ASSESSEMENT OF CO-INOCULATION OF *BRADYRHIZOBIUM JAPONICUM* AND *BACILLUS SUBTILLS* ON YIELD AND METABOLIC PROFILE OF BAMBARA GROUNDNUT AND COWPEA UNDER GLASSHOUSE CONDITIONS

By

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I declare that the dissertation is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

SIGNATURE

08/06/20

DATE

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DEDICATION

I would like to dedicate this dissertation to my grandmother, Nelwamondo Nyadzanga Ellah, and my precious daughter, Nemaungani Phindulo.

ABSTRACT

Bambara groundnut and cowpea are essential legumes that are well adapted to unfavourable environmental conditions and have high dietary values for humans. However, they are under-researched and under-utilised. Thus, there are limited records on yields and metabolic profiling of these leguminous crops co-inoculated with *B. japonicum* and *Bacillus subtilis*. Generally, very few studies have reported on the effects of co-inoculation of other plant growth-promoting rhizobacteria and rhizobia strains on leguminous plants. This study therefore assessed the effects of *B. subtilis* (strain BD233) on germination of Bambara groundnut under different temperature regimes, and evaluated the effects of co-inoculation of *B. japonicum* and *B. subtilis* on yields of cowpea under glasshouse conditions. The study also assessed the metabolite profile of the crops using 1H nuclear magnetic resonance (NMR) spectroscopy. The data showed that inoculation of *Bacillus subtilis* on Bambara groundnut landraces under different temperatures enhanced germination (germination percentage, germination rate indices and plumule length). Furthermore, co-inoculation with *B. japonicum* and *Bacillus subtilis* (strain BD233) improved plant yield of cowpea plants. Partial least squares-discriminant analysis (PLS-DA) revealed distinct separations between treatments (co-inoculation of *B. japonicum* and *Bacillus subtilis*, inoculation *of B. japonicum*, uninoculated plus NO3 and zero inoculation) on Bambara groundnut and cowpea plants. The VIP score revealed that co-inoculation with *B. japonicum* and *Bacillus subtilis* (strain BD233) resulted in low concentrations of metabolites in Bambara groundnut plants and in contrast, high concentrations of metabolites in cowpea plants. Co-inoculation with *B. japonicum* and *Bacillus subtilis* (strain BD233) has a potential of improving yield of both Bambara groundnut and cowpea in sustainable agriculture. The metabolic profile of Bambara groundnut and cowpea subjected to co-inoculation has shown that both crops metabolic composition and profile are highly dependent on co-inoculation.

MANWELEDZO

Phonda na ṋawa ndi mangaṋawa a ndeme ane a kona u tea zwavhuḓi kha nyimele dza vhupo vhune ha si vhe havhuḓi na ndeme ya nṱha ya pfushi kha vhathu. Naho zwo ralo, a hu athu u itwa ṱhoḓisiso dzo linganaho nga hadzo na u sa shumiswa Nga zwenezwo hu na rekhodo dzo pimiwaho nga ha khaṋo na u ela tshileme tsha molekulu ṱhukhu dza methaboḽiki dza zwiliṅwa izwi zwa mangaṋawa u khetha na *B. japonicum na Bacillus subtilis*. Nga u angaredza, ndi ngudo dzi si nngana dzo no vhigwaho nga ha masiandaitwa a khetha nyaluwo ya zwimela zwine zwa ṱuṱuwedza bakitheria dzine dza baḓekanywa na midzi na bakitheria dzine dza shandukisa naiṱirodzheni u vha amonia kha zwimela zwa mangaṋawa. Ngudo heyi nga zwenezwo yo asesa masiandaitwa a *B. subtilis* (tshiliṅwa tsha BD233) kha mumelo wa phonda nga fhasi ha ndaulo ya thempheretsha dzo fhambanaho, na u ela masiandaitwa a u khetha *B. japonicum* na *B. subtilis* kha khaṋo dza phonda na ṋawa nga fhasi ha nyimele ya fhethu hune ha ṱavhiwa zwimela nga fhasi ha tsireledzo kana ndangulo. Ngudo dzo dovha dza ela tshileme tsha molekulu ṱhukhu dza methaboḽiki dza zwiliṅwa hu tshi shumiswa ^{1}H maanḓa a u tzwonzwiwa ha nyukiḽia nga eḽekiṱhironiki maginethe (NMR) nga u ṱanganelana ha radiesheni ya eḽekiṱhironiki maginethe. Data yo sumbedza u ḓivhadzwa ha *Bacillus subtilis* kha tshiliṅwa tshapo tsha phonda fhasi ha thempheretsha dzo fhambanaho u khwinisa mumelo (phesenthedzhi ya mumelo, zwisumbi zwa phimo ya muelo na vhulapfu ha pulumule). U isa phanḓa, u ḓivhadzwa hafhu ha *B. japonicum* na *Bacillus subtilis* (tshiliṅwa tsha BD233) khaṋo yo khwiniswaho ya tshiliṅwa kha zwimela zwa ṋawa. Musaukanyo wa u khethekanya zwitatisiṱika (Partial least squares-discriminant analysis) (PLS-DA) wo sumbedza khethekanyo dzo fhambanaho vhukati ha kushumisele (u ḓivhadzwa hafhu ha *B. japonicum* na *Bacillus subtilis*, u ḓivhadzwa ha *B. japonicum*, i songo ḓivhadzwaho hafhu na NO_3 na ziro i songo ḓivhadzwa hafhu) kha phonda na zwiliṅwa zwa ṋawa. Tshikoro tsha VIP tsho wanulusa uri u ḓivhadzwa hafhu ha *B. japonicum* na *Bacillus subtilis* (kha tshiliṅwa tsha BD233) zwo bveledza mutzwonzwo wa fhasi wa methobolaithisi kha zwiliṅwa zwa phonda na phambano, ya mutzwonzwo wa nṱha wa methobolaithisi kha zwiliṅwa zwa ṋawa. U khetha ha *B. japonicum na Bacillus subtilis* (kha tshiliṅwa tsha BD233) zwo vha na ndeme ya u khwinisa khaṋo ya vhuvhili hazwo phonda na ṋawa kha vhulimi vhu sa nyeṱhi. U ela tshileme tsha molekulu ṱhukhu dza methaboḽiki dza phonda na ṋawa tenda u ḓivhadzwa hafhu ho sumbedza uri vhuvhili ha kubveledzele kwa methaboḽiki ya zwiliṅwa na muelo zwo ḓitika nga maanḓa nga u khetha.

SETSOPOLWA

Ditloo tša Bambara ke dipeu tše bohlokwa tšeo di kgonago go phela gabotse go maemo a tikologo yeo e sego ya loka e bile di na le boleng bja godimo bja dijo tšeo di lekanego go batho. Le ge go le bjalo, gona le dinyakišišo tša fase ka tšona le gore ga di šomišwe kudu. Ka gona, go na le direkhoto tše dinnyane ka ga pego ya mehola le tšhomišo ya yona ka ga dibjalo tše tša go dira dipeu tšeo di kopantšhwago le *B. japonicum le Bacillus subtilis*. Ka kakaretšo, dinyakišišo tše dinnyane kudu di begile ka ga dikhuetšo tša kopantšho ya mehlare e mengwe ya go huetša go gola ga pakteria ya medu (rhizobacteria) le dingangego tša pakteria ya ka gare ga medu (rhizobia) go dibjalo tša dipeu. Nyakišišo ye ka gona e lekotše dikhuetšo tša *B. subtilis* (strain BD233) go melo ya ditloo tša Bambara ka fase ga maemo a dithempereitša tša go fapana, le go lekola dikhuetšo tša kopantšho ya *B. japonicum* le *B. subtilis* go mehola ya ditloo tša Bambara le dinawa ka fase ga maemo a ntlo ya digalase. Nyakišišo gape e lekotše pego ya tšhomišo ya dibjalo go šomišwa sedirišwa sa go laetša maatlakgogedi sa ^{1}H (NMR). Tshedimošo e bontšhitše gore tsenyo ya *Bacillus subtilis* go ditloo tša Bambara tša tlwaelo ka fase ga dithempereitša tša go fapana go kaonafaditše go mela (phesente ya go mela, lebelo la dikelo tša melo le botelele bja kutu ya sebjalo). Gape, kopantšho le *B. japonicum* le *Bacillus subtilis* (strain BD233) go kaonafaditše mehola ya dibjalo tša mehlare ya dinawa. Tshekatsheko ya go hwetša tswalano ya dithišu tše pedi (PLS-DS) e utollotše ditlogelano tša go fapana magareng ga mekgwa (kopantšho ya *B. japonicum* le *Bacillus subtilis*, tsenyo ya *B. japonicum,* yeo e sego ya tsenywa le NO_3 le tsenyo ya lefeela) go ditloo tša Bambara le dibjalo tša dinawa. Dipoelo tša VIP di utollotše gore kopantšho ya *B. japonicum* le *Bacillus subtilis* (strain BD233) e tlišitše dipoelo tša fase tša ditšweletšwa tša dimolekule tša dibjalo tša ditloo tša Bambara e bile gape ge re dira phapanyo, bontšhi bjo bo lego godimo bja ditšweletšwa tša dimolekule ka go dibjalo tša dinawa. Kopantšho ya *B. japonicum* le *Bacillus subtilis* (strain BD233) e na le kgonagalo ya go kaonafatša mehola ya bobedi ditloo tša Bambara le dinawa ka go temo ya sa ruri. Seemo sa ditšweletšwa tša ditloo tša Bambara le dinawa tšeo di dirilwe kopantšho se bontšhitše gore bobedi tlhamotšweletšo le seemo sa dibjalo tše di ithekgile kudu mo go kopantšho.

ABBREVIATIONS

ANOVA	……	Analysis of Variance
ARC	……	Agriculture Research Council
BNF	……	Biological Nitrogen Fixation
DAS	……	Days After Sowing
G%	……	Germination Percentage
GI	……	Germination Index
GRI	……	Germination Rate Index
mM	……	Millimolar
NRM	……	^{1}H Nuclear Magnetic Resonance
˚C	……	Degree Celsius
PGPR	……	Plant Growth Promoting Rhizobacteria
PL	……	Plumule Length

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CHAPTER 1: GENERAL INTRODUCTION

1.1 INTRODUCTION

Bambara groundnut (*Vigna subterranea* (*L*)*. Verdc*) is known to be the third most essential legume crop in African countries after cowpea and groundnut in agricultural cropping systems. Cowpea (*Vigna unguiculata L*. Walp) is a significant nourishing legume plant cultivated worldwide. In South Africa, production of these grain legumes is mainly performed by small-holder farmers and subsistence and plays a crucial role in improving rural diets and food security. As leguminous crops, Bambara groundnut and cowpea are also valued for their ability to fix atmospheric nitrogen (Hall & Ehlers, 1997) and adaptability to harsh environmental conditions (Gomez, 2004). Additionally, these legumes are high in protein, vitamins, complex carbohydrates, minerals and consumable oils, thus play a role in poverty and malnutrition alleviation in the African rural communities.

Legumes have a symbiotic relationship with rhizobia and contribute significant amounts of nitrogen (N) when in association with compatible rhizobia. Rhizobia are often used as inoculants in less fertile soils. The use of rhizobial inoculants as biofertilizers in most cropping systems has gained popularity recently due to negative effects of synthetic fertilizers (De Resende *et al.,* 2006). Rhizobial inoculants were found to be the most affordable and environmentally friendly soil improvement approach for sustainable agriculture (Wange, 1989). Furthermore, legume yields and bacterial populations in the soil were increased remarkably through inoculation of legumes with compatible and effective rhizobia (Ankomah *et al.,* 1996; Anuar *et al.,* 1995; Abaidoo *et al.,* 2007).

Plant-growth promoting rhizobacteria (PGPR) are soil bacteria that inhabit plant's rhizosphere and have been found to improve the plant's tolerance to abiotic and biotic factors (Radhakrishnan *et al*., 2017). Several studies have demonstrated that inoculation of crops with PGPR such as *Bacillus spp*. mitigated the effects both biotic and abiotic stresses (Radhakrishnan *et al*., 2017). *Bacillus spp*. were found to stabilize phosphates, and stimulate phytohormones, growth, mineral nutrition, yield and fix nitrogen, thus, improving plant growth, mineral nutrition and yield (Kumar *et al.,* 2012). Addition of bacterial strain *Bacillus* in soybean has significantly increased plant growth and accumulation of seeds (Bai *et al*., 2003). Thus, *Bacillus subtilis* is a ubiquitous microorganism with a potential to substitute costly artificial chemical fertilizers

(Bhattacharyya & Jha, 2012) due to its potential to reduce abiotic factors thus enhancing plant growth (Felici *et al*., 2008).

Co-inoculation of rhizobia inoculants with PGPR was reported to improve plant physiology and nitrogen fixation and plant biomass of legume crops (Bai *et al*., 2003; Schwartz *et al*., 2013). Additionally, studies have shown that co-inoculation of legume crops with PGPR such as *Pseudomonas* and *Bacillus* and rhizobial strains remarkably increased yield and nutrient absorption (Kuan *et al*., 2016).

1.2 PROBLEM STATEMENT

Traditionally, small-holder and subsistence farmers in the rural communities of South Africa cultivate both Bambara groundnut and cowpea annually to feed the ever-growing population. However, crop yields are often low partly as a result of poor soil fertility (with very low N and P). Soil fertility management remains a major problem in rural areas in sub-Sahara Africa, as most soils are experiencing significant decline in nutrients due to nutrient mining by plants over the years. The utilisation of PGPR and native rhizobia as inoculants is regarded as feasible symbiotic organic pathway to deal with biotic and abiotic factors in crop production and enhance legume yield due to growth promoting stimulators (Mwangi *et al*., 2011). PGPR play critical role in the soil as biofertilizers and have a variety of mechanisms such as mitigation of environmental constrains, enhance yield, accumulation of plant metabolites and remediation of contaminants in the agricultural soil (Timmusk *et al*., 2014). Thus, the use of cheap alternative method for soil improvement with the potential to increase crop yield and improve soil fertility could play a crucial role in household food and nutrition security in rural communities with annual cultivations of these leguminous crops. However, to date there is limited information the effects of co-inoculation of *B. japonicum* and *B. subtilis* (strain BD233) on the nodulation and yield of Bambara groundnut and cowpea.

1.3 RATIONAL OF THE STUDY

Earlier studies indicated that soil fertility continued to decline, which results to low crop yield especially in rural communities where smallholder and subsistence farmers have limited resources (Ali *et al*., 2018). This study has a potential to contribute to the body of knowledge the effects of the use of an alternative biofertilizers which is a combination of both PGPR (*B. subtilis*) and compatible rhizobia (*B. japonicum*) would result in improved

nutrients solubilisation, uptake and accumulation which would improve grain yield and plant metabolites of Bambara groundnut and cowpea. Literature has shown that inoculation with *Bradyrhizobium japonicum* increases K in the rhizosphere, as a results this mechanism enables it to solubilise K in soils. Correspondingly, there is evidence of increase concentration of plant metabolite in rhizospheric Fe, N and P. Therefore, their improved uptake by the legumes would increase their growth compared to yield of plants grown without inoculation as these nutrient increase grain yield. The use of environmentally friendly bacteria as a biofertilizers in this study is a sustainable way of improving soil nutrition and yields, thus, provides valuable data and understanding on role of PGPR and rhizobia on both Bambara groundnut and cowpea.

1.4 AIM OF THE STUDY

The aim of this study was to evaluate the effects of co-inoculation of *B. japonicum* and *Bacillus subtilis* on the germination, nodulation, yield and metabolic profile of Bambara groundnut and cowpea under glasshouse conditions.

1.5 OBJECTIVES

- To assess the effects of *Bacillus subtilis* on germination of Bambara groundnut under different temperature regimes.
- To evaluate the effects of co-inoculation of *B. japonicum* and *Bacillus subtilis* on yield of cowpea under glasshouse conditions.
- To determine the effects of co-inoculation of *B. japonicum* and *Bacillus subtilis* on metabolic profile in Bambara groundnut and cowpea.

1.6 HYPOTHESES

- Inoculation with *Bacillus subtilis* will enhance germination and plumule length of Bambara groundnut under different temperatures.
- Co-inoculation of *Bacillus subtilis* and *Bradyrhizobium japonicum* will enhance nodulation and yield of cowpea.
- Co-inoculation with *Bacillus subtilis* and *Bradyrhizobium japonicum* will affect the metabolic profiles of Bambara groundnut and cowpea.

1.7 STUDY LAYOUT

This dissertation contains six chapters, the research chapters included. Chapter summaries are offered to allow readers to follow and understand discussions on issues and research findings were applicable are presented in each chapter.

Chapter one consists of a background to the study and offers the aim and objectives for undertaking the same, including the research problem and rationale of the study.
Chapter two discusses literature review relative to the production, growth requirements, uses and nutritional contents of both Bambara groundnut and cowpea. It also covers literature relating to biological nitrogen fixation including its importance and limiting factors. In addition, the chapter provides a brief overview on the significance of rhizobia in cropping systems and use of PGPR in agriculture. The role of *Bacillus subtilis* in crop production including co-inoculation with PGPR is also reviewed. In closing the chapter, a brief account of metabolomics in legumes is provided. In summary, the chapter provides the drive of conducting literature review and conclusions from the literature in relation with the topic.
Chapters three, four and five presents the research discoveries for separate experiments. Explicitly, chapter three presents and discusses findings on the effect of elevated temperature and *Bacillus subtilis* (BD233) strain inoculation on Bambara groundnuts landraces seed germination indices. Chapter four describes and discusses findings on the effect of co-inoculation of *B. japonicum* and *Bacillus subtilis* on yield of cowpea under glasshouse conditions. Chapter five describes and discusses findings on the effects of co-inoculation of *B. japonicum* and *Bacillus subtilis* (strain BD233) on Bambara groundnut and cowpea metabolites using ^{1}H nuclear magnetic resonance spectroscopy (NMR). Chapter six presents the general discussion, conclusions of the whole study and implications and recommendations thereof.

CHAPTER 2: LITERATURE REVIEW

2.1 INTRODUCTION

Leguminous crops such as cowpea (*Vigna unguiculata* L. Walp) and Bambara groundnut (*Vigna subterrenea* L. Verdc) are known for their ability to fix atmospheric nitrogen and enhancing nutritional balances of low-income communities. These legumes are high in protein, vitamins, complex carbohydrates, minerals, and consumable oils. Regardless of their significance, yields of these crops remain low averaging <500 kg/ha (Abudulai *et al*., 2017). The main factors leading to low yield include diseases, insect pests attack, low soil fertility, lack of inputs, drought, late planting and high population densities (Ajeigbe *et al*., 2010a; Mabika, 1991; Mbewe *et al.,* 1995).

2.2 BAMBARA GROUNDNUT PRODUCTION

The production of Bambara groundnut worldwide is approximate at 330 000 tons annually (DAFF, 2011). West African countries including Nigeria, Chad, Togo, Ghana, Niger, and Benin produces 50% of the tons (DAFF, 2011). In South Africa, Mpumalanga, Limpopo, KwaZulu-Natal, Eastern Cape and Northern provinces, produce the crop in large scale (Swanevelder, 1998). Thus, Bambara groundnut has different names depending on the population tribe, in Limpopo it is known as phonda by Venda, izindlubu in Zulu and indlubu in Xhosa (Bamshaiye *et al*., 2011)

2.3 GROWTH REQUIREMENTFOR BAMBARA GROUNDNUT

Bambara groundnut requires a well-drained soil, which make it easier to harvest (DAFF, 2016). The temperature requirement for this crop ranges as of 20°C to 28°C. Bambara groundnut thrives well in harsh conditions which include drought and hot weather. The growth period of this legume crop ranges from 130-174 days (Mabhaudhi *et al.,* 2013). Germination starts at 7-14 days depending on the genotype (Berchie *et al*., 2010). Vegetative propagation of this legume crop initiates from germination to flowering thirty days subsequent to planting and proceeding pending vegetative growth ceases.

2.4 USES OF BAMBARA GROUNDNUT AND NUTRITIONAL CONTENT

Bambara groundnut is mainly intercropped with maize. This legume crop is also be used as animal supplements, for example, the grains have been utilized as chick feed and

haulms for livestock and foliage for appropriate elevated content of nitrogen and phosphorus (Linnemann, 1991; Bamshaiye *et al*., 2011). Bambara groundnut contains protein approximately 18%, 65% carbohydrate and 6.5% fats (Mazahib *et al*., 2013). The grains can be eaten as stamp, roasted or steamed (Bamshaiye *et al*., 2011). The storing up of the grains has been practised in many ways; the grains are canned into potage or can be produced into processed food. In addition, Bambara groundnut contains important nutrients such as potassium, calcium, iron and zinc, thus, making it a nutritious balanced meal. Interestingly, the crop has the ability of enhancing food security in rural communities due to its relative high protein content (Murevanhema & Jideani, 2013). This legume crop can be used to supplement cereals, flour for bread and biscuits for nutrition improvement for weaning infants (James *et al*., 2017; Okafor, Okafor, Leelavathi, Bhagya & Elemo, 2015).

2.5 COWPEA PRODUCTION

Globally, Brazil, USA and West Indies in America are the largest producers with 2.4 hectares. The major producers of cowpea in Africa are West and Central Africa which includes Ghana, Senegal, Cameroon, Mali, Burkina Faso, Niger, and Nigeria. They contribute 64% of the 3 million tons produced every year. Nigeria is the leading producer of cowpea worldwide contributing half of total grains worldwide. In South Africa, small-holder farmers intercrop cowpea with other field crops such as maize (DAFF, 2011), only 6% is cultivated as sole crop (Asiwe, 2009). The crop is mainly crucial in rural communities of the subtropics and tropics in developing countries, (Quin, 1997). The production of cowpea in South Africa remains low in comparison to other crops such as maize. In South Africa, small holder farmers cultivate the crop in dry lands where less water is required. Furthermore, the production area and quantity production of the crop remains unknown (DAFF, 2009).

2.6 GROWTH REQUIREMENT FOR COWPEA

The development habitat of cowpea differs depending on the genotype where they vary from erect, semi-erect, bushy, sprawling, and prone, to rising while day length as well as growth surroundings can additionally distress crop structures (Timko *et al*., 2007). Furthermore, maturity of cowpea is grouped into three levels depending on the genotype, for example, where 60-75 days regarded as early maturity, 80-85 day as medium maturity

and 110-130 days as late maturity (Singh *et al*., 2003).The vegetative propagation of the crop initiates from germination to development of flowers, which is approximately forty days subsequent to sowing, depend on the genotype (Pandey *et al*., 1987). The genotype of cowpea is important more especially on differentiation leaves (Pottorff *et al*., 2012). Furthermore, the leaf petioles vary from 5-25 cm long and are dark green, shiny to smooth and the stems vary from slightly hairy to fine and some have purple shades (DAFF, 2009). The temperature, genotype, form of growth and photoperiod has an impact on the formation of the plant (Timko & Singh, 2008). According to Ige et al. (2011) the roots varies according to the genotypes, where some genotypes have strong tap-root and lateral roots. In order for seed germination to initiate the temperature ranges from 20-30°C (Quass, 1995). When compared with other crops cowpea thrives in environmental conditions. Thus, makes rainfall requirement of 400 to 700 mm annually ideal for production (DAFF, 2011). Harvesting of crops grains is mostly done after the moistness content is approximately 12-14% to reduce the damage and cracking of the grains (Mullen *et al*., 2003). This crop can be harvested in three different phases of escalation maturity from fresh emerald shell, matured to dread pods (Davis *et al*. 1991). Cowpea has different types of seed colours and textures depending on the genotype (Timko & Singh, 2008).

2.7 USES OF COWPEA AND NUTRITIONAL CONTENT

In South Africa, the crop is known in different languages, in Limpopo Province is known as Munawa, Imbumba and Dinawa, whereas in KwaZulu Natal it is known as Isihlumaya. The crop is utilised in several ways for example, leaves and green shells are used as vegetable, compost and hay as feed for animals during dry seasons, and the seeds as snacks and main meals in most parts of Africa (Singh *et al*., 2002, Davis *et al*., 1991, Kiari *et al*., 2011, Heuze *et al*., 2015). Cowpea is highly nutritious and comprises of 24-25% proteins, 63.6% carbohydrates and 50-60% starch, thus making a most complete balance diet across the African continent (Akyaw *et al*., 2014).

2.8 NITROGEN FIXATION IN LEGUMES

Soil fertility management is major a problem in rural areas in sub-Sahara Africa, as most soils are experiencing serious decline in nutrients due to nutrient mining by plants over the years. Leguminous crops play a significant role in soil fertility management due to the biological nitrogen fixation in symbiotic relationship with rhizobia available in the soil. Legume crops provide the available rhizobia in the soil with organic sources such as

carbon while the rhizobia fix atmospheric N_2 (Giller *et al*., 2013). Legume crops are also integrated in cropping systems as mono-crops intercrops and as double up (Snapp *et al*., 2010). Alternatively, incorporating legume crops in cropping systems can offer affordable and sustainable nitrogen than synthetic fertilisers.

2.9 FACTORS LIMITING BIOLOGICAL NITROGEN FIXATION

Development especially nodule forming legumes have limiting factors for symbiotic nitrogen fixation and includes nutrient deficiency, high salt content, pH, plant infection, wild plant rivalry, harsh high temperature, mineral toxicity and dampness (Giller,2001).

Soil acidity decreases *Rhizobium* survival in the soil and reduces nodulation thus affects the process for symbiotic nitrogen fixation (Taylor *et al.,* 1991). Duncan (2002) demonstrated that white clover plants root development and photosynthesis is disrupted when the soil has of pH 4.8, or less. The most significant mineral nutrient required by legumes is phosphorus (Chaudhary *et al*., 2008). Rhizobial population and root growth of legumes are restricted due to low soil P levels, and negatively affects biological nitrogen fixation (Yakubu *et al.,* 2010). Nevertheless, most subsistence farmers use high P cowpea to address P limitation in the soil (Magani & Kuchinda, 2009). Harsh environmental conditions including drought reduces nitrogen fixation and infection. The rhizobial population declines when soil water content is low (Giller, 2001). Salt affects the rhizobial movement to the roots (Singleton & Bohlool, 1984), thus, hinders the initiation of fresh root nodules and reduces the efficacy of well-grown nodules (Rao *et al*., 2002). Symbiotic nitrogen fixation is also threatened by surplus salt (Rao *et al.,* 2002).

2.10 SIGNIFICANCE OF RHIZOBIA IN CROPPING SYSTEMS

Rhizobia are a group of the microorganisms that are capable for nitrogen fixation and benefits carbohydrates from the crop. Rhizobia belong to six genera namely *Bradyrhizobium Mesorhizobium Azorhizobium*, *Allorhizobium Sinorhizobium and Rhizobium* (Hardarson & Atkins, 2003). Positive results of symbiotic nitrogen and yield increases when suitable strains of rhizobia added to legumes (Gueye, 1990; Dadson *et al*., 1988). Rhizobia colonise the roots of legumes, which enables symbiotic nitrogen fixation to occur.

2.11 THE USE OF PLANT GROWTH PROMOTING RHIZOBACTERIA IN AGRICULTURE

Plant growth promoting rhizobacteria (PGPR) are environmentally friendly microorganisms used for improvements of soil fertility and yield (Kilian *et al*., 2000).They present an ability to improve nutrient uptake, supply sufficient phytohormones and provide superior resistance against phytopathogens that enhance plant growth (Kumar *et al.,*2012). The plant growth promoting rhizobacteria belong to *Acetobacter, Azospirillum, Azotobacter, Bacillus, Burkholderia, Klebsiella, Pseudomonas,* and *Serratia genera* (Glick, 1995; Jones *et al*., 2007). *Pseudomonas* and *Bacillus spp.* are the most known and utilized species amongst the PGPR group (Kang *et al*., 2015a).

Bacillus species are positive gram, ubiquitous in nature and used in production of medical, industrial and agriculture products (Lyngwi & Joshi, 2014). PGPR such *Bacillus spp.* are associated with roots along with increased biofilms to enhance vegetative development (Beauregard *et al*., 2013). Furthermore, *Bacillus spp.* are used as biofertilizers to enhance nutrient uptake from the rhizospheres and minimize pathogenic microbial developments (Kang *et al*., 2015b).

2.11.1 *BACILLUS* SPP. PROMOTE PLANT GROWTH

Studies have shown that incorporating *Bacillus spp*. as a biofertilizers in cropping systems improved plant biomass production (Ashraf *et al*., 2004; Barnawal *et al*., 2013; Radhakrishnan & Lee, 2016). In addition, the fruits quantity in tomato and cucumber were also improved by application of *Bacillus spp*. (Kilian *et al*., 2000; Dursun *et al*., 2010). *Bacillus spp*. converts important complex form nutrients, to simple obtainable form that are required through nutrient uptake (Kuan *et al*., 2016). *Bacillus spp.* synthesise plant hormones, such as IAA, gibberellins, cytokinin and spermidines, and enhance roots and shoots in rice seedlings (Xie *et al*., 2014).

2.11.2 EFFECTS OF *BACILLUS SPP*. INOCULATION IN DROUGHT PRONE AREAS

Drought occurs as a result of low annual precipitation resulting in low soil moisture and plant stress which leads to poor productivity and plant death. The utilisation of drought resistance PGPR to the soil enhanced population of microorganisms in rhizosphere and improved root and plant growth (Vardharajula *et al*., 2011). It has been found that roots occupied by PGPR effectively absorbed more water, which improved plant's mechanism

for defence against biotic and abiotic conditions (Marulanda *et al*., 2009). Furthermore, nutrient uptake like N, P and K was reported to decrease in plants exposed to drought compared to *Bacillus* spp. -treated plants which showed increased uptake of macronutrients (Barnawal *et al*., 2013). *Bacillus* spp. application enhanced chlorophyll synthesis and photosynthesis on plants subjected to drought (Hashem *et al*., 2015).

2.11.3 APPLICATION OF *BACILLUS* TO IMPROVE PLANTS HEALTH IN SALINE SOIL

Climate change has negatively affected rainfall patterns and distribution and lead to prolong periods of high temperature and inconsistent rainfall patterns. Consequently, agricultural soils have become saline and less fertile (Al-Karaki, 2006). Salinity affects germination rates and yield of crops (Radhakrishnan & Lee, 2014). Application of *Bacillus* spp. inoculant has been reported to improve plant growth during salt exposure (Hashem *et al*., 2016). Bacterial strains such as *Bacillus* minimized harmful results of salt in the soil by inhibiting lipids peroxidation in soybean (Han *et al*., 2014) and improving lipid synthesis in Indian bassia (*Bassia indica)* grown on saline soils (Hashem *et al*., 2015).

Most of the farming land is faced with polluted and outlined metals from industrialized runoffs as well as fine chemicals, which contains a harmful impact on crop production and microorganisms commune in the soil (Ashraf *et al*., 2017). Trace elements such as cadmium, manganese, and zinc are main contaminants in water and soil; these metals are hard to be transformed into non-hazardous material (Arthur *et al*., 2012). Microorganisms have the ability to transfer lethal metals to non-lethal forms, which is an appropriate administration of heavy metal phytoextraction (Kang *et al*., 2015c). In addition, bacterial immunization was found to enhance water uptake and decrease electrolyte escape to alleviate cadmium stress, thus improving plant growth (Ahmad *et al*., 2014). Plant growth promoting rhizobacteria were reported to promote nutrient uptake in the plants and prevented effects of larger amounts of cadmium which decreased the absorption of P, Fe, Zn and Mn (Malekzadeh *et al*., 2012).

2.11.4 *BACILLUS* INOCULANTS AS A BIOLOGICAL CONTROL FOR PESTS

Plant growth promoting rhizobacteria such as *B. amyloliquefaciens, B. cereus*, and *B. subtilis*, are amongst the bacterial pesticides utilised for pest control (Gadhave & Gange, 2016). In addition, *Bacillus* spp. as a pesticide agent in soil and roots sustain development

of the plant and enhance the acceptance and total transfer of pesticides right through the whole plant to manage pest infestation (Myresiotis *et al*., 2015).

2.11.5 ANTI-MICROBIAL EFFECTS OF *BACILLUS* SPP. IN PLANTS

Pathogens are a serious thread in crop production and have been controlled using synthetic chemicals which resulted in environmental pollution and toxicity (Narasimhan & Shivakumar, 2015). The use of PGPR as substitutes for synthetic fungicides, bactericides have been found to be efficient green ecological approach to managing numerous plant diseases (Egamberdieva *et al*., 2014). Plant growth promoting rhizobacteria colonize pathogenic bacteria growth in the soil or in tissues of the plant and minimizes the negative effects of pathogens in plants. *Bacillus* spp. was also found to destroy pathogenic bacteria inhabitants and decreases their occurrence in plants by formation of biofilms around root surface (Hinarejos *et al*., 2016).

2.12 INOCULATION OF LEGUMES WITH BOTH RHIZOBIUM STRAINS AND PLANT GROWTH PROMOTING RHIZOBACTERIA

PGPR play an important role in promoting plant growth through various mechanisms and result in improved plant physiology (Remans *et al*., 2008). Dey *et al.* (2004) reported that PGPR positively affects plant growth through nitrogen fixation, and its ability to enhance water and nutrients absorption.

The utilisation of commercial inoculants on legume plants have shown to improve yield (Tena *et al*., 2016). Inoculation of legumes is subjected to the availability of compatible rhizobia present in the soil and their efficiency (Kantar *et al*., 2010). Plant growth, biomass, grain yield and nitrogen fixation were increased when legumes were inoculated with effective rhizobial strains (Funga *et al*., 2016). Farmers in many parts of the world observed yield augmentation when inoculating legumes with rhizobial strains (Cigdem & Merih, 2008). The use of co-inoculation of rhizobial strains and PGPR, enormously improved yields on common bean compared to single rhizobia inoculation (Korir *et al.,* 2017). Stajkovic et al. (2011) reported that co-inoculation of bean with *Rhizobium* plus *Bacillus* strains Nji and *Rhizobium* plus *Bacillus* strains Bx positively influenced nodule number compared to single inoculation of *Rhizobium.* It has been shown that nodulation and plant biomass increased when legumes were subjected to *Bacillus* and *Bradyrhizobium* co-

inoculation (Medeot *et al.,* 2010). However, there were still limitations in yields and quality of crops at farm level of most indigenous and under-researched legume crops.

2.13 METABOLOMICS IN LEGUMES

Metabolomics is a fast-emergent technology and at current biometric authentication and metabolic profiling methods that are used, for assortment of present metabolites, numerous investigative methods entailing partition and findings are executed (Doerfler *et al.,* 2014). The partition methods of metabolomics in legumes were conducted using gas chromatography-time of flight mass spectrometry (GC-TOF-MS) and LC-Orbitrap-MS (Scherling *et al.,* 2010), capillary electrophoresis-time of flight mass spectrometry (CE-TOF-MS) (Sato *et al.,* 2013) and nuclear magnetic resonance (NMR) (Charlton *et al*., 2008).

2.14 IMPORTANCE OF PRIMARY AND SECONDARY METABOLITES IN LEGUMES

Primary metabolites are particles that play a major role in the development, growth and reproduction of plants; they contain amino acids, organic acids, carbohydrates, nucleotides and lipid; and secondary metabolites tiny particles attained from primary metabolites and they consist of flavonoids and polyphenols (Mechri *et a*l., 2015). Metabolites have crucial responsibility in plant protection and shield mechanism against biotic and abiotic stress (Akula & Ravishankar, 2011). In addition, Bambara groundnut and cowpea contain antioxidative capacity, therefore forage free radical ions and shield human beings against cancer sickness, as well as anti-inflammatory and antimicrobial properties (Nijveldt *et al*., 2001). Legumes such as cowpea can be eaten as green vegetables, sun dried or fermented (Wafula *et al*., 2016) and contain vitamins, macro and micro minerals, flavonoids, antioxidants, ß-carotene, fatty acids, amino acids, carbohydrates and dietary fibre (Goncalves *et al*., 2016).

CHAPTER 3: EFFECTS OF *BACILLUS SUBTILIS* (STRAIN BD233) ON GERMINATION OF BAMBARA GROUNDNUT SUBJECTED TO DIFFERENT TEMPERATURE REGIMES

Abstract

Bambara groundnut is one of the significant underutilized indigenous African crops grown primarily for human consumption However, at farm level, yields decline due to rising temperatures as result of climate change. Plant growth promoting rhizobacteria (PGPR) have been reported to mitigate heat stress effects on crop and improve yields. Therefore, the aim of this study was to assess the effects of *Bacillus subtilis* (BD233) strain on germination of Bambara groundnuts landraces under elevated temperatures. Three Bambara groundnut landraces (red, cream and brown) were inoculated with *Bacillus subtilis* (strain BD233) and incubated at three different temperature levels (24°C, 27°C and 35°C) for germination test. Generally, BD233 strain and temperature significantly improved germination and plumule length of Bambara groundnut landraces. At 24°C and 35°C, inoculation of brown Bambara groundnut landrace with *Bacillus subtilis* (BD233 strain) increased germination percentage (95.83%), germination index (7.90) and plumule length (5.42 cm). The lowest germination percentage and the lowest plumule length were observed when Bambara groundnut landraces were not inoculated with strain BD233 at different temperatures. This study, therefore, suggests that the inoculation of Bambara groundnut with *Bacillus subtilis* (strain BD233) has a potential to minimize the effect of high temperatures on seed germination and could thus, be recommended for yield improvement of this crop under global warming scenarios.

3.1 INTRODUCTION

Air temperatures were reported to increase significantly as a result of climate change (Hartmann *et al.,* 2013) and this abiotic factor has a straight consequence on growth and development of plants (Waraich *et al*., 2012). Consequently, yield loss and declined production on most crops were observed (Wheeler *et al.,* 2000). Studies have shown that high temperatures ranging from 30 to 34°C can have negative effects on plants depending on the species and area resulting in low yields (Porter *et al*., 2014). For example, reduced germination percentage, abnormal seedling and decreased plumule length were reported in grain yield legumes exposed to high temperature (Hasanuzzaman *et al.,* 2013). In

addition, studies reported that seed germination at elevated temperatures was reduced in leguminous crops such as mung bean (Piramila *et al.,* 2012).

Bambara groundnut is an indigenous African crop that has been underutilized for consumption by humans and animals and under-researched. In sub-Sahara Africa, the crop is cultivated as a cash crop for its grains and leaves by smaller holder farmers in rural communities (Anderman et al., 2014). However, its production at farmers fields is often affected by severely high temperatures which lead to low yield (Abudulai *et al*., 2017). Thus, this yield decline of this important African indigenous legume crop offers an opportunity for researcher to investigate ways to combat effects of severe heat through the use of environmentally friend biofertilizers (bacterial inoculant).

Positive gram species such as *Bacillus* are mainly found in the soil and have been successfully used within the agriculture industries. Additionally, *Bacillus* being one of the plant growth promoting rhizobacteria (PGPR) can be used as commercial inoculant to improve plant growth and yield (Choudhary, 2011). PGPR are known for production of plant hormones such as auxins, cytokinins, gibberellins and ethylene, which are noted to excite the growth of plants (Park *et al*., 2017). In addition, PGPR were stated to enhance plant growth by dropping various environmental stresses (Tiwari *et al*., 2016). Notably, *Bacillus* spp. produces hormones that regulate the level of phytohormones in plants (Kundan *et al.,* 2015).

Future climate changes are associated with significant increases in air temperatures (Hartmann *et al.,* 2013). Heat stresses can affect seed germination in particular plant growth and development in general. For example, elevated temperatures (>35^{o}C) can lead to production of reactive oxygen species (ROS) that are harmful to seed germination of most important economic crops (Pukacka & Ratajczak, 2005). During seed germination, high temperatures can enhance ROS production, which can affect cell wall formation and radical elongation (Bailly, 2004; Müller *et al*., 2009) and have an effect on germination and development of important crops thus causing threats to future well-balanced diets and food security especially in developing areas. Despite the significance of *Bacillus spp.* in seed germination and of yields on crops under various adverse environmental conditions (Paredes-Páliz *et al.*, 2016), there is lack of literature on the effects of *Bacillus subtilis* (BD233) on Bambara groundnut landraces under different temperatures regimes.

Therefore, the objective of this study was to evaluate the effect of *Bacillus subtilis* (BD233 strain) on seed germination of different Bambara groundnut landraces subjected to different temperature regimes.

3.2 MATERIALS AND METHODS

3.2.1 EXPERIMENTAL DESIGN AND INOCULUM

Seeds of three Bambara groundnut landraces (red, brown and cream) were bought from a local market at Pretoria were used to conduct a germination trial. A total of 145 seeds per landrace were selected and sterilized using 3.5% of sodium hypochlorite for 30 minutes, followed by 70% ethanol and rinsed 4 times with sterile distilled water and soaked overnight with Mili-Q water. Lysogeny broth was used to grow the bacterial strain (*Bacillus subtilis* BD233) overnight at 37°C and sterile distilled water was used to adjust the culture to 0.5 McFarland Standards before inoculation.

The study consisted of two treatments, seeds inoculated with 2ml of *Bacillus subtilis* BD233 strain (B+) and seeds without BD233 (B-). Both treatments were incubated in growth chambers with 3 different temperatures (24, 27 and 35°C) for a period of 14 days. Whatman No.1 filter paper disc and 9 cm petri dishes were used throughout the experiment. The moisture content of the filter paper disc was maintained throughout the experiment. The number of germinated seeds was recorded at 2 days interval for a period of two weeks and thereafter, the plumule length was measured r using vernier caliper (Model DC-515A). Germination percentage (G%), germination index (GI) and germination rate index (GRI) were calculated from germination data according to Olisa *et al.* (2010) as follow:

$$G\% = \frac{\text{No of emerged seedling at final count}}{\text{total number of seeds planted}} \times 100$$

- Where G is the germination percentage.

$$GI = \frac{\Sigma(\text{Nx})(\text{DAS})}{\text{total number of seedlings that emerged at final count}}$$

- Where GI is the germination index, *Nx* is the number of seedlings that emerged on the days after planting and the DAS is the days after planting.

$$GRI = \frac{\mathrm{GI}}{G\%}$$

- Where GRI is the germination rate index.

3.4 STATISTICAL ANALYSIS

The collected data were subjected to three-way analysis analysis of variance (ANOVA) using the STATISTICA software version 10 (StatSoft Inc., Tulsa, OK, USA). Treatment means were separated using Duncan's multiple range test at $p<0.05$.

3.5 RESULTS

Table 3.1: Effects of *Bacillus subtilis* inoculation and temperature on plumule length and germination indices of Bambara groundnut landrace incubated at different temperature regimes.

	Growth parameter and germination Indices			
Landrace	**Plumule Length (cm)**	**Germination %**	**Germination index**	**Germination rate index**
Cream	1.33±0.12b	47.90±4.66c	4.63±0.64b	0.08±0.01a
Red	2.39±0.27a	63.19±5.30b	5.61±0.47ab	0.09±0.01a
Brown	2.19±0.29a	77.08±4.77a	6.36±0.36a	0.08±0.01a
Inoculation				
B+	2.56±0.24a	73.14±3.82a	6.79±0.27a	0.09±0.01a
B-	1.38±0.09b	52.31±4.43b	4.28±0.42b	0.08±0.01a
Temperature				
24°C	2.06±0.21b	65.97±5.42a	5.83±0.51a	0.09±0.01a
27°C	1.12±0.07c	57.64±6.16a	5.04±0.64a	0.07±0.01a
35 °C	2.72±0.31a	64.57±5.28a	5.73±0.39a	0.09±0.01a
F-Statistics				
Landrace (L)	39.5**	10.6**	5.71**	0.13^{ns}
Inoculation (IN)	80.4**	16.2^{ns}	35.60^{ns}	1.85^{ns}
Temperature (T)	129.7***	1.0**	1.38^{ns}	1.74^{ns}
L x IN x T	6.5*	0.9^{ns}	2.46^{ns}	0.47^{ns}

Values (M±S.E.) followed by followed by similar letters in a column are not significantly different at p≤0.05 and ns= not significant

Table 3.2: Interactive effects of *Bacillus subtilis* inoculation and temperature on plumule length and germination indices of Bambara landraces incubated under different temperature regimes.

Landrace	Inoculation	Temperature	Growth parameter and germination indices			
			Plumule Length (cm)	Germination %	Germination index	Germination rate index
Cream	**B+**	24°C	1.82±0.40cde	62.50±7.22bcde	6.30±1.46abc	0.10±0.02a
		27°C	0.70±0.09g	45.83±4.17efg	6.22±0.78abc	0.08±0.04b
		35°C	1.94±0.10cd	54.07±4.07cdefg	6.30±1.46abc	0.11±0.02a
Cream	**B-**	24°C	1.00±0.07fg	37.50±7.22fg	2.33±1.17de	0.06±0.03abc
		27°C	1.10±0.27fg	29.17±11.02g	1.17±1.17de	0.02±0.02c
		35°C	1.40±0.29def	58.33±20.83cdefg	5.44±0.78abc	0.10±0.02a
Red	**B+**	24°C	4.10±0.40b	70.83±15.02abcde	7.47±0.47a	0.11±0.02a
		27°C	1.28±0.09efg	75.00±7.22abcde	7.13±0.70ab	0.09±0.02ab
		35°C	4.32±0.38b	79.17±8.33abcde	5.87±0.13abc	0.07±0.01ab
Red	**B-**	24°C	2.32±0.18c	58.33±15.02cdefg	5.89±0.68abc	0.11±0.02a
		27°C	1.18±0.0fg	45.83±18.16efg	2.72±1.40de	0.04±0.02bc
		35°C	1.12±0.06fg	50.00±7.22efg	4.59±0.6bcd	0.09±0.02a
Brown	**B+**	24°C	1.98±0.25cd	95.83±4.17a	6.75±0.80abc	0.07±0.01ab
		27°C	1.46±0.09def	83.33±11.02abcd	7.13±0.70ab	0.09±0.02a
		35°C	5.42±0.19a	91.67±4.17ab	7.90±0.49a	0.08±0.00bc
Brown	**B-**	24°C	1.16±0.14fg	70.83±4.17abcde	6.22±0.78abc	0.09±0.01a
		27°C	1.00±0.18fg	66.67±15.02abcdef	5.89±0.68abc	0.09±0.02a
		35°C	2.12±0.12c	54.17±11.02cdefg	4.27±0.39cd	0.08±0.02b

Values (M±S.E.) followed by similar letters in a column are not significantly different at p≤0.05. B+ (inoculation with *B*. *subtilis*), B- (not inoculated with *B*. *subtilis*) and incubated under different temperature regimes.

The plumule length and germination indices of the tested Bambara groundnut landraces differed significantly in response to *Bacillus subtilis* inoculation and different temperatures. Among the landraces red landrace had the highest plumule length (2.39 cm), followed by brown (2.19 cm) and the least was in cream (1.33 cm) (Table 3.1). The result showed that brown had higher germination percentage of (77.08 %), germination index (6.36), followed by red (64.19 %) and the least was in cream (47.90 %).

According to Table 3.1, the results showed that bacterial inoculation notably affected Bambara groundnut plumule length, germination per cent and germination. Seeds inoculated with *B*acillus *subtilis* (BD233) had higher plumule length, germination percentage and germination index in contrast with those not inoculated with the bacteria. The interactive effects of *Bacillus subtilis* and temperature showed significant differences at ($p<0.05$) among Bambara landraces in plumule length (Table 3.2). Inoculation with *Bacillus subtilis* (strain BD233) enhanced plumule length under all temperature regimes compared to uninoculated seeds. plumule length was highest in brown landrace with 5.42 cm at 35°C, followed by red landrace with the plumule length of 4.32 cm, after inoculation with *Bacillus subtilis* strain (BD233). At 24°C, the red landrace recorded the highest plumule length of 4.10 cm while the cream landrace yielded the lowest plumule length, when *Bacillus subtilis* was added. The cream landrace yielded the lowest plumule length in all different temperature levels compared to the red and brown landrace after inoculation. The uninoculated cream landrace exhibited the lowest plumule length of 1.00 cm at 24°C.

Landraces assessed differed significantly in the observed germination indices under different temperature regimes and inoculation. The highest germination percentage (95.83%) was exhibited by inoculated brown landrace at 24°C, while the lowest germination (29.17%) was observed in the cream landrace at 27°C. The overall observations on germination test revealed that the cream landrace with or without inoculation performed poorly compared to brown and red landraces (inoculated or not inoculated) under all temperature regimes.

3.6 DISCUSSIONS

Heat stress as a result of high temperatures causes harsh agriculture losses, which poses a threat to food security (Masipa, 2017). Seed quality is an indication of the seed value in agriculture and includes factors such as germinability and vigour (Hampton, 2002). In

addition, seed quality can be negatively affected by environmental conditions throughout seed development (Hampton *et al*., 2013). In this study, inoculation with *Bacillus subtilis* (strain BD223) significantly enhanced plumule length of two of the three Bambara landraces subjected to different temperature regimes. These findings could be attributed to ability of *Bacillus subtilis* to increase synthesis of hormones plant growth regulators such as auxins, gibberellins, and cytokinins (Bottini *et al*., 2004; Bloemberg & Lugtenberg, 2001). Erturk (2010) reported that the plumule length was increased by 40.00 to 47.50% when inoculation of *Bacillus* RC03 and *Bacillus complex* RC19 was applied to kiwi fruit. Nevhulaudzi et al. (2017) reported that inoculation with *Bacillus subtilis* (strain BD223) significantly enhanced plumule length and germination indices of cowpea.

Both brown and red Bambara landraces performed better than cream landrace in terms of all the recorded parameters, this is consistent with findings by Zulu & Modi (2010) who reported that seed colour was associated with seed quality. These findings may be due to associated tannins and polyphenols present in dark-coloured seeds (Mabhaudhi & Modi, 2013). Polyphenols have antioxidant properties and provide the plants with a defence mechanism against stress, consequently promoting plant tolerance to harsh conditions (Chibarabada, 2014).

Germination indices which determine the rate at which the plants germinate was also evaluated amongst the treatments and the brown and red landraces performed better than cream in all germination indices parameters. This could also be a result of the genetic variations among the landraces, which were reported to lead to differences among overall performance of landraces with dark coloured being more vigorous than coloured seeds (Mabhaudhi, 2012). Inoculation of Bambara groundnuts with *Bacillus subtilis* (strain BD233) improved germination under all temperatures and in all landraces. These findings are consistent with previous studies on crops such as rice (Ashrafuzzaman *et al*., 2009), soybean (Naz *et al*., 2009), chickpea (Mahejibin & Patel, 2007; Mishra *et al*., 2010) and Bambara groundnut (Sinefu, 2011). Moreover, germination index in this study did not differ significantly among the different landraces under different temperature regimes, a finding similar to Sinefu (2011) who reported no significant differences among the red, brown and cream landraces.

In general, inoculation of *Bacillus subtilis* (strain BD233) on Bambara groundnut seeds improved growth parameter (plumule length) and enhanced all germination indices. These

findings could be due to increased plant hormones such as auxins, cytokinins, gibberellins and ethylene that were stimulated by inoculation with PGPR (Park *et al*., 2017). Plant growth promoting rhizobacteria were reported to enhance plant growth by reducing numerous environmental stresses (Tiwari *et al*., 2016), thus, this study is consistent with these previous findings. *Bacillus* produces plant hormones thereby modulating the entire plant's hormonal balance and its reaction to stress by regulating endogenous level of phytohormones in plants (Kundan *et al.,* 2015).

3.7 CONCLUSION

This study revealed that germination percentage and plumule length were highly influenced by temperature, landrace and inoculation with *Bacillus subtils* (strain BD233). Inoculation with *Bacillus subtils* (strain BD233) Bambara seeds enhanced plumule length and germination percentage under high temperature, indicating that strain BD233 has a potential to mitigate high temperature effects and improve crop yield under future climate change scenarios.

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CHAPTER 4: EFFECTS OF CO-INOCULATION OF *B. JAPONICUM* AND *BACILLUS SUBTILIS* ON YIELD, CHLOROPHYLL CONTENT AND STOMALTAL CONDUCTANCE OF COWPEA GROWN UNDER GLASSHOUSE CONDITIONS

Abstract

Cowpea (*Vigna unguiculate* L. Walp) plays an important role in everyday nutritional intake of rural communities, supplementing as a basis of energy, protein and minerals, in emerging nations. However, improved yield for cowpea is one of the main concerns in agriculture. Improved yield of various crops due to co-inoculation with rhizobia and some strains of *Bacillus* have been reported but not for cowpea. Therefore, this study evaluated effects of co-inoculation of *B. japonicum* and *Bacillus subtilis* on yield of cowpea under glasshouse conditions. A glasshouse experiment was carried out using a randomized complete block design with four treatments: co-inoculation with *B. japonicum* and *Bacillus subtilis* (strain BD233), inoculation with *B. japonicum* only, zero inoculation +NO_3 and zero inoculation, with each treatment replicated twelve times. Co-inoculation of *B. japonicum* and *Bacillus* recorded the highest plant height, number of leaves, chlorophyll content, stomata conductance, nodulation, seed and pod number, seed weight, and plant biomass compared to other treatments throughout the study. This study suggests that co-inoculation of *B. japonicum* and *Bacillus subtilis* (strain BD233) increase yield of cowpea and could be an ideal biofertilizers.

4.1 INTRODUCTION

Soil nutrient depletion has resulted in huge financial losses of about 18% in agricultural arable land (Nkonya *et al*., 2011). Khosro & Yousef (2012) reported that soil fertility is amongst the major limiting factor that leads to reduction of crop yields, especially in these small holder farming systems. Thus, incorporating practices minimizing soil degradation, enhancing soil fertility and improving yields is of paramount importance. Agricultural land continues to be contaminated by toxic trace metals which have notably become a serious global challenge (Gratão *et al*., 2015). The main contributors of soil contaminations are use of synthetic fertilisers, mining and chemical processing industries (Chen *et al*., 2018). Application of synthetic fertiliser has been practised for decades to improve soil nutrition and plant growth, but their continuous use has deteriorated soil health status, contaminated the environment through air, soil and water pollution. Accumulation of toxic

trace metals in soil are hard to be eradicated from the plant soil structures and in addition, the build-up in plant tissue has an undesirable effect in plant growth (Wang *et al*., 2017). Thus, the usage of environmentally friendly biofertilizers has appeared to gain more popularity in sustainable agricultural practices (Choudhary, 2011).

Several studies have reported that incorporating N_2-fixing legumes such as cowpea, Bambara groundnut and soybean into cropping systems is an inexpensive and durable method of tapping atmospheric N_2 for improved crop yields (Kyei-Boahen *et al*., 2017). Leguminous plants are able to establish a symbiotic relationship with bacteria belonging to genera *Rhizobium, Bradyrhizobium, Sinorhizobium, Azorhizobium, Mesorhizobium, and Allorhizobium* (Sawada *et al*., 2003; Bottomley, 2007), and through a biological nitrogen fixation process, these crops contributed significant amounts of N to the soil (Mohale *et al*., 2014). Thus, replenishing soil fertility and improving crop production (Roychowdhury *et al*., 2013). However, the yields of legumes remain low due to lack of effective rhizobia in the soil, therefore, inoculating legumes with compatible rhizobial strains is crucial for enhanced yield, improved nodulation and nutrient uptake (Tena *et al*., 2016; Kyei-Boahen *et al*., 2017).

PGPR are rhizosphere-inhabiting bacteria that affect positively plant growth and development through bacterial production of plant growth regulators such as auxins, gibberellins, and cytokinins (Bottini *et al*., 2004; Bloemberg & Lugtenberg, 2001). Most of *Bacillus* that colonizes plant roots has been reported to have growth-promoting assets (Idris *et al*., 2007). Furthermore, inoculation of plants with microbial inoculants such as PGPR can improve plant growth directly through tolerance to harsh biotic stress by regulating root improvement, advance soil microbial growth and increase nutrient accessibility (Ma *et al*., 2016; Bhattacharyya & Jha, 2012). Combination of plant growth promoting rhizobia with rhizobium enhanced nodulation, yield and growth compared to single inoculation in pea (Schwartz *et al*., 2013), and soybean (Dashti *et al*., 1998; Araujo *et al*., 2009; Patra *et al.,*2012). However, there is lack of information on the effects of co-inoculation of *B. japonicum* and *B. subtilis* on the nodulation and yield of cowpea. Hence, this study's objective was to examine effects of co-inoculation of *B. japonicum* and *B. Subtilis* (strain BD233) on yield of cowpea under glasshouse conditions.

4.2 MATERIAL AND METHODS

4.2.1 SITE DESCRIPTION

The glasshouse which is located at the Horticultural center, Florida campus, UNISA (Johannesburg, South Africa) hosted the study. Latitude: S26 °9.501 and Longitude: E27 °54.113, during the 2018 summer season. The site has a humid subtropical climate (warm, frequently moist summers and mild to cool winters).

4.2.2 SOURCE OF COWPEA CULTIVAR

Seeds of cowpea (Nyira cultivar) were collected from ARC Agricultural Research Council, Plant Protection Research Institute, Roodeplaat, South Africa.

4.2.3 SEEDS STERILIZATION AND PLANT NUTRITION

Seeds of cowpea were sterilized using sterilized using 3.5% of sodium hypochlorite for 30 minutes, followed by 70% ethanol and rinsed 4 times with sterile distilled water and soaked overnight with Mili-Q water. Lysogeny broth was used to grow the bacterial strain (*Bacillus subtilis* BD233) overnight at 37°C and sterile distilled water was used to adjust the culture to 0.5 McFarland Standards before inoculation. Planting pots were sterilized by spraying 70% of ethanol before planting. Tap water was used to wash out nutrients from the Acid-washed sand and micro-waved at 70°C for 72 h. The plants were irrigated twice a week using distilled water and a standard nutrient solution of 10ml per pot containing N-free nutrient.

Table 4.1: N-free nutrient solution (Broughton and Dilworth, 1971).

Stock Solution	Form	Element	MV	Chemical g/L	M
1	CaCl2.2H2O	Ca	147.03	294.0	2
2	KH2PO4	P	136.09	136.1	1
3	Fe C6H5O7. 3H2O	Fe	355.04	6.700	0.02
	MgSO4. 7H2O	Mg	246.5	123.3	0.5
	K2SO4. H2O	K	174.06	87.00	0.5
	MnSO4. H2O	Mn	169.02	0.338	0.002
4	H3BO3	B	61.84	0.247	0.004
	ZnSO4. 7H2O	Zn	287.56	0.228	0.001
	CuSO4. 5H2O	Cu	249.69	0.100	0.0004
	CoSO4. 7H2O	Co	281.12	0.056	0.0002
	Na2MoO2. 2H2O	Mo	241.98	0.048	0.0002

After preparing the stock solutions, 10 L of nitrogen-free nutrient solution was prepared by mixing 5 mL of each stock solution with 5 L of distilled water in a container and further diluted to 10 L by adding another 5 L of distilled water (Table 4.1). The pH of the solution was adjusted to 6.8 with NaOH or HCl.

4.2.4 EXPERIMENTAL DESIGN

The experiment was conducted using a randomized complete block design (RCBD) with 12 replicates for each treatment. Sterilized forceps were used to select healthy seeds and one seed was planted per 15 cm pot containing sterilized acid-washed sand. Live legume bacteria for cowpea group (*Bradyrhizobium japonicum*) 6.5×10^8 viable cells/g were used as inoculant. The experimental treatments included i) *B. japonicum* inoculation, ii) *B. japonicum* and *Bacillus subtilis* (strain BD233) co-inoculation, iii) zero inoculation $+NO_3$ and iv) zero inoculation.

4.2.5 INOCULUM PREPARATION

Lysogeny broth was used to grow the *Bacillus* bacterial strains (strain BD233) overnight at 37°C. The cultures were adjusted with distilled water to 0.5 McFarland Standards. About 500 g of *Bradyrhizobium japonicum* was added into 1800 mL of sterile distilled water and then 2 mL of *Bradyrhizobium japonicum* inoculants and 2 mL of *Bacillus subtilis* and *Bradyrhizobium japonicum* was inoculated to the seeds at planting. Nitrogen was supplied in the form of 0.5 mM ammonium nitrate (NO_3 treatment).

4.3 DATA COLLECTION

Twelve cowpea plants were harvested at 100 days of planting (DAP). Yield parameters (plant fresh and dry weight, plant height, and grain yield) and nodulation were determined. The plant height and number were recorded at 30, 44, 58 and 72 days after planting. The stem diameter was also measured using a digital vernier calliper (Model DC-515), root fresh and dried weight were determined. stomatal conductance was determined using a porometer (SC–1 Leaf Porometer, Pullman, USA) at 80 DAP and Chlorophyll content was measured using a handheld chlorophyll meter (Opti-Sciences model CCM-300, Hudson, USA).

4.4 STATISTICAL ANALYSIS

The collected data were subjected to one-way analysis of variance (ANOVA) using the STATISTICA software version 10 (StatSoft Inc., Tulsa, OK, USA). Treatment means were separated using Duncan's multiple range test at $p<0.05$.

4.5 RESULTS AND DISCUSIONS

Table 4.2: Effects of co-inoculation on whole plant fresh weight (WPFW), whole plant dried weight (WPDW), root fresh weight (RFW), root dried weight (RDW), stem diameter (SD), Number of pods/plant (NP/P), Number of seeds/plant (NS/P), seeds weight/plant, nodules/plant (N/P) and nodule fresh weight/plant

	Yield								**Nodulation**	
Treatment	WPFW (g)	WPDW (g)	RFW(g)	RDW (g)	SD (cm)	NP/P	NS/P	SW/P (g)	N/P	NFW(g)
Bacillus+Brady	175.18±9.33a	19.13±0.64a	51.10±3.44a	12.46±0.40a	8.32±0.47a	7.90±0.67a	81.30±8.38a	10.46±0.89a	8.90±1.03a	1.47±0.12a
Brady	165.38±8.03b	18.85±0.40a	44.56±1.97b	11.49±0.32b	7.46±0.41a	6.10±0.53a	63.60±5.41b	8.05±0.39a	6.30±0.78b	1.42±0.11a
Zero inoculation +NO_3	127.33±5.46c	16.97±0.34b	40.57±0.88c	11.29±0.29bc	5.44±0.27b	5.70±0.68ab	49.50±6.15c	5.6±0.71b	0.00±0.00c	0.00±0.00b
Zero inoculation	61.23±4.88d	12.38±0.59c	25.93±1.82d	9.73±0.46d	4.58±0.33b	3.40±0.34b	36.80±2.89d	4.65±0.40b	0.00±0.00c	0.00±0.00b

Values (M±S.E.) followed by dissimilar letters (in lower case) within a column for each treatment are significantly different at $p<0.05$.

4.5.1 Effects of co-inoculation on whole plant fresh (WPFW) and dry weight (WPDW), root fresh (RFW) and dry weight (RDW), stem diameter (SD), number of pods/plant (NP/P), number of seeds/plant (NS/P), seeds weight/plant (SW/P), nodules/plant (N/P) and nodule fresh weight (NFW) of cowpea grown under glasshouse conditions

Co-inoculation of cowpea with *B. japonicum* and *Bacillus subtilis* (strain BD233) significantly increased whole plant fresh weight (175.18 g), followed by inoculation *B. japonicum* (165.38 g), and NO_3 (127.33 g) and the least was the uninoculated cowpea (61.23g) (Table 4.2). Inoculation with plant growth promoting rhizobacteria slightly improved whole plant dried weight compared to other treatments. Dashti *et al*. (1998) and Patra *et al.* (2012) demonstrated that dual inoculation of rhizobial strain *and Bacillus* spp. increased soybean dry matter yield than single strain inoculation. When cowpea was co-inoculated with *B. japonicum* and *Bacillus subtilis* (strain BD233), the root dry matter was increased significantly compared to other treatments. Similar findings were reported in common bean (*Phaseolus vulgaris*) co-inoculated with rhizobial strain IITA-PAU 987 and *B. megaterium* that yielded high root dry weight (Korir *et al*., 2017). In this study, the inoculation of cowpea with both *B. japonicum* and *Bacillus subtilis* (strain BD233) noticeably increased the stem diameter (8.32 mm), followed by inoculation of *B. japonicum* (7.46 mm), and NO_3 (5.44 mm), and the lowest was the no inoculation treatments (4.58 mm).

Co-inoculation with *B. japonicum* and *Bacillus subtilis* (strain BD233) significantly enhanced grain yield in cowpea. For example, number of pods per plant was considerably increased with co-inoculation of *B. japonicum* and *B. subtilis* (strain BD233) than *B. japonicum* alone, NO_3 and no inoculation (Table 4.2). Co-inoculation of *B. japonicum* and *B. Subtilis* (strain BD233) enhanced seed number per plant compared to other treatments. Seed weight significantly differed amongst the treatments; with co-inoculation of *B. japonicum* and *B. Subtilis* (strain BD233) recording the highest while the lowest was observed on the uninoculated cowpea. Similar trends were observed when co-inoculation of *Rhizobium* and *Pseudomonas* and *Rhizobium* and *Bacillus* enhanced pod and seed number (Mathivanan *et al*. 2014). In common bean, the co-inoculation of rhizobial strain Rb133 + *Pseudomonas* strain increased pod, seed number and seed weight (Yadegari & Rahmani, 2008).

Cowpea nodulation was enhanced by co-inoculation of *B. japonicum* and *Bacillus subtilis* than inoculation of *B. japonicum* alone. Nodule number was high on the co-inoculated, followed by inoculation of *B. japonicum* alone, and the no nodules recorded on no inoculation + NO_3 and zero inoculation cowpea (Table 4.2). In terms of nodule fresh weight, there were no significant difference between the co-inoculation of *B. japonicum* and *Bacillus subtilis and* single inoculation of *B. japonicum.* Co-inoculation of *B. japonicum* and *Bacillus subtilis* recorded the highest nodule fresh weight (1.47 g), followed by inoculation with *B. japonicum* (1.42 g), and NO_3 (0.00 g) and the least was uninoculated cowpea (0.00 g). This finding is consistent with a study by Stajkovic et al. (2011) who reported that dual inoculation with *Rhizobium* and *Bacillus* strains increased nodule number per plant compared to inoculation with *Rhizobium* alone. Additionally, nodulation was improved when *Bacillus meagterium* and rhizobial strain IITA-PAU 987, and *B. megaterium* and rhizobial strain CIAT 899 were co-inoculated in common bean (Korir *et al*., 2017). All these findings are consistent with our study, indicating that co-inoculation has a potential to improve cowpea yield and nodulation.

Bacillus spp. has the ability to convert critical nutrients that are required in the process absorption by root (Kuan *et al*., 2016). *Bacillus spp.* synthesises plant-growth-promoting hormones, such as IAA, gibberellins, cytokinins and spermidines, and enhance root and shoot elongation and cellular division (Xie *et al*., 2014; Radhakrishnan & Lee, 2016). Furthermore, *Bacillus spp*. produces ACC deaminase (EC 4.1.99.4) that prevents ethylene production in plants and excites plant growth.

4.5.2 Effects of *B. japonicum* and *Bacillus subitilis* on growth of cowpea under glasshouse

Co-inoculation of *B. japonicum* and *B. subtilis* (strain BD233) significantly improved the plant height at 30, 44, 58 and 72 days after planting (Figure 4.1). Co-inoculation of *B. japonicum* and *B. subtilis* (strain BD233) resulted in a plant height of 20.60 cm, followed by inoculation with *B. japonicum,* which yielded 18.10 cm while NO_3 treated uninoculated cowpea plants recorded 18.30 cm and the uninoculated negative control exhibited 17.70 cm at 30 days after planting. At 44 days after co-inoculation resulted in 27.1 cm followed inoculation of *B. japonicum* 25.4 cm. Generally, plant height did not differ for NO_3 application and the negative control (zero inoculation) (Figure 4.1). Similar results were observed when co-inoculation of *Bacillus subtilis* and *B. japonicum* drastically increased

plant height of soybean (Tilak *et al.,* 2006). In addition, Mathivanan et al. (2014) reported the effects of co-inoculation of plant growth promoting rhizobacteria (*Bacillus spp*) with rhizobia (*Rhizobium*) on plant growth improvement.

At 30, 44, 58 and 72 days after planting, the amount of leaves per plant was expressively improved by co-inoculation of *B. japonicum* and *B. subtilis* (strain BD233), single inoculation with *B. japonicum* and other treatments (NO_3 and negative control). Overall, at all growth stages, leaf number was highest on the *B. japonicum* and *B. subtilis* (strain BD233) co-inoculated cowpea and lowest on the uninoculated plants (Figure 4.2). Similar trends were also stated by Iličić *et al*. (2017) on soybean co-inoculated with *B. japonicum* 526 and *Bacillus sp. Q*10.

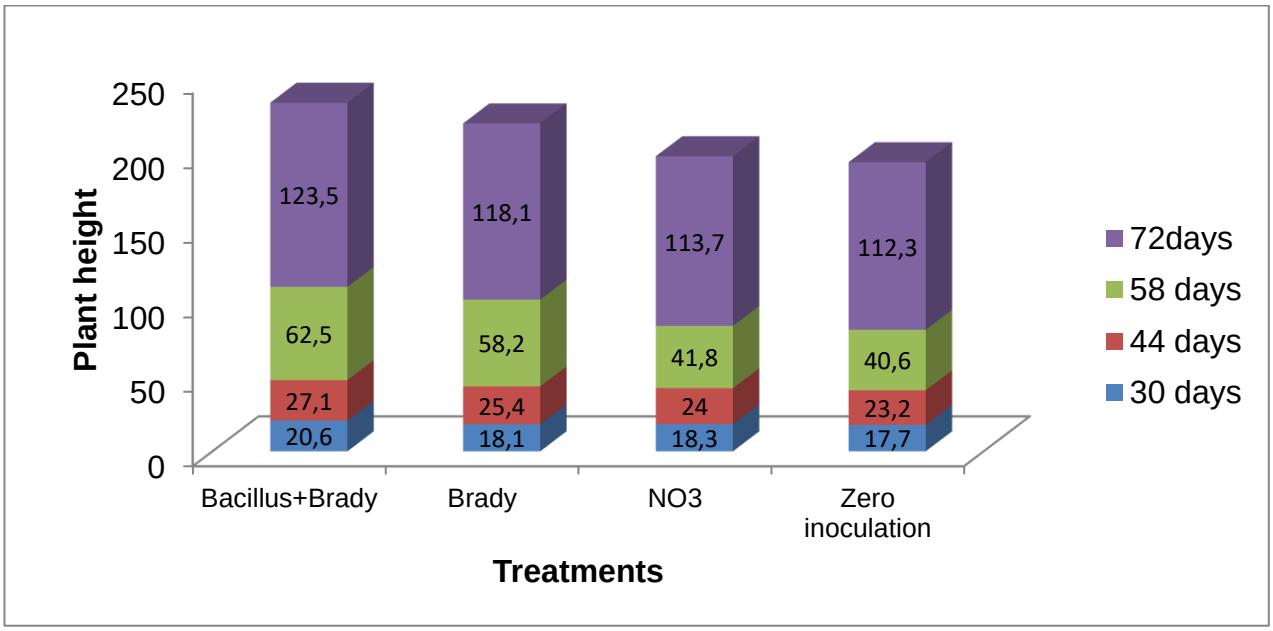

Figure 4.1: Effects of co-inoculation of *B. japonicum* + *Bacillus subtils* on plant height (cm) at 30, 44, 58, and 72 days after planting.

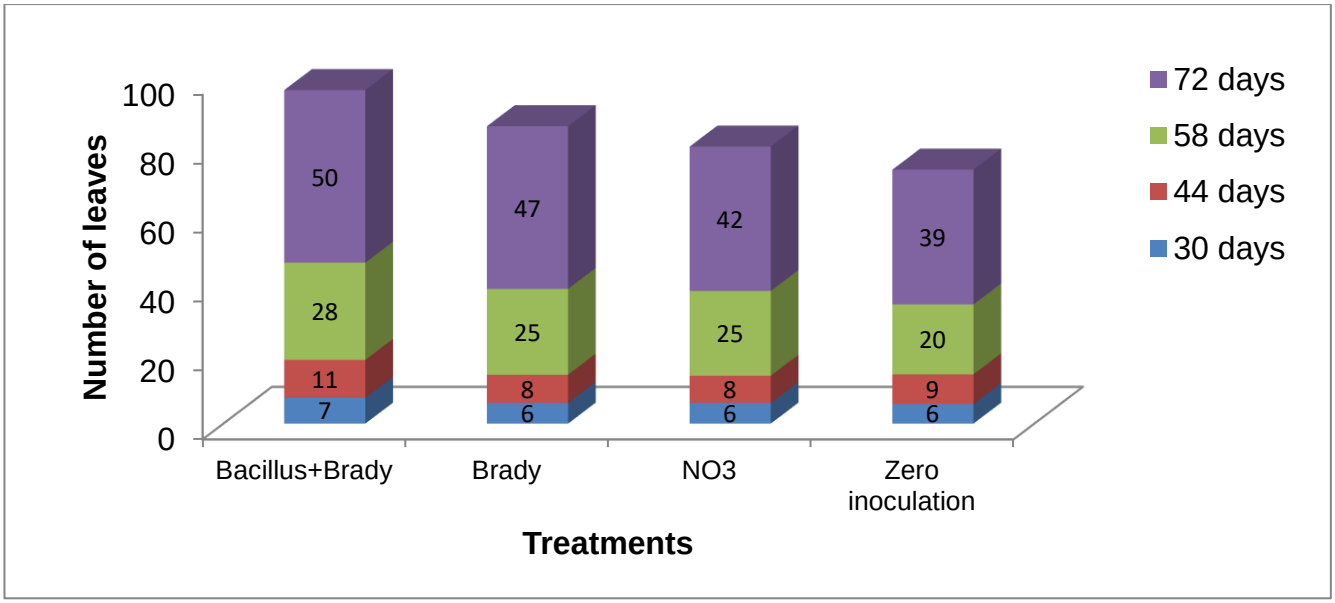

Figure 4.2: Effects of co-inoculation of *B. japonicum* + *Bacillus subtilis* on number of leaves at 30, 44, 58 and 72 days after planting.

4.5.3 Effects of *B. japonicum* and *Bacillus subtilis* on chlorophyll content and stomatal conductance of cowpea grown under glasshouse condition

Chlorophyll content was remarkably increased by co-inoculation of *B. japonicum* and *B. subtilis* (strain BD233) increased than the other treatments (Figure 4.3). This could be due to PGPR which is reported to enhance accumulation of chlorophyll through process synthesis of plant-growth-promoting hormones (Radhakrishnan & Lee, 2016). Similar findings were observed by (Aseri *et al*., 2008) who reported that the highest total chlorophyll was observed in dual inoculated plants of *Punica granatum* with *G. mosseae* and *A. brasilense* after 4 months. Stomatal conductance was significantly increased when co-inoculation of *Bacillus subtilis* and *B. japonicum* was applied compared to the other treatments (Figure 4.4). Positive strains are reported to enhance plant physiology through providing promotive enzymes and metabolites (Ali *et al*., 2009) augmenting nutrient absorption from plant rhizosphere soils (Schwartz *et al*., 2013).

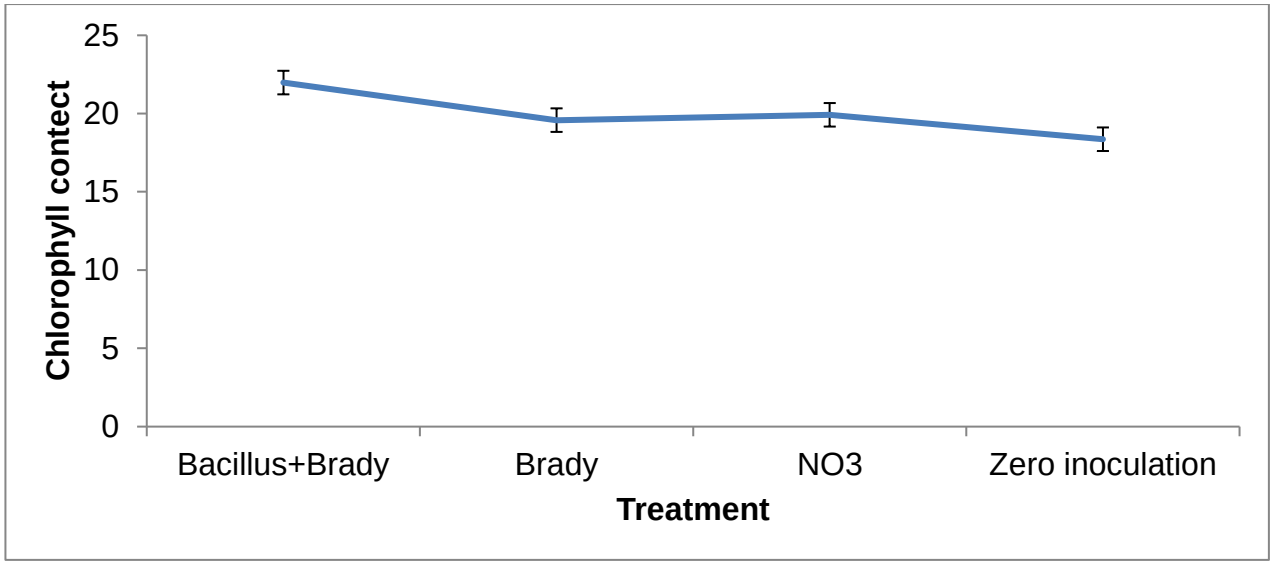

Figure 4.3: Effects of co-inoculation of *B. japonicum* + *Bacillus subtilis* on cowpea leaf chlorophyll content at 80 days after planting.

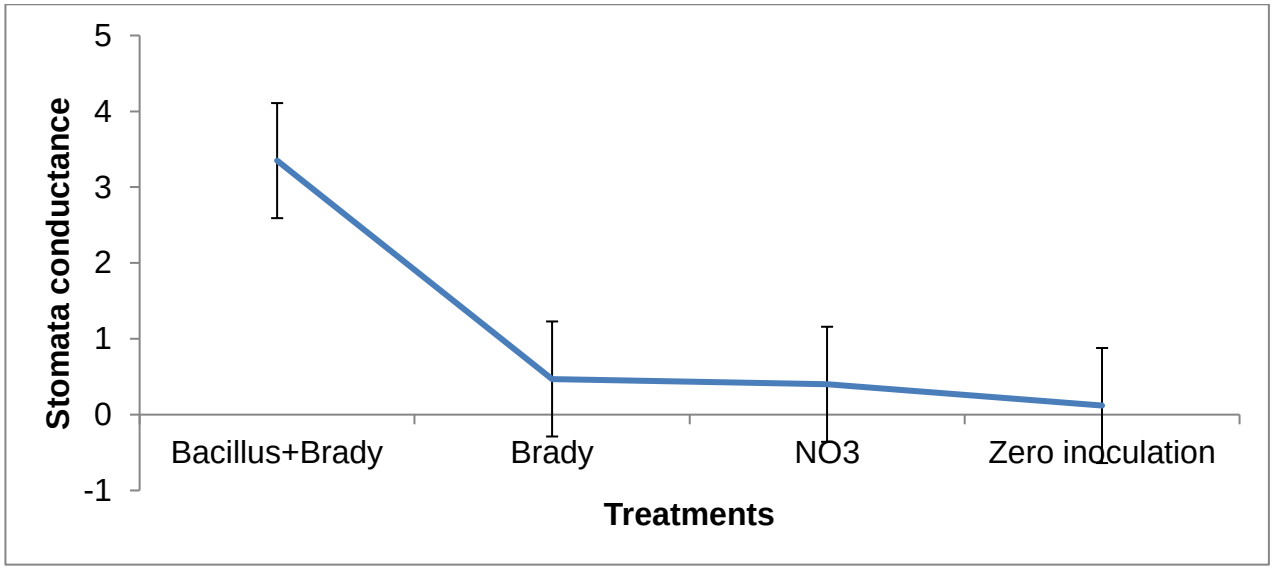

Figure 4.4: Effects of co-inoculation of *B. japonicum* + *Bacillus subtilis* on stomata conductance at 80 days after planting.

4.6 CONCLUSION

This study revealed that co-inoculation with *B. japonicum* and *B. subtilis* (strain BD233) increased growth (number of leaves, plant height, and stem diameter), nodulation (nodules

numbers and weight) and yield (seed and pod number, and seed weight), chlorophyll content, and stomatal conductance of cowpea grown under glasshouse conditions. The utilisation of PGPR and rhizobial inoculants as bio-fertilisers has positive impact on yield of cowpea for sustainable agriculture and food security especially in rural communities.

CHAPTER 5: EFFECTS OF CO-INOCULATION OF *B. JAPONICUM* AND *BACILLUS SUBTILIS* ON METABOLITES OF BAMBARA GROUNDNUT AND COWPEA GROWN UNDER GLASSHOUSE CONDITION

Abstract

Co-inoculation of *B. japonicum* and *Bacillus subtilis* has been reported to improve yields and quality of various crops. However, there is still lack of information on the effects of co-inoculation of *B. japonicum* and *Bacillus subtilis* on metabolites of Bambara groundnut and cowpea. This study, therefore, evaluated the effects of co-inoculation of *B. japonicum* and *Bacillus subtilis* (strain BD233) on Bambara groundnut and cowpea metabolites using ^{1}H nuclear magnetic resonance spectroscopy (NMR). The treatments consisted of co-inoculation of *B. japonicum* and *Bacillus subtilis* (strain BD233), *B. japonicum* inoculation, zero inoculation + NO_3 and zero inoculation. The Partial Least Squares -Discriminant Analysis (PLS-DA) demonstrated four distinct groups of Bambara groundnut and cowpea samples of four different treatments. The variable importance in projection (VIP) and relative concentrations corresponding metabolite of Bambara groundnut treatments revealed that co-inoculation of *B. japonicum* and *Bacillus subtilis* (strain BD233) accumulated low concentration of metabolites. However, cowpea co-inoculated with *B. japonicum* and *Bacillus subtilis* (strain BD233) accumulated high concentration of metabolites. The findings of this study suggest that metabolites of Bambara groundnut and cowpea were greatly dependant on the inoculation. Co-inoculation enhanced cowpea metabolites and could be recommended as biofertilizers for cowpea production.

5.1 INTRODUCTION

Indigenous legumes are a prospective solution for insecurity and recuperating livelihoods in Sub-Saharan Africa (Grivetti & Ogle, 2000). Malnutrition crisis due to shortage of nutrients, such as minerals, proteins, and microelements, has been a health problem throughout centuries in Sub-Saharan Africa (Okonya & Maass, 2014). Legumes have been recognized as the inexpensive and sustainable sources of crucial nutrients for a stable nutritious diet (Goncalves *et al*., 2016; Avanza *et al*., 2013). Leguminous crops such as cowpea and Bambara groundnut have numerous economic, environmental, and agronomic advantages therefore they remain the largely cultivated legumes across the African continent (Goncalves *et al*., 2016; Linguya *et al*., 2015). The residual squander of

the harvest is utilised as scavenge, silage or hay for farm animal supplement (Goncalves *et al*., 2016). The foliage, are sun-dried or fermented, can be utilised as immature vegetables (Saidi *et al*., 2010; Wafula *et al*., 2016; Ibrahim *et al*., 2002). Cowpea foliage is consisting of vitamins, macro and micro minerals, flavonoids, antioxidants, ß-carotene, fatty acids, amino acids, carbohydrates, and dietary fibre (Okonya & Maass, 2014; Goncalves *et al*., 2016). Leguminous plants contain antioxidative capacity, therefore forage free radical ions and shield humans against cancer sickness, as well as anti-inflammatory and antimicrobial assets (Nijveldt *et al*., 2001).

Application of PGPR and rhizobia convert essential complex form of nutrients, to simple obtainable form that are required during absorption through roots (Kuan *et al*., 2016; Kang *et al*., 2015a). Recent studies have demonstrated that plant biomass was increased when legumes were subjected to *Bacillus* and *Bradyrhizobium* co-inoculation (Medeot *et al.,* 2010). PGPR such as *Bacillus* produce plant hormones and regulate their level in the tissues, thus controlling the entire hormone in plants stability and how it react to stress (Kundan *et al.,* 2015). Hence forth, metabolomics profiling of co-inoculation of PGPR and rhizobia can assist to identify metabolites involved in beneficial plant-microbe interaction in sustainable agriculture. This study evaluated effects of co-inoculation on metabolites of Bambara groundnut and cowpea using ^{1}H nuclear magnetic resonance (NMR) spectroscopy.

5.2 MATERIAL AND METHODS

5.2.1. SITE DESCRIPTION

As described in Chapter 4, section 4.2.1.

5.2.2. EXPERIMENTAL DESIGN

As described in Chapter 4, section 4.2.2, with 6 replicates

5.2.3. INOCULUM PREPARATION

As described in Chapter 4, section 4.2.3

5.2.4. SAMPLE PREPARATION

Matured leaves were harvested randomly with 6 replicates in each treatment from cowpea and Bambara groundnut plants. Cowpea leaves were air dried at room temperature and

Bambara groundnut leaves were freeze dried. Samples were grounded into fine powder using pestles and mortars.

5.2.5. ^{1}H NUCLEAR MAGNETIC RESONANCE (NMR) ANALYSIS

Fifty milligrams of every sample (six replicates for each treatment) were measured in a 2 mL Eppendorf tube. 1 mL of the solvent of 75% Methanol D4 and 25% (Deuterium oxide (D_20) (750 µl), 750 µl of 0.2M (pH 6) sodium phosphate buffer in 0.1% (TSP) was added to dissolve the samples then vortexed for one minute and placed in a sonicator water bath for 30 minutes. Samples were then centrifuged for five minutes at 10 000 rpm on a benchtop centrifuge. Seven hundred and fifty microliters of the supernatant were then pipetted and transferred into 5mm NMR tubes.

The ^{1}H NMR spectra were acquired on a Varian 600 MHz spectrometer (CSIR, Pretoria) using a method demonstrated by Maree and Vijoen (2012) by operating at a proton NMR frequency of 600 MHz, with 48 scans recorded. All NMR spectra were phase- and baseline-corrected using MestReNova 12.01 (Mestrelab Research). MetaboAnalyst was used with Pareto scaling method to conduct multivariate data analysis.

5.3 RESULTS

5.3.1 Metabolomic analysis of Bambara groundnut samples co-inoculated with *B. japonicum* and *Bacillus subtilis*

Bambara groundnut samples with different treatments were divided from each other by the principal component 1 (15%), principal component 2 (12.5%) and principal component 3 (21.2%). The PLS-DA revealed that four separated groups, Bambara groundnut co-inoculated with *B. japonicum* and *Bacillus subtilis* (strain BD233) (red) grouped together on the far right of the PLS-DA score plot followed by single inoculation of *B. japonicum* Bambara groundnut plants (green). The uninoculated samples (light blue) gathered in the middle and the NO_3 (dark blue) Bambara groundnut samples grouped narrowly from the middle towards the bottom of the PLS-DA score plot.

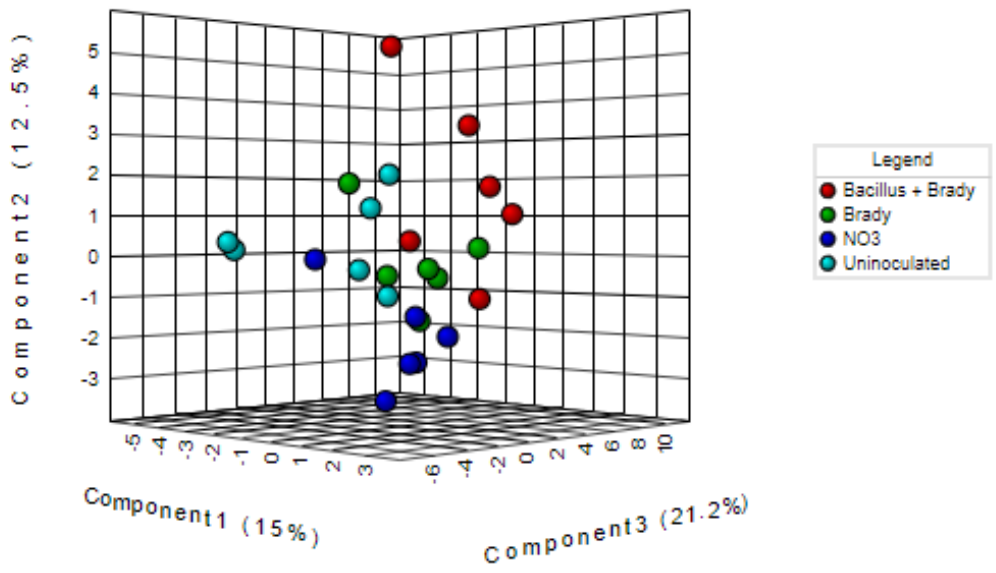

Figure 5.1: Partial Least Squares - Discriminant Analysis (PLS-DA) from ^{1}H NMR peak intensity of Bambara groundnut treatments (co-inoculation, inoculation, uninoculated + NO_3, and uninoculated).

MetaboAnalyst was used to determine VIP scores and discriminating of metabolites. The shaded boxes demonstrated that the relative concentration of each corresponding metabolites under each treatment. The VIP scores showed that uninoculated Bambara groundnut samples obtained the highest concentration of metabolites with chemical shifts (0.22, 0.18, 0.26, 3.02, 3.10, 3.06, 2.94, 2.94, 2.98, 0.62, 3.14, 2.90, and 0.42 ppm) followed by uninoculated + NO_3 with chemical shift of (8.85 and 9.17 ppm). Co-inoculation resulted low concentration of metabolites with one high chemical shift (8.41 ppm). Generally, inoculation of *B. japonicum* resulted in accumulation of one metabolite concentration (Figure 5.2).

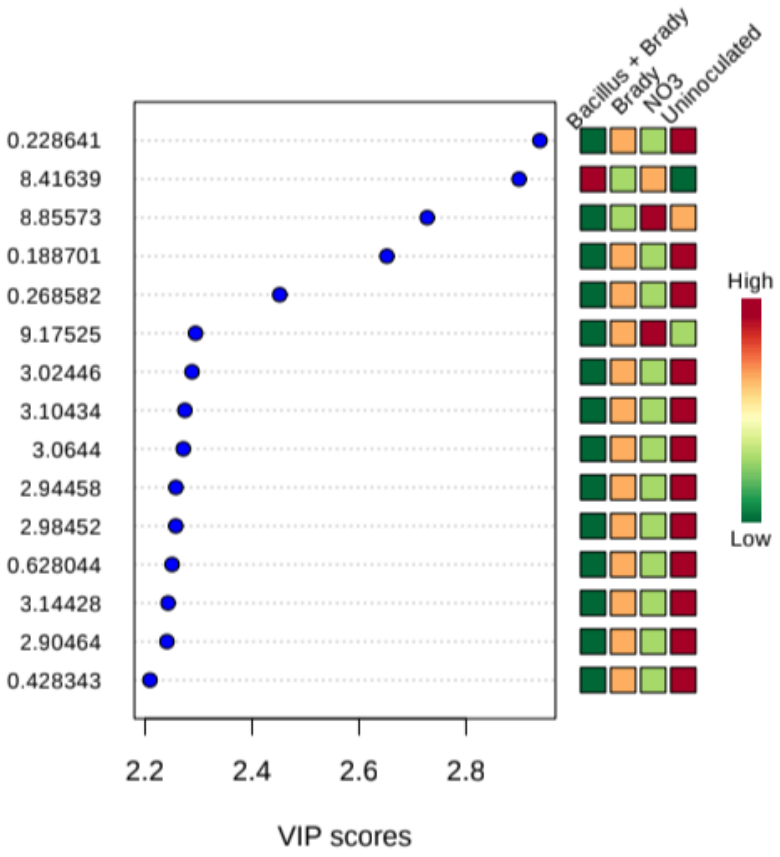

Figure 5.2: Variable importance in projection (VIP) and relative concentrations corresponding metabolite per Bambara groundnut treatments.

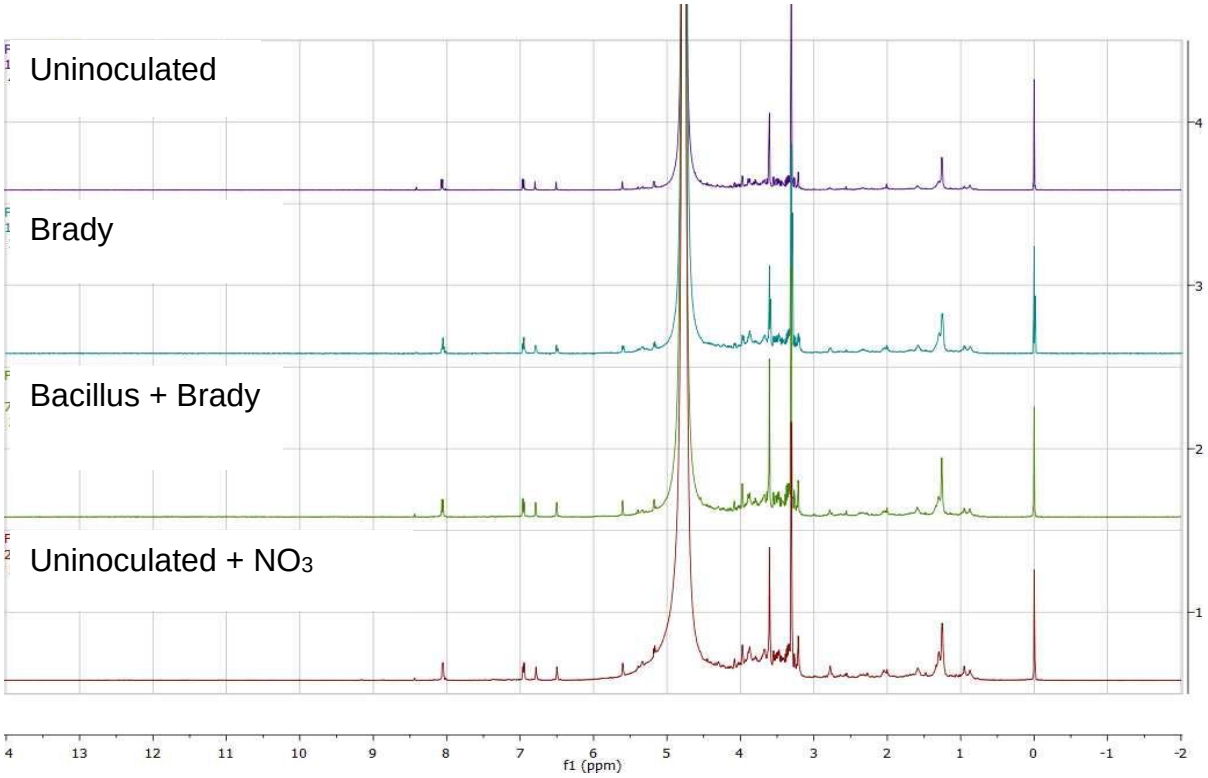

Figure 5.3: ^{1}H NMR spectra (600MHz) of for Bambara groundnut showing the four treatments namely (co-inoculation, inoculation, uninoculated and NO_3, and uninoculated).

5.3.2 Metabolomic analysis of cowpea samples co-inoculated with *B. japonicum* and *Bacillus subtilis*

Cowpea plant samples with different treatments were divided from each other by the principal component 1 (12.4%), principal component 2 (35.5%) and principal component 3 (8.6%) (Figure 5.4). The PLS-DA showed that four separated groups, cowpea plants co-inoculated with *B. japonicum* and *Bacillus subtilis* (strain BD233) (red) grouped together on the left of the PLS-DA score plot followed by single inoculation of *B. japonicum* cowpea plants towards the middle bottom (green). The NO_3 (dark blue) cowpea samples grouped in the middle-right and uninoculated samples (light blue) gathered towards the right of the PLS-DA score plot (Figure 5.4).

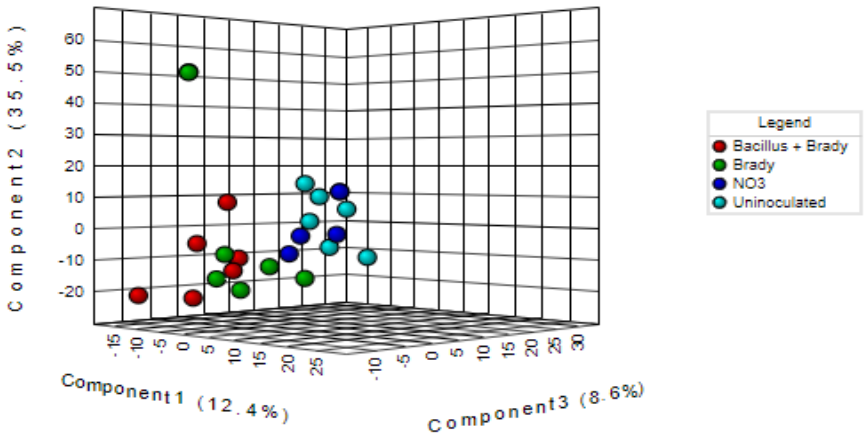

Figure 5.4: Partial Least Squares - Discriminant Analysis (PLS-DA) from ^{1}H NMR peak intensity of cowpea plants treatments (co-inoculation, inoculation, uninoculated and NO_3, and uninoculated).

The VIP scores were obtained using MetaboAnalyst. The shaded boxes showed that the comparative concentration of each corresponding metabolites under each treatment. The VIP scores revealed that co-inoculation of *B. japonicum* and *Bacillus subtilis* accumulated high concentration of metabolites with chemical shift (8.89, 8.09, 9.17, 8.13, 7.49, 6.45 and 6.4 ppm) followed by uninoculated + NO_3 with chemical shifts of (9.33, 5.81, 5.97 and 8.93 ppm). While uninoculated cowpea samples obtained the highest chemical shift concentration at (8.41, 6.81 and 5.89 ppm). Overall *B. japonicum* inoculated cowpea accumulated the lowest contractions and co-inoculation of *B. japonicum* and *Bacillus subtilis* recorded the highest concentration than other treatments (Figure 5.5).

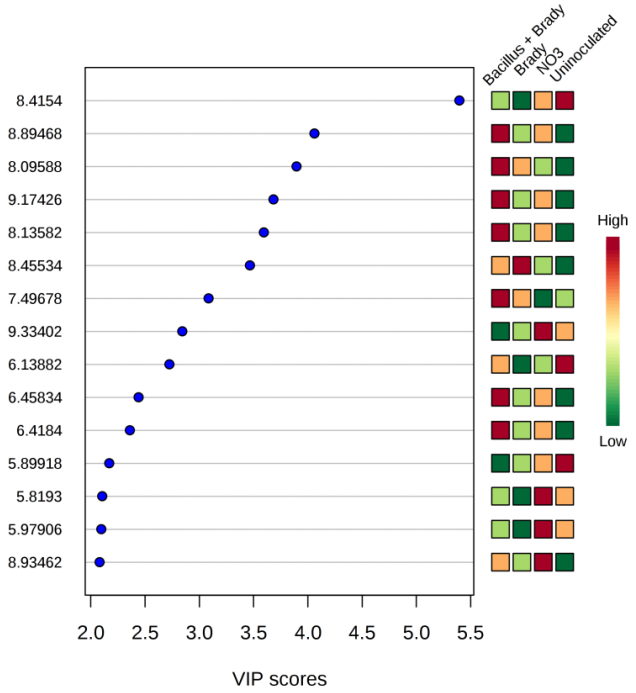

Figure 5.5: Variable importance in projection (VIP) and relative concentrations corresponding metabolite per cowpea treatments.

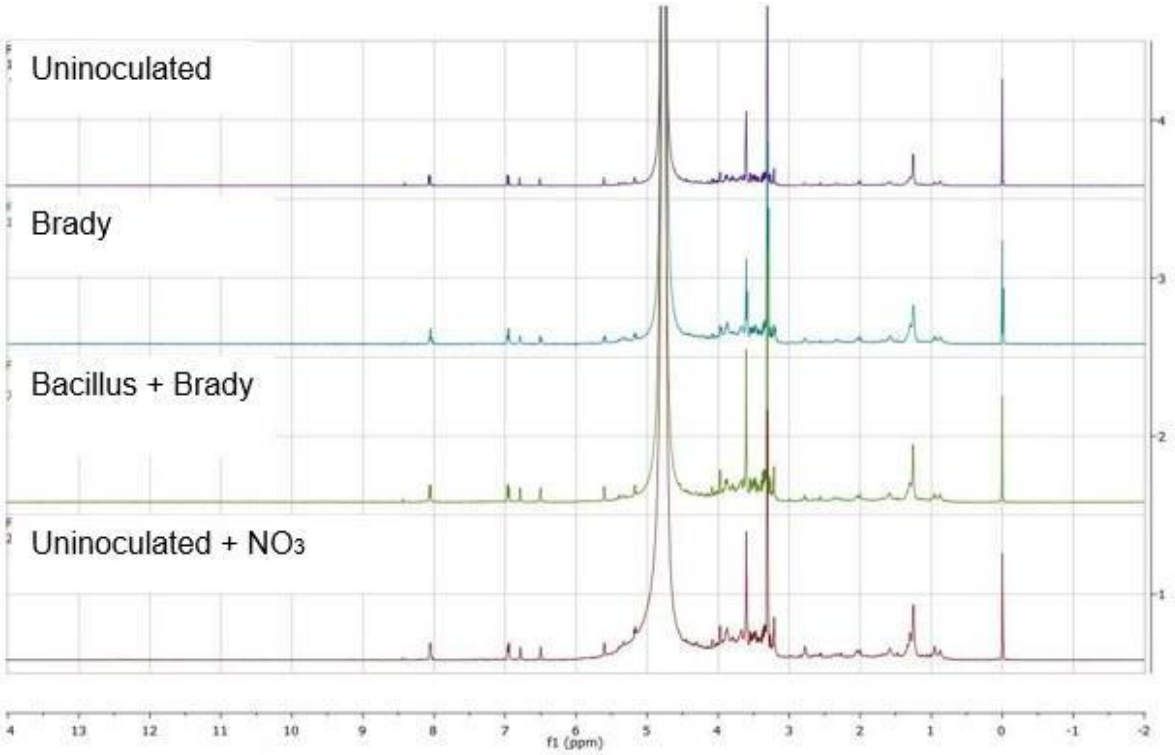

Figure 5.6: ^{1}H NMR spectra (600MHz) of for cowpea showing the four treatments namely (co-inoculation, inoculation, uninoculated and NO_3, and uninoculated).

Figure 5.3 and 5.6 presents a presentative one dimensional ^{1}H NMR fingerprint of Bambara groundnut and cowpea subjected to co-inoculation with *B. japonicum* and B. subtilis, single inoculation of *B.subtilis* NO_3 and Zero inoculation.

5.4 DISCUSSIONS

Bacillus spp. is known to promote plant growth and stimulating of synthesis of phytohormones (Shao *et al*., 2015). Application of *Bacillus* spp. increased the accumulation of metabolite biosynthesis in plants (Kloepper *et al*., 2004). In this study, the ^{1}H NMR data analysis revealed the differences in metabolic profile patterns per inoculation treatment. The VIP score showed that co-inoculation with *B. japonicum and Bacillus subtilis* (strain BD23) increased concentration of metabolites in cowpea extract and the accumulation of metabolites. The findings are consistent with (Chamam *et al.,* 2013) who reported that accumulation of metabolites was increased when *Azospirillum* strains were inoculated in rice genotypes. Pagnani et al. (2018) reported that inoculation of PGPR such as increased metabolite accumulation in hemp plants.

Metabolites are synthesised by plants and play important roles in plant defence mechanism and plant-microbe interactions (Mierziak *et al*., 2014). In this study, co-inoculation of *B. japonicum* and *Bacillus subtilis* (strain BD233) in Bambara groundnut resulted in decreased metabolites accumulation. Previous studies reported that plant growth promoting rhizobacteria alter metabolites in plant (Mishra *et al*., 2018). The findings in this study are consistent with Chamam *et al.* (2013) who reported that inoculation of PGPR *Azospirillum* strains on decreased accumulation of metabolites in rice genotypes. Similar findings were also observed when *Bacillus velezensis* 5113 was inoculated in wheat plants (El-Daim *et al*., 2019). These findings may be associated with combination of plant genotype and bacterial strain.

5.5 CONCLUSION

This study showed that co-inoculation of *B. japonicum* and *Bacillus subtilis* (strain BD233) accumulated low concentration of metabolites in Bambara groundnut but increased metabolites accumulation in cowpea. The findings of this study suggest that metabolites of Bambara groundnut and cowpea were greatly dependant on the inoculation. Co-inoculation enhanced cowpea metabolites and could be recommended as biofertilizer for cowpea production.

CHAPTER 6: GENERAL DISCUSSIONS AND CONCLUSIONS

6.1 GENERAL DISCUSSIONS

Sustainable agricultural practices are the fundamental solutions for the declined crop yield due to soil degradation and climate change (Suzuki *et al*., 2014). One of the sustainable agricultural approaches is the use of PGPR inoculants as biofertilizers as these groups of microorganisms have improved yield and quality of many crops (Barnawal *et al*., 2013). In this study, inoculation of brown and red landraces of Bambara groundnut with *Bacillus subtilis* and subjected to low (24°C) and high (35°C) resulted in greater growth (plumule length) and germination percentage and index. Thus, inoculation with *Bacillus subtilis* (strain BD233) has a potential of improving yield when grown in high temperatures.

In order to continue feeding the ever-growing population world, it is of great importance to use more efficient sustainable ways of improving crop yields. An efficient sustainable alternative recently used in agricultural crop production is the application of plant growth promoting rhizobacteria in combination with compatible rhizobial strains (Korir *et al.,* 2017). Inoculation of plant growth promoting rhizobacteria together with rhizobial strains in legume crops has been reported to enhance symbiotic nitrogen fixation, increasing nodulation, and plant growth of (Mathivanan *et al*., 2014; Korir *et al*., 2017). Consistently, in this study (Chapter 4), co-inoculation improved yield, nodulation, chlorophyll content and stomatal conductance compared with other treatments (*B. japonicum* single inoculation, no inoculation). This could be attributed to the fact that PGPR such as *Bacillus* species are known to produce hormones (auxins, cytokinins, gibberellins and ethylene) which are responsible for plant growth and development (Park *et al*., 2017).

Quality indicators for crops are determined by their nutritional and biochemical composition which reflects the metabolic profile of a plant. The metabolic profile and patterns are assessed through metabolomic analysis using the LC-MS and NMR techniques. This study successfully assessed the metabolic profile of both Bambara groundnut and cowpea using ^{1}H NMR technique. The data analysed for each crop demonstrated that the inoculation with *Bacillus subtilis* has an effect metabolic profile and pattern as shown in the PLS-DA (see chapter 5). This finding was consistent with Kloepper et al. (2004) who reported that application of *Bacillus* spp. improved the biosynthesis of metabolite in plants. In this study,

accumulation of selected metabolites of important from VIP score demonstrated that concentration of metabolite was influenced by inoculation. Furthermore, co-inoculation of *B. japonicum* and *Bacillus subtilis* (strain BD233) increased metabolite accumulation in cowpea cultivars but not in Bambara groundnut landraces. The high metabolite concentrations as a result of co-inoculation suggest that combination of both *Bradyrhizobia japonicum* and *Bacillus subtilis* have a potential of improving crop quality and can thus be used as an effective green biofertilizers or inoculant. However, the low metabolite concentrations obtained after co-inoculation with both *Bradyrhizobia japonicum* and *Bacillus subtilis* of Bambara groundnut landraces could be due to the lack of compatibility between the landrace and strain.

6.2 CONCLUSIONS

This study successfully evaluated the effects of inoculation with *Bacillus subtilis* (strain BD233) on germination of Bambara groundnut landraces and suggested that *Bacillus subtilis* (strain BD233) could be used to mitigate the effects of heat stress. Furthermore, co-inoculation of *B. japonicum* and *Bacillus subtilis* (strain BD233) on cowpea improved yield and nodulation under glasshouse conditions. The metabolic profile of cowpea and Bambara groundnut subjected to co-inoculation was assessed through metabolomic analysis and has shown that both crops metabolic composition and profile are highly dependent on co-inoculations. However, accumulation of metabolites after co-inoculation of *B. japonicum* and *Bacillus subtilis* (strain BD233) was observed in cowpea than Bambara groundnut landraces.

It ought to be noted that the inoculation of seeds or plants with biofertilizers including *Bacillus* and *Bradyrhizobium* is not meant to replace the application of fertilizers. It should therefore be noted that although their performance resulted in increased seed germination, and the growth and development of seedlings, including plant growth and yield, they ought to be co-applied with fertilizers. Also, it ought to be noted that research is yet to reveal whether continued application of or inoculation with biofertilizers (especially on low-nutrient soils) has a sustainable positive effect on soil fertility or grain yield. Therefore, caution should be taken and a statement included that the long-term effect of their usage/application is not yet known. Growth media used to carry out the research were chosen because they are nutrient free and to avoid soil microorganisms and soil nutrients.

Lastly, biofertilizers have the capacity to improve the cleavage (particle-bound nutrients) and solubilisation of native nutrients in soils or other growth media but also the efficiency of fertilizers and therefore increase their availability. It is interesting and worth noting that the performance of biofertilizers is not exclusive to soil but other growth media as well. It is possible that the cleavage and solubilisation of nutrients involve those inside seeds and plant organs, which can explain the performance in this study, especially in the first research study.

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