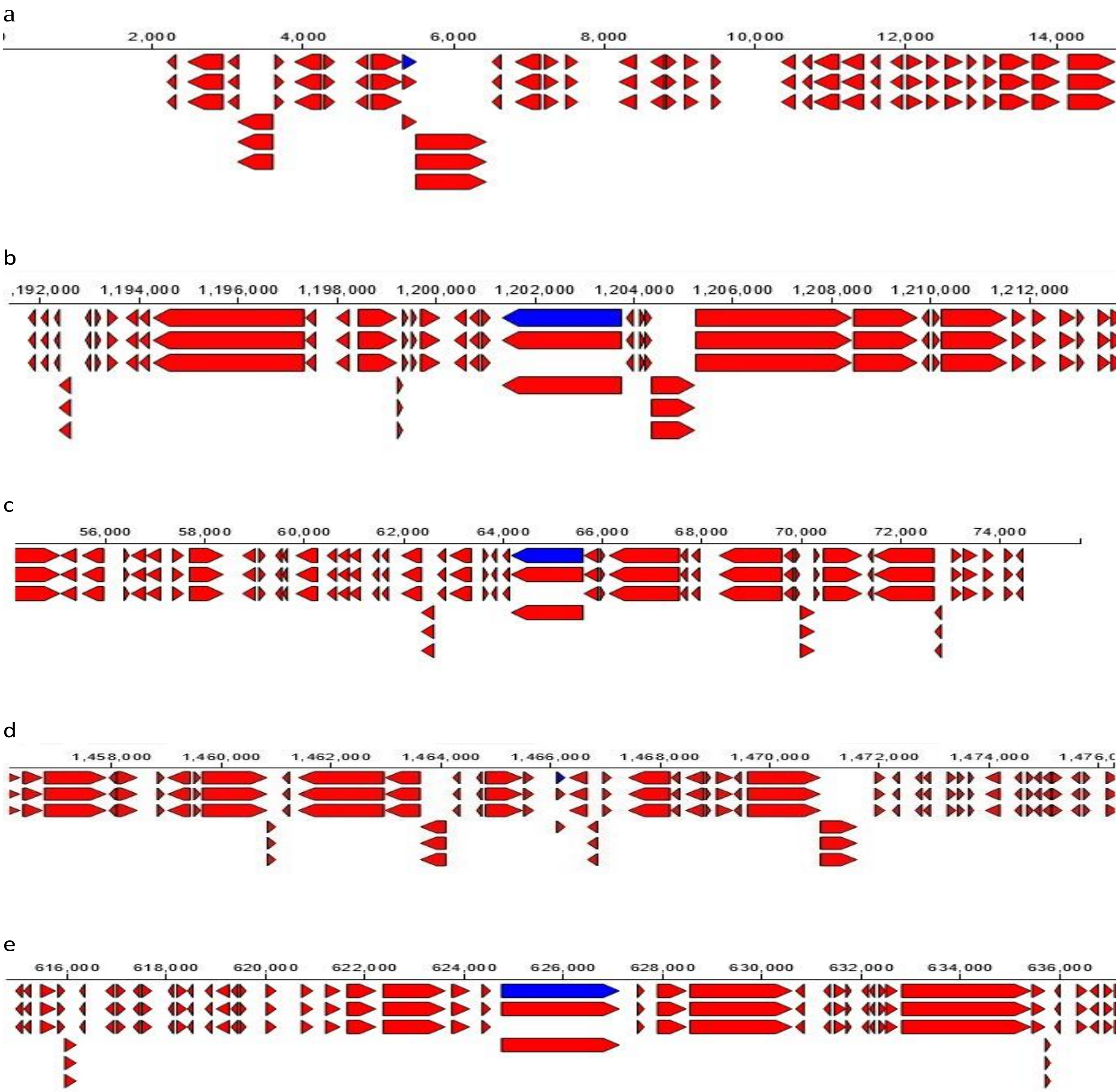


Figure 4.5: Plant probiotic influencing genes and their location in the genome of endophytic *Aspergillus minisclerotigenes* (a). Gamma glutamyl phosphate reductase gene (b). Indole 3-glycerol phosphate synthetase gene (c). Siderophore synthetase acetyl transferase gene (d). Glucose -1- dehydrogenase gene (e). Diphthine-ammonia gene.

4.4 Discussion and concluding remarks

Therefore, this study detailing the whole genomic sequence insight on *Aspergillus minisclerotigenes* strain NWUFe10 is a good foundation in identifying important plant growth promoting secondary metabolites, the genes involved in plant growth enhancement and lays good baseline information for further comparative genomics and pangenomics study. However, a simple assay like the use of neutral red desiccated coconut agar (NRDCA) cultural screening for determination of *Aspergillus* strain ability to produce aflatoxins or not is a necessity before moving forward to certify it as bioinoculating agent.

Data Availability

A. minisclerotigenes NWUFe10 has been deposited at the NCBI database under the 18S rRNA gene accession number OK012380, SAMN 24519002 and BioProject number PRJNA793232. The Sequence Read Archive raw reads are deposited under accession number SRR17406350.

The genome of *Aspergillus minisclerotigenes* strain NWUFe10 can be publicly assessed via: https://www.ncbi.nlm.nih.gov/sra/PRJNA793232

CHAPTER FIVE

5.0 GENOMIC INSIGHTS INTO ENDOPHYTIC STRAINS *STENOTROPHOMONAS GENICULATA* NWUBE21 AND *PSEUDOMONAS CARNIS* NWUBE30 FROM COWPEA WITH PLANT GROWTH-STIMULATING ATTRIBUTES

Abstract

Endophytic microbiota are being researched as a vital source of beneficial attributes that are of immense importance in enhancing agroecological crop productions. This is due to their presence in all living plants worldwide, and the fact that they have shown unique agro-biotechnological attributes indicate that they can be deployed as natural microbial plant probiotic resources for achieving sustainable improvements in agroecological crops. In this study, endophytic strains *Pseudomonas carnis* NWUBe30 and *Lysinibacillus xylanilyticus* NWUBe21 were isolated from cowpea tissues (seed and root) and their plant growth-promoting attributes were assessed by following standard biological procedures. The various biotechnological assays used in characterizing and confirming the plant growth-promoting characteristics of these isolated strains include their ability to solubilize phosphates, to fix nitrogenand to produce ammonia, indole acetic acid, siderophore, aminocarboxylase and deaminase acid, exopolysaccharide and hydrogen cyanide. Furthermore the 16S rRNA gene, as well as the key plant growth-promoting genes that they contain are amplified by polymerase chain reactions (PCRs). As part of this study a growth chamber assay was also deployed to confirm the growth -promoting properties of these endophytic isolates. The two endophytic isolates – *P. carnis* strain NWU Be30 and *S. geniculata* strain NWU Be21 were found to possess multiple plant-growth promoting attributes including the solubilization of phosphates, the production of auxin, siderophore, hydrogen cyanide, exopolysaccharide, ammonia and acids. The various plant growth-promoting genes were amplified by the PCR at their expected base pair value. In order to further elucidate the agronomically enhancing biotechnological potency of this isolate, the genome of the organism was sequenced using the Illumina NovaSeq 6000 systems platform. The genome analysis conducted in terms of the bioinformatics protocol revealed genes responsible for promoting plant growth phytopathogen resistance and biosynthetic gene clusters all of which are involved in the synthesis of various secondary metabolites. Amongst other important genes that are present in the genome, putative genes are responsible for plant growth enhancement. They play a role, amongst others, phosphate solubilization, auxin production and siderophore production. Genes responsible for formation of biofilm, are also present in the genome. This study highlights those agronomic traits improving the biotechnological potential of both *Stenotrophomonas geniculata* strain NWUBe21 and *Pseudomonas carnis* strain NWU Be30 that can be harnessed to achieve improvements in sustainable agroecological crop production.

Keywords: whole genome explorations; illumina sequencing platform; plant growth-enhancing genes; endophytic *Stenotrophomonas geniculata*; endophytic *Pseudomonas carnis*; agroecological crop improvement; leguminous crop—*Vigna unguiculata*; plant probiotic endophytes

5.1 Introduction

The quest for achieving sustainable and an environmentally balanced improvement in food productivity, food safety and security in a challenging; and climatic changing world of global vulnerabilities is of utmost importance. In the light of the crisis around the world - Corona virus, disruptions in business supply-chain logistics, war and famine outbreaks, the task of enhancing crop productivity through agroecologically balanced, renewable microbial resources that are sustainable, cheaper and ecofriendly is an attractive research focus theme (Berendsen *et al.*, 2018; Kumawat *et al.*, 2022; Morella *et al.*, 2020). Research into the deployment of biostimulants, biopesticides and biofertilizers as effective bioinoculating agents is on the upward trend (Barea, 2015; Kumawat *et al.*, 2021). These bioinoculating agents improve crop productivity as an alternative/complement to synthetic agrochemicals as crop production inputs to achieve sustainable agroproducts in the form of enhanced outputs. Therefore, in order to bioprospect and characterize potent bioinoculants, high throughput and /advanced sequencing technologies are being deployed as a necessity in characterizing and elucidating the potential of microbial strains with better prospects as bioinoculating agents. Hence, the genomic analysis of these microbial agents, throughthe entire body of genomic sequencing technology is of utmost importance in characterizing the genomic potential of microbial resources that have shown appreciable prospects as potential bioinoculants that could in fact be applied as plant-improving microbial strains and could thus achieve higher crop productivity.

Cowpea *Vigna unguiculata* L. Walpers is of immense benefit to humankind, biodiversity conservation, soil health and environmental sustainability. It is also highly beneficial to the animal husbandry/fodder industries across the globe, but most importantly in the tropical climatic regions of the globe (Ayalew *et al.*, 2021a; Cardona-Ayala *et al.*, 2020; Omomowo & Babalola, 2021).

Likewise, endophytic microorganisms have beneficial associations with all living plants through their presence in the innermost tissues of their host plants, and impotantly without harmful consequences (Afzal *et al.*, 2019; Compant *et al.*, 2021; Omomowo & Babalola, 2019).

Owing to the positive influence of most endophytic microbes in their host plant interrelationships, there are numerous literature sources on their positive attributes as plant immune boosters, in their biocontrolling activities and their fight against phytopathogens and in their contributions to improving plant growth (Adeleke *et al.*, 2022a; del Carmen Orozco-Mosqueda & Santoyo, 2021; Kaewkla *et al.*, 2022).

Thus it can be said that, the research in recent times on endophytic microbial resources is on the upward trend. The highly potent and superior biopesticidal, biostimulating and biofertilizing eco-friendly natural resources that are currently accessible could infact be deployed as complements or alternatives to synthetic agrochemical usage in crop production (Ahmad *et al.*, 2022; Cui *et al.*, 2022).

Hence, following on the standardized genomic protocol and bioinformatics downstream analysis, the elucidation of the genomic features and potentialities of two endophytic bacterial strains from cowpea tissues with their multifaceted plant-improving attributes, is highlighted in this study.

5.2 Materials and methods

5.2.1 The Isolation, morpho-genomic authentication and plant-growth-stimulating screening of cowpea endophytic bacterial isolates

Standardized microbiological protocols were followed in the course of isolating, characterizing, and assessing growth-improvement both under *in-vitro* onditions and in growth chamber study. In brief, 33 endophytic bacterial strains were isolated from cowpea seed and root tissue; the isolates were then subjected to morphological, biochemical, molecular, as well as plant growth- improving protocols. Following these experimental processes, two endophytic bacterial strains namely *Stenotrophomonas geniculata* NWUBe21 and *Pseudomonas carnis* NWUBe30, both displaying highly potent growth-enhancing traits, were selected for further genomic exploration. The standardized *invitro* protocols that were deployed in confirming the plant growth- promoting characteristics of these bacterial endophytic isolates, and the various assays and methodologies deployed in achieving these objectives include phosphate solubilization (Nautiyal, 1999), and the production of ammonia (Cappucino & Sherman, 1992) indole acetic acid (Matsuda *et al.*, 2018), siderophore (Schwyn & Neilands, 1987), exopolysaccharide production (Khan & Bano, 2019), and hydrogen cyanide (Dinesh *et al.*, 2015), as well as the fomation of biofilm (Allkja *et al.*, 2021). The aforementioned functions were performed in addition to analysis that was conducted in a growth chamber to confirm the growth-promoting properties of the endophytic isolates (data unpublished). For the molecular studies, the active growth overnight of a nutrient broth culture was performed to extract the genomic DNA. Zymoclean bacteria and a fungi extraction kit were used and the directives from the manufacturer were meticulously followed. The ribosomal DNA (rDNA), containing the 16S rRNA gene for identifying the bacterial strains as well as selected key plant growth- promoting genes, was amplified by using both universal and specific primers (Chen *et al.*, 2016; Fukuda *et al.*, 2016; Raddadi *et al.*, 2008) to orchestrate a polymerase chain reactions (PCR). The phylogenetic relations to other bacterial strains in the GenBank repository were determined by deploying NCBI blasting and Molecular Evolutionary Genetics Analysis (MEGA7) software (Kumar *et al.*, 2016).

5.2.2 Genomic DNA extraction, whole-genome sequencing and annotation

A miniprep DNA extraction kit from ZR (Zymo Research Company, USA) was used to obtain from the DNA from the cultures in the liquid medium that had been grown overnight. The quality check of the extracted DNA was assessed using gel electrophoresis and Nanodrop spectrophotometry (Thermo Fisher, USA). Genomic sequencing was carried out in the USA at the Molecular Research Laboratory (MR DNA) in Shallowater, Texas, using the Novaseq 6000 illumina platform. Library preparation was carried out using the DNA Prep, (M) Tagmentation library preparation kit (Illumina) and by following the manufacturer's step-wise guide. Fifty (50) ng DNA was used in preparing the libraries. The samples simultaneously underwent fragmentation and the addition of adapter sequences. These adapters were utilized during a limited-cycle PCR in which unique indices were added to the sample. Following the library preparation, the final concentration of the libraries was measured using the Qubit® dsDNA HS Assay Kit (Life Technologies), and the average library size was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies). The initial and final concentration of libraries for *Stenotrophomonas geniculata* NWUBe21 and *Pseudomonas carnis* NWUBe30 amounted to (108.0 ng/µL: 318.0 ng/µL) and (30.40 ng/µL: 21.00 ng/µL) respectively. The average library sizes for *Stenotrophomonas geniculata* NWUBe21 and *Pseudomonas carnis* NWUBe30, were 704 bp and 735 bp respectively. The libraries were then pooled in equimolar ratios of 0.6nM, and sequenced in terms of paired ends for 300 cycles using the NovaSeq 6000 system (Illumina). Since the post sequencing processing entails use of different platforms, the bioinformatics parameters deployed were set at the default. The Department of Energy Systems biology knowledgebase (Kbase) was the main platform that was deployed (Cottingham, 2014). The trimming of the sequences read, the reads quality assessment was determined using Trimmomatic v0.36, FastQC v.1.0.1,(Arkin *et al.*, 2016; Bolger *et al.*, 2014). The genome assembling and annotation were achieved using SPAdes v.3.12.0, and (PGAP) version (v4.7), i.e the Prokaryotic Genome Annotation Pipeline (Aziz *et al.*, 2008; Nurk *et al.*, 2013). For genome mining in order to detect important metabolites and their biosynthetic gene clusters, the antiSMASH version 6.0 (Blin *et al.*, 2021) was used. Using subsystems Technology (RAST version 2.0) and PATRIC version 3.6.12 , rapid annotation was used to determine the functions of the genes (Overbeek *et al.*, 2014; Wattam *et al.*, 2017).

5.3 Results

5.3.1 Identification, characteristics and plant-growth stimulating attributes of endophytic strains

The result obtained from carrying out the exercise to determine the morphological and molecular characteristics of the isolated endophytic strains indicated that the two isolates are *Stenotrophomonas geniculata* strain NWUBe21 and *Pseudomonas carnis* strain NWUBe30. The Gram reaction for *S. geniculata* NWUBe21 indicated that it is Gram-positive and rod in shape while *P. carnis* strain NWUBe30 was found to be Gram-negative and rod in shape. The two endophytic strain possess the ability to use starch, maltose, glucose, fructose and sucrose. They were also found to be nitrate reducer and oxidase positive. Moreover, the biochemical profile of the isolates revealed their ability to use citrate as a carbon source, and that they are catalase and nitrate positive. Both organisms are also indole and Voges-Proskaeur negative, and do not ferment mannose, sorbitol, melibiose, maltose and lactose. The ribosomal ribonucleic acid (rRNA) 16S gene amplification of *S. geniculata* NWUBe21 and *P. carnis* strain NWUBe30 yielded the expected 1400 bp amplicon size, also the expected base size for plant-growth-improving glucose dehydrogenase (GCD) and petrobactin siderophore. The (Asb) biosynthetic gene was amplified

(Figure 5.1). The phylogeny relatedness of the two endophytic strains *S. geniculata* NWUBe21 and *P. carnis* NWUBe30 with other closely related bacterial strains in the Genbank is presented in (Figure 5.2 and 5.3), which shows 100% similarity with other similar strains. The beneficial *invitro* plant-growth stimulating attributes are likewise represented in (Table 5.1).

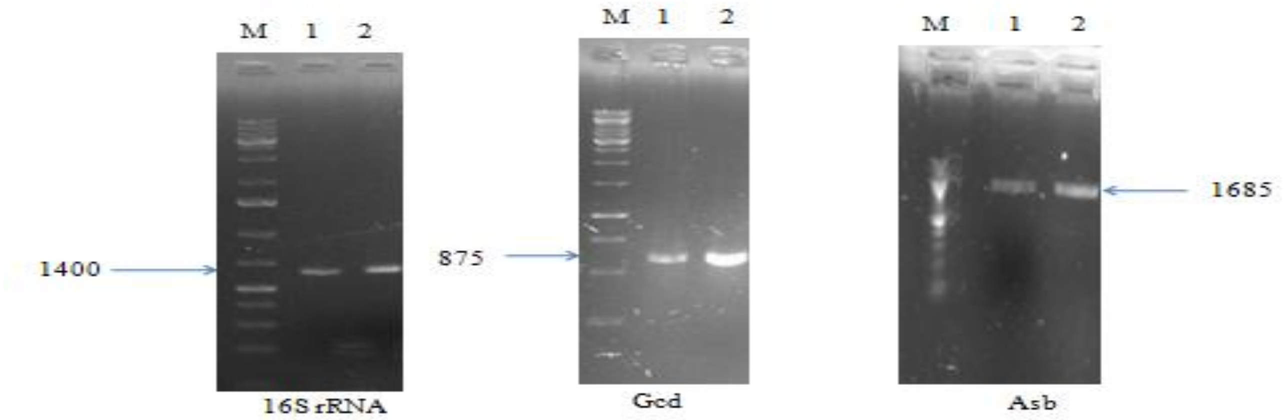


Figure 5.1: Endophytic bacterial strains *S. geniculata* NWUBe21 and *P. carnis* NWUBe30 amplified genes for 16S rRNA, the glucose dehydrogenase gene (Gcd) and the petrobactin siderophore biosynthetic gene (Asb).

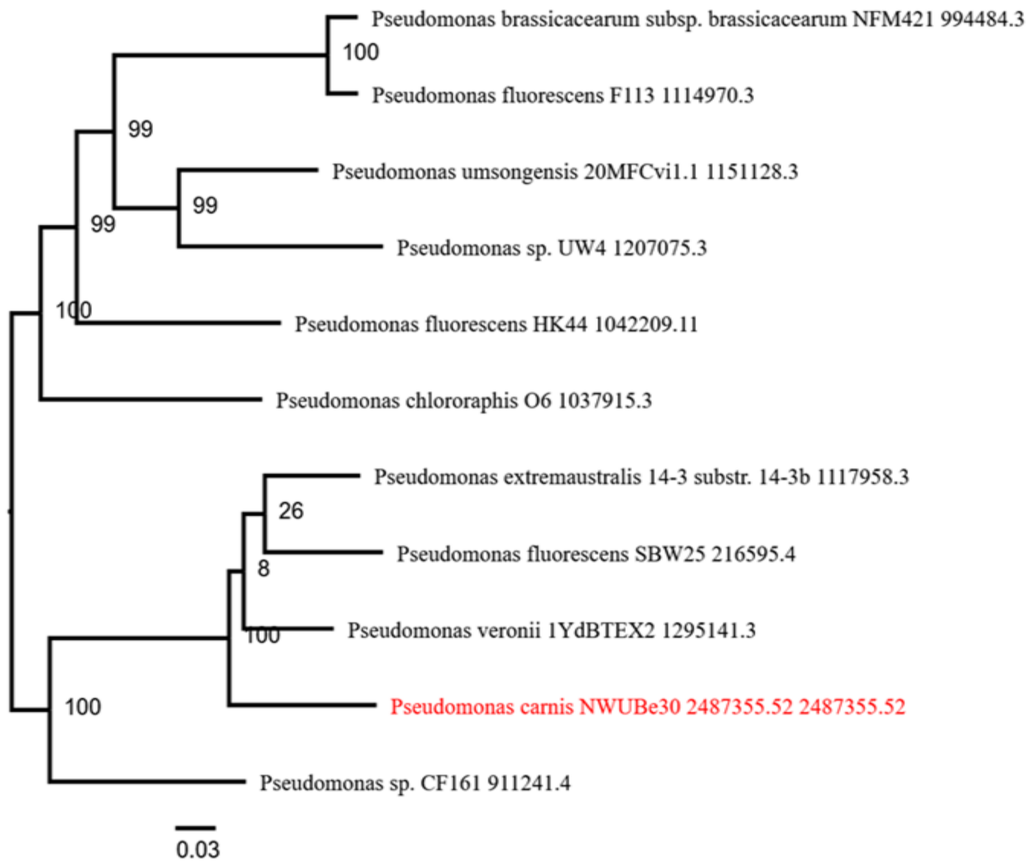


Figure 5.2: Phylogenetic relatedness of endophytic *Pseudomonas carnis* strain NWUBe30 with other isolates in GenBank.

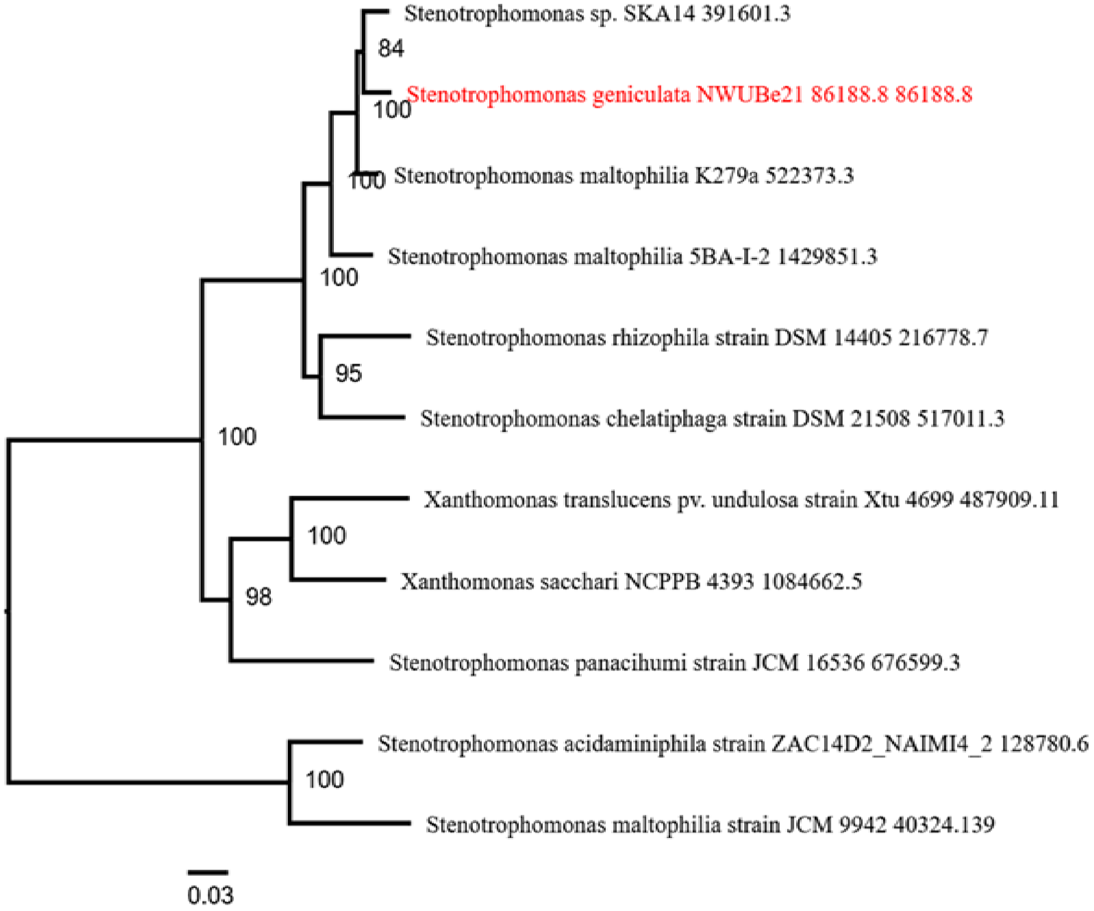


Figure5.3: Phylogenetic relatedness of endophytic *Stenotrophomonas geniculata* strain NWUBe21 with other isolates in GenBank.

Table 5.1**:** Growth promoting *in-vitro* attributes of endophytic bacteria *P. carnis* NWUBe30 and *S. geniculata* NWU Be21 strains

Endophytic bacteria	*P. carnis* NWUBe30		*S. geniculata* NWUBe21	
Plant-growth promoting assay	Qualitative screening output	Quantitative screening output	Qualitative screening output	Quantitative screening output
Exopolysaccharide production	++		++	
Ammonia production	+++		++	
Indole acetic acid (IAA) production	+++	17.46±0.08	+++	15.67±0.05
Phosphate solubilization	++	35.85±0.12	++	32.15±0.05
Siderophore production	++	898.25±0.23	++	89.60±0.42
Biofilm formation	+	10.46±0.11	+	11.29±0.03
Acds production	+		+	
Hydrogen cyanide production	++		++	

Key: Highly active = +++; High activity = ++; Slightly high = +; None active = -

5.3.2 Genomic features of endophytic *Pseudomonas carnis* strain NWUBe30 and *Stenotrophomonas geniculata* strain NWUBe21

The genome of *Pseudomonas carnis* strain NWUBe30 was assembled into 23 contigs consisting of 5,901,107 bp and an average G+C content of 60.2%. The total genes that was predicted amounted to 6306, while pseudogenes was 280 and tRNA operons 87. In addition, the genomic features of Stenotrophomonas geniculata strain NWUBe21 were assembled into 961 contigs and a bp size of 5120194, with the G+C percentage content being 64.79. More features of the genome are presented in (Table 2). The RAST annotation categorises the genes into 427 and 377 subsystems for Stenotrophomonas geniculata strain NWUBe21 and Pseudomonas carnis strain NWUBe30 respectively. An overview of these two genomes as shown in (Figure 5.4 and 5.5) was created by means of the PATRIC online platform. The circular view shows the contigs and the coding and non-coding features, the antimicrobial resistance genes, the drug targets and the G+C content of the genome. Different genes for the respective metabolisms namely carbohydrate and protein, amino acid derivatives, aromatic compounds, iron and its acquisition secondary metabolisms and several others, and also for stress response and membrane transport were present. AntiSMASH predicted the presence of 15 biosynthetic gene clusters in the genomes of Pseudomonas carnis strain NWUBe30 and for Stenotrophomonas geniculata strain NWUBe21 genome, It also predicted six biosynthetic gene clusters that are responsible for secondary metabolite synthesis. The predicted genes included fengycin, lassopeptide, siderophore, viscosin, pyoverdin, arylpolyene, hserlactone and betalactone (Figure 5.6 and 5.7).

Table 5.2: Genomic overview of endophytic strains *S. geniculata* NWUBe21 and *P. carnis* NWUBe30

Genome	*S. geniculata* strain NWUBe21	*P. carnis* strain NWUBe30
Size	5120194	5901107
GC value	64.79	60.2
N50	13089	401656
L50	105	6
Number of contigs	961	23
Number of subsystems	427	377
Number of coding sequences	4986	5300
Number of tRNA	63	52

Number of rRNA	5	3
Non coding repeat	169	189
Genes with SEED annotation	3934	2117

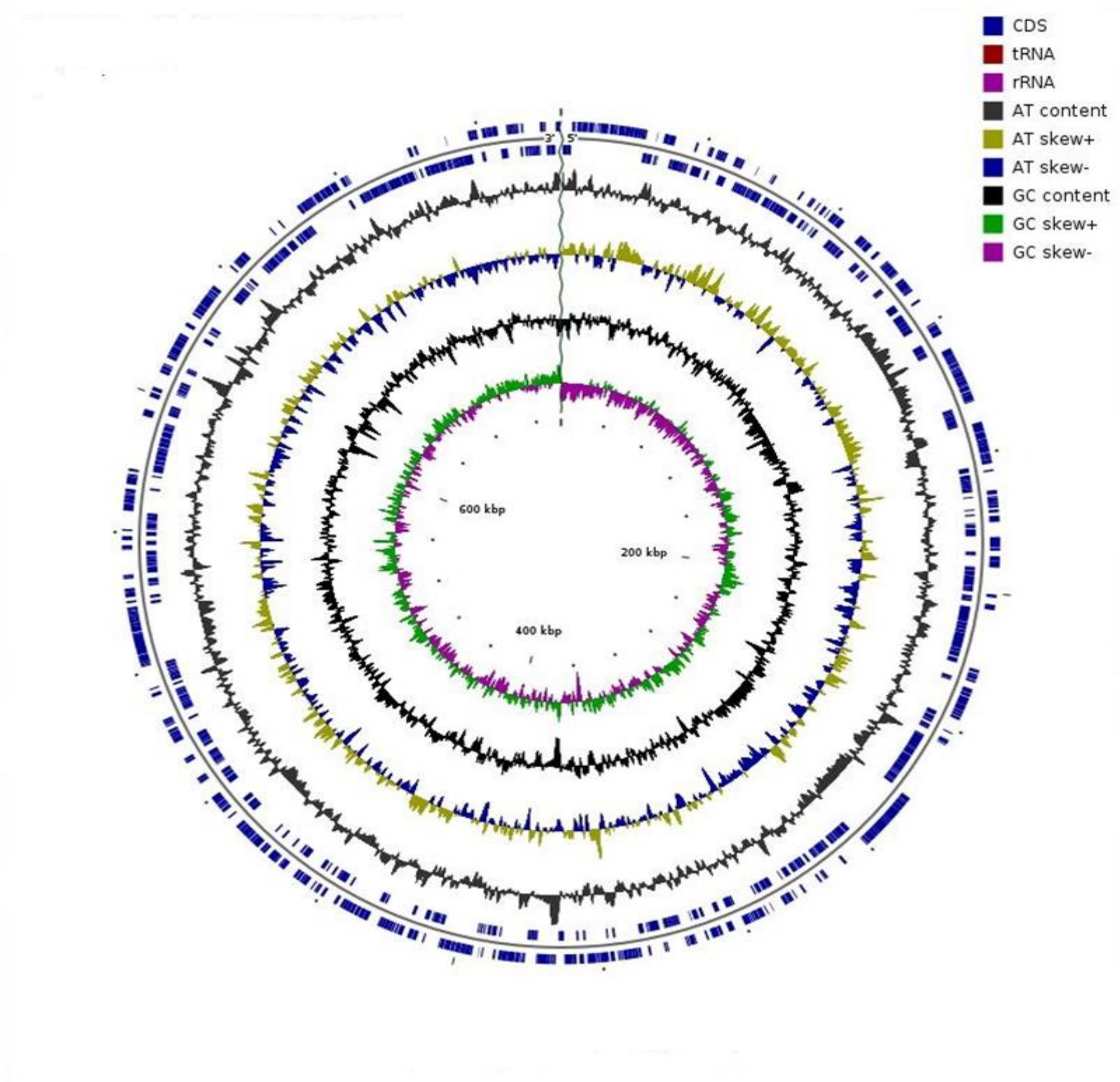


Figure 5.4: Circular overview of the genome of endophytic *Pseudomonas carnis* strain NWUBe30

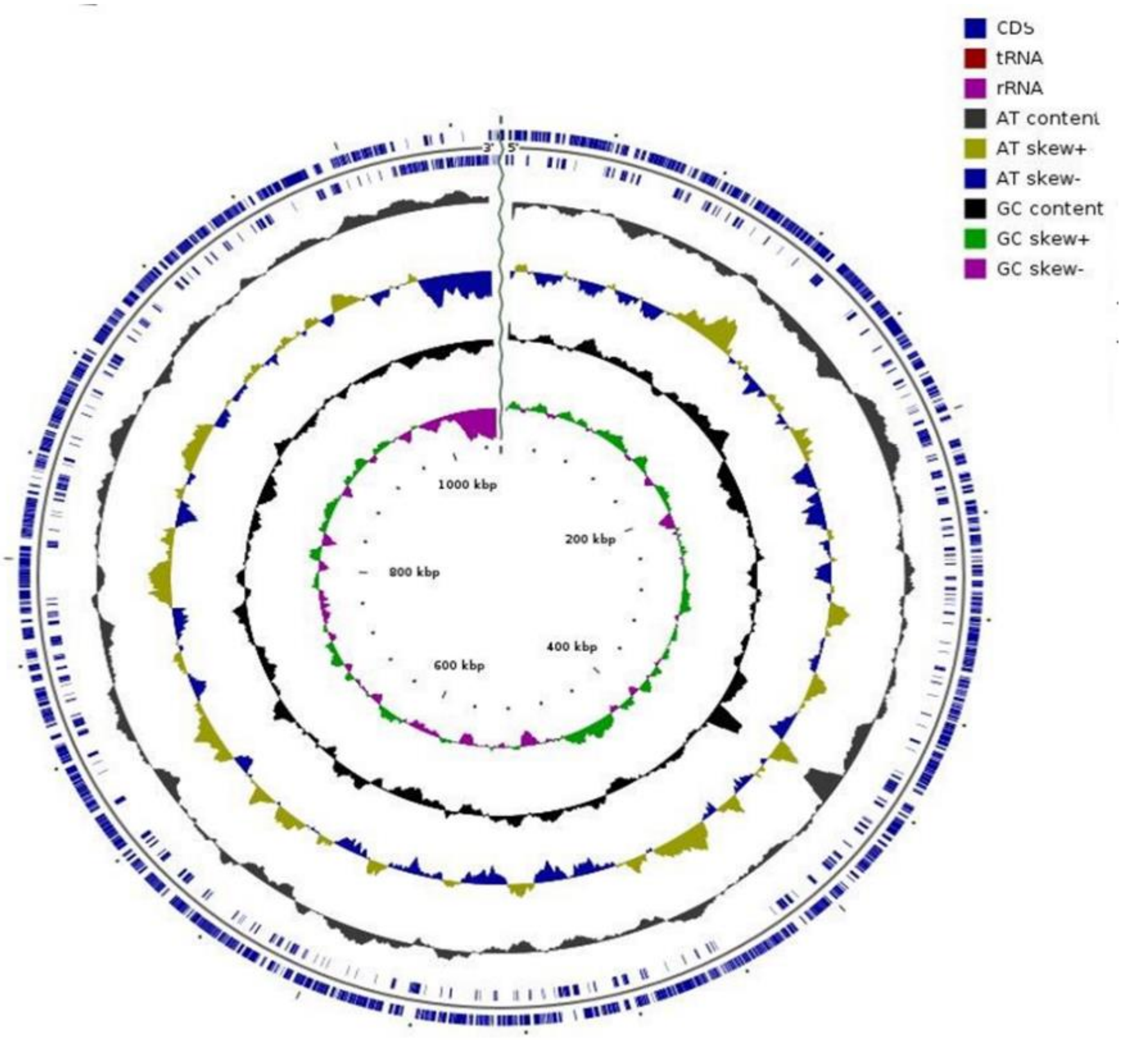
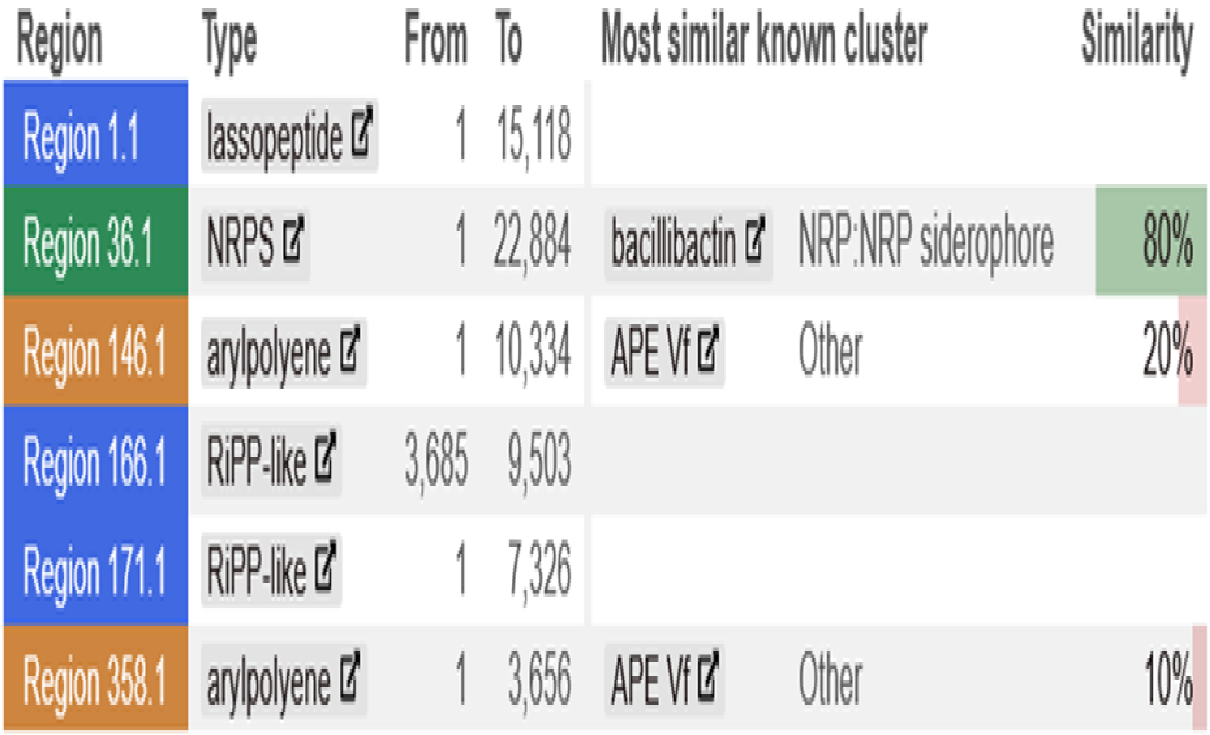


Figure 5.5: Circular overview of the genome of endophytic *Stenotrophomonas geniculata* strain NWUBe21

Region	Type	From	To	Most similar known cluster		Similarity
Region 1.1	RiPP-like	155,250	166,095			
Region 1.2	redox-cofactor	672,575	694,722	lankacidin C	NRP + Polyketide	13%
Region 2.1	arylpolyene	270,954	314,529	APE Vf	Other	40%
Region 2.2	NRPS-like	652,869	678,749	fragin	NRP	25%
Region 3.1	siderophore	223,605	235,530			
Region 3.2	NRPS	325,134	378,030	pyoverdin	NRP	10%
Region 3.3	NAGGN	411,424	426,287			
Region 3.4	hserlactone	501,435	522,010			
Region 4.1	NRPS-like, betalactone	314,009	357,485	pyoverdin	NRP	1%
Region 5.1	NRPS	101,465	167,357	pyoverdin	NRP	11%
Region 7.1	terpene	110,842	133,067			
Region 10.1	NRPS	87,482	151,717	viscosin	NRP	43%
Region 12.1	RiPP-like	6,218	17,096			
Region 13.1	NRPS	53,810	100,079	tolaasin I / tolaasin F	NRP:Lipopeptide	50%
Region 15.1	betalactone	57,611	80,785	fengycin	NRP	13%

Figure 5.6: *Pseudomonas carnis* strain NWUBe30 biosynthetic gene clusters metabolites from antiSmash



Region	Type	From	To	Most similar known cluster		Similarity
Region 1.1	lassopeptide	1	15,118			
Region 36.1	NRPS	1	22,884	bacillibactin	NRP:NRP siderophore	80%
Region 146.1	arylpolyene	1	10,334	APE Vf	Other	20%
Region 166.1	RiPP-like	3,685	9,503			
Region 171.1	RiPP-like	1	7,326			
Region 358.1	arylpolyene	1	3,656	APE Vf	Other	10%

Figure 5.7: *Stenotrophomonas geniculata* strain NWUBe21 biosynthetic gene clusters for secondary metabolites from antiSmash

5.3.3. Abundance of plant growth improving genes in the genomes of endophytic bacteria strains

The genomic annotation, namely, genome mining and PCR exploration of the genetic composition of the two endophytic bacteria confirmed the presence of diverse plant growth stimulating genes in their genomes. The presence of different genes, as well as the functional/metabolic roles that are responsible for the plant growth stimulation of the endophytic bacteria were predicted using both the Kbase and RAST server for the genome annotation. Thus, the highlighted results is presented (Table 5.3, Figures 5.8, 5.9, 5.10 and 5.11).

Table 5.3: Plant growth stimulating genes in the genome of *P. carnis* strain NWUBe30 and *S. geniculata* strain NWUBe21

Contigs tag	Product role	Gene	Pathway
Stenotrophomonas geniculata NWUBe21 Node_255	Biofilm PGA outer membrane secretin	PgaA	Biofilm Adhesin biosynthesis
Node_21	Indole-3-Pyruvate decarboxylase	IPDC	Pyruvate metabolism
Node_17	Intermediate for the synthesis of Tryptophan	Indole-3-glycerol phosphate synthase	Tryptophan biosynthesis
Node_1	Glucose-6-phosphate 1-dehydrogenase	GCD	Phosphate pentose
Node_120	Ferri-Bacillibactin esterase (iron transport and uptake)	BesA	Iron metabolism
Node_67	Ammonia/Ammonium transporter	amtB	Ammonia metabolism
Node_39	Phosphoribosylanthranilate isomerase	trpC	Tryptophan biosynthesis
Node_89	Exopolyphosphatase monomer	Ppx	Phosphorus metabolism

Node_20	Alkaline phosphatase	phoA2	Organic phosphorus metabolism
Node_5	Nitrate reductase	narG	Nitrate metabolism
P. carnis NWUBe30 Node_2	Biofilm PGA synthesis auxillary protein	PgaD	Biofilm Adhesin biosynthesis
Node_5	Pellicle/biofilm biosynthesis protein	PslA	Biofilm Adhesin biosynthesis
Node_5	Iron siderophore receptor protein	FecA	Iron metabolism
Node_2	Nitrogen regulation protein	NtrC	Nitrogen metabolism
Node_10	Exopolysaccharide protein	ExoZ	Polysaccharide degradation
Node_8	Auxin efflux carrier family protein		Auxin biosynthesis
Node_8	Tryptophan synthase	trpA	IAA production
Node_6	Nitrogen fixing	NifU	Nitrogen fixation
Node_6	Ammonia monooxygenase		Ammonia metabolism
Node_1	Indole-3-glycerol phosphate synthase		Tryptophan biosynthesis

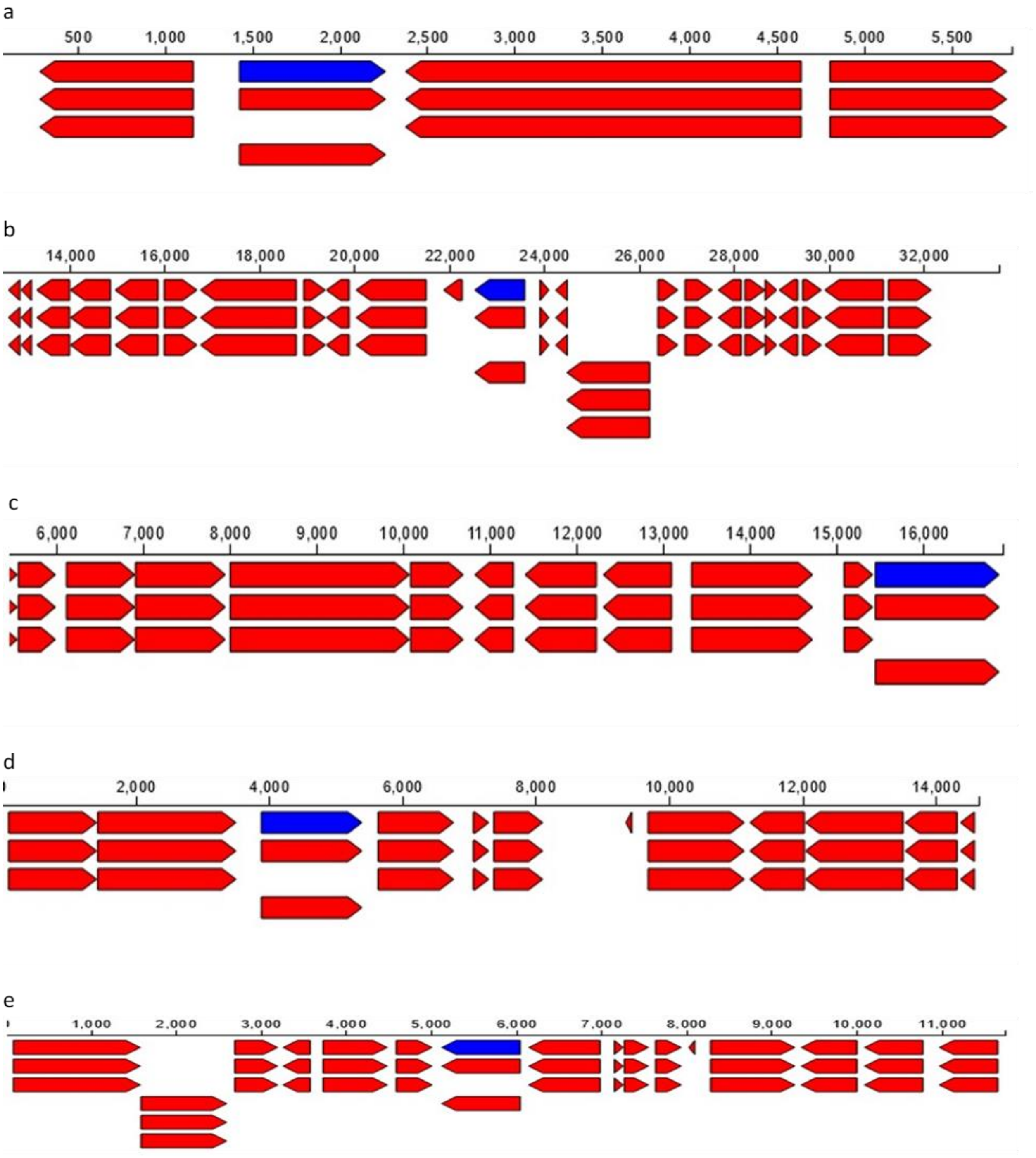


Figure 5.8: Annotation of plant probiotic-influencing genes and their location in the genome of endophytic *Stenotrophomonas geniculata* strain NWUBe21 (a). Acid phosphatase gene (b). Alkaline phosphatase gene (c). Ammonia/Ammonium transporter gene (d). Exopolyphosphatase gene (e). Ferribacilibactin gene

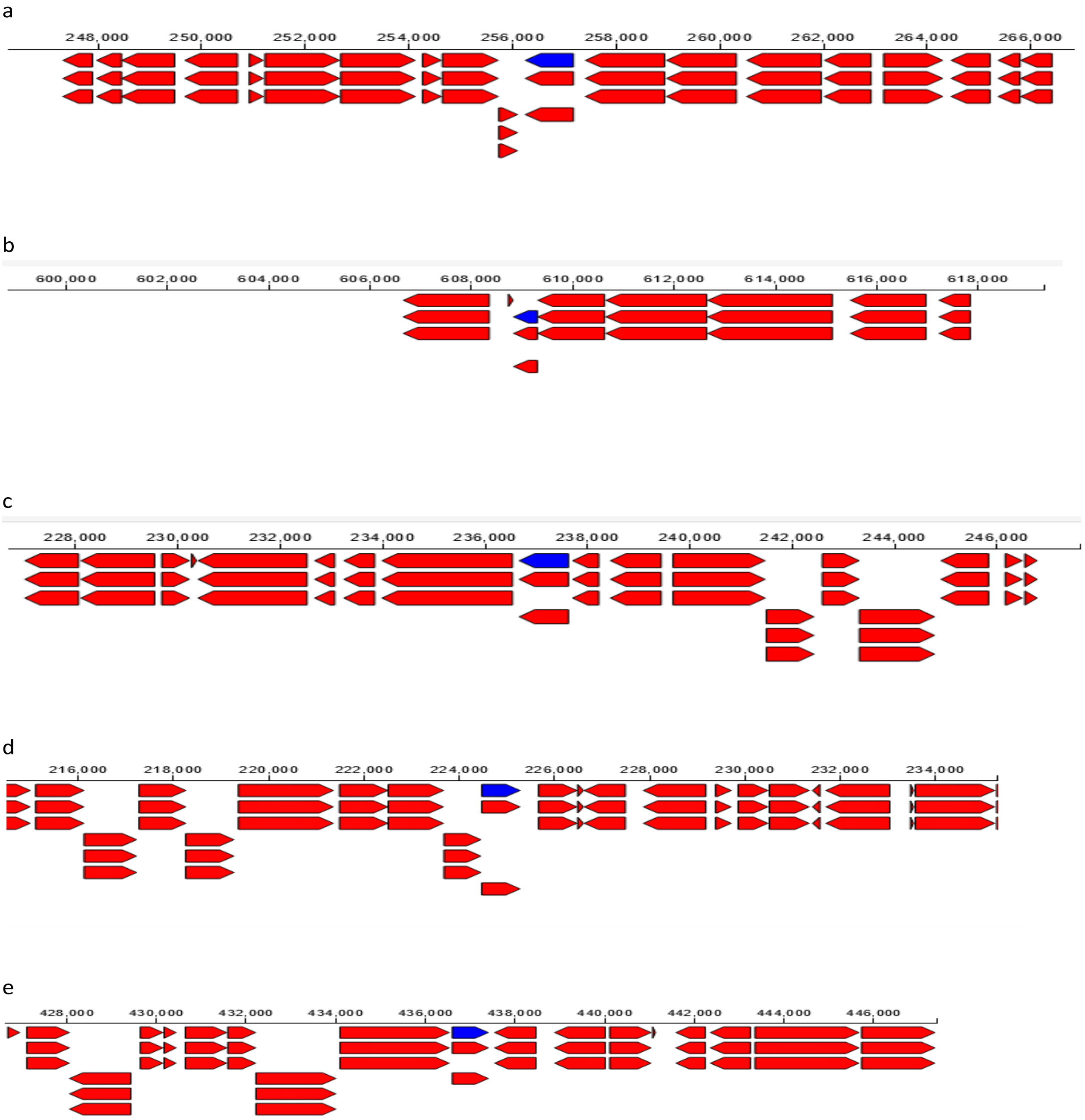


Figure 5.9: Annotation of plant probiotic influencing genes and their location in the genome of endophytic *Pseudomonas carnis* NWUBe30 (a). Auxin efflux carrier protein gene (b). Biofilm PGA auxillary protein gene (c). Iron sideropohore protein gene (d). Nitrogen fixing nifU gene (e). Pyrroloquinoline quinone (Coenzyme PQQ) biosynthesis protein gene

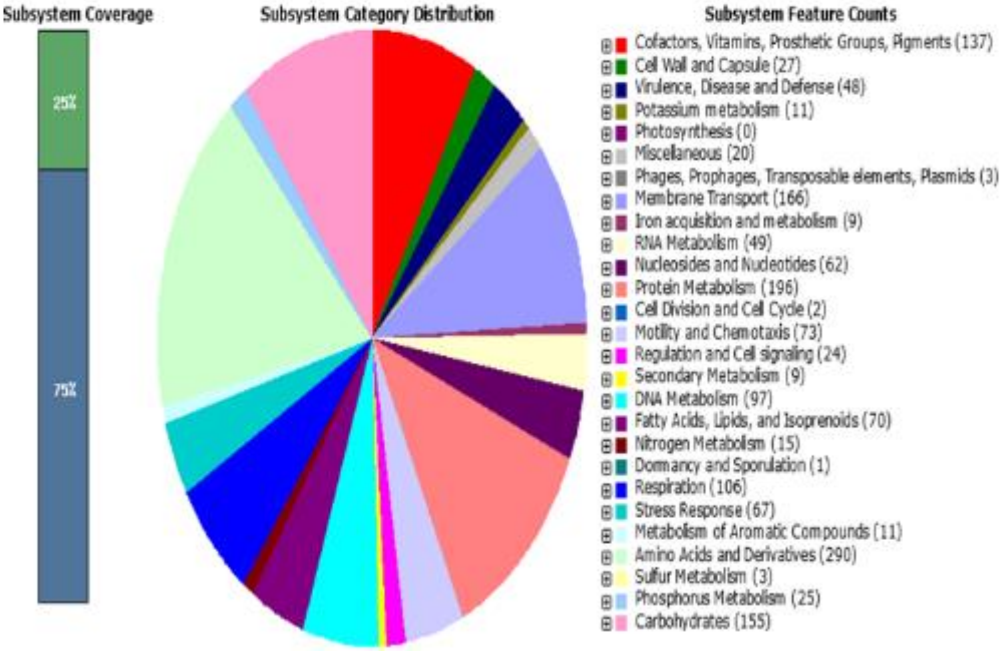


Figure 5.10: Endophytic *Stenotrophomonas geniculata* strain NWUBe21 subsystem overview showing different functional/metabolic roles

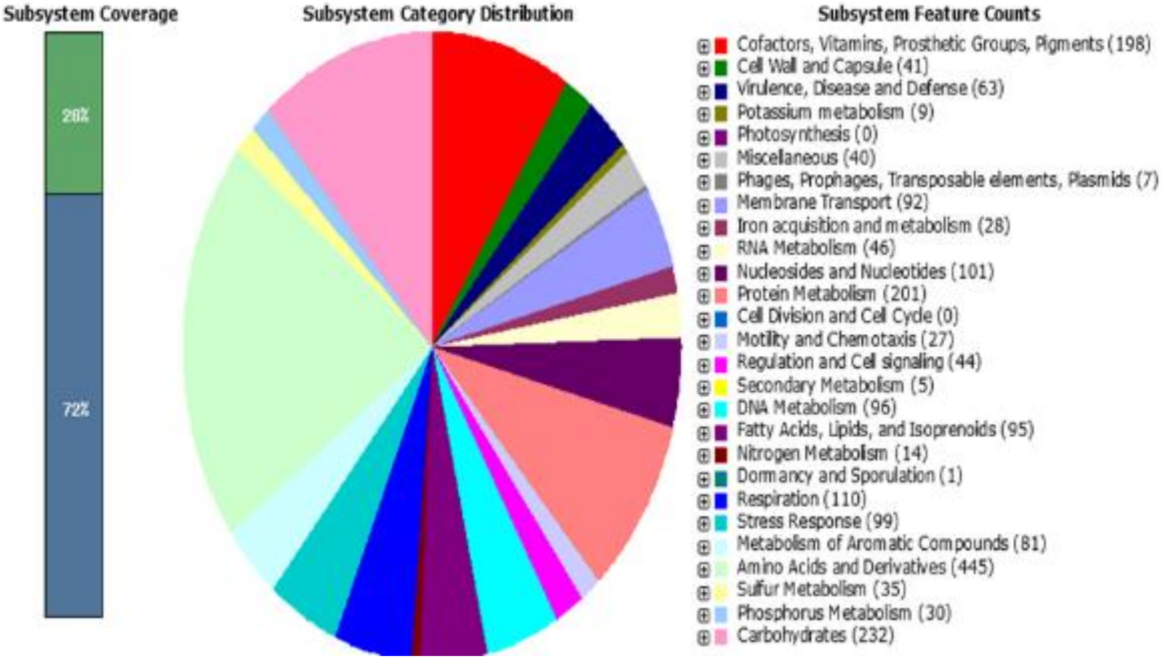


Figure 5.11: Endophytic *Pseudomonas carnis* strain NWUBe30 subsystem overview showing different functional/metabolic roles

5.4. Discussion

Authentication of the endophytic bacteria strains as plant growth enhancers

The quest for cheaper, readily available natural resource based alternatives to synthetic agrochemicals as a means of achieving highly intensified improvements in sustainable agricultural productivity is a must in the midst of, amongst others declining agricultural input resources, climatic change variations, famine, urbanization, war and the increasing number of people in the world to feed. Endophytic microbiota research is being looked into as a veritable means of achieving the objective of boosting food safety and security in a sustainable manner (Quintas-Nunes *et al.*, 2022). In this study, endophytic bacterial strains *Stenotrophomonas geniculata* NWUBe21 and *Pseudomonas carnis* NWUBe30, isolated from the tissues of *Vigna unguiculata* were authenticated as possessing plant growth-improving characteristic features. Results obtained from both endophytic strains indicated their potency to solubilize phosphate, produce siderophore, secrete plant hormones and to, produce auxin and ammonia, as well as other plant growth enhancing metabolites. Similar results of endophytic bacterial strains that possess plant growth-stimulating features are reported (Di *et al.*, 2022; Tran *et al.*, 2022; Wang *et al.*, 2022b).

To further examine the genetic basis of the plant growth-promoting traits of *Stenotrophomonas geniculata* strain NWUBe21 and *Pseudomonas carnis* strain NWUBe30, the genomic exploration of the two endophytic bacteria was conducted in order to determine the genes responsible for the important secondary metabolites secreted, as well as the genes responsible for the plant growth-stimulating features. The highlights of the beneficial features in the genome of the endophytic bacterial strains that confer plant-growth- promoting attributes are summarized below

Genetic components that are involved in plant growth enhancement

The genome exploration of the two endophytic bacterial strains *L. xylanilyticus* NWUBe21 and *P. carnis* NWUBe30 indicated the presence of various genes that play vital roles in enhancing plant-growth traits.

Iron aquisition and metabolism

The genomes of the two endophytic bacterial strains contain genes that are responsible for siderophore production and iron metabolism. The include iron siderophore receptor protein, the iron siderophore sensor protein, the ferrous iron transport periplasmic protein EfeO, the ferrous iron transport permease EfeU, and the ferrous iron transport peroxidase EfeB. Iron is of vital importance for plant growth and survival. The presence of various iron acquisition and siderophore genes in the genome of these two bacterial strains is responsible for the development of plants by making iron readily available for utilization by the plants (Fadiji & Babalola, 2020; Olanrewaju *et al.*, 2019).

Motility and Chemotaxis

The movement and attachment of microorganisms to plants is crucial for effective colonization (Xu *et al.*, 2019b). Different genes that aid the attachment and movement of these two microbes in plants are present in their genomes. They include the chemotaxis protein, CheV, the flagellar L-ring protein, FlgH, the flagellar biosynthesis protein, FlhA, the predicted signal transduction protein and the flagellar motor rotation protein MotA.

Nitrogen Metabolism

Nitrogen remains a key macro-nutrient that is essential for plant growth; hence, it is regarded as a key synthetic fertilizer that is generally deployed for agricultural production (Alori & Babalola, 2018). Various nitrogen metabolism genes are present in the genome of the two endophytic bacteria, such as NorD – the nitric oxide reductase gene, the ammonium transporter, nitrilase, NorR, and the glutamate-ammonia-ligase adenylyltransferase gene.

Phosphorus Metabolism

Phosphorus is another key mineral that is essential for plant growth and vitality. Phosphate solubilization and the genes that are responsible for the solubilization and metabolism of phosphorus in usable forms are critical to growth in plants (Wan *et al.*, 2020)Olanrewaju and Babalola, 2019).The genes that are associated with phosphorus metabolism that and that are in the genome of these strains include, amongst others, the phosphate regulon sensor protein, PhoR, the phosphate transport system regulatory protein, PhoU, exopolyphosphatase, alkaline phosphatase, the phosphate starvation-inducible protein, PhoH, and the polyphosphate kinase protein.

Plant hormone-auxin biosynthesis

The presence of the tryptophan synthase alpha chain, the tryptophan synthase beta chain, phosphoribosyl anthranilate isomerase and, anthranilate phosphoribosyl transferase are precursors to the plant hormone, auxin biosynthesis, and are present in the genomes of the two endophytic strains. Auxin plays a key role in the fitness of a plant and in promoting its growth (Shahzad *et al.*, 2017).

Stress Response

The ability to cope appropriately with different stressors and responses is inherent in the genome of the two endophytic strains. Different stress response genes include aquaporin Z, betaine aldehyde dehydrogenase, the OpgC protein, the glycine betaine transporter, OpuD, the ferric uptake regulation protein FUR, the organic hydroperoxide resistance protein and, the organic hydroperoxide resistance protein, superoxide dismutase. These stress tolerance genes aid plants to mitigate stressful conditions and ultimately lead to plant growth enhancement (Hantke *et al.*, 2019; Woo *et al.*, 2020).

Moving forward, there are different biochemical and physiological mechanisms deployed by microorganisms in achieving positive changes that leads to plant growth enhancement in plant-microbe interrelationships. The data reported in this study indicated that *Pseudomonas carnis* strain NWUBe30 and *Stenotrophomonas geniculata* strain NWUBe21 possess plant-growth-stimulating attributes that aid in the growth and development of plants. These results are aligned with a report on endophytes as plant growth improving microbial resources for attaining sustainable agroecological results (Adeleke *et al.*, 2021; Ribeiro *et al.*, 2022; Santoyo *et al.*, 2021).

In addition, the exploration into the genomes of the two endophytic isolates, *Pseudomonas carnis* strain NWUBe30 and *Stenotrophomonas geniculata* strain NWUBe21 showed the presence of important metabolites and genes that play key/functional roles in amongst others chemotaxis and motility, siderophore secretion and iron metabolism, phosphate/phosphorus solubilization and metabolism, nitrogen metabolism, plant hormonal secretions, stress responses and adaptions. All these highlighted genes confer functional attributes on the two endophytic bacterial strains that are crucial to their plant growth-stimulating traits through effective root colonization, the ability to use complex organic and inorganic substrates, thus making them available to plants as usable metabolites, the ability to adapt and cope with stressful conditions in the plant environment, the sequestration of iron and the metabolization of plant use, phosphate solubilization; the fixing of nitrogen and nitrogen metabolism. The genomic composition of the two endophytic strains confers functional roles on specific genes that regulates plant vigour, fitness and growth. These results agree with the outcomes of previous studies (Belaouni *et al.*, 2022; del Carmen Orozco-Mosqueda & Santoyo, 2021; Jayakumar *et al.*, 2021; Kong *et al.*, 2022; Mukherjee *et al.*, 2021; Olanrewaju *et al.*, 2021).

5.5. Conclusions

Findings from this research highlighted the plant-growth-stimulating features of two endophytic bacterial strains, *Stenotrophomonas geniculata* NWUBe21 and *Pseudomonas carnis* NWU Be30. This study; further elucidated the genomic insights that are responsible for the beneficial plant-growth-stimulating features of their plant host. The exploration of the genomes of these strains indicated multi-faceted genes that confer the ability to effectively colonize and enhance plant growth. These findings confirmed the potential of these two endophytic bacterial isolates as candidates for producing bioinoculants for optimizing sustainable agroecological enterprises.

CHAPTER SIX

6.0 BIOASSESSMENT OF PHYLOGENETIC RELATEDNESS AND PLANT GROWTH ENHANCEMENT OF ENDOPHYTIC BACTERIAL ISOLATES FROM COWPEA (*VIGNA UNGUICULATA*) SEEDS AND PLANT TISSUES

Abstract

Cowpea is of great importance to an array of people in most tropical countries of the world, especially in Africa and Latin America, and in selected countries in Europe and the rest of the world. It is the preeminent indigenous African legume and a frontline option for meeting the nutritional demands of poor people and livestock for protein. The benefits of cowpea production include its role as a means of creating wealth through income generated by diverse stakeholders that are involved in the value chain. Also, it replenishes the quality of the soil nutrients through its ability to fix atmospheric nitrogen. The benefits that are associated with the production of cowpea make it a topmost research focus area for improving the sustainability of the crop and its ecologically balanced productivity. The use of an eco-friendly and sustainable alternative to synthetic fertilizers and agro-pesticides, requiring fewer, and therefore cheaper inputs, has in recent times become an attractive research theme. Therefore, bioprospecting for effective endophytic bacteria isolates as potential bioinoculants for enhancing cowpea productivity makes this research a priority. In this study, cowpea seeds and the aerial parts of the plant, including the leaves, shoots and near root tissue were used to isolate and characterize endophytic bacteria through morpho-genotypic techniques. In all, 33 endophytic bacterial species were authenticated by sequencing the 16S rRNA, and determining their identity through the National Centre for Biotechnology Information (NCBI) database. These isolates were then assessed for their in-vitro growth-promoting potential, as well as their in-planta growth in chamber experiments. The research findings highlight that some of the authenticated endophytic bacterial isolates are potential bioinoculants that can be applied in further studies to improve the agronomic growth yield and productivity of the cowpea.

Keywords: Effective endophytic bacterial strains; low-input sustainable alternative; cowpea growth improvement; agro-ecological sustainability; poor people nutritional demands; indigenous leguminous crop

6.1 Introduction

The bioprospecting investigation of endophytic microbiota from both unusual and challenging, and otherwise normal ecological niches to identify and characterize strains with unique attributes for supporting and enhancing sustainable agricultural productivity has been on the rise lately (Gupta *et al.*, 2022; Jain *et al.*, 2021). In fact, endophytic microbiota-formulated bioinoculating agents will be forming the foundation of a newer Green Revolution to achieve agroecological sustainability. Endophytic microbiota have demonstrated efficacy as renewable and sustainable natural microbial resources that are cheaper to produce and that can be used as field inoculants in the form of biofertilizers, biopesticidal agents, and biostimulating agents, to achieve enhanced outputs of agroproducts and environmental balance (Omomowo & Babalola, 2019; White *et al.*, 2019). Therefore, they can be used in crop production as complementary to or an alternative to synthetic agrochemicals. The versatility of endophytic microbiota in tackling abiotic and biotic stresses in plants through diverse mechanistic actions, such as the secretion of vital metabolites, the expression of beneficial gene responses and antagonistic actions, thereby optimizing agricultural outputs is reported (Abd_Allah *et al.*, 2018; Bokhari *et al.*, 2019).The plant-growth-stimulating traits and plant productivity enhancement of endophytic microbiota are achieved through diverse mechanisms, that include the ability to convert or fix atmospheric nitrogen, to convert insoluble phosphates to soluble phosphates, to produce vital enzymes and, important phyto-metabolites, and to suppress or inhibit microbial pathogens and pests (Shah *et al.*, 2021; Wang *et al.*, 2020). The firm establishment of endophytic microbiota to the colonized innermost tissues of plants confers on them multiple beneficial traits that aid in promoting plant-growth. Endophytic microbiota constitute a goldmine of bioactive metabolites that influence their actions as immune boosters in plants, stimulate plant-growth, help in the biocontrol of pests and microbial pathogens, as well as enable plants to cope with environmental stressors (Potshangbam *et al.*, 2017; Sahu *et al.*, 2019; Singh & Gaur, 2017). However, endophytic microbial research has lately proven promising as an alternative option or complement to synthetic agrochemicals in achieving, in a sustainable manner, the optimization of agri-system outputs (Agarwal *et al.*, 2020; Ahmad *et al.*, 2022; Faria *et al.*, 2021). Endophytic bacteria are the inhabitants of the innermost tissues of plants (Compant *et al.*, 2021; Hardoim *et al.*, 2015), and confer beneficial support for the symbiotic-associations with their plant host in diverse ecological niches and under varying environmental conditions. Numerous studies have confirmed the plant-growth-stimulating potency of bacteria endophytes on planted crops, thereby alluding to their potential as bioinoculating agents that can enhance the sustainability of agricultural productivity (Dubey *et al.*, 2021; Morales-Cedeño *et al.*, 2021; Xu *et al.*, 2019a). The mechanisms used by these beneficial endophytic microbiota include the conferral of an appropriate stress response and, the production of vital nutrients and bioactive metabolites, thus inducing, amongst others, allelopathic effects on plants (Adeleke & Babalola, 2021a; Santoyo *et al.*, 2016b).

(*Vigna unguiculata* (L.) Walp.) is well known as cowpea, and is a foremost African leguminous crop that is of great importance in terms of its nutritional, economic and environmental attributes. It is grown mainly in the warmer regions of Africa and in other semi-arid zones worldwide. It is an annual crop and also a diploid with 2n=2x=22. The genomic size of cowpea is estimated at 620 million base pairs (Lonardi *et al.*, 2019). In terms of its importance, it is a vital source of dietary nutrients for humans and also provides fodder for livestock. Moreso, a key trait of cowpea includes its role in maintaining a soil-ecological balance by fixing atmospheric nitrogen in symbiotic association with nodulating bacteria (Ravelombola *et al.*, 2017). Cowpea is strategically valuable to Africa in terms of the economy of scale associated with its production (Muñoz-Amatriaín *et al.*, 2017). The protein and essential amino acid content of cowpea is between 23% and 32% (Carvalho *et al.*, 2017). Cowpea provides an alternative means to ensuring a balanced diet in most African countries in that it provides a cheaper way of accessing the necessary dietary nutrients and for positively influencing the well-being and health of the populace(Gonçalves *et al.*, 2016). All the parts of the cowpea plant are valuable: from the leaves, that can be used as vegetables, to the haulms (cowpea pod walls, stems, and leaves), which are used as livestock fodder for animals, thus generating an income for farmers (Kebede & Bekeko, 2020). *Vigna unguiculata* L. Walp is a key legume in the tropics and arid regions of the world (Xiong *et al.*, 2016). Importantly, it is useful in agro-ecological conservation. In terms of importance, cowpea production contributes significantly to economic productivity and environmental sustainability in Africa (Cardona-Ayala et al., 2020; (Olajide & Ilori, 2017; Ovalesha *et al.*, 2017). In contrast to the huge benefits and potential of cowpea, its productivity is limited in Africa, and its status as an under-utilized leguminous crop persists. Thus, the task ahead is to find a way out of these dire circumstances by maximizing the potential use of natural resource such as endophytic microbiota in an efficient and agro-ecologically sustainable manner to enhance cowpea productivity. Therefore, this research aims at circumventing the low productivity constraints of cowpea production in North West Province, South Africa, by bioprospecting endophytic bacterial isolates with effective plant probiotic-promoting traits, that could be deployed as potential bioinoculants to improve cowpea production.

6.2. Materials and Methods

6.2.1. Cowpea (*Vigna unguiculata* L Walps) sampling/collection

Prior to the initiation of this research, healthy cowpea plant samples, including roots, stems and leaves, with no external symptoms of disease, were already available in that they had been taken from cowpea farms in Mafikeng and its environs, and kept in cold conditions in plastic bags at 4 °C before being processed for isolation and characterization.

However, the cowpea seeds used in this study were collected from the Agricultural Research Council (ARC), in Pretoria. The locations of the sampling and experimentation sites, indicates the GPS coordinates (25°47’19.1”S 25°37’05.1 “E).

6.2.2 Isolation of endophytic microbiota from leaves, seeds and near root tissue zones of cowpea

Healthy cowpea seeds, and aerial tissue, both from below and above the surface were rinsed under running tap water to remove the attached debris. Further surface sterilization was carried out by washing the cowpea plant samples with 70% ethanol and then three percent (3%) sodium hypochlorite for three minutes. This was followed again by sterilizing the samples with 70% ethanol and, then washing it thoroughly three times with sterilized water to remove epiphytic microorganisms from it. The efficacy of the surface sterilization was assessed by plating the final rinse water on growth media. The surface sterilized cowpea plant tissues, as well as further processed cowpea tissues that were appropriately crushed with mortar and pestle and diluted using a phosphate buffer saline, were subsequently plated on varied microbiological growth media. The petri-plates were replicated and incubated according to standard microbiological protocol. Further purification of the putative isolated endophytes was achieved through several subculturing processing with the pure isolates finally being stored for further analysis on storage media incorporating 20 % glycerol.

6.2.3. Molecular characterization of endophytic bacterial isolates

Following the manufacturer’s instructions, the total genomic DNA of the pure culture endophytic bacterial isolates were extracted using Zymoclean kits for bacteria and fungi. The purity of the DNA extracted was assessed on 0.8 % agarose using UV-visual gel documentation.

The conserved 16S rRNA genes of the bacterial isolates were amplified by applying PCR amplification techniques and also using universal primers 341 forward and 907 reverse for bacterial identification (Fukuda *et al.,* 2016).

The PCR reaction cocktails of 25 µl volume contained the following: 2X Master Mix: 12.5 µl; molecular grade water 8.5; forward and reverse primers of one µl each; and a DNA template of two µl.

The PCR cycling conditions using a Biorad 1000 thermocycler USA, included an initial denaturing at 95 ˚C for five minutes, followed by 34 cycles of denaturing at 95˚C for 30 seconds, annealing at 59˚C for one minute, an extension at 72˚C for 45 seconds, and a final extension at 72˚C for seven minutes, with the resultant amplicon finally being held at 4˚C.

Note: Following the gradient PCR protocol, the annealing temperature for the optimized PCR amplification of DNA of the different microbial isolates varied from 50˚C to 59˚C. The various primers used for the PCR experiments are shown in Table 1.

Table 6.1: Primers (16S and PGP genes) used for the identification of endophytic bacterial isolates, *ACPHO,* ASB and *ACDS* gene amplification, indicative of their plant growth related attributes.

Target Gene	Primer Name	Nucleotide Sequence (5′ -------→ 3′)	Product Size (bp)	Reference
16S	341-F	AGAGTTTGATCCTGGCTCAG	1300–1500	(Fukuda et al., 2016)
	907-R	AAGGAGGTGATCCAGCCGCA		

ACDS	ACDS-F3	ATCGGCGGCATCCAGWSNAAYCANAC	750-755	(Li. 2011)
	ACDS-R3	GTGCATCGACTTGCCCTCRTANACNGGRT		
IPDC	IPDC-F	CAYTTGAAAACKCAMTATACTG	1715-1809	(Raddadi et al., 2008)
	IPDC-R	AAGAATTTGYWKGCCGAATCT		
ACPHO	ACPHO-F	AAGAGGGGCATTACCACTTTATTA	360	(Poly et al., 2001)
	ACPHO-R	CGCCTTCCCAATCRCCATACAT		
GCD	GCD-F	GACCTGTGGGACATGGACGT	750-755	(Li. 2011)
	GCD-R	GTCCTTGCCGGTGTAGSTCATC		
ASB	ASB-F	GAGAATGGATTACAGAGGAT	1685	(Raddadi et al., 2008)
	ASB-R	TTATGAACGAACAGCCACTT		

The success of the amplification protocol was assessed through electrophoresis using a 1.2 % agarose gel in a TAE buffer, stained with ethidium bromide, and run for one hour in the same TAE buffer, and finally visualized using UV gel documentation. The amplified products were quality checked and sequencing, in both forward and reverse directions, was carried out at Inqaba Biotech, Pretoria, South Africa.

6.2.4. Molecular authentication and phylogenetic assessment

Investigation of the molecular authenticity of the isolated bacteria endophytes that were sequenced was performed using different bioinformatics tools that include chromas-lite - for sequence trimming, and Bioedit – for sequence editing for correction and alignment. Thereafter, the correctly trimmed, edited and aligned sequences were subjected to blasting using the National Centre for Biotechnological Information’s (NCBI) nucleotide blast platform for the identification and authentification of the isolates and for referencing with similar strains in the Genbank database. The Molecular Evolutionary Genetics Analysis software (MEGA7) (Kumar *et al.*, 2016) was deployed for identifying, through phylogenetic analyses, the relatedness of endophytic bacterial strains and other closely related bacterial strains in Genbank. The maximum likelihood method, was deployed for the construction of a phylogenetic tree.

6.2.5. An *in-vitro* assay of the endophytic bacterial plant growth-stimulating attributes

Potency for solubilizing inorganic phosphate

Based on (Nautiyal, 1999) protocols, the potency of the bacterial endophytic isolates to solubilize phosphate was assessed. After adjusting the concentration of insoluble calcium tri-phosphate to 0.5, endophytic cultures freshly grown in Luria Bertani (LB) broth were used as an aliquot to inoculate the National Botanical Research Institute's Phosphate (NBRIP) solubilization agar plate containing the insoluble calcium tri-phosphate. The plates were replicated thrice and incubated for seven (7) days at 30°C. The appearance of a transparent halo-zone around the culture colony confirmed that the isolates did indeed have the potential to solubilize phosphate.

Potency of bacterial isolates to produce Indole acetic acid

The ability of the endophytic bacterial isolates to produce indole acetic acid was assessed using the assay protocol devised by (Matsuda *et al.*, 2018). Overnight cultures of the endophytic bacterial isolates were used to inoculate peptone water that contained five (5) mM L-tryptophan, and the liquid media incubated on a rotary shaker at 150 rpm for 48 hours and at 30°C. Thereafter, the cultures were processed and centrifuged to obtain a supernatant; Salkowski reagent was then added to the supernatant in a ratio of 2:1 and the resultant solution was incubated in the dark for 30 minutes before the absorbance was measured with the aid of a spectrophotometer at 530 nm. A standard curve of IAA was also plotted using various concentrations of indole acetic acid in order to quantify the amount of IAA produced by the endophytic isolates.

Potency of endophytic bacterial isolates to produce siderophore

The ability of the endophytic bacterial isolates to produce siderophore was assessed qualitatively on chrome azural (CAS) agar plates (Schwyn & Neilands, 1987). The endophytic isolates were freshly grown in the Luria Bertani (LB) medium to obtain a 0.5 concentration at OD600. A diffusible disc was then placed on a freshly prepared CAS-blue agar plate. Thereafter, 10 µl aliquots of each endophytic culture were inoculated onto the plate and incubated for 72 hours. The appearance of a yellowish-orange halo around the culture colony indicated a positive potency to produce siderophore.

Potency of endophytic bacteria isolates to promote ACC deaminase activity (ACCD)

The assay protocol, as described by Glick *et al*. (1995), was used to assess the potentials of the endophytic bacterial isolates for promoting ACC deaminase activity. A fresh culture was grown overnight and then used as the inoculum by harvesting and centrifuging the broth culture to obtain a culture pellet. Thereafter, saline water that had been previously sterilized, was used to wash the pellet thoroughly and, then, re-suspended in saline water before spot inoculation on three (3) mM ACC incorporating minimal media petri-plates. A minimal media plate that had been incorporated with ammonium sulphate was used as the positive control, while the minimal media plates without any nitrogenous source served as the negative control. All plates were in triplicate and cultured for 72 hours at 30°C. The growth of the endophytic bacteria on the three (3) mM ACC incorporating minimal media petri-plates indicated the potential of the endophytic bacterial isolates to promote ACC deaminase activity.

Ammonia production

The endophytic isolates were assessed for their potency in producing ammonia (Cappucino & Sherman, 1992). This involved using a freshly grown overnight culture of endophytic isolates to inoculate 10 ml sterilized peptone water in test tubes and the incubation of this liquid medium on a rotary incubator for 48 hours at 30° C. The development of yellowish to brownish colouration subsequent to the addition of Nessler's reagent (0.5 ml) was a positive indication of the potential of the isolates to produce ammonia.

Exopolysaccharide production

The abilitiy of the endophytic isolates to produce exopolysaccharides was assessed using the methodology proposed by Khan and Bano (2019), but with a few modifications. A 10% sucrose-supplemented Luria Bertani (LB) agar was prepared and the pH of the medium was adjusted to seven before sterilization. Also, freshly grown overnight cultures of the endophytic isolates were used to impregnate sterile filter paper and placed carefully inside the LB medium plates before incubation for 48 hours at 30°C. The potential of the isolates to produce exopolysaccharide was indicated by the formation of a mucoid colony on the filter paper.

Hydrogen cyanide production

The ability of the isolated bacterial endophytes to produce hydrogen cyanide (HCN) was determined by the methodology proposed by Dinesh *et al.* (2015). The endophytic isolates were streaked on the LB agar medium that was incorporated with glycine (4.4 g/L). Thereafter, sterilized filter paper was dipped into a solution of picric acid, then carefully placed on the lid of the petri-plates and sealed up with parafilm before incubation for 96 hours at 30°C. The isolates proved positive for hydrogen cyanide production when, after incubation, the filter paper changed colour from a yellowish to a reddish-brown.

6.2.6 Seed germination and seedling growth

Two varieties of cowpea seeds (PAN311 and Bechuana white) were used to perform this experiment. The seeds were surface sterilized through several steps of disinfection, the process involving three (3 %) sodium hypochlorite, sterile water, 70 % ethanol and finally, cleaning with sterile water, to remove any epiphytic microbes. Thereafter, seed germination assays were conducted for seven days using 10 seeds per plate with tissue toweled bioprimed endophytic treatment. The control seed plate was inoculated with distilled water and replications in the growth chamber were conducted thrice. Based on the calculated readings, the percentage germination rate and biomass results for shoot length, root length and weight were recorded.

Potentials of endophytic bacterial isolates to inhibit phytopathogenic fungal activity

The inhibitory activity of endophytic bacterial isolates against selected phytopathogenic organisms was assessed on petri-plates through a confrontational/dual assay protocol (Vinale et al. 2008).

The phytopathogenic fungi were grown on PDA petri-plates at room temperature for five days. Thereafter, the overnight culture of endophytic bacterial isolates grown on the LB broth was streaked on a freshly prepared yeast malt extract agar plate at the periphery before confrontation with a five millimetre agar disc of phytopathogenic fungi placed perpendicular to the streaked bacterial isolates. (The control plate contained only the fungal pathogens on the yeast malt extract agar plates and not the bacterial endophytes). The plates were replicated three times, and after seven days of incubation, the percentage inhibitory potential was estimated, using the formula, $Control - treated \div control \ \times 100$.

The control represented the diameter of the phytopathogenic fungal growth on the yeast malt extract agar without the endophytic bacterial isolates, while the treated represented the diameter of the phytopathogenic fungal that was confronted by the endophytic bacterial isolates on the yeast malt extract plate.

Potentials of endophytic bacterial isolates to tolerate environmental stress

For the external stress tolerance potential of the endophytic bacterial isolates, salinity induced with NaCl was deployed at one percent (1%), three percent (3%) and five percent (5%), respectively. Temperature stress was determined at 40, 35 and 20, respectively, while the pH deployed was at ten (10), seven (7) and five (5) respectively (Vyas et al. 2009). The endophytic bacterial isolates were incubated for 48 hours and the experimental readings were replicated thrice.

6.3. Results

6.3.1 Morpho-genotypical authentication

Data obtained in this current study on the morphological and, biochemical characteristics and the genotypical authentication of the isolated endophytic microbiota associated with the cowpea plant indicated that 33 diverse bacterial strains, consisting of 11 genera, namely, *Stenotrophomonas, Brevundimonas, Staphylococcus, Paenibacillus, Bacillus, Myroides, Lynsinibacillus, Pseudomonas, Mammalicoccus, Micrococcus and Ignatzscineria*, had been isolated. The most common endophytic strains in terms of occurrence were found to be *Staphylococcus* strains (10), *Bacillus* strains (nine), *Brevundimonas* strains (four), *Stenotrophomonas* strains two, *Myroides* strains two, while *Pseudomonas*, *Lysinibacillus*, *Paenibacillus*, *Ignatzscineria*, *Mammalicoccus* and *Micrococcus* presented with only one strain each. Also, (24) of the isolated endophytic bacterial strains were found to be gram negative, while nine of the isolated bacterial strains were gram positive. The taxonomic phyla indicated that most of the endophytic strains were firmicutes, while others fell into the phyla – proteobacterial class, mainly the gammaproteobacterial class, and others belonged to the phyla high G+C and CFB classes. The microscopic shapes of the endophytic bacterial strains were found to be mainly cocci, slim rods, short rods and long rods. The distinct reactions of the isolated cowpea endophytic microbial strains to various forms of morphometric characterization for their morphological authentication are represented below in Table 6.2.

In addition to their morphological characteristics, genotypical identification, based on 16S rRNA gene sequencing, and further Bioinformatics processing resulted in a genotypical authentication, composed of molecularly diverse bacterial strains, as shown in Table 6.3. The phylogenetic relatedness of the endophytic bacterial strains with similar strains in the GenBank is presented in Figures 6.1 to 6.5. The phylogenetic inference indicates that the various endophytic strains are diverse, but have ancestral linkages with similar species in the GenBank repository.

Table 6.2: Morphological and biochemical characteristics of endophytic bacteria isolates from seeds and near root tissue zones of cowpea (*Vigna unguiculata*)

Bacteria code	Gram reaction	Cell morphology	Catalase	Oxidase	Casein hydrolysis	Gelatin hydrolysis	Methyl red	Voges Proskaeur	Nitrate reductase	Putatively identified
Be1	-	R	+	-	+	+	-	-	-	*Stenotrophomonas maltophilia*
Be2	-	R	+	-	+	+	-	-	-	*Stenotrophomonas maltophilia*
Be3	-	R	+	+	-	+	-	-	+	*Brevundimonas bullat*
Be4	+	R	+	-	+	+	-	+		*Bacillus wiedmannii*
Be5	+	R	+	-	+	+	-	+	+	*Bacillus anthracis*
Be6	+	C	+	+		+	-	-	-	*Micrococcus luteus*
Be7	-	R	+	+					+	*Myroides pelagicus*
Be8	+	R	+	+	+	+	-	+	+	*Bacillus tropicus*
Be9	-	R	+	+	-	-	-	-	-	*Ignatzschineria indica*
Be10	+	R	-	-	+	+	-	-	+	*Bacillus thuringiensis*
Be11	+	C	+	-	+	+	-	-	+	*Staphylococcus saprophyticus*
Be12	+	C	+	-	+	-	-	+	+	*Staphylococcus edaphi*
Be13	+	C	+	-	+	+	+	-	+	*Staphylococcus nepale*
Be14	+	R	+	-	+	+	+	+	+	*Staphylococcus xylosu*
Be15	+	C	+	-	+	-	+	-	+	*Staphylococcus cohnii*
Be16	+	C	+	+	+	+	-	-	+	*Mammaliicoccusstepa*
Be17	+	C	+	-	+	+	-	-	+	*Staphylococcus succin*
Nwa1	+	C	+	-	+	+	-	-	+	*Staphylococcus casei*
Nwa2	+	C	+	-	+	+	+	+	+	*Staphylococcus arletta*
Nwa3	+	C	+	-	+	+	-	-	+	*Staphylococcus saprophyticus*
Nwa5	+	R	-	-	-	-	-	-	-	*Lysinibacillus xylanily*
Nwa6	+	R	+	+	+	+	+	-	-	*Bacillus altitudinis*
Nwa7	+	R	+	-	+	+	-	-	-	*Paenibacillus illinoise*
Nwa8	-	R	+	+	-	+	-	-	+	*Brevundimonas bullat*
Nwa9	+	R	+	+	+	+	-	+	+	*Bacillus tropicus*
Nwa11	-	R	+	+	+	-	-	-	-	*Brevundimonas terrae*
Nwa12	-	R	+	+	+	+	-	-	+	*Myroides odoratimimu*

Nwa13	+	R	+	+	+	+	+	-	+	*Bacillus paramycoides*
Nwa16	-	R	+	+	-	+	-	-	+	*Brevundimonas bullat*
Nwa17	-	R	+	+	+	+	+	+	-	*Pseudomonas fluoresc*
Nwa18	+	R	+	-	+	-	-	+	+	*Bacillus cereus*
Nwa21	+	R	+	-	+	-	-	+	+	*Bacillus bingmayonge*
Nwa22	+	C	+	-	+	+	+	-	+	*Staphylococcus kloosi*

Table 6.2: continued

Bacteria code	Urease	Starch hydrolysis	Citrate	Motility	Indole	Glucose	Fructose	Maltose	Lactose	Sucrose	Putatively identified
Be1	-	-	+	+	-	+	-	+	+	+	*Stenotrophomonas malt*
Be2	-	-	+	+	-	+	-	+	+	+	*Stenotrophomonas pav*
Be3	-	-	-	+		+	+	-	-	-	*Brevundimonas bullata*
Be4	+	+	+	+	-	+	+	+	+	+	*Bacillus wiedmannii*
Be5	-	-	-	-	+	+	+	+	+	+	*Bacillus anthracis*
Be6	+	+	-	-	-	-	+	-	+	+	*Micrococcus luteus*
Be7	-	+	-	-	-		-	-	-	+	*Myroides pelagicus*
Be8	-	+	+	+	-	-	+	+	-	-	*Bacillus tropicus*
Be9	-	-	-	-	-	-	-	-	-	-	*Ignatzschineria indica*
Be10	+	-	-	+	-	+	+	+	+	+	*Bacillus thuringiensis*
Be11	+	-	-	-	+	+	+	+	+	+	*Staphylococcus saproph*
Be12	-	-	-	-	-	+	+	+	+	+	*Staphylococcus edaphi*
Be13	+	-	-	-	-	+	+	+	+	+	*Staphylococcus nepale*
Be14	+	-	-	-	-	+	+	+	+	+	*Staphylococcus xylosus*
Be15	-	-	-	-	-	+	+	+	+	+	*Staphylococcus cohnii*
Be16	+	-	+	-	-	+	+	+	-	+	*Mammaliicoccusstepan*
Be17	+	-	-	-	-	+	+	+	+	+	*Staphylococcus succin*
Nwa1	+	-	-	-	-	+	+	+	+	+	*Staphylococcus casei*
Nwa2	+	-	-	-	-	+	+	+	+	+	*Staphylococcus arletta*

Nwa3	+	-	-	+	-	+	+	+	+	+	*Staphylococcus saproph*
Nwa5	+	-	-	+	-	-	-	+	-	-	*Lysinibacillus xylanilyti*
Nwa6	+	+	+	+	-	+	+	+	+	+	*Bacillus altitudinis*
Nwa7	-	+	+	+	-	+	+	+	-	-	*Paenibacillus illinoisen*
Nwa8	+	+	+	+	-	+	+	+	+	+	*Brevundimonas bullata*
Nwa9	-	+	+	+	-	-	+	+	-	-	*Bacillus tropicus*
Nwa11	-	-	+	+	-	-	-	-	-	+	*Brevundimonas terrae*
Nwa12	+	-	-	-	-	-	+	-	+	-	*Myroides odoratimimus*
Nwa13	-	+	+	-	-	+	+	+	-	+	*Bacillus paramycoides*
Nwa16	+	+	+	+	-	+	+	+	+	+	*Brevundimonas bullata*
Nwa17	-	+	-	+	-	-		+	-	-	*Pseudomonas fluoresce*
Nwa18	-	+	+	+	-	+	+	+	-	+	*Bacillus cereus*
Nwa21	-	+	+	+	-	+	+	+	-	+	*Bacillus bingmayongen*
Nwa22	+	-	-	-	-	+	+	+	+	+	*Staphylococcus kloosii*

Table 6.3: Molecular authentication of endophytic bacterial strains isolated from cowpea seeds and root tissues based on the 16S rRNA gene sequence.

S/N	Culture Code	Molecularly identified strains	Similarity %	Bacteria Phyla	GenBank Accession Number
1.	Be1	*Stenotrophomonas maltophilia* strain NwuBe01	100	Proteobacteria	OK050078
2.	Be2	*Stenotrophomonas pavanii* strain NwuBe02	100	Proteobacteria	OK050079
3.	Be3	*Brevundimonas bullata* strain NwuBe03	100	Proteobacteria	OK050080
4.	Be4	*Bacillus wiedmannii* strain NwuBe04	100	Firmicute	OK050081

5.	Be5	*Bacillus anthracis* strain NwuBe05	100	Firmicute	OK050082
6.	Be6	Micrococcus luteus strain NwuBe06	100	High G+C	OK050083
7.	Be7	*Myroides pelagicus* strain NwuBe07	100	CFB	OK050084
8.	Be8	*Bacillus tropicus* strain NwuBe08	100	Firmicute	OK050085
9.	Be9	*Ignatzschineria indica* strain NwuBe09	100	Proteobacteria	OK050086
10.	Be10	*Bacillus thuringiensis* strain NwuBe10	100	Firmicute	OK050087
11.	Be11	*Staphylococcus saprophyticus* strain NwuBe11	100	Firmicute	OK050088
12.	Be12	*Staphylococcus edaphicus* strain NwuBe12	100	Firmicute	OK050089
13.	Be13	*Staphylococcus nepalensis* strain NwuBe13	100	Firmicute	OK050090
14.	Be14	*Staphylococcus xylosus* strain NwuBe14	100	Firmicute	OK050091
15.	Be15	*Staphylococcus cohnii* strain NwuBe15	100	Firmicute	OK050092
16.	Be16	*Mammaliicoccus stepanovicii* strain NwuBe16	100	Firmicute	OK050093
17.	Be17	*Staphylococcus succinus* strain NwuBe17	100	Firmicute	OK050094
18.	Nwa1	*Staphylococcus casei* strain NwuBe18	100	Firmicute	OK050095
19.	Nwa2	*Staphylococcus arlettae* strain NwuBe19	100	Firmicute	OK050096
20.	Nwa3	*Staphylococcus saprophyticus* strain NwuBe20	100	Firmicute	OK050097
21.	Nwa5	*Lysinibacillus xylanilyticus* strain NwuBe21	100	Firmicute	OK050098

22.	Nwa6	*Bacillus altitudinis* strain NwuBe22	100	Firmicute	OK050099
23.	Nwa7	*Paenibacillus illinoisensis* strain NwuBe23	100	Firmicute	OK050100
24.	Nwa8	*Brevundimonas bullata* strain NwuBe24	100	Proteobacteria	OK050101
25.	Nwa9	*Bacillus tropicus* strain NwuBe25	100	Firmicute	OK050102
26.	Nwa11	*Brevundimonas terrae strain* NwuBe26	100	Proteobacteria	OK050103
27.	Nwa12	*Myroides odoratimimus strain* NwuBe27	100	CFB	OK050104
28.	Nwa13	*Bacillus paramycoides strain* NwuBe28	100	Firmicute	OK050105
29.	Nwa16	*Brevundimonas bullata strain* NwuBe29	100	Proteobacteria	OK050106
30.	Nwa17	*Pseudomonas fluorescens strain* NwuBe30	100	Proteobacteria	OK050107
31.	Nwa18	*Bacillus cereus strain* NwuBe31	100	Firmicute	OK050108
32.	Nwa21	*Bacillus bingmayongensis strain* NwuBe32	100	Firmicute	OK050109
33.	Nwa22	*Staphylococcus kloosii strain* NwuBe33	100	Firmicute	OK050110

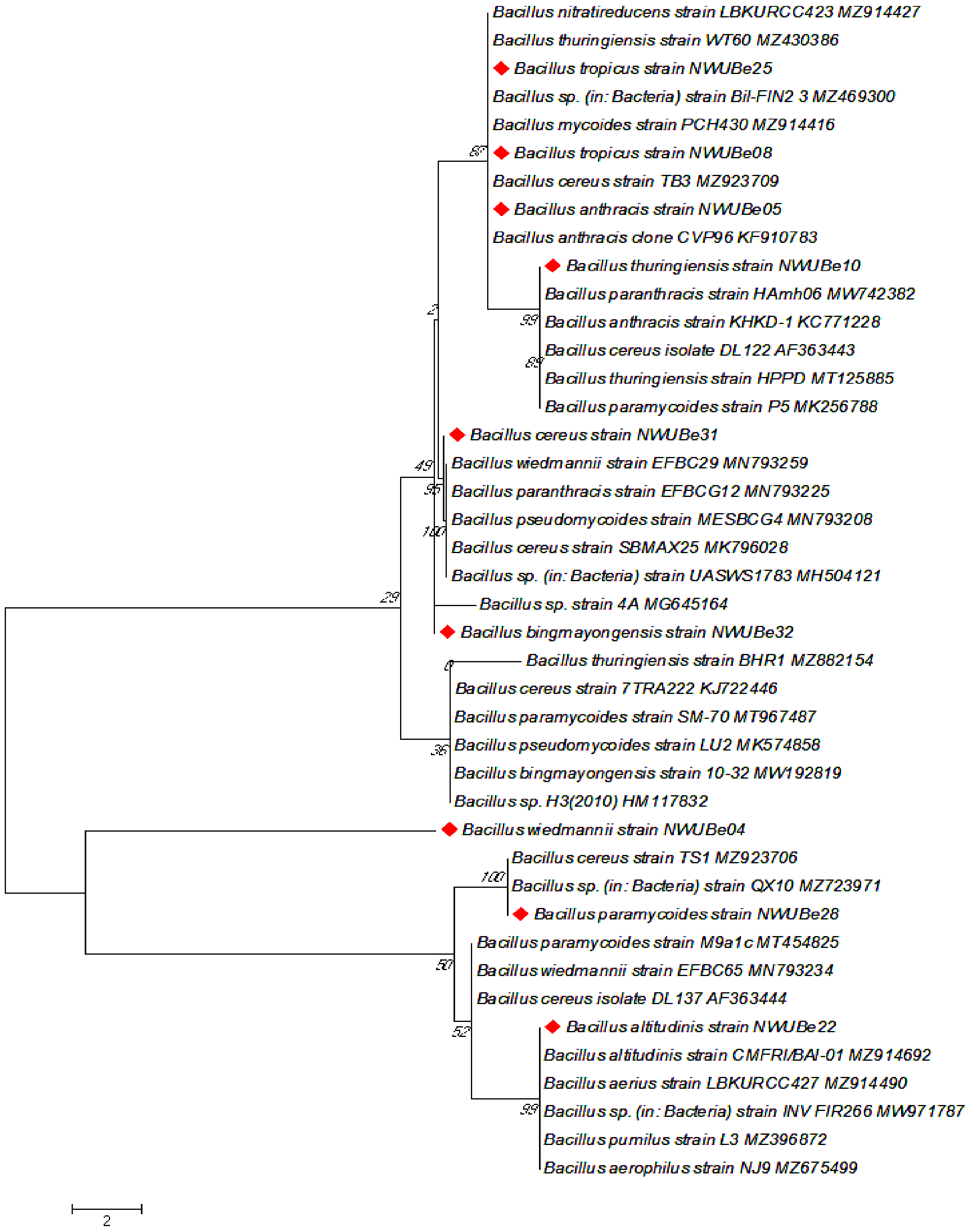


Figure 6.1. Evolutionary relationships of endophytic *Bacillus* strains taxa from cowpea plants

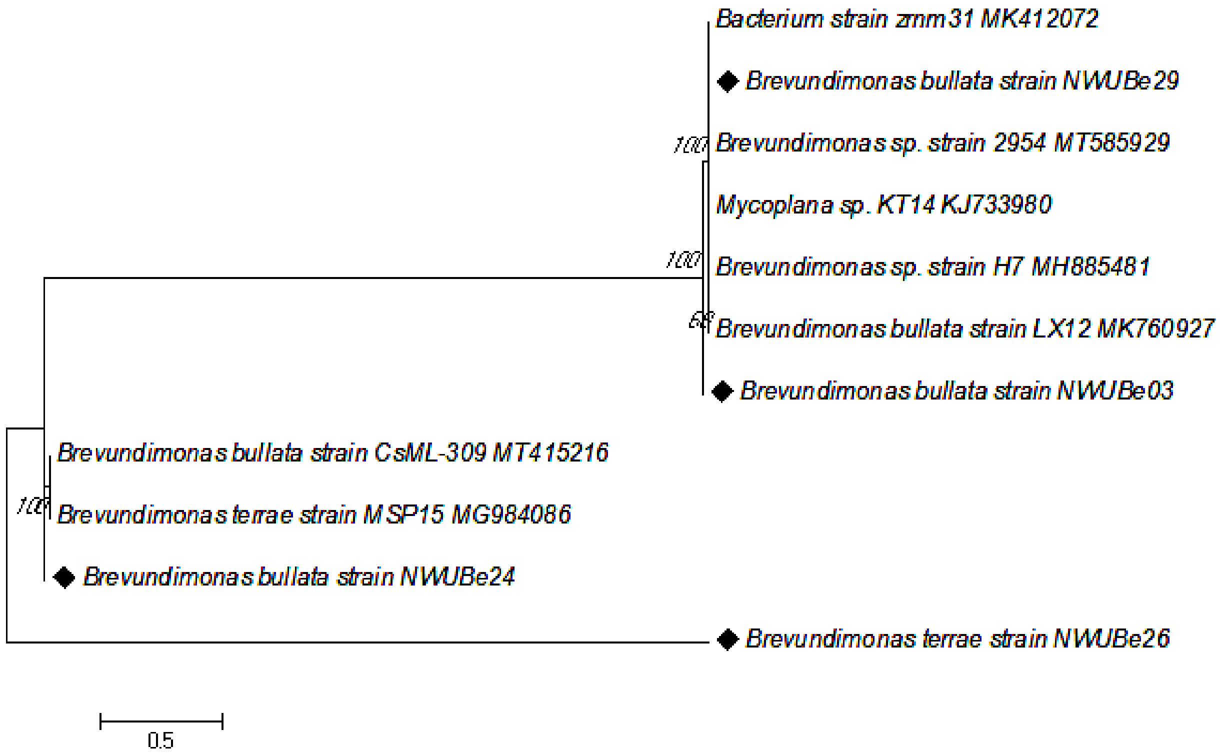


Figure 6.2. Evolutionary relationships of endophytic *Brevundimonas* strains taxa from cowpea plants

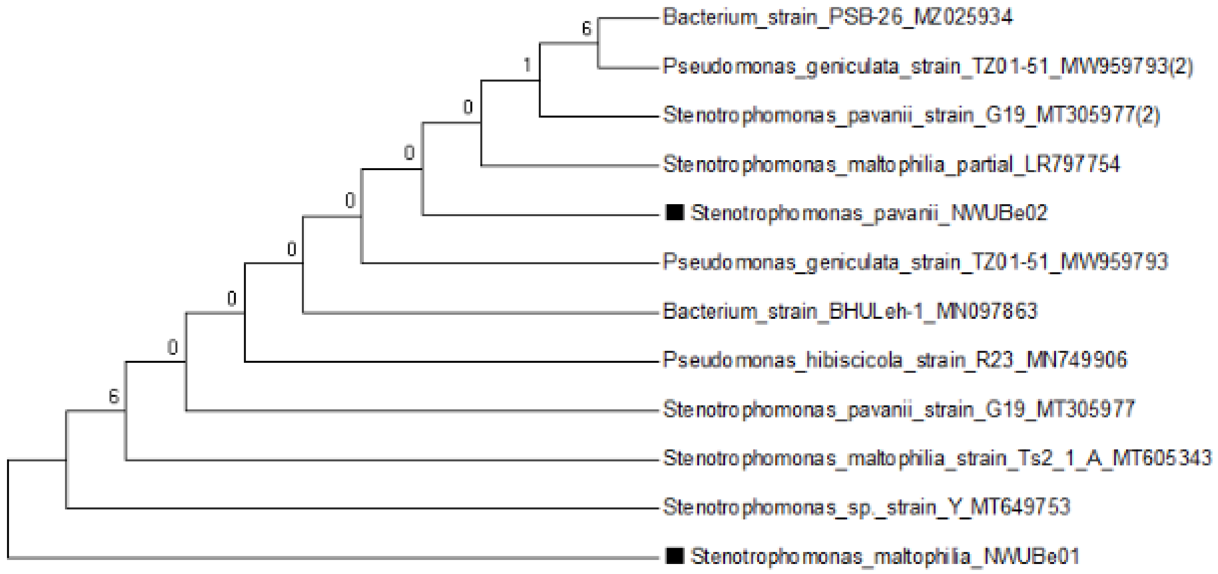


Figure 6.3. Evolutionary relationships of endophytic *Stenotrophomonas* strains taxa from cowpea plants

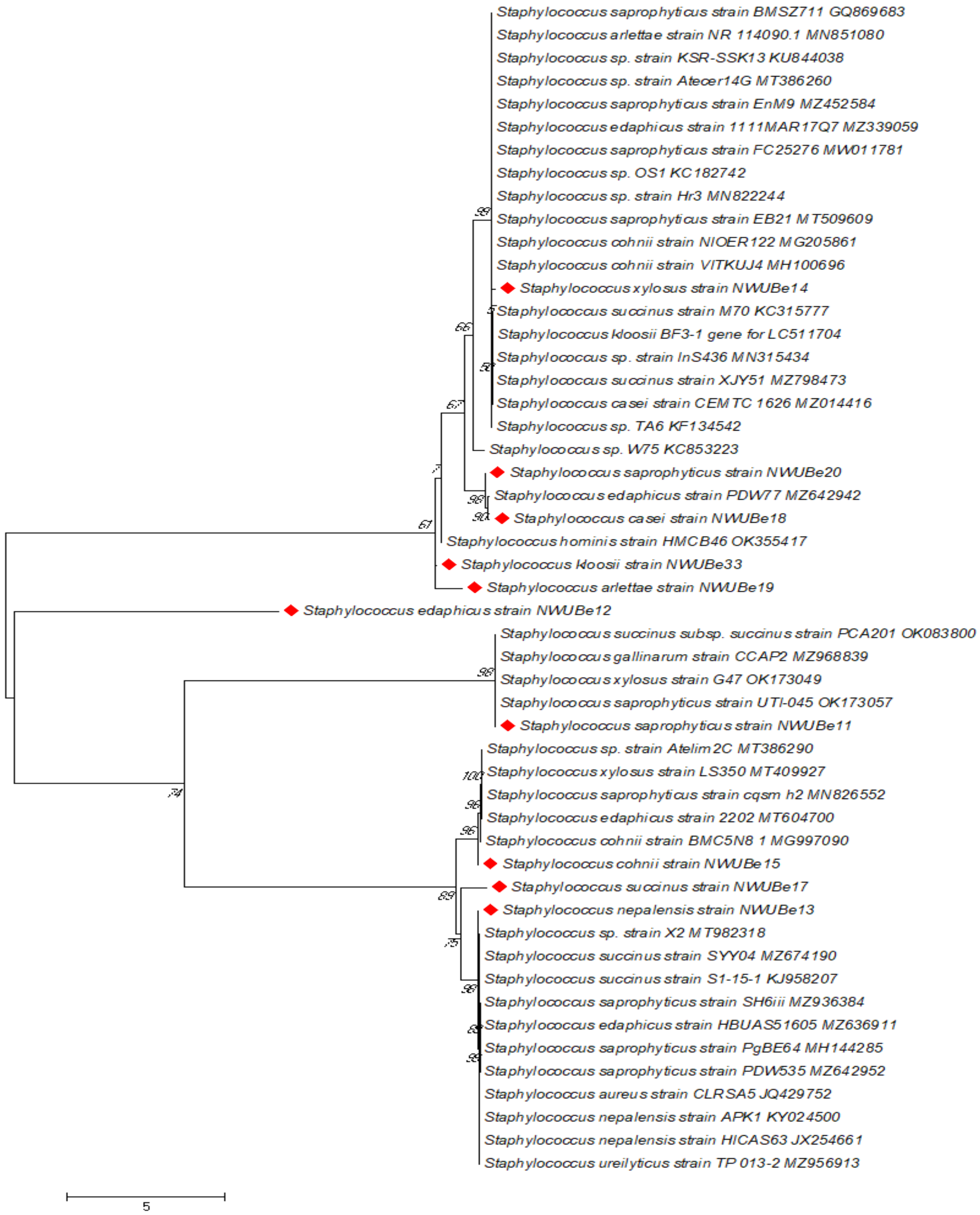


Figure 6.4. Evolutionary relationships of endophytic *Staphylococcus* strains taxa from cowpea plants

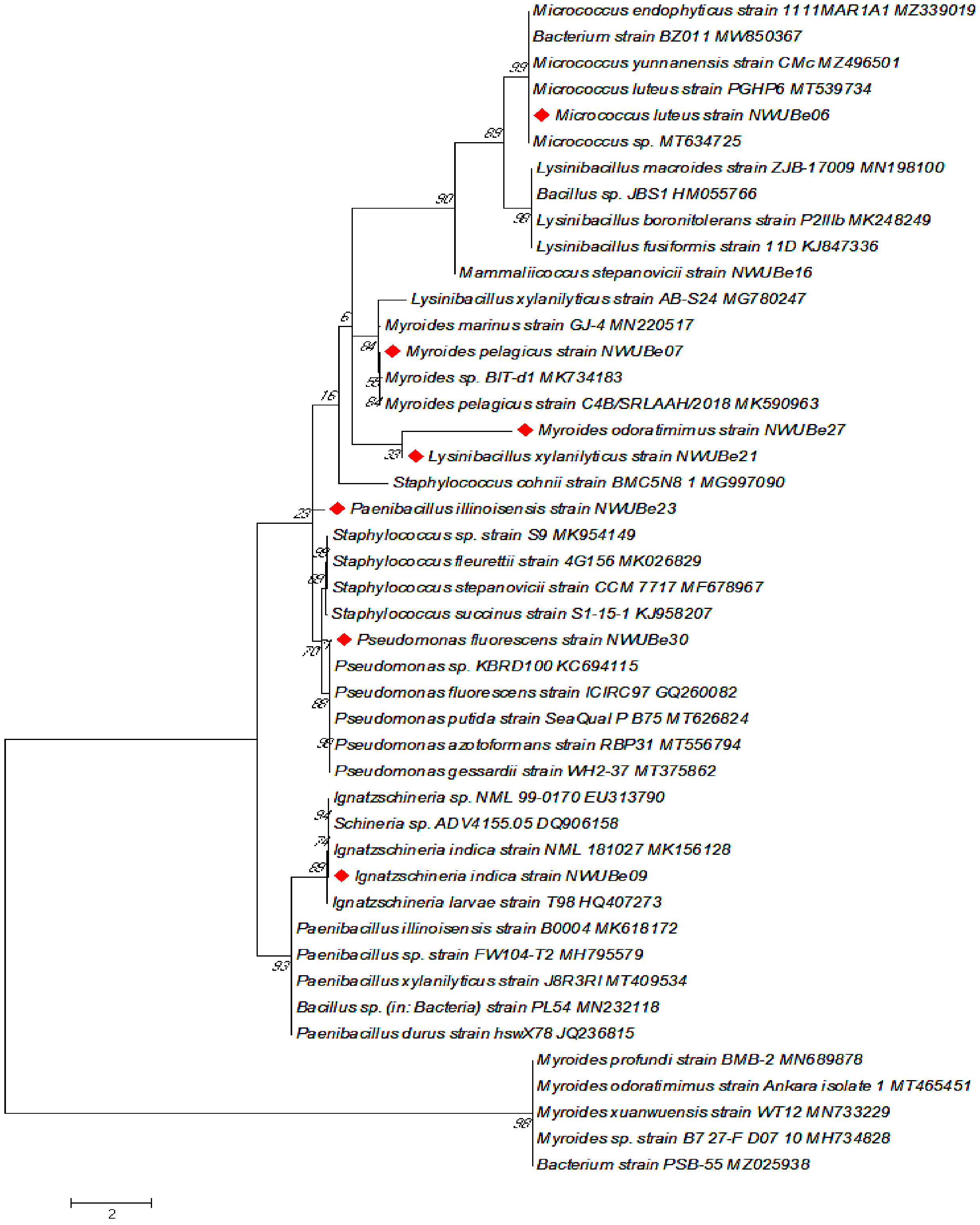


Figure 6.5. Evolutionary relationships of different endophytic bacteria strains taxa from cowpea plants

6.3.2. Plant growth-stimulating attributes of endophytic bacterial strains

The results of the plant growth-enhancing attributes of the isolated cowpea endophytic strains indicate that they are potent in promoting the growth of plants. All 33 of the isolates possess the ability to produce exopolysaccharide, IAA, ammonia and siderophore. More-so, the ability to solubilize phosphate into assimilable forms for plant utilization was identified in 70% of the isolated strains. The highlights of the plant growth-stimulating potential of the endophytic bacterial strains include their production of hydrogen cyanide, and also their capacity to produce 1-aminocyclopropylane 1-carboxylate enzyme are shown in Table 6.4.

Table 6.4: Plant growth-promoting *in-vitro* attributes of endophytic bacterial isolates from cowpea tissue.

Bacteria Code	ACDS Production	Ammonia Production	Auxin Production	Exopolysaccharide Production	HCN Production	Phosphate Solubilization	Siderophore Production
Be1	-	++	++	+	++	-	++
Be2	+	+++	+++	+	++	++++	++
Be3	+	+++	+++	+	++	++	++
Be4	+	++	++	+	++	-	++
Be5	+	++	+++	+	++	++	++
Be6	-	++	++	+	-	-	-
Be7	-	++	++	+	-	-	-
Be8	-	++	++	+	-	+++	++
Be9	-	++	++	+	-	-	-
Be10	+	+++	+++	+	++	++	++
Be11	-	++	++	+	++	-	++
Be12	+	+++	+++	+	+++	+++	++
Be13	-	++	++	+	++	-	-
Be14	+	+++	+++	+	+++	++	++
Be15	-	++	++	+	-	-	-
Be16	-	++	++	+	-	-	++

Be17	-	++	++	+	-	-	-
Nwa1	-	++	++	+	-	-	++
Nwa2	-	++	++	+	-	-	-
Nwa3	-	++	++	+	-	-	++
Nwa5	+	++	++++	+	++	++	++
Nwa6	+	+++	+++	+	++	++	-
Nwa7	+	+++	+++	+	++	++	++
Nwa8	-	++	++	+	-	-	++
Nwa9	+	+++	+++	+	++	-	-
Nwa11	-	++	++	+	-	-	-
Nwa12	-	++	++	+	-	-	++
Nwa13	+	+++	+++	+	-	-	-
Nwa16	-	++	++	+	-	-	-
Nwa17	+	+++	+++	+	++	++	++
Nwa18	+	+++	+++	+	++	++	++
Nwa21	+	+++	+++	+	++	++	++
Nwa22	-	++	++	+	-	++	++

Key: Highly active = +++; High activity = ++; Slightly high = +; None active = -

6.3.3. Tolerance to environmental stress

In this study, the results obtained in relation to the environmental stress tolerance potential of the endophytic bacterial strains to salinity stress, temperature and pH varied. The temperature of growth at 30° and 35°C supported the growth of the endophytic strains optimally, while at 40°C, the growth responses varied. Likewise, as shown in Tables 6.5, 6.6, and 6.7, varied growth responses were also observed among all the endophytic strains at different salinity and pH levels.

Table 6.5: Environmental stress tolerance of endophytic bacterial isolates to different pH levels

S/N	Endophytic bacteria strains	pH 5	pH 7	pH 10
1	*Stenotrophomonas maltophilia* strain NwuBe01	++	+++	+
2	*Stenotrophomonas pavanii* strain NwuBe02	++	++	+
3	*Brevundimonas bullata* strain NwuBe03	+	++	+
4	*Bacillus wiedmannii* strain NwuBe04	+	++	+
5	*Bacillus anthracis* strain NwuBe05	++	++	+
6	Micrococcus luteus strain NwuBe06.	++	++	+
7	*Myroides pelagicus* strain NwuBe07	+	++	+
8	*Bacillus tropicus* strain NwuBe08	++	+++	++
9	*Ignatzschineria indica* strain NwuBe09	++	+	+
10	*Bacillus thuringiensis* strain NwuBe10	++	++	+
11	*Staphylococcus saprophyticus* strain NwuBe11	+	+	+
12	*Staphylococcus edaphicus* strain NwuBe12	++	+++	++

13	*Staphylococcus nepalensis* strain NwuBe13	+	+	+
14	*Staphylococcus xylosus* strain NwuBe14	++	+	+
15	*Staphylococcus cohnii* strain NwuBe15	+	++	+
16	*Mammaliicoccus stepanovicii* strain NwuBe16	+	+	+
17	*Staphylococcus succinus* strain NwuBe17	+	+	+
18	*Staphylococcus casei* strain NwuBe18	++	+	+
19	*Staphylococcus arlettae* strain NwuBe19	+	+	+
20	*Staphylococcus saprophyticus* strain NwuBe20	++	++	+
21	*Lysinibacillus xylanilyticus* strain NwuBe21	++	+++	++
22	*Bacillus altitudinis* strain *NwuBe22*	++	++	++
23	*Paenibacillus illinoisensis* strain NwuBe23	++	+++	++
24	*Brevundimonas bullata* strain NwuBe24	+	+	+
25	*Bacillus tropicus* strain *NwuBe25*	++	++	++
26	*Brevundimonas terrae strain* NwuBe26	+	+	+
27	*Myroides odoratimimus strain* NwuBe27	+	+	+

28	*Bacillus paramycoides strain* NwuBe28	++	++	+
29	*Brevundimonas bullata strain* NwuBe29	++	+	+
30	*Pseudomonas fluorescens strain* NwuBe30	++	+++	++
31	*Bacillus cereus strain NwuBe31*	++	++	+
32	*Bacillus bingmayongensis strain* NwuBe32	+	++	+
33	*Staphylococcus kloosii strain* NwuBe33	++	++	+

Key: Highly active = +++; High activity = ++; Slightly high = +; None active = -

Table 6.6: Environmental stress tolerance of endophytic bacterial strains to varying salinity levels

S/N	Endophytic bacteria strains	NaCl 1 %	NaCl 3 %	NaCl 5%
1	*Stenotrophomonas maltophilia* strain *NwuBe01*	+	+	+
2	*Stenotrophomonas pavanii* strain *NwuBe02*	++	+	+
3	*Brevundimonas bullata* strain *NwuBe03*	+	+	-
4	*Bacillus wiedmannii* strain *NwuBe04*	++	+	+
5	*Bacillus anthracis* strain *NwuBe05*	++	+	+
6	Micrococcus luteus strain *NwuBe06*.	++	+	+

7	*Myroides pelagicus* strain *NwuBe07*	+	+	-
8	*Bacillus tropicus* strain *NwuBe08*	++	++	+
9	*Ignatzschineria indica* strain *NwuBe09*	++	+	-
10	*Bacillus thuringiensis* strain *NwuBe10*	++	+	+
11	*Staphylococcus saprophyticus* strain *NwuBe11*	+	+	-
12	*Staphylococcus edaphicus* strain *NwuBe12*	++	+	+
13	*Staphylococcus nepalensis* strain *NwuBe13*	+	+	-
14	*Staphylococcus xylosus* strain *NwuBe14*	++	+	-
15	*Staphylococcus cohnii* strain *NwuBe15*	++	+	-
16	*Mammaliicoccus stepanovicii* strain *NwuBe16*	+	+	-
17	*Staphylococcus succinus* strain *NwuBe17*	+	+	-
18	*Staphylococcus casei* strain *NwuBe18*	++	+	-
19	*Staphylococcus arlettae* strain *NwuBe19*	+	+	-
20	*Staphylococcus saprophyticus* strain *NwuBe20*	++	+	+
21	*Lysinibacillus xylanilyticus* strain *NwuBe21*	++	++	+

22	*Bacillus altitudinis* strain *NwuBe22*	++	+	+
23	*Paenibacillus illinoisensis* strain *NwuBe23*	++	+	+
24	*Brevundimonas bullata* strain *NwuBe24*	+	+	-
25	*Bacillus tropicus* strain *NwuBe25*	++	+	+
26	*Brevundimonas terrae strain NwuBe26*	+	+	-
27	*Myroides odoratimimus strain NwuBe27*	+	+	-
28	*Bacillus paramycoides strain NwuBe28*	++	+	-
29	*Brevundimonas bullata strain NwuBe29*	++	+	-
30	*Pseudomonas fluorescens strain NwuBe30*	++	++	+
31	*Bacillus cereus strain NwuBe31*	++	+	+
32	*Bacillus bingmayongensis strain NwuBe32*	++	+	+
33	*Staphylococcus kloosii strain NwuBe33*	++	+	-

Key: Highly active = +++; High activity = ++; Slightly high = +; None active = -

Table 6.7: Environmental stress tolerance of endophytic bacterial strains to varying temperatures

S/N	Endophytic bacteria strains	25°C	30°C	40°C
1	*Stenotrophomonas maltophilia* strain *NwuBe01*	+	+	+
2	*Stenotrophomonas pavanii* strain *NwuBe02*	++	+	+
3	*Brevundimonas bullata* strain *NwuBe03*	+	+	-
4	*Bacillus wiedmannii* strain *NwuBe04*	++	+	+
5	*Bacillus anthracis* strain *NwuBe05*	++	+	+
6	Micrococcus luteus strain *NwuBe06*.	++	+	+
7	*Myroides pelagicus* strain *NwuBe07*	+	+	-
8	*Bacillus tropicus* strain *NwuBe08*	++	++	+
9	*Ignatzschineria indica* strain *NwuBe09*	++	+	-
10	*Bacillus thuringiensis* strain *NwuBe10*	++	+	+
11	*Staphylococcus saprophyticus* strain *NwuBe11*	+	+	-
12	*Staphylococcus edaphicus* strain *NwuBe12*	++	+	+
13	*Staphylococcus nepalensis* strain *NwuBe13*	+	+	-
14	*Staphylococcus xylosus* strain *NwuBe14*	++	+	-

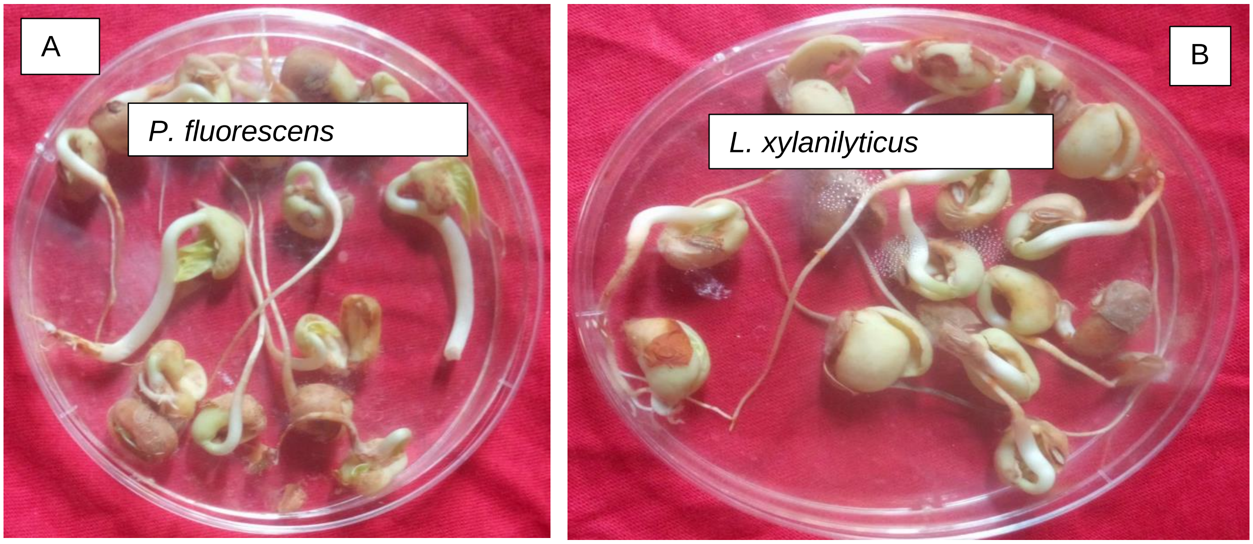
15	*Staphylococcus cohnii* strain *NwuBe15*	++	+	-
16	*Mammaliicoccus stepanovicii* strain *NwuBe16*	+	+	-
17	*Staphylococcus succinus* strain *NwuBe17*	+	+	-
18	*Staphylococcus casei* strain *NwuBe18*	++	+	-
19	*Staphylococcus arlettae* strain *NwuBe19*	+	+	-
20	*Staphylococcus saprophyticus* strain *NwuBe20*	++	+	+
21	*Lysinibacillus xylanilyticus* strain *NwuBe21*	++	++	+
22	*Bacillus altitudinis* strain *NwuBe22*	++	+	+
23	*Paenibacillus illinoisensis* strain *NwuBe23*	++	+	+
24	*Brevundimonas bullata* strain *NwuBe24*	+	+	-
25	*Bacillus tropicus* strain *NwuBe25*	++	+	+
26	*Brevundimonas terrae strain NwuBe26*	+	+	-
27	*Myroides odoratimimus strain NwuBe27*	+	+	-
28	*Bacillus paramycoides strain NwuBe28*	++	+	-
29	*Brevundimonas bullata strain NwuBe29*	++	+	-

30	*Pseudomonas fluorescens strain NwuBe30*	++	++	+
31	*Bacillus cereus strain NwuBe31*	++	+	+
32	*Bacillus bingmayongensis strain NwuBe32*	++	+	+
33	*Staphylococcus kloosii strain NwuBe33*	++	+	-

Key: Highly active = +++; High activity = ++; Slightly high = +; None active = -

6.3.4. Influence of seed biopriming of endophytic bacterial strains on cowpea seed germination

The growth chamber study of endophytic bacterial strains for bio-priming of cowpea seeds indicated positive seed germination enhancement over the control (Figure 6.6). The best seed germination performance was recorded for *Pseudomonas fluorescens* strain NWUBe30, *Lysinibacillus xylanilyticus* strain NWUBe21 and *Bacillus cereus* strain NWU Be31 respectively (Figure 6.7).



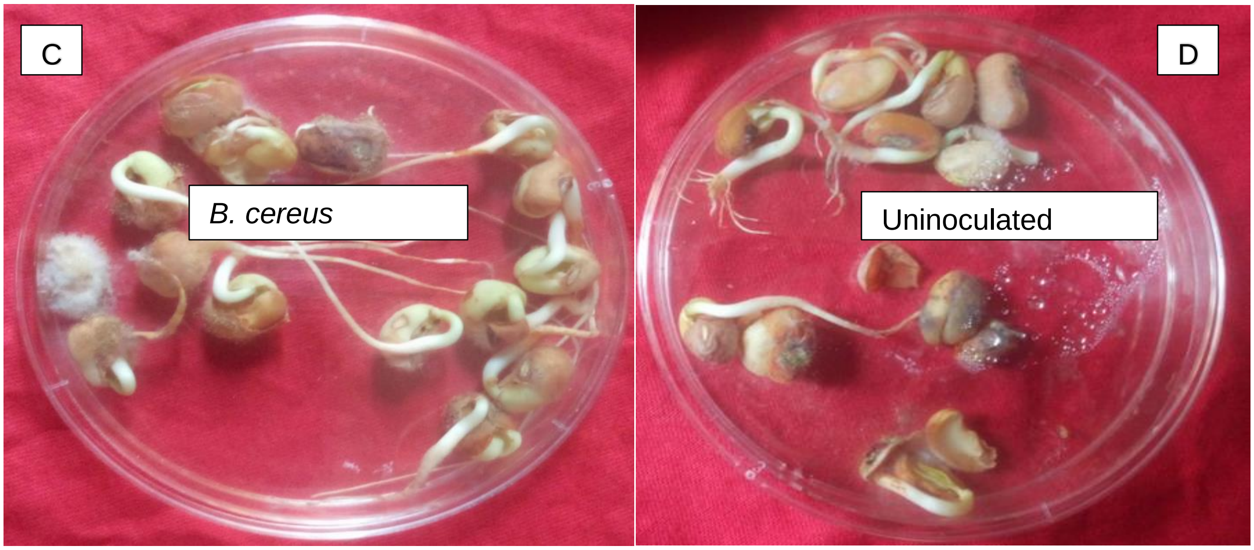


Figure 6.6: Endophytic bacterial strains bio-primed cowpea seeds germination on petri-plates

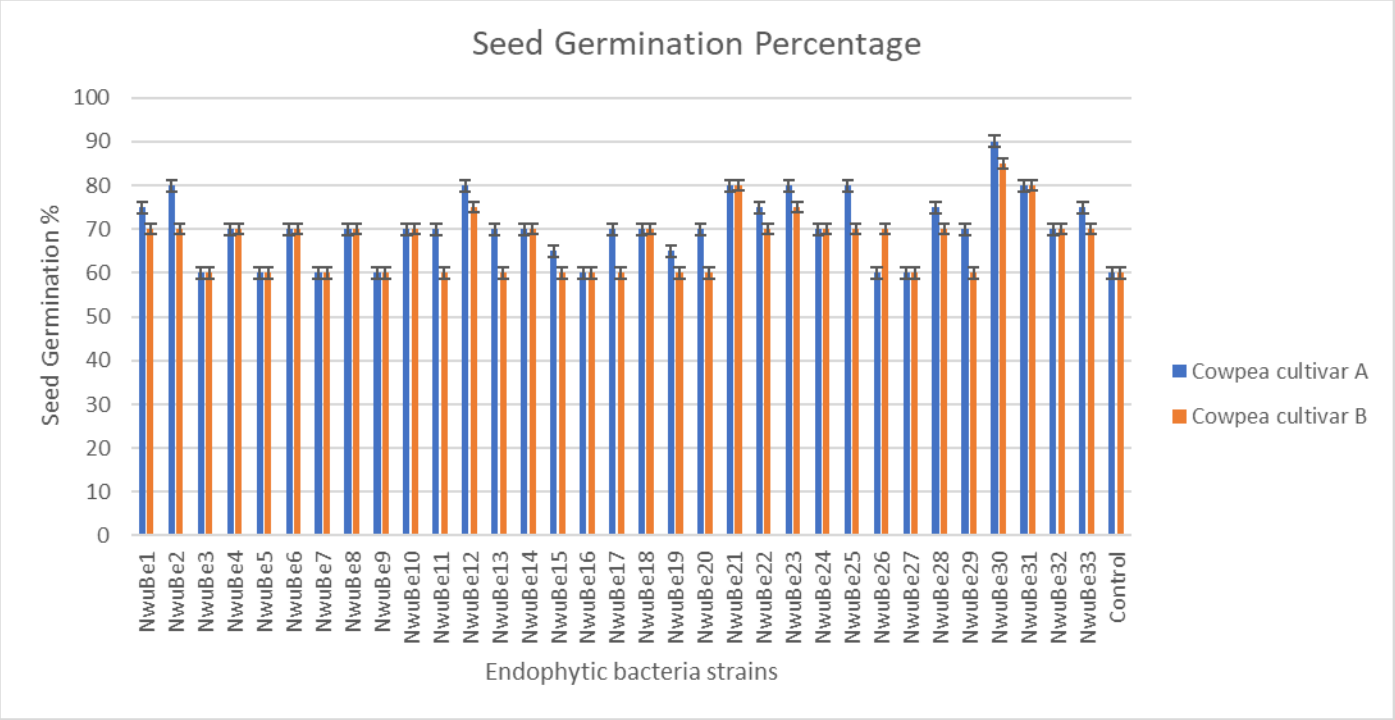


Figure 6.7: Endophytic bacterial strains seed germination percentage of two cowpea cultivars

6.3.5 Endophytic bacterial strains inhibitory action against phytopathogenic fungal

The results of endophytic bacterial strains inhibitory action against phytopathogenic fungi indicated positive inhibitory activity against some selected phytopathogens in dual confrontational assay. The percentage inhibitory value over the control phytopathogens ranges from 50 % to 80 %. This is represented in Figures 6.8 and 6.9.

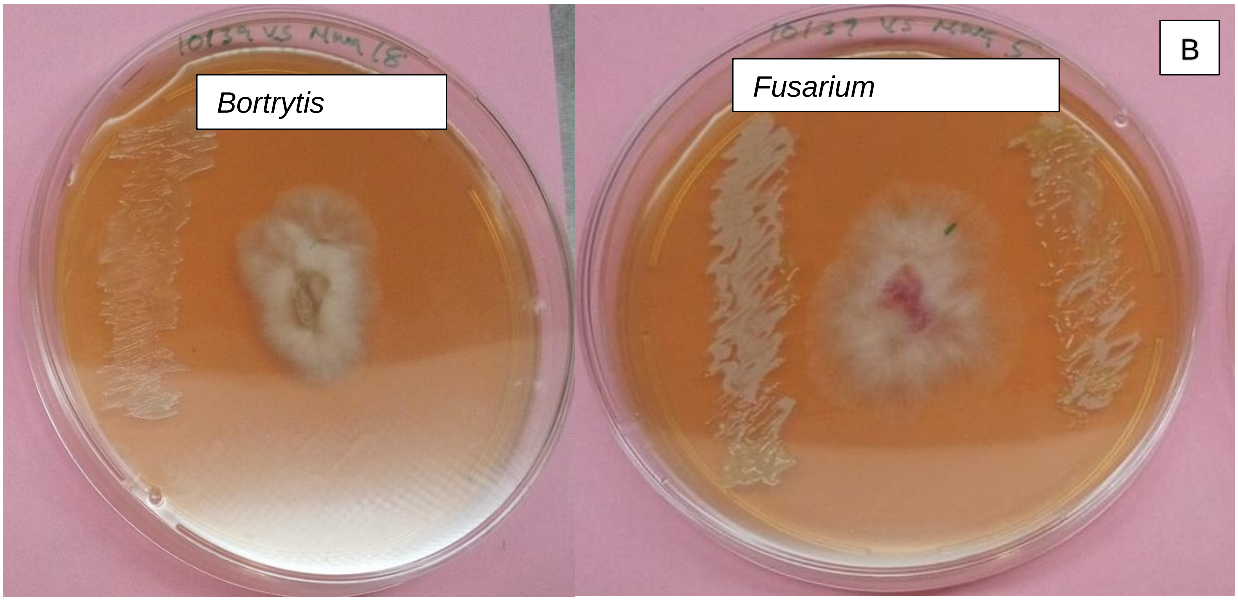


Figure 6.8: Anti-phytopathogenic potency of endophytic bacterial strains in confrontational culture assay

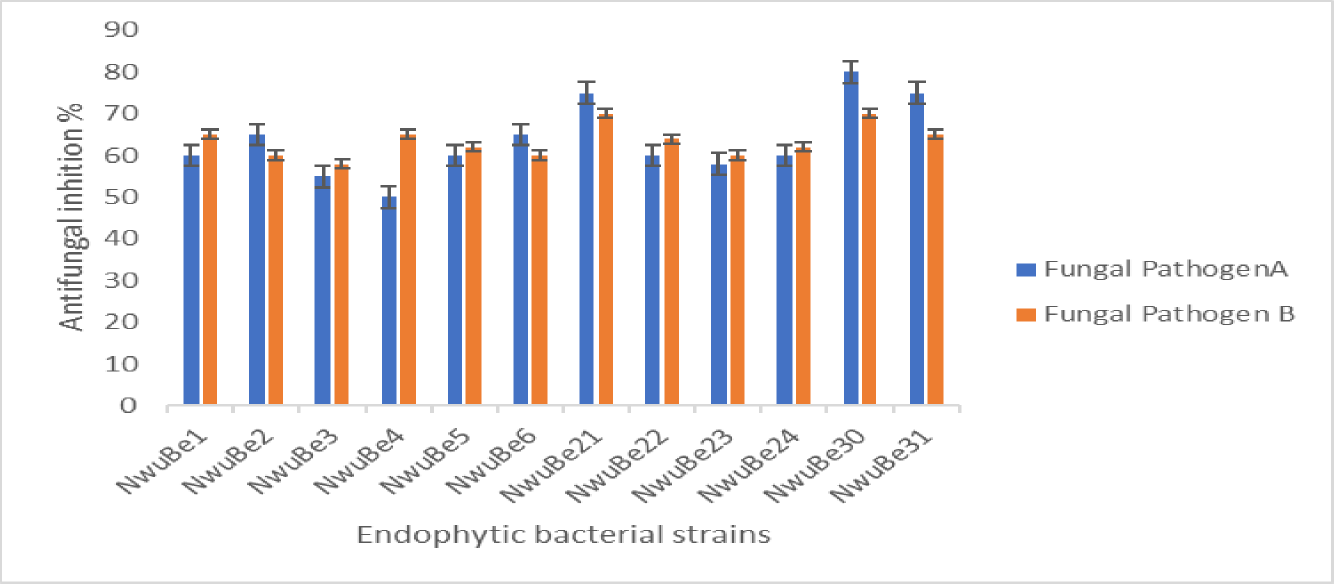


Figure 6.9: Antipathogenic fungal percentage inhibitory potency of endophytic bacterial strains

6.3.6 Amplification of plant growth promoting genes by polymerase chain reactions

The results obtained when the genomic DNA of selected endophytic bacterial strains were used to amplify PGP genes by PCR including ASB, ACPHO, IPDC, GCD, indicated that about 70 % of the selected strains confirmed positive gene presence for both ACPHO and GCD genes at the expected band size. However, about 30 % of the selected strains confirmed the positive possession of all four of the genes, namely, ACCD, ASB, IPDC, and GCD, respectively.

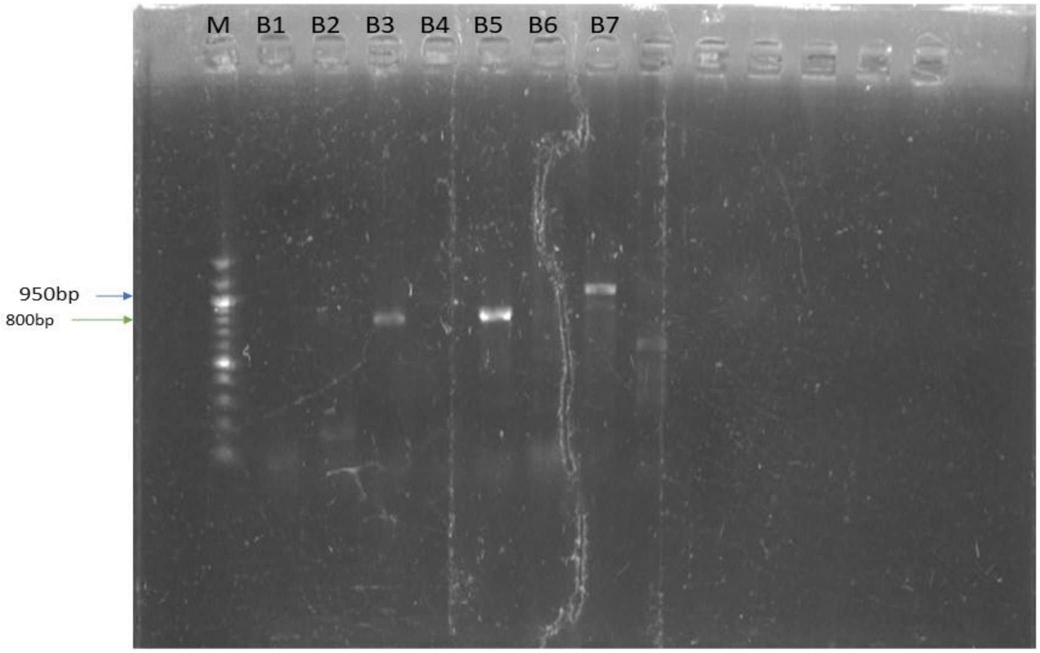


Figure 6.10: ACCD gene amplified by endophytic bacterial strains at the expected base pair sizes by PCR

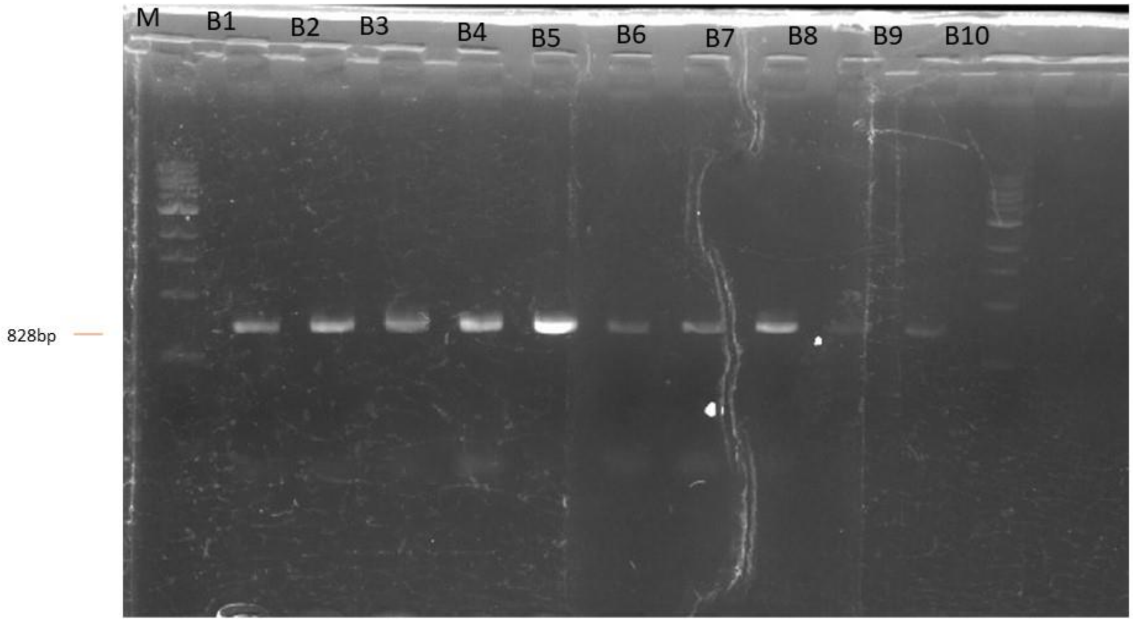


Figure 6.11: ACPHO gene amplified by endophytic bacterial strains at 828 base pair size by PCR

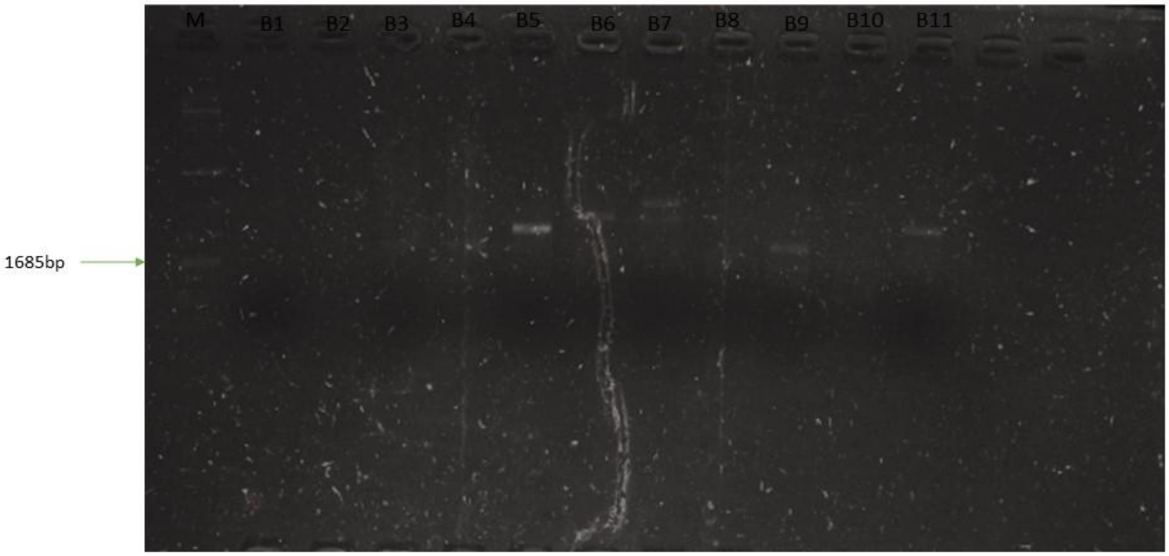


Figure 6.12: ASB gene amplified by endophytic bacterial strains at 1685 base pair size by PCR

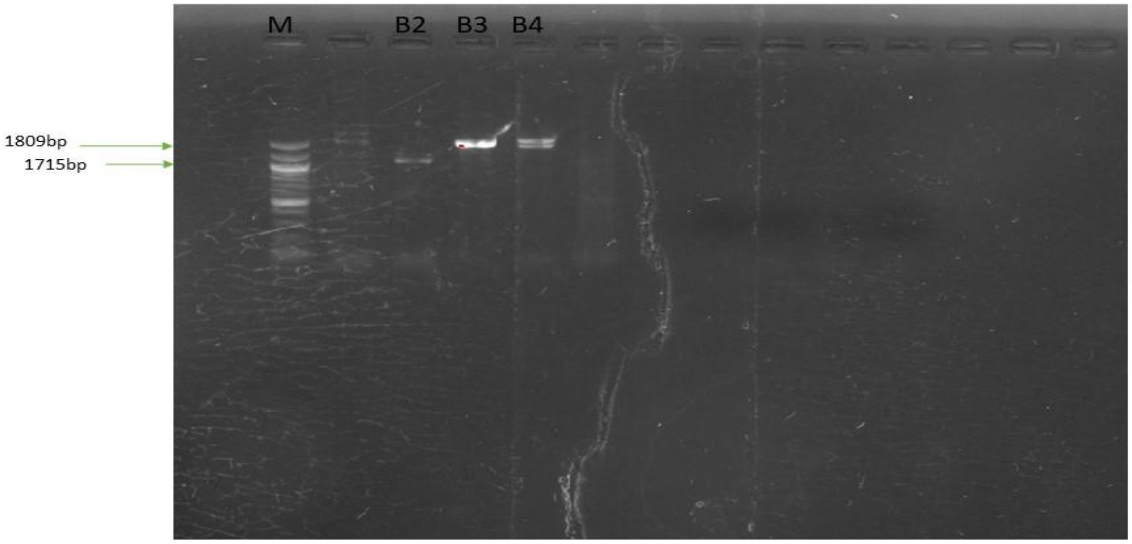


Figure 6.13: IPDC gene amplified by endophytic bacterial strains at expected base pair sizes by PCR

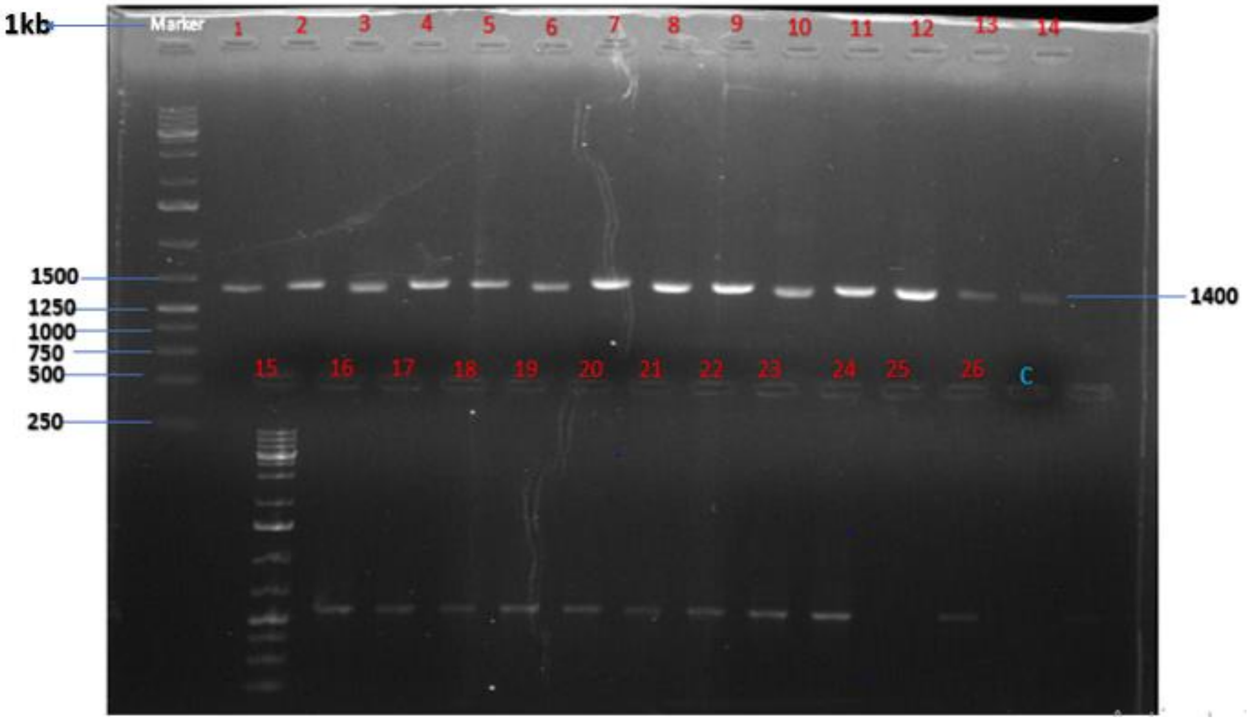


Figure 6.14: Agarose gel electrophoretic representation of the 16S rRNA gene of cowpea endophytic bacterial strains amplified by a polymerase chain reaction using the 1k bp molecular ladder

6.4. Discussion

Endophytic microbial research studies in recent times have shown remarkable roles of endophytic bacterial isolates as important partners in the plant-microbiome interactions, with beneficial consequences in relation to plant growth, fitness and functionality. In studies that cover diverse plant types, numerous endophytic bacterial isolates have been reported to have beneficial attributes that enhance plant growth (Bhutani *et al.*, 2022; Maheshwari *et al.*, 2022; Phurailatpam *et al.*, 2022).

Highlights from the findings of some of these studies indicate that endophytic microbiota positively influence plant growth and vitality through mechanistic approaches, that include amongst others, their ability to favourably compete for the available nutrients, their secretion of vital plant hormones, their confrontations with destructive phytopathogens, and their metabolization of important complex nutrients that are in forms not available as nutrients (ALKahtani *et al.,* 2020; Kushwaha *et al.*, 2020a). These earlier reports that have shown promising research outcomes with regard to the deployment of endophytic microbiota as bioinoculating agents and as viable alternatives to synthetic agrochemicals for sustainable crop management, have prompted this research effort.

Thus, our study focused on bioprospecting endophytic bacterial strains from cowpea seeds and tissues as microbial agents that could be deployed in improving cowpea productivity as viable and sustainable substitutes to synthetic agrochemicals.

Hence, based on their morphometric characteristics, biochemical traits and molecular sequencing, 33 endophytic bacterial isolates were morpho-genetically authenticated to be potent in enhancing plant-growth. The taxonomy, phylogeny, and metabolic functions indicated that the isolated endophytic bacterial strains are highly diverse and multifaceted.

Compositional diversity of endophytic microbiota

The endophytic isolates are composed of 11 genera that include *Pseudomonas, Lysinibacillus, Bacillus, Paenibacillus, Stenotrophomonas, Brevundimonas, Staphylococcus, Micrococcus, Myroides, Ignatzscineria* and *Mammalicoccus*. The results concerning the nucleotide blast and phylogenetic relatedness of the isolated endophytic bacterial strains indicated a 97 to 100% species similarity, with related strains in the Genbank. Our findings corroborate with findings from (Maheshwari *et al.*, 2021; Vo *et al.*, 2021)(Adeleke et al. 2022; De Almeida Lopes et al. 2018; Flores-Duarte et al. 2022; Jia et al. 2022; Khanna et al. 2022; Maheswari et al. 2021; Mukhtar et al. 2020; Vo et al. 2021;) which reported that diverse endophytic bacterial strains can be isolated and characterized from the different tissues of plants including leguminous crops.

Functional characterization of endophytic microbiota

The stimulation of plant-growth has been attributed, through a diversity of approaches, to endophytic microbiota (Bhutani *et al.*, 2021; Pandey *et al.*, 2022; Tran *et al.*, 2022). This present investigation affirms the efficacy of endophytic bacterial strains in achieving plant growth-stimulating activities in cowpea through multifarious metabolic traits that include, phosphate solubilization, the production of indole acetic acid, siderophore, exopolysaccharide, ammonia and ACCD. All the endophytic bacterial isolates possess at least three plant growth-improving traits. The potential of these effective microbial endophytic strains to elicit PGP traits has been attributed to their plant growth-enhancing capacity through direct and indirect mechanistic approaches (Aeron *et al.*, 2021; Afzal *et al.*, 2019). For instance, the siderophore-producing abilities of endophytic microbes makes for the chelating of iron from unavailable to usable forms for plant absorption, thus enhancing growth (Etminani & Harighi, 2018; Gorai *et al.*, 2021). Likewise, the abilities of endophytic microbiota to solubilize phosphate, to produce auxin-like IAA, to regulate ethylene biosynthesis through the production of the ACC deaminase enzyme, and to fix atmospheric nitrogen confer, amongst others, growth-promoting attributes on planted crops (Adhikari Dhungana *et al.*, 2018; Bhutani *et al.*, 2018; Brígido *et al.*, 2019; Eid *et al.*, 2021).

Endophyte-mediated phosphate solubilization via organic acid secretion is a veritable means of making phosphorus available for plant uptake (Lucero *et al.*, 2021; Mei *et al.*, 2021). Also, endophytic microbial mediated phyto-hormonal production has been reported to stimulate growth in planted crops (Adeleke *et al.*, 2022b; Chen *et al.*, 2017a). These positive attributes by endophytic microbiota are mediated through the beneficial regulation of the stressful conditions induced by ethylene, as well as through the signaling of molecules for positive plant-microbe interactions.

Generally, the improvement in the growth of planted crops results from endophytic microbe inoculation through different mechanistic approaches, such as the production of siderophore, IAA, and the ACC deaminase enzyme, nitrogen fixation, biofilm formation and other growth-promoting traits. These benefits are the result of various metabolic and physiological changes that are induced in the host plant and include such functions as phytohormonal production, the modification of plant root architecture to enhance water and nutrient uptake and retention, the antagonization and evasion of phytopathogens, as well as the ability to alleviate abiotic stressors in the environment.

In addition to the data obtained from the *in-vitro* growth assessment assay, seed germination studies have indicated that all the endophytic bacterial strains possess multiple plant growth-improving traits, which significantly enhance, the seed germination percentage rate, to a greater extent than those of the control plant. The enhancement in seed germination and in the seedling growth of the cowpea plant that were observed in this study concurs with the previous report in the literature that supports bioinoculating microbial agents as a veritable means of mediating physiological changes and growth improvement in planted crops (Adeleke *et al.*, 2022a; Khanna *et al.*, 2022; Kumar & Dubey, 2022).

6.5. Conclusion

The salient findings of this study affirm that the resident endophytic bacterial from cowpea plant tissue have the potential to improve cowpea productivity. These diverse and efficient endophytic microbial isolates employ different growth-promoting traits such as, amongst others, the secretion of siderophore and auxin, the ability to chelate iron, to solubilize phosphate, to produce ammonia, to take, action to inhibit the growth of phytopathogens, and thus to improve cowpea growth. Therefore, these functions have shown good prospects for the endophytic bacterial isolates as a means to improving the sustainability of cowpea production. However, these isolates need to be subjected to further genomic exploration studies and multiple experimental trials in the field.

CHAPTER SEVEN

7.0 MORPHO-GENOTYPICAL DIVERSITY AND GROWTH-PROMOTING POTENTIAL OF CULTURABLE ENDOPHYTIC FUNGAL ISOLATES FROM THE TISSUES OF *VIGNA UNGUICULATA* IN NORTH WEST PROVINCE, SOUTH AFRICA

Abstract

Cowpea is a frontline indigenous African legume with prospects as a vital source of nutrition, for solving both human and livestock protein demands. It is also a means for generating an income by the different stakeholders involved in the value chain. Furthermore, it also aids in improving the soil nutrient status through its potential for nitrogen fixation. The benefits that are associated with the production of cowpea, make it a topmost research focus area for promoting sustainability and for intensifying ecologically balanced productivity. The search for alternatives for the huge volume of synthetic agro-chemicals that are deployed for cowpea production; makes the investigations into a sustainable and, nutritional product, the cowpea, the crux of this study. That the cowpea plant is; environmentally and agro-ecologically balanced and that it has through the bioprospecting of its cultivable endophytic fungal isolates, the potential to improve in terms of its productivity, are added incentives for focusing on it as a research topic. Using morphological and molecular techniques This study succeeded in isolating and identifying endophytic fungi from the seeds, leaves and near root tissues of cowpea plants. This research further assessed the growth promoting-potential of the isolates in vitro and also in growth chamber experiments. A total of 26 endophytic fungal isolates were molecularly characterized using internal transcribed spacer regions and further phylogenetically grouped according to their genotypic diversity. All the fungal isolates demonstrated growth enhancing traits in vitro, and two of the isolates were further deployed in an assay to improve seedlings in a growth chamber environment. At the conclusion of our study, it was possible to identify and characterize diverse endophytic fungal isolates from cowpea root zone tissues, leaves and seeds that would have the potential using agro-ecologically sustainable approach; to promote the growth of the cowpea plants and improve on its productivity.

Keywords: fungal endophytes; protein demands; synthetic agrochemicals; agroecological sustainability; cowpea tissues; biostimulants

7.1. Introduction

Multiple constraints challenge the optimization of agricultural production efforts to achieve food security and safety in the modern world. Hence, urgent attention is needed in the field of research to leap beyond these multiple constraints in order to intensify agroproduction and sustainability (Glaser & Lehr, 2019; Kumar *et al.*, 2021c; Omomowo & Babalola, 2021).Achieving secure and safe foods supplies to feed the burgeoning global population is difficult and very challenging in the light of amongst other constraints, the huge and devastating corona virus pandemic,changing weather patterns and the negative anthropogenic actions of man on a global scale (Jámbor *et al.*, 2020; Shahzad *et al.*, 2021).The conventional and modernized means of achieving agricultural productivity to feed the world population rely on the application of expensive and non-renewable agro-chemicals such as synthetic fertilizers and numerous pesticides(Duan *et al.*, 2016; Prashar & Shah, 2016). Indeed, the intensive use of agrochemicals has proved to be very expensive, and is actually threatening the well-being of man and the ecological environment (Jacquet *et al.*, 2022; Luan *et al.*, 2020; Mahanty *et al.*, 2017; Meena *et al.*, 2020).In contrast to the intensive use of agrichemicals in enhancing crop productivity, microbial-based formulations are reported as the potent option to achieved a renewed and greener revolution in agroecological production in the quest to achieve improved crop growth and development(Kumawat *et al.*, 2022; Liu-Xu *et al.*, 2022; Pandey *et al.*, 2022).

Indeed, a viable and sustainable alternative/complement to synthetic fertilizers and other pesticides for achieving improved agroecological productivity is through the deployment of microbial inoculants to revitalizing soil health and confer specific functional traits on plants to mitigate their abiotic and biotic stressors (Adeleke *et al.*, 2022c; Baron & Rigobelo, 2022; Yan *et al.*, 2019).Nevertheless, in recent times, as a partner in the plant-phytomicrobiome interactions endophytic microbial research has lately proven potent as an alternative option or complement to synthetic agrochemicals in achieving in a sustainable manner the optimization of agri-systemic outputs (Ikram *et al.*, 2022; Kapoor *et al.*, 2022; Putri, 2022)The of endophytic microbiota in stimulating plant growth and in enhancing plant productivity has been achieved through diverse mechanisms that include their ability to fix atmospheric nitrogen, and to convert insoluble phosphate to soluble phosphate, the production of vital enzymes and; important phyto-metabolites and the suppression/inhibition of microbial pathogens and pests (Mehmood *et al.*, 2019a; Nuangmek *et al.*, 2021; Poveda *et al.*, 2021).

The firm establishment of endophytic microbiota to colonize the innermost tissues of plants confers on them multiple beneficial traits that aid in improving plant-growth. Endophytic microbiota incorporate a goldmine of bioactive metabolites that influence their actions as immune boosters in plants, stimulate plant-growth, help in the biocontrol of pests and microbial pathogens, and in coping with environmental stressors (Potshangbam *et al.*, 2017; Singh *et al.*, 2021).The endophytic fungi inhabit the innermost tissues of the plant without any adverse impact; instead they confer benefits on the plant (Hardoim *et al.*, 2015; Lugtenberg *et al.*, 2016). Research reports have ascertained the effectiveness of endophytic fungi as beneficial bioagents that when applied to plants, can mitigate and contain the destructive impacts of pests and phytopathogens, ameliorate both the abiotic and the biotic stressors affecting planted crop, boost the immunity status of the plant and secrete vital plant hormones, and other bioactive metabolites. In their entirety all the bioactivities of the fungal endophytic microbiota eventually enhance plant-growth (Afandhi *et al.*, 2019; Akhtar *et al.*, 2022; Asim *et al.*, 2022; Bilal *et al.*, 2018; Farias *et al.*, 2020).

The common name for *Vigna unguiculata* (L.) Walp. is the cowpea, which is a foremost African leguminous crop that is of great importance in terms of its the nutritional, economic and environmental attributes. It is grown mainly in the warmer regions of Africa and other semi-arid zones worldwide. It is an annual crop and also a diploid with 2n=2x=22. The genomic size of cowpea is estimated at 620 million base pairs (Lonardi *et al.*, 2019). In terms of its importance, it is a vital source of dietary nutrients for humans and also, provides fodder for livestock. More so, a key trait of the cowpea includes its role in maintaining the soil-ecological balance by fixing atmospheric nitrogen in symbiotic association with nodulating bacteria (Ravelombola *et al.*, 2017). The protein and essential amino acid content of cowpea varies between 23% and 32% (Carvalho *et al.*, 2017). All parts of the cowpea plant are valuable from the leaves that can be used as vegetables, to the haulms (the cowpea pod walls, stems, and leaves) which are used as livestock fodder, and thereby generating an income farmers (Kebede & Bekeko, 2020). Cowpea is strategically valuable leguminous crop to Africa in terms of the economy of scale associated with its production (Muñoz-Amatriaín *et al.*, 2017).

In the light of above-mentioned facts, this research aims at circumventing the low productivity constraints of cowpea production in North West Province South Africa, by bioprospecting endophytic fungal isolates to harness their probiotic promoting traits in order to deployed them as potential bioinoculants to improve cowpea production.

7.2 Materials and Methods

7.2.1. Cowpea (*Vigna unguiculata* (L) Walps) sampling/collection

Healthy cowpea plant samples including roots, stems and leaves with no external symptoms of disease were obtained from farms in Mafikeng, Northwest province, South Africa . It was appropriately transported in cold conditions in plastic bags at 4 °C before processing for isolation of endophytes. However, the cowpea seeds used in the study was obtained from Agricultural research council (ARC), Pretoria

7.2.2. Endophytic fungal isolation from leaves, seeds and near root tissue zones of (*Vigna unguiculata*) cowpea plant

Healthy cowpea seeds and tissues were firstly rinsed with tap water to eliminate debris. Further, several steps of ensuring surface sterilization was achieved through washing with 70 % ethanol, then cleaning with 3 % sodium hypochlorite for 3 minutes. This was again, followed by cleaning with 70 % ethanol, then washing thoroughly with sterile water upto 5 times to eliminate any epiphytic microbes in the cowpea samples. The efficacy of the surface sterilization process was assessed via plating the last rinsed water used on culture media. The sterilized cowpea tissues, were crushed appropriately with sterile pestle-mortar, diluted using phosphate buffer, then plated on varied mycological culture media. The petri-plates were incubated following standard protocol. Furthermore, several subculturing steps on PDA were carried out to obtain pure endophytic fungi isolates, and stored using 20 % glycerol for further analysis.

7.2.3. Genomic extraction, PCR amplification and sequencing of ITS and PGP genes of endophytic fungal isolates

The genomic DNA of the endophytic fungal isolates were extracted using Zymoclean kits following specifications of the manufacturer. The extracted DNA purity was checked on 0.8 % agarose gel using UV-visual gel documentation. The ITS region genes of the fungi endophytes were amplified through PCR amplification techniques with the use of ITS 1 forward and ITS 4 reverse primers for fungi (White *et al.*, 1990). The pcr reaction cocktails of 25 ul consist: 2X master mix: 12.5 ul, molecular grade water 8.5, forward and reverse primer 1 ul each, and DNA template 2 ul. The PCR cycling conditions using (Biorad 1000 thermocycler, USA), consists of initial denaturing at 95 °C for 5 mins, followed by 30 cycles of denaturing at 94°C for 30 sec, annealing at 52°C for 1 min, extension at 72°C for 45 seconds, and final extension at 72°C for 7 min and holding at 4°C. Table1 below represents the primers deployed and references used in the amplification protocol. The pcr amplification product was checked by electrophoresis using 1.2 % agarose gel in TAE buffer stained with ethidium bromide, run for 1 hour in the same TAE buffer, and finally visualized using UV gel documentation equipment. The amplified products were quality checked and sequence unidirectionally at Inqaba biotech, Pretoria, South Africa.

Table 7.1: Primers for internal transcribe spacer region and plant growth promoting genes (ITS and PGP genes) used for the identification of endophytic fungal strains isolated from cowpea tissue for amplification.

Target Gene	Primer Name	Nucleotide Sequence (5′ -------→ 3′)	Product Size (bp)	Reference
ITS	ITS1-F ITS4-R	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	300-1000	(White et al., 1990)
IPDC	IPDC-F IPDC-R	CAYTTGAAAACKCAMTATACTG AAGAATTTGYWKGCCGAATCT	1715 - 1809	(Raddadi et al., 2008)
ACPHO	ACPHO-F ACPHO-R	AAGAGGGGCATTACCACTTTATTA CGCCTTCCCAATCRCCATACAT	360	(Poly et al., 2001)
GCD	GCD-F GCD-R	GACCTGTGGGACATGGACGT GTCCTTGCCGGTGTAGSTCATC	750-755	(Li. 2011)

7.2.4. Molecular authentication and phylogenetic assessment

The molecular characterization and authentication of the isolated fungal endophytes after sequencing were carried out using appropriate Bioinformatics tools for trimming, bioediting and aligning sequences before subjecting them to the National Centre for Biotechnological Information (NCBI) nucleotide blast platform for proper referencing with similar fungal strains in the Genbank database.The sequences were deposited in the Genbank repository and accession numbers were obtained. (MEGA 7) (Molecular Evolutionary Genetics Analysis) software (Kumar *et al.*, 2016) was used to infer the relatedness of the fungal strains and the maximum likelihood model was used for the construction of a phylogenetic tree.

7.2.5. In vitro assays of the plant growth-stimulating attributes of Endophytic fungi

The standardized *invitro* assay protocols that was deployed to confirm the plant growth-promoting attributes of these fungal endophytic isolates, and the various analyses and methodologies deployed in achieving this objetive included the solubilization phosphates (Nautiyal, 1999) the production of ammonia (Cappucino & Sherman, 1992) indole acetic acid (Matsuda *et al.*, 2018), siderophore (Schwyn & Neilands, 1987), exopolysaccharide (Khan & Bano, 2019), and hydrogen cyanide (Dinesh *et al.*, 2015). The potential for biofilm and biomass formation was yet another aspect that was established (Allkja *et al.*, 2021), as was the confirmation of the growth-promoting properties of the endophytic fungal isolates through a seeding growth chamber assay.

Potency of fungal isolates for solubilizing Inorganic phosphates

Based on Mehta and Nautiyal (2001) protocols,the potency of the fungal endophytic isolates to solubilize phosphate was assessed. After the concentration has been adjusted to 0.5, Fresh grown endophytic fungal cultures grown in a potato dextrose (PD) broth were used as an aliquot to inoculate the National Botanical Research Institute's Phosphate (NBRIP) solubilization agar plate containing insoluble calcium tri-phosphate. The plates were replicated thrice and incubated for seven days at 30 °C. The appearance of a transparent halo-zone around the culture colony confirmed that the isolates have the potential for phosphate-solubilization.

Potency of fungal isolates for producing Indole acetic acid

The ability of the endophytic fungal isolates to produce indole acetic acid was assessed on the basis of assay protocol proposed by (Matsuda *et al.*, 2018) . Three day cultures of the endophytic isolates were used to inoculate yeast-enriched peptone water that contained five (5) mM L-tryptophan. This liquid medium was then incubated on a rotary shaker at 150 rpm, for 48 hours and at 30°C. Thereafter, the cultures were processed and centrifuge to obtain a supernatant. Salkowski reagent was added to the supernatant at a ratio of 2:1 and the liquid was incubated in the dark for 30 minutes before the absorbance was measured with the aid of spectrophotometer at 530 nm. The standard curve of IAA was also plotted on the basis of the various concentrations of indole acetic acid in order to quantify the amount of IAA produced by the endophytic fungal isolates.

Potency of endophytic fungal isolates for producing siderophore

The ability of the endophytic fungal isolates to produce siderophore was assessed qualitatively on Chrome Azural (CAS) agar plates (Schwyn and Neilands,1987). The endophytic isolates were freshly grown in a yeast extract glucose medium to obtain a 0.5 concentration at OD600. A diffusible disc was placed on a freshly prepared CAS-blue agar plate. Thereafter 10 µl of aliquot from each endophytic fungal culture was inoculated onto the plate and incubated for 72 hours. The appearance of a yellowish-orange halo around the culture colony indicated a positive potency for producing siderophore.

Potency of endophytic fungal isolates for promoting ACC deaminase activity (ACCD

The assay protocol as described by Glick et al. (1995), was used in assessing the potentials of the endophytic fungal isolates to promote ACC deaminase activity by harvesting and centrifuging the broth culture to obtain a culture pellet. Freshly grown cultures were produced and then used as inoculum. There-upon, the culture pellet was washed thoroughly in sterile saline water and re-suspended in saline water before a spot inoculation was conducted on three (3) mM ACC incorporated minimal media petri-plates. The minimal media plate that was incorporated with ammonium sulphate was used as a positive control while the minimal media plates without any nitrogenous sources served as the negative control. All plates were in triplicate and cultured for 72 hours at 30 ^{o}C. The growth of the endophytic fungal isolates on the Three (3) mM ACC-incorporated minimal media petri-plates indicated the potential of endophytic fungal isolates for promoting ACC deaminase activity.

Ammonia production

By using the protocol proposed by Cappuccino and Sherman (1992). The endophytic isolates were assessed for their potency in producng ammonia. This involved the use of a freshly grown culture of endophytic isolates to inoculate 10 ml of sterilized peptone water in test tubes which was then incubated on a rotary incubator for 48 hours at 30 ^{o}C . The development of yellowish to brownish colouration subsequent to the addition of Nessler’s reagent (0.5 ml) proved to be a positive indication of the potential of endophytic fungal isolates for producing ammonia .

Exopolysaccharide production

The abilitiy of the endophytic fungal isolates exopolysaccharides was assessed in accodance with the methodology devised by (Khan and Bano, 2019), but with a few modifications. A 10% sucrose-supplemented yeast extract agar (YEA) was prepared and the pH of the medium adjusted to seven before sterilization. Also, freshly grown cultures of the endophytic fungal isolates were used to impregnate sterile filter paper and placed carefully on the YEA medium plates before the liquid was incubated for 72 hours at 30 ^{o}C. The potential to produce exopolysaccharide was indicated by the formation of a mucoid colony around the filter paper.

Hydrogen cyanide production

The ability of the isolated fungal endophytes to produce hydrogen cyanide (HCN) was determined in accordance with the protocol proposed by Dinesh *et al.* (2015). The endophytic isolates were inoculated on the YEA medium that was incorporated with glycine (4.4 g/L). Thereafter, sterilized filter paper was dipped into a solution of picric acid and then carefully placed on the lid of the petri-plates and sealed up with parafilm before incubation for 96 hours at 30 ^{o}C. The isolates proved to be positive for the production of hydrogen cyanide when after incubation the filter paper changed colour from a yellowish to a reddish-brown.

7.3. Results

7.3.1. Morphological and molecular authentication

The results obtained in this present investigation on morphological characteristics and molecular authentication of isolated cowpea endophytic fungi indicated that there were twenty-six different fungal strains, they are composed of 5 fungi genera and all are of the phyla Ascomycota. The endophytic fungi isolates authenticated include: *Aspergillus* sp, *Aspergillus minisclerotigenes*, *Aspergillus sigurros*, *Aspergillus lentulus*, *Aspergillus ochraceus, Aspergillus sclerotiorum*, *Aspergillus carneus*, *Aspergillus persii*, *Aspergillus calidoustus*, *Aspergillus ostianus*, *Aspergillus bridgeri*, *Fusarium solani*, *Fusarium oxysporum*, *Fusarium phaseoli*, *Penicillium rubens*, *Penicillium palitans*, *Penicillium dipodomyicola*, *Penicillium sp*, *Penicillium oxalicum, Penicillium chrysogenum, Penicillium commune, Penicillium fuscoglaucum,* Epicoccum nigrum, *Epicoccum sorghinum*, *Alternaria sp*. Also, the isolated cowpea endophytic fungi strain distinct morphometrics characters for their morphological authentication is represented in (Table 7.2).

In addition to their morphological characteristic features, genotypic identification based on ITS gene sequencing and amplification by PCR, as well as further bioinformatics processing resulted in their genotypic authentication as composed of molecularly diverse fungi strains, in which 84 % of the isolated fungi strains were identified up to species level, while the remaining 16 % were identified to the genus level. This result is shown in (Table 7.3). The phylogenetic relatedness of the endophytic fungi strains with similar strains in the GenBank is presented in (Figures 7.1 to 7.6). The phylogenetic inference was based on the maximum likelihood method, and indicated that the various endophytic fungi strains were diverse, but have ancestral linkages with similar species in the GenBank repository forming distinct clades.

Table 7.2: Endophytic fungal strains Isolated from cowpea tissues and their morphological characteristics

S/N	Endophytic Fungal strains	Morphometrics features	Phyla
1	*Fusarium solani* strain NwuFe01	Colony on PDA attained a diameter of 4 cm in 7 days. Aerial mycelium is dense, floccose and greyish-white. Abundant micro-conidia that are oblong, formed from elongated conidiospores	Ascomycetes
2	*Penicillium rubens* strain NwuFe02	PDA colony attain a diameter of 4 cm within 7 days. Has numerous erect conidiophores forming a dense velvety felt, furrows arranged in radial pattern are seen. Colour is pale blue-green changing to darker green with age. Pale yellow exudates are produced. Conidiophore are quarter-verticillate branched, stipes are hyaline, smooth walled, branched metulae with thick walled. Conidia subglobose becoming globose with age, produced in loose columns	Ascomycetes
3	*Aspergillus sigurros strain* NwuFe03	The colony features on PDA are floccose, whitish mycelia, moderately dense sporulation and reverse colour being yellowish-white to olive. The conidiophores are biseriate with smooth brown stipes. Conidia are globose to wart-like and the conidia are radiate	Ascomycetes
4	*Alternaria sp strain* NwuFe04	Colonies are fast growing, black to olivaceous-black suede-like. Conidiophores are pale brown, and branched, bearing catenulate conidia at the apex and apical fertile parts. Conidia catenulate, mostly up to 9 in a chain, often branched. Conidia developed, brown, spindle-shaped, with cylindrical beaks	Ascomycetes
5	*Aspergillus sp* strain NwuFe05	The colonies on PDA grow up to 4-7cm within 7days. The conidiophore is the term Aspergillum, which is aseptate with vesicles on stipes having Phialides and metulae.	Ascomycetes

6	*Aspergillus lentulus* strain NwuFe06	Colonies on Potato Dextrose Agar are whitish and cottony attaining a diameter of 2cm in 7 days. Conidiophores have subclavate uniseriate vesicles. Hyphae are hyaline and septate	Ascomycetes
7	*Penicillium palitans* strain NwuFe07	The colonies on PDA are fast growers, with floccose texture. They have terverticillate conidiophores that are rough-walled. The conidia are globose or ellipsoidal.	Ascomycetes
8	*Aspergillus ochraceus* strain NwuFe08	Colonies on Potato dextrose agar attained a diameter of 4-5cm at 7days, sporulating densely with a felt of conidiophores intermixed with aerial mycelia. Colour is pale greenish yellow becoming light to dull brown. Conidiophores are hyaline and rough walled, vesicles are subglobse. Phialades are borne directly on the vesicle or metulae. Conidia is globose to subglobose with rough wall.	Ascomycetes
9	*Epicoccum sorghinum* strain NwuFe09	Colonies on PDA are greyish green with tufted appearance. Produces distinct red pigmentation that discolours the medium and forms exudate droplets. Conidia are ovoid – ellipsoidal in shape. Pycnidia are subglobose They form single celled chlamydospores which mostly occur in intercalary positions.	Ascomycetes
10	*Aspergillus minisclerotigenes* strain NwuFe10	Colonies on PDA are velvety, floccose with white mycelium and sparse conidial heads and dense felt of dark sclerotia at the center, reverse is brownish-orange. Conidiophore stipes are hyaline, coarsely roughened. Conidial heads are biseriate. Vesicles are subglobose, conidia are ellipsoidal, pale green and smooth walled	Ascomycetes
11	*Fusarium phaseoli* strain NwuFe11	Colony on PDA had a diameter of 4-5 cm in 7 days. Growth was fluffy and white with a purple tint. Scanty aerial mycelial growth is observed. Abundant sickle	Ascomycetes

		shaped a septate micro conidia and moderate septate macroconidia are present	
12	*Epicoccum nigrum* strain NwuFe12	Colony on PDA is green-yellow color, fast growing, suede-like, with a strong yellow diffusible pigment. Reverse side of plate is reddish toward the center. Spores are not produced on PDA	Ascomycetes
13	*Aspergillus sclerotiorum* strain NwuFe13	Colonies are cream colour on PDA. Conidiophore are rough walled, conidial heads are biseriate, conidia and sclerotia are yellow	Ascomycetes
14	*Penicillium dipodomyicola* strain NwuFe14	On PDA the colony growth rate was quite rapid, with fasciculate texture. The conidiophores are quarterverticillate and have short phialides with smooth-wall.	Ascomycetes
15	*Penicillium* sp strain NwuFe15	The colony on PDA grows fast, with a yellowish-green colour that change to darker-green. The Conidiophores are hyaline and rough-wall. The phialides are flask-shape and metulae are branch. Conidia are globose to subglobose	Ascomycetes
16	*Aspergillus carneus* NwuFe16	On PDA, colonies are fast growing, they are white becoming brownish red with age. Conidiophores are smooth and brown to colourless. Conidia are smooth, unpigmented and spherical conidial heads are columnar in shape. Vesicles are globose and sterigmata are biseriate. Hyphae are thick walled; brown exudates are present	Ascomycetes
17	*Penicillium oxalicum* strain NwuFe17	Colonies on PDA grow rapidly and are spreading, they have a velvety texture, are blue-green in colour, reverse is yellowish green. Conidial heads are irregularly biverticillate. Conidiophores are smooth, metulae are appressed in verticils of 2-4. Conidia are elliptical, smooth and very large	Ascomycetes
18	*Penicillium chrysogenum* strain NwuFe18	Colony attains a diameter of 5 cm within 10 days with numerous erect single conidiophores forming a dense velvety	Ascomycetes

		felt. Colonies have yellowish aerial mycelia with flocculent conidiophores. Yellow-green in colour changing to a darker green. Has aromatic odour. Conidiophores arising from substrate, quaterverticillate and branched. Stipes are hyaline, smooth walled bearing 3 or 4 phialides. Conidia are globose, hyaline and smooth walled produced in loose columns	
19	*Penicillium commune* strain NwuFe19	Colonies on PDA are soft and velvety. Growth is granular and reverse side is yellow. Conidia is smooth and spherical, dull green in colour, borne in disordered chains on conidiophores with rough walled stipes.	Ascomycetes
20	*Aspergillus persii* strain NwuFe20	Colonies on Potato dextrose agar attained a diameter of 4 cm in 7days colour is pastel to light yellow in colour, while reverse is orange brown. Conidiophores are hyaline and smooth walled conidial heads are loosely columnar to radiate, biserate. Phialades are borne directly on the vesicle. Conidia are globose and smooth. Exudates are present	Ascomycetes
21	*Aspergillus calidoustus* strain NwuFe21	Colonies are white becoming brownish – yellow with age. Has velvety texture, purple exudates are present on the surface of colony, reverse is light yellow with olive brown center. Conidiophores are curved and smooth- walled. Vesicles are small and spherical; they are biseriate with metulae slightly shorter than phialides. Conidia are echinulate. Hyphae are septate and Stipes are short. Irregular shaped hulle cells are present.	Ascomycetes
22	*Aspergillus ostianus* strain NwuFe22	Colony on PDA attains a diameter of 2.5 cm in 7 days. It is cream coloured turning brown at the middle with age. Conidiophore are smooth, light-brown and hyaline. Conidial head is large, globose and radiate, phialides are borne on metulae. Conidia are smooth walled	Ascomycetes

23	*Penicillium fuscoglaucum* strain NwuFe23	The colonies on PDA spreads moderately. The conidiophores are biverticillate and are finely-roughened. The conidia vary from greenish shades to a blue shade.	Ascomycetes
24	*Aspergillus bridgeri* strain NwuFe24	The PDA colonies spreads very fast; the conidia are ochre or light yellow, and their conidiophores are biseriate. They produce sclerotia that could be yellow, white or cream colour.	Ascomycetes
25	*Fusarium oxysporum* strain NwuFe25	Colony on PDA attained a diameter of 5 cm in 4 days. Floccose, becoming felty, whitish with a peach tinge. Reverse is purple. Septate, slightly curved Micro-conidia borne on lateral simple phialides. Macro conidia are sparse. Chlamydospores in hyphae are hyaline, smooth and in pairs	Ascomycetes
26	*Alternaria* sp strain NwuFe26	Colony on PDA attained a diameter of 5cm in 7 days. Grey colour on the surface ang ochraceous on the reverse. Conidiophore are single from hyphae, curved smooth walled, pale brown with 1- 10 septa. Conidia are in short or moderately long chains of 2-8 conidia, which are ellipsoidal with transverse septa and beakless	Ascomycetes

Table 7.3**:** Molecular identification of endophytic fungi isolates from seeds and near root zone tissues of *Vigna unguiculata* L. walps based on the ITS rRNA gene sequence.

S/N	Culture Code	Molecular authentication	Percentage similarity	Accession Number
1.	Fe1	*Fusarium solani* NwuFe01	100	OK012371
2.	Fe2	*Penicillium rubens* NwuFe02	100	OK012372
3.	Fe3	*Aspergillus sigurros* NwuFe03	100	OK012373
4.	Fe4	*Alternaria sp* NwuFe04	100	OK012374
5.	Fe5	*Aspergillus sp* NwuFe05	99.31	OK012375

6.	Fe6	*Aspergillus lentulus* NwuFe06	100	OK012376
7.	Fe7	*Penicillium palitans* NwuFe07	98.84	OK012377
8.	Fe8	*Aspergillus ochraceus* NwuFe08	100	OK012378
9.	Fe9	*Epicoccum sorghinum* NwuFe09	99.86	OK012379
10.	Fe10	*Aspergillus minisclerotigenes* NwuFe10	99.85	OK012380
11.	Fe11	*Fusarium phaseoli* NwuFe11	99.71	OK012381
12.	Fe12	*Epicoccum nigrum* NwuFe12	100	OK012382
13.	Fe13	*Aspergillus sclerotiorum* NwuFe13	99.86	OK012383
14.	Fe14	*Penicillium dipodomyicola* NwuFe14	99.96	OK012384
15.	Fe15	*Penicillium sp* NwuFe15	100	OK012385
16.	Fe16	*Aspergillus carneus* NwuFe16	99.47	OK012386
17.	NFe17	*Penicillium oxalicum* NwuFe17	99.77	OK012387
18.	NFe18	*Penicillium chrysogenum* NwuFe18	99.46	OK012388
19.	NFe19	*Penicillium commune* NwuFe19	99.69	OK012389
20.	NFe20	*Aspergillus persii* NwuFe20	99.62	OK012390
21.	NFe21	*Aspergillus calidoustus* NwuFe21	99.92	OK012391
22.	NFe22	*Aspergillus ostianus* NwuFe22	99.71	OK012392
23.	NFe23	*Penicillium fuscoglaucum* NwuFe23	99.87	OK012393
24.	NFe24	*Aspergillus bridgeri* NwuFe24	99.72	OK012394
25.	NFe25	*Fusarium oxy*sporum NwuFe25	100	OK012395
26.	NFe26	*Alternaria* sp NwuFe26	99.86	OK012396

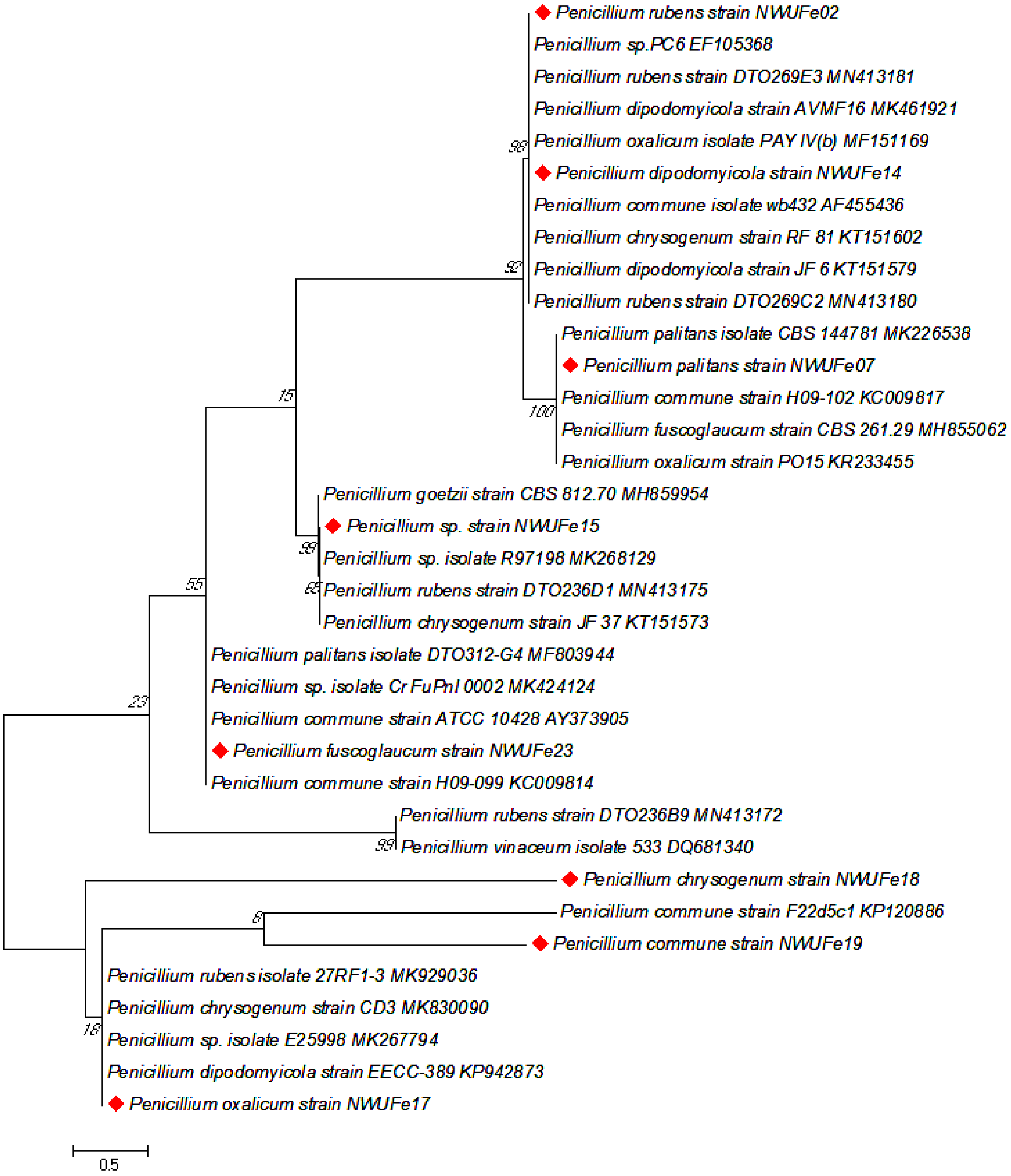


Figure 7.1: Evolutionary relationships of endophytic *Penicillium* strains taxa from cowpea plants

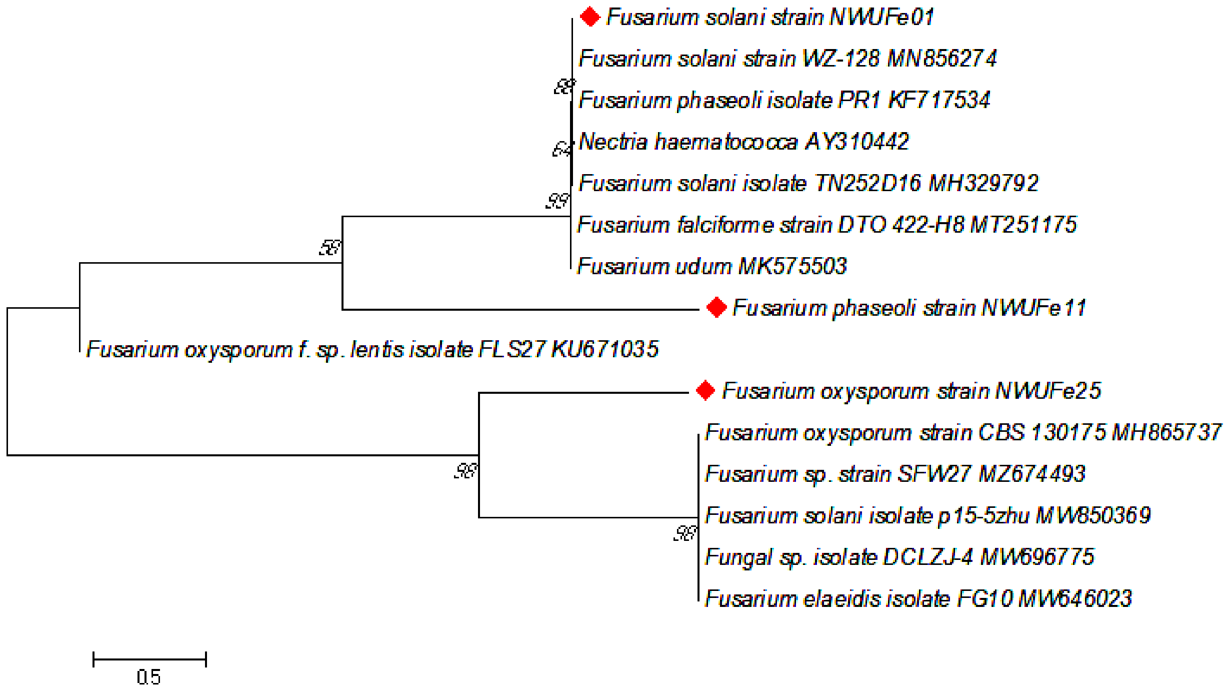


Figure 7.2: Evolutionary relationships of endophytic *Fusarium* strains taxa from cowpea plants

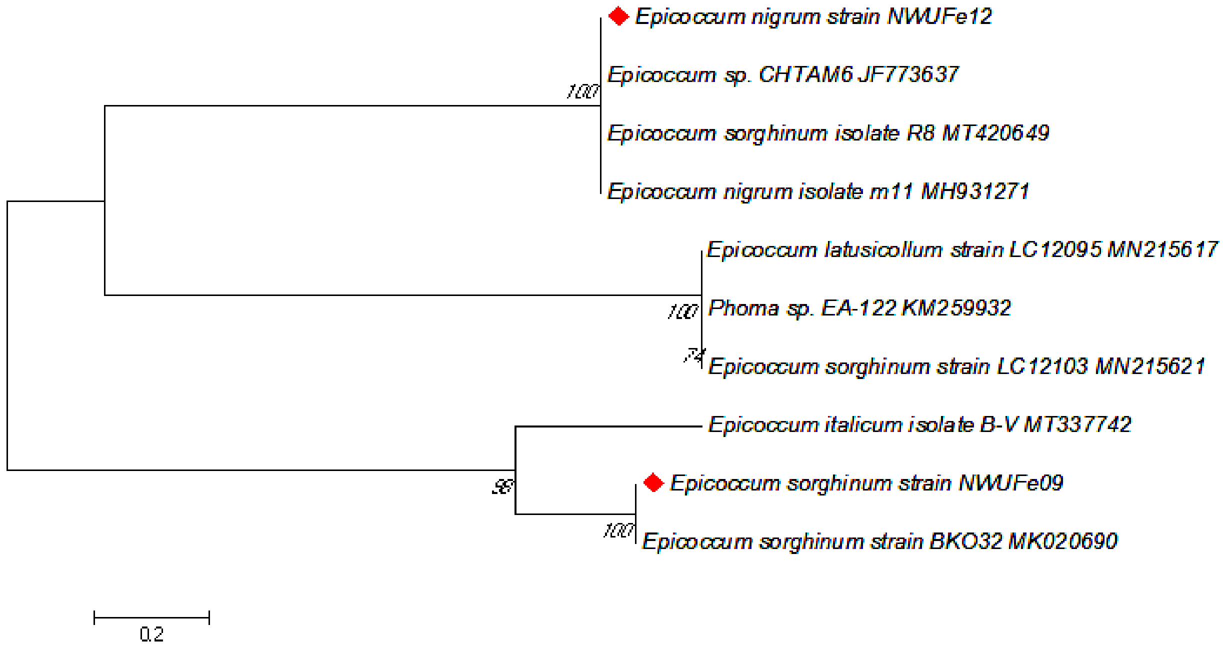


Figure 7.3: Evolutionary relationships of endophytic *Epicoccum* strains taxa from cowpea plants

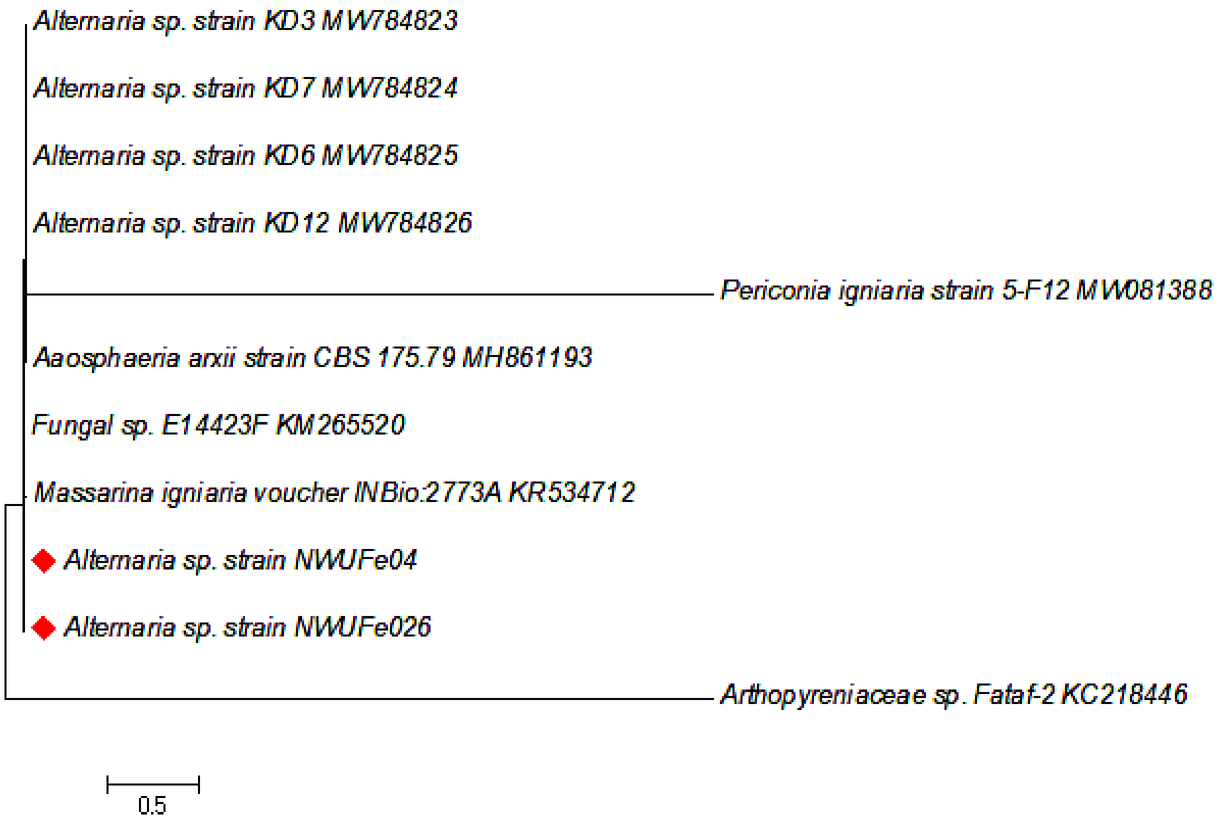


Figure 7.4: Evolutionary relationships of endophytic *Alternaria* strains taxa from cowpea plants

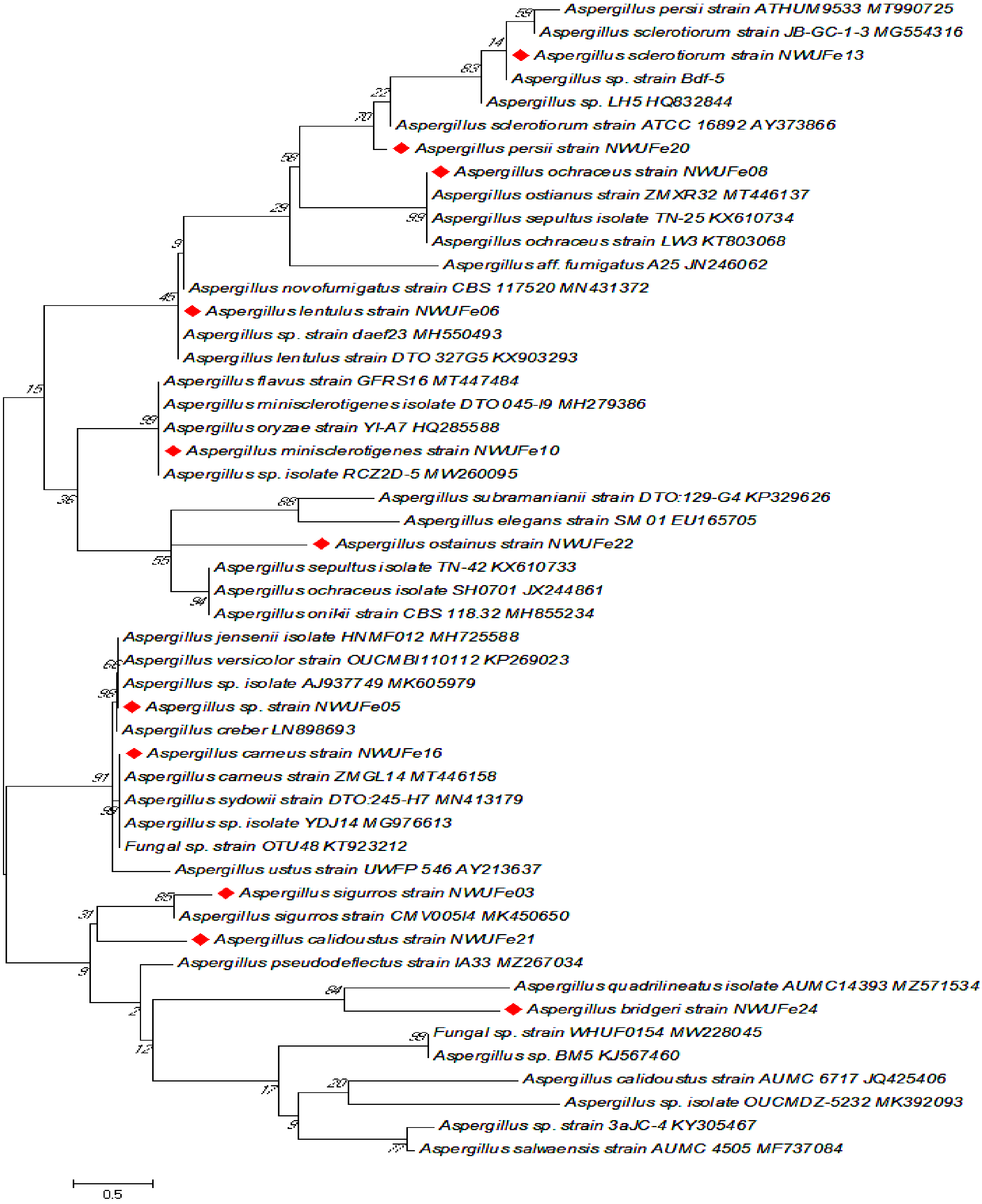


Figure 7.5: Evolutionary relationships of endophytic *Aspergillus* strains taxa from cowpea plants

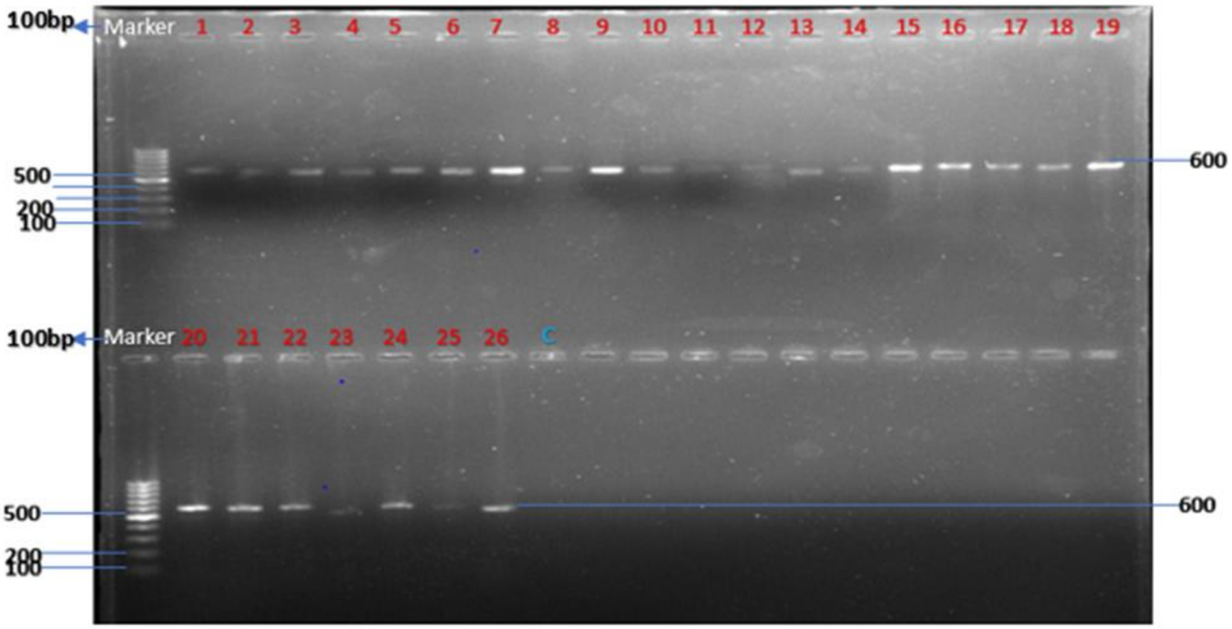


Figure 7.6: ITS spacer region genes of cowpea endophytic fungi strains amplified by polymerase chain reaction using 100 bp molecular ladder

7.3.2. Plant-growth stimulating attributes of endophytic fungi strains

The results of the plant-growth enhancing attributes of the isolated cowpea endophytic fungal strains indicated that they are potent in promoting the growth of plants. All the 26 fungi isolates possess the ability to produce Exopolysaccharide, IAA, Ammonia and siderophore. Moreso, the ability to solubilize phosphate into assimilable forms for plant utilization was identified in 70% of the isolated strains. The results highlighting the multifarious plant-growth stimulating potentials of the cowpea endophytic fungi strains, is shown in (Table 7.4).

Table 7.4**:** Plant growth promoting in-vitro attributes of endophytic fungal isolates from cowpea tissues.

Bacteria Code	Acds Production	Ammonia Production	Auxin Production	Exopolysaccharide Production	HCN Production	Phosphate Solubilizing	Siderophore Production
Fe1	-	++	++	+	++	-	++
Fe2	+	+++	+++	+	++	++++	++

Fe3	+	+++	+++	+	++	++	++
Fe4	+	++	++	+	++	-	++
Fe5	+	++	+++	+	++	++	++
Fe6	-	++	++	+	-	-	-
Fe7	-	++	++	+	-	-	-
Fe8	-	++	++	+	-	+++	++
Fe9	-	++	++	+	-	-	-
Fe10	+	+++	+++	+	++	++	++
Fe11	-	++	++	+	++	-	++
Fe12	+	+++	+++	+	+++	+++	++
Fe13	-	++	++	+	++	-	-
Fe14	+	+++	+++	+	+++	++	++
Fe15	-	++	++	+	-	-	-
Fe16	-	++	++	+	-	-	++
NFe17	-	++	++	+	-	-	-
NFe 18	-	++	++	+	-	-	++
NFe19	-	++	++	+	-	-	-
NFe20	-	++	++	+	-	-	++
NFe21	+	++	++++	+	++	++	++
NFe22	+	+++	+++	+	++	++	-
NFe23	+	+++	+++	+	++	++	++
NFe24	-	++	++	+	-	-	++
NFe25	+	+++	+++	+	++	-	-
NFe26	-	++	++	+	-	-	-

Key: Highly active = +++; High activity = ++; Slightly high = +; None active = -

7.3.3 Tolerance to environmental stress

The results obtained in this study in relation to the environmental stress tolerance potentials of the endophytic fungi strains to salinity stress, temperature and pH indicated varied results. The temperature of growth at 30 and 35 degrees centigrade supported the growth of all the endophytic fungi strains optimally, while at 40 C, the growth response varies. Likewise, varied growth response was also observed among all the endophytic fungi strains at different salinity level and pH as shown (Tables 7.5 to 7.7).

Table 7.5: Environmental stress tolerance of endophytic fungal strains to varying salinity

S/N	Endophytic Fungal strains	NaCl 1 %	NaCl 3 %	NaCl 5%
1	*Fusarium solani* strain NwuFe01	+	+	+
2	*Penicillium rubens* strain NwuFe02	++	+	+
3	*Aspergillus sigurros strain* NwuFe03	+	+	-
4	*Alternaria sp strain* NwuFe04	++	+	+
5	*Aspergillus sp* strain NwuFe05	++	+	+
6	*Aspergillus lentulus* strain NwuFe06	++	+	+
7	*Penicillium palitans* strain NwuFe07	+	+	-
8	*Aspergillus ochraceus* strain NwuFe08	++	++	+
9	*Epicoccum sorghinum* strain NwuFe09	++	+	-
10	*Aspergillus minisclerotigenes* strain NwuFe10	++	+	+
11	*Fusarium phaseoli* strain NwuFe11	+	+	-
12	*Epicoccum nigrum* strain NwuFe12	++	+	+
13	*Aspergillus sclerotiorum* strain NwuFe13	+	+	-
14	*Penicillium dipodomyicola* strain NwuFe14	++	+	-
15	*Penicillium sp* strain NwuFe15	++	+	-
16	*Aspergillus carneus* NwuFe16	+	+	-

17	*Penicillium oxalicum* strain NwuFe17	+	+	-
18	*Penicillium chrysogenum* strain NwuFe18	++	+	-
19	*Penicillium commune* strain NwuFe19	+	+	-
20	*Aspergillus persii* strain NwuFe20	++	+	+
21	*Aspergillus calidoustus* strain NwuFe21	++	++	+
22	*Aspergillus ostianus* strain NwuFe22	++	+	+
23	*Penicillium fuscoglaucum* strain NwuFe23	++	+	+
24	*Aspergillus bridgeri* strain NwuFe24	+	+	-
25	*Fusarium oxysporum* strain NwuFe25	++	+	+
26	*Alternaria* sp strain NwuFe26	+	+	-

Key: Highly active = +++; High activity = ++; Slightly high = +; None active = -

Table 7.6: Environmental stress tolerance of endophytic fungal strains to varying temperature

S/N	Endophytic Fungal strains	25°C	30°C	40°C
1	*Fusarium solani* strain NwuFe01	+	+	+
2	*Penicillium rubens* strain NwuFe02	++	+	+
3	*Aspergillus sigurros strain* NwuFe03	+	+	-
4	*Alternaria sp strain* NwuFe04	++	+	+
5	*Aspergillus sp* strain NwuFe05	++	+	+
6	*Aspergillus lentulus* strain NwuFe06	++	+	+
7	*Penicillium palitans* strain NwuFe07	+	+	-

8	*Aspergillus ochraceus* strain NwuFe08	++	++	+
9	*Epicoccum sorghinum* strain NwuFe09	++	+	-
10	*Aspergillus minisclerotigenes* strain NwuFe10	++	+	+
11	*Fusarium phaseoli* strain NwuFe11	+	+	-
12	*Epicoccum nigrum* strain NwuFe12	++	+	+
13	*Aspergillus sclerotiorum* strain NwuFe13	+	+	-
14	*Penicillium dipodomyicola* strain NwuFe14	++	+	-
15	*Penicillium sp* strain NwuFe15	++	+	-
16	*Aspergillus carneus* NwuFe16	+	+	-
17	*Penicillium oxalicum* strain NwuFe17	+	+	-
18	*Penicillium chrysogenum* strain NwuFe18	++	+	-
19	*Penicillium commune* strain NwuFe19	+	+	-
20	*Aspergillus persii* strain NwuFe20	++	+	+
21	*Aspergillus calidoustus* strain NwuFe21	++	++	+
22	*Aspergillus ostianus* strain NwuFe22	++	+	+
23	*Penicillium fuscoglaucum* strain NwuFe23	++	+	+
24	*Aspergillus bridgeri* strain NwuFe24	+	+	-
25	*Fusarium oxysporum* strain NwuFe25	++	+	+
26	*Alternaria* sp strain NwuFe26	+	+	-

Key: Highly active = +++; High activity = ++; Slightly high = +; None active = -

Table 7.7: Environmental stress tolerance of endophytic fungal strains to varying pH

S/N	Endophytic Fungal strains	pH 3	pH 5	pH 10
1	*Fusarium solani* strain NwuFe01	++	+++	+
2	*Penicillium rubens* strain NwuFe02	++	++	+
3	*Aspergillus sigurros strain* NwuFe03	+	++	+
4	*Alternaria sp strain* NwuFe04	+	++	+
5	*Aspergillus sp* strain NwuFe05	++	++	+
6	*Aspergillus lentulus* strain NwuFe06	++	++	+
7	*Penicillium palitans* strain NwuFe07	+	++	+
8	*Aspergillus ochraceus* strain NwuFe08	++	+++	++
9	*Epicoccum sorghinum* strain NwuFe09	++	+	+
10	*Aspergillus minisclerotigenes* strain NwuFe10	++	++	+
11	*Fusarium phaseoli* strain NwuFe11	+	+	+
12	*Epicoccum nigrum* strain NwuFe12	++	+++	++
13	*Aspergillus sclerotiorum* strain NwuFe13	+	+	+
14	*Penicillium dipodomyicola* strain NwuFe14	++	+	+
15	*Penicillium sp* strain NwuFe15	+	++	+
16	*Aspergillus carneus* NwuFe16	+	+	+
17	*Penicillium oxalicum* strain NwuFe17	+	+	+
18	*Penicillium chrysogenum* strain NwuFe18	++	+	+

19	*Penicillium commune* strain NwuFe19	+	+	+
20	*Aspergillus persii* strain NwuFe20	++	++	+
21	*Aspergillus calidoustus* strain NwuFe21	++	+++	++
22	*Aspergillus ostianus* strain NwuFe22	++	++	++
23	*Penicillium fuscoglaucum* strain NwuFe23	++	+++	++
24	*Aspergillus bridgeri* strain NwuFe24	+	+	+
25	*Fusarium oxysporum* strain NwuFe25	++	++	++
26	*Alternaria* sp strain NwuFe26	+	+	+

Key: Highly active = +++; High activity = ++; Slightly high = +; None active = -

7.3.4. Influence of endophytic fungi strains seed biopriming on cowpea seed germination

The growth chamber study of cowpea seeds that were bio-primed with endophytic fungi strains indicated positive seed germination enhancement over the control (Figure 7.7). The best seed germination performance was recorded for *Aspergillus minisclerotigenes* NWUFe10, *Aspergillus* sp NWUFe5 and *Penicillium chrysogenum* NWUFe18 respectively (Figure 7.8).

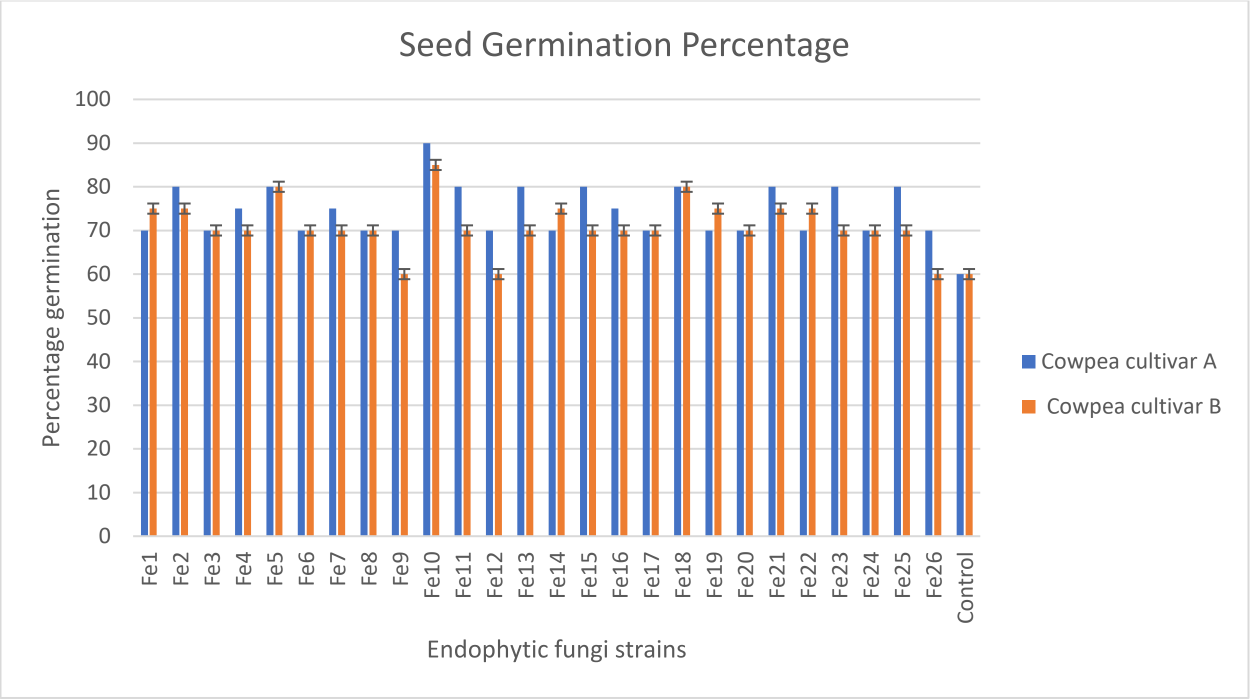


Figure 7: Endophytic fungal strains seed germination percentage of two cowpea cultivars

Figure 7.8: Endophytic fungal strains bio-primed cowpea seeds germination on petri-plates

7.3.5. Amplification of plant growth promoting genes by Polymerase chain reaction

The results obtained when the genomic DNA of selected endophytic fungal strains were used to amplify PGP genes including ACPHO, IPDC, GCD indicated that about 70 % of the selected strains confirmed positive to both ACPHO and GCD genes at the expected band size. However, in all about 30 % of the selected strains confirmed positive possession of all the three genes viz ACPHO, IPDC and GCD respectively. This is presented in (Figures 7.9 to 7.11).

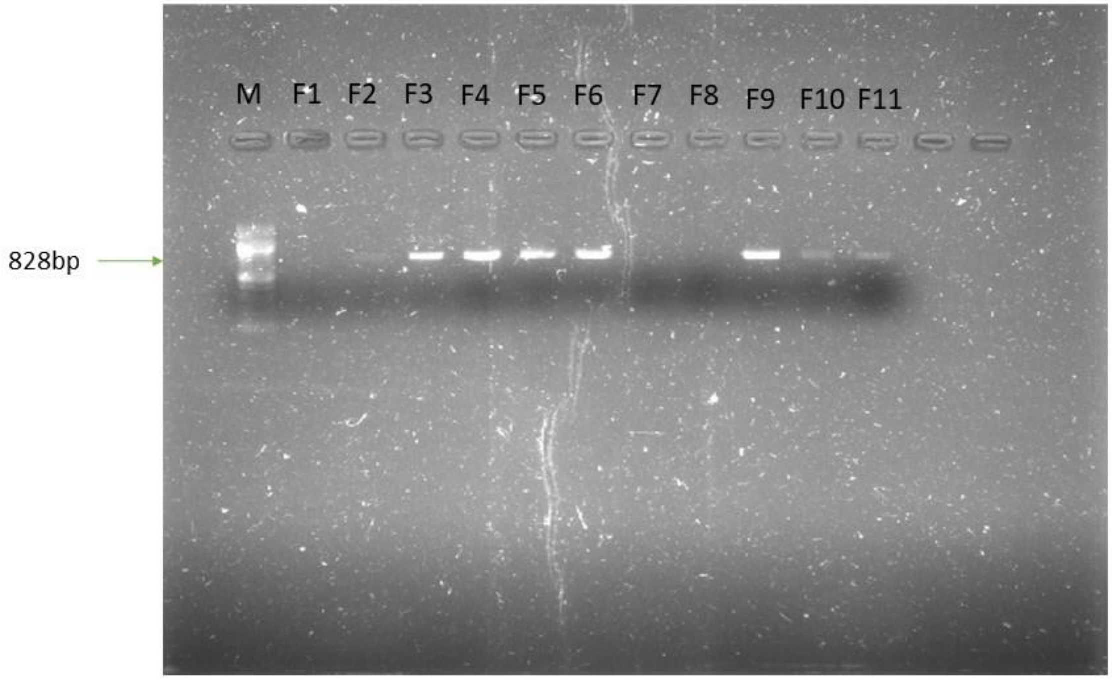


Figure 7.9: Acid phosphatase gene amplified by endophytic fungi strains at 828 bp size by PCR

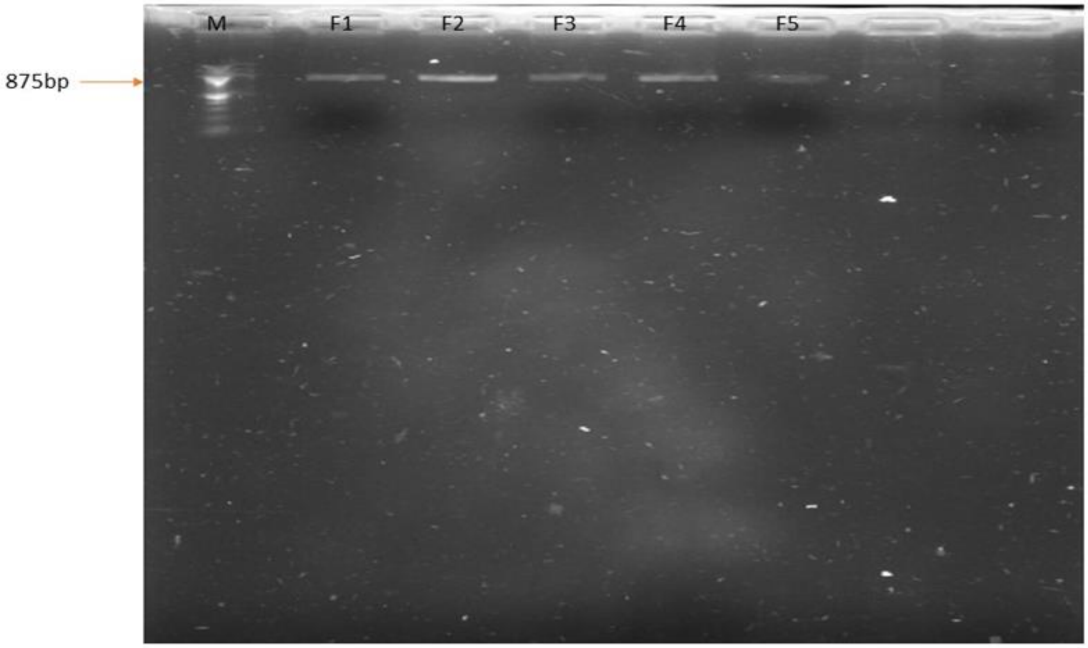


Figure 7.10: Glucose dehydrogenase gene amplified by endophytic fungi strains at 875 bp size by PCR

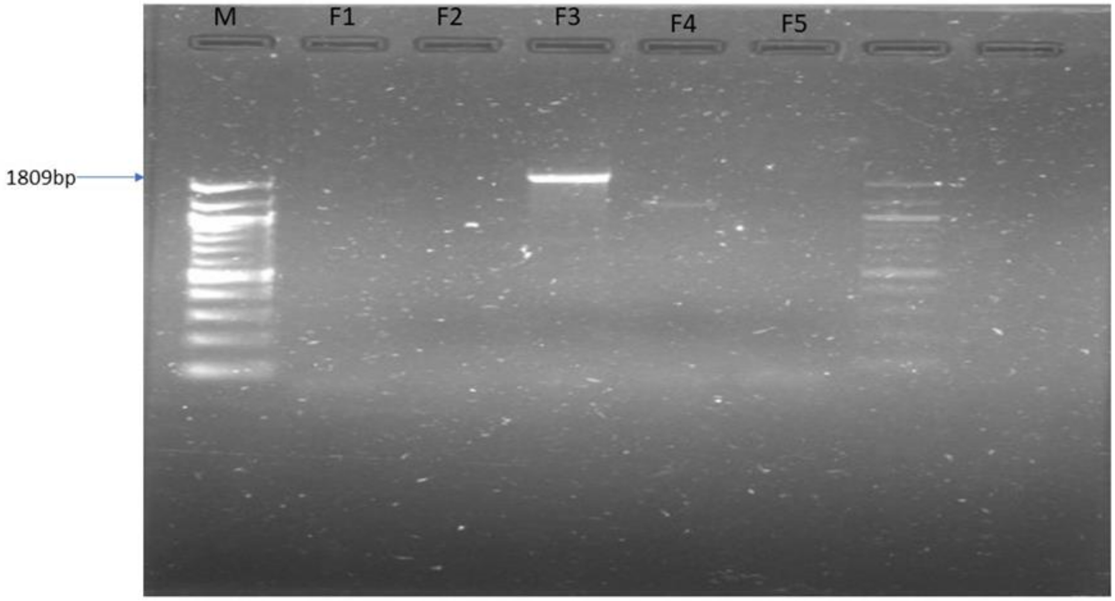


Figure 7.11: Indole pyruvate dehydrogenase gene amplified by endophytic fungi strains 1809 bp size by PCR

7.4. Discussion

The advantages of harnessing renewable microbial resources as bioinoculants in achieving plant productivity enhancement, while maintaining agroecological and environmental sustainability is of top priority in agricultural biotechnology. The trending narrative is the deployment of beneficial partners of the plant-microbe interrelationship to improve plant growth and development. Endophytes have shown remarkable prospects as potent plant-growth stimulating partners in their interactions with planted crops. A lot of research investigation have reported beneficial roles of endophytic fungi isolates in improving growth and development in diverse planted crops (Grabka *et al.,* 2022; Liu-Xu *et al.,* 2022; Verma *et al.*, 2022). Endophytic microbial research studies in recent time have shown their remarkable roles as important partner in the plant-microbiome interactions with beneficial consequences in relations to plant growth, fitness and functionality. Numerous endophytic fungi isolates have been reported in studies that covers diverse plant types to have beneficial attributes that enhance plant growth (Ali *et al.*, 2022a; Chowdappa *et al.*, 2020; Hussain *et al.*, 2022; Rustamova *et al.*, 2022). These findings implies that endophytic fungi isolates are viable alternative to synthetic agrochemicals in intensifying agricultural productivity to achieve food security for all. Hence, we hypothesized that endophytic fungi strains bio-prospected and characterized from cowpea seeds and aerial tissues are veritable natural resource to improve cowpea plant growth in our investigation. The data obtained in our study showed that 26 different fungi strains with diverse biochemical traits, morpho-genetics characters were authenticated as having plant-growth improving attributes.

Compositional diversity of endophytic fungi isolates

The endophytic fungal strains in this study are composed of five genera that included 11 Aspergillus strains, 8 Penicillium strains, 3 Fusarium strains and 2 strains each of Alternaria and Epicoccum. This shows that endophytic Aspergillus strains are the most prevalent, followed by Penicillium and Fusarium strains. Similar results corroborate our findings indicating the dominance of ascomycota phyla in isolated endophytic fungi from plant tissues are reported in literature (Li *et al.*, 2020; Silva *et al.*, 2018; Suryanarayanan *et al.*, 2018; Xia *et al.*, 2019). The phylogenetic relatedness of the isolated endophytic fungi strains indicated between 98-100% species similarity with related strains in the GenBank. Our result correlates with findings from (Lubna et al. 2022;) that reported diverse endophytic fungi strains were isolated and characterized from leguminous crops, with Aspergillus and Penicillium isolates being more abundant.

Functional characterization of endophytic fungi strains

Our investigation highlighted the potency of endophytic fungi strains in stimulating cowpea plant-growth using multifaceted traits such as; phosphate solubilization, indole acetic acid production, siderophore production, exopolysaccharide, ammonia production, ACCD production. All the endophytic fungi strains possess up to 3 plant-growth improving traits. The capability of the endophytic fungi strains that possess PGP traits used in promoting plant-growth enhancement is achieved by direct and indirect mechanistic approaches (Ali *et al.*, 2022b; Santiago *et al.*, 2017; Singh *et al.*, 2021). For instance, the siderophore producing abilities of endophytic microbes makes the chelating of iron from unavailable forms to utilizable forms for plant absorption to enhance growth (Ripa *et al.*, 2019). Likewise, the abilities of endophytic microbiota to solubilize phosphate, produce auxin-like IAA, to regulate ethylene biosynthesis through production of ACC deaminase enzyme, and to fix atmospheric nitrogen among others confers growth-promoting attributes in planted crops (Lacriola *et al.*, 2020; Mantzoukas & Eliopoulos, 2020; Mehta *et al.*, 2019). These positive attributes by endophytic fungi microbiota are mediated through beneficial regulation of stressful conditions induced by ethylene, as well as through signaling molecules for positive plant-microbes' interactions (Mitter *et al.*, 2021; Pirttilä *et al.*, 2021). Endophyte mediated phosphate solubilization via organic acid secretion is a veritable means of making Phosphorus available for plant uptake (Adhikari & Pandey, 2019). Also, endophytic microbiota mediated phyto-hormonal production has been reported to influence plant-growth stimulation in planted crops (Turbat *et al.*, 2020). In addition, the data from the invitro growth assessment assay, seed germination and seed vigor studies indicated that all the endophytic fungi strains possess multiple plant-growth improving traits and enhanced significantly, the seed germination and seed vigor index more than the control plant. Also, this positive cowpea seed germination percentage and seedling growth improvement due to inoculation with competent endophytic fungal inoculants in our study, is similar to findings by (Silva *et al.*, 2021; Wang *et al.*, 2022a) that also attributed improvement in seed germination and seedling growth performance due to mechanistic plant growth traits of endophytic fungal inoculants.

7.5. Conclusion

This present investigation confirmed the growth-stimulating features of endophytic fungi strains isolated from tissues of cowpea plant. The exploration of bioactivities of these potent endophytic isolates indicated they possess multiple PGP traits deployed to directly and indirectly enhance cowpea productivity improvement such as indole acetic acid secretion, siderophore secretion, ammonia production, solubilization of phosphate among others. Therefore, the endophytic fungi strains are good candidates as alternatives to agrochemical fertilizer usage to improve cowpea production in a sustainable and ecologically balanced way. There is however, need for further in-depth genomic insight and field experimental research of these potential effective bioinoculants.

CHAPTER EIGHT

8.0 AGRONOMIC GROWTH AND YIELD IMPROVEMENT POTENTIAL OF COWPEA PLANT BY CULTURABLE BACTERIAL AND FUNGAL ENDOPHYTES IN FIELD EXPERIMENT IN MAFIKENG, NORTHWEST PROVINCE, SOUTH AFRICA

Abstract

The astronomical increase in the demographic statistics of the global human population, demands urgent response to achieving food and nutritional security by deploying cheaper and sustainable means of improving crop productivity. Cowpea (vigna unguiculata L. Walps), an under exploited indigenous African legume fits perfectly in changing this narrative if sustainable means of improving its yield potential is actualized by utilizing cheaper, readily available and eco-friendly bioinoculating agents. A field trial experiment was conducted for two planting seasons by applying plant growth improving endophytic *Stenotrophomonas geniculata* NWUBe21, *Pseudomonas carnis* NWUBe30 and *Aspergillus minisclerotigenes* NWUFe10 as a single and mixed inoculants on cowpea in Mafikeng, Northwest province, South Africa. A randomized 2 by 5 factorial design, and completely block experimentation that involves two cowpea cultivars, five treatments combinations were replicated three times for assessing the agronomic growth and yield improving potential of the bioinoculating endophytic microbial agents on cowpea growth and yield parameters over two planting seasons. The data observed were subjected to various statistical analysis and the significance level was set at 5% probability. The results indicated marked agronomic growth and yield productivity improvement due to the bioinoculating treatments in both the singly applied and co-inoculation of the microbial agents. The 100 seed weights, number of pods and pod weight were significantly higher at ($P<0.05$) probability level compare to the uninoculated control. The application of endophytic microbial agents *Stenotrophomonas geniculata* NWUBe21, *Pseudomonas carnis* NWUBe30 and *Aspergillus minisclerotigenes* NWUFe10 led to cowpea agronomic growth and yield enhancement in the field experimentation trials, and this confirms the huge potential of promoting the deployment of natural, renewable and agro-ecological balanced indigenous endophytic microbial resources as a means of improving the productivity of cowpea in order to ensure food and nutritional security.

Keywords: Indigenous microbial resources; eco-friendly bioinoculating agents; *Vigna unguiculata*; cowpea growth enhancement; sustainable agriculture; bacterial endophyte; fungal endophyte

8.1. Introduction

The human race is face with dire constraints to solve food insecurity, hunger and malnutrition for the majority poor in the light of the rising global human populace, climatic vulnerabilities, covid-19 pandemic, high cost of energy amongst others. A glimpse into recent statistical data on human population from the literature estimate that there will be an additional 2.3 billion humans on planet earth by 2050, thus adding to the challenge of tackling food insufficiency and hunger (Fasolin *et al.*, 2019; Putelat *et al.*, 2021; van Dijk *et al.*, 2021). Worthy of note is the fact that enormous quantities of costly synthetic agrochemicals, pesticides and fertilizers are applied for intensifying agricultural production to meet food demand globally. However, the cost implication is very high in relation to environmental imbalance and public health safety (Buvaneshwari *et al.*, 2020; Jang *et al.*, 2021; Xu *et al.*, 2020). The Africa continent faces a more difficult scenario in tackling food and nutritional insecurity due to lack of technological advancement and financing challenges (Adedeji *et al.*, 2020).

Therefore, there is a need to sustainably intensify agricultural productivity through the deployment of efficient, renewable, low-input, natural resources to ensure agro-ecological balance. Endophytic microbes are found inhabiting/residing in the innermost tissues in all plants, and confers beneficial association with plants that are recently seen as a means of improving crop productivity, achieving reduce or chemical-free crop production and also, a means of ensuring environmental and agro-ecological sustainability (Dwibedi *et al.*, 2022; Omomowo & Babalola, 2019; Rani *et al.*, 2022).

(*Vigna unguiculata* (L.) Walp.) known globally as Cowpea or Black-eye pea, is of huge benefits and significance to a lot of people globally that cut across different continents of Asia, America, Europe and most importantly Africa (Ferreira *et al.*, 2022; Kumar *et al.*, 2022). Cowpea possesses multi-faceted beneficial attributes that includes; relevance to human and animal nutritional, agroecological/environmental beneficial attributes, trade and economic benefits among others (Ayalew & Yoseph, 2022; Gerrano *et al.*, 2022; Omomowo & Babalola, 2021). This strategic and unique African legume is diploid (2n=2x=22), cowpea is an annual crop, and the genome is estimated at 620 million nucleotide sequences reads (Lonardi *et al.*, 2019).

Cowpea originates from Africa, but is now an important pulse crop globally in most countries (Herniter *et al.*, 2020). Cowpea is a multi-beneficial crop and helps the poorer populace to balance their dietary nutrition as source of nutritious grain or vegetable (Gonçalves *et al.*, 2016). It is also valuable in the livestock sector as source of fodder / feed-stock in animal husbandry (Alemu *et al.*, 2016b).

In relation to agroecological sustainability and soil health improvement, cowpea serves as soil cover, it adds nitrogen to soil through its atmospheric N fixation capability, reduces weeds and serves as a foremost choice for mixed cropping and intercropping helps in proliferating beneficial soil microbes (Das *et al.*, 2018; Sun *et al.*, 2019; Yahaya, 2019).

However, cowpea productivity in Africa is low and sub-optimal due to a myriad of constraints, that include amongst others, a changing pattern of climate, issue of drought, the depletion of soil nutrients, the constraints of the excessive and expensive synthetic agrochemicals, problem of poor seed cultivars, and also, challenges of phytopathogens and pest's infestation (Afutu *et al.*, 2017).

The cultivation of cowpea occurs in the warmer months of the year in a planting year; thus, this constraint also limits breeding cycle production of cowpea plant in a year. Despite the fact that Africa countries lead other nations with regards to cowpea production quota, there still exist a huge deficit in terms of low productivity of this vital and resilient legume. The grain yield and productivity of cowpea is very low in most small-holder farmers of cowpea in Africa (Bolarinwa *et al.*, 2021; Kebede & Bekeko, 2020). To worsen the challenge of cowpea low yield output and productivity, expensive agrochemicals and pesticides are required to achieve this low yield. This thus leads to environmental imbalance and potential threat to public health.

In an era of devastating impacts of climate vulnerabilities, the degradation of soil physical and chemical properties and the depletion of vital nutrients in soil, cowpea plant production can be promoted as a resilient and multi-beneficial legume to ameliorate food insecurity and nutritional deficiency in Africa (Animasaun *et al.*, 2015; Gerrano *et al.*, 2022).

To effectively combat these highlighted challenges, there is the need to formulate simple biological alternatives to take care of these highlighted constraints. This is imperative because the plant microbiome is a fundamental partner in protecting plant from stresses by synthesizing enzymes or metabolites that can negatively affect plant pathogens, it produces important phytohormones and ensure / enable plant tolerance to environmental stress (Braga *et al.*, 2016).

Moreover, the search for sustainable alternatives to boost crop productivity is promising with regards to endophytic microbial studies. Hence, it is hypothesized in this study that the application of endophytic bacterial and fungal isolates as bioinoculating microbes can improve the agronomic growth and yield parameters of cowpea plants in an agro-ecologically and ecofriendly sustainable field trial experiments in Mafikeng, Northwest Province, South Africa.

8.2. Materials and methods

8.2.1. Field trial location, history, experimental design, cowpea cultivars and endophytic bacterial and fungal isolates

The experimental planting of cowpea was performed over 2 seasons between 2020 and 2021 in Mafikeng, North West province in South Africa (25° 49' 20" S and25° 36' 45" E). The used field had a history of being used for organic farming of maize plant without synthetic fertilizer application. The experimental field used in this study, based on the physical and chemical properties of soil was of the sandy-loamy soil texture. The experimental layout of the field was five rows, the plot size was 3m by 2.5m, and had an intra and inter row spacing of 25cm by 75cm.

The experimental design used was a 2 X 5 factorial in a completely randomized block outlook and replicated thrice. The five (5) inoculating treatments used are designated as treatment with - water (Tn0), *Pseudomonas carnis* NWUBe30 (Tn1), *Stenotrophomonas geniculata* NWUBe21 (Tn2), *Aspergillus minisclerotigenes* NWUFe10 (Tn3), and mixed inoculant treatment with *P.carnis* NWUBe30 + *S. geniculata* NWUBe21 + *A. minsclerotigenes* NWUFe10 (Tn4).

Two cowpea genotype (PAN 311, Bechuana white) were used and designated as Cv1 and Cv2. Two seeds per hole were sown and then thinned to one after 7 days of sowing.

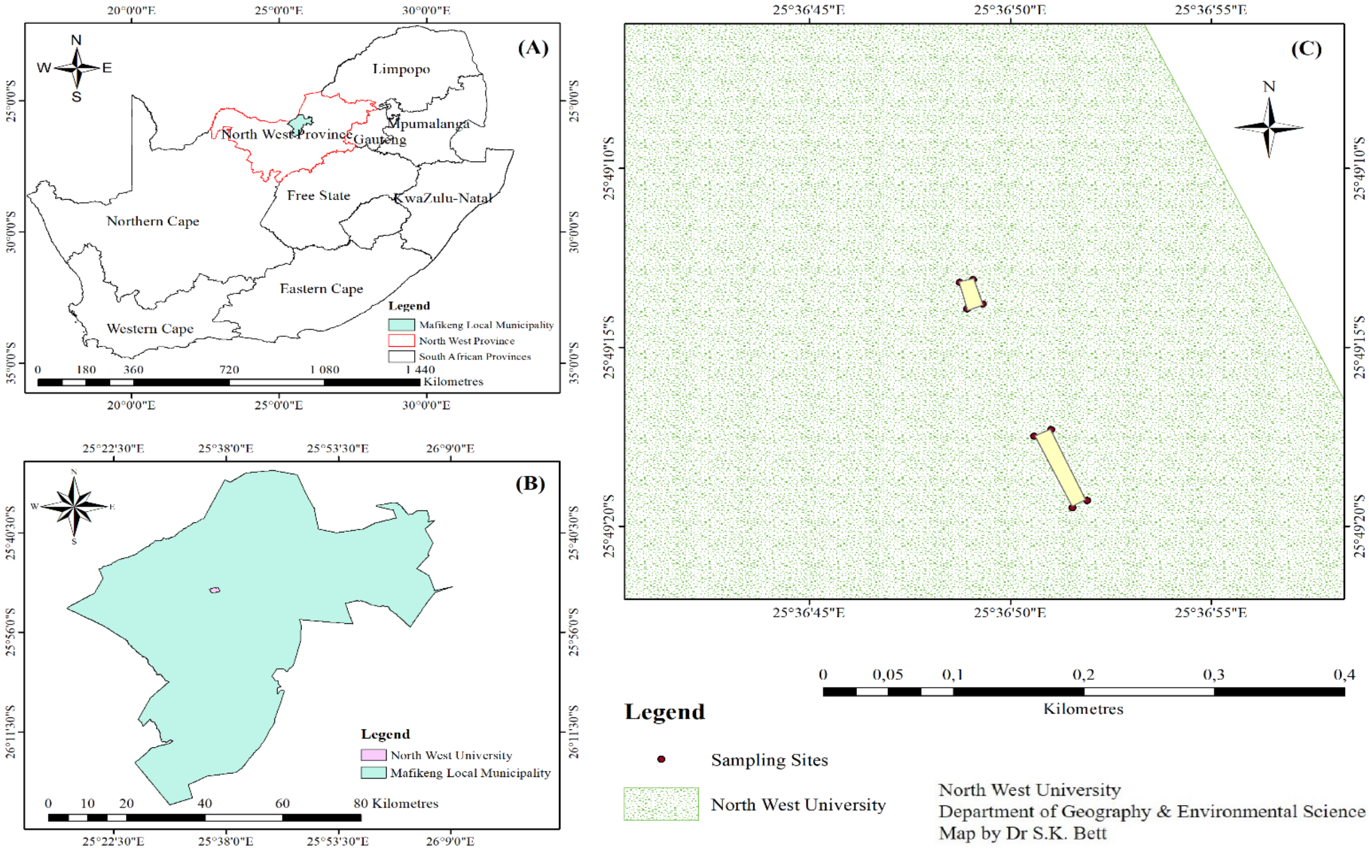


Figure 8.1: Diagrammatic representation of map of the North west province of South Africa indicating experimental location and the GPS coordinates.

8.2.1.1. Endophytic bacterial and fungal strains seed inoculant treatment

Prior to seed inoculation treatment, overnight culture of endophytic bacterial strains *Stenotrophomonas geniculata* NWUBe21 and *Pseudomonas carnis* NWUBe30 were grown in Luria Bertani (LB) broth, the concentration was adjusted to (10^6 CFU/ml) using UV-VIS spectrophotometer at optical sensity (OD) 610 nm wavelength. Likewise, endophytic *Aspergillus minisclerotigenes* strain NWUFe10 was cultured in yeast extract glucose broth medium for 5 days, the concentration was adjusted to (10^6 CFU/ml) with spectrophotometer at (OD) 610 nm. The two endophytic bacterial strains and the fungal strain were in earlier studies molecularly characterized and their genomes assembled and annotated as potent plant-growth improving microbial resources.

8.2.1.2. Cowpea seeds disinfection and endophytic inoculation treatments

The two (2) cowpea cultivar seeds used for planting were initially disinfected using sodium hypochlorite, 70 % ethanol, sterile distilled in multiple step process to eliminate any contaminants. Thereafter, the inoculation treatment of the cowpea seeds entails immersing the cowpea seeds in the endophytic bacterial and fungal strains medium solution containing 1% carboxymethylcellulose for 10 minutes, as single inoculant and mixed inoculant treatment respectively. The bio-inoculated cowpea seeds were allowed to air dried before taking to the field for planting.

8.2.2. Cowpea plant agronomic growth analysis

During the course of the field experimentation trials, agronomic vegetative growth parameters were determined for plant height, number of leaves and leaf area index at 21, 42 and 63 days after planting of cowpea plant. Also, the yield parameters of the cowpea plant due to endophytic bacterial and fungal strains inoculation treatment was recorded for the number of pods, pods length, pods weight, number of seeds Per Pod, 100 Seeds weight and the grain yield per hectare.

The cowpea seeds endophytic inoculant treatments that were deployed for the field trial experimental layout are illustrated below:

Treatment 1-Cowpea cultivar 1 + water = CV1T0

Treatment 2- Cowpea cultivar 1 + *Pseudomonas carnis* strain NWUBe30 = CV1T1

Treatment 3– Cowpea cultivar 1 + *Stenotrophomonas geniculata* strain NWUBe21= CV1T2

Treatment 4- Cowpea cultivar 1 + *Aspergillus minsclerotigenes* strain NWUFe10 = CV1T3

Treatment 5- Cowpea cultivar 1 + *Pseudomonas carnis* NWUBe30 + *Stenotrophomonas* *geniculata* NWUBe21 + *Aspergillus minisclerotigenes* NWUFe10 = CV1T4

Treatment 6 - Cowpea cultivar 2 + water = CV2T0

Treatment 7 - Cowpea cultivar 2 + *Pseudomonas carnis* strain NWUBe30 = CV2T1

Treatment 8 – Cowpea cultivar 2 + *Stenotrophomonas geniculata* strain NWUBe21= CV2T2

Treatment 9 - Cowpea cultivar 2 + *Aspergillus minsclerotigenes* strain NWUFe10 = CV2T3

Treatment 10 - Cowpea cultivar 2 + *Pseudomonas carnis* NWUBe30 + *Stenotrophomonas* *geniculata* NWUBe21 + *Aspergillus minisclerotigenes* NWUFe10 = CV2T4

These endophytic inoculant treatments were carried out in triplicates following the design as highlighted above.

8.2.3. Statistical Analysis

The observed field data in this study were analyzed using one-way ANOVA, with a P-value set at 0.05 or less taken as significance. Also, the data means were compared using Least Square Difference (L.S.D) set at 0.05 Significance level using SPSS Software of version 20.0.

8.3. Results

8.3.1. Soil physico-chemical properties

The soil physical and chemical analysis result is presented in Table 8.1. The soil granulomy indicated the sandy-silt nature of the soil sample. The soil pH indicated alkaline to slight acidity values of 7.80 to 6.70. Also, the soil sample values for total nitrogen, organic matter and ECEC are 0.90 to 0.83, 10.60 to 10.40 and 13.84 and 12.11 respectively.

Table 8.1: Physicochemical properties of soil samples of experimental location

Soil properties	Sample A	Sample B
pH in H_2O	7.80 ± 0.16	6.70 ± 0.39
Organic Carbon (g/kg)	10.60 ± 0.33	10.40 ± 0.11
Total - Nitrogen "	0.78 ± 0.12	0.70 ± 0.23
Available Phosphorus (mg/kg)	4.50 ± 0.02	4.50 ± 0.01
Exchangeable Bases (cmol/kg)		
Calcium "	7.75 ± 0.18	5.12 ± 0.11
Magnesium "	5.98 ± 0.11	5.93 ± 0.12
Potassium "	0.74 ± 0.26	0.63 ± 0.11
Sodium "	0.25 ± 0.03	0.23 ± 0.01
Exchangeable Acidity "	0.02 ± 0.01	0.03 ± 0.02
ECEC "	13.84 ± 0.21	12.11 ± 0.30
Base Saturation (g/kg)	1000 ± 2.41	988 ± 3.12

Micronutrients (mg/kg)			
Mn	"	104.00 ± 2.22	106.00± 2.31
Fe	"	82.00 ± 0.54	89.00 ± 0.59
Zn	"	1.30 ± 0.02	1.20 ± 0.01
Cu	"	2.70 ± 0.00	2.67 ± 0.01
Particle Size Distribution (g/kg)			
Sand	"	762.00 ± 4.31	731.00 ± 3.32
Silt	"	177.00 ± 1.22	104.00 ± 2.24
Clay	"	61.00 ± 0.52	60.00 ± 0.41

8.3.2. Endophytic microbiota inoculated treatments effects on cowpea plant agronomic growth enhancement at different vegetative growth stage

Endophytic microbiota treatments using single and mixed inoculation with *Pseudomonas carnis* NWUBe30, *Stenotrophomonas geniculata* NWUBe21 and *Aspergillus minisclerotigenes* NWUFe10 positively influence the plant height, number of leaves and leaf area index over the un-inoculated control cowpea plant. The average agronomic growth data at different days after cowpea sowing; 21 DAS, 42 DAS and 63 DAS over the two-planting season, indicated that plant height, the number of leaves and the leaf index are significantly enhanced due to endophytic microbiota inoculant treatments compared to the un-inoculated for the two cowpea cultivars. The endophytic inoculant treatment using Cv1TPc performed best with a plant height of 38.5cm; number of leaves – 45; and a leaf area index of 56 cm^2 for 21 days (DAS) of cowpea plant. A similar trend of results was recorded for the plant height, number of leaves and leaf area index for 42 DAS and 63 DAS respectively for the inoculant treatment Cv1TPc. At 42 DAS, the result for plant height was 42.4cm, the number of leaves 47 and the leaf area index was 59 cm^2. However, the results obtained at 63 DAS indicated that endophytic inoculant treatment with CV1TSPA gave better results with respect to plant height 50.6 cm; number of leaves – 50 and the leaf area index of 63 cm^2. In summary, the endophytic inoculant treatment improved the agronomic growth traits of the planted cowpea plant significantly at ($P<0.05$) as shown in (Tables 8.2 and 8.3).

Table 8.2: Endophytic microbiota inoculant treatment influence on agronomic growth characteristics of two *Vigna unguiculata* cultivar at different vegetative stages for 2019/2020

Cowpea cultivar 1 (CV1)

	Plant height (cm)			Number of leaves			Leaf Area Index (cm^2)		
Bioinoculant Treatment	21 DAS	42 DAS	63 DAS	21 DAS	42 DAS	63 DAS	21 DAS	42 DAS	63 DAS
CV1T0 (control)	11.5	16.4	22.4	21.2	22.4	26.2	40.3	47.3	54.2
CV1T1	15.3	21.5	24.5	26.2	25.6	32.4	58.3	59.6	62.6
CV1T2	17.9	22.9	26.9	17.8	23.2	43.2	53.7	63.7	84.7
CV1T3	22.8	22.8	23.8	25.2	24.9	35.9	63.8	65.8	73.8
CV1T4	21.5	23.8	24.8	22.4	22.8	50.8	50.6	70.3	80.3
Mean	29.5	29.5	29.5	20.8	21.7	34.7	52.9	50.9	52.9
LSD	2.58	3.14	4.43	2.44	3.12	4.37	5.48	4.95	6.32
P level	***	***	***	***	***	***	***	***	***

Cowpea cultivar 2 (CV2)

	Plant height (cm)			Number of leaves			Leaf Area Index (cm^2)		
Bioinoculant Treatment	21 DAS	42 DAS	63 DAS	21 DAS	42 DAS	63 DAS	21 DAS	42 DAS	63 DAS
CV2T0 (control)	17.4	18.6	20.4	19.4	24.1	34.4	50.3	60.3	62.7
CV2T1	22.9	26.4	27.9	16.5	26.5	27.2	54.2	64.2	67.6
CV2T2	24.3	25.3	29.3	17.0	32.0	41.6	63.6	78.6	82.3
CV2T3	26.5	26.5	30.5	19.8	27.45	29.8	58.4	69.4	75.2

CV2T4	25.4	24.4	32.4	16.9	23.9	46.9	77.4	82.3	85.6
Mean	20.5	21.4	24.6	15.7	24.7	14.7	52.9	56.8	62.4
LSD	2.43	2.53	4.12	1.73	3.14	3.94	4.91	5.04	6.10
P level	***	***	***	***	***	***	***	***	***

Data represents average of 10 replicates, ***= indicates mean squares are significant at $P < 0.05$

Table 8.3: Endophytic microbiota inoculant treatment influence on agronomic growth characteristics of two *Vigna unguiculata* cultivar at different vegetative stages for 2020/2021

Cowpea cultivar 1 (CV1)

	Plant height (cm)			Number of leaves			Leaf Area Index (cm^2)		
Bioinoculant treatment	21 DAS	42 DAS	63 DAS	21 DAS	42 DAS	63 DAS	21 DAS	42 DAS	63 DAS
CV1T0 (control)	14.60	19.50	25.52	24.5	26.2	30.12	44.25	54.5	56.7
CV1T1	18.43	23.24	27.20	29.2	31.7	37.0	75.80	74.45	81.3
CV1T2	21.5	25.40	28.5	19.5	28.4	46.5	59.45	65.42	89.45
CV1T3	26.7	28.45	29.35	30.4	29.5	38.24	68.5	69.41	79.5
CV1T4	25.31	27.10	32.6	27.1	28.6	55.3	70.	76.26	85.6
Mean	21.24	24.14	28.5	23.25	24.5	35.2	56.1	62.3	68.46
LSD	3.22	3.14	3.71	3.15	3.65	4.16	5.96	5.25	5.60
P level	***	***	***	***	***	***	***	***	***

Cowpea cultivar 2 (CV2)

	Plant height (cm)			Number of leaves			Leaf Area Index (cm^2)		
Bioinoculant treatment	21 DAS	42 DAS	63 DAS	21 DAS	42 DAS	63 DAS	21 DAS	42 DAS	63 DAS
CV2T0 (control)	19.10	20.2	22.5	22.5	26.5	35.0	60.5	65.0	68.5

CV2T1	24.5	30.0	29.5	30.8	32.5	29.5	64.5	68.0	74.2
CV2T2	26.45	28.45	32.4	29.5	35.7	42.0	65.0	79.2	85.4
CV2T3	30.40	28.25	31.10	26.4	32.25	31.5	62.5	68.2	79.2
CV2T4	27.5	27.60	38.2	19.5	28.0	48.0	78.5	86.0	86.5
Mean	26.5	26.5	29.5	25.7	29.2	34.4	62.4	72.8	75.4
LSD	2.74	2.80	4.62	3.80	3.65	3.86	4.91	5.04	6.10
P level	***	***	***	***	***	***	***	***	***

Data represents average of 10 replicates, ***= indicates mean squares are significant at $P < 0.05$

8.3.3. Endophytic microbiota inoculated treatments influence on cowpea plant agronomic yield parameters at harvest stage

The yield parameters of cowpea plant at harvest due to endophytic inoculation treatments using single and mixed inoculants with *Pseudomonas carnis* strain NWUBe30, *Stenotrophomonas geniculata* strain NWUBe21 and *Aspergillus minisclerotigenes* strain NWUFe10 positively influence the yield parameters of planted cowpea cultivars. At harvest, the endophytic inoculation treatments significantly differ from the un-inoculated control in the number of seeds per pod, the pod length, pod weight and the seed yield. With respect to the endophytic inoculant treatment deployed, the treatment with *P. carnis* strain NWUBe30 significantly at ($P<0.05$) increased the number of seeds, pods, the pod length, the pods weight and the 100 seed weight value respectively. The 100 seed weight, weight of the pods, the number of seeds and the number of pods were also, significantly improved due to endophytic treatment with *S. geniculata*. The mixed endophytic inoculant treatment likewise had a similar positive improvement influence on the weight of pods, the pod length, number of pods produce and the seed yield parameters. The results obtained showed that the cowpea plant un-inoculated control had the lowest yield parameters with respect to the number of seeds, number of pods, pods weight and the seeds weight respectively (Tables 8.4 and 8.5).

Table 8.4: Endophytic microbiota inoculant treatment influence on agronomic yield characteristics of Vigna unguiculata plant at harvest during Planting season 2019/2020 field trial

Cowpea cultivar 1 (CV1)

Bioinoculant treatment	Number of pods	Pods length	Pods weight	Number of seeds Per Pod	100 Seed weight	Grain yield per hectare (Kg/ha)
CV1T0	12.4	13.34	10.68	11.63	10.52	47.85
CV1T1	21.9	17.46	15.26	15.35	10.97	69.58
CV1T2	16.3	15.85	18.54	13.25	12.46	77.36
CV1T3	16.85	22.75	22.24	14.5	13.26	75.28
CV1T4	12.5	22.15	27.16	12.82	14.54	86.58
Mean	13.75	15.26	15.65	11.74	11.85	44.95
LSD	1.47	0.58	1.76	0.68	0.46	1.28
P level	***	***	***	***	***	***

Cowpea cultivar 2 (CV2)

Bioinoculant treatment	Number of pods	Pods length	Pods weight	Number of seeds Per Pod	100 Seed weight	Grain yield per hectare (Kg/ha)
CV2T0	11.1	13.54	9.20	11.8	9.95	63.5
CV2T1	18.5	20.21	10.4	15.8	11.76	69.1
CV2T2	16.3	18.73	18.52	16.7	12.24	76.7

CV2T3	15.6	24.12	22.10	14.25	12.79	79.5
CV2T4	26.7	22.45	27.65	13.75	14.64	86.56
Mean	13.4	12.94	13.95	11.95	12.38	60.63
LSD	1.35	0.56	1.43	0.58	2.13	2.64
P level	***	***	***	***	***	***

Data represents average of 10 replicates, ***= Indicates mean squares are significant at $P < 0.05$

Table 8.5: Endophytic microbiota inoculant treatment influence on agronomic yield characteristics of *Vigna unguiculata* plant at harvest during Planting season 2020/2021 field trial

Cowpea cultivar 1 (CV1)

Bioinoculant treatment	Number of pods	Pods length	Pods weight	Number of seeds Per Pod	100 Seed weight	Grain yield per hectare (Kg/ha)
CV1T0	10.7	12.25	9.42	10.4	9.84	45.6
CV1T1	22.1	16.6	14.68	14.5	10.56	68.42
CV1T2	15.7	15.5	17.21	12.6	11.24	75.16
CV1T3	16.4	23.21	21.50	13.2	12.50	73.5
CV1T4	11.1	21.54	26.59	11.7	13.8	84.38
Mean	12.4	14.7	14.25	10.4	10.2	41.7
LSD	1.23	0.39	1.24	0.35	0.39	0.45
P level	***	***	***	***	***	***

Cowpea cultivar 2 (CV2)

Bioinoculant treatment	Number of pods	Pods length	Pods weight	Number of seeds Per Pod	100 Seed weight	Grain yield per hectare (Kg/ha)
CV2T0	13.5	14.23	10.54	13.46	11.24	67.42
CV2T1	19.14	22.5	12.62	16.5	13.21	68.74

CV2T2	16.8	19.25	20.14	17.3	13.58	77.9
CV2T3	17.1	23.5	23.68	15.14	13.64	79.86
CV2T4	24.52	21.85	26.49	14.20	15.82	88.73
Mean	14.24	13.75	13.98	12.72	13.26	62.54
LSD	1.56	0.65	1.79	0.62	2.58	3.21
P level	***	***	***	***	***	***

Data represents average of 10 replicates, ***= Indicates mean squares are significant at $P < 0.05$

8.4 Discussion

Cowpea is a versatile, resilient and multi-beneficial grain legume with a huge potential to mitigate the challenges of climate variations, depletion of soil nutritional components, drought issues, human nutritional deficiency in relation to protein as well as fodder and forage for animal husbandry. In recent times, more attention and research efforts are being given to this orphaned and underexplored African foremost indigenous grain legume with immense potentials to ameliorate human and animal protein deficiency, restore soil nutrients depletion, fix atmospheric nitrogen through nodulation, maintain agro-ecological balance and also serve as a veritable means of ensuring food and nutritional security (Cullis & Kunert, 2017; Edet & Ishii, 2022; Natasha Muchemwa *et al.*, 2022).

Cowpea productivity is very low in most producing and consuming nations mainly in sub saharan Africa due to a myriad of constraints that include low seed cultivars, changing climatic conditions, high demands for expensive synthetic agrochemicals, due to microbial pathogens and pest amongst other challenges. To combat and overcome these challenges, the searchlight have been beam to the application of microbial inoculating agents to ensure sustainable agroecological productivity improvement. Research reports have shown promising results with regards to microbial enhancement of cowpea agronomic growth and yield improvement using microbial inoculants (Omomowo *et al.*, 2009; Omomowo *et al.*, 2018; Omomowo *et al.*, 2020; Pereira *et al.*, 2020). A lot of research effort have been directed towards the intensification of cowpea production through the use of mycorrhiza fungi and nodulating bacteria like rhizobia and bradyrhizobia (Haro *et al.*, 2018; Kavadia *et al.*, 2021; Oliveira *et al.*, 2017; Omirou *et al.*, 2016), all the cited references have highlighted prospects and potency of these bioinoculating agents as a sustainable means of improving cowpea productivity in an agro-ecological sustainable means. Lately, endophytic microbiota application as bioinoculating agents have shown promising prospects in supporting diverse crop growth both in pot and field experimental studies (Adeleke *et al.*, 2022a; Adeleke *et al.*, 2022b; Agarwal *et al.*, 2020; Lucero *et al.*, 2021; Vo *et al.*, 2021).

The deployment of endophytic microbial agents as sustainable and ecofriendly inoculants to improve planted crops growth attributes, have affirmed their potency as alternatives to costly and import dependent synthetic agrochemicals that are threats to public health and environmental sustainability. Therefore, the major focus of this study was aimed at improving cowpea agronomic growth and yield enhancement potential in Field experimental trials in Mafikeng, Northwest, Province, South Africa using effective endophytic bacteria and fungi strains.

The influence of deploying endophytic bacterial strains *Pseudomonas carnis* NWUBe30, *Stenotrophomonas geniculata* NWUBe21 and *Aspergillus minisclerotigenes* NWUFe10 were applied as single inoculants and as mixed inoculatings to assess the agronomic growth and yield enhancement potency of planted cowpea.

The results showed that application of endophytic bacterial strains *P.carnis* NWUBe30 and *S.geniculata* NWUBe21, as well as endophytic fungal strain *A.minisclerotigenes* NWUFe10 improved the agronomic growth and yield parameters of cowpea plants significantly at ($P<0.05$) when deployed singly or as mixed culture inoculants compared to un-inoculated control. There was significantly marked growth improvement in the plant height, number of leaves and the leaf area index of the cowpea cultivars attributed to endophytic microbiota inoculation treatment. Also, the cowpea agronomic yield data recorded an enhancement in the number of pods, pods length, pods weight, the number of seeds as well as the seeds weight when compared to the un-inoculated control. This significant improvement in both the agronomic growth and yield improvement parameters of cowpea due to endophytic microbiota inoculation is made possible through the physiological and metabolic transformative capabilities of the endo-microbiota to make nutrients available to their host plant, to produce important plant-hormonal metabolites, to break down complex nutrients to available nutrients for plant use, as well as to antagonize competing phytopathogens of plants (Aeron *et al.*, 2021; Eid *et al.*, 2021; Gorai *et al.*, 2021). All the mention traits of the deployed endophytic microbiota are the reasons for the improvement in cowpea agronomic growth and yield improvement. Various research outcomes have reported similar growth, development and functionality improvement on planted crops due to the deployment of bioinoculating microbiota (Afandhi *et al.*, 2019; Dubey *et al.*, 2021; Mei *et al.*, 2021).

The deployment of endophytic microbiota strains as single inoculants lead to the significant improvement at ($P<0.05$) for agronomic growth parameters - plant height, number of leaves, leaf area index, as well as yield parameters – Number of pods, pods length, pods weight, number of seeds as well as seeds weight. These findings are similar to results of (Ayalew *et al.*, 2021b; Shet & Garg, 2022) on cowpea growth improvement due to application of bioinoculating microbials.

Furthermore, our findings confirmed the positive synergistic influence of mixed endophytic microbiota deployment on cowpea plant in the improvement of the agronomic growth parameters of the number of leaves, leaf area index and the height of cowpea plants. This mixed endophytic culture inoculants, also improve the yield parameters of number of seeds, the seeds weight, the number of pods, the pods length and the weight of pods. Several similar research outcomes such as our findings are also reported in literature (de Oliveira *et al.*, 2022; do Nascimento *et al.*, 2021; Rocha *et al.*, 2020; Souza-Alonso *et al.*, 2021; Surendirakumar *et al.*, 2022), using multiple microbial agents as single and co-inoculants in improving cowpea productivity. In general, the influence of endophytic microbiota strains - *Pseudomonas carnis* NWUBe30, *Stenotrophomonas geniculata* NWUBe21 and *Aspergillus minisclerotigenes* NWUFe10, deployed in this field trial experiment in Mafikeng, Northwest Province, South Africa, resulted in the enhancement of agronomic growth and yield response of cowpea plants. Our findings affirmed that endophytic microbiota strains can be deployed to improve cowpea production and they are promising natural microbial resources to enhance cowpea production output and ensure agroecological sustainability.

8.5. Conclusion

These findings have shown promising positive influence of endophytic microbiota strains on planted cowpea agronomic growth and yield enhancement in field experimental trials. The deployment of endophytic *Pseudomonas carnis* strain NWUBe30, *Stenotrophomonas geniculata* strain NWUBe21 and *Aspergillus minisclerotigenes* strain NWUFe10 as single inoculating agents, and as mixed culture inoculating agents enhanced the seeds weight, number of seeds, pods weight, pods length and the number of pods. Also, these bioinoculating agents improve the plant height, number of leaves and the leaf area index of cowpea plant. Therefore, these natural microbial resources can be used by small-scale subsistence/marginal farmers to improve the output of cowpea production. However, multiple field trials in different environments are suggested for future research.

CHAPTER NINE

9.0. CONCLUDING REMARKS AND RECOMMENDATIONS

9.1 Concluding remarks

In this current investigative study on improving cowpea productivity in Northwest Province, South Africa through the use of bacterial and fungal endophytic microbial inoculants, it was affirmed that cowpea production can be improved through biostimulation with effective endophytic bacterial strains like *Pseudomonas carnis* NWUBe30 and *Stenotrophomonas geniculata* NWUBe21, as well as potent endophytic fungal strain *Aspergillus minisclerotigenes* NWUFe10.

However, members of the *Aspergillus* genera are known prolific producers of aflatoxins that are injurious to humans and animals, hence the need for effective screening to ascertain their aflatoxigenic potency before deployment as bioinoculating agents. A simple assay like the use of neutral red desiccated coconut agar (NRDCA) cultural screening for determination of *Aspergillus* strain ability to produce aflatoxins or not is a necessity before moving forward to certify it as bioinoculating agent.

The plant growth-improving traits of the bacteria and fungi endophytic strains are attributed to their potential to produce exopolysaccharides, indole acetic acid, siderophore, biofilm, solubilize phosphate, as well as abilities to antagonize phytopathogens under in vitro conditions. In addition to the in vitro study results, the genomic exploration investigations of endophytic microbiota strains *Pseudomonas carnis* NWUBe30, *Stenotrophomonas geniculata* NWUBe21 and *Aspergillus minisclerotigenes* NWU Fe10 demonstrated that they possess plant growth-enhancing genes in their genomes that are responsible for their cowpea productivity improvement. Moreover, the plant-growth data obtained when *Pseudomonas carnis* NWUBe30, *Stenotrophomonas geniculata* NWUBe21, and *Aspergillus minisclerotigenes* NWUFe10 were deployed as bioinoculating agents in cowpea field experiment as single or mixed inoculants indicated cowpea production improvement over the un-inoculated control. The deployment of these effective endophytic microbial resources led to cowpea agronomic yield and growth enhancement.

Therefore, this study has contributed to knowledge in that:

Effective and phylogenetically diverse endophytic microbiota (bacterial and fungal strains) from cowpea seeds and aerial tissues possessing plant-growth stimulating attributes were authenticated and characterized.

Also, there were abundance of plant-growth stimulating genes and several secondary metabolite gene clusters in the genomes of *Pseudomonas carnis* NWUBe30, *Stenotrophomonas geniculata* NWUBe21, and *Aspergillus minisclerotigenes* NWUFe10 elucidated in this study. Moreover, single and mixed culture inoculation using endophytic *Pseudomonas carnis* strain NWUBe30, *Stenotrophomonas geniculata* strain NWUBe21, and *Aspergillus minisclerotigenes* strain NWUFe10 confirmed the cowpea agronomic yield and growth enhancing effectiveness of these endophytic bioinoculants compared to the un-inoculated control.

9.2. Recommendations

Although the baseline information obtained in this study shows positive prospect that support the deployment of indigenous endophytic microbiota as a tool to improve cowpea production, however, more research investigation on field trial studies of various cowpea genotypes in different agroecological environmental settings are a required necessity. In addition to this, endophytic strain improvement studies are further recommended for these effective endophytic bioinoculants that showed good prospect as alternative to synthetic agrochemicals in achieving sustainable cowpea productivity improvement.

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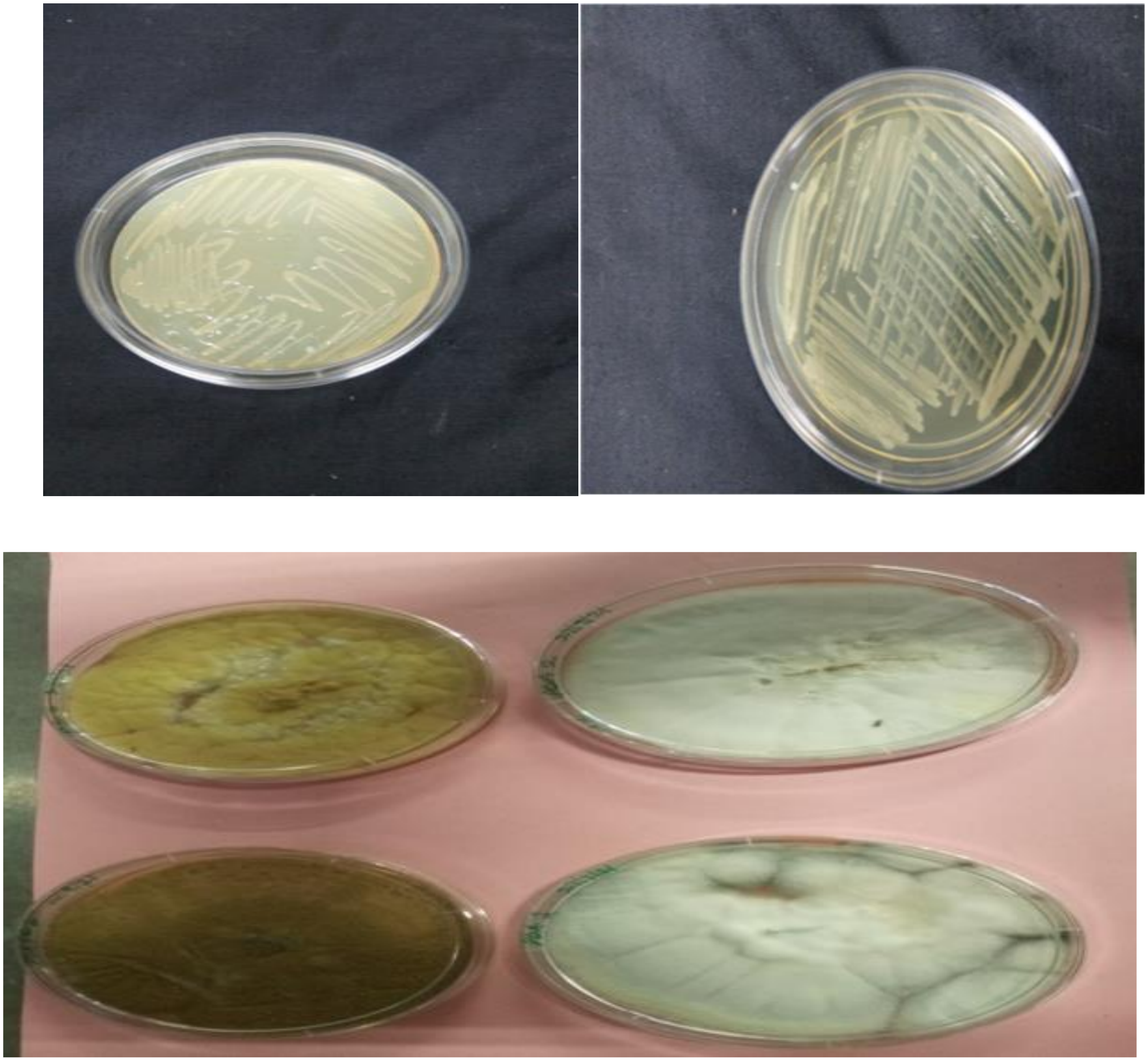
APPENDIX

Figure A: Endophytic bacteria culture and fungi culture isolates on petri-plates



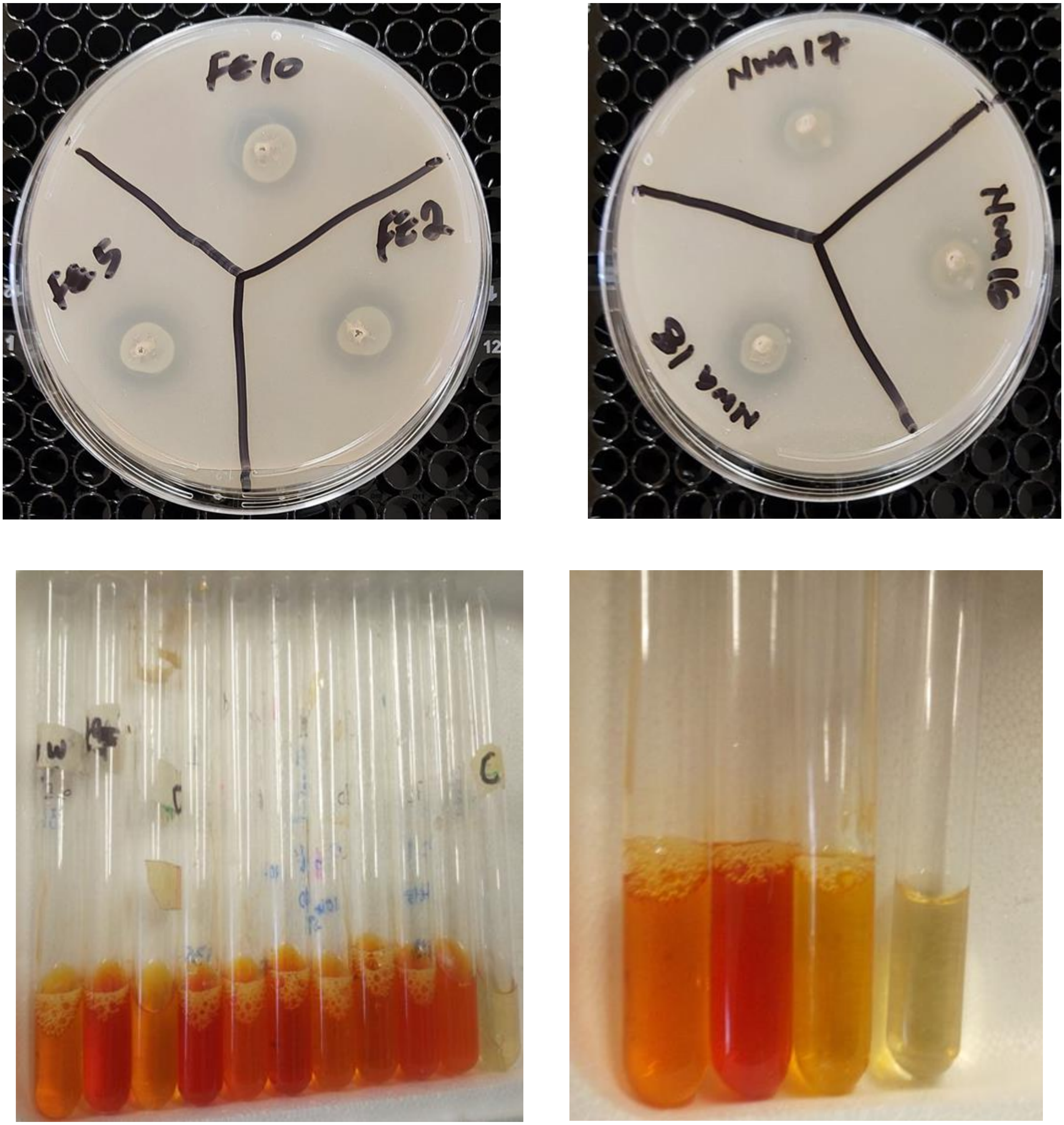


Figure B: Plant growth promoting assay for IAA and Phosphate solubilization

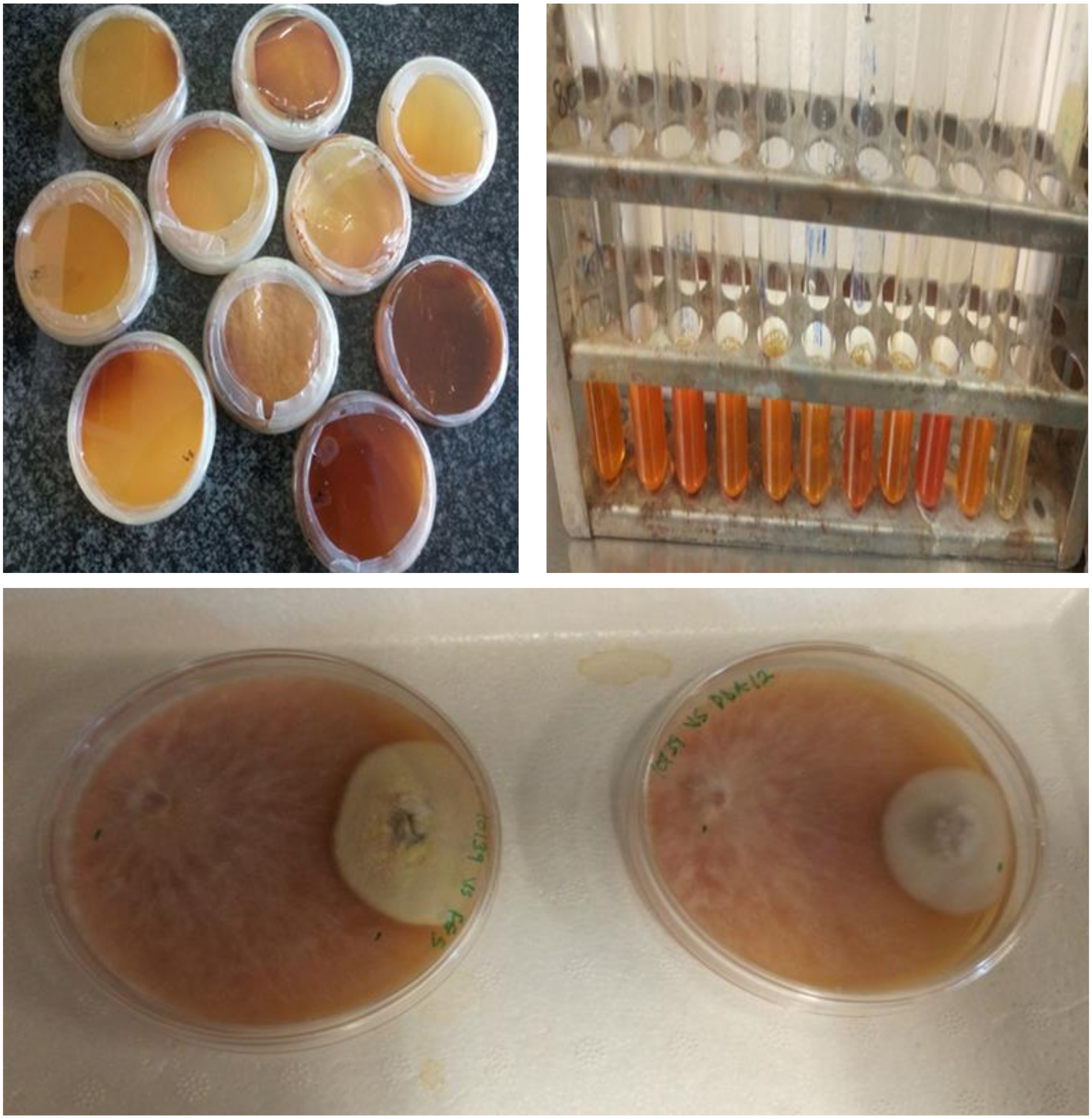


Figure C: Plant growth promoting assay for Ammonia production and antifungal inhibition