

Interactive effects of *Bacillus subtilis* and elevated temperature on germination, growth and grain quality of cowpea irrigated with acid mine drainage

by

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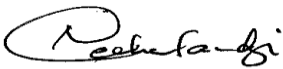
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DECLARATION

I, Thalukanyo Nevhulaudzi, hereby declare that the work presented in this dissertation entitled “INTERACTIVE EFFECTS OF *BACILLUS SUBTILIS* AND ELEVATED TEMPERATURE ON GERMINATION, GROWTH AND GRAIN QUALITY OF COWPEA IRRIGATED WITH ACID MINE DRAINAGE” is original work done by myself under the mentorship of my supervisors. Additionally, I declare that the work presented herein has not been published or submitted at any other institution as part of the requirements for any degree programme. All cited literature in this dissertation from other individuals or institutions has been acknowledged and listed in the reference section. I also certify that I have complied with the rules, requirements, procedures and policies of the University of South Africa.

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DEDICATION

To my loving family, father, mother, sister, brothers and son (Roana), I dedicate this dissertation.

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ABBREVIATIONS

ANOVA	Analysis of Variance
AMD	Acid Mine Drainage
<i>B.subtilis</i>	<i>Bacillus subtilis</i>
Ca	Calcium
Cd	Cadmium
cm	Centimetre
Cr	Chromium
Cu	Copper
DAP	Days after Planting
DAS	Days after Sowing
DWAF	Department of Water Affairs and Forestry
FAO	Food and Agriculture Organisation
Fe	Iron
G%	Germination Percentage
GCMs	General Circulation Models
GHG	Greenhouse Gas
GI	Germination Index
GRI	Germination Rate Index
h	Hour
Hg	Mercury
IPCC	Intergovernmental Panel on Climate Change
K	Potassium
Mg	Magnesium
Min	Minute
mL	Millilitre
mm	Millimetre
mM	Millimolar
Mn	Manganese
Ni	Nickel
°C	Degree Celsius

Pb	Lead
PGPR	Plant Growth Promoting Bacteria
UNFCCC	United Nations Framework Convention on Climate Change
UNISA	University of South Africa
Zn	Zinc

ABSTRACT

This study's main goal was to evaluate *Bacillus subtilis* inoculation and mine water irrigation effect on germination, growth, nodulation, physiology and shoot/grain quality of cowpea genotypes exposed to extreme climatic conditions (elevated temperatures). The first experiment evaluated the interactive effect of *Bacillus subtilis* (BD233) inoculation and elevated temperature on germination indices and plumule lengths of three genotypes (Asetanapa, Soronko and Nyira) of cowpea. The results showed that interaction between *B. subtilis* (BD233) and temperature significantly ($p < 0.05$) influenced the germination indices (germination percentage (G%), germination index (GI) and germination rate index (GRI)) and plumule length of cowpea seedlings and genotype responses were significantly different. At elevated temperature (35°C), inoculation with *B. subtilis* (BD233) enhanced seed germination and growth of cowpea. The second experiment evaluated the effect of temperature on growth and nutritional content of cowpea incubated for seven days in a growth chamber. The results showed that when cowpea genotype, Soronko, was incubated at different temperature regimes, the whole plant biomass, shoot carbon and crude protein contents were significantly affected with temperature increases at all three stages of the plants' life cycle. The results suggest that the pre-flowering (40 DAP) and flowering (90 DAP) stages of cowpea compared to post-flowering (123 DAP) are more susceptible to elevated temperatures (30-35°C). The third experiment evaluated *Bacillus subtilis* inoculation and mine water irrigation effect on growth, nodulation, physiology and nutritional content of cowpea under glasshouse conditions. The results revealed that the interaction of *B. subtilis* (BD233) inoculation and mine water (75% AMD) irrigation was significant for the growth, nodulation, stomatal conductance, chlorophyll contents and shoot/grain nutritional quality of cowpea genotypes. In comparison with control, generally, *B. subtilis* inoculation enhanced the growth, nodulation and yield of all tested cowpea genotypes and irrigation with mine water significantly influenced the mineral contents in both shoot and grain of cowpea. Taken together, findings in this study have implications for cultivation of cowpea, an important candidate for food/nutrition security in Africa, under future climate change scenarios.

CHAPTER 1: GENERAL INTRODUCTION AND STUDY BACKGROUND

1.1 INTRODUCTION

Water for irrigation of crops is a scarce resource and most surface waters are being contaminated due to mining activities, especially in developing countries such as South Africa, a water-scarce country with a long mining history. The advent of climate change has compounded the problem with frequent occurrences of droughts, erratic rainfall patterns (less rains) and elevated temperatures, which have the potential to impact the growth of most crops. This potential threat to the security of household food and nutrition, posed by climate change and elevated temperatures, is worrisome and therefore, there is a need to clean up contaminated surface waters especially acid mine water and mitigate climate change. Microorganisms including some *Bacillus* spp. have been reported as bio-remediators of contaminated waters and soils (with heavy metals) and also as plant growth enhancers.

The overall goal of this study was to evaluate the interactive effect of *Bacillus subtilis* and mine water irrigation on the germination indices, nodulation, growth, physiology and nutritional quality of three cowpea genotypes exposed to elevated temperature regimes. This chapter provides the study background, research problem, aim and objectives, significance of the study, research hypothesis, and the overall organization of the dissertation.

1.2 BACKGROUND TO THE STUDY

Acid mine drainage (AMD) generated from mining activities remains to be a significant water pollution problem worldwide (Coetzee et al., 2010; Naidoo, 2017). Exposure to air and water of sulphide bearing minerals, often in the form of pyrite, generates AMD. The oxidation of sulphide in AMD produces acids such as H_2SO_4 and heavy metals (Fe, Zn, Mn, Pb, Cd, Cu, Ni). AMD generated through entrance of water into mine voids, is characterized by the following: low pH (2-4), high sulphur concentration (1-2 g/l) and levels of heavy metals (Fe, Zn, Mn, Pb, Cd, Cu, Ni, Hg and Cr) (Vadapali et al., 2008). These heavy metals in water systems can persist and bio-accumulate in living organisms such as plants, especially if plants are exposed to higher concentrations over time and thereafter consumed by animals and humans in the food web, ultimately posing health hazards and environmental risk in the ecosystem

(Naicker et al., 2003). The discharge of heavy metals into aquatic ecosystems thereby causing contamination of surface water and groundwater systems has become a matter of concern (Mamba et al., 2009). In addition, changes in pH brought about by AMD tends to significantly impact the chemistry of mine waters and rivers into which they are discharged. Based on the metal being mined and mining process, the pH level can vary from one area to another, for instance, a comparison of the pH of surface water from the precincts a gold (extremely acidic (pH<2)) and coal (pH >4)) mines showed significant differences (Nevhulaudzi et al., 2014).

In South Africa, mining activities have a long history and has left behind huge amounts of AMD in the mining industry's precincts. The mining activities associated with income generation opportunities have attracted a huge population of migrant workers in these mining areas. This development has led to increased demand for water from surface waters by the resident population for both industrial and domestic uses including irrigation of crops. In South Africa, the close proximity of mining industries to water catchment areas and therefore surface waters is an additional threat to ecosystems due to pollution by AMD derived from the pyritic materialisations in coal and gold deposits. The country has a little arable land percentage and the rainfall pattern is erratic and low annually, therefore the country is considered water-scarce (Jovanovich et al., 1998). In addition, most surface waters are polluted by wastewaters from industries and urbanization including municipal waste (Du Plessis, 1983). Therefore, good quality water scarcity in the country especially for crop irrigation by farmers is of major concern. Recently, the country experienced droughts in some provinces (Western Cape and Free State) and the looming climate change due to the El Nino phenomenon makes the situation very worrisome. One of the main threats of climate change is rise in global average temperatures, which poses additional problems for seed germination, crop development and other living organisms (Ewing, 1981). Furthermore, erratic rain patterns (less rains) and frequent occurrence of droughts are also associated with climate change. Therefore, the possible utilization of available AMD contaminated surface waters for agricultural crop irrigation and the impending physiological threats due to increases in temperature/climate change are subjects of interest to the scientific community in South Africa in particular and the world in general. In South Africa, the amelioration of acid mine water using lime for the irrigation of crops has been reported (Du Plessis, 1983). However, the amelioration of AMD water with lime is not only expensive but can also release pollutants in soils following irrigation of crops with lime-treated acid mine water. On the other hand, although

reverse osmosis has been demonstrated to possibly treat badly polluted water such as acid mine water to drinking quality and crop irrigation standards, the cost of ameliorating mine water by this process can be however colossal (McCarthy, 2011). Thus, there is a dire need to search for cheaper ameliorating alternatives or available bio-remediators to de-contaminate AMD water without posing any threat to ecosystems or agronomic systems. At present, clean-up processes of heavy metal pollution are costly and environmentally damaging. Interestingly, researchers have started generating cost-effective technologies that include the use of microorganisms for the cleaning process of pollutants in mine water (Abou-Shanab et al., 2003; Hooda, 2007), for possible use in crop irrigation. One good example of such technologies is bioremediation. Bioremediation is a technology that uses organisms to breakdown contaminants and thereby decontaminate polluted natural environments (Agarwal, 1998).

Cowpea (*Vigna unguiculata* L. Walp) is an important nutritious crop that belongs to the family Fabaceae/Leguminosae. It is reported to have evolved in West Africa (Smartt, 1985) but nowadays it is cultivated in most regions of the world including East and Southern Africa, Southeast Asia, Southern United States and Latin America. The crop is mostly cultivated because of its ability to contribute nitrogen to nutrient poor soils in addition to providing cheap dietary protein, carbohydrate, minerals and vitamins for humans (Bressani, 1985, Belane, 2011). Cowpea grain is rich in protein (42%) and carbohydrate (57%) and the green leaves and young pods are capable of supplying up to 35% protein when consumed as vegetables (Kadam et al., 1984; Belane, 2011). In South Africa, the crop is cultivated and consumed mostly by smallholder farmers in rural communities in all provinces in the country and therefore, has a potential role to play in securing household food and nutrition.

1.3 RESEARCH PROBLEM

South Africa is a water-scarce country and climate change will compound the water crisis as evident by the recent droughts in some regions in the country (Western Cape, Free State and Mpumalanga provinces). However, the country has huge deposits of minerals with pyritic formations, which is the source of AMD pollution in most surface waters including dams and rivers due to its long mining history especially in the

Witwatersrand Basin (McCarthy, 2011). AMD water is toxic (with heavy metals and low acidity) and when used for irrigation can affect the growth and nutritional quality of crops. As well, the exposure to extreme climatic conditions (such as elevated CO₂ and temperatures) poses a threat to the growth and yield of crops such as cowpea and vegetables. Increases in global temperatures (elevated temperature) caused by climate change can influence seed germination and growth of important economic crops such as cowpea thereby posing threats to future household food and nutrition security. In future, there would be changes in agricultural crop production/food security due to climate change as a results of high temperatures, which can have adverse effects on crop yields because of the overall reduction in seed germination and plant growth (Ewing, 1981). Furthermore, the need to mitigate climate change cannot be over emphasized especially in developing countries in sub-Sahara Africa such as South Africa, which is a water-scarce country. There is a dire need to ameliorate AMD water with cheaper alternatives that are environmentally-friendly and readily available. The use of microorganisms in the amelioration of badly polluted water such as AMD has been proposed. Therefore, the suitability of *B. subtilis* as a potential heavy metal resistant bacteria in soils polluted with mine water was explored in this study.

1.4 AIM AND OBJECTIVES OF THE STUDY

1.4.1 Research Aim

The main goal of this study was to evaluate seed germination, growth, nodulation, physiology and shoot and grain quality of cowpea genotypes inoculated with *Bacillus subtilis* and irrigated with mine water and exposed to extreme climatic conditions (elevated temperatures).

1.4.2 Research Objectives

- To assess and compare the seed germination indices and growth of three cowpea genotypes (Asetenapa, Soronko & Nyira) inoculated with *B. subtilis* (BD233) and exposed to elevated temperature regimes.
- To determine the growth and shoot/grain nutritional quality responses of cowpea to elevated temperatures (>30°C).

- To evaluate the interactive effect of inoculation with *B. subtilis* (BD233) and mine water irrigation on the growth, nodulation and physiology (stomatal conductance and leaf chlorophyll content) of cowpea genotypes.
- To assess and compare the shoot and grain nutritional quality of the three cowpea genotypes irrigated with mine water and inoculated with *B. subtilis* (BD233).

1.5 HYPOTHESES

- Inoculation with *B. subtilis* (BD233) and exposure to elevated temperatures have no effect on the seed germination indices and growth of three cowpea genotypes.
- There are no changes in the growth and shoot/grain nutritional quality responses of cowpea to elevated temperatures.
- Interaction of *B. subtilis* (BD233) inoculation and mine water irrigation has no effect on the growth, nodulation and physiology of cowpea genotypes.
- There are no differences in the nutritional content of shoot and grain of cowpea genotypes irrigated with mine water and inoculated with *B. subtilis* (BD233).

1.6 SIGNIFICANCE OF THE STUDY

The practice of utilizing *Bacillus* species in agriculture is on the increase steadily mainly because it offers a substitute to expensive environmentally-unfriendly fertilizers (Bhattacharyya & Jha, 2012). Certain strains of *Bacillus* spp. are described to stimulate plant growth mainly by secreting siderophores and phytohormones e.g. auxins, cytokinins, gibberellins etc.) in plant rhizosphere soils (Ali et al., 2009a, Galaviz et al., 2018) or augmenting nutrient utilization (solubilisation of minerals) in plants (Bowen & Rovira, 1999; Zhang et al., 2010; Meng et al., 2016). Studies have found that *B. subtilis* can quench soil ills (such as nutrient deficiencies) and activities of plant pathogens to boost plant growth (Kloepper et al., 2004; Adesemoye et al., 2008; Felici et al., 2008). On the other hand, elevated temperatures due to future climate change can drastically affect the seed germination and growth of crops (Ewing, 1981) such as cowpeas. However, few or no studies have explored the interactive effect of *B. subtilis* and elevated temperatures on seed germination and growth of cowpea genotypes, an important crop in Africa and other regions in the world (Freire-Filho, 2011). Findings from this study have implications for the choice of cowpea genotypes grown especially

by resource-poor farmers in areas experiencing climate change (elevated temperatures) and irrigated with ameliorated AMD water. Among the tested cowpea genotypes, Soronko was revealed to have superior traits with regards to adaptation to climate change. At elevated temperatures (30-35°C) and without *B. subtilis* inoculation, Nyira followed by Soronko had the highest germination percentage, fastest germination rates (lowest GI & GRI values) and enhanced growth. Co-inoculation of Soronko with *B. subtilis* and a commercial inoculant, enhanced nodulation and yield compared to control even when irrigated with mine water. The interaction of *B. subtilis* inoculation and mine water irrigation (75% AMD) had a significant effect on the nutritional quality of cowpea. This study has demonstrated the potential of *B. subtilis* as a suitable metal resistant bacteria in soils polluted with acid mine water.

1.7 ETHICAL CONSIDERATION

This study was carried out in line with UNISA's policy on ethics, the research proposal was approved by CAES committee on 04 November 2016 (2016/CAES/093) and the approval was reviewed annually until the completion of the experiment.

1.8 STUDY LAYOUT

This dissertation consists of six chapters including three research chapters. Each chapter summary is presented here to guide the readers through the deliberations on topics presented.

Chapter one provides a background to the study and presents the aim and objectives for undertaking the study, including the research problem and significance of the study.

Chapter two deliberates the literature studied in relation to crop production and climate change and the role of microorganisms in bioremediation of AMD polluted water sources. It offers the purpose of conducting a literature review and findings from the literature in relation to the topic.

Chapters three, four and five present research findings for separate experiments. Specifically, chapter three describes and discusses findings on the effect of elevated temperature and *Bacillus subtilis* inoculation on cowpea seed germination indices and growth. Chapter four describes and discusses findings on the effect of elevated temperature (>30°C) on the growth and nutritional quality of cowpea at three growth

stages. Chapter five describes and discusses findings on the interactive effect of *Bacillus subtilis* inoculation and mine water irrigation on the growth, nodulation, physiology and nutritional content of shoot and grain of cowpea genotypes under glasshouse conditions.

Chapter six presents the general discussion and recommendations thereof.

CHAPTER 2: LITERATURE REVIEW

2.1 INTRODUCTION

The previous chapter gave the background and research problem as an overview of the study. It also highlighted the study aim and objectives including its significance and concludes with the structure of the dissertation. This chapter is a review of the literature related to irrigation of crops with contaminated water and role of microorganisms in bioremediation of the badly polluted water such as AMD water. The literature on climate change (elevated temperature) effects on plant physiology and crop production and cowpea cultivation as food/nutrition security are also reviewed. The chapter closes with a summary of the gaps identified in the literature reviewed.

2.2 ACID MINE DRAINAGE AS CONTAMINANT OF WATER SOURCES AND SOILS

Surface waters are being contaminated due to mining activities resulting in clean water for crop irrigation becoming a very scarce commodity. Mining of gold and coal is associated with AMD responsible for long-term loss to watercourses and biodiversity including entire ecosystems (Akcil & Koldas, 2006). Mostly, AMD occurs as a result of reactive sulphide minerals (pyrite) stored in waste rock dumps reacting with water and oxygen (Strenen-Joyce et al., 2013). Acid mine drainage has high specific conductivity, concentrations of iron, aluminium, and manganese, and low pH (2-4), and concentrations of toxic heavy metals (Pb, Hg, Cr, Zn & Ni) (Strenen-Joyce et al., 2013).

In developing countries such as South Africa, wastewater disposal has become problematic because increased urbanization and industrialization are producing large quantities of municipal waste and industrial effluent (acid mine drainage) (Alloway & Ayres, 1993). In South Africa, the disposal of AMD derived from mining activities is a serious environmental problem. Among the problems of disposing mine water from mines (gold and coal) and old underground workings, is the acidification of water sources and soils when mine water is discharged from the mine treatment plants into the environment over time. Acid mine drainage water is highly acidic (pH ranging typically from 2 to 4.4) and can therefore increase soil acidity. According to statistics provided by Venter et al. (2001), a huge proportion (about 5 million ha) of soils in South Africa are severely acidic and about 11 million ha are moderately acidic. Considering the country's long gold and coal mining history and vast deposit of pyritic formations,

it is highly probable that the prevalence of the reported high soil acidity may be due to AMD reaching water pathways or river catchments. Soil acidification due to either AMD or natural driving forces (hydrolytic displacement of base cations or oxidation reactions) can change soil properties and processes such as contraction of cation exchange capacity and expansion of anion exchange capacity (Fey, 2001). In addition, it can decrease the concentrations of some important basic cations (Ca, Mg, K) and solubility of nutrients (phosphorous and molybdenum) essential for the growth of plants and can also increase the concentration of some toxic elements (aluminium and manganese) (Sumner et al., 1990). The loss of essential nutrients in soils exposed to AMD and soil accumulation of heavy metals will translate into poor productivity if such soils are used for crop cultivation.

In most countries, one of the possibilities for disposing wastewater/mine water is to irrigate crops in agricultural land (Oloya & Tagwira, 1996). Agriculture utilises about 70% of water withdrawal hence it is expected that in times of water scarcity farmers will turn into wastewater/mine water as water source especially by poor-resource farmers in rural communities (United Nation's Food and Agriculture Organization (FAO), 2010). Long-term utilization of wastewater for irrigation of important economic crops such as cowpea may result in heavy metal accumulation in humans through the food web and contamination of soils in particular and the environment in general (Chipo et al., 2011). Long-term irrigation of agricultural soils with wastewater/mine water will ultimately results to soil contamination in addition to compromising the safety and quality of food crops (Muchuweti et al., 2006). Soil health may be endangered when wastewater/mine water are used for crop irrigation (Barman et al., 2000). Dougherty and Hall (1995) also stated that the use of wastewater/mine water in agricultural lands for a long-term has resulted to soil contamination with metals. Clearly, irrigation of crops with wastewater/mine water can bring changes in the quality of soil as trace element contributions are sustained for a long period. Heavy metals in wastewater/mine water are one of the factors negatively affecting natural environments (Ahmad Wani et al., 2007; Marchel et al., 2015). Heavy metal contamination in agricultural soils has become a thoughtful risk to global sustainability of crop production (Chen et. al., 2007; Ahmad Wani et al., 2007). These heavy metals include Cd, Pb, Zn, Cu, Hg, Cr, Mn, Fe, Zn & Ni.

Amongst the heavy metals, some are non-biodegradable and can persist in the environment over a long period. For example, cadmium is of a great concern due to

its toxic effects on flora and fauna even at low concentrations (Hu et al., 2009). Cadmium is categorised by high toxicity and high capacity to bio-accumulate in plants because it is persistent and non-biodegradable. Cadmium has a high phytotoxic impact when accumulated even at low concentrations and the impact on plants ranges from a reduction in growth, increased chlorosis and wilting to death of cells (Gallego et al., 2012). In addition, this heavy metal has a comparative high movement within the soil-plant system, which suggests it can go through the food chain and subsequently, build-up to toxic levels in living organisms thereby posing threats to their health in particular and that of the ecosystem in general (Burger, 2008) because it is persistent over a long period. A previous study found that high concentrations of cadmium in soils can arise naturally however often are due to industrialization or over use of fertilizers containing cadmium (Tiller et al., 1997). Lead is another heavy metal of great concern as it can also bio-accumulate and thereby cause health-related problems in living organisms including humans over time when consumed through the food chain. The uptake of heavy metals from the rhizosphere by plants is the main path of exposing humans to them in soils (Wang et al., 2007). However, irrigation of crops with ameliorated mine water or wastewater has its benefits, which include but not limited to water conservation. In addition, there are beneficial heavy metals e.g., Zn, Mn & Fe for plant growth especially when at the adequate concentrations but others (e.g. Cd, Pb) are not, even at low concentrations, because after their addition into soils they could be transferred through the food chain to humans and over time can bio-accumulate and persist and cause harmful effects when they reach toxic levels (Aziz et al., 1996). Recognition of the detrimental effects of some heavy metals has prompted the need to clean-up or ameliorate acid mine water or wastewaters containing heavy metals. Environmentally friendly processes need to be established and implemented to clean-up and protect the environment by means of bioremediation techniques (Stratten, 1987). One such environmentally-friendly process is the use of microorganisms in the amelioration of badly polluted waters such as AMD. Interestingly, some microorganisms are also known to enhance plant growth (Compant et al., 2010; Dary et al., 2010; Román-Ponce et al., 2017) through several mechanisms.

2.3 MICROORGANISMS AS POTENTIAL BIO-REMIEDIATORS OF ACID MINE DRAINAGE AND PLANT GROWTH ENHANCERS

Bioremediation is a recent technology gaining popularity in the clean-up of polluted natural environments. The technology is considered as one of the most cost-effective and environmentally conscious management tool to recover polluted soils in their natural stance and manage the polluted environment. The technology is relatively cheaper than conventional methods (reverse osmosis, chemical fixation, physical treatment) because it relies on promoting the growth of indigenous microorganisms capable of breaking down contaminants on-site (Agarwal, 1998). Bioremediation process involves transmuting (biotransformation and biodegradation) contaminants into non-hazardous or less hazardous products (Singh et al., 2014). Biotransformation involves any modification of the structure of molecules/compounds by microorganisms into a less harmful molecule/compound. Biodegradation process involves the breaking down of a molecule/compound into environmentally-friendly or non-toxic products. Heavy metal resistant bacteria having plant growth promotion traits are currently being used as eco-friendly and less cost-effective technologies for bioremediation of metal contaminated soils (Khan et al., 2009). Microorganisms capable of biodegrading or bioremediation are recognized members of microbial consortiums and they include *Bacillins* (Singh et al., 2014), rhizobacterial strains of *Alcaligenes*, *Bacillus*, *Curtobacterium*, and *Microbacterium*, with plant growth-enhancing traits (Román-Ponce et al., 2017). In particular, legume plants associated with microorganisms (plant growth-promoting rhizobacteria (PGPR)) have been used for bioremediation of soils contaminated with heavy metals (Carrasco et al., 2005; Zhuang et al., 2007). For example, *Bacillus* PZ3 is a PGPR, was reported to reduce Cr in pea plants when inoculated in chromium amended soil and also enhance the plant's growth, nodulation, leghaemoglobin, chlorophyll and protein content compared to control plants (Wani & Zainab, 2016). These bacteria (PGPR) types are suggested to have the ability to secrete plant stimulating metabolites in heavy metal polluted soils (Wani et al., 2007) and in the process accelerate phytoremediation of such soils by enhancing the health and growth of plants (Compant et al., 2010; Dary et al., 2010). In essence, they can be referred to as metal resistant bacteria because they have the ability to effectively sequester metals in heavy metal contaminated soils and also enhance plant growth (Fatnassi et al., 2014).

Bacillus subtilis is a ubiquitous Gram-positive, rod-shaped bacteria noted to enhance the development and growth of plants including providing defense against pathogens. Microorganisms with the ability to grow in the rhizosphere soils of plants are best for use as biocontrol agents since the rhizosphere serves as an interface for pathogens to attack plant roots. Many bacteria species, including *Bacillus*, are thought to have the potential for biocontrol against plant pathogens (Weller, 1988). There are reports of *Bacillus* spp. having the ability to control diseases on a wide variety of plant species and also bio-remediate contaminated soils (Sheng & Xia, 2006). Furthermore, the biological control approach using microorganisms was successfully used to control many potato diseases and the possibilities for biological control of the potato brown rot disease have been studied by many workers (Weller, 1988; Jacobsen, 2002; Verma et al., 2010). Many rhizosphere organisms referred to as “plant growth promoting rhizobacteria (PGPR)” are noted to positively influence plant growth and plant health (Schippers, 1992). Studies in the Netherlands suggest that PGPR stimulated potato growth mainly by quashing cyanide-producing compounds (Weller, 1988). There are few benefits for applying PGPR mechanism thus include but not limited to increases in germination rates, nodulation, etc. An earlier study showed that chickpea seed inoculation with PGPR increased nodulation, N uptake, growth and yield (Verma et al., 2010). PGPR can also be used for biocontrol of plant disease and phytoremediation of soils polluted with heavy metals. Inoculation with PGPR and *rhizobium* has been shown to increase the biomass of shoot and root, N₂ fixation and grain yield in chickpea (Verma et al., 2010). For example, inoculation of lentil with the *Rhizobacterium* strain RL9 in Zn contaminated soils caused an increase in dry matter (150%), nodule numbers (15%), nodule dry mass (27%), leghaemoglobin (30%), seed yield (10%) and grain protein (8%), compared with control (Ahmad Wani et al., 2007). However, climatic variability has been reported to impact the effectiveness of PGPR (Okon & Labandera-Gonzalez, 1994) especially under unfavourable field conditions, where they are expected to function normally such as in agricultural systems. Studies under different climatic conditions conducted by Bustamante and Ciampi (1989) also described *Pseudomonas fluorescens* as a soil treatment that protected 90% of potato seedlings against infection with bacterial wilt disease and lowered the population of the pathogenic bacteria. Other studies have demonstrated the potential of *Pseudomonas* strains to control soil-borne pathogens and enhance growth and yield of potato and radish plants (Burr et al., 1978; Klopper et al., 1980; Howie & Echandi, 1983). In addition, in a study by Prathibha and Siddalingeshwara (2013), they reported

that *Pseudomonas fluorescens* and *Bacillus subtilis* considerably improved seed germination, vigor index and nutritional quality (protein and carbohydrate content) of sorghum. In a recent study, Román-Ponce et al. (2017) reported that inoculation of *Brassica nigra* seeds with microorganisms such as strains of *Microbacterium* sp., and *Curtobacterium* sp. considerably improved seed germination and root development in soils loaded with heavy metal (Zn). These authors concluded that these rhizobacterial strains have potential as efficient bio-inoculants in phytoremediation of multiple heavy metals contaminated soils (Román-Ponce et al., 2017). However, the suitability of *B. subtilis* as a metal resistant bacteria especially in soils polluted with AMD water is yet to be explored including the impact of temperature on its activities as variations in climatic conditions can affect the activities of microorganisms and a plant's physiology as well (Okon & Labandera-Gonzalez, 1994).

2.4 EFFECT OF TEMPERATURE ON GROWTH AND PHYSIOLOGY OF PLANTS

By 2100, the current global temperature is predicted to increase by 1.8-4°C (Intergovernmental Panel on Climate Change (IPCC), 2007). Among environmental factors, temperature is considered as the most detrimental stress affecting plant growth and development. This is the case because numerous biochemical processes involved in plant activities are sensitive to heat stress due to elevated temperature. However, when exposed to changes in temperature regimes, plants have coping mechanisms that involve stress-induced biochemical and physiological alterations including shifts in primary and secondary metabolites profiles (Kaplan et al., 2004). The responses and coping mechanisms of plants to temperature vary across genera, species and sometimes between genotypes of the same species.

Generally, temperature is noted to affect various processes involved in a plant's growth and development starting from germination to seedling emergence and establishment and through to plant maturity including the yield and other biochemical and physiological processes. Therefore, it is important to investigate the effect of temperature on physiological and biochemical processes including photosynthesis, chlorophyll content, and stomatal conductance in order to enhance the productivity of crops exposed to elevated temperature (>30°C). For instance, pollination is severely affected in maize with exposure to an elevated (35°C) temperature from 30°C. Pollen viability was noted to decrease significantly with maize exposure to a temperature above 35°C (Dupuis & Dumas, 1990) and exposure to temperatures above 30°C was

noted to decrease grain yield in maize (Commuri & Jones, 2001) and rice (Kim et al., 2013).

So far, several investigations have established that amongst the environmental factors, temperature significantly stimulated seed germination (Rivers & Webber, 1971; White & Monstes, 1993; Craufurd et al., 1996), and the rate of seed germination and seedling emergence is influenced substantially by temperature (Garcia-Huidobro et al., 1982; Covell et al., 1986). For example, low temperatures at higher latitudes and highland tropical sites frequently caused a low percentage of seed germination resulting in poor stand formations (White & Monstes, 1993). Other studies showed that at 5°C or 10°C cowpea seeds failed to germinate however, at higher temperature (20°C-30°C), maximum germination (90%) was reached and only 85 to 88% at these temperatures; 15°C, 35°C and 40°C (Covell et al., 1986). Similar results were obtained showing that cooler temperatures (<15°C) reduced cowpea seeds germination rate and above 80% seed germination was obtained at constant temperatures (18°C-40°C), whereas elevated temperatures (>40°C) severely reduced seed germination and above 43.5°C, there were zero percentage of seed germinations for all genotypes (Craufurd et al., 1996). It is important to highlight that seed storage environment greatly influences the period of seed survival and the optimal temperature for germination is significantly affected by previous treatment of seed or its age (Auld & O'Connell, 1991). A study conducted on the effect of temperature seed germination of common bean found that among 16 genotypes, the base temperature varied from 5-8°C to 8-9°C with lowest germination percentage whereas optimum temperature ranged from 25-5°C to 29-3°C and provided high germination percentages (White & Monstes, 1993). It was also revealed that temperature affected seed germination rate; at 4.4°C germination was delayed until the 26th day but at 26.6°C, the germination began within one day (Rivers & Webber, 1971). Further reports showed that high temperatures generated by a fire could promote germination in species with a hard seed cover, by inducing the breaking of seed coats, thereby facilitating the subsequent embryo imbibition and radicle expansion (Auld & O'Connell, 1991).

In other studies, elevated temperatures have been noted to influence potato plant's growth and yield (Hijmans, 2003; Levy & Veilleux, 2007; Hancock et al., 2014) because several processes in potato physiology are impacted. For instance, the processes affected include tuber development, carbon transport/partitioning, CO₂

fixation, chlorophyll content and the photosynthetic performance of the potato plant in general thus leading to lower yields (Hancock et al., 2014). Furthermore, in response to elevated temperature there can be shifts in the leaf and tuber primary and secondary metabolites profiles of potato (Hancock et al., 2014). It is important to note that elevated temperature is associated with climate change, which if not mitigated, will significantly impact household food and nutrition security especially in poor rural communities in developing countries such as South Africa and other African countries.

2.5 CLIMATE CHANGE AND CROP PRODUCTION

In general, climate change may denote fluctuations in average weather conditions, or weather variations within the longer-term average context. According to Mcilveen (2010), climate change can be defined as climatic dissimilarities lasting for a period of time. The Intergovernmental Panel on Climate Change (IPCC), describes climate change as “any variation in climate over time, whether due to natural variability or as a result of human action” (IPCC, 2007). The IPCC definition, however, contrasts that of the United Nations Framework Convention on Climate Change (UNFCCC) (UNFCCC, 2013), where climate changes is the result of direct or indirect human activities modifying atmospheric composition over a period of time. Two main practical methods to address climate change are mitigation and adaptation (Harrison et al., 2010; UNFCCC, 2013). Modelling of climate change has become a modern tool in addressing this new phenomenon.

Analysis of climate model results seems to suggest that anthropogenic changes, particularly increases in greenhouse gases (GHG), are probably responsible for alterations in average weather conditions. Lately, global issues related to climate change are dominating environmental agenda forums as well as platforms grabbing with agricultural concerns, as shown by the upsurge of scientific articles dealing with climate change (Adams, 1989). Even if the course of greenhouse gases concentrations in the atmosphere could be forecast with confidence, future climates fate would still be indeterminate. Analysis of current climate models seems to suggest that they are not too reliable except global general circulation models (GCMs), which are large-scale mathematical depictions of the climate system (Kaiser, 1991). Forecast from GCMs on the climate system indicate that a doubling of atmospheric CO₂ will increase global average temperature from 1.5 to 4.5°C. In addition, it will modify precipitation amounts and frequency, however, other current climate models predict

an average increase of 2.8 to 5.2°C in average global temperature over the next century (Karl et al., 1990). Regardless of average global temperature increase, changes in weather is expected to impact crop and livestock production including other constituents of agricultural systems (Eitzinger et al., 2013). It is quite clear now that changes in the average global weather conditions alter rainfall patterns, evaporation levels, runoff and soil moisture storage, which as a consequence affect the growth and development of plants. In agreement with this view, it is reported that the incidence of inadequate moisture during different growth stages of plants (flowering, pollination and grain-filling) is harmful to most grain crops (e.g. cowpea, maize, soybean and wheat) (Eitzinger et al., 2013). Furthermore, extant literature shows that crop and livestock yields are directly affected by changes in climatic factors such as temperature and precipitation and the frequency and severity of extreme instances like droughts, floods, and wind storm (Adams, 1989). For instance, rice yield declines have been reported in south China as a result of climate change. Climatic change is expected to be less favourable for spring wheat and winter wheat yields in south China but opposite compared to northern region (Houxuan et al., 1993). Other studies in the east and central regions of China conclude that maize yields would decline if there is no increase in available moisture (Houxuan et al., 1993). In another interesting study conducted in China, modelling of soybean growth indicates yield expansions in the far north-east, but, notwithstanding faster growth, yield declines in most other areas (Smit & Yunlong, 1996). Clearly, the advent of climate change will significantly impact crop production directly and household food and nutrition indirectly. It is important to highlight that the climate change impact on crop yields, agricultural productivity and food security vary depending on the types of agricultural practices and methods (Watson et al., 1997). African agricultural production systems may significantly be affected by the increase in global warming (Watson et al., 1997). Developing countries i.e. India, Brazil, South Africa etc. rely more on farming for food and nutrient acquisition, and the majority of the farms are situated in areas already too hot or dry and the poor farmers are less able to adapt to changes in climate (Mendelsohn et al., 1994). The effects of global climate changes on agricultural production are likely to be small to moderate and in particular, large commercial farms are therefore more vulnerable to global warming than small household farms (Aydinalp & Cresser, 2008). In the advent of climate change, small household farmers dependent on subsistence farming are particularly vulnerable to food and nutrition insecurity. Notably, many of these at-risk populations are located in developing countries (Aydinalp & Cresser,

2008). Climate can be a significant determinant for future price trends therefore the steadiness of whole food systems may be at severe risk under climate change. It is easy to speculate that future food and crop prices will increase due to problems associated with climate change. According to Kaiser (1991), the prices for corn and soybeans will tend to be higher than for wheat and coarse grains and these price increases will be due to declines in overall production in developed countries e.g. European regions, U.S and Canada. Ultimately, these circumstances will result in food security being threatened. According to the United Nation's Food and Agriculture Organization (FAO) (2002), "food security refers to a "situation that exists when all people, at all times, have physical, social, and economic access to sufficient, safe, and nutritious food that meets their dietary needs and food preferences for an active and healthy life". Accordingly, everyone has a basic right to enough diet (FAO, 2002). All four dimensions of food security are affected by climate change i.e. food production and availability, the stability of food supplies, access to food and food consumption (IPCC, 2007). Over and above climate change, socio-economic impacts on food production, food security also depends on economic growth, changes to trade flows, stocks, and food aid policy (Prajapati et al., 2011). Climate change does not only have an impact on crop production but livestock as well. Analysis conducted exposes that the impacts of climate change differ across countries. Countries with cool environment are likely to encounter livestock losses from warmer temperatures due to the loss of beef cattle, and irrigated crops in presently hot regions such as Ethiopia and West Africa will suffer from global warming, although irrigated crops in the Nile Delta and Kenyan highlands will gain (Kurukulasuriya et al., 2011). In these instances, where global warming is increasing incredibly, farmers should consider growing crops such as cowpea which are resistance or adaptable to warm climatic conditions. Cowpeas have the ability to grow under difficult and stressful conditions in marginal areas (Ntombela, 2012). Cowpea's resilience in the different agro-ecological regions in sub-Saharan Africa is the main reason why it is the cheap number one protein source in the human diet. In an interesting field study conducted in Ghana and South Africa, with different agro-ecological climatic conditions, Belane (2011) investigated the effect of interaction of genotype and environment on plant growth and N₂ fixation of 30 cowpea genotypes. The author reported significant differences in the interaction effect on cowpea biomass yield, symbiotic parameters, grain yield and nutritional contents in both leaf and grain among the studied genotypes. Furthermore, the study revealed that cowpea leaves had more minerals (3-fold of Fe) compared to spinach, which is a

common vegetable in South Africa (Belane & Dakora, 2010; Belane, 2011). Notably, Fe is among the micronutrients that are deficient in most African diets. In Africa, cowpea is an essential component of food and nutrition security (Seed World Cowpeas (SWC), 2017).

2.6 COWPEA CULTIVATION FOR FOOD SECURITY

Cowpea (*Vigna unguiculata* L. Walp) is an important nutritious legume grain crop that is cultivated under different agro-ecological regions around the globe. In sub-Saharan Africa, it is a staple food for most households that provides cheap and rich nutrients such as protein, minerals and vitamins (Bressani, 1985) and fodder (Tarawali et al., 2002). Cowpea could be grown as a multi-purpose crop providing high protein grain for human consumption and crop residues of high nutritive value for animal feed and as a forage crop. In addition, as a staple food crop in sub-Saharan Africa, cowpea is commonly cultivated as an intercrop with other crops such as maize and sorghum including some vegetables such as okra and spinach, and recently in some traditional farms, it is intercropped with cassava and cotton (Rusinamhodzi et al., 2006). Cowpea as a legume, can contribute to soil fertility, through its nitrogen fixing abilities. One of the advantages of using cowpea in intercropping and rotational cropping systems in Africa is because of its ability to fix a large amount of N (up to 201 kg N ha⁻¹) (Dakora et al., 1987). Cowpea's contribution in terms of nitrogen provision and other benefits to cropping systems are well documented in Africa (Dakora et al., 1987; Ofori & Stern, 1987; Dakora & Keya, 1997; Ayisi et al., 2000; Caskey et al., 2001; Bado et al., 2006; Ncube et al., 2007; Adjei-Nsiah et al., 2008).

Cowpea is an important forage crop with high yield and quality and it is adaptable to harsh environmental conditions (Ayan et al., 2012). In semi-arid tropics covering Asia, Africa, Central and South America, cowpea is a valuable food legume and component of the traditional cropping systems. Cowpea has many uses: the young leaves, green pods and green seeds are used as vegetables and dry seeds are used in various food preparations (Singh et al., 2003). Cowpea hay is a nutritious balanced feed for animals in West Africa during the dry season and has an ample purpose in feeding animals (Tarawali et al., 2002). For a higher yield and quality, cowpea can be intercropped with other crops compared to single cropping. Cowpea is a drought resistant crop that has potential to withstand future climate scenarios associated with climate change and therefore, can be cultivated as legumes with low water requirement to replace the

water intensive conventional species in forage production systems (Rao & Shahid, 2011).

In sum, cowpea has a huge potential for symbiotic N₂ fixation and fits well into traditional cropping systems in most regions in Africa. The crop matures faster than most other crops and it's a cheap alternative source of nutrients and food that bridges the hunger-gap between previous and current harvests, therefore, it can serve as a food/nutrition security crop for rural communities in the advent of climate change in sub-Saharan Africa in particular and the world in general.

2.7 CONCLUSIONS

This chapter has identified AMD as a source of pollutant for surface water sources and irrigating of crops with contaminated water such as mine water has a long-term effect on both crop productivity and soil properties. Long-term irrigation with AMD water can bring changes in the quality of soil as trace element inputs are continued over a long period of time. Heavy metal contamination in agricultural soils has become a serious threat to crop production globally. Microorganisms (e.g. *B. subtilis*) have the potential for bioremediation of badly polluted waters such as AMD water. Climate change (elevated temperature) can significantly impact a plant's physiology and the production of important economic crops such as cowpea. The next chapter present findings on cowpea seed germination as affected by elevated temperature.

CHAPTER 3: SEED GERMINATION INDICES OF COWPEA EXPOSED TO ELEVATED TEMPERATURE AND *BACILLUS SUBTILIS* (BD233) INOCULATION

3.1 ABSTRACT

This experiment investigated the interactive effect of *Bacillus subtilis* (BD233) and elevated temperature treatment on seed germination indices and plumule lengths of three cowpea genotypes (Asetanapa, Soronko and Nyira) from northern Ghana. The experimental treatments included cowpea seeds treated with *B. subtilis* (BD233) (B+) and without *B. subtilis* (BD233) (B-), and each treatment was incubated at six different temperature regimes (10, 25, 30, 35, 40 and 45°C) for seven days. A standard germination test (three replicates of 30 seeds per genotype per treatment) repeated twice was carried out to determine the following germination indices; germination percentage (G%), germination index (GI) and germination rate index (GRI). The interaction of *B. subtilis* (BD233) and temperature significantly ($P < 0.05$) influenced the germination indices and plumule length of cowpea seedlings and the differences in genotype responses were also significant. The highest G% was for Nyira (98.6%) followed by Soronko (84.8%) seeds treated with *Bacillus* (B+) and without *Bacillus* (B-) respectively and incubated at 35°C. With or without *B. subtilis* (BD233), all three genotypes incubated at either 10, or 40 or 45°C had the lowest G% compared to the other temperatures. In general, Nyira seeds treated with *B. subtilis* (BD233) and Soronko seeds without the bacteria obtained the fastest germination rates (lowest GI & GRI values) compared to Asetanapa seeds treated either with or without the bacteria. Soronko seeds inoculated with *B. subtilis* (BD233) and incubated at 35°C for seven days had the longest plumule (1.40 cm) compared to the other genotypes under similar conditions. *B. subtilis* (BD233) inoculation has the potential to positively influence germination and growth of cowpea, an important candidate for food/nutrition security in Africa.

3.2 INTRODUCTION

Globally, the climate is changing rapidly and regional temperatures especially in tropical Africa are predicted to rise by 2-5°C over the next 50-100 years (IPCC, 2007). The impact of this forecasted rise in regional temperatures on vegetation requires a serious attention (Sershen et al., 2014) especially in tropical Africa where the majority of the population depends on subsistence farming for their livelihood, nutritional needs

and food/nutrition security. In particular, elevated temperatures associated with climate change have been reported to affect seed germinability (seedling viability and vigour) and seedling establishment in a number of crops (Han et al., 2009) including cowpea, which is believed to have evolved in tropical West Africa (Smartt, 1985).

Cowpea (*Vigna unguiculata* L. Walp) is an important crop that belongs to the family Fabaceae/Leguminosae (Padulosi & Ng, 1997). The crop is mostly cultivated because of its ability to contribute nitrogen to nutrient-poor soils in addition to providing affordable dietary protein, carbohydrate, minerals and vitamins for humans (Bressani, 1985). Cowpea grain is rich in protein (42%) and carbohydrate (57%) and the green leaves and young pods are capable of supplying up to 35% protein when consumed as vegetables (Kadam et al., 1984). The crop is cultivated in tropical and sub-tropical regions with relatively high temperatures (28-30°C). However, elevated temperatures (>35°C) can lead to the production of reactive oxygen species (ROS) that are harmful to plant seeds undergoing germination (Pukacka & Ratajczak, 2005). During seed germination, elevated temperatures due to the predicted global climate change scenarios can enhance ROS production, which can affect cell wall formation and radicle elongation (Bailly, 2004; Müller et al., 2009) and influence germination and growth of this important crop thereby posing threats to future food/nutrition security. In future, there would be changes in agricultural crop production/ food security due to climate change. High temperatures can have adverse effects on crop yields where crops suffer overall reduction in growth (Ewing, 1981). There is need to mitigate impact of elevated temperature on seed germination especially on important economic crops such as cowpea and cultivation of crops in general.

Interestingly, a ubiquitous microorganism, *Bacillus subtilis*, and temperature have been reported to stimulate seed germination in some plants (Paredes-Páliz et al., 2016). The use of *Bacillus species* in agriculture is increasing steadily as it offers an alternative to expensive synthetic chemical fertilizers. Certain strains of *Bacillus* spp. are noted to increase the growth and development of plant through several mechanisms but mainly by providing siderophores and phytohormones (auxins, cytokinins, gibberellins etc.) (Ali et al., 2009a) or enhancing nutrient uptake (solubilisation of minerals) from plant rhizosphere soils (Schwartz et al., 2013; Meng et al., 2016). A study has found that *B. subtilis* has the ability to quench soil ills to boost plant growth (Adesemoye et al., 2008). However, no studies have explored the interactive effect of *B. subtilis* and temperature on seed germination indices of cowpea,

an important crop in Africa and other regions of the world (Freire-Filho, 2011). The experiment aimed to investigate the effect of *B. subtilis* and elevated temperature on seed germination indices and plumule lengths of three cowpea genotypes (Asetanapa, Soronko and Nyira) and compare genotype responses across treatments.

3.3 MATERIALS AND METHODS

3.3.1 Seed Materials and Measurements

Seeds of three cowpea genotypes (Asetanapa, Soronko and Nyira) were obtained from the Crop Research Institute, Kumasi, Ghana. Thirty randomly selected seeds per genotype were used for measurement of their grain lengths and widths. The length and width of the seed were measured using a digital vernier caliper (Model DC–515).

3.3.2 Bacterial Strain

The bacterial strain (*Bacillus subtilis* (BD233)) used in this study was obtained from the National Collection of Bacterial Species of the Agricultural Research Council, Plant Protection Research Institute, Roodeplaat, South Africa. *Bacillus subtilis* (BD233) was cultivated in lysogeny broth at 37°C overnight and the pure cultures adjusted to 0.5 McFarland Standards with sterile distilled water before the germination experiment.

3.3.3 Germination Experiment and Experimental Design

In all experiments and for each genotype, 30 cowpea seeds were surface-sterilized by soaking in 70% ethanol for 90 s, and then in 1.5% bleach for 15 min, and then rinsed six times with sterile distilled water (Pule-Meulenberg et al., 2010). The experimental treatments included the following: 1) seeds treated with *B. subtilis* (BD233) (B+) and 2) seeds not treated with *B. subtilis* (BD233) (B-) and each treatment was incubated in darkness at six different temperature regimes (10, 25, 30, 35, 40 and 45°C) for seven days. A standard seed-germination experiment arranged in a complete randomized block design (three replicates of 30 seeds per genotype per treatment) was carried out under sterile conditions using 9 cm plastic Petri dishes and Whatman No. 1 filter paper discs. The filter paper discs were all kept moistened with sterile distilled water throughout the experiment. Each set of experiments was repeated twice.

In each experiment and for each treatment, the number of germinated seeds (radicle/plumule emergence) was recorded every day for seven days and the seedling plumule length measured (cm) after seven days. Normal seedling produced was used for seed germination indices evaluation (ISTA, 2006). Germination indices including germination percentage (G%), germination index (GI) and germination rate index (GRI) were calculated from germination data according to Olisa et al. (2010) as follows:

$$G\% = \frac{\text{No of emerged seedlings at final count}}{\text{Total number of seeds planted}} \times 100 \quad 1$$

$$GI = \frac{\sum(Nx)(DAS)}{\text{Total number of seedlings that emerged at the final count}} \quad 2$$

Where Nx is considered the number of seedlings that emerged on days after planting and DAS is the day after planting.

$$GRI = \frac{GI}{G\%} \quad 3$$

3.3.4 Data Analysis

The collected data were subjected to 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 $p < 0.05$.

3.4 RESULTS AND DISCUSSION

The aim of this experiment was to evaluate the interactive effect of *B. subtilis* and elevated temperature on the seed germination indices and growth of an important leguminous grain and forage crop, cowpea. One objective was to compare the responses of the different cowpea genotypes to *B. subtilis* inoculation and exposure to different temperature regimes. Clearly, cowpea seed characteristics analysis showed that the grains were different in seed coat colour and size. Nyira seeds were reddish-brown in colour and the smallest in size (5.87 mm (length) and 4.33 mm (width)) compared to the brown and largest Asetenapa seeds (8.33 mm (length) and 5.73 mm (width)) (Table 3.1). The differences in seed coat colour can be attributed to types of anthocyanin and other flavonoids synthesized by the plant (Holton & Cornish, 1995). Anthocyanin genes are found in almost all plants and are known to control the pigmentation of multiple plant parts (Chandler et al., 1989) including the seed coat colour in cowpea, which has an influence on consumer preference. In a classical

germination experiment in which seed sizes of cowpea genotypes from different African countries were compared in relation to germination, Craufurd et al. (1996) found a correlation between seed size and rate of seed germination. The authors reported that the smallest-seeded cowpea cultivar, TVu 946, had the fastest seed germination rate compared to the largest-seeded cultivar, TVu 8342, which had the slowest germination rate (Craufurd et al., 1996). Similar to this report, findings in this experiment revealed that the largest-seed genotype, Asetanapa, had a lower germination percentage/index and rate index when compared to the smallest-seeded genotype, Nyira (Tables 3.2 & 3.3, Figure 3.1).

Table 3. 1: Cowpea genotype seed measurements (n=30).

Genotype	Length (mm)	Width (mm)	Seed Coat Colour
Asetanapa	8.33±0.2a	5.73±0.2a	brown
Soronko	7.20±0.2b	5.07±0.1b	white
Nyira	5.87±0.2c	4.33±0.2c	Reddish-brown

Values (M±S.E.) followed by dissimilar letters within a column are significantly different at $p \leq 0.05$.

Another objective was to evaluate the interactive effect of *B. subtilis* (BD233) inoculation and incubation temperature on the germination indices (G%, GI, & GRI) of three cowpea genotypes obtained from Ghana in tropical West Africa. According to findings in this experiment, the interaction of *B. subtilis* (BD233) and temperature significantly ($p < 0.05$) influenced the germination indices of all three cowpea genotypes. The highest G% was obtained for Nyira seeds (98.6%) treated with *B. subtilis* and incubated at an elevated temperature of 35°C (Table 3.2). Elevated temperature significantly affected seed germination percentages as all three cowpea genotypes incubated at 10°C and supra temperature (45°C) had the lowest seed germination percentages (0%) compared to those incubated at temperatures of 25°C, 30°C and 35°C (Table 3.2). The cardinal germination temperatures of cowpea seeds are 25°C-40°C but in this experiment, treating seeds of cowpea genotypes with *B. subtilis* (BD233) showed enhanced germination percentages when incubated at a temperature range of 25-35°C. For example, Nyira seeds treated with *B. subtilis* (BD233) and incubated at 35°C had a significantly higher germination percentage (98.6%) compared to those incubated at an ambient temperature of 30°C (50.3%). For both Nyira and Soronko genotypes, the temperature range for optimum seed germination percentage was 25-35°C regardless of the treatment, but all three

genotypes showed 0% seed germination at 10°C in this experiment (Table 3.2). Similar to this finding, Covell et al. (1986) and Craufurd et al. (1996) reported lack of seed germination of some cowpea genotypes from West Africa at temperatures below 10°C and maximum seed germination at temperatures of 35.8°C and 35°C respectively. In another study, Motsa et al. (2015) reported optimum seed germination of cowpea at 36°C with a cardinal temperature of 30-36°C. The noted differences in optimum and cardinal temperatures of these studies when compared to the present study findings could be attributed to genetic variability among the seeds used in each of these studies (Mohammed et al., 1988) or to seed accession differences in latitude (Saeidnejad et al., 2012).

In this experiment, treating seeds of both Nyira and Soronko with *B. subtilis* (BD233) enhanced germination percentage (98.6%) when compared to those not treated with the bacteria (50.8%) and incubated at optimum elevated temperature (30 and 35°C) (Table 3.2). In agreement with findings in this experiment, Manjula and Podile (2001) reported that *Bacillus subtilis* AF 1 in peat formulations significantly stimulated percentage seed germination of two leguminous crops (groundnut and pigeon pea). Similarly, Niranjan et al. (2003) reported a positive influence of two strains of *Bacillus* on the germination and growth of pearl millet.

Table 3. 2: Seed germination percentage (G%) of cowpea genotypes inoculated with *B. subtilis* (B+) or without *B. subtilis* (B-) and incubated at different temperatures.

Temp. (°C)	Germination (%)					
	Asetenapa		Soronko		Nyira	
	(B+)	(B-)	(B+)	(B-)	(B+)	(B-)
0	0.0±0.0dA	0.0±0.0bA	0.0±0.0cA	0.0±0.0cA	0.0±0.0dA	0.0±0.0dA
25	14.1±0.9bC	5.3±0.3aC	80.0±6.9aA	81.1±4.4aA	37.8±2.9cB	12.2±1.2cC
30	30.2±2.4aC	6.2±0.7aD	78.4±7.3aA	84.4±2.1aA	50.3±5.5bB	38.9±2.9bC
35	9.4±0.8cC	6.7±0.5aC	78.7±5.8aA	84.8±2.2aA	98.6±1.4aA	50.8±5.4aB
40	0.0±0.0dC	0.0±0.0bC	19.8±0.1bA	8.9±0.1bB	0.0±0.0dC	0.0±0.0dC
45	0.0±0.0dA	0.0±0.0bA	0.0±0.0cA	0.0±0.0cA	0.0±0.0dA	0.0±0.0dA

Values (M±S.E.) followed by dissimilar letters (in lower case) within a column for each genotype are significantly different at $p \leq 0.05$. Values (M±S.E.) followed by dissimilar letters (in upper case) within a row for each genotype are significantly different at $p \leq 0.05$.

Similarly, the rate of seed germination was affected by inoculation with *B. subtilis* (BD233) as significant differences in other germination indices were revealed after four days' incubation at 35°C temperature (Table 3.3). Inoculation with *B. subtilis* (BD233) enhanced the germination rate of Soronko seeds compared to Nyira. The smaller the GI and GRI values of seeds, the faster is their rate of germination (Adetumbi et al., 2011). Seeds of Nyira inoculated with *B. subtilis* (BD233) and incubated at 35°C for four days showed a higher G% (67.3%) compared to Soronko (55.5%) and Asetenapa (7.7%) under the same conditions (Table 3.3). This finding implies that Nyira seeds (smallest in size) germinated at a faster rate compared to Soronko seeds. Similar to this finding, the germination indices of two elite cowpea genotypes (Ife BPC and Ife Brown) were affected significantly by storage condition (Adetumbi et al., 2011). In their study, Ife BPC had smaller GI and GRI values with the fastest germination rate but a lower final germination percentage compared to Ife Brown, which also indicated differences in cultivar responses. In this experiment, differences in genotype responses to *B. subtilis* (BD233) inoculation and incubation temperature were also noted.

Table 3. 3: Seed germination indices of cowpea genotypes inoculated with *B. subtilis* (B+) or without *B. subtilis* (B-) and incubated for four days at 35°C temperature.

Cultivar	G(%)		GI		GRI	
	(B+)	(B-)	(B+)	(B-)	(B+)	(B-)
Asetenapa	7.7c	5.0c	2.23c	0.04b	0.06a	0.06a
Soronko	55.5b	44.4a	3.08a	0.03b	0.06a	0.06a
Nyira	67.3a	38.8b	2.93b	0.14a	0.03b	0.07a

Values (M±S.E.) followed by dissimilar letters within a column are significantly different at $p \leq 0.05$.

Interestingly, genotypic differences were noted in the cumulative seed germination percentages for cowpea inoculated with *B. subtilis* (BD233) over the experimental period. There were significant differences in rate of achieving over 50% seed germination amongst the three genotypes at all incubation temperatures (Figure 3.1). At both 25°C and 30°C, only Soronko seeds inoculated with *B. subtilis* (BD233) attained above 50% seed germination after four days' incubation compared to seeds of the other two genotypes. At 35°C, both Soronko and Nyira seeds achieved 50% germination at four days of incubation, and at 40°C, the highest seed germination was obtained for Soronko but it was below 50% at seven days of incubation (Figure 3.1).

Similarly, the interaction of *B. subtilis* and temperature also significantly ($p < 0.05$) influenced the growth of cowpea seedlings. Soronko seeds inoculated with *B. subtilis* (BD233) and incubated at 35°C for seven days had the longest seedling plumule (1.40 cm) compared to seedlings of the other genotypes under similar conditions. At 25°C, with or without *B. subtilis* (BD233) inoculation, Soronko seedling plumule was significantly longer than those of the other genotypes. Inoculation with *B. subtilis* (BD233) markedly increased the plumule length of the test genotypes when compared to those evaluated at different temperature regimes without the bacteria (Table 3.4). For example, Asetenape inoculated with the bacteria showed greater plumule length at 30°C while Soronko at 35 and 40°C, and Nyira at 25°C, 30°C, and 35°C (Table 3.4). The enhanced plumule length of Soronko and Nyira seeds inoculated with the bacteria is a clear indication that *B. subtilis* (BD233) has the potential to influence growth of cowpea even at a higher temperature. In agreement, *Bacillus* sp. was also reported to enhance the growth and yield of French beans (Saxena et al. 2013), sorghum

(Prathibha & Siddalingeshwara, 2013), groundnut and pigeon pea (Manjula & Podile, 2001), soybean (Bai et al., 2003) and pearl millet (Niranjan et al., 2013).

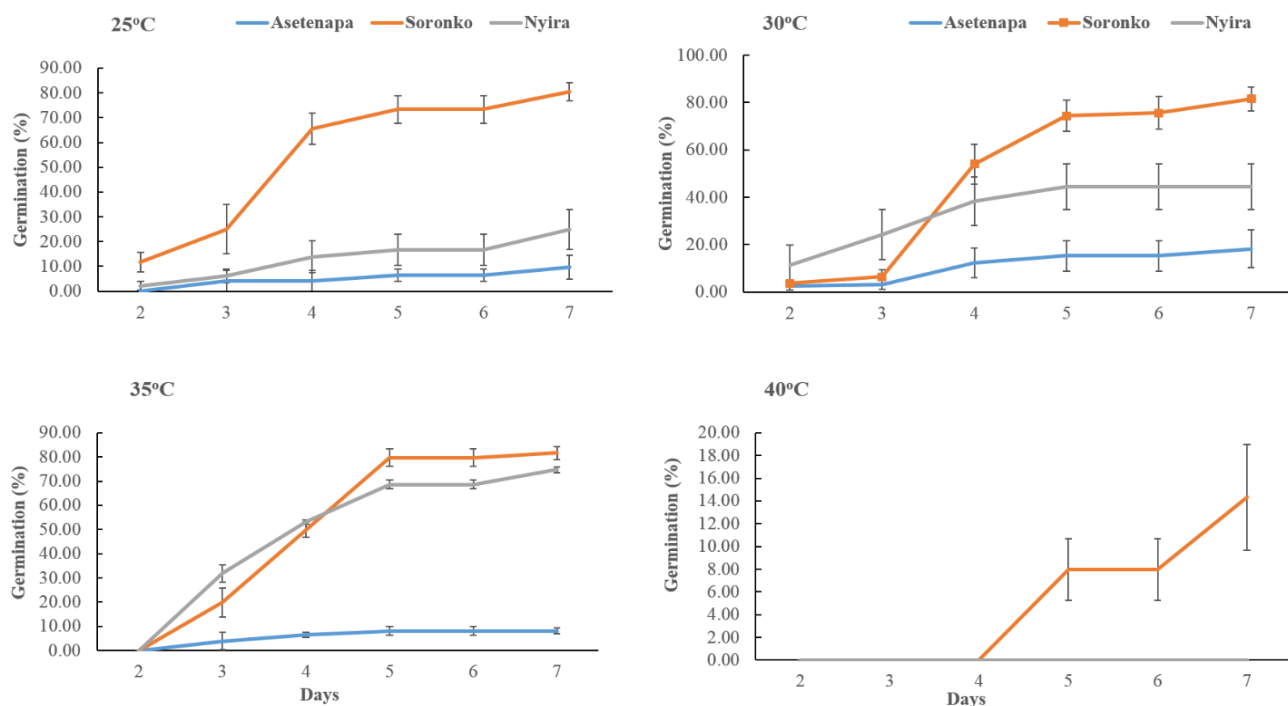


Figure 3.1: Cumulative germination percentages (G%) of cowpea genotypes inoculated with *B. subtilis* (BD233) and incubated at different temperatures (25, 30, 35 and 40°C).

Table 3. 4: Seedling plumule length of cowpea genotypes inoculated with *B. subtilis* (B+) or without *B. subtilis* (B-) and incubated at different temperatures.

Temp. (°C)	Plumule length (cm)					
	Asetenapa		Soronko		Nyira	
	(B+)	(B-)	(B+)	(B-)	(B+)	(B-)
25	0.13±0.0bC	0.08±0.0aC	0.92±0.1bA	1.11±0.1aA	0.56±0.0bB	0.17±0.0bC
30	0.48±0.0aB	0.07±0.0aC	0.93±0.1bA	1.08±0.1aA	0.61±0.0bB	0.15±0.0bC
35	0.08±0.0cD	0.05±0.0bD	1.40±0.1aA	1.14±0.1aB	1.08±0.1aB	0.56±0.0aC
40	0.00±0.0cC	0.00±0.0cC	0.42±0.0cA	0.24±0.0bB	0.00±0.0cC	0.00±0.0cC

Values (M±S.E.) followed by dissimilar letters (in lower case) within a column for each genotype are significantly different at $p \leq 0.05$. Values (M±S.E.) followed by dissimilar letters (in upper case) within a row for each genotype are significantly different at $p \leq 0.05$.

3.5 CONCLUSIONS

The interaction of *B. subtilis* (BD233) and temperature significantly influenced the germination indices and plumule lengths of all three cowpea genotypes included in this experiment. Findings in this experiment show that the cardinal temperature for the three West African cowpea genotypes was 25-35°C and the seed germination percentage ranged from 78.4 to 98.6%. Clearly, inoculation with *B. subtilis* (BD233) significantly influenced seed germination and growth of all three cowpea genotypes at elevated temperatures. *B. subtilis* (BD233) inoculation has potential to positively influence the germination and growth of cowpea, an important candidate for food/nutrition security in Africa under future climate change scenarios.

CHAPTER 4: GROWTH AND NUTRITIONAL RESPONSES OF COWPEA (Cv. Soronko) TO SHORT-TERM ELEVATED TEMPERATURE

4.1 ABSTRACT

An evaluation of the effect of elevated temperature on the growth and nutritional quality of cowpea was undertaken in this experiment. Surface sterilized seeds of cowpea (Cv. Soronko) were germinated in pots in the glasshouse. At different growth stages (pre-flowering (40 DAP), flowering (90 DAP) and post-flowering (123 DAP)), plants were incubated in growth chambers set at three different temperature regimes (25, 30, & 35°C) for a period of seven days. Whole plant biomass (fresh and dry weight), and shoot carbon and crude protein contents were significantly affected with increase in temperature at all three stages of the plants' growth. At an elevated temperature (35°C), the pre-flowering and flowering stages of cowpea were most affected compared to control (25°C). The results suggest that the pre-flowering and flowering stages of cowpea compared to post-flowering are more susceptible to elevated temperatures (30-35°C).

4.2 INTRODUCTION

Cowpea (*Vigna unguiculata* L. Walp) is an important nutritious legume cultivated in most regions in Africa including South Africa. In South Africa, rural farmers cultivate cowpeas for subsistence and source of income and nutrients but also to a lesser extent as feed for livestock (Kritzing, 2004), whereas the crop is utilised largely for other purposes in other countries in Central and West Africa (Singh et al. 2002). For example, in West Africa, the leaves are eaten as vegetables. The seeds can be cooked and eaten with as a meal. When the young green leaves of cowpea are eaten as a vegetable, it can provide between 27.1 and 34.7% protein to humans and animals (Jasper & Norman, 1983; Ahenkora et al., 1998). Mature seeds contain per edible portion (100 g): 56-66 g carbohydrate, 22-24 g protein, 11 g water, 5.9-7.3 g crude fibre, 3.4-3.9 g ash, 1.3-1.5 g fat, and minerals such P, Ca, and Fe (0.146 g, 0.104-0.076 g, & 0.005 g respectively) (Asante et al., 2006). The edible grain of cowpea is suggested to contain a protein range of 22-25% (Oyenuga 1959; Platt, 1962), and carbohydrates and ash contents of 56.8% and 3.6%, respectively (Platt, 1962). A research conducted on the nutritional value of the cowpea leaves and immature pods, recommends that the leaves have similar nutrition value to black nightshade and sweet potatoes leaves, while the green pods have less anti-nutritional factors than the

dried seeds (Gonçalves et al., 2016). Clearly, the nutritional benefit associated with the grain and leaves of cowpea is not well documented, however, the crop is a cheap source of nutrients for rural communities in South Africa. This legume crop has the advantage of growing in poor-nutrient soils in marginal areas in the country because of its ability to perform symbiotic nitrogen fixation with microorganisms (rhizobacteria) (Belane, 2011). In addition, the crop is tolerant to heat and drought associated with climate change in marginal areas, and therefore can be considered as a climate-change friendly crop (Awika & Duodu, 2017).

Globally, changes in climate can alter rainfall patterns, evaporation, runoff and soil moisture storage in most regions including areas under agricultural cultivation, which as a result affects the growth and development of crops. It is reported that the occurrence of insufficient moisture during flowering, pollination and grain-filling is harmful to most crops (Eitzinger et al., 2010). In addition, increases in average temperatures or short-term temperature increases causing high-temperature stress on plants are associated with changes in climate change. Changes in temperature are noted to affect the physiological processes and metabolite profile of plants including nutritional status, which ultimately affects their growth and development. Earlier studies have reported on the sensitivity of the different growth stages of plants to climatic conditions (Prasad et al., 2002; Hatfield et al., 2008; Hatfield & Prueger, 2015) including legumes. Agricultural systems in the tropics are expected to be impacted by drastic short-term changes in temperature and soil moisture. In particular, African agricultural production may significantly be affected by the increase in global warming associated with climate change (Watson et al., 1997). In developing sub-Saharan countries such as South Africa, majority of cowpea cultivation is located in places that are already too hot or dry, and poor farmers in rural areas, are less able to adapt (Mendelsohn et al., 1994). In Sub-Saharan Africa, cowpea has received more research attention leading to breeding for earliness, high grain yield, and pest and disease resistance (Singh & Ntare, 1985). However, there is need for rural farmers to cultivate cowpea genotypes that are tolerant to future elevated temperatures. In addition, literature is scarce on the effect of elevated temperature on the crop's nutrition especially at different growth stages. Therefore, the aim of the experiment was to evaluate the effect of short-term elevated temperature on the nutritional quality of cowpea at different growth stages.

4.3 MATERIALS AND METHODS

4.3.1 Plant Material

Among the three cowpea genotypes employed in this study, the Soronko genotype was noted to have the highest germination percentage and longest plumule length at elevated temperature (35°C) and was therefore selected for this experiment. The seeds were obtained as described in Chapter 3, section 3.31.

4.3.2 Plant Growth Media and Experimental Conditions

Acid washed sand was obtained from Masstores (Pty) Ltd, Sunnyhill, Johannesburg, South Africa. The acid sand was thoroughly washed using tap water to remove nutrients. One litre planting pots (14-inch) were used for the experiment. Surfaced-sterilized cowpea (cv Soronko) seeds (3) were germinated in pots containing acid washed sand and the emergent seedlings were thinned to one per pot. The seedlings were grown in a glasshouse set at a day/night temperature regime of 25/18°C with a supplementary light intensity of 300 $\mu\text{mol m}^{-2}\text{s}^{-1}$ and an overhead irrigation system was used with each plant receiving 63 mL of water a day split into three regimes of 5 mins each. The experiment was conducted at the University of South Africa (Unisa) Florida campus, Gauteng Province (26° 10' 30" S, 27° 55' 22.8" E).

4.3.3 Fertilisation of Cowpea

Seedlings of cowpea in pots were grown with modified ¼-strength N-free solution as a source of nutrients (Broughton & Dilworth, 1970). Stock solutions of the N-free nutrient was prepared as described by Broughton and Dilworth (1970) and details of salts used are shown in Table 4.1 below.

Table 4. 1: N-free plant nutrients and their concentrations in stock solutions.

Stock Solutions	Form	Source/Element	Mass/Litre	Concentration
A	CaCl ₂ .2H ₂ O	Ca	294.1	2.0 M
B	KH ₂ PO ₄	P	136.1	1.0 M
C	Fe-citrate	Fe	607	20.0 mM
	MgSO ₄ .7H ₂ O	Mg	123.3	0.5 M
	K ₂ SO ₄	K	87.0	0.5 M
	MnSO ₄ .2H ₂ O	Mn	0.338	2.0 mM
D	H ₃ BO ₃	B	0.247	4.0 mM
	ZnSO ₄ .7H ₂ O	Zn	0.288	1.0 mM
	CuSO ₄ .5H ₂ O	Cu	0.100	0.4 mM
	CoSO ₄ .7H ₂ O	Co	0.056	0.2 mM
	NaMoO ₂ .2H ₂ O	Mo	0.048	0.2 mM

For each 10 L of full strength stock solution, 5.0 mL of each of solutions (A-D) was taken and added to 5 L of water, then diluted to 10 L. One N NaOH was used to adjust the pH to 6.6-6.8 (Broughton & Dilworth, 1970). The seedlings were irrigated with the N-free nutrients solutions twice during the seven days in growth chambers (Nüve test cabinet TK 120, China) (Figure 4.1).

Commercial inoculants for cowpea were obtained from STIMUPLANTS CC in Pretoria. Specifically, a packet of the powdered inoculant with *Bradyrhizobium spp* CB756 strains was used for inoculation of cowpea seedlings. About 2 g of the powder inoculant was mixed with 500 mL of distilled water and a sticker and applied as a slurry to cowpea seeds before planting. Two weeks after seedling establishment the same mixture of inoculant was applied close to the root of seedlings to ensure inoculation of each plant.

4.3.4 Experimental Treatments: Biomass Determination and Nutrient Analysis

4.3.4.1 Cowpea growth and nutritional responses to elevated temperature

For this experiment, three seedlings of cowpea (cv Soronko) were exposed to three temperature regimes (25°C (control), 30 & 35°C (elevated)) as treatments for a period of seven days. For each of the above-mentioned treatments, three seedlings of similar phenology were randomly selected from seedlings grown in the glasshouse at three different growth and developmental stages (pre-flowering (40 days after planting (DAP)), flowering (90 DAP) and post-flowering (123 DAP)). The plants were incubated in three separate growth chambers set at the different temperatures for a one-week period (Figure 4.1). In each growth chamber, the humidity was set and maintained at 70% and the lights were on (00h00-12h00) and off (12h01-23h59) for 12 h for seven days. Seedlings were irrigated with tap water every 24 h for seven days and fertilized with modified ¼-strength N-free solution twice in seven days. The three different stages of the plant's development (pre-flowering, flowering and post-flowering) exposed to the different temperature regimes were assessed after seven days of incubation for the effect of temperature on the growth and nutritional quality of cowpea.

Throughout the experiment there were no incidences of plant diseases and pest and the plants were healthy before and during the 7-day incubation period.



Figure 4.1. Growth chamber- Nüve TK Series Test Cabinets (Nüve test cabinet (model TK 120))

4.3.4.2 Plant biomass

For each treatment, the fresh and dry weights of the whole plant (shoot and root) were determined using a weighing balance. For whole plant fresh weight (FW) determination, after seven days' incubation all three plants were thoroughly washed with tap water and immediately dried using paper towels before weighting. For dry weight (DW) determination, the harvested plants were oven-dried at 60°C for 72 h to constant weight and weighed. The plant biomass (whole plant fresh and dry weights) was determined for each growth stage exposed to the treatments.

4.3.5 Determination of Nitrogen (Crude Protein) and Carbon

4.3.5.1 Sample preparation

For this experiment, finely ground oven-dried shoot of plants exposed to the three different temperature regimes (treatments) were used. Sample grinding was done using a mechanical mill (Knifetec 1095 Sample Mill) and passed through an 80-mesh sieve to obtain uniform particles. Samples were uniform powder finely ground and homogenized for repeatable and consistent results. Typically, samples should be ground to a fineness of <0.5 mm. About 0.1-0.5 g of sample was weighed for each sample analysis.

4.3.5.2 Sample analysis

Determination of nitrogen/protein and carbon in samples was done using LECO Trumac CN analyser (832 series, USA) (Figure 4.2). Approximately 0.3 g of plant sample was weighed into a crucible transferred to a suitable position of the autoloader. These steps were repeated for each sample to be analysed.



Figure 4.2. LECO Trumac C/N analyser

Before each sample analysis, pre-weighed homogeneous samples were placed into a large ceramic boat and loaded into the purge chamber located in the front of the horizontal ceramic high-temperature furnace. After the entrained atmospheric gas was purged from the sample, the ceramic boat was introduced into the furnace regulated at a temperature of 1100 to 1450°C. Complete oxidation of the macro sample was ensured by a pure oxygen environment within the furnace, with additional oxygen being directed onto the sample via a ceramic lance. The ceramic boat and all ash from the sample were removed from the furnace at the end of combustion, leaving the furnace free of ash build-up. For each treatment applied to the three different growth stages, three replicate shoot samples were analysed for the concentration of its crude protein/nitrogen and carbon contents.

4.3.6 Data Analysis

Data on growth parameters (whole plant biomass (FW & DW)) and nutritional contents (CN) of cowpea shoot were analyzed using one-way ANOVA analysis. All parameters and concentration measurements were tested at $p < 0.05$ significance level and the Duncan multiple range test was used for separation between treatment means.

Statistica v. 10, StatSoft (StatSoft Inc., Tulsa, OK, USA)) was used for all statistical analysis.

4.4 RESULTS AND DISCUSSION

4.4.1 Cowpea Growth Response to Short-term Elevated Temperature

In this experiment, short-term elevated temperature significantly ($p < 0.05$) affected all three growth stages of cowpea (cv Soronko) incubated in growth chambers (Table 4.2). For each cowpea growth stage exposed to a 7-day elevated temperature (30–35°C), the whole plant (stem + leaf + root) biomass decreased significantly compared to control (25°C). For example, at 40 DAP (pre-flowering stage), in comparison with control, the whole plant fresh and dry weights of cowpea exposed to elevated temperature (35°C) was reduced by 42 and 29%, respectively, and at 30°C, cowpea whole plant fresh and dry weights reduced by 30 and 52% respectively (Table 4.2). At 90 DAP (flowering/anthesis stage), in comparison with control, the cowpea whole plant fresh and dry weights exposed to elevated temperature (35°C) was reduced by 47% and 63% respectively and at 30°C, the fresh and dry weights reduced by 31% and 60% respectively (Table 4.2). Similarly, at 123 DAP (post-flowering stage) the growth response of cowpea was affected by a higher temperature (30°C) compared with control. The whole plant fresh and dry weights of cowpea reduced by 31% and 60% respectively when exposed to a high temperature (30°C) compared with control, however, no significant difference in whole plant biomass was noted between elevated temperature (35%) and control (25°C) (Table 4.2).

Table 4. 2: Effects of temperature on the plant biomass (g/plant) (fresh weight (FW) and dry weight (DW)) at different growth stages of cowpea grown in a growth chamber.

Temp (°C)	Whole plant biomass (g/plant)					
	Pre-flowering (40 DAP)		Flowering (90 DAP)		Post-flowering (123 DAP)	
	FW	DW	FW	DW	FW	DW
35	5.6±0.8b	3.2±0.5b	47.3±9.3c	6.3±1.4b	92.6±8.8a	18.6±4.8a
30	6.7±1.2b	2.3±0.9b	62.0±1.3b	7.0±2.9b	81.3±8.8b	14.6±2.7b
25	9.6±2.4a	4.8±1.6a	89.6±8.6a	17.3±4.3a	93.6±34.8a	18.3±6.3a

Values (M±S.E. (n=3)) followed by similar letters in a column are not significantly different at $p \leq 0.05$.

As several authors have reported, plant growth rate throughout their life cycle is affected by temperature extremes (Prasad et al., 2002; Hatfield et al., 2008; Hatfield & Prueger, 2015; Kondache et al., 2018) and the range differs among species (Lafta & Lorenzen, 1995). Across all plant species, the reproductive growth stage is more sensitive to temperature extreme and pollination is one of the most susceptible phenological stages (Hatfield & Prueger, 2015). In general, in the plant's life cycle, anthesis and early grain filling stages are more sensitive to heat shock events for most crops (Stone & Nicolas, 1994; Barlow et al., 2015). In this experiment, the pre-flowering and flowering stages of cowpea (cv Soronko) were more affected by temperature extremes compared to post-flowering. At 90 DAP (flowering/anthesis), plants exposed to elevated temperature (35°C) showed the highest reduction in whole plant biomass (47% and 63% in FW & DW respectively) compared with those exposed to the same temperature at other growth stages (42 and 29% at pre-flowering and 1% and 0% at post-flowering). A recent study showed that heat stress impacted the reproductive stage; the flower bud initiation, decreases the number and size of flowers, deformed floral organs resulting in loss of flowers and young pods therefore reduction in seed yield of legumes (Sita et al., 2017). Similar to this finding, the vegetative and reproductive growth stages of maize development were most affected by exposure to elevated temperature (30-35°C), and in particular, because of decreased pollen viability (Hatfield et al., 2008), the grain yield was reduced by 80-90% compared to control (Hatfield & Prueger, 2015). In another related study, high temperature (31/29°C day/night) in growth chambers significantly reduced plant biomass of two potato genotypes (total dry weights reduced by 44% and 72% in cv Norchip and cv Up-to-date, respectively) and the tuber growth was more affected compared to shoot growth (Lafta & Lorenzen, 1995). Similarly, the growth stages of wheat plant were affected differently when exposed to heat shocks over short periods. Grain number reduction was highest when heat shock was applied two days prior to anthesis but was lower by two levels when applied two days after anthesis. The grain number was reduced significantly by 12-22% in five individual genotypes of wheat when exposed to heat shock 10-30 days after anthesis compared to no significant reduction in the remaining 75 genotypes tested (Stone & Nicolas, 1995). The insignificant change in whole plant biomass at post-flowering when the control was compared with exposed 35°C (Table 4.2) would suggest that the plant has the capacity to acclimate to short-term heat events (Barlow et al., 2015). Heat stress tolerance mechanisms in plants has been linked to production of heat shock proteins and in wheat plants, heat shock was

associated to reduction of grain yield and translocation of photosynthates to grains as well as starch synthesis (Acevedo et al., 2002; Barlow et al., 2015).

4.4.2 Effect of Short-term Elevated Temperature on Cowpea Crude Protein and Carbon Content

Similarly, short-term elevated (30-35°C) temperature significantly ($p < 0.05$) affected the nutritional quality of cowpea at different growth stages. The crude protein content (Nitrogen value (%)) in shoot of cowpea (cv Soronko) was significantly affected compared to control at both the pre-flowering and flowering stages after a seven-day period incubation in growth chambers (Figure 4.3).

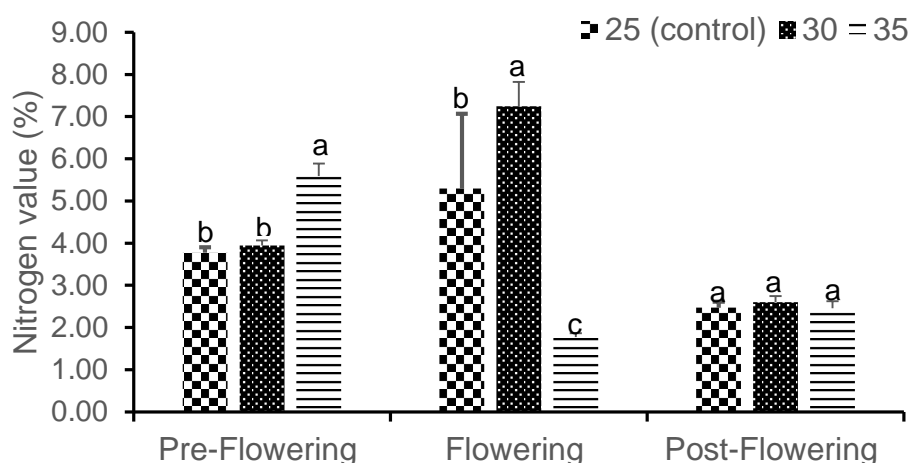


Figure 4.3: Effect of short-term elevated temperature on the nutritional content (Nitrogen value (%)) of cowpea cv Soronko shoot at different growth stages.

At 40 DAP (pre-flowering), the shoot crude protein content (5.59 N%) of cowpea cv Soronko exposed to short-term elevated temperature (35°C) was increased significantly compared to control (3.77 N%). In contrast, at the flowering stage (90 DAP), elevated temperature (35°C) significantly reduced the crude protein content (1.77%) of the shoot compared to control (5.59%). Interestingly, at 30°C, the nitrogen level (%) was significantly higher in the shoots compared to control during the flowering stage. However, at post-flowering (123 DAP), elevated temperatures (30°C & 35°C) compared to control had no significant effect on the crude protein content in shoots of cowpea cv Soronko plants (Figure 4.3). Clearly, this finding further showed the sensitivity to elevated temperature of the flowering stage in cowpea plants' growth and development. During the flowering stage, the crude protein content in shoots was

drastically increased at 30°C and reduced at 35°C compared to control. In agreement with this finding, short-term/abrupt heat stress reduced the total protein content, nutrient-uptake levels and assimilation proteins in roots of tomato plants compared to control (Giri et al., 2017) especially at the flowering stage.

Similarly, the carbon content in shoot of cowpea (cv Soronko) at both the pre-flowering and flowering stages were affected by short-term elevated temperature compared to control. At the pre-flowering stage, the carbon content in shoot of cowpea (cv Soronko) was increased significantly at 30°C (16.7%) compared to control (15%), however, at both the flowering and post-flowering stages no significant changes in shoot carbon contents were noted but there was a slight reduction and increase at the flowering stage and post-flowering (respectively) compared to control (Figure 4.4).

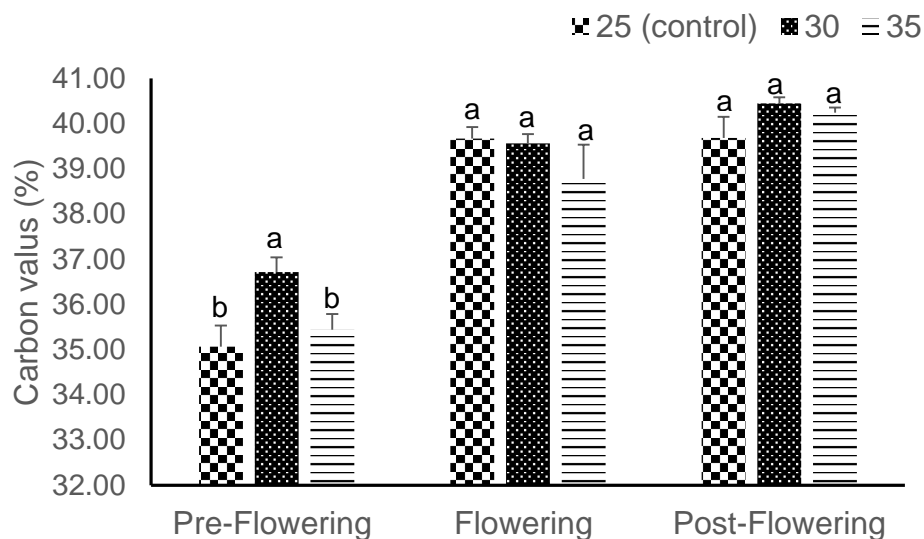


Figure 4.4: Effect of short-term elevated temperature on the nutritional content (Carbon value (%)) of cowpea cv Soronko shoot at different growth stages.

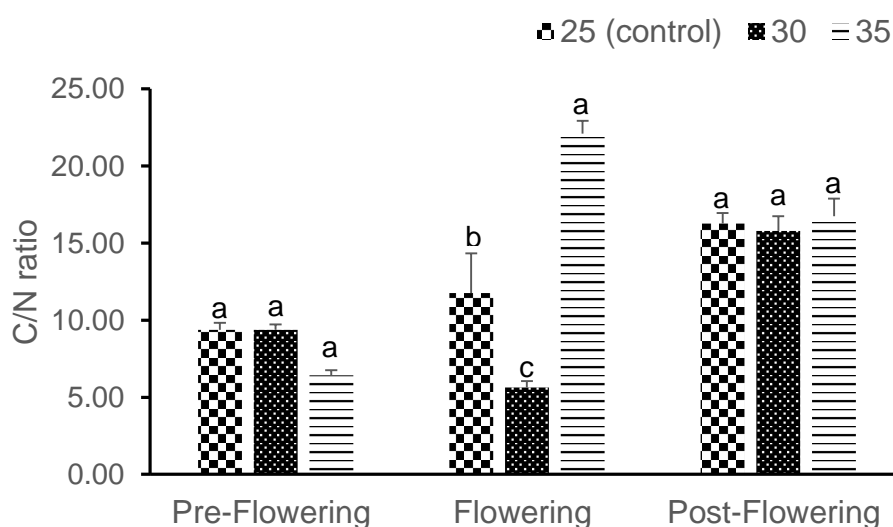


Figure 4.5: Effect of short-term elevated temperature on the nutritional content (C/N ratio) of cowpea cv Soronko shoot at different growth stages.

Comparison of the C/N ratios in shoots of cowpea cv Soronko showed that short-term elevated temperature similarly affected accumulation of nutrients during the different growth stages. For example, at the pre-flowering growth stage, elevated temperature (35°C) slightly reduced the C/N ratio compared to control and at the flowering stage, the C/N ratio was significantly increased at 35°C (22.1%) compared to control (11.8%). The C/N ratio in shoots of cowpea cv Soronko exposed to short-term elevated temperature showed significant differences only at the flowering growth stage (Figure 4.5). During both the pre-and post-flowering stages, there were no significant changes in the C/N ratio in shoot of cowpea cv Soronko incubated at elevated temperature compared to control. Clearly, the CN ratio results further demonstrated the susceptibility of the flowering stage to elevated temperature in the tested crop.

In this experiment, the susceptibility of the flowering stage (90 DAP) to short-term elevated temperature (Figures 4.3-4.5) noted in changes in cowpea cv Soronko's shoot crude protein and carbon content has been demonstrated and it has implications for the crop's grain nutritional quality and yield. At anthesis, the plant's pollination process can be impacted by elevated temperature, which can result in changes in the number of grains (Barlow et al., 2015). Heat shock is reported to impact mostly grain yield during anthesis to early grain filling in wheat crop (Barlow et al., 2015).

Although nutrients in cowpea shoots were determined in this experiment, notably, elevated temperature is reported to negatively affect the nutrient sink-source relationship between the root and shoot (Huang et al., 2012), which implies that decreases in root nutrients will reduce nutrient transfers to shoot. If the growth and mass of root is affected by elevated temperature as reported by many authors (Rennenber et al., 2006; Wahid et al 2007; Huang et al., 2012; Heckathorn et al., 2014), the growth of above-ground parts (shoot) will also be affected because the supply of water and nutrients will be affected (Hao et al., 2012; Giri et al., 2017).

4.5 CONCLUSIONS

In this experiment, exposure to short-term (seven days) elevated temperature (35°C) significantly affected the growth and nutritional quality of cowpea at the pre-flowering (40 DAP) and flowering growth stages (90 DAP). Compared to other growth stages, the whole plant biomass (Table 4.2) and nutrient contents (Figures 4.3-4.5) of cowpea

at the flowering stage (125 DAP) were most affected by elevated temperature. Rural farmers cultivating cowpea should adopt cropping management strategies for the protection of the flowering stage in the crop's growth cycle especially during periods of changes in climatic conditions (temperature extremes over short periods) under climate change scenarios.

CHAPTER 5: INTERACTION EFFECT OF *BACILLUS SUBTILIS* INOCULATION AND MINE WATER IRRIGATION ON COWPEA'S GROWTH, PHYSIOLOGY AND NUTRITIONAL QUALITY

5.1 ABSTRACT

The aim of study was to evaluate the interaction of *Bacillus* inoculation and acid mine water irrigation on the growth, physiology and nutritional quality of cowpea. Three cowpea genotypes (Asetenapa, Soronko & Nyira) were exposed to four treatments namely i) Inoculation with *B. subtilis* (BD233) (B+) and irrigation with mine water (75% AMD) (+), ii) Inoculation *B. subtilis* (BD233) inoculation (B+) and no mine water irrigation (-), iii) No *B. subtilis* (BD233) inoculation (B-) and irrigation with mine water (+) and iv) No inoculation with *B. subtilis* (BD233) (B-) and no irrigation with mine water (-) (control). A factorial (three factors: genotype x *B. subtilis* inoculation x AMD irrigation) experiment was conducted using a randomized complete block design with three replications. The results show that interaction of *Bacillus* inoculation and acid mine water irrigation was significant on the growth, nodulation, physiology and nutritional quality of cowpea's shoot and grain. *Bacillus* inoculation enhanced the growth, nodulation and yield of the tested cowpea genotypes and acid mine water irrigation negatively influenced the growth and nutritional quality of cowpea grains. Genotype responses to the treatments were significantly different.

5.2 INTRODUCTION

Rural communities rely on vegetables and legume crops such as cowpea to meet their mineral and protein requirements. Cowpea is deliberated as the second most important researched food grain legume in sub-Saharan Africa and constitutes a cheap source of plant protein for humans. As a food, the fresh leaves, seeds and immature pods of cowpea are consumed as vegetables. Cowpea leaves and seeds provide a rich source of proteins and calories, minerals and vitamins. In semi-arid regions of Africa, cowpea is widely grown and eaten in many households. The crop's genetic diversity enables its adaptation to different soil ecologies throughout the continent (Ehlers & Hall, 1997; Singh et al., 2003). It matures faster than most crops and therefore, can fill the hunger gap between cropping seasons, especially in rural communities where farmers are dependent on subsistence farming for their livelihoods. In West African countries that grow cowpeas as a major food crop, this

complements the main cereal diet (Langyintuo et al., 2003). The crop has the potential to serve as a food and nutrition security crop in sub-Saharan Africa.

In general, symbiotic legumes, including cowpea, do not only provide plant protein but also have the ability to supply adequate amounts of macro/micronutrients through their capacity to take up and concentrate minerals in their tissues (Belane, 2011). In addition, the crop has a unique ability to fix atmospheric N in symbiosis with bacteria (*Rhizobium*) thus, improving soil N fertility, which is important for system productivity and therefore fits well in the African traditional cropping systems.

In South Africa, cowpea cultivation has the potential to play a role in mitigating the threat posed by climate change on household food and nutrition security especially for poor-resource/smallholder farmers in rural communities. However, as a water-scarce country, for example, in Gauteng Province most surface waters are contaminated with AMD and there is a dire need to either clean/bio-remediate or use acid mine water for the irrigation of crops. Poor-resource farmers cannot afford expensive technologies to decontaminate or bio-remediate acid mine water discharging into freshwater sources used for irrigation of crops in their communities. In the advent of water scarcity during future climate change scenarios, it is expected that farmers will turn into wastewater/acid mine water as water source for irrigation of crops especially by resource-poor farmers in rural communities (FAO, 2010). Interestingly, the use of heavy metal resistant bacteria including the *Bacillus* consortium (Singh et al., 2014) having plant growth promotion traits (Román-Ponce et al., 2017) have been reported as eco-friendly and less cost-effective technologies for bioremediation of heavy metal polluted soils (Khan et al., 2009).

Bacillus subtilis is reported to enhance plant growth and also bio-remediate polluted soils. For example, *Bacillus* PZ3 was reported to reduce Cr in pea plants when inoculated with the bacteria in chromium amended soils and also enhanced the plant's growth, nodulation, leghaemoglobin, chlorophyll and protein content compared to control plants (Wani & Zainab, 2016). *Bacillus* spp. and other microorganisms are known to produce compounds such as siderophores and indol-3-acetic acid (IAA), which enhances the plant's growth and resistance to some heavy metals (Román-Ponce et al., 2017). However, no study has assessed the interactive effect of inoculating cowpea with any *Bacillus subtilis* strain and irrigation with acid mine water under field or glasshouse conditions. Therefore, the aim of study was to evaluate the

interaction of *Bacillus* inoculation and mine water irrigation on the growth, physiology and nutritional quality of cowpea. The first objective was to assess the growth, nodulation, chlorophyll content and stomatal conductance of cowpea genotypes inoculated with *B. subtilis* and irrigated with acid mine water. The second objective was to assess the nutritional contents of grains and shoots of three cowpea genotypes irrigated with acid mine water and exposed to *B. subtilis* inoculation under glasshouse conditions.

5.3 MATERIALS AND METHODS

5.3.1 Plant Material

Seeds of three cowpea genotypes (Asetanapa, Soronko and Nyira) were obtained from the Crop Research Institute, Kumasi, Ghana. The seeds were stored in storage facilities in Eureka Building, Florida campus, University of South Africa.

5.3.2 Bacterial Strains

The bacterial strain (*Bacillus subtilis* (BD233)) used in this study was obtained from the National Collection of Bacterial Species of the Agricultural Research Council, Plant Protection Research Institute, Roodeplaat, South Africa. Strains previously grown on Luria-Bertani solid media was sub-cultured aseptically in a sterile laminar flow and grown on Luria-Bertani broth at 37°C overnight. Thereafter, pure strains of the bacteria cultures were diluted and the broth adjusted to 0.5 McFarland Standards with sterile distilled water before use for inoculation of cowpea plants.

5.3.3 Plant Growth Material and Cowpea Fertilization

As described in Chapter 4, Sections 4.3.2 and 4.3.3.

5.3.4 Co-Inoculation of Cowpea

As described in Chapter 4, Section 4.3.3, a commercial powered inoculant with *Bradyrhizobium spp* CB756 strains was used for inoculation of cowpea seedlings. In addition, 2 mL of the adjusted (0.5 McFarland standards) *B. subtilis* broth media was applied to roots of each cowpea seedling grown in the glasshouse for the experiment.

5.3.5 Acid Mine Drainage

Acid mine drainage was acquired from an abandoned gold mine in Randfontein in Gauteng Province, South Africa. For the acid mine water (AMD) irrigation treatment, 75% mine water was prepared by mixing 750 mL of AMD with 250 mL tap water and was used to irrigate cowpea seedlings two weeks after planting in the glasshouse. The seedlings were thereafter irrigated with 75% AMD on a weekly basis until termination of the experiment. A previous study showed that 75% AMD considerably enhanced the growth of two potato genotypes although tubers were contaminated with some heavy metals (Nemutanzhela et al., 2017).

5.3.6 Experimental Treatments: *B. subtilis* Inoculation and AMD Irrigation

Prior to planting, seeds were surface-sterilized as described by Pule-Meulenberg et al. (2010). The seeds were divided into batches and each batch was planted in pots filled with acid washed sand. In each pot, three seeds of each cowpea genotype were planted and after seedling establishment were thinned to one plant per pot. The pots containing seedlings with the treatments were placed on top of tables in the glasshouse. In this experiment, the following four treatments were employed; i) Inoculation with *B. subtilis* (BD233) (B+) and irrigation with mine water (75% AMD) (+), ii) Inoculation *B. subtilis* (BD233) inoculation (B+) and no mine water irrigation (-), iii) No *B. subtilis* (BD233) inoculation (B-) and irrigation with mine water (+) and iv) No inoculation with *B. subtilis* (BD233) (B-) and no irrigation with mine water (-) (control).

A factorial (three factors: genotype x *B. subtilis* inoculation x AMD irrigation) experiment was conducted using a randomized complete block design with three replications. The experiment was carried out in an automated glasshouse at the UNISA's Florida science campus.

Data on growth parameters was collected at seven-day intervals. Cowpea nodulation, yield (number of pods, pod weight (fresh and dry) and number of grains per plant and its nutritional quality (elemental composition in shoot and grains) were assessed for each treatment.

5.3.7 Cowpea Growth, Nodulation and Yield Determination

For the first objective, data on growth parameters was collected at seven-day intervals. Cowpea biomass (shoot and root), nodulation (number of nodules per plant), yield (the

number of pods, pod weight (fresh and dry)) and the number of grains per plant were determined at 125 DAP.

5.3.7.1 Number of branches, plant height and stem diameter

The number of branches per plant was counted physically every week. The plant height was measured using a measuring tape and the stem diameter using a Vernier calliper at seven-day intervals throughout the experimental period.

5.3.7.2 Plant biomass (shoot and root weights)

In this experiment, six plants for each treatment were randomly harvested 125 days after planting for the determination of the plant's shoot and root fresh and dry weights. A weighing balance was used to determine the shoot and root fresh weights immediately after washing with tap water of all the harvested plant parts (shoot and roots). For dry weight determination, the plant parts were oven-dried at 60°C for 72 h to constant weight and the weight measured using a weighing balance.

5.3.7.3 Nodulation and yield

Four plants were randomly selected and carefully uprooted at 125 DAP, with root system intact to determine nodulation. Harvested roots were washed with water and nodules were carefully detached and their total numbers counted per treatment. The detached nodules were kept frozen at -10°C in a refrigerator. For yield determination for each treatment, the number of pods per plant were counted and the number of grains per pod for each plant also determined.

5.3.8 Measurement of Stomatal Conductance and Chlorophyll Content

The measurement for stomatal conductance was done at 77 DAP and for chlorophyll content at 70 DAP and 84 DAP. For each treatment, a porometer (SC-1Leaf Porometer, Pullman, USA) was used to measure the leaf stomatal conductance (on the abaxial and adaxial sides of a mature leaf). The same fourth leaf from the apex (identified with a marker) of the plant was used to determine both the stomatal conductance and chlorophyll content. A handheld chlorophyll meter (Opti-Sciences model CCM-300, Hudson, USA) was used for measurements. From 70 DAP through to 120 DAP, chlorophyll content was measured every week.

5.3.9 Determination of Mineral Content in Mine Water (75% AMD), Cowpea Shoot and Grain

Three replicate acid mine water (75%) samples were analysed using Inductively Coupled Plasma Mass Spectrometer (ICP-MS) and inductively coupled plasma atomic emission spectroscopy (ICP-AES) to determine the concentration of some elements. For elemental determination in mine water, an aliquot of a well-mixed, homogeneous aqueous sample was accurately measured for sample processing. For total recoverable analysis of an aqueous sample containing undissolved material, analytes were first solubilized by gentle refluxing with nitric and hydrochloric acids. After cooling, the sample was made up to volume, mixed and centrifuged or allowed to settle overnight prior to analysis. For elemental determination in plant organs, three plants per treatment were harvested at 125 DAP and the parts (shoot and grains) separated. Each part was oven-dried (60°C), weighed, and each ground to fine powder (0.85 mm mesh size) prior to analysis for mineral density. To measure the mineral elements (P, Ca, Cu, Zn, Mn, Fe, and Al) in cowpea shoot and grain, 1 g of ground sample was ashed in a porcelain crucible at 500°C overnight. This was followed by dissolving the ash in 5 mL of 6 M HCl (analytical grade) and placing it in an oven at 50°C for 30 min and 35 mL of deionized water was added. The mixture was filtered through Whatman no. 1 filter paper. Mineral element concentration in plant extracts was determined from three replicate samples using inductively coupled plasma-mass spectrometer (ICP-MS) (Ataro et al., 2008). The quality of data collected was checked using standard solutions with certificate of analysis. In place of analyte isotopes to monitor each element, known sample as standard was used after every 10 samples. Sulphur was determined by wet digestion procedure using 65 % nitric acid (high purity grade). In each case, 1 g of milled plant material was digested overnight with 20 mL of 65 % nitric acid in a 250 mL glass beaker. The beaker containing the extract was then placed on a sand bath and gently boiled until approximately 1 mL of the extract was left. After that, 10 mL of 4 M nitric acid (high purity grade) was added and boiled for 10 min. The beaker was removed from the sand bath, cooled, and the extract washed completely in a 100 mL volumetric flask and the extract filtered through Whatman no.2 filter paper. The S in the sample was then determined by direct aspiration on the calibrated simultaneous ICP-MS.

5.3.11 Data Analysis

Data on growth parameters, nodulation, physiological measurements (leaf stomatal

conductance and chlorophyll content) and elemental quality of cowpea were subjected to analysis of variance (ANOVA) using Statistica v. 10, StatSoft (StatSoft Inc., Tulsa, OK, USA). A 3-way ANOVA was performed to compare the interactive effect of genotype x *B. subtilis* inoculation x AMD irrigation on the growth, nodulation, physiology and nutritional quality of cowpea and 1-way ANOVA for comparing mineral nutrients among genotypes and between inoculation and irrigation. All parameters and measurements were tested at $p < 0.05$ significance level and the Duncan multiple range test was used for separation of means among treatment.

5.4 RESULTS AND DISCUSSION

5.4.1 Interactive Effect of *Bacillus subtilis* (BD233) and Acid Mine Water Irrigation on the Growth, Nodulation and Physiology of Cowpea

Climate change has resulted in increases in dry conditions in most regions of South Africa, a water-scarce country. In the advent of a water crisis, it is highly likely that resource-poor farmers will use acid mine water for crop irrigation. Acid mine drainage is noted to contain pollutants such as heavy metals that can affect the growth of crops. In this study, analysis of the mine water (75% AMD) showed relatively low concentrations of some heavy metals (Table 5.1) compared to the South African National guidelines for the irrigation of crops (Department of Water Affairs and Forestry (DAWF), 1996).

Table 5.1 The concentration of heavy metals in mine water (75% AMD)

Element	Elemental composition (mg/L)	South African Standards (mg/L)
Cd	0.0005	0.01–0.05
Li	1.597	>2.5
Se	0.010	0.02–0.05
As	0.002	0.01–2.0
Zn	0.008	1.0–5.0
Ni	0.210	0.20–2.0
Mn	1.350	0.02–10.0
Cr	0.003	0.10–1.0
Pb	0.0002	0.2–2.0

However, long-term irrigation of plants with this treatment (low concentrations of heavy metals as was the case in this study) cannot only lead to accumulation of heavy metals in soils but also their uptake by plants (Nemutanzhela et al., 2017). There is a need to bio-remediate AMD for irrigation of crops as a cheaper and environmentally-friendly alternative. The aim of this study was to evaluate the interactive effect of *Bacillus subtilis* (BD233) inoculation and mine water irrigation on the growth, nodulation, physiology and shoot/grain elemental quality of cowpea. The results show that the interactive effect of *B. subtilis* (BD233) inoculation and mine water irrigation was significant for cowpea's growth, nodulation, physiology and nutritional quality (shoot and grain) of all three genotypes at 125 DAP under glasshouse conditions. Among the genotypes, with exposure to *B. subtilis* (BD233) inoculation and mine water irrigation, Soronko had a significantly higher number of branches (16.33), plant height (42.58 cm) and biomass (fresh and dry weights of shoot (63.25 g & 11.25 g) and root (35.14 g & 4.06 g) respectively) compared to control. The stem girth of Nyira (115.67 mm) was the biggest followed by Soronko (111.00 mm) and Asetenapa had the lowest values of all measured growth parameters (Table 5.2). Although genotypic differences in cowpea's growth under different conditions have been reported, Belane (2011) found a significant interaction effect of genotype and environment on the growth parameters of 30 cowpea genotypes grown in different agro-ecological climatic conditions. However, in this study, inoculation with *B. subtilis* (BD233) had a significant effect on the growth of cowpea. At 125 DAP, plants inoculated with *B. subtilis* (BD233) had a higher number of branches, plant height and biomass (fresh and dry weights of shoot and root) compared to those not inoculated with the bacteria (Table 5.2). In agreement with this finding, Ogugua et al. (2018) reported that *B. subtilis* (BD233) inoculation significantly enhanced seedlings' growth of sweet pepper (36.3%) and lettuce (14.0%) relative to control. The whole plant biomass (dry weight) of sweet pepper increased by 70.7% after soil inoculation with *B. subtilis* (BD233) compared with control. Similarly, *B. subtilis* strain GB03 improved shoot (35%) and root (42%) length and biomass (133%) in 20 days old *Codonopsis pilosula* seedlings (Wu et al., 2016). Furthermore, when tomato was inoculated with *B. subtilis* BEB-ISbs, marked increases were reported in the saleable yield, and weight and length of fruit relative to control (Mena-Violante & Olalde-Portugal, 2007). Jeong et al. (2013) reported a 349% increase in plant dry weight after eight weeks, when *Brassica juncea* inoculated with P-solubilizing *Bacillus* spp. was compared to control. In addition, Shishido et al. (1996) reported enhanced root growth (18%) in two conifer species inoculated with three

strains of *Bacillus polymyxa* (L6-16R, S20-R, and Pw-2R) when compared to control. Interestingly, all three strains stimulated the growth of both conifer species. A study conducted by Ali et al. (2009b) showed enhanced growth in sorghum when inoculated with a strain of *Pseudomonas* sp. under high temperature conditions compared to those not inoculated. Interestingly, when some legume species (common bean, peanut, soybean and pigeon pea) were co-inoculated with rhizobia and several strains and species of *Bacillus*, marked changes in root architecture and increased nodulation were reported (Schwartz et al., 2013). Generally, microorganisms including *Bacillus subtilis* are known to influence plant growth directly (hormone (auxins, cytokinins and gibberellins) secretion (Ali et al., 2009a; Galaviz et al., 2018)) and indirectly (production of stress inhibiting enzymes, siderophore production and P-solubilization) (Schwartz et al., 2013; Meng et al., 2016).

In this study, irrigation with mine water (75% AMD) had a significant effect on the growth of the tested cowpea genotypes. At 125 DAP, the measured growth parameters of plants irrigated with mine water were significantly lower compared to those irrigated with tap water with the exception of the dry root weight (Table 5.2). Mine water is a source of heavy metals (see Table 5.1) and long-term irrigation of crops can subsequently lead to metal toxicity stress in plants (Ali et al., 2013).

Table 5.2: Effects of *B. subtilis* inoculation and acid mine water (75% AMD) irrigation on the growth parameters of cowpea genotypes at 125 DAP in a glasshouse.

Genotype	Growth parameters						
	Branches /plant	Plant height (cm)	Stem diameter (mm)	Shoot FW (g)	Shoot DW (g)	Root FW (g)	Root DW (g)
Soronko	16.33a	42.58a	111.00b	63.25a	11.25a	35.14a	4.06a
Nyira	15.33b	28.25b	115.67a	58.94b	8.81b	34.75b	2.13b
Asetenapa	7.33c	17.33c	60.50c	30.06c	3.56c	19.19c	1.13c
Inoculation							
<i>B. subtilis</i>	15.61a	31.39a	97.44a	60.92a	9.96a	33.38a	3.67a
No <i>B. subtilis</i>	10.39b	27.39b	94.00b	40.58b	5.79b	26.00b	1.21b
Irrigation							
Acid mine water (AMD)	9.89b	24.00b	90.00a	42.46b	6.67b	25.71b	2.17a
No AMD	16.11a	34.78a	1.44b	59.04a	9.08a	33.67a	2.71a
F-Statistics							
Genotype (G)	244.44***	283.92***	1698.71***	9.65***	10.22***	6.30***	3.39*
Inoculation (IN)	205.52***	21.34***	16.27***	9.81***	8.63**	2.97**	6.90**
Irrigation (IR)	281.73***	154.31***	178.32***	6.11***	2.90*	3.46**	0.31 ^{ns}
G x IN x IR	44.69***	12.86***	2264.49***	15.36***	6.61**	13.98***	1.61*

Values (M±S.E. (n=3)) followed by similar letters in a column are not significantly different at *p≤0.05, **p≤0.01, ***p≤0.001 and ns = not significant.

Similarly, at 125 DAP, the interactive effect of *B. subtilis* (BD233) inoculation and mine water irrigation was significant on the growth of each cowpea genotype. For example, the highest number of branches per plant was obtained by Asetenapa (21.67) followed by Nyira (21.00) when not inoculated with *B. subtilis* and irrigated mine water compared to the same genotypes exposed to other treatments. In contrast, with or without *B. subtilis* (BD233) inoculation, Asetenapa irrigated with mine water had zero number of branches (0.00) because the seedlings did not survive exposure to mine water (Table 5.3). Accumulation of toxic concentration of some heavy metals in mine water (Table 5.1) could have caused death of seedlings. For example, even at low concentrations, cadmium can cause growth reduction, wilting, chlorosis and cell death in plants (Gallego et al., 2012). However, when Soronko and Nyira were exposed to the same treatment, they showed some degree of tolerance although the mechanism involved may be still unknown.

At 125 DAP, the measured growth parameters such as plant height, stem diameter, shoot fresh (FW) and dry (DW) weight and root fresh (FW) and dry (DW) weight were

affected by the interaction of *B. subtilis* (BD233) inoculation and mine water irrigation. For example, without mine water irrigation, Soronko inoculated with *B. subtilis* (BD233) had the tallest plants (111.0 cm) compared to those not inoculated with the bacteria (6.7 cm). In essence, inoculation with the bacteria enhanced Soronko plant height by over 60% compared to uninoculated plants. Similarly, Asetenapa (108.0 cm) inoculated with *B. subtilis* (BD233) but not irrigated with mine water had the second tallest plants. However, Asetenapa not inoculated with the bacteria and not irrigated with mine water had a significantly wider stem diameter (128.0 mm) compared to the other treatments (Table 5.3). Biomass determination also showed the interaction effect of *B. subtilis* (BD233) inoculation and mine water irrigation. For example, Soronko inoculated with *B. subtilis* (BD233) and not irrigated with mine water had the highest shoot fresh and dry weights (104.5 g & 18.3 g respectively) and root fresh and dry weights (54.5 g & 8.0 g respectively) compared to other treatments. Similarly, the biomass of Nyira was enhanced when inoculated with *B. subtilis* (BD233) but not irrigated with mine water compared to plants inoculated with the bacteria and irrigated with mine water (Table 5.3).

Table 5.3: Interactive effects of *B. subtilis* inoculation and acid mine water (75% AMD) irrigation on the growth parameters of cowpea at 125 DAP in a glasshouse.

Genotype	Inoculation	Irrigation	Growth parameters						
			Branches/ plant	Plant height (cm)	Stem diameter (mm)	Shoot FW (g)	Shoot DW (g)	Root FW (g)	Root DW (g)
Soronko	B+	+	18.0ba	69.7ab	102.0g	89.5ab	17.0ab	41.5ab	6.5ab
		-	17.0c	111.0a	117.0c	104.50a	18.3a	54.7a	8.0a
Soronko	B-	+	12.3d	103.0a	120.0bc	47.3cd	9.8bcd	30.8bc	1.8bc
		-	18.0bc	6.7c	105.0fg	11.8de	1.0f	13.5cd	1.0c
Nyira	B+	+	10.3c	106.0a	123.0b	44.0cd	8.8cde	27.5bc	2.8bc
		-	19.0b	37.3b	108.0ef	90.8ab	14.3abc	46.0ab	3.8abc
Nyira	B-	+	11.0dc	72.0ab	120.7bc	37.32cd	3.0def	24.0bc	1.5c
		-	21.0a	72.0ab	111.0dc	63.8bc	9.3bcd	41.5ab	2.0bc
Asetenapa	B+	+	0.0g	0.0c	0.0h	0.0e	0.0f	0.0d	0.0c
		-	7.7f	108.0a	114.0d	36.8cd	1.5ef	30.5bc	1.0c
Asetenapa	B-	+	0.0g	0.0c	0.0h	0.0e	0.0f	0.0d	0.0c
		-	21.7a	74.3ab	128.0a	83.5ab	12.8abc	46.2ab	3.5abc

Values (M±S.E. (n=3)) followed by similar letters in a column are not significantly different at *p≤0.05, **p≤0.01, ***p≤0.001 and ns = not significant. B+ (inoculation with *B. subtilis* (BD233)), B- (not inoculated with *B. subtilis* (BD233)) and + (irrigation with mine water (75% AMD) and – (not irrigated with mine water).

The nodulation and yield of the tested cowpea genotypes varied significantly in response to *B. subtilis* inoculation and irrigation with mine water. Among the genotypes, Soronko had the highest number of nodules per plant (15.75), followed by

Nyira (12.94) and the least was in Asetenapa (3.94) (Table 5.4). Also, the result showed that Soronko had significantly higher number of pods per plant (5.06), pod fresh and dry weights (12.88 g & 3.88 g respectively), number of grains per plant (23.81) and grain fresh weight per plant (3.56 g) compared to Asetenapa that had the lowest values of these measured parameters (Table 5.4).

According to Table 5.4, one-way analysis revealed that inoculation with *B. subtilis* (BD233) significantly affected cowpea nodulation and yield. Plants co-inoculated with *B. subtilis* (BD233) had significantly higher number of nodules compared to those not inoculated with the bacteria. Similarly, the number of grains and grain fresh weight/plant of plants co-inoculated with *B. subtilis* (BD233) were significantly higher compared to the those not co-inoculated with *B. subtilis* (BD233) and the number of pods and pod biomass (fresh and dry weights) per plant were slightly more than those of plant not co-inoculated with *B. subtilis* (BD233) (Table 5.4).

Similarly, irrigation with mine water (75% AMD) had a significant effect on the nodulation and yield of the tested cowpea genotypes. The nodulation (number of nodules/plant) and measured yield parameters (number of pods/plant, pod fresh and dry weights, number of grains and grain fresh weight/plant) of plants irrigated mine water were significantly lower compared to those not irrigated with mine water (Table 5.4).

Table 5.4: Effects of *B. subtilis* inoculation and acid mine water (75% AMD) irrigation on nodulation and yield of cowpea genotypes at 125 DAP in a glasshouse.

Genotype	Nodulation and yield					
	No. of nodules/plant	No. of pods/plant	Pods FW (g)	Pods DW (g)	No. of grains/plant	Grain FW (g)/plant
Soronko	15.75±5.9a	5.06±0.75a	12.88±2.12a	3.88±0.2a	23.81±2.5a	3.56±0.7a
Nyira	12.94±5.1b	4.88±1.28b	10.88±0.12b	2.13±0.2a	23.75±2.3a	3.38±0.8a
Asetenapa	3.94±2.1c	1.88±0.89c	4.13±0.37c	1.50±0.1a	7.75±6.8b	1.31±0.1b
Inoculation						
<i>B. subtilis</i>	15.00±4.5a	4.13±0.87a	10.54±2.37a	3.29±0.2a	22.88±2.1a	3.33±0.2a
No <i>B. subtilis</i>	6.75±2.8b	3.75±0.85a	8.04±0.74a	1.71±0.8a	14.00±1.3b	2.17±0.2b
Irrigation						
Acid mine water (AMD)	4.67±1.8b	2.25±0.56b	6.46±1.65b	0.96±0.1b	9.21±0.9b	2.04±0.2b
No AMD	17.08±4.8a	5.63±0.96a	12.13±2.54a	4.04±0.3a	27.67±2.6a	3.46±0.3a
F-Statistics						
Genotype (G)	2.24*	7.08*	4.89*	2.37 ^{ns}	6.68**	5.79**
Inoculation (IN)	3.30*	0.23 ^{ns}	1.09 ^{ns}	2.93 ^{ns}	4.73*	3.79*
Irrigation (IR)	7.43*	18.91***	5.59*	11.13*	20.47***	5.59*
G x IN x IR	1.85*	5.62**	3.63*	6.37**	5.71**	9.61***

Values (M±S.E. (n=3)) followed by similar letters in a column are not significantly different at *p≤0.05, **p≤0.01, ***p≤0.001 and ns = not significant.

Similarly, the interactive effect of *B. subtilis* (BD233) and mine water irrigation was significant on the nodulation and yield of the tested cowpea genotypes. For example, at 125 DAP, without mine water irrigation, Soronko (41.5) followed by Nyira (25.3), had the utmost nodule counts per plant when inoculated with *B. subtilis* compared to the same genotypes exposed to other treatments or their respective controls (Table 5.5). Again, without mine water irrigation, the same genotypes, Soronko and Nyira, when inoculated with *B. subtilis* had the uppermost pod counts per plant (8.0 & 8.5 respectively), pod fresh weight (19.0 g & 21.3 g respectively), pod dry weight (10.3 g & 6.3 g respectively), number of seeds per plant (53.0 & 40.8 respectively) and seeds fresh weight (6.8 g & 5.3 g respectively) compared to the other treatments and their respective controls (Table 5.5). In agreement with this finding, preliminary investigation indicated that the interactive effect of *B. subtilis* (BD233) inoculation and elevated temperature was significant for the germination and plumule length of cowpea (Nevhulaudzi et al., 2017). Taken together, these results are in consonant with other related studies in which the interactive effect of bacteria on growth of plants has been

reported. For example, the interaction of some plant growth promoting bacteria and P fertilizers promoted the production of dry matter in chickpea (Gupta et al., 2009), rice and wheat (Sharma & Prasad, 2003), and sunflower (Joe & Sivakumaar, 2009). Pea plants co-inoculated with *Bacillus* PZ3 was reported to boost the plant's growth, nodulation, leghaemoglobin, chlorophyll and protein content compared to control plants (Wani & Zainab, 2016). Similarly, the interaction of *Bacillus subtilis* and seaweed (kelpak) was reported to be significant for potato's growth and yield (Kanu & Ntushelo, 2015).

Table 5.5: Interactive effects of *B. subtilis* inoculation and acid mine water (75% AMD) irrigation on nodulation and yield of cowpea genotypes at 125 DAP in a glasshouse.

Genotype	Inoculation	Irrigation	No. of nodules/ plant	No. of pods/ plant	Pods FW (g)	Pods DW (g)	No of seeds/plant	Seeds FW/pl ant
Soronko	B+	+	18.2ab	6.0ab	16.3ab	2.8bc	24.3bcd	3.5ab
		-	41.5a	8.0a	19.0a	10.3a	53.0a	6.8a
Soronko	B-	+	2.7d	4.8abc	14.3b	2.5bc	11.8cde	3.5ab
		-	0.5d	1.5cd	2.0c	0.1c	6.3de	0.5bc
Nyira	B+	+	3.2d	2.0bcd	5.0c	0.5c	19.3bcde	4.5a
		-	25.3ab	8.5a	21.3a	6.3ab	40.8ab	5.3a
Nyira	B-	+	0.0d	0.5cd	1.5c	0.0c	0.0e	0.0c
		-	23.0ab	8.5a	15.8b	1.8bc	35.0ab	3.8a
Asetenapa	B+	+	0.0d	0.0d	0.0d	0.0c	0.0e	0.0c
		-	3.8d	0.3cd	1.8c	0.0c	0.0e	0.0c
Asetenapa	B-	+	0.0d	0.0d	0.0d	0.0c	0.0e	0.0c
		-	12.0c	7.3a	14.8b	6.0ab	31.0bc	5.3a

Values (M±S.E. (n=3)) followed by similar letters in a column are not significantly different at *p≤0.05, **p≤0.01, ***p≤0.001 and ns = not significant. B+ (inoculation with *B. subtilis* (BD233)), B- (not inoculated with *B. subtilis* (BD233)) and + (irrigation with mine water (75% AMD) and – (not irrigated with mine water).

Plants use stomata to regulate transpiration, metabolite flux, and water retention. In this study, the data obtained showed that the interaction of *B. subtilis* (BD233) and mine water (75% AMD) irrigation significantly affected leaf stomatal conductance of cowpea genotypes. For example, when irrigated with tap water, Nyira inoculated with *B. subtilis* (BD233) had the highest leaf stomatal conductance on the adaxial ($120.00 \pm 12.1 \text{ m}^2/\text{s mol}^{-1}$) and abaxial ($123.00 \pm 12.8 \text{ m}^2/\text{s mol}^{-1}$) sides of the leaf compared to when inoculated and irrigated with mine water (adaxial: $106.00 \pm 10.5 \text{ m}^2/\text{s mol}^{-1}$ and abaxial: $108.00 \pm 10.8 \text{ m}^2/\text{s mol}^{-1}$). Similarly, when Soronko was inoculated with the bacteria and treated with mine water, both the adaxial and abaxial stomatal conductance were reduced compared to Soronko plants inoculated with the bacteria but irrigated with tap water (Table 5.6). Compared with all other treatments, Soronko not inoculated with *B. subtilis* (BD233) and irrigated with mine water had the lowest

stomatal conductance. In general, all three genotypes with or without *B. subtilis* (BD233) inoculation had lower stomatal conductance when irrigated with mine water compared to when not irrigated AMD. Interestingly, several authors (Du Plessis, 1983; Jovanovic et al., 1998; Pandey & Sharma, 2002; Ali et al., 2013; Özkay et al., 2014; Nemutanzhela et al., 2017) have reported that long-term irrigation with mine water can increase concentrations of metals in soils, which plants can subsequently take-up and bio-accumulate in their organs. In particular, accumulation of high concentration of heavy metals especially at toxic levels can results in changes in plant physiological processes. For instance, high accumulation of Cd in *Brassica napus* leaf drastically reduced water balance, stomatal conductance and CO₂ concentrations (Ali et al., 2013). This report is in line with findings in the current study as long-term irrigation with mine water with heavy metals (Table 5.1) reduced the stomatal conductance of two cowpea genotypes (Soronko and Nyira) regardless of inoculation with or without *B. subtilis* (BD233) and most probably resulted in death of Asetenapa seedlings (represented as not determined (nd)) (Table 5.6). Furthermore, the results seem to suggest that inoculation with *B. subtilis* (BD233) could have influenced concentration of heavy metals uptake from the rhizosphere soils, as cowpea plants inoculated with the bacteria had higher stomatal conductance compared to those not inoculated. For example, Soronko irrigated with mine water had higher stomatal conductance when inoculated with *B. subtilis* (BD233) (102.00±10.5 (adaxial) and 105.00±10.5 (abaxial)) compared to without inoculation with the bacteria (71.33±7.1 (adaxial) and 102.00±10.5 (abaxial)) (Table 5.6). This finding is in agreement with reports that *Bacillus* spp. can remediate heavy metals in contaminated soils (Wani et al., 2007; Fatnassi et al., 2014).

Table 5.6: Interactive effects of *B. subtilis* inoculation and mine water (75% AMD) irrigation on leaf stomatal conductance ($\text{m}^2/\text{s mol}^{-1}$) of cowpea grown in a glasshouse at 77 DAP.

Genotype	Inoculation	Irrigation	Stomatal conductance ($\text{m}^2/\text{s mol}^{-1}$)	
			Adaxial	Abaxial
Soronko	B+	+	102.00±10.5c	105.00±10.5h
		-	115.00±11.5ab	117.00±11.8c
Soronko	B-	+	71.33±7.1d	102.00±10.5g
		-	116.67±15.8ab	120.00±11.5b
Nyira	B+	+	106.00±10.5bc	108.00±10.8f
		-	120.00±12.1a	123.00±12.8a
Nyira	B-	+	107.00±10.5bc	111.00±10.5e
		-	109.00±10.5bc	125.00±12.5e
Asetenapa	B+	+	nd	nd
		-	112.00±11.5b	114.00±11.5d
Asetenapa	B-	+	nd	nd
		-	120.00±12.2a	123.33±12.1a
F-Statistics				
G x IN x IR			49.09***	79.28***

Values ($M \pm S.E.$, $n=3$) followed by similar letters in a column are not significantly different at $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ and ns = not significant. B+ (inoculation with *B. subtilis* (BD233)), B- (not inoculated with *B. subtilis* (BD233)) and + (irrigation with mine water (75% AMD)) and - (not irrigated with mine water) and nd (not determined).

Similarly, the interaction of *B. subtilis* (BD233) inoculation and acid mine water (75% AMD) irrigation significantly affected leaf chlorophyll content in cowpea. For example, at 70 DAP, with mine water irrigation, Asetanapa not inoculated with *B. subtilis* (BD233) recorded the highest chlorophyll content ($123.00 \pm 11.8 \text{ mg/m}^2$) (chlorophyll a) and $121.00 \pm 0.58 \text{ mg/m}^2$ (chlorophyll b)) compared to when it was inoculated with the bacteria ($107.33 \pm 10.1 \text{ mg/m}^2$) (chlorophyll a) and $71.00 \pm 7.1 \text{ mg/m}^2$ (chlorophyll b)) (Table 5.7). Two weeks later, at 84 DAP, a similar trend was noted. Asetanapa not inoculated with the bacteria but irrigated with mine water recorded the highest chlorophyll content ($124.00 \pm 11.58 \text{ mg/m}^2$) (chlorophyll a) and $124.33 \pm 12.33 \text{ mg/m}^2$ (chlorophyll b)) compared to when inoculated with *B. subtilis* (BD233) ($110.00 \pm 10.5 \text{ mg/m}^2$) (chlorophyll a) and $80.33 \pm 8.1 \text{ mg/m}^2$ (chlorophyll b)) (Table 5.7). At both 70 and 84 DAP, with or without *B. subtilis* (BD233) inoculation, irrigation with mine water reduced the chlorophyll contents in all three tested cowpea genotypes. This finding is in consonant with reports by several authors (Pandey & Sharma, 2002; Zengin & Munzuroglu, 2006; Elloumi et al., 2007; John et al., 2009; Nemutanzhela et al., 2017) that long-term exposure of plants to heavy metal contaminated soils can induce stress known to reduce chlorophyll content due to metal toxicity. Furthermore, the interaction of metals could also negatively influence the leaf chlorophyll content. For example, Soltangheisi et al. (2014) reported that at 28 days after transplanting, the interactive effect of Zn and Fe in rhizosphere soil decreased the chlorophyll content of sweet corn.

Table 5.7: Interactive effects of *B. subtilis* inoculation and acid mine water (75% AMD) irrigation on leaf chlorophyll content (mg/m²) of cowpea grown in a glasshouse.

Genotype	Inoculation	Irrigation	Leaf chlorophyll (mg/m ²)			
			70 DAP		84 DAP	
			Chlorophyll a	Chlorophyll b	Chlorophyll a	Chlorophyll b
Soronko	B+	+	70.67±6.8c	102.00±10.5b	41.33±4.1d	102.00±10.5b
		-	76.67±6.3c	77.00±7.5c	112.33±10.3ab	114.33±11.3ab
Soronko	B-	+	43.00±3.8d	72.33±7.1c	73.33±7.1c	105.00±10.5b
		-	114.00±10.8ab	111.33±11.9b	115.00±11.5ab	117.00±10.5ab
Nyira	B+	+	105.00±10.5b	72.33±7.1c	105.00±10.5b	108.00±10.8b
		-	117.00±11.5ab	116.00±11.5b	118.00±11.8ab	120.00±12.5a
Nyira	B-	+	106.67±10.8b	81.33±7.9c	105.67±11.6b	107.33±10.8b
		-	120.00±12.5a	108.33±10.3b	121.00±12.0a	116.67±11.8ab
Asetenapa	B+	+	nd	nd	nd	nd
		-	107.33±10.1b	71.00±7.1c	110.00±10.5b	80.33±8.1c
Asetenapa	B-	+	nd	nd	nd	nd
		-	123.00±11.8a	121.00±0.58a	124.00±11.58a	124.33±12.33a
F-Statistics						
G x IN x IR			13.64***	8.61**	35.49***	39.33***

Values (M±S.E. (n=3)) followed by similar letters in a column are not significantly different at *p≤0.05, **p≤0.01, ***p≤0.001 and ns = not significant. B+ (inoculation with *B. subtilis* (BD233)), B- (not inoculated with *B. subtilis* (BD233)) and + (irrigation with mine water (75% AMD) and – (not irrigated with mine water) and nd (not determined).

5.4.1 Effect of *Bacillus subtilis* (BD233) and Acid Mine Water Irrigation on the Nutritional Content of Cowpea's Shoot and Grain

One-way analysis of variance revealed significant differences in the nutritional content among shoots of the tested cowpea genotypes in response to *B. subtilis* (BD233) inoculation and mine water irrigation. For example, the highest concentrations of P (0.49%), Ca (5.65%), Fe (271.13 mg/kg), Cu (2.51 mg/kg), Zn (80.54 mg/kg) and Mn (103.38 mg/kg) were found in Nyira shoot compared to Asetenapa, which had the lowest concentrations of these elements (P=0.17±0.05%, Ca=2.48%, Fe=143.61 mg/kg, Cu=1.19 mg/kg, Zn=31.99 mg/kg, and Mn=40.14 mg/kg respectively) (Table 5.8). The concentration of Al was similar in the shoot of both Soronko and Nyira but was markedly higher compared to Asetenapa. Interestingly, inoculation with *B. subtilis* (BD233) significantly affected the concentration of Fe, Al and Mn in cowpea shoot. For example, plants inoculated with *B. subtilis* (BD233) had lower concentration of Fe (185.75 mg/kg) and Al (60.11 mg/kg) compared to those not inoculated with the bacteria (241.17 and 72.66 mg/kg respectively). In contrast, Mn concentration was markedly higher in shoot of cowpea plants inoculated with *B. subtilis* (BD233) (77.10 mg/kg) compared to those not inoculated with the bacteria (67.59 mg/kg) (Table 5.8).

Similarly, irrigation with mine water (75% AMD) affected the concentration of some heavy metals in cowpea shoot. For example, plants irrigated with mine water had marked concentrations of Fe (244.64 mg/kg), Zn (64.79 mg/kg) and Al (71.75 mg/kg) compared to those not irrigated with mine water (182.28, 54.18 and 61.02 mg/kg respectively) (Table 5.8). In contrast, Cu concentration was higher in shoot of plants not irrigated with mine water (2.15 mg/kg) compared to those irrigated with mine water (1.91 mg/kg). However, the concentrations of P, Ca and Mn were not different when plants irrigated with mine water was compared to those not exposed to the same treatment (Table 5.8). The high concentrations of these metals (Fe, Zn and Al) found in cowpea shoot could have been derived from the mine water (75% AMD) (Table 5.1). Long-term irrigation of crops with polluted waters such as acid mine water could lead to high concentrations of heavy metals in rhizosphere soils, and subsequently, their uptake and accumulation in plant organs will be enhanced. Compared to control, the lower concentrations of these metals in shoot of cowpea inoculated with *B. subtilis* (BD233) would suggest their metal toxicity tolerance and potential to bio-remediate heavy metal polluted soils as reported by other authors (Khan et al., 2009; Jeong et al., 2013; Singh et al., 2014).

Table 5.8: Effect of inoculation and irrigation on the mineral composition in shoot (dry matter (DM)) of cowpea genotypes under glasshouse conditions.

Genotype	Mineral composition in shoot (DM)						
	P (%)	Ca (%)	mg/kg				
			Fe	Cu	Zn	Mn	Al
Soronko	0.32b	5.29b	225.64b	2.39b	65.92b	73.51b	79.24a
Nyira	0.49a	5.65a	271.13a	2.51a	80.54a	103.38a	79.17a
Asetenapa	0.17c	2.48c	143.61c	1.19c	31.99c	40.14c	40.75b
Inoculation							
<i>B. subtilis</i>	0.32a	4.37a	185.75b	2.07a	60.77a	77.10a	60.11b
No <i>B. subtilis</i>	0.33a	4.58a	241.17a	1.98a	58.20a	67.59b	72.66a
Irrigation							
Mine water (75% AMD)	0.34a	4.19a	244.64a	1.91b	64.79a	69.76a	71.75a
No AMD	0.30a	4.75a	182.28b	2.15a	54.18b	74.93a	61.02b
F-Statistics							
Genotype (G)	116.01***	31.29***	14.79***	68.93***	95.85***	44.45***	21.10**
Inoculation (IN)	0.05 ^{ns}	0.24 ^{ns}	8.16***	0.77 ^{ns}	0.76 ^{ns}	3.01 ^{ns}	5.06*
Irrigation (IR)	5.26***	2.49 ^{ns}	10.32**	5.32*	13.01**	0.89 ^{ns}	3.69*
G x IN x IR	48.14***	40.11***	17.03***	69.54***	84.55***	38.58***	15.22***

Values (M±S.E. (n=3)) followed by similar letters in a column are not significantly different at *p≤0.05, **p≤0.01, ***p≤0.001 and ns = not significant.

In response to *B. subtilis* (BD233) inoculation and mine water irrigation, significant differences were recorded in grain elemental concentrations among the tested cowpea genotypes (Table 5.9). Soronko grain had the highest concentrations of P (0.53%), Ca (0.13%), Fe, (163.89 mg/kg), Cu (3.47 mg/kg), Zn (55.20 mg/kg), Mn (15.04 mg/kg) and Al (2.70 mg/kg) compared to the other genotypes (respectively) (Table 5.9). Inoculation with *B. subtilis* (BD233), as a single factor, significantly affected the accumulation of some heavy metals in grains of cowpea. For example, concentrations of Fe (133.11 mg/kg) and Zn (37.43 mg/kg) were markedly higher in grains of cowpea plants not inoculated with *B. subtilis* (BD233) compared to those inoculated with the bacteria (51.81 and 33.98 mg/kg respectively) (Table 5.9). However, inoculation with *B. subtilis* (BD233) did not significantly impacted grain accumulation of some elements (P, Ca, Cu, Mn and Al) as slight differences were noted compared to uninoculated plants (respectively). In general, plants not inoculated with *B. subtilis* (BD233) accumulated more heavy metals in their grains compared to those inoculated with the bacteria.

Table 5.9: Effect of inoculation and irrigation on the mineral composition in grain (dry matter (DM)) of cowpea genotypes under glasshouse conditions.

Genotype	Mineral composition in grain (DM)						
	P (%)	Ca (%)	mg/kg				
			Fe	Cu	Zn	Mn	Al
Soronko	0.53a	0.13a	163.89a	3.47a	55.20a	15.04a	2.70a
Nyira	0.42b	0.07b	75.27b	2.29b	40.42b	11.73b	0.45b
Asetenapa	0.13c	0.03b	38.21c	0.83c	11.49c	2.91c	0.25b
Inoculation							
<i>B. subtilis</i>	0.36a	0.06a	51.81b	1.93a	33.98b	9.28a	0.43a
No <i>B. subtilis</i>	0.36a	0.09a	133.11a	2.46a	37.43a	10.51a	1.83a
Irrigation							
Mine water (75% AMD)	0.45a	0.10a	137.57a	3.05a	46.23a	12.82a	1.88a
No AMD	0.28b	0.05a	47.34b	1.35b	25.17b	6.97b	0.38a
F-Statistics							
Genotype (G)	929.21***	5.02*	3.27**	27.47***	327.35***	21.09***	1.51*
Inoculation (IN)	0.24 ^{ns}	1.12 ^{ns}	3.88*	3.26 ^{ns}	5.91*	0.60 ^{ns}	1.20 ^{ns}
Irrigation (IR)	464.28***	4.02 ^{ns}	4.78*	33.65***	220.36***	13.81**	1.37 ^{ns}
G x IN x IR	173.68***	0.04 ^{ns}	0.13*	0.32 ^{ns}	36.33***	2.05 ^{ns}	0.97*

Values (M±S.E. (n=3)) followed by similar letters in a column are not significantly different at *p≤0.05, **p≤0.01, ***p≤0.001 and ns = not significant.

A comparison of the elemental content of cowpea shoot and grain in response to the interactive effect of *B. subtilis* (BD233) inoculation and mine water irrigation at 125 DAP revealed significant differences between and among genotypes. For example, without mine water irrigation, Soronko accumulated more Fe (359.42 mg/kg) in grain (dry matter) compared to shoot (240.8 mg/kg) without inoculation with *B. subtilis* (BD233) but accumulated more Fe in the shoot (217.0 mg/kg) compared to grain (83.69 mg/kg) when inoculated with the bacteria (Figure 5.1A). In contrast, without mine water irrigation, Nyira accumulated more Fe in shoot compared to grain with or without *B. subtilis* (BD233) inoculation (Figure 5.1A).

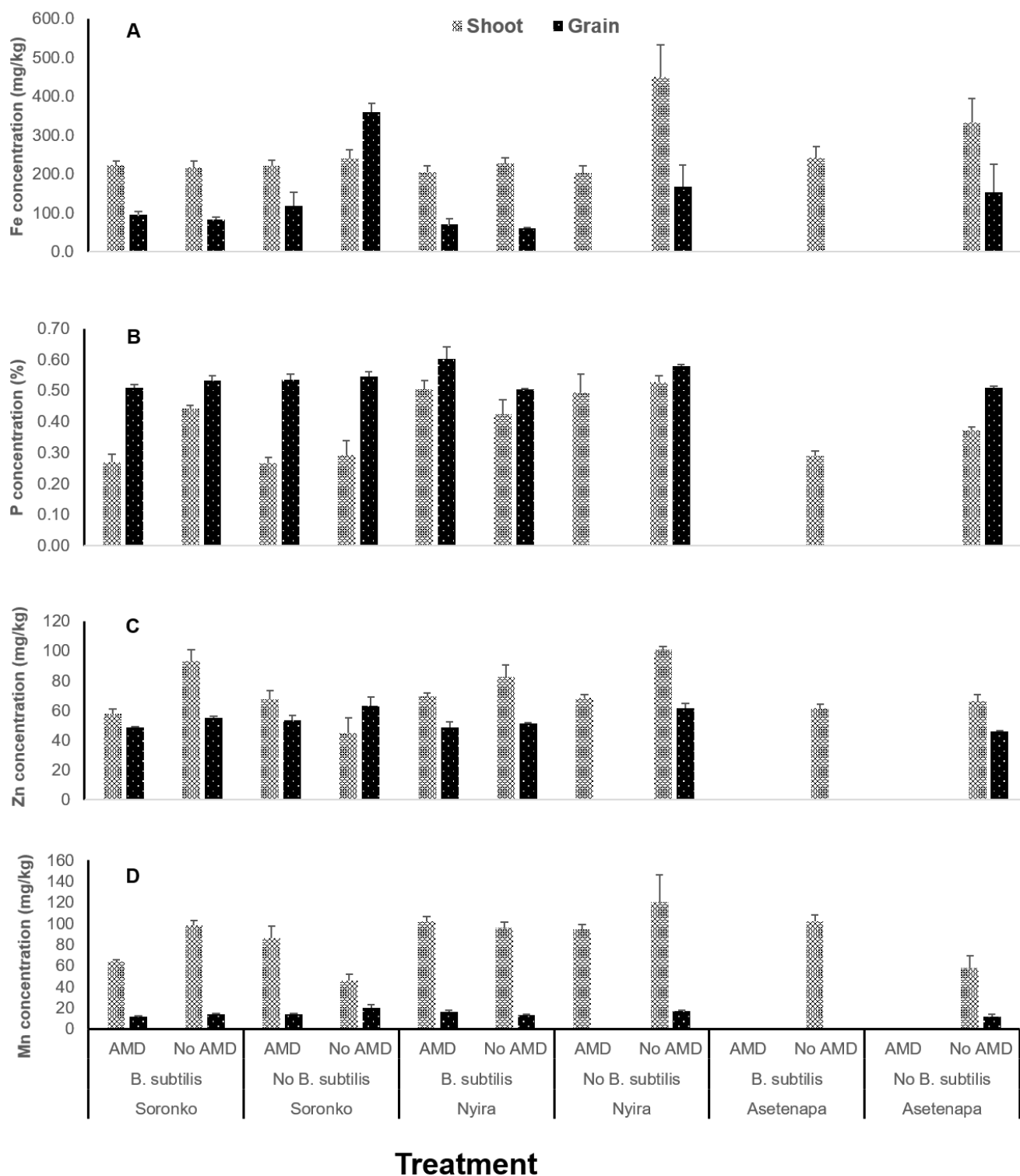


Figure 5.1: Comparison of elemental content in cowpea shoot and grain in response to the interactive effect of *B. subtilis* (BD233) inoculation and mine water irrigation. A) Iron, B) Phosphorus, C) Zinc and D) Manganese.

In general, with or with *B. subtilis* (BD233) inoculation and mine water irrigation, the grain of all cowpea genotypes accumulated more P compared to shoot. However, without *B. subtilis* (BD233) inoculation, when Nyira was irrigated with mine water, more P was accumulated in shoot than in the grain and when Asetenapa was irrigated with

mine water and inoculated with *B. subtilis* (BD233), the shoot also accumulated more P compared to the grain (Figure 5.1B). In contrast to P accumulation, more Zn was accumulated in shoot compared to grain for all genotypes regardless of the treatment except when Soronko without inoculation with the bacteria and mine water irrigation was the opposite i.e. more Zn in grain than in shoot (Figure 5.1C). Similarly, the concentration of Mn was more in shoot compared to grain for all tested genotypes regardless of the treatment (Figure 5.1D).

The cowpea crop serves as important sources of cheap protein, carbohydrates and nutrients for human nutrition. In this study, with or without mine water irrigation, inoculation with *B. subtilis* (BD233) showed concentration of some heavy metals (Fe, Zn, & Mn) in shoot and grains that are comparable to those reported in leaf and grain of most cowpea genotypes by Belane (2011). It will be interesting to test *B. subtilis* inoculation of cowpea under field conditions because microbes are known to help plant take-up nutrients (Schwartz et al., 2013). In this study, plants not inoculated with *B. subtilis* (BD233) accumulated more heavy metals when irrigated with mine water compared to those inoculated with the bacteria. The shoot of cowpea plants irrigated with mine water had marked concentrations of Fe, Zn, and Al compared to those not irrigated with mine water (Table 5.8). Among the genotypes, the grains of Soronko had more nutrients (P, Ca, Fe, Cu, Zn, Mn and Al) compared to the other genotypes (respectively) (Table 5.8). In general, inoculation with *B. subtilis* (BD233) affected accumulation of minerals in the shoot and grains of all genotypes and genotypic variations were noted in this study. The noted cowpea genotypic differences in mineral content in their organs are consistent with the findings of other studies for various crop species (Quintana et al., 1996; Grusak & DellaPenna, 1999; Belane & Dakora, 2010; Balane, 2011).

5.5 CONCLUSIONS

In conclusion, this study has showed that inoculation with *B. subtilis* (BD233) and irrigation with mine water (75% AMD) significantly influenced the growth (plant height, stem diameter, plant biomass), nodulation and yield of all the tested cowpea genotypes. However, genotypic variation was noted for all measured parameters in response to the treatments employed in the study. Soronko and Nyira showed some level of tolerance to metal toxicity at 125 DAP compared to the Asetenapa plants that did not survive when irrigated with mine water regardless of inoculation with or without

B. subtilis (BD233). The interaction of *B. subtilis* (BD233) inoculation and mine water irrigation affected the physiology (chlorophyll content and stomatal conductance) of cowpea and genotypic variation was also noted. Furthermore, the study showed that with or without *B. subtilis* (BD233) inoculation there was accumulation of heavy metals (Fe, Zn & Al) in cowpea shoot possibly from the mine water and genotypic variation was also noted. Notably, inoculation with *B. subtilis* (BD233), with or without mine water irrigation, affected the accumulation of heavy metals in cowpea grains. This finding clearly indicate that *B. subtilis* (BD233) has ability to sequester heavy metals in soils polluted with mine water.

CHAPTER 6: GENERAL DISCUSSION AND RECOMMENDATIONS

6.1 GENERAL DISCUSSION

Water for irrigation of crops is a scarce resource especially in areas that have a history of mining in South Africa. Mining of gold and coal over the years in the country has resulted in the pile-up of huge amounts of mine tailings close to water catchment areas (Jovanovich et al., 1998). AMD generated from mine tailings, and through entrance of water into mine voids, is generally characterized by low pH (2-4), high sulphur concentration (1-2 g/l) and high levels of metals, particularly heavy metals such as Zn, Cd, Cu, Ni, Pb, Mn, Fe, Hg and Cr (Vadapali et al., 2008). When these heavy metals are eventually discharged into water systems (surface and ground waters), they can persist and bio-accumulate in living organisms such as plants, ultimately posing health hazards and environmental risk in the ecosystem (Naicker et al., 2003). Ultimately, when water polluted with acid mine drainage are used for crop irrigation it changes soil properties and processes such as contraction of cation exchange capacity and expansion of anion exchange capacity (Fey, 2001) due to soil acidification. In addition, it can decrease the concentrations of some important basic cations (Ca, Mg, K) and solubility of nutrients that are essential for plant growth and also increases the concentration of some toxic elements (Al and Mn) (Sumner et al., 1990). Following heavy metal build-up in rhizosphere soils, plants will subsequently take up, translocate and accumulate them in their organs (Nemutanzhela et al., 2017). The accumulation of heavy metals in food crop organs have health risk implications if human consume such crops. For example, Khan et. al (2008) found significantly higher concentrations of heavy metal in food crop plants grown in wastewater-irrigated soils compared to those on soils not irrigated with the treatment. The concentrations of heavy metal in the food crop were also reported to exceed the accepted parameters stipulated by the State Environmental Protection Administration (SEPA) in China and the World Health Organization (WHO) (Khan et al. 2008). The accumulation of heavy metals in plant organs (with the associated health risk when consumed by humans) and loss of essential nutrients in soils exposed to acid mine water will eventually translate into poor crop productivity and nutrient quality, thereby posing threats to the security of food and nutrition. Therefore, the contamination of surface waters (streams, rivers, dams) and soils with acid mine water has become a matter of global concern (Mamba et al., 2009). In South Africa, a huge proportion (about 5 million ha) of soils are severely acidic and about 11 million ha are moderately acidic (Venter et al., 2001) possibly due

to pyritic formations in mineral deposits and acid mine acidification. In addition, the percentage of arable land and average annual rainfall for crop irrigation is low (Jovanovich et al., 1998). In the country, agriculture is a major economic activity and availability of clean water is a major concern for both commercial and poor-resource farmers. Agriculture utilises about 70% of water withdrawal hence it is expected that in times of water scarcity farmers will turn into polluted waters as water source especially by poor-resource farmers in rural communities (FAO, 2010). Therefore, the scarcity of good quality water in the country especially for irrigation of crops by resource-poor farmers is a major concern especially for rural communities within and around the precinct of mining industries. Recently, the country experienced severe drought in some provinces (Western Cape and Free State) and the looming climate change due to the El Nino phenomenon makes the situation very worrisome. One of the major threats associated with climate change is increase in global average temperatures, which poses additional problems for seed germination and crop development (Ewing, 1981). Temperature is noted to significantly affect seed germination (Rivers & Webber, 1971; White & Monstes, 1993; Craufurd et al., 1996), the rate of seed germination and seedling emergence of most crops including cowpea (Garcia-Huidobro et al., 1982; Covell et al., 1986). This additional threat to household food and nutrition security associated with climate change is worrisome and therefore, there is a need to mitigate climate change scenarios such as short-term elevated temperatures. To this end, agricultural management systems should adopt new strategies or approaches to mitigate climate change and increase food productivity. One suggested strategy is to clean-up contaminated surface waters especially those polluted by acid mine water for crop irrigation. However, the cost is colossal for cleaning AMD contaminated water using conventional methods (reverse osmosis, use of wet lands and lime) beside the negative impact of such technologies on the environment (McCarthy, 2011). There is need to search for cheaper and environmentally-friendly alternatives. Interestingly, modern technologies use plants and microorganisms for decontamination of polluted soils or waters and are therefore considered to be cost-effective and environmentally-friendly (Abou-Shanab et al., 2003; Hooda, 2007; Zhuang et al., 2007). *Bacillus* spp. are among the microorganisms used to bio-remediate contaminated waters and soils (with heavy metals) (Carrasco et al., 2005; Zhuang et al., 2007) and also as enhancers of seed germination and plant growth (Prathibha & Siddalingeshwara, 2013). Another strategy to mitigate climate change is to cultivate genotypes with traits adaptable to rapid and excessive short-

term changes in climatic conditions. This study evaluated the interactive effects of *Bacillus subtilis* inoculation and elevated temperature on the germination indices (germination percentage (G%), germination index (GI) and germination rate index (GRI)) and growth of three cowpea genotypes. The results obtained showed that the interaction of *B. subtilis* (BD233) inoculation and elevated temperature significantly ($p < 0.05$) influenced the germination indices and plumule length of cowpea seedlings. For example, Nyira (98.6%) followed by Soronko (84.8%) had the highest germination percentage when their seeds were treated with *B. subtilis* (BD233) and without *B. subtilis* (BD233) respectively, and incubated at an elevated temperature (35°C). With or without *B. subtilis* (BD233), all three genotypes incubated at either 10°C, or 40°C or 45°C had the lowest germination percentages compared to the other temperatures (Table 3.2). In general, Nyira seeds inoculated with *B. subtilis* (BD233) and Soronko seeds without the bacteria obtained the fastest germination rates (lowest GI & GRI values) compared to Asetanapa seeds treated either with or without the bacteria (Table 3.3). Soronko seeds inoculated with *B. subtilis* (BD233) and incubated at 35°C for seven days had the longest plumule (2.06 cm) compared to the other genotypes under similar conditions (Table 3.4). Clearly, this study has demonstrated the potential of *B. subtilis* (BD233) inoculation to positively influence the germination and growth of cowpea, an important candidate for food/nutrition security in Africa.

In addition, the study showed that elevated temperature (>35°C) can significantly affect the growth and nutritional quality of cowpea at different growth stages (pre-flowering (40 DAP), flowering (90 DAP) and post-flowering (123 DAP)). For example, at an elevated temperature (35°C), the whole plant biomass (fresh and dry weights), and shoot carbon and crude protein contents at the pre-flowering and flowering stages of cowpea were most affected compared to control (25°C) (Table 4.2 and Figs. 4.3 & 4.4). The results obtained in this study suggest that the pre-flowering and flowering stages of cowpea compared to post-flowering are more susceptible to elevated temperatures (30-35°C). According to these results, under future climate change scenarios, rural farmers cultivating cowpea should adopt cropping management strategies that will protect the flowering stage in the crop's growth cycle especially during periods of changes in climatic conditions (temperature extremes over short periods associated with climate change).

Furthermore, this study has showed that interaction of *B. subtilis* (BD233) inoculation and mine water (75% AMD) irrigation can significantly influence cowpea's growth

(plant height, stem diameter, plant biomass) (Table 5.3), nodulation and yield (Table 5.5). Interestingly, there were genotypic variations noted in response to treatments employed in the study as Soronko and Nyira genotypes showed some level of tolerance to metal toxicity at 125 DAP compared to Asetenapa that did not survive when irrigated with mine water regardless of inoculation with or without *B. subtilis* (BD233) (Tables 5.3 & 5.5). The interaction of *B. subtilis* (BD233) inoculation and mine water irrigation similarly affected the physiology (chlorophyll content and stomatal conductance) of cowpea (Tables 5.6 & 5.7). Additionally, the study showed that the interaction of *B. subtilis* (BD233) inoculation and mine water (75% AMD) irrigation can influence the nutritional content of cowpea. For example, with or without *B. subtilis* (BD233) inoculation, irrigation with mine water caused accumulation of heavy metals (Fe, Zn & Al) in cowpea shoot and inoculation with *B. subtilis* (BD233), with or without mine irrigation, affected the accumulation of heavy metals in cowpea grains. These results are in agreement those reported by in a recent study by Israr et al. (2016). Their results showed that the interactive effect of phosphorus fertilization and inoculation with a plant growth promoting rhizobacteria (*Pseudomonas putida*) was significant for plant nutrient uptake (N, P, and K) that resulted in growth enhancement in chickpea (Israr et al., 2016). In this study, the results showed genotypic variations in response to the interactive effect of *B. subtilis* (BD233) inoculation and mine water (75% AMD) irrigation, which seems to suggest that farmers should consider cultivating cowpea genotypes with metal tolerance when using acid mine water for irrigating their crops under water crisis situations associated future climate change. Additionally, the effect of *B. subtilis* inoculation should inform their choice of the cowpea genotypes.

In conclusion, according to the results obtained in this study, the interactive effect of *B. subtilis* inoculation and mine water irrigation can influence the growth, nodulation, yield, physiology and nutritional quality of cowpea. Furthermore, inoculation with *B. subtilis* has potential to enhance seed germination, growth and nutritional quality of cowpea even when irrigated with acid mine water under future climate change scenarios characterised with short-term extreme temperatures. Taken together, the results in this study suggest that *B. subtilis* (BD233) has the potential to serve as an effective heavy metal resistant bacteria especially in soils polluted with mine water.

6.2 RECOMMENDATIONS

- Soronko genotype seeds inoculated with *B. subtilis* (BD233) and incubated at 35°C was found to be better than the other genotypes with respect to germination rates and growth. However, more research is required to compare it with other elite genotypes and determine if the superior seed quality (vigour) of Soronko would interpret to higher yields under field conditions.
- Additionally, the interactive effect of *B. subtilis* (BD233) inoculation and mine water (75% AMD) irrigation on cowpea's growth, nodulation, yield and nutritional quality should be evaluated under field conditions.
- There is need to decipher the mechanisms involved in the metal resistance of any *Bacillus* spp. in soils polluted acid mine water.
- The study findings may be of significance to resource-poor farmers interested in cowpea cropping under future climate change scenarios.

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