BACILLUS SUBTILIS (BD233) *AND BRADYRHIZOBIUM* SP. (CB756) CO-INOCULATION EFFECTS ON GERMINATION, GROWTH, SYMBIOTIC PERFORMANCE AND ECO-PHYSIOLOGY OF LOCAL COWPEA GENOTYPES EXPOSED TO MINE WATER

by

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DECLARATION

I, Thalukanyo Nevhulaudzi, hereby certify that the research described in this thesis "*Bacillus subtilis* (BD233) and *Bradyrhizobium* sp. (CB756) co-inoculation effect on germination, growth, symbiotic performance and eco-physiology of local cowpea genotypes exposed to mine water," is original work that I completed under the guidance of my supervisors. Furthermore, I certify that the material provided here has never been published or submitted as part of a degree program requirement at any other university. The reference section of this thesis contains an acknowledgement and list of all mentioned works from other people or organizations. Additionally, I attest to my adherence to the University of South Africa's policies, procedures, rules, and standards.

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DEDICATION

This thesis is dedicated to my loving family including my son (Roana), father, mother, and siblings.

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ABBREVIATIONS

ANOVA	.....	Analysis of Variance
AMD	.....	Acid Mine Drainage
B. subtilis	.....	*Bacillus subtilis*
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	.....	Gram
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

ICP-MS	.....	Inductively Coupled Plasma-mass Spectrometer
K	.....	Potassium
LB	.....	Luria-Bertani
Mg	.....	Magnesium
Min	.....	Minute
mL	.....	Millilitre
mm	.....	Millimetre
mM	.....	Millimolar
Mn	.....	Manganese
MW	.....	Mine Water
Ni	.....	Nickel
^{o}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 examined the interactive effects of co-inoculating *Bacillus subtilis* (BD233) and *Bradyrhizobium* sp. CB756 on the germination, growth, and symbiotic performance of three local cowpea genotypes (Glenda, Pan 311, and Sudan) under exposure to mine water. The experimental treatments consisted of seeds treated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756, as well as three levels of mine water (0%, 25%, and 75%). Seeds not treated with either *B. subtilis* (BD233) or *Bradyrhizobium* sp. CB756, subjected to same three mine water levels. A standard germination test was conducted in duplicate over a period of seven days to evaluate the germination indices. In this study, four treatments were employed and include the following: i) seeds treated with *B. subtilis* (BD233) alone, ii) seeds inoculated with *Bradyrhizobium* sp. CB756 alone, iii) seeds co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 and iv) seeds not inoculated with either bacterium and exposed to 0% mine water (control). In a greenhouse, a factorial experiment with three replications and a complete randomized design was carried out. The study showed that co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 under varying mine water levels significantly influenced germination, growth and symbiotic performances of local cowpea genotypes. Cowpea irrigated with mine water and co-inoculated showed resistance to heavy metal stress with respect to their germination and symbiotic performance and genotypic differences noted. Pan 311 with the lowest $\delta^{15}N$ and highest amount of N-fixed was found to be more dependent on nitrogen fixation compared to Glenda and Sudan, and it also had higher soil N uptake with heaviest dry shoot biomass. The results showed that cowpea's carbon assimilation physiology was notably resilient to both mine water irrigation and microbial inoculation treatments. Intercellular CO_2 concentration increased significantly at low mine water dilution (25%). The study further revealed that co-inoculating Pan 311 with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 improved the shoot nutrient contents by 24% (Ca), 16% (Mg), 24% (Mn), 17% (B) and 21% (Al) compared to when not inoculated and genotypic differences noted in response to dual or single inoculation. These findings highlight the potential of tailored microbial applications to enhance cowpea productivity in mine water contaminated soils.

Keywords: Cowpea, Genotypes, Germination, Co-inoculation, *Bradyrhizobium* sp., Mine Water, *Bacillus Subtilis,* Temperature, Heavy Metals, Nitrogen Fixation.

CHAPTER 1: ORIENTATION TO THE STUDY

1.1 INTRODUCTION

Acid mine drainage, or mine water, continues to be a major global source of surface and ground water contamination (Coetzee et al., 2010; Naidoo, 2017). When Minerals containing sulfides, frequently in the form of pyrite, are exposed to the atmosphere when water is present, sulphuric acid is produced, and heavy metals and other contaminants are released into the drainage system. This process creates mine water. Low pH levels (2-4), elevated sulfur concentration (1-2 g/L), and high metal concentrations, especially heavy metals like Zn, Cd, Cu, Ni, Pb, Mn, Fe, Hg, and Cr, are typical characteristics of mine water produced by water entering the mine voids (Vadapali et al., 2008). Naicker et al. (2003) and Win et al. (2024) claim that these heavy metals in water systems can linger and bioaccumulate in living things, especially if plants are exposed to higher concentrations and subsequently ingested by humans and animals in the food chain, ultimately posing risks to human health and the environment. Concerns have been raised about the release of heavy metals into aquatic environments, which contaminate surface water and groundwater systems especially in regions with mining activities (Mamba et al., 2009).

South Africa has a long history of mining activities with huge amounts of mine water left behind the mining industry's precincts. Due to acid mine drainage from the pyritic formations in coal and gold resources, the mining industry's close proximity to surface waters and water catchment areas poses an extra risk to ecosystems. It is believed that the nation has a low percentage of arable land, poor rainfall, and a water shortage (Jovanovich et al., 1998). Thus, the lack of high-quality water throughout the nation, particularly for commercial and resource-poor farmers to irrigate their crops, is a serious issue. The situation is quite concerning given the previous droughts and the onset of climate change brought on by the El Nino phenomena. Rising global average temperatures are one of the main concerns posed by climate change, and they create additional challenges for crops and other living things. Moreover, frequent droughts and unpredictable rainfall patterns are linked to climate change. Therefore, the scientific community is interested in the potential use of surface waters (rivers, lakes, and dams) contaminated by mine water for agricultural crop irrigation as well as the

imminent physiological risks brought on by temperature changes. Du Plessis (1983) highlighted the advantages of managing the country's wastewater disposal from mines and demonstrated the feasibility of irrigating crops using acid mine water treated with lime. However, using lime to improve mine water is not only costly, but it can also release contaminants into the soil after crops are irrigated with acid mine water that has been treated with lime. In order to decontaminate mine water without endangering ecosystems or agronomic systems, it is imperative to find more affordable ameliorating alternatives or readily available bioremediators. Currently, heavy metal contamination clean-up procedures are expensive and harmful to the environment. It is interesting to note that scientists have begun developing affordable solutions that use microbes to clean up contaminants (Hooda, 2007).

It is noted that plant growth-promoting bacteria can enhance the productivity, nutrient uptake and resilience of plants, particularly under resource-limited or stress-prone conditions. Recent reports indicate that co-inoculating plant growth-promoting rhizobacteria (PGPR) and rhizobia can enhance the symbiotic performance of soybean, improve its yield and suppress disease incidence (Xing et al., 2022). A study by Singh et al. (2021) showed that co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 can enhance nitrogen fixation, nutrient uptake, and metal stress tolerance of cowpea genotypes. *Bacillus subtilis* is an omnipresent microorganism that has reportedly improved plant growth and development including providing defense against plant pathogens. The use of *Bacillus* species in agriculture is increasing steadily as it offers an alternative to expensive synthetic chemical fertilizers (Bhattacharyya and Jha, 2012). Certain strains of *Bacillus* sp. are reported to enhance plant growth through several mechanisms but mainly either by providing promotive metabolites (siderophores and phytohormones e.g. auxins, cytokinins, gibberellins) (Ali et al., 2009) or enhancing nutrient uptake (solubilisation of minerals) from plant rhizosphere soils (Meng et al., 2016). Studies have found that *B. subtilis* has ability to quench soil ills to boost plant growth (Adesemoye et al., 2008; Felici et al., 2008). Similarly, *Bradyrhizobium* sp. CB756, a nitrogen-fixing bacterium, is noted to improve plant growth and nutrient acquisition. Research has demonstrated that when cowpea plants are cultivated in acidic soils with high Al concentrations, co-inoculation with these two microorganisms (*B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756) can greatly increase the uptake of vital micronutrients like iron (Fe), zinc

(Zn), and copper (Cu) (Dutta et al., 2019). This is explained by the fact that *B. subtilis* (BD233) produces siderophores, which aid in the chelation of iron and other micronutrients, increasing their availability to the plants. Furthermore, *B. subtilis* (BD233) improves the nodulation and nitrogen fixation abilities of *Bradyrhizobium* sp. CB756, which increases plant biomass and nutrient uptake (Nelwamondo et al., 2026).

The potential of *B. subtilis* as a bioremediator, particularly concerning mine water, remains to be thoroughly investigated in its functional activities. The cowpea (*Vigna unguiculata* L. Walp), a member of the Fabaceae/Leguminosae family, is a vital and nutrient-dense crop. The crop is primarily grown for its capacity to supply inexpensive dietary protein, carbohydrates, minerals, and vitamins to humans as well as nitrogen to nutrient-poor soils (Belane, 2011). When eaten as vegetables, the green leaves and immature pods can provide up to 35% of the protein in cowpea grain, which is high in both protein (42%) and carbohydrates (57%) (Belane, 2011). The crop has the ability to help provide household food and nutrition because it is primarily grown and consumed by smallholder farmers in remote South African provinces. Since 1880, global temperatures have risen by about 1°C, with projections of a 1.5°C increase by 2050 and 2-4°C by 2100 (Zhao et al.,2017; IPCC,2014) subject to increase in greenhouse gas emissions. Wheeler and von Braun (2013) stated that crops are affected by climate change, including shifts in temperature, precipitation, and increased CO_2 levels. However, cowpeas' resilience in harsh conditions makes them essential for food and nutrition security in tropical and subtropical regions (Chakravaram et al., 2025).

1.2 PROBLEM STATEMENT

Water shortage is a problem in South Africa (Muller et al., 2009), and as previous droughts in the provinces of Mpumalanga, the Free State, and the Western Cape have shown, climate change will make the situation worse (Botai et al., 2017). However, because of its extensive mining history, particularly in the Witwatersrand Basin, the nation has the majority of surface waters, including dams and rivers that are contaminated with massive volumes of minerals with pyritic forms (McCarthy, 2011). When used for irrigation, mine water can have an adverse effect on crop development and nutritional quality since it is hazardous (containing heavy metals and low acidity). Additionally, the growth and output of crops like vegetables such as cowpeas are

threatened by exposure to extreme weather conditions (such as high temperatures and CO_2). Future household food and nutrition security may be threatened by rising global temperatures brought on by changes in climate, which can affect the germination of seeds and the growth of significant economic crops like cowpea. Global climate change impacts agriculture through both direct and indirect effects, such as altered crop growth, fluctuating yields, extreme weather, shifting precipitation, soil degradation, and changes in pest and disease patterns (Yuan et al., 2024). These changes reduce soil fertility and land resources, challenging sustainable agricultural development (Eekhout and De Vente, 2022). Furthermore, it is impossible to overstate the importance of mitigating climate change, particularly in developing sub-Saharan African nations like water-scarce South Africa. There is an urgent need to improve mine water with more affordable, widely available, and ecologically friendly substitutes. It has been suggested that microorganisms be used to improve severely contaminated water, such as mine water. Additionally, no research has been done on the effects of co-inoculation of *Bradyrhizobium japonicum* and *B. subtilis* on the germination, growth, symbiotic performance and eco-physiology of local cowpea genotypes irrigated with mine water. Thus, this study will investigate the appropriateness of co-inoculating *B. subtilis* and *B. japonicum* as prospective bacteria that can withstand high metals in soils contaminated by mine water.

1.3 AIM AND OBJECTIVES

1.3.1 Aim

To evaluate the effects of co-inoculation *B. subtilis* and *B. japonicum* on growth, physiological performance, nitrogen fixation and nutrients accumulation of local cowpea genotypes irrigated with mine water.

1.3.2 Objectives

I. To determine the effects of *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 co-inoculation on germination, growth and yield of local cowpea genotypes subjected to mine water.

II. To determine the effects of *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 co-inoculation on symbiotic performance of local cowpea genotypes subjected to mine water.

III. To evaluate the effect of co-inoculation of *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 on the eco-physiological parameters and carbon assimilation of local cowpea genotypes subjected to mine water.

IV. To determine the effect of co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 on nutritional composition of local cowpea genotypes subjected to mine water.

1.4 HYPOTHESES

I. Co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 strains has no effects on germination, growth and grain yield of local cowpea genotypes irrigated with mine water.

II. Mine water has no effect on the biological N_2 fixation (BNF) of cowpea inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 strains.

III. Mine water has no effect on the eco-physiological parameters and carbon assimilation of local cowpea genotypes co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 strains.

IV. Co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 strains has no effects on nutritional composition of local cowpea genotypes subjected to mine water.

1.5 THE STUDY'S SIGNIFICANCE

Co-inoculating plant growth-promoting rhizobacteria (PGPR) and rhizobia can enhance the symbiotic performance of soybean, improve its yield and suppress disease incidence (Xing et al., 2022). A study by Singh et al. (2021) showed that co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 could enhance nitrogen fixation, nutrient uptake, and metal stress tolerance of cowpea genotypes. Generally, microbes are noted to influence plant growth in several ways even when inoculated alone or in combinations. The usage of *Bacillus* species in agriculture has been steadily growing because they offer an inexpensive substitute for expensive, environmentally harmful fertilizers (Jha and Bhattacharyya, 2012). Auxins, cytokinins, gibberellins, and other phytohormones are secreted by some strains of *Bacillus* sp. in plant rhizosphere soils (Ali et al., 2009a, Galaviz et al., 2018), or they increase nutrient utilization (solubilisation of minerals) in plants (Bowen & Rovira, 1999; Zhang et al.,

2010; Meng et al., 2016). According to research, *B. subtilis* can suppress plant pathogen activity and soil problems such nutrient deficits to promote plant growth (Kloepper et al., 2004; Adesemoye et al., 2008; Felici et al., 2008). Heat stress significantly restricts legumes yields and is expected to worsen as climate change continues (Mohammed et al., 2024). Microbial inoculation functions as an effective climate adaptation strategy by promoting soil health, optimising nutrient cycling, and strengthening plant resilience against environmental stressors, pests, and diseases (Díaz-Rodríguez et al.,2025). The application of microbial inoculants is consistent with the objectives of sustainable agriculture. It mitigates environmental pollution, decreases greenhouse gas emissions, and enhances soil biodiversity, thereby supporting resilient and productive farming systems amid climate change challenges. The impact of co-inoculation of *B. subtilis* and *Bradyrhizobium* sp. and elevated temperatures on cowpea genotype development and seed germination, a significant crop in Africa and other parts of the world, has, however, not been thoroughly studied (Freire-Filho, 2011). Transcriptome analysis of chrysanthemum showed that co-inoculating multiple PGPR strains improves growth and quality more than single strain inoculation, as it triggers gene expression related to metabolism, signal transduction, and beneficial compound synthesis (Wang et al., 2024). The findings of this study inform the selection of cowpea genotypes, especially for farmers with limited resources in areas experiencing climatic shifts, such as increased temperatures, and utilizing improved mine water for irrigation. This research seeks to promote sustainable agricultural practices, improve crop yields through the use of mine water, and offer valuable support to smallholder farmers.

1.6 ETHICAL CLEARANCE

This study was carried out in line with UNISA's policy on ethics, the research proposal was approved by CAES committee on 05 August 2021 (2021_CAES_HREC_116 FR) and the approval reviewed yearly until the experiment's conclusion.

1.7 LAYOUT OF STUDY

This thesis consists of eight chapters, five of which are dedicated to research. To assist readers in thinking through the issues discussed, a summary of each chapter is provided here.

The first chapter presents the study by outlining its background, defining its purpose and objectives, identifying the research problem, and explaining why the study is important. The second chapter reviews the literature on agricultural productivity and climate change, as well as the role of microorganisms either singly or in co-inoculations in the bioremediation of water sources contaminated by mine water. The objectives of the literature review are articulated, and relevant conclusions pertaining to the topic are presented whereas the third chapter is dedicated to an outline of the materials and methodologies employed in this study.

The results of multiple experiments are detailed in chapters four through to seven in the findings on the effect of *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 co-inoculation on germination, growth and grain biomass production and grain yield of local cowpea genotypes subjected to mine water under glasshouse conditions are described and discussed in Chapter four. Chapter five defines and debates findings on the symbiotic performance of local cowpea genotypes when subjected to co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 strains. Chapter six evaluated the effects of co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 on the eco-physiological parameters and carbon assimilation of local cowpea genotypes subjected to mine water. Chapter seven outline results on the interactive effects of co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 on the nutritional composition of local cowpea genotypes subjected to mine water.

Chapter eight provides a comprehensive discussion, draws conclusions, and offers corresponding recommendations.

CHAPTER TWO: LITERATURE REVIEW

2.1 Acid Mine Drainage as water pollutant

Extracting minerals such as gold is related with acid mine drainage issues which can cause long-term damage to watercourses and biodiversity (Akcil & koldas, 2006). Acid mine drainage occurs due to reactive sulphide minerals stored in waste rock dumps. This has caused widespread pollution especially in surface waters around and within mining industries' precincts. Additionally, some effluents produced by the metal mining industry comprise large quantities of toxic substances that include heavy metals, which have serious effects on human health and ecology.

2.2 Wastewater for crop irrigation

In developing countries, the management of wastewater has emerged as a significant challenge due to rising urbanization and industrialization, which have led to substantial increases in municipal waste and industrial effluent production (Alloway and Ayres, 1993). In many countries, treated wastewater is utilised for irrigation purposes on agricultural land as an effective method of disposal (Oloya & Tagwira, 1996). The use of wastewater for crop irrigation offers several advantages, including water conservation among other potential benefits. Irrigation of agriculture soils with wastewater may impact food safety and quality in addition to contaminating the land. (Muchuweti et al., 2006). Agriculture accounts for approximately 70% of global water withdrawals; therefore, during periods of water scarcity, it is expected that farmers may increasingly rely on wastewater as an alternative water source. The continued use of wastewater for irrigation could lead to plants accumulating heavy metals (Chipo et al., 2011). Furthermore, the long-term application of wastewater to agricultural land may lead to soil contamination with heavy metals (Dougherty and Hall, 1995).

Heavy metals either from wastewater or acid mine drainage water are one of the factors negatively affecting natural environment (Marchel et al., 2015). Heavy metal contamination in agricultural soils has emerged as a significant concern for crop production on a global scale (Chen et al., 2007). These heavy metals such as Zn, Cd, Cu, Ni, Pb, Mn, Fe, Hg, and Cr pose various risks; cadmium is particularly concerning because of its toxicity to plants and animals even at low concentrations (Hu et al., 2009). Cadmium is categorised by its high toxicity and high ability to bio-accumulate

in plants. Tiller et al. (1997) indicated that while elevated cadmium concentrations in soils may occur through natural processes, they are frequently a result of industrial activities or excessive use of agricultural fertilizers with significant cadmium content. Soil-to-plant transmission of heavy metals is the major pathway of human exposure to soil contamination (Wang et al., 2007). According to the Department of Agriculture and Water Affairs (DWAF, 1996), heavy metals including cadmium (Cd) and lead (Pb) may pose risks to human health. Prolonged consumption of foods enriched with cadmium can lead to the accumulation of this metal at levels potentially harmful to human health. Plants are nonetheless capable of accumulating lead at concentrations that are potentially hazardous to humans and plants, for example, potatoes, lettuce and hay.

Vegetables cultivated in wastewater-irrigated soils can take up heavy metals in large enough quantities to cause potential health risks to the consumers. For example, an experiment carried out by Khan et al. (2008) showed that plants grown in wastewater-irrigated soils were significantly higher in concentration of heavy metals than plants grown in the reference soil.

2.3 Microorganisms as bioremediators for acid mine drainage

According to Stratten (1987), bioremediation should be used to establish and implement eco-friendly methods for cleaning and protecting the environment. Bioremediation can be defined as any process that uses micro-organisms, fungi, green plants or their enzymes to return the natural environment altered by contaminants to its original condition. Bioremediation serves as an efficient approach for managing polluted environments and facilitating the restoration of contaminated soil. This technology relies on promoting the growth of specific microflora or microbial consortia that are indigenous to the contaminated sites that are able to perform desired activities (Agarwal, 1998). Bioremediation involves biotransformation and biodegradation processes by transforming contaminants to non-hazardous or less hazardous chemicals (Singh et al., 2014). Biotransformation is any alteration of the molecule or structure of a compound by micro-organisms. Biodegradation is the breaking down of organic or bioaccumulation and biotransformation of inorganic compounds into environmentally friendly compounds. A number of microorganisms that can carry out

biodegradation in many different environments are identified as active members of microbial consortiums; these microorganisms include *Bacillus* sp. (Singh et al., 2014).

Microorganisms with the ability to proliferate in the rhizosphere are excellent for use as biocontrol agents, because the rhizosphere provides the first line of defense for roots against pathogen attack. Bacteria thought to have potential for biocontrol occur in many species including *Bacillus* (Singh et al., 2014). Interestingly, *Bacillus* sp. has been tested on a wide variety of plant species for ability to control diseases and numerous studies have investigated the potential for applying these approaches to control of potato brown rot disease (Kloepper et al., 2004; Pal and McSpadden Gardener, 2006).

Furthermore, many microorganisms from the rhizosphere can influence plant growth and health positively and are therefore often referred to as “plant growth promoting rhizobacteria” (Xing et al., 2022). Studies in the Netherlands suggest that Plant Growth Promoting Rhizobacteria (PGPR) advance potato development primarily through suppressing cyanide-producing deleterious rhizosphere microorganisms (Kloepper et al., 2004).

An alternative and economically friendly process for the decontamination of mine water is metal precipitation using anaerobically generated sulphide. Sulphate-reducing bacteria (SRB) can use the sulphate present in the mine water as the terminal electron acceptor during the metabolism of the organic matter resulting in the production of H_2S, which can be used as a metal precipitating agent (Gibert et al., 2002). According to Gibert et al. (2002), mine water treatment using sulphate-reducing bacteria (SRB) prefer environmental requirements such as anaerobic condition, high pH, sufficient electron donors and appropriate substrate for immobilization. In a study undertaken by Nevhulaudzi et al. (2020), their results indicated that nodulation and yield of the tested cowpea genotypes varied significantly in response to *B. subtilis* inoculation and irrigation with mine water. Similar results were obtained for plants dual-inoculated with *B*. *subtilis* (BD233), which had significantly higher number of nodules compared to those not inoculated with the bacteria. So far, no study has explored the potential of co-inoculation of *Bacillus sp.* and *Bradyrhizobium* sp*.* to ameliorate mine water or its suitability to bioremediate heavy metal in contaminated water for crop irrigation.

2.4 Biological Nitrogen Fixation (Nitrogen Source)

Most of the nitrogen in the soil occurs in an organic form and only a slight fraction is available to higher plants as inorganic mineral N in the form of ammonium or nitrate. Majority of plants and other living organisms require large amounts of nitrogen than they need essential elements other than carbon, oxygen and hydrogen (Belane 2011). Nitrogen is regularly considered as the most important nutrient for plants in terrestrial ecosystems (Diekmann and Falkengren-Grerup, 1998). Nitrogen may be introduced into any natural and/ or agricultural ecosystems from commercial fertilizers, biological N_2 fixation, green manures, N deposition from precipitation, and combustion of fossil fuel (Belane 2011). Biological nitrogen fixation can sustainably increase agricultural productivity especially when legumes are included in the cropping systems. As far as literature is concerned, cowpea can fix large amount of N (up to 201 kg N ha^{-1}) in a cropping season (Dakora et al., 1987) and a study carried out by Belane and Dakora (2009) stated that cowpea tested fixed over 100 kg N ha^{-1} but so far, no study has assessed the biological nitrogen fixation of cowpea co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 strains..

2.5 Beneficial Microorganisms (Plant Growth-Promoting Rhizobacteria (PGPR) and their Role in Plant Growth and Stress Tolerance

Plant growth-promoting rhizobacteria (PGPR) are a group of beneficial bacteria that can colonize the plant rhizosphere and confer various beneficial effects on their host plants (Kloepper et al., 1980). PGPR can enhance plant growth and productivity through a range of mechanisms, including the fixation of atmospheric nitrogen, solubilization of inorganic phosphates, production of phytohormones, and induction of systemic resistance against biotic and abiotic stresses (Xing et al., 2022). These mechanisms allow PGPR to improve nutrient acquisition, alleviate stress responses, and ultimately enhance the overall performance of the plant (Bhattacharyya & Jha, 2012).

2.5.1 *Bradyrhizobium japonicum* and its Symbiotic Relationship with Legumes

Bradyrhizobium japonicum is a well-known nitrogen-fixing bacterium that forms a symbiotic relationship with leguminous crops, including cowpea (Kaneko et al., 2002). This bacterium has the unique ability to colonize the root system of legumes and establish root nodules, where it can fix atmospheric nitrogen and provide it to the host

plant in a form that can be readily utilised (Okubo et al., 2012). The symbiotic relationship between *B. japonicum* and legumes, such as cowpea, is of great agronomic importance, as it can enhance the plant's access to nitrogen and reduce the need for synthetic nitrogen fertilizers (Burdman et al., 2000), which are environmentally unfriendly.

2.5.2 *Bacillus subtilis* and its Plant Growth-Promoting and Stress-Alleviating Abilities

Bacillus subtilis is another PGPR that has been extensively studied for its beneficial effects on plant growth and stress tolerance (Ryu et al., 2004). This bacterium can produce a wide range of secondary metabolites, including phytohormones, siderophores, and antimicrobial compounds, which can stimulate plant growth and development, as well as enhance the plant's defence mechanisms against biotic and abiotic stresses (Kloepper et al., 2004). Additionally, *B. subtilis* has been shown to induce systemic resistance in plants, priming their physiological and biochemical responses to better cope with environmental challenges, such as drought (Pineda et al., 2010).

The co-inoculation of cowpea with both *B. japonicum* and *B. subtilis* has the potential to leverage the synergistic effects of these two beneficial microorganisms, leading to improved nutrient acquisition, enhanced drought tolerance, and ultimately, increased yield of the crop (Rodrigues et al., 2013).

2.6 Co-inoculation of Cowpea with Bradyrhizobium japonicum and Bacillus subtilis: Synergistic Effects of Co-inoculation on Plant Growth and Stress Tolerance

The co-inoculation of cowpea with both *B. japonicum* and *B. subtilis* has been shown to have synergistic effects on plant growth and stress tolerance. By combining the nitrogen-fixing powers of *B. japonicum* with the plant growth-promoting and stress-alleviating characteristics of *B. subtilis*, the co-inoculation can offer a more comprehensive set of benefits to the host plant (Rodrigues et al., 2013). Studies have demonstrated that co-inoculation of cowpea with these two beneficial microorganisms can lead to improved root and shoot development, increased biomass production, and improved tolerance to various abiotic stresses, including drought (Kakar et al., 2016). The synergistic interaction between the two bacteria can be attributable to their

complementary modes of action, such as the provision of fixed nitrogen by *B. japonicum* and the production of phytohormones, siderophores, and antimicrobial compounds by *B. subtilis*, which can collectively enhance the plant's overall fitness and resilience (Bhattacharyya & Jha, 2012).

2.7 Growth response and nutrient uptake of cowpea genotypes imperilled to co-inoculation of *B. japonicum* and *B. subtilis* exposed to environmental stress

Cowpea (*Vigna unguiculata* L. Walp.) is a crucial legume crop widely cultivated in the arid and semi-arid regions of the world, predominantly in sub-Saharan Africa, where it is the principal source of dietary protein, carbohydrates, and other essential nutrients for the local population (Timko & Singh, 2008). However, the productivity of cowpea is often constrained by various abiotic stresses, including drought and heavy metal, which is a substantial challenge in many regions where the crop is grown (Fatokun et al., 2012; Win et al., 2024).

Drought stress can significantly impair cowpea plants' growth, development, and total yield by inducing physiological and biochemical changes that impair nutrient uptake, photosynthesis, and other essential processes (Anyia & Herzog, 2004). To address this challenge, researchers have investigated the potential of beneficial microorganisms, such as plant growth-promoting rhizobacteria (PGPR), to enhance the drought tolerance and nutrient acquisition capabilities of cowpea genotypes, including landraces which are often further resilient to environmental strains (Egamberdieva et al., 2017).

Among the PGPR, *B. japonicum* and *B. subtilis* have been widely studied for their capability to establish symbiotic relationships with legumes, including cowpea, and to confer various growth-promoting and stress-alleviating effects (Burdman et al., 2000; Kakar et al., 2016, Win et al., 2024). The co-inoculation of cowpea with these two beneficial bacteria has the potential to synergistically improve the ability of plants to withstand heavy metals stress and enhance its nutrient uptake, thereby improving overall growth performance and productivity (Rodrigues et al., 2013). This study aims to investigate the growth and yield responses, symbiotic performance and nutrient uptake of local cowpea genotypes when co-inoculated with *B. japonicum* and *B. subtilis* and exposed to heavy metal stress conditions due to mine water. The results of this investigation are expected to advance the development of sustainable and

resilient cowpea production systems, particularly in regions where mine water pollution is a major constraint.

CHAPTER THREE: GENERAL MATERIALS AND METHODS

3.1 COWPEA GERMINATION

3.1.1 Seed Materials and Measurements

Three local cowpea genotypes (Glenda, Sudan and Pan 311) were sourced from local markets in Pretoria, South Africa and stored at Eureka Building, Florida campus, University of South Africa. Hard/damaged seeds were removed and seed viability test was done prior to experimentation, and only viable seeds of equal sizes were used for the seed germination experiment. All seeds were surfaced sterilized before experiments were carried out.

3.1.2 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. Strains previously grown on Luria-Bertani solid media were 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 seedlings (Nevhulaudzi et al., 2020). The bacterial cells were extracted from LB agar plates and transferred into liquid LB media, yielding a minimum 8.547x109 cells per mL. Only, 2 mL of strain was pipetted on the cowpea seedling. About 500 g of *Bradyrhizobium* sp. in commercial powdered inoculant Simbiose Nod Soja® (Simbiose: Biological Agrotechnology) containing the CB756 strains (minimum concentration of 7.2 × 109 viable cells per mL) added to 1800 mL of sterile distilled water according to the producer's instruction and was used for the germination experiment.

3.2 COWPEA YIELD AND GROWTH

3.2.1 Experimental Conditions and Data Collection

The experiment was conducted at the University of South Africa (Unisa) Florida campus, Gauteng Province (26° 10′ 30″ S, 27° 55′ 22.8″ E) in the controlled-environment glasshouse. The mean air temperature was 26.4°C during the day and 23.4°C at night, and mean relative humidity was 70%.

The experiment was repeated under same glasshouse conditions, the first experiment was carried out from October 2021 to January 2022 while the second experiment was conducted in January 2022 to April 2022, to validate the first experimental results. Data on growth parameters (number of leaves per plant, stem diameter and plant height) was collected at seven-day intervals. Nodule numbers per plant, shoot weight (fresh and dry), root weight (fresh and dry), number pods per plant, and grain dry weight were recorded after harvest**.** Cowpea biomass (shoot and root), nodulation (number of nodules per plant), yield (the number of pods) and the number of grains per plant were determined at 90 Days after Planting (DAP). The number of leaves per plant were 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. Plants for each treatment were harvested randomly at 90 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 freeze-dried for 72 h to constant weight and the weight measured using a weighing balance. Plants were carefully uprooted at 90 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. For yield determination for each treatment, the number of pods per plant were counted.

3.2.2 Cowpea Co-Inoculation

A commercial powered inoculant containing *Bradyrhizobium* sp. CB756 strains was utilised to inoculate cowpea seedlings. Furthermore, 2 mL of *B. subtilis* broth media, adjusted to 0.5 McFarland standards, was administered to the roots of each cowpea seedling cultivated in the glasshouse for this experiment.

3.2.3 Acid Mine Drainage

The AMD was collected from a gold mine site situated in Randfontein, 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 25% mine water was prepared by mixing 250 mL of AMD with 750 mL tap water which was used

to irrigate cowpea seedlings two weeks after planting in the glasshouse. This was done to allow seedlings overcome transplant shock and prevent seedling death. Elemental analysis of the mine water was carried out as defined by APHA et al. (2023).

Table 3.1. Elemental composition of mine water and control (0% AMD) used for irrigating cowpea

Element	Mine Water (AMD) (mg/L)			South African Standards (mg/L)	Agricultural Water Use Limit (mg/L)
	0%	25%	75%		
Al	0.021	0.009	0.112	0.3	5
B	0.053	0.072	0.0160	2.4	0.5
Ba	<0.010	0.055	0.070	0.7	-
Be	<0.010	<0.002	<0.002	-	0.1
Cd	<0.005	<0.006	<0.008	0.003	10
Co	<0.005	0.096	0.285	-	0.5
Cu	0.008	0.003	0.004	2	0.2
Fe	0.012	0.070	0.055	0.3	5
Mn	0.003	8.981	22.83	0.004-0.1	0.02
Pb	<0.010	<0.017	<0.027	0.01	0.1
Sr	0.111	0.335	0.438	-	-
Ni	<0.005	0.043	0.197	≤0.7	-
Zn	0.012	0.052	0.133	≤5.0	1
Cr	<0.005	0.007	0.006	≤0.005	0.1
Mg	8.83	63.21	120.9	-	500
K	3.50	10.83	14.39	-	50
Ca	19.03	257.8	408.4	-	1,000
Na	13.9	79.06	147.7	≤200	70

Source - Department of Water Affairs and Forestry (DWAF) (1996)

3.2.4 Plant Growth Media

Acid washed sand was obtained from a local factory in Germiston, South Africa. The acid sand was thoroughly washed using tap water to remove nutrients. The sand was placed in the autoclave for 72 h at 60^0C. One litre planting pots (14-inch) were used for the experiment. A packet of the powdered inoculant with *Bradyrhizobium sp.* CB756 strains was used for inoculation of cowpea seedlings. About 2g 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. Surfaced-sterilized cowpea 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 µmol $m^{-2}s^{-1}$ and plants were irrigated with each plant receiving three levels (0, 25, 75% AMD) of mine water a day.

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 3.2 below.

Table 3.2. N-free plant nutrients and their concentrations in stock solutions

Stock Solutions	Form	Source/Element	Mass/Litre	Concentration
A	$CaCl_2.2H_2O$	Ca	294.1	2.0 M
B	KH_2PO_4	P	136.1	1.0 M
C	Fe-citrate	Fe	607	20.0 mM
	$MgSO_4.7H_2O$	Mg	123.3	0.5 M
	K_2SO_4	K	87.0	0.5 M
	$MnSO_4.2H_2O$	Mn	0.338	2.0 mM
D	H_3BO_3	B	0.247	4.0 mM
	$ZnSO_4.7H_2O$	Zn	0.288	1.0 mM
	$CuSO_4.5H_2O$	Cu	0.100	0.4 mM
	$CoSO_4.7H_2O$	Co	0.056	0.2 mM
	$NaMoO_2.2H_2O$	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 plants were irrigated with the N-free nutrients solutions once a week.

3.3 DATA ANALYSIS

The data were analysed using a three-way analysis of variance (ANOVA) with STATISTICA software version 10 (Stat Soft Inc., Tulsa, OK, USA). Differences between treatment means were determined by Duncan's multiple range test at $p \leq 0.05$.

CHAPTER FOUR: Effect of *Bacillus subtilis* (BD233) and *Bradyrhizobium* sp. CB756 co-inoculation on germination, growth and yield of local cowpea genotypes irrigated with mine water.

4.1 INTRODUCTION

Africa's arid and semi-arid regions as well as other areas worldwide are facing a massive challenge in agriculture because of climate variations. Stresses like mine water (heavy metal) and drought are particularly detrimental to plant growth and yield in agricultural production, affecting the world's food security (Vurukonda et al., 2016), and if current trends continue, mine water and drought affected areas are expected to double, and water resources will decrease by 30% by 2050 (Kasim et al., 2013). Globally, mining is a human activity known to provide revenue while also polluting the environment, particularly surface waterways that receive mine waste. Specifically, acid mine drainage that is produced by mine tailings and water entering mine cavities is often categorised by low pH (2-4), high sulphur concentrations (1-2 g/L), and elevated metal levels, especially heavy metals as Zn, Cd, Cu, Ni, Pb, Mn, Fe, Hg, and Cr (Vadapalli et al., 2008). The release of heavy metals into aquatic environments (including both surface and groundwater) poses significant health and environmental risks, as these substances are persistent and have the potential to bioaccumulate in organisms such as plants (Fey, 2001). The depletion of essential soil nutrients due to exposure to mine water, combined with the accumulation of heavy metals in plant tissues, which can pose health risks to humans ultimately leads to reduced crop yields and diminished nutrient quality, thereby threatening food and nutrition security. Consequently, the contamination of surface waters including streams, rivers, and dams as well as soils by mine water has emerged as an issue of international significance (Mamba et al., 2009). In South Africa, mining of gold and coal over the years has resulted in the pile-up of huge amounts of mine tailings close to water catchment areas (Jovanovic, et al., 1998). Furthermore, a significant amount of the nation's soils (roughly 5 million hectares) is extremely acidic, and another 11 million hectares are moderately acidic (Venter et al., 2001). This may be because of acid mine acidification and pyritic formations in mineral deposits, and the country has low percentage of arable land and average annual rainfall for crop irrigation (Jovanovic et al., 1998). Climate change-related temperature rises have made much of sub-Saharan Africa drier, which has made matters worse for South Africa, a country with limited

water resources. Recently, South Africa experienced droughts in some provinces, where agriculture is a major economic activity and therefore, availability of clean water has become 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 to polluted waters as water source especially by poor-resource farmers in rural communities (FAO 2010). Therefore, the scarcity of good quality water especially for irrigation of crops by resource-poor famers is a major concern especially for rural communities within and around the precinct of mining industries in South Africa. In mining industries' precinct, most rural communities rely on vegetables and legume crops such as cowpea to meet their mineral and protein requirements.

Cowpea is considered the second most important food grain legume in sub-Saharan Africa and are an inexpensive source of plant protein for people. Many households in semi-arid parts of Africa produce and consume cowpeas. A crop that can adapt to various soil ecologies throughout the continent thanks to its genetic diversity and is known as a "climate smart crop" (Tripathi et al., 2022). Because it can bridge the hunger gap between cropping seasons and develops more quickly than most crops, it has the potential to be a crop that ensures food and nutrition security in sub-Saharan Africa (Belane 2011). Leguminous crops also typically have a special capacity to adjust atmospheric nitrogen in symbiosis with bacteria (Rhizobium), hence, thereby increasing soil nitrogen fertility. This is crucial for system productivity and is why it works well in traditional cropping systems in Africa. Rainwater is the main source of crop irrigation for farmers with limited resources in the majority of traditional farming systems in Africa. In certain areas, like South Africa, mining water contamination and recent changes in climate conditions linked to climate change are making it more difficult to get clean irrigation water. The production of cowpeas in South Africa may help lessen the danger climate change presents to the security of household food and nutrition, particularly for smallholder and low-resource farmers in rural areas. However, because the nation lacks water, there is an urgent need to either clean/bio-remediate or use mine water for agricultural irrigation. For instance, the majority of surface waters in Gauteng Province are contaminated by acid mine drainage (AMD). Costly technologies (such as liming and reverse osmosis) to bio-remediate or detoxify mine effluent discharged into freshwater sources utilized for crop irrigation in their

communities are beyond the means of poor resource farmers (McCarthy, 2011). The *Bacillus* consortium (Singh et al., 2014) and other heavy metal-resistant bacteria are noteworthy because they demonstrated to exhibit features that promote seed germination and plant development and can be used as inexpensive and more environmentally friendly solutions for the bioremediation of heavy metal-polluted soils (Khan et al., 2009). In chromium-amended soils, for instance, *Bacillus* PZ3 has been shown to decrease Cr in pea plants when co-inoculated with the bacteria. Notably, studies have reported significant alterations in root architecture and enhanced nodulation in certain legume species (including common bean, peanut, soybean, and pigeon pea) when co-inoculated with rhizobia and various strains and species of *Bacillus* (Schwartz et al., 2013). Furthermore, studies have showed that several strains of *B. subtilis* can significantly enhance growth of some crops relative to control (Jeong et al., 2013, Ogugua et al., 2018; Wu et al., 2016). *B. subtilis* (BD233) inoculation significantly enhanced seedlings' growth of sweet pepper and lettuce relative to control (Ogugua et al., 2018), and co-inoculation with same bacterial strain affected the seed germination and plumule length of exotic cowpea genotypes (Nevhulaudzi, 2019). Interestingly, little or no study has reported on the potential effect of *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 co-inoculation on cowpea germination, growth and yield under varying mine water. The study by Nemutanzhela et al. (2017) showed that irrigation with 75% AMD (Fly ash-treated AMD) considerably enhanced the growth and tuber yield of two potato genotypes although tubers were contaminated with unsafe levels of Zn, Ni and Sr relative to control. In the study of Nevhulaudzi (2019), co-inoculation with *B. subtilis* (BD233) enhanced the germination and growth of exotic cowpea genotypes. Specifically, no study has assessed the interactive effects of *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 co-inoculation on the germination, growth and yield of local cowpea genotypes subjected to mine water. Therefore, the objective of study was to assess the effects of *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 co-inoculation on the germination indices, growth and yield of local cowpea genotypes irrigated with mine water.

4.2 MATERIALS AND METHODS

4.2.1 Seed Materials and Measurements

As outlined in section 3.1.1 of chapter 3.

4.2.2 Bacterial Strains

As outlined in section 3.1.2 of chapter 3.

4.3 GERMINATION EXPERIMENT AND EXPERIMENTAL DESIGN

In all experiments and for each genotype, 30 cowpea seeds were surface-sterilized by immersion in 70% ethanol for 90 s, followed by treatment with 1.5% bleach for 15 mins, and rinsed six times with sterile distilled water to prevent contamination (Pule-Meulenberg et al., 2010). The microbial inoculation treatments included i) seeds treated with *B. subtilis* (BD233) alone, ii) seeds inoculated with *Bradyrhizobium* sp. CB756 alone, iii) seeds co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 and iv) seeds not inoculated with either bacterium (control). Each treatment was incubated in the growth chamber at room temperature for seven days.

The germination experiment was conducted between November and December 2021 in growth chambers placed at the University of South Africa, Florida campus (26° 10′ 30″ S, 27° 55′ 22.8″ E)

Two experimental trials were performed employing a complete randomized design (CRD), consisting of three replicates of 30 seeds for each genotype and treatment, under sterile conditions. The experiments utilized 9 cm plastic Petri dishes and Whatman No. 1 filter paper discs. Petri dishes were sealed with laboratory film (Pechiney Plastic Packaging) to prevent loss of moisture. Additional three levels of mine water (2 mL) were added to keep filter paper discs moistened at counting dates if required, and to ensure that water availability would not be mystified with other treatment factors.

At each consequent counting date, newly germinated seeds were added to previously recorded germination counts so that the final count at 7 days after initiation (DAI) was a collective germination number of all prior counting dates.

In each experiment and for each microbial inoculation treatment, seed germination counts (radicle/plumule emergence) were evaluated and recorded daily 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:

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

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

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

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

4.4 COWPEA YIELD AND GROWTH

4.4.1 Experimental Conditions

As outlined in section 3.2.1 of chapter 3.

4.4.2 Bacterial Strains

As outlined in section 3.1.2 of chapter 3.

4.4.3 Cowpea Co-Inoculation

As outlined in section 3.2.2 of chapter 3.

4.4.4 Acid Mine Drainage

As outlined in section 3.2.3 of chapter 3.

4.4.5 Plant Growth Media

As outlined in section 3.2.4 of chapter 3.

4.5 COWPEA YIELD AND GROWTH EXPERIMENTAL DESIGN AND TREATMENT

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 same four germination treatments were used, and the seedlings were irrigated regularly with the three regimes of mine water. For this growth experiment, the control refers to seedlings raised from seeds not inoculated and exposed to 0% mine water.

A factorial (three factors: Genotype x inoculation x level of mine water) experiment was conducted using a complete randomized design (CRD) with three replications. Three hundred and twenty-four (324) viable seeds of same sizes were selected and sterilized using 70% ethanol and rinsed six times with sterile distilled water. Planting pots were also sterilized by spraying 70% of ethanol before planting. Thirty-six (36) pots per treatment and 108 pots were used.

4.6 DATA COLLECTION

Data on growth parameters (number of leaves per plant, stem diameter and plant height) was collected at seven-day intervals. Shoot weight (fresh and dry), root weight (fresh and dry), number pods per plant, and grain dry weight were recorded after harvest. Growth parameter data was collected at seven-day intervals. Measurements included cowpea biomass (shoots and roots, yield (number of pods), and the number of grains per plant, all assessed at 90 days after planting (DAP).

4.6.1 Number of Plant leaves, plant height and stem diameter

Growth parameter data were collected at seven-day intervals. Measurements included cowpea biomass (shoots and roots), yield (number of pods), and the number of grains per plant, all assessed at 90 days after planting (DAP).

4.6.2 Plant biomass (shoot and root weights)

Plants from each treatment were randomly collected 90 days after planting to measure both shoot and root fresh and dry weights. After washing all harvested shoots and roots with tap water, their fresh weights were immediately recorded using a weighing balance. For dry weight measurements, these plant parts were freeze-dried for 72 hours until reaching a constant weight, after which their mass was determined with a weighing balance.

4.7 RESULTS AND DISCUSSION

4.7.1 Germination indices of cowpea co-inoculated with *B. subtilis* (BD 233) and *Bradyrhizobium* sp. CB756 at seven days incubation.

Bacillus subtilis (BD 233) and *Bradyrhizobium* sp. CB756 co-inoculation was tested on cowpea's seed germination indices under varying mine water irrigations (0, 25 and 75%) and across different genotypes (Glenda, Sudan, Pan 311) under glasshouse conditions. According to Table 4.1, irrespective of genotype, cowpea's seed germination indices was not significantly affected by exposure to mine water, however, those treated with moderate mine water (25%) had a slightly higher G% (28.06%) compared to control (24.44%) and, high mine water (75%) (19.81%), which had a significantly higher GRI (0.13) compared to the 25% mine water with the least GRI (0.02). The results implies that cowpea seeds treated with 25% mine water had the fastest germination rate that resulted to a slightly higher germination percentage (24.44%) compared to those treated with 75% mine water with the slowest germination rate.

Similarly, regardless of mine water level, no significant genotypic differences were noted with respect to germination of cowpea seeds, however, Glenda exhibited slightly higher germination (G% (32.04%), GI (0.48) and GRI (0.05)) compared to Sudan with the least values (G% (20.00%), GI (0.45) and GRI (0.02), respectively) (Table 4.1). However*, B. subtilis* (BD 233) and *Bradyrhizobium* sp. CB756 co-inoculation significantly affected seed germination of cowpea genotypes at seven days of incubation. Co-inoculated Glenda and Pan 311 exhibited the highest seed germination (G% (36.11%) and G% (38.33%), respectively) compared to indices of genotypes without co-inoculation (Table 4.1). Similarly, the germination rate of seed was significantly influenced by microbial inoculation as Pan 311 with *B. subtilis* (BD 233) and *Bradyrhizobium* sp. CB756 co-inoculation exhibited the lowest GRI value (0.00) (fastest rate) that resulted in highest germination percentage (G% (36.11%) in contrast to other microbial inoculations. These results are in agreement with previous reports by Nevhulaudzi et al. (2017) that inoculation with *B. subtilis* (strain BD223) significantly affected the germination indices of exotic cowpea genotypes including genotypic differences in response to microbial inoculation. Similarly, seed germination and plume length of kiwi plants were influenced by co-inoculation with *Bacillus* RC03 and *Bacillus* complex RC19 w (Erturk 2010). Microbes release hormones (auxins, cytokinins,

gibberellins and ethylene), metabolites (sidedophores) and biosynthesise osmolytes for growth of different crops (rice (Ashrafuzzaman et al., 2009), soybean (Naz et al., 2009), chickpea (Mahejibin & Patel, 2007; Mishra et al., 2010) and Bambara groundnut (Sinefu, 2011; Nelwamondo, 2020). Additionally, by releasing exopolysaccharides, rhizobia are noted to influence movement of water and soil nutrients essential for enhanced seed germination around the rhizosphere due to increased root adherence to soil (Fahde et al., 2023). However, interactions of mine water x genotype x microbial inoculation were not found to be significant (Table 4.1), which is in contrast to reports by Nelwamondo (2020) for three genotypes of Bambara groundnut inoculated with *B. subtilis* (strain BD223).

Table 4.1 Germination indices of cowpea co-inoculated with *B. subtilis* (BD 233) and *Bradyrhizobium* sp. *CB756* at seven days of incubation

	Germination Percentage (%)	**Germination Index (GI)**	**Germination Rate Index (GRI)**
Mine Water (%)			
0	24.44±2.3a	0.64±0.29a	0.03±0.02b
25	28.06±2.6a	0.34±0.08a	0.02±0.01b
75	19.81±0.9a	0.99±0.23a	0.13±0.05a
Genotype			
Glenda	32.04±2.8a	0.48±0.14a	0.05±0.02a
Sudan	20.00±1.9a	0.45±0.12a	0.02±0.01a
Pan 311	20.29±1.8a	1.04±0.33a	0.12±0.05a
Treatment			
Glenda+*B. subtilis*	30.78±2.9ab	1.12±0.59a	0.05±0.04b
Glenda+*B.japonicum*	27.96±8.1b	0.43±0.11a	0.03±0.02b
Glenda+*B.stubtilis+B.japonicum*	36.11±9.1a	0.53±0.27a	0.06±0.04b
Sudan+*B. subtilis*	21.11±1.7b	0.38±0.15a	0.02±0.01b
Sudan+*B.japonicum*	18.89±0.9bc	0.52±0.20a	0.02±0.01b
Sudan+*B.stubtilis+B.japonicum*	25.00±0.3b	0.41±0.20a	0.02±0.01b
Pan 311+*B. subtilis*	10.37±0.1c	0.96±0.35a	0.18±0.09a
Pan 311+*B.japanicum*	30.78±0.3a	1.12±0.59a	0.05±0.04b
Pan 311+*B.stubtilis+B. japanicum*	38.33±0.4a	0.15±0.07a	0.00±0.00b
F-Statistics			
Mine water (%)	0.542^{ns}	2.201^{ns}	3.594^{*}
Genotype (G)	1.482^{ns}	2.296^{ns}	2.540^{ns}
Treatment (T)	4.542^{**}	2.201^{ns}	3.594^{*}
% x G	0.101^{ns}	1.051^{ns}	2.118^{ns}
% x T	0.452^{ns}	0.928^{ns}	1.296^{ns}
G x T x %	1.620^{ns}	0.115^{ns}	0.902^{ns}

G x % x T	0.118^{ns}	0.822^{ns}	0.703^{ns}

Values (Mean ±Std. Err.) 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. subtilis* = *B. subtilis* (BD 233) and *Brady.* sp. = *Bradyrhizobium* sp. *CB756.*

4.7.2 Growth, nodulation and yield of cowpea co-inoculated with *Bradyrhizobium* sp. CB756 and *B. subtilis* (BD 233) at physiological maturity.

According to Table 4.2, irrespective of genotype and inoculation, cowpea irrigated with 0% mine water (control) exhibited superior growth, with the thickest stem girth (4.39±0.15 cm), heaviest shoot fresh weight (16.51 ± 0.38 g), shoot dry weight (3.01 ± 0.14 g), root fresh weight (6.40 ± 0.46 g), whole plant biomass (Fresh (12.76±0.56 g) and dry (2.63±0.17 g) and highest pod production per plant (2.57 ± 0.11). In contrast, increase in mine water to 25% and 75%, resulted in a notable decrease in these parameters; particularly shoot biomass indicating that mine water stress negatively affects cowpea productivity. However, no significant differences were noted in plant height as well as number of leaves per cowpea plant (Table 4.2).

Notably, irrespective of mine water irrigation and inoculation treatment, there was also significant differences among genotypes with respect to growth and cowpea yield. Pan 311 demonstrated the tallest plants (10.84 ± 0.76 cm) with thicker stems (4.84 ± 0.29 cm), higher number of leaves per plant (18.78 ± 0.10), and heavier shoot weights fresh (16.72±1.00 g) and dry (2.742.74 g) while Glenda and Pan 311 produced higher shoot biomass compared to Sudan with decreased parameters, respectively. Pan 311 (8.25±0.64 g) and Glenda (8.89±0.98 g) had heavier fresh root weight compared to Sudan (6.91±0.84 g) but no significant differences in dry root weight. Among the genotypes Glenda exhibited the heaviest whole plant biomass (fresh (7.84±0.90 g) and dry (1.44±0.12 g). Interestingly, inoculation treatments significantly influenced cowpea growth and yield. Plants treated with *Bradyrhizobium* sp. CB756 alone or in combination with *B. subtilis,* generally outperformed those inoculated with *B. subtilis* alone in terms of plant height, shoot biomass, and pod yield. For instance, Sudan plants co-inoculated with *Bradyrhizobium* sp. CB756 achieved the tallest height (18.43 ± 1.05 cm) and highest pod yield (3.11 ± 0.12). In this study, the effect of the two-level *Rhizobium* inoculation and their potential to promote growth and yield of cowpea of different local genotypes grown under varying mine water (0%, 25% 75%) was assessed. The results revealed that *B. subtilis* (BD 233) and *Bradyrhizobium* sp.

CB756 co-inoculation improved the growth and yield of local cowpea genotypes under controlled glasshouse conditions. In a related study, Nevhulaudzi et al. (2017) reported that an exotic cowpea genotype, Nyira co-inoculated with *B. subtilis* (BD 234) had taller plants and higher biomass compared to when not co-inoculated, which together with Asatenapa (not co-inoculated) had the highest number of branches. In agreement with these findings, 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. Nelwamondo (2020) reported similar results for three genotypes of Bambara groundnut inoculated with *B. subtilis* (strain BD223) and exposed to different temperatures under glasshouse conditions. Rhizobia inoculation has been regarded as a sustainable and cost-effective tool used to supplement the plants' nitrogen needs, and several studies have reported that inoculation with rhizobia improves legume growth, nutrition, and production (Tena et al., 2016; Koskey et al., 2017; Nyaga et al., 2020). 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-lSbs, marked increases were reported in the saleable yield, and weight and length of fruit relative to control (Mena-Violante and Olalde-Portugal 2007). In this study, regardless of co-inoculation with or without *B. subtilis* and *Bradyrhizobium* sp. CB756, irrigation with mine water (75% MW) had a significant effect on the growth of the tested local cowpea genotypes and genotypic responses varied. Mine water is a source of metals (see Table 3.1) and long-term irrigation of crops leading to accumulation of excess amounts can subsequently lead to metal toxicity stress in plants (Ali *et al*., 2017; Nemutanzhela *et al*., 2017).

4.7.3 Genotype x treatment x mine water interaction effects on the growth, nodulation and yield of local cowpea genotypes

Based on the results attained in this study, the interactive effects of genotype x inoculation x mine water with respect to cowpea's stem girth, fresh and dry weights of shoot, root and whole plant biomass and number of pods per plant were significant relative to control (0% mine water and no inoculation) (Table 4.2).

4.7.3.1 Growth (Stem girth)

According to Figure 4.1, genotype x treatment x mine water interaction significantly influences the expansion of cowpea's stem girth, when co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756. From the results, the effect was particularly obtained under 75% mine water irrigation, where co-inoculated Sudan plants showed 30% and 24% increases in stem diameter compared to those with single inoculation. In the study, Sudan genotype exhibited exceptional metal tolerance with co-inoculated plants maintaining wider stem girth even at 75% mine water concentration compared to Pan 311, which showed wider variability in metal tolerance even with co-inoculation or single inoculation.

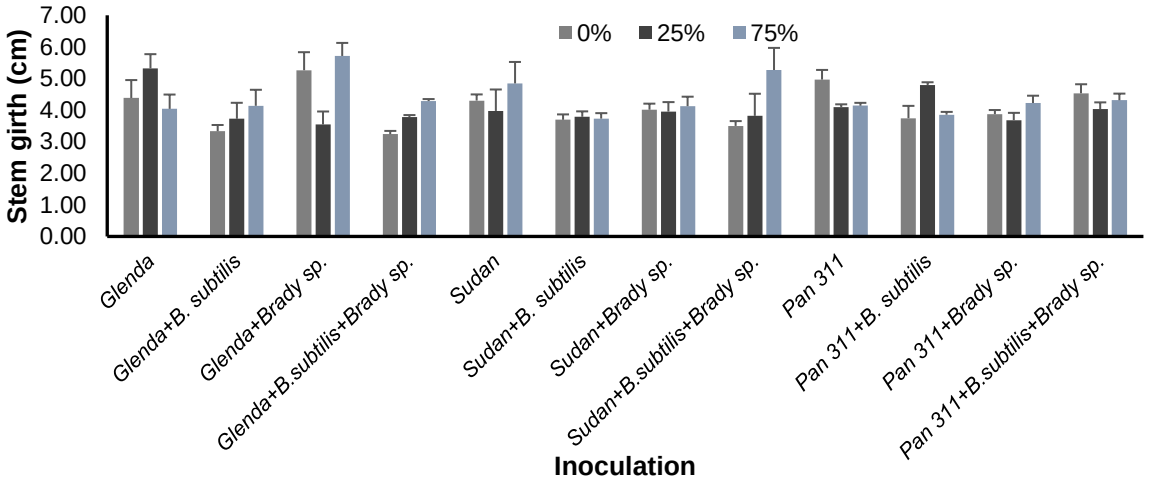

Figure 4.1 Genotype x inoculation x mine water interaction effects on the stem diameter (cm) of cowpea under glasshouse conditions.

Table 4.2 Growth, nodulation and yield of cowpea co-inoculated with *B. subtilis* (BD 233) and *Bradyrhizobium sp. CB756* at physiological maturity

	Plant Height (cm)	Stem diameter (cm)	No. of leaves per plant	Shoot FW (g)	Shoot DW (g)	Root FW (g)	Root DW (g)	Whole Plant FW (g)	Whole Plant DW (g)	No. of nodules per plant	No of pods per plant
Mine Water (%)											
0	14.27±0.43a	4.39±0.15a	15.99±0.37a	16.51±0.38a	3.01±0.14a	6.40±0.46a	1.36±0.04a	12.76±0.56a	2.63±0.17a	47.04±2.84a	2.57±0.11a
25	14.52±0.48a	4.04±0.10b	17.03±0.43a	14.96±0.55b	2.45±0.10b	6.32±0.37a	0.88±0.10a	7.34±0.45c	1.29±0.08c	37.35±2.39b	1.90±0.11b
75	14.19±0.46a	4.07±0.12b	16.74±0.41a	15.19±0.47b	2.54±0.11b	5.24±0.29b	0.86±0.45a	9.19±0.51b	1.78±0.11b	36.13±2.19b	2.09±0.13b
Genotype											
Glenda	10.38±0.55a	4.58±0.35a	19.19±0.90a	15.82±0.94b	2.54±0.18b	8.89±0.98a	0.87±0.08a	7.84±0.90a	1.44±0.12a	46.44±3.90c	2.06±0.21a
Sudan	9.53±0.43b	3.73±0.19b	18.11±0.89b	14.82±1.12c	2.17±0.17c	6.91±0.84b	0.79±0.06a	5.63±0.87b	1.13±0.18b	49.17±4.35b	1.72±0.25b
Pan 311	10.84±0.76a	4.84±0.29a	18.78±0.89b	16.72±1.00a	2.74±0.27a	8.25±0.64a	0.92±0.06a	5.89±0.79b	0.93±0.12c	64.44±4.55a	1.67±0.18b
Treatment											
Glenda+B. subtilis	11.25±0.81d	3.77±0.13c	19.06±0.88a	16.23±0.88b	2.62±0.15b	11.36±0.92a	1.59±0.35a	6.34±0.76	1.14±0.11c	83.33±4.38a	1.83±0.16b
Glenda+Brady.sp.	15.38±0.90bc	4.37±0.28a	16.24±0.83b	17.14±0.58ab	2.94±0.37b	5.40±0.56b	0.75±0.07a	12.02±1.07ab	2.05±0.21b	28.28±3.85c	2.72±0.23b
Glenda+B.stubtilis+ Brady.sp.	16.97±0.92abc	3.74±0.11c	14.89±0.76c	17.00±0.46ab	2.97±0.34b	5.02±0.37b	0.64±0.06a	10.72±1.30c	2.23±0.36ab	20.22±2.96d	2.06±0.28bc
Sudan+B. subtilis	17.97±0.93ab	4.03±0.14b	16.25±0.78b	17.83±0.90ab	3.24±0.21a	5.94±0.50b	0.99±0.08a	9.11±1.20d	1.59±0.23c	37.63±4.31b	1.92±0.26b
Sudan+ Brady.sp.	18.43±1.05a	4.19±0.28b	17.14±0.87ab	18.28±0.83a	3.22±0.24a	5.15±0.61b	0.72±0.06a	14.25±0.73a	2.85±0.24a	37.78±3.45b	3.11±0.12a
Sudan+B.stubtilis+ Brady.sp.	14.28±0.74c	4.40±0.14a	15.28±0.68bc	14.38±0.93c	2.62±0.15b	4.22±0.64c	0.57±0.05a	11.14±0.93c	2.12±0.21ab	25.67±3.26cd	2.44±0.21b
Pan 311+B. subtilis	15.92±0.98abc	4.13±0.19b	14.35±0.64c	12.07±0.92c	2.61±0.15b	4.26±0.45c	0.75±0.05a	11.11±1.28c	2.61±0.44a	25.11±3.28cd	2.22±0.32b
Pan 311+ Brady.sp.	15.46±0.97bc	3.92±0.11c	14.65±0.62c	13.24±0.65c	2.45±0.28b	2.69±0.16d	3.01±1.77a	12.10±0.94ab	2.50±0.25a	28.44±3.22c	2.28±0.18b
Pan 311+B.stubtilis+ Brady.sp.	15.50±0.94bc	4.29±0.14ab	15.11±0.62bc	13.15±1.19c	1.90±0.18d	3.74±0.36c	0.75±0.08a	11.11±1.00c	2.20±0.34ab	35.60±3.60b	2.23±0.21b
F-Statistics											
Mine Water	0.165ns	3.765*	1.88ns	4.86**	7.245***	5.688**	1.184ns	42.554***	41.956***	11.495***	10.051***
Genotype	2.625***	2.825**	2.235*	3.735**	4.635**	6.535**	1.112ns	8.825**	12.125**	9.565**	2.005**
Treatment	12.969***	3.122***	5.291***	6.775***	2.984***	20.221***	1.762ns	10.865***	9.207***	27.199***	3.74**
Mine Water x Treatment	0.931ns	2.735***	0.974ns	5.298***	1.881*	5.028***	1.947*	2.734***	3.510***	2.172**	1.203ns

Values (Mean ±Std. Err.) 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. subtilis = *B. subtilis* (BD 233) and *Brady.* sp. = *Bradyrhizobium* sp. *CB756.*

4.7.3.2 Yield (pod production)

According to Figure 4.2, generally, pod production was negatively affected by increase in mine water stress. However, co-inoculation with *B. subtilis* (BD 233) and *Bradyrhizobium* sp. *CB756* generally improved pod production compared to single-strain treatments, particularly at moderate stress (25%). Concerning the genotypes, Glenda showed the best response to dual inoculation, while Sudan and Pan 311 showed varying levels of stress tolerance. These results suggest that microbial co-inoculation may enhance cowpea resilience to mine water stress, though genotype-specific effects were observed. Further analysis is needed to confirm these trends statistically.

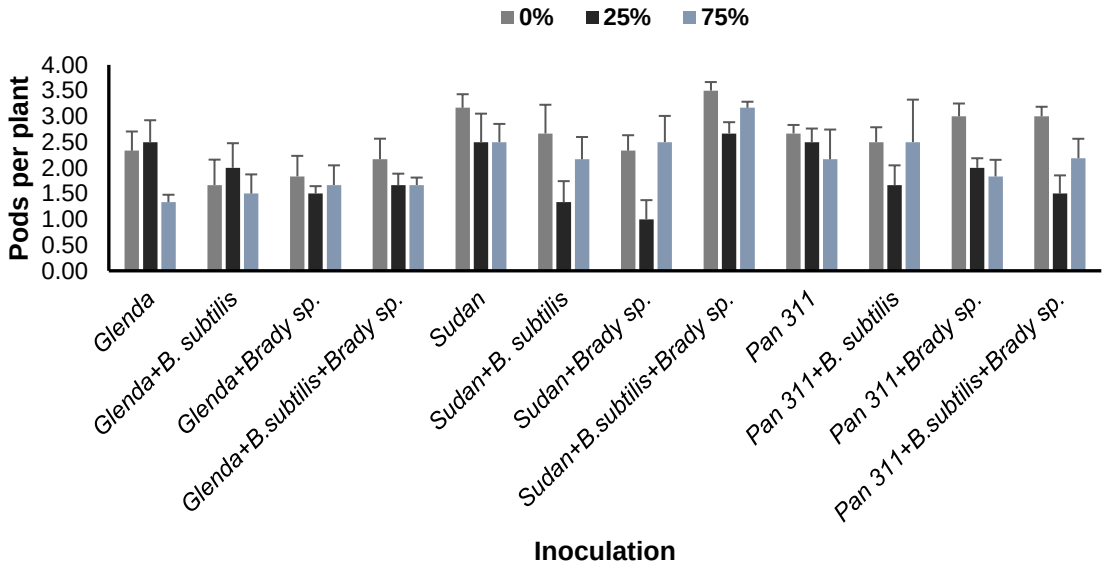

Figure 4.2 Genotype x inoculation x mine water interaction effects on the number of pods per cowpea plant under glasshouse conditions.

4.7.3.3 Yield (Shoot biomass- fresh and dry weight (g))

Results attained in Figure 4.3 shows the effect of microbial co-inoculation on the shoot fresh weight of cowpea under different mine water regimes. From the study, all three varieties and combined application of *B. subtilis* (BD 233) and *Bradyrhizobium* sp. CB756 consistently enhanced shoot fresh weight compared to the single inoculated or un-inoculated controls. From the results, the effect was particularly obtained under moderate (25%) mine water irrigation, where co-inoculated plants showed 28-35% greater biomass than control. In the study, Sudan variety exhibited exceptional metal tolerance with co-inoculated plants maintaining 85% of their maximum biomass even

at 75% mine water concentration. On the other hand, Pan 311 exhibited greater variability in response; however, given the limited information on the genetic background of the market-sourced genotypes, this variability cannot be conclusively attributed to genetic heterogeneity in metal detoxification pathways and warrants further investigation.

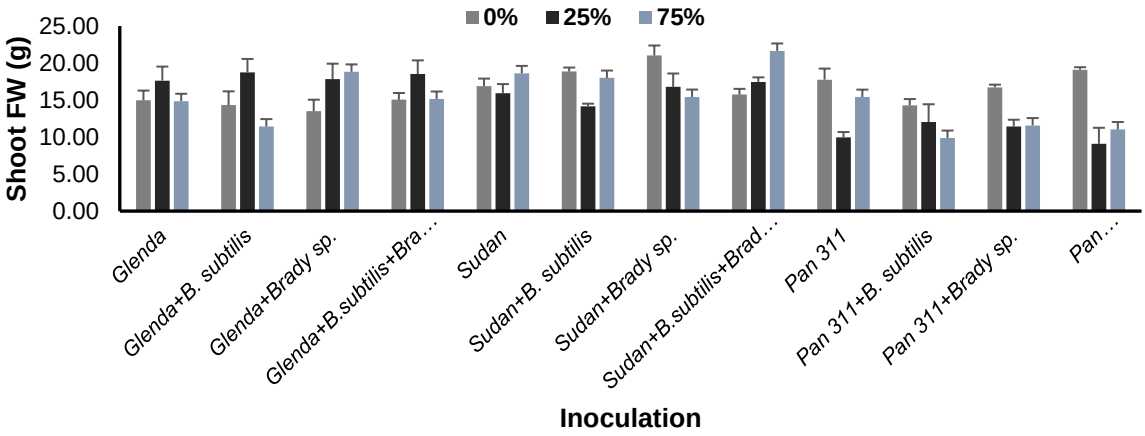

Figure 4.3 Genotype x inoculation x mine water interaction effects on the fresh shoot weight (g) of cowpea under glasshouse conditions.

The results obtained in the current study further confirm a significant improvement in shoot dry weight across all three local cowpea genotypes when co-inoculated with *B. subtilis* (BD 233) and *Bradyrhizobium* sp. CB756 under different mine water irrigation regimes (Fig. 5.5). The findings shows that Sudan variety maintained 78-85% of maximum biomass production even at 75% mine water concentration under co-inoculation, which shows a remarkable metal tolerance conferred possibly by the microbial partnership (Nevhulaudzi et al., 2017; Nelwamondo, 2020). This resilience aligns with findings by Etesami & Maheshwari (2018), who documented similar protective mechanisms in legumes subjected to heavy metal stress. In addition, Glenda exhibited optimal growth response (34% increase over control) at 25% mine water irrigation with co-inoculation.

Taken together, these results align well with recent reports by Buthelezi et al. (2025) that *Bradyrhizobium japonicum* and *Pseudomonas fluorescens* co-inoculation of mung

bean led to notable increases in shoot and root biomass under glasshouse conditions. In another earlier related study, do Nascimento (2020) reported enhanced dry shoot biomass (15–64%) and dry root biomass (>100%) compared to the single elite strains BR 3262 and BR 3267, when cowpea was co-inoculated with BR 3262 and 28 *Bradyrhizobium* sp.

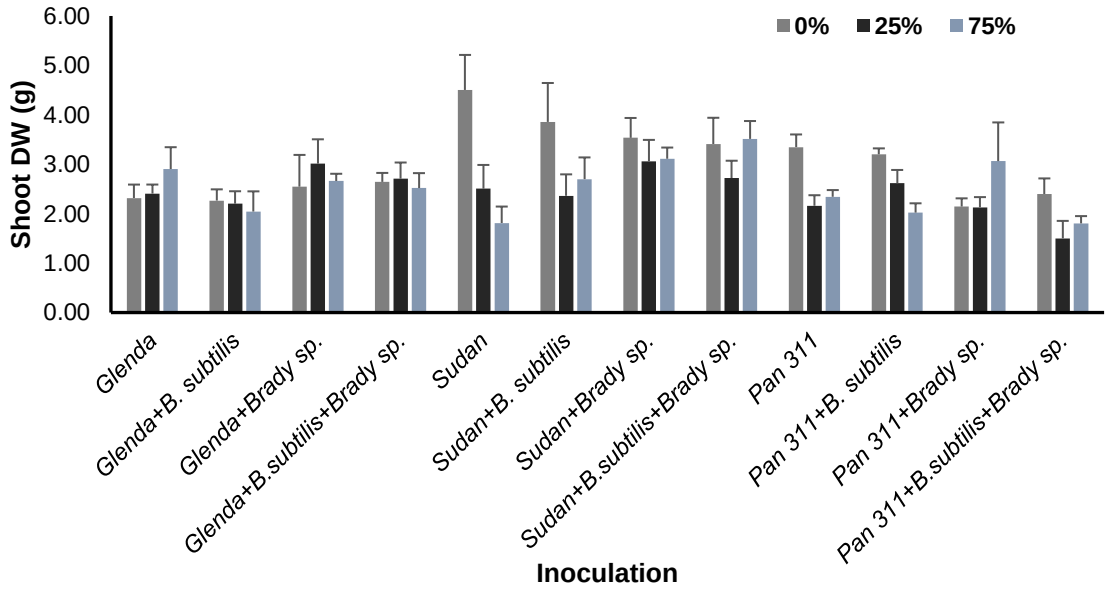

Figure 4.4 Genotype x inoculation x mine water interaction effects on the dry shoot weight (g) of cowpea under glasshouse conditions.

4.7.3.4 Yield (Root biomass- fresh and dry weight (g))

The current study results in Figure 4.5 shows fresh root weight responses of three cowpea genotypes to microbial inoculation under different mine water irrigation levels. The findings shows that co-inoculation with *B. subtilis* (BD 233) and *Bradyrhizobium* sp. CB756 significantly enhanced root growth across all local cowpea genotypes, with the most pronounced effects observed with moderate mine water (25%) irrigation. Glenda showed particularly strong response to co-inoculation, exhibiting a 42% increase in fresh root weight compared to controls. However, Sudan displayed remarkable metal tolerance, maintaining 78-82% of maximum root growth even at highest (75%) mine water concentration when co-inoculated. This finding is consistent with reports of varietal differences in metal stress adaptation (Etesami & Maheshwari, 2018).

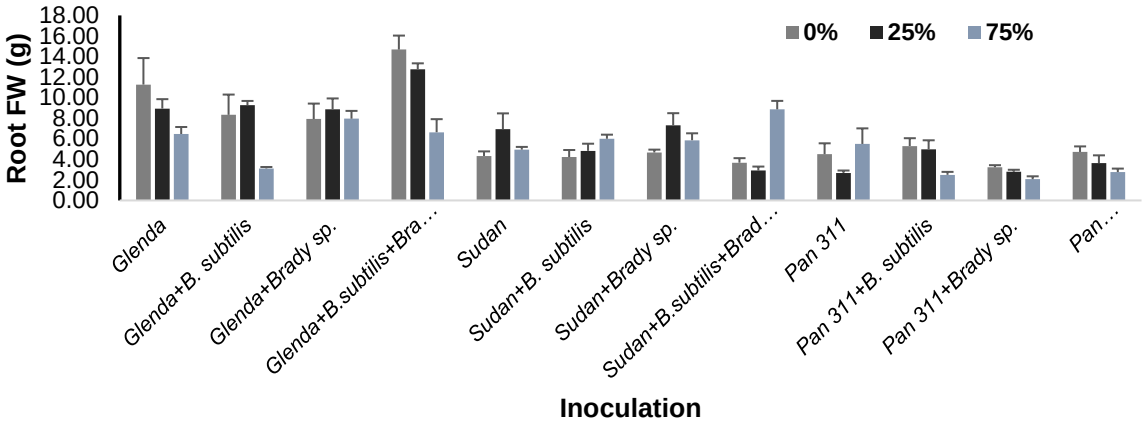

Figure 4.5 Genotype x inoculation x mine water interaction effects on the fresh root weight (g) of cowpea under glasshouse conditions.

Similarly, results in Figure 4.6 shows the effect of *B. subtilis* (BD 233) and *Bradyrhizobium* sp. CB756 co-inoculation on dry root weight (DW) in local cowpea genotypes under varying mine water stresses. However, in the absence of mine water (0%), all plant genotypes exhibited higher dry root weight when co-inoculated with *B. subtilis* (BD 233) or *Bradyrhizobium* sp. CB756 compared to their untreated controls, suggesting that these bacteria promote root growth under normal conditions. The combination of both bacteria further enhanced dry root weight in some cases, particularly in Glenda, indicating a potential synergistic effect. However, for Sudan and Pan 311, the dual-bacterial treatment did not always outperform single-strain applications, possibly due to competitive interactions or strain-specific compatibility (Etesami & Maheshwari, 2018). Notably, with 25% mine water irrigation, dry root weight generally decreased across all treatments, but bacterial inoculation mitigated this decline. *B. subtilis* (BD 233) appeared more effective than *Bradyrhizobium* sp. CB756 in sustaining root growth under stress, particularly in Sudan and Pan 311. Also, at the highest mine water concentration (75%), root DW was severely reduced in all genotypes with the exception of co-inoculated Pan 311, reflecting the inhibitory effects of heavy metal toxicity. However, bacterial treatments, especially *B. subtilis* (BD 233), still provided some protective benefits, as inoculated plants maintained marginally higher root DW compared to untreated controls. Notably, the combination of both bacteria did not offer additional advantages under severe stress, possibly due to

reduced bacterial viability or overwhelmed plant defence mechanisms (Nevhulaudzi et al., 2017; Nelwamondo, 2020).

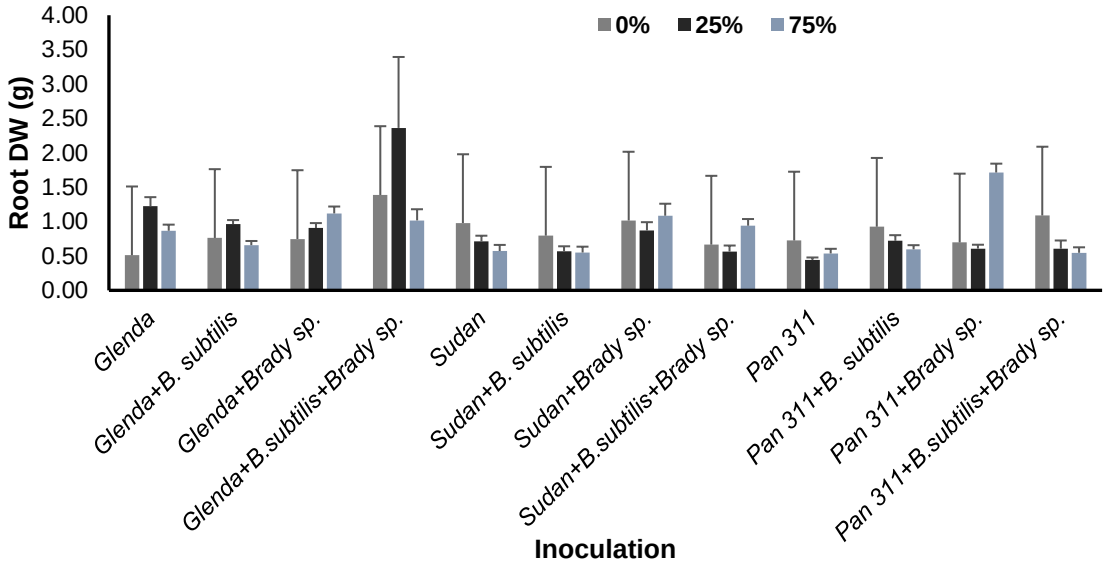

Figure 4.6 Genotype x inoculation x mine water interaction effects on the dry root weight (g) of cowpea under glasshouse conditions.

4.7.3.5 Yield (Whole plant biomass- fresh and dry weights (g))

Figure 4.7 below also shows the effect of *B. subtilis* (BD 233), *Bradyrhizobium* sp. CB756, and their co-inoculation on fresh whole plant weight (FW) of three local cowpea genotypes under varied mine water stress. However, under non-stressed conditions (0% mine water), bacterial inoculation generally enhanced whole plant FW, with *B. subtilis* (BD 233) having more consistent growth promotion across all genotypes compared to *Bradyrhizobium* sp. CB756. From the results, the combined inoculation shows additive effects in Glenda, suggesting the potential relationship between the two bacterial strains in this genotype. However, in Sudan and Pan 311, the dual-inoculation results were comparable to or slightly lower than single-strain treatments, possibly due to competitive interactions between the microbes or host-specific compatibility issues (Etesami & Maheshwari, 2018). But with 25% mine water stress, there was a clear decline in whole plant FW across all the treatments, which reflect the initial inhibitory effects of contamination. Notably, Sudan exhibited the greatest resilience to moderate stress when treated with *B. subtilis* (BD 233), compared with Glenda and Pan 311. However, under severe stress (75% mine water), slow plant growth was experienced in all genotypes, with whole plant FW reduction

compared to control. In the study, *B. subtilis* (BD 233) maintained a slight growth over *Bradyrhizobium* sp. CB756, particularly in Pan 311, where it helped preserve marginally higher biomass (Fig. 4.8).

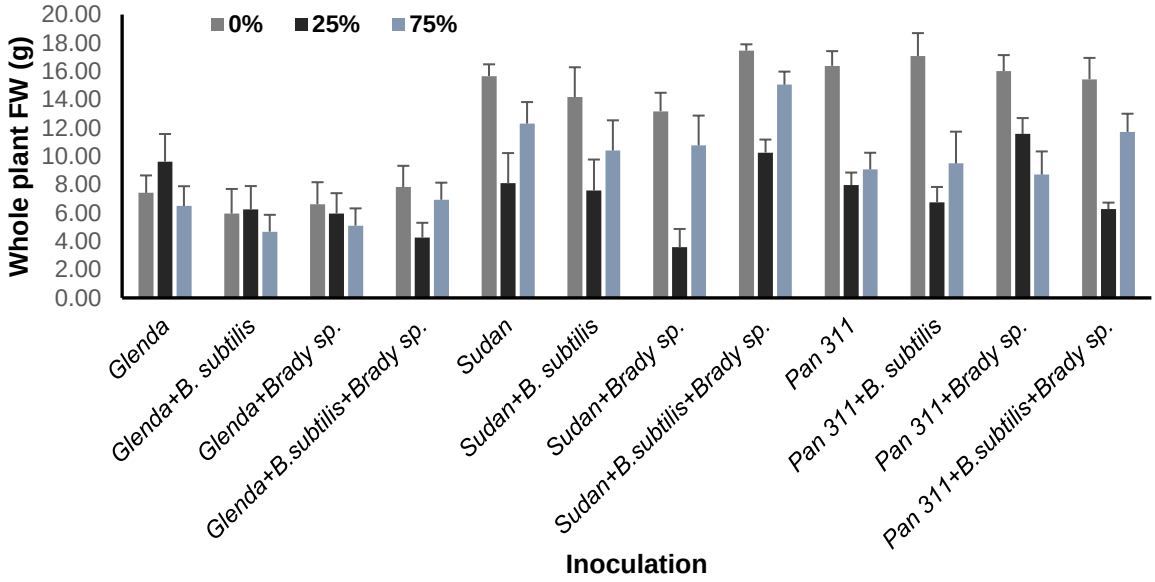

Figure 4.7 Genotype x inoculation x mine water interaction effects on the fresh whole plant weight (g) of cowpea under glasshouse conditions.

Figure 4.8 demonstrates same interaction effect of co-inoculating *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 on dry whole plant weight of local cowpea genotypes when exposed to varying levels of mine water stress. With 0% mine water (control), dry whole plant biomass is generally highest, as expected under no heavy metal stress, compared to moderate stress (25% mine water) and highest stress level (75%). Compared to control, co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 mitigated the heavy metal stress posed by the 75% mine water treatment for all genotypes. When irrigated with 75% mine water, co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 led to increases in dry whole plant weight in Glenda (4%), Sudan (31%) and Pan 311 (20%) compared to when these genotypes were treated with 0% mine water.

In sum, the interactive effects on fresh whole plant biomass seems to suggest that co-inoculation may help mitigate the negative effects of mine water, particularly when concentrations are high. These results highlight the potential of microbial inoculants (single or combinations) to enhance cowpea resilience under environmental stress,

though further statistical validation is needed to confirm the significance of these interactions.

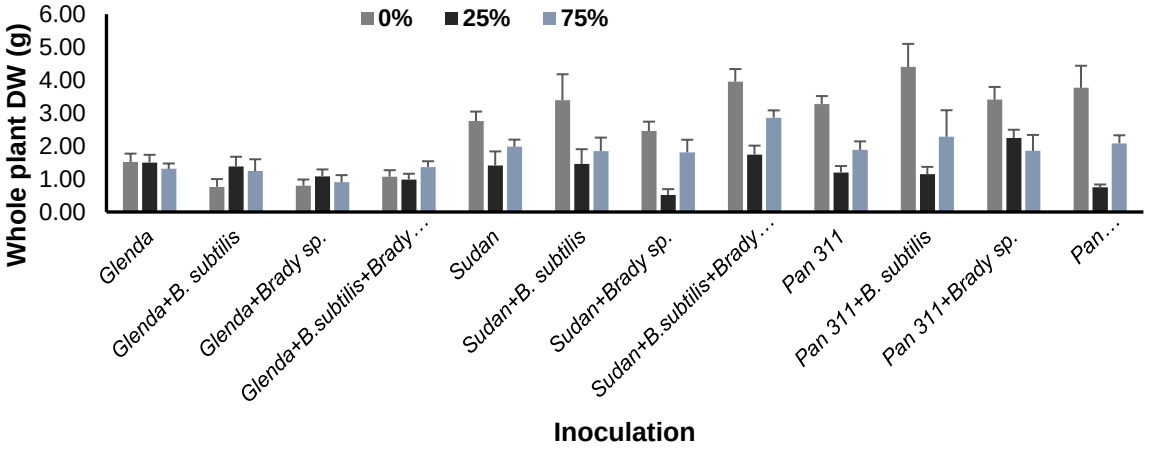

Figure 4.8 Genotype x inoculation x mine water interaction effects on the dry whole plant weight (g) of cowpea under glasshouse conditions.

Taken together, results obtained in this study have shown the potential mitigating effect of co-inoculating local cowpea genotypes with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 watered with mine water (heavy metal stress) under glasshouse conditions. This finding agrees with reports by Abou-Shanab et al. (2017), who showed that co-inoculating with indigenous *Bradyrhizobium japonicum* ORGS3 and ORGS5 and reference USDA 110 strains improved the growth and yield performance of soybean when grown in different field conditions.

4.8 SUMMARY

In summary, the study showed that dual inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 under varying mine water stress significantly influenced the germination, growth (plant height, stem diameter, plant biomass) and yield components of all three tested local cowpea genotypes. However, genotypic variation was noted for all measured parameters in response to the treatments employed in the study. The interaction of *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 co-inoculation of local cowpea genotypes under varying mine water irrigation significantly affected the growth and yield of all tested local cowpea genotypes and

genotypic variation was also noted. Furthermore, the study shows that mine water stress significantly reduces cowpea germination, growth, and yield, with severe declines observed with the highest mine water stress (75%). Co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 mitigated stress effects, particularly at moderate levels (25%), enhancing shoot biomass, root development, and pod yield. Genotypic variation was evident, with Pan 311 and Glenda showing superior stress tolerance and symbiotic efficiency, while Sudan exhibited metal resilience under co-inoculation. *Bradyrhizobium* sp. CB756 primarily improved yield-related traits, whereas *B. subtilis* (BD233) boosted nodulation, suggesting distinct roles in stress adaptation. These results highlight the potential of tailored microbial applications to enhance crop productivity in soils contaminated by mine water.

CHAPTER FIVE: Effects of *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 co-inoculation on symbiotic performance of local cowpea genotypes irrigated with acid mine water

5.1 INTRODUCTION

Human population increase and industrialization has had a negative effect on crop production and soil fertility. Soils polluted with heavy metals due to acid mine water (AMD) exposure from mining activities and sustained chemical over-fertilization are noted to result in increased soil acidity, poor crop yields due to oxidative stress (Dimkpa et al., 2009) and metal toxicity in the food web, thereby compromising human health (Stambulska et al., 2018). Additionally, AMD can affect the relationships between the plant and associated microbes, alter soil chemistry, and negatively impact plant health, in general. Microbes have been reported to alleviate heavy metal stress and environmental stresses in plants especially when they are in a symbiotic relationship (Zhang et al., 2020). Recent studies reported synergistic effects of dual inoculation (multiple beneficial microorganisms) on the symbiotic performance and overall growth of plants (Mohammed et al., 2019; Kumar & Singh, 2020; Patel et al., 2022; Thompson et al., 2024). The complimentary processes by which multiple beneficial microorganisms promote plant growth are responsible for their synergistic effects. For example, co-inoculation of *Bacillus subtilis* and *Bradyrhizobium* sp. has demonstrated significant enhancement in the symbiotic performance of cowpea (Muindi et al., 2021). In this dual inoculation, *B. subtilis* is known to produce a variety of compounds that promote plant growth, including exopolysaccharides, siderophores, and indole-3-acetic acid (IAA) (Spaepen et al., 2014) that can improve root architecture and development, nutrient acquisition and nodule morphology (Schwartz et al., 2013). Conversely, *Bradyrhizobium* species exhibit high efficiency in fixing atmospheric nitrogen and transferring it to the host plant, thereby enhancing nitrogen nutrition (Dakora et al., 2015).

Co-inoculation of *B. subtilis* and *Bradyrhizobium* sp. CB756 creates a synergistic relationship that improves various aspects of plant growth, nodulation, and nitrogen fixation efficiency in cowpea (Muindi et al., 2021; Olorunmaiye et al., 2021). Studies conducted by Mohammed et al. (2019) revealed that cowpea co-inoculated with *B. subtilis* and *Bradyrhizobium* sp., had a 35-45% increase in nodule count and a 40-50% enhancement in nodule dry weight compared to the single inoculation treatments.

Furthermore, co-inoculation synergistic effect was linked to improved physiological performances, such as enhanced chlorophyll content and photosynthetic rates. Research by Adesemoye et al. (2019) indicated that cowpea plants treated with the combination of *B. subtilis* and *Bradyrhizobium* sp. CB756 exhibited significantly higher chlorophyll a and b levels, leading to increased photosynthetic efficiency. This improvement translates to better biomass accumulation and, ultimately, higher grain yields. Kumar & Singh (2020) showed a 30-40% increase in leghemoglobin content in nodules from co-inoculated plants, indicating enhanced nitrogen fixation efficiency, compared to single inoculated cowpea. Research by Patel et al. (2022) demonstrated that co-inoculation resulted in significant improvements in root development (25-35% increase in root length and mass), shoot growth (30-40% increase in shoot biomass), and overall plant vigour. Furthermore, Ahmed & Hassan (2023) reported that local cowpea genotypes co-inoculated with *B. subtilis* and *Bradyrhizobium* sp. CB756 exhibited improved nitrogen fixation rates, with acetylene reduction activity showing a 45-55% increase compared to single strain treatments. The researchers also noted enhanced phosphorus solubilisation and uptake, contributing to better overall plant nutrition. Research conducted by Rodriguez et al. (2023) reported a 30-40% rise in grain yield and a 15-20% enhancement in grain protein content across different local cowpea genotypes. These improvements were consistent across various soil types and environmental conditions, indicating the robust nature of this microbial partnership and its importance in sustainable agriculture. However, these studies were conducted under field conditions and to the best of how knowledge; few studies have been done under controlled conditions. For example, Egamberdieva et al. (2010) reported a positive effect of co-inoculating *Rhizobium galegae* and root colonising *Pseudomonas* strains on the growth of fodder galega (*Galega orientalis* Lam.) under greenhouse conditions. Using rhizotrons, Galbieri et al. (2023) showed the positive synergistic effect of co-inoculating *B. subtilis*, isolate IMA Bs/170005 and *B. japonicum* on the growth and yield of soybean.

So far, few studies have examined the effect of dual inoculation on symbiotic performance of legumes under heavy metal stress due to acid mine water irrigation. This study aimed to assess the effects of co-inoculating *B. subtilis* (BD233) with *Bradyrhizobium* sp. CB756 on the symbiotic performance of local cowpea genotypes exposed to mine water under glasshouse conditions. The specific objectives include;

i) to assess cowpea's dependency on nitrogen fixation when exposed to dual inoculation (*B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756) and mine water stress and ii) to assess the effects of co-inoculation on nodulation, biomass and nutrients (N & C) accumulation by local cowpea genotypes irrigated with mine water.

5.2 MATERIALS AND METHODS

5.2.1 Seed Materials

As outlined in section 3.1.1 of chapter 3.

5.2.2 Experimental Conditions

As outlined in section 3.2.1 of chapter 3.

5.2.3 Bacterial Strains

As outlined in section 3.1.2 of chapter 3.

5.2.4 Cowpea Co-Inoculation

As outlined in section 3.2.3 of chapter 3.

5.2.5 Acid Mine Drainage

As outlined in section 3.2.4 of chapter 3.

5.2.6 Plant Growth Media

As outlined in section 3.2. of chapter 3.

5.2.7 Experimental Design

As outlined in section 4.5 of chapter 4.

5.3 DATA COLLECTION AND MEASUREMENTS

5.3.1 Cowpea Nodulation and Yield Determination

At 90 days after planting, plants were gently removed from the soil with their root systems preserved to assess nodulation. The harvested roots were rinsed with water,

then the nodules were delicately separated and counted for each treatment. Yield was measured by recording pod count per plant for each treatment.

5.3.2 Measurement of Nitrogen Fixation

5.3.2.1 $^{15}N/^{14}N$ and $^{13}C/^{12}C$ isotope ratios

Shoot samples of cowpea were oven dried at 60, grind and weighed (2-3 mg) into Al tin capsules and run on a mass spectrometer and $^{15}N/^{14}N$ ratio using a Carlo Erba NA1500 elemental analyzer (Fisons Instruments SpA, Strada, Rivoltana, Italy) linked to a Finnigan MAT252 mass spectrometer via Conflo II Open-split device.

The ^{15}N natural abundance was expressed as (delta) notation, which is the deviation of the sample's ^{15}N natural abundance from atmospheric N_2 (0.36637 atom % ^{15}N), using the following relationship (Unkovich et al., 2008):

$$\delta^{15}N\ ‰ = \frac{(^{15}N / ^{14}N)_{sample} - (^{15}N / ^{14}N)_{atm}}{(^{15}N / ^{14}N)_{atm}} \times 100$$

To analyse $^{13}C/^{12}C$ rations, the shoot samples were weighed into tin capsules and ran on the mass spectrometer as described for $^{15}N/^{14}N$ ratio. The ratio of $^{13}C/^{12}C$ in each sample was used to calculate the ^{13}C natural abundance, or $\delta^{13}C$ (‰), as (Stout and Rafter, 1978):

$$\delta^{13}C = \frac{\left(^{13}C/^{12}C\right)_{sample} - \left(^{13}C/^{12}C\right)_{standard}}{\left(^{13}C/^{12}C\right)_{standard}} * 1000$$

Where $\left(^{13}C/^{12}C\right)_{sample}$ is the isotopic ratio of the sample, and $\left(^{13}C/^{12}C\right)_{standard}$ is the isotopic ratio of PDB, a universally accepted standard from belemnite Pee Dee limestone formation (Craig, 1957). Shoot C content per plant was calculated as:

Shoot C content = %C x shoot dry matter per plant

5.3.2.2 Shoot N content

The N content of the plant samples was determined as the product of %N and sample weight (Mohale et al*.,* 2014)

Shoot N Content (g. plant^{-1}) = %N_{shoot} × dry $mass_{shoot}$

5.3.2.3 Percent N derived from fixation (%Ndfa)

The percent N derived from atmospheric N_2 fixation (%Ndfa) was estimated using equation (Mohale et al*.,* 2014):

$$\%\,\text{Ndfa} = \frac{\delta^{15}\text{N}_{\text{ref}} - \delta^{15}\text{N}_{\text{leg}}}{\delta^{15}\text{N}_{\text{ref}} - \text{B}} \times 100$$

Where the $\delta^{15}N_{ref}$ is the mean $\delta^{15}N$ natural abundance of reference plant, $\delta^{15}N_{leg}$ is the Mean $\delta^{15}N$ natural abundance of legume and the B value is the $\delta^{15}N$ natural abundance of test legume wholly dependent on N_2 fixation for its N nutrition. The B value replaces the value of atmospheric N as it incorporates the isotopic fractionation associated with N_2 fixation (Unkovich et al., 2008). For cowpea shoots, a B value of -1.759 was used in calculating %Ndfa (Pule-Meulenberg & Dakora, 2009).

The amount of N-fixed was calculated according to the method of Maskey et al. (2001) and Mohale et al. (2014) as follow:

N-fixed = %Ndfa × legume biomass N

Where legume biomass N is the N content of Bambara groundnut shoots

5.3.2.4 Soil N uptake

Soil N uptake was calculated as (Unkovich et al., 2008).

Soil N uptake = Total legume biomass N – amount of N – fixed

5.3.2.5 Isotopic analysis of plant samples for $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratios

About 2.0 mg of each milled (0.85 mm mesh) sample was weighed into a tin capsule (Elemental Microanalysis Ltd, Okehamptom, UK), and the samples were loaded and run on a Thermo Finnigan Delta Plus XP stable light isotope mass spectrometer coupled via a Conflo III device to a Thermo 1112 Flash elemental analyzer, against an internal reference material (*Nasturtium* sp.). The reference materials had been calibrated against IAEA standards (Air for N, Pee Dee Belemite (or PDB) for C) and

the results expressed relative to these standards. The natural abundance of ^{15}N was calculated as (Mariotti et al, 1981):

$$\delta^{15}N = \frac{\left[{}^{15}N/{}^{14}N\right]_{sample} - \left[{}^{15}N/{}^{14}N\right]_{standard}}{\left[{}^{15}N/{}^{14}N\right]_{standard}} \times 1000 ,$$

where $^{15}N/^{14}N_{sample}$ and $^{15}N/^{14}N_{standard}$ are the abundance ratios of the sample and the standard, air, respectively.

Whole plant ^{15}N natural abundance was calculated as an average of the ^{15}N natural abundance values of all plant parts weighted by their respective total N (Robinson *et al*., 2000):

$$\delta^{15}N_{whole\ \ plant} = \frac{\sum\left(\delta^{15}N_{root} \times N_{root} + \delta^{15}N_{shoot} \times N_{shoot}\right)}{\sum\left(N_{root} + N_{shoot}\right)}$$

The N content of each organ was determined as the product of %N and sample weight (Pausch *et al*., 1996):

N amount (mg. plant^{-1}) = $\%N_{organ}$ x dry $weight_{organ}$

The percent plant N derived from fixation of atmospheric N_2 (Ndfa) was calculated from the ^{15}N abundance of the legume species and a non-N_2-fixing reference plant according to Shearer & Kohl, (1986):

$$\%Ndfa = \frac{\left[\delta^{15}N_{ref} - \delta^{15}N_{leg}\right]}{\left[\delta^{15}N_{ref} - B\ value\right]} x\,100$$

Where $\delta^{15}N_{ref}$ is the mean ^{15}N natural abundance of non-N_2-fixing reference plant shoots (Table 5.1), $\delta^{15}N_{leg}$ is the mean ^{15}N natural abundance of the legume (cowpea) shoots, and the B value is the ^{15}N natural abundance of cowpea shoots which were dependent solely on symbiotic N_2 fixation for their N nutrition.

The amount of N-fixed was calculated as (Maskey et al., 2001):

$$\mathrm{N-fixed} = \left(\frac{\mathrm{Ndfa}}{100}\right) \times \mathrm{legume\ shoot\ N}$$

For the determination of cowpea's symbiotic dependence on N fixation (N-Fixed (mg. Plant^{-1}) and %Ndfa), several reference plants were used and their δ^{15}N (‰) values ranged from 3.28 to 7.42 δ^{15}N (‰) (Table 5.1).

Table 5.1 The δ^{15}N (‰) of reference plant sampled from Unisa greenhouse for estimation of % Ndfa in cowpea

Common name	**Botanical name**	**δ^{15}N (‰)**
Lettuce	*Lactuca sativa*	5.12
Spinach	*Spinacia oleracea L.*	3.28
Cabbage	*Brassica oleracea*	5.55
Night shade	Solanaceae	7.42
Unknown plant		7.01

5.4 DATA ANALYSIS

The experiment was repeated in two seasons, the first experiment was carried out from October 2021 to January 2022 while the second experiment was conducted in January 2022 to April 2022, to validate the first experimental results. No differences in the results were noted so the data was pooled for analysis. Data was subjected to three-way analysis of variance (ANOVA) using the STATISTICA software version 10 (Stat Soft Inc., Tulsa, OK, USA). Treatment means were separated using the Duncan's multiple range test at $p \leq 0.05$.

5.5 RESULTS AND DISCUSSION

5.5.1 Effect of *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 co-inoculation on symbiotic performance of local cowpea genotypes exposed to mine water

In this study, the influence of dual inoculation on the symbiotic dependence of three local cowpea genotypes irrigated with mine water under glasshouse conditions was determined. A 3-Way ANOVA analysis revealed no significant differences in the measured parameters (δ^{15}N (‰), %N, N-fixed, %Ndfa and Soil N uptake (mg Plant^{-1})) with respect to the synergistic effect of co-inoculation of *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 across the genotypes, mine water levels and treatments

employed in this study (Table 5.2). These results are not in agreements with reports by Udeh et al. (2023) indicating that Bambara groundnut (*Vigna Subterranea* (L.) Verdc) landraces co-inoculated with *B. japonicum* and *B. subtilis* under drought stress showed variation in the measured symbiotic performance parameters. Similarly, Mohammed et al. (2022) reported marked differences in the shoot DM, $\delta^{15}N$ (‰), %N, Ndfa (%), Shoot/N, N-fixed, and Soil N uptake of cowpea plant under co-inoculation with rhizobacteria.

5.5.1.1 $\delta^{15}N$ (‰)

According to Table 5.2, dual inoculation of *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 did not significantly affected the $\delta^{15}N$ (‰) of local cowpea genotypes irrigated with mine water under glasshouse conditions. However, mine water level and genotype had a significant effect on cowpea's $\delta^{15}N$ (‰). Regardless of genotype and treatment, cowpea not irrigated with mine water and with no inoculation (control) had the lowest $\delta^{15}N$ (‰) value (-1.16) compared to those irrigated with mine water (25% (-0.78) & 75% (-0.84)). Irrespective of mine water and inoculation, there were significant differences in $\delta^{15}N$ (‰) among cowpea genotypes. Glenda had the lowest $\delta^{15}N$ (‰) (-1.10) compared to Sudan (-0.88) and Pan 311(-0.78). Since N_2 fixation is determined by $\delta^{15}N$ values, which indicates how dependent is the legume crop for its N nutrition, crops with lower $\delta^{15}N$ can fix more nitrogen through biological nitrogen fixation (Belane et al., 2011). This implies that Glenda is more dependent on nitrogen fixation for N nutrition compared to Pan 311 and Sudan. Similar genotypic differences in dependence on N fixation were noted among landraces of Bambara groundnut, when exposed to simulated drought stress conditions (Udeh, 2023).

However, in the current study, no significant differences were noted among the inoculation treatments with respect to $\delta^{15}N$ (‰). Cowpea inoculated with either with single inoculation (*Bradyrhizobium* sp. CB756) or co-inoculation (*B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756) had slightly higher $\delta^{15}N$ (‰) values (-0.94‰ and -0.96‰) compared to single (*B. subtilis* (BD233)) (-0.86‰) or without inoculation (-0.95‰). Similarly, the interactions of mine water, genotype and inoculation treatments showed no significant differences in the $\delta^{15}N$ (‰) values of cowpea (Table 5.2). In contrast to these findings, Udeh (2023) reported marked differences among

treatments, and Bambara groundnut co-inoculated with *B. japonicum* and *B. subtilis* under drought stress showed the lowest $\delta^{15}N$ (‰) value compared to control.

5.5.1.2 N content (%)

According to Table 5.2, dual inoculation of *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 did not significantly affected **%**N content of shoots of local cowpea genotypes irrigated with mine water under glasshouse conditions. However, slight differences were noted in N contents of cowpea shoots. Irrespective of genotype and inoculation treatment, cowpea irrigated with mine water (25% & 75%) had slightly higher %N content (0.03%) compared to those not exposed to mine water (0.02%). Similarly, regardless of inoculation treatment and mine water level, Glenda had a slightly higher shoot N content (0.03%) compared with either Sudan or Pan 311 with lower %N content (0.02%). Similarly, inoculation treatment had a slight effect on %N content in shoots of cowpea regardless of genotype and mine water level. Co-inoculation of *B. subtilis* and *B. japonicum* resulted in slightly higher %N content (0.03%) in cowpea shoots compared to those not inoculated (0.02%) and single inoculated with *B. subtilis* (0.02%). However, the interaction among mine water, genotype and inoculation had no significant effects on %N of cowpea shoots (Table 5.2). Contrary to these findings, Bambara groundnut co-inoculated with *B. japonicum* and *B. subtilis* (BD 234) under simulated drought stress was noted to significantly affect N concentration and the interaction effects of the treatments were also significant, although no differences were noted among the landraces (Udeh 2023).

5.5.1.3 N-Fixed (mg Plant^{-1}) and %Ndfa

According to Table 5.2, genotypic response was significant with respect to amount of nitrogen fixed when cowpea was exposed to mine water treatments. For example, Pan 311 exhibited highest amount of N-fixed (49.20 mg Plant^{-1}) compared to Sudan with least N-fixed (37.28 mg Plant^{-1}). Similarly, Sudan recorded the least %Ndfa (86.85%) compared with Glenda that exhibited the highest %Ndfa (90.07%), followed by Pan 311 (87.01%). However, the treatment had no significant effect on %Ndfa regardless of genotype and mine water level. Cowpea not irrigated with mine water (0%) had a slightly higher N-fixed (46.57 mg Plant^{-1}) and %Ndfa (91.05%) compared to those treated with mine water (25% (42.97 mg Plant^{-1}) and %Ndfa (85.43%), and 75% (37.69 mg Plant^{-1}) and %Ndfa (87.47%), respectively). Regardless of genotype and mine

water level, cowpea either with single inoculation (*Bradyrhizobium* sp. CB756) or co-inoculation (*B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756) had slightly higher N-fixed (48.40 mg.Plant^{-1} and 46.94 mg.Plant^{-1}, respectively) and %Ndfa (88.61% and 88.98%, respectively), compared with cowpea without inoculation (N-fixed (40.09 mg.Plant^{-1}) and %Ndfa (88.48%). Similarly, the interaction among mine water, genotype and inoculation had no significant effects on N-Fixed (mg Plant^{-1}) and %Ndfa values of cowpea shoots (Table 5.2). The slight differences in N content and %Ndfa noted among local cowpea genotypes could be attributed to the presence of heavy metals in mine water (Nemutanzhela et al., 2017) and microbes (*B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756), known to remediate metals in soils (Sprent et al., 1988). Analysis of the acid mine water showed the presence of some heavy metals (Al, Cd, Co, Cu, Cr, Fe, Ni, Pb, Sr, & Zn) although majority were below the South African standards for drinking water and agricultural water use limit (mg/L) (Table 3.1). Bio accumulation over time can affect the growth and development of plants. However, microbes are known to supress environmental stresses on plants by secreting phytohormones and exopolysaccharides that can absorb and chelate heavy metals thereby reducing their bioavailability in soils (Zhang et al., 2024). Park et al. (2017) used gas chromatography mass spectrometry analysis to show that *Bacillus aryabhattai* strain SRB02 produced significant amounts of phytohormones such as abscisic acid, indole acetic acid, cytokinin and different gibberellic acids in culture. The authors reported that soybean inoculated with *Bacillus aryabhattai* strain SRB02 was able to tolerate heat stress better than those not treated with same microbe. Similar to our finding, the amount of N-fixed and %Ndfa were relatively high compared to control implying that the heavy metal stress was supressed by *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756.

5.5.1.4 Soil N uptake (mg. Plant^{-1})

According to Table 5.2, dual inoculation of *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 did not significantly affected soil N uptake (mg. Plant^{-1}) by local cowpea genotypes irrigated with mine water under glasshouse conditions. However, regardless of genotype and treatment, cowpea irrigated with mine water (25%) exhibited the highest soil N uptake (7.05 mg. Plant^{-1}) compared to those not irrigated with mine water (0%) (4.37 mg. Plant^{-1}). Irrespective of mine water and treatment, there were significant differences in soil N uptake among cowpea genotypes. Pan 311

had the highest soil N uptake (7.44 mg. Plant^{-1}) compared to Sudan (4.21 mg. Plant^{-1}) and Glenda (4.87 mg. Plant^{-1}). Similar to this finding, Udeh (2023) found significant differences in N uptake among landraces of Bambara groundnut under drought stress conditions. However, in the current study, no significant differences were noted among the treatments with respect to soil N uptake (mg. Plant^{-1}). Cowpea inoculated with either with single inoculation (*Bradyrhizobium* sp. CB756) or co-inoculation (*B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756) had slightly higher soil N uptake (6.76 mg. Plant^{-1} and 5.66 mg Plant^{-1}) compared to single (*B. subtilis* (BD233) (5.04 mg Plant^{-1}) or without inoculation (4.51 mg. Plant^{-1}). Similarly, the interactions of mine water, genotype and treatments showed no significant differences in soil N uptake (mg. Plant^{-1}) by local cowpea genotypes (Table 5.2).

Table 5.2: Effect of co-inoculation of *B. subtilis* (BD233) with *Bradyrhizobium* sp. CB756 on symbiotic performance of cowpea exposed to acid mine water under glasshouse conditions

Factor	δ^{15}N (‰)	%N	N-Fixed (mg Plant^{-1})	%Ndfa	Soil N uptake (mg
MINE WATER (%)					
0	-1.16±0.09b	0.02±0.00a	46.97±3.86a	91.05±1.13a	4.37±0.54b
25	-0.78±0.11a	0.03±0.00a	42.57±3.17a	85.43±1.44b	7.05±0.53a
75	-0.84±0.06a	0.03±0.00a	37.69±5.03a	87.47±0.72b	5.76±1.02ab
Genotype (G)					
Glenda	-1.10±0.07b	0.03±0.00a	42.60±2.65a	90.07±0.84a	4.87±0.57b
Sudan	-0.88±0.13a	0.02±0.00a	37.28±4.08b	86.85±1.72b	4.21±0.44b
Pan 311	-0.78±0.06a	0.02±0.00a	49.20±5.26a	87.01±0.68b	7.44±1.02a
Treatment					
Control	-0.95±0.14a	0.02±0.00a	40.09±5.12a	88.48±1.86a	6.49±1.44a
G + *S. subtilis*	-0.86±0.12a	0.02±0.00a	36.60±3.93a	85.91±1.56a	4.93±0.42a
G+ *B. japonicum*	-0.94±0.07a	0.03±0.00a	48.40±5.83a	88.61±0.74a	5.92±0.68a
G + *S. subtilis* + *B.*	-0.96±0.08a	0.03±0.00a	46.94±3.85a	88.98±1.08a	5.56±0.47a
F-Statistics					
MINE WATER (A)	**19.543*** **	1.662^{ns}	1.130^{ns}	**15.376*** **	**3.418***
Genotype (G)	**13.600*** **	1.855^{ns}	1.212^{ns}	**10.616*** **	**4.811****
Treatment (T)	0.772^{ns}	1.176^{ns}	1.382^{ns}	0.643^{ns}	0.607^{ns}
A x G	2.356^{ns}	0.681^{ns}	0.482^{ns}	2.127^{ns}	0.405^{ns}
A x T	0.370^{ns}	0.581^{ns}	1.319^{ns}	0.399^{ns}	1.316^{ns}
G x T	1.568^{ns}	0.722^{ns}	1.199^{ns}	1.377^{ns}	0.770^{ns}
A x G x T	0.525^{ns}	0.572^{ns}	0.950^{ns}	0.550^{ns}	0.867^{ns}

Values (M±S.E. (n=5)) followed by similar letters in a column are not significantly different at p≤0.05*, p≤0.01** and p≤0.001***.

5.5.2 Effect of *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 co-inoculation on nodulation, biomass and nutrients (N&C) accumulation by local cowpea genotypes exposed to acid mine water

In this study, the influence of co-inoculation on nodulation, biomass and nutrients (N&C) accumulation by three local cowpea genotypes irrigated with different levels of acid mine water under glasshouse conditions was determined. A 3-Way ANOVA analysis revealed no significant differences in the measured parameters (Dry Biomass (g), N content (g), N content/plant (mg), $\delta^{13}C$ (‰), and %C) with respect to the synergistic effect of co-inoculation of *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 across the genotypes, mine water levels and treatments employed in this study. However, significant differences were not among treatments independent of cowpea genotype and level of mine water and the results are described, interpreted and discussed as follows.

5.5.2.1 Shoot biomass (dry weight) (g)

According to Table 5.3, dual inoculation of *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 did not significantly affected the dry shoot biomass of local cowpea genotypes irrigated with mine water under glasshouse conditions. Regardless of genotype and treatment, cowpea not irrigated with mine water (0%) had a slightly higher shoot biomass (1.83 g) compared to those irrigated with mine water (25% (1.65 g) & 75% (1.62 g)). However, irrespective of mine water and treatment, there were significant differences in shoot biomass among cowpea genotypes. Pan 311 had the heaviest shoot biomass (2.36 g) compared to Sudan (1.81 g) and Glenda (1.74). Although, there were no significant differences noted among the treatments, cowpea inoculated either with single inoculation (*Bradyrhizobium* sp. CB756) or co-inoculation (*B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756) had slightly higher shoot biomass values (2.14 g and 1.97 g) compared to those inoculated with single (*B. subtilis* (BD233) (1.84 g) or without inoculation (1.90 g), irrespective of the genotype and mine water level. Similarly, the interactions of among mine water levels, genotypes and treatments showed no significant differences in the dry shoot biomass of cowpea (Table 5.3). This result presents challenges for interpretation, and there is limited literature available to substantiate it.

5.5.2.2 Number of nodules per plant

According to Table 5.3, mine water had no significant effect on number of nodules regardless of cowpea genotype and treatment. Cowpea not irrigated with mine water had a slightly higher number of nodules (30.61 Plant^{-1}) compared to those irrigated with mine water (25% (27.64 Plant^{-1}) and 75% (25.06 Plant^{-1})). However, genotypic response was significantly affected with respect to number of nodules per plant when cowpea was exposed to dual inoculation of *B. subtilis* and *B. japonicum* and mine water treatments. According to Table 5.3, Glenda exhibited the highest number of nodules (52.28 Plant^{-1}) compared to Pan 311 with the least number of nodules (14.83 Plant^{-1}), irrespective of mine water and treatment. Similarly, cowpea co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 had more nodules per plant (40.85 Plant^{-1}) compared to control (no inoculation) with 20.63 nodules per plant, regardless of cowpea genotype and mine water level.

The interaction of mine water (A) x genotype (G) x treatment (T) had a significant effect on the number of nodules per plant. For instance, maximum nodule count per plant (46.44) was obtained when cowpea was not exposed to mine water (0%) but co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 compared to those without inoculation (T1) (21.78 nodules Plant^{-1}) or with single (T2- *B. subtilis* (BD233) (28.33 nodules Plant^{-1}) or T3- *Bradyrhizobium* sp. CB756 (25.89 nodules Plant^{-1})) (Fig. 5.1).

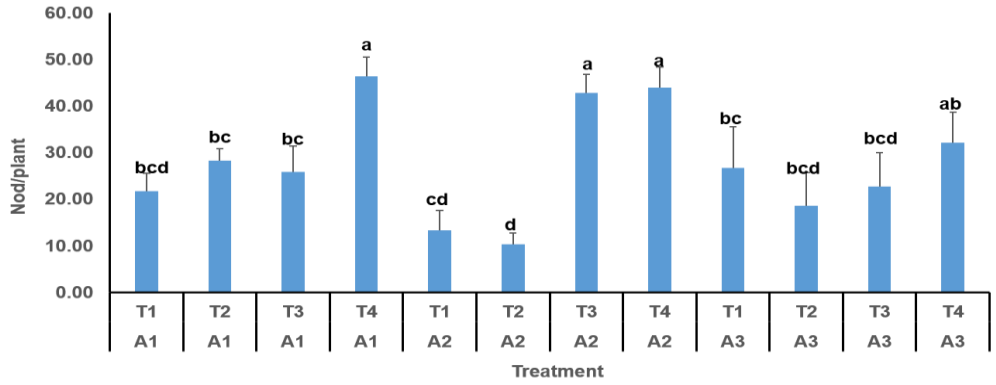

Figure 5.1 Interactive effect of mine water (A) and treatment (T) on number of nodules per cowpea plant.

Similarly, the interactive effect of genotype (G) and treatment (T) on number of nodules per plant was significant. The highest number of nodules per plant (75.56) was exhibited when Glenda (G1) was co-inoculated with *B. subtilis* (BD233) and

Bradyrhizobium sp. CB756 compared to when the same genotype was without inoculation (T1)(35.67) and with single inoculation (T2- *B. subtilis* (BD233) (40.89 nodules Plant^{-1}) or T3- *Bradyrhizobium* sp. CB756 (57.00 nodules Plant^{-1})) (Fig. 5.2).

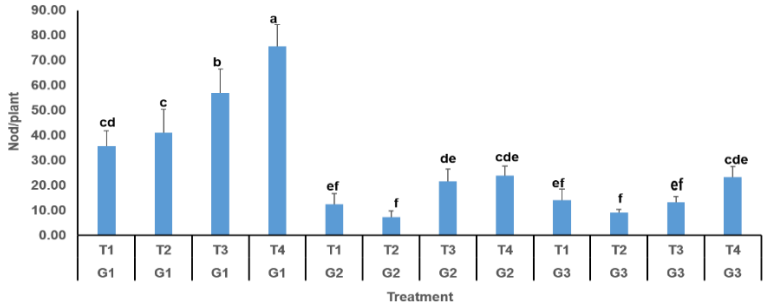

Figure 5.2 Interactive effect of genotype (G) and treatment (T) on number of nodules per cowpea plant.

Additionally, the interactive effect of mine water (A) x genotype (G) x treatment (T) on number of nodules per plant was significant. For example, the highest number of nodules per plant (90.33) was exhibited when Glenda (G1) was not irrigated with mine water (A1) but co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 compared to when the same genotype was not inoculated (T1) and irrigated with 0% mine water (A1) with 26.00 nodules per plant (Fig. 5.3).

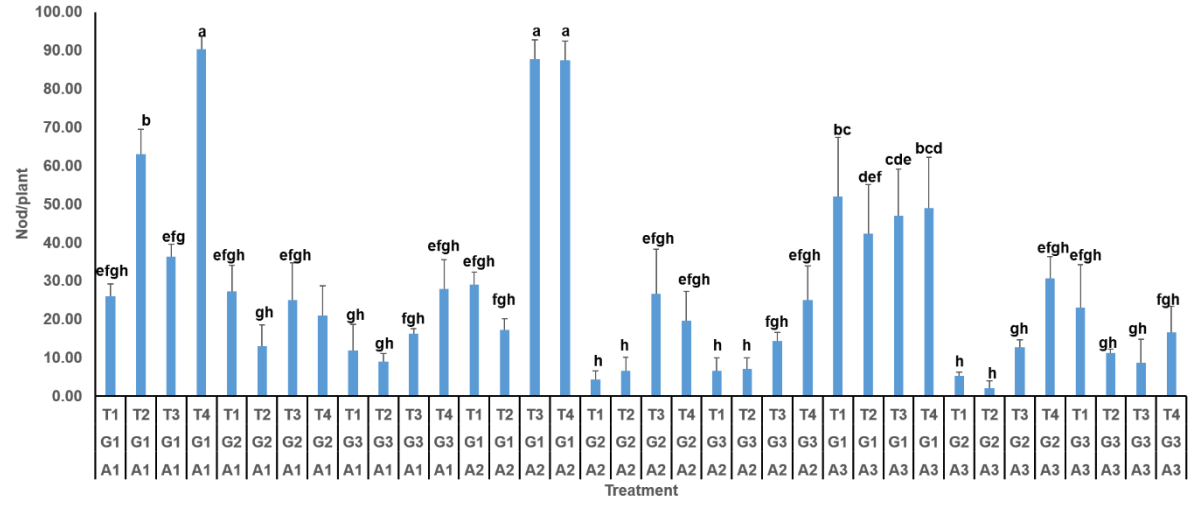

Figure 5.3 Interactive effect of mine water (A) x genotype (G) x treatment (T) on number of nodules per cowpea plant.

5.5.2.3 N content (g) and N content/plant (mg)

Co-inoculation of *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 did not significantly affected the N content (g) and N content/plant (mg) of local cowpea genotypes irrigated with mine water under glasshouse conditions. Regardless of genotype and treatment, cowpea not irrigated with mine water (0%) had a slightly higher N content (0.05 g) and N content/plant (47.34 mg) compared to those irrigated with mine water (25%) (0.04 g & 43.46 g, respectively) (Table 5.3). Similarly, there were no significant differences in genotype response, independent of mine water level and treatment, Pan 311 had a slightly higher N content (0.06 g) and N content/plant (55.27 g) compared with Sudan with lower N content (0.04 g) and N content/plant (44.23 g). Although, there were no significant differences noted among treatments, cowpea inoculated either with single inoculation (*Bradyrhizobium* sp. CB756) or co-inoculation (*B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756) had slightly higher N content (0.06 g & 0.05 g, respectively) and N content/plant (47.96 g & 48.32 g, respectively) compared to those inoculated with single (*B. subtilis* (BD233) (0.04 g & 40.39 g, respectively), irrespective of genotype and mine water level. Similarly, the interactions among mine water levels, genotypes and treatments showed no significant differences in the N content (g) and N content/plant (g) of cowpea shoots (Table 5.3).

5.5.2.4 $\delta^{13}C$ (‰)

Similarly, co-inoculation of *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 did not significantly affected the $\delta^{13}C$ (‰) of local cowpea genotypes irrigated with mine water under glasshouse conditions. However, regardless of genotype and treatment, cowpea not irrigated with mine water (0%) had a significantly higher $\delta^{13}C$ (‰) (-26.07‰) compared to those irrigated with mine water (25%) (-26.72 ‰) (Table 5.3). Similarly, there were significant differences in genotype response, independent of mine water level and treatment, Glenda had higher $\delta^{13}C$ (‰) (-25.42 ‰) compared with Sudan with lower $\delta^{13}C$ (‰) (-25.96 ‰). Although, there were no significant differences noted among treatments, cowpea inoculated either with single inoculation (*Bradyrhizobium* sp. CB756) or co-inoculation (*B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756) had slightly higher N content (-25.65 ‰ & -25.44 ‰, respectively) compared to those inoculated with single inoculation (*B. subtilis* (BD233)

(-25.92 ‰), irrespective of genotype and mine water level. Similarly, the interactions among the mine water levels, genotypes and treatments showed no significant differences in the $\delta^{13}C$ (‰) of cowpea shoots (Table 5.3).

5.5.2.5 %C

According to Table 5.3, co-inoculation of *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 did not significantly affected the %C of local cowpea genotypes irrigated with mine water under glasshouse conditions. Regardless of genotype and treatment, cowpea irrigated with mine water (25%) had a slightly higher %C (0.44%) likened to those not irrigated with mine water (0%) (0.40%). Similarly, irrespective of mine water level and treatment, there were no significant differences in %C among cowpea genotypes. Glenda had a slightly higher %C (0.40%) compared to Sudan (0.39%) and Pan 311 (0.39%). Additionally, there were no significant differences noted among the treatments, cowpea without inoculation had a slightly higher %C (0.46%) contrasted to those inoculated either with single inoculation (*Bradyrhizobium* sp. CB756 (0.40%) and *B. subtilis* (0.39%) or with co-inoculation (*B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756) (0.40%), irrespective of the genotype and mine water level. Similarly, the interactions of among mine water levels, genotypes and treatments showed no significant differences in the %C of cowpea shoots (Table 5.3).

5.5.2.6 C/N ratio

Co-inoculation of *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 significantly affected the C/N ratio of local cowpea genotypes irrigated with mine water under glasshouse conditions. However, regardless of genotype and treatment, cowpea not irrigated with mine water (0%) had a slightly higher C/N ratio (18.16) compared to those irrigated with mine water (25% (17.58) & 75% (17.18)) (Table 5.3). On the other hand, irrespective of mine water level and treatment, there were significant differences in C/N ratios among cowpea genotypes. Sudan had the higher C/N value (19.61) compared to pan 311 (17.57) and Glenda (15.74). Similarly, there were significant differences noted among the treatments, cowpea inoculated either with single inoculation (*Bradyrhizobium* sp. CB756) or with co-inoculation (*B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756) had slightly lower C/N values (16.58 and 16.31) compared to those inoculated with single (*B. subtilis* (BD233) (19.61) or without inoculation (18.07), irrespective of the genotype and mine water level. Also, the interactions

among mine water levels, genotypes and treatments showed significant differences in the C/N value of cowpea shoots (Table 5.3).

Table 5.3 Effect of co-inoculation of *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 on biomass and nutrient accumulation by cowpea exposed to acid mine water under glasshouse conditions

Factor	Shoot Biomass (g)	No. of nodules/Plant	No. of pods/Plant	N content (g)	N content/Plant (mg)	$\delta^{13}C$ (‰)	%C	C/N
Mine Water (%)								
0	1.83±0.12a	30.61±4.45a	2.89±0.13a	0.05±0.01a	47.34±2.75a	-26.07±0.12a	0.40±0.00a	18.16±0.43a
25	1.65±0.09a	27.64±4.92a	1.50±0.18c	0.05±0.01a	53.63±7.28a	-26.72±0.16b	0.44±0.05a	17.58±0.43a
75	1.62±0.14a	25.06±3.67a	2.33±0.15b	0.04±0.00a	43.46±3.44a	-26.40±0.17ab	0.39±0.00a	17.18±0.43a
Genotype (G)								
Glenda	1.74±0.11b	52.28±4.87a	2.28±0.18a	0.04±0.00a	44.91±2.61a	-26.08±0.13a	0.40±0.00a	15.74±0.43c
Sudan	1.81±0.19b	16.19±2.23b	2.17±0.20a	0.04±0.00a	44.23±2.81a	-26.48±0.14ab	0.39±0.00a	19.61±0.43a
Pan 311	2.36±0.24a	14.83±1.84b	2.28±0.16a	0.06±0.01a	55.27±7.57a	-26.62±0.18b	0.44±0.05a	17.57±0.43b
Treatment (T)								
Control	1.90±0.24a	20.63±3.51c	2.22±0.21a	0.06±0.01a	55.90±9.78a	-26.32±0.17a	0.46±0.07a	18.07±0.43b
G + *S. subtilis*	1.84±0.17a	19.07±4.38c	2.22±0.21a	0.04±0.00a	40.39±2.82a	-26.54±0.16a	0.39±0.00a	19.61±0.43a
G + *B.*	2.14±0.28a	30.52±5.12b	2.07±0.21a	0.05±0.00a	47.96±4.04a	-26.43±0.18a	0.40±0.00a	16.58±0.43c
G + *S. subtilis* + *B*	1.97±0.17a	40.85±5.86a	2.44±0.21a	0.05±0.00a	48.32±2.90a	-26.29±0.20a	0.40±0.00a	16.31±0.43c
F-Statistics								
MINE WATER	1.029^{ns}	1.369^{ns}	**30.645*****	1.102^{ns}	1.102^{ns}	**4.994****	0.859^{ns}	0.879^{ns}
Genotype (G)	**4.812***	**79.906*****	0.258^{ns}	1.601^{ns}	1.601^{ns}	**3.718***	0.887^{ns}	**13.724*****
Treatment (T)	0.137^{ns}	**13.523*****	1.096^{ns}	1.257^{ns}	1.257^{ns}	0.449^{ns}	1.000^{ns}	**6.380*****
A x G	1.852^{ns}	0.552^{ns}	**6.645*****	0.457^{ns}	0.457^{ns}	1.487^{ns}	0.884^{ns}	1.761^{ns}
A x T	1.865^{ns}	**4.023****	**3.419****	1.328^{ns}	1.328^{ns}	1.054^{ns}	0.965^{ns}	1.05^{ns}
G x T	1.253^{ns}	**2.520***	**2.452***	1.139^{ns}	1.139^{ns}	1.515^{ns}	0.982^{ns}	0.913^{ns}
A x G x T	0.919^{ns}	**3.503****	1.677^{ns}	0.933^{ns}	0.933^{ns}	1.522^{ns}	0.976^{ns}	**1.893***

Values (M±S.E. (n=5) followed by similar letters in a column are not significantly different at p≤0.05*, p≤0.01** and p≤0.001***.

The interaction of mine water (A) x genotype (G) x treatment (T) had a significant effect on the C/N ratio of cowpea shoots. For example, when Sudan (G2) was inoculated with *B. subtilis* (BD233) (T2) and irrigated with 25% mine water, cowpea shoots recorded the peak C/N value (27.04) compared to when same genotype (G2) was co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 (T4) and exposed to 25% mine water (15.78) (Fig. 5.4).

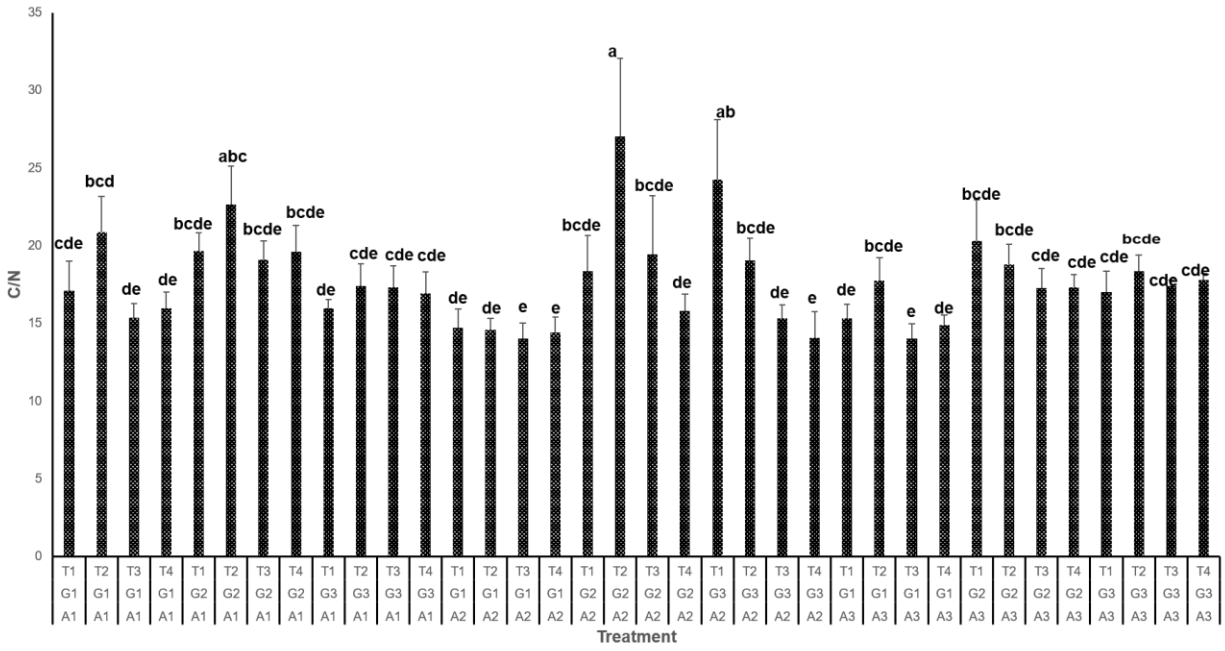

Figure 5.4 Mine water x genotype and treatment interactive effect on the C/N ratios in shoot of cowpea

5.6 SUMMARY

South Africa is characterised by limited water resources and has a longstanding history of gold and coal mining, and AMD has polluted most surface waters including lakes, streams and rivers in provinces where there is need for agricultural water use. Elemental analysis of the mine water used to irrigate cowpea revealed the presence of heavy metals (Al, Cd, Co, Cu, Cr, Fe, Ni, Pb, Sr, & Zn) and their concentrations were below the South African standards for drinking water and agricultural water use limit. Cowpea irrigated with mine water and co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756

showed resistance to heavy metal stress with respect to their symbiotic performance and genotypic differences were also noted. Glenda with the lowest $\delta^{15}N$ and highest amount of N-fixed was found to be more dependent on nitrogen fixation for N nutrition compared to Pan 311 and Sudan, and it also had highest soil N uptake with the heaviest dry shoot biomass. Among genotypes, Glenda seems to exhibit more resistance to heavy metal stress. Similarly, cowpea co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 had more nodules per plant and lower C/N values compared to control (no inoculation) or with single inoculation. According to these findings, co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 is a promising technology for the bioremediation of AMD polluted water bodies and soils in mining communities in South Africa, especially for the cultivation of leguminous crops such as the local cowpea genotypes. However, more studies using more local genotypes under field conditions are required to verify the findings in the current study.

CHAPTER SIX: *Bacillus subtilis* (BD233) and *Bradyrhizobium* sp. CB756 co-inoculation effect on eco-physiological parameters and carbon assimilation of local cowpea genotypes irrigated with mine water

6.1 INTRODUCTION

Researchers are looking at novel ways to increase crop production while reducing environmental effects due to the growing demand for sustainable farming methods. Research on the interactions between plant growth-promoting rhizobacteria (PGPR) and their host plants is essential for sustainable agriculture, especially in areas where soil and water contamination are a problem. Effective PGPR that improve plant health and nutrient uptake include *Bacillus subtilis* and *Bradyrhizobium* sp. According to recent research, co-inoculation of these advantageous bacteria may enhance plant performance in stressful situations, as those brought on by irrigation with mine water (Smith et al., 2021; Johnson & Lee, 2020; Buthelezi et al., 2025). Irrigating crops with mine water presents unique challenges, as it often contains elevated concentrations of heavy metals and salinity, which can impede plant growth and carbon assimilation (Zhao et al., 2018).

Because leguminous plants like cowpea can fix atmospheric nitrogen, improving soil fertility and lowering the need for synthetic fertilizers, they are essential to sustainable agriculture (Sanginga et al., 2003). Cowpea is a drought-tolerant legume that is essential to food security, especially in arid and semi-arid areas. Due to possible pollutants, using mine water for irrigation presents substantial hurdles; nonetheless, microbial co-inoculations may improve the resilience of native cowpea genotypes (Smith et al., 2021; Johnson & Lee, 2020). Prior studies have shown that microbial co-inoculation can have a positive impact on eco-physiological indices, such as photosynthetic efficiency and water consumption efficiency (Davis et al., 2019; Wang et al., 2022). Additionally, it has been observed that co-inoculation of rhizobia and bacteria that promote plant development, enhances nodulation and nitrogen fixation in a synergistic way, improving plant performance (Kloepper et al., 2004; Raaijmakers et al., 2009; Rudrappa et al., 2012). Under stress conditions, co-inoculation has proven especially beneficial. Zhang et al. (2020) reported that plants inoculated with multiple microorganisms showed superior tolerance to various environmental stresses. Their research demonstrated improved drought tolerance (15-30% better survival), enhanced salt stress resistance (20-35%

improved tolerance), and better adaptation to heavy metal stress (25-40% reduced toxicity). The combined effect of different microorganisms helps plants maintain better physiological functions under adverse conditions. The main goal of this study was to evaluate the effect of co-inoculation of *B. subtilis* strain BD233 and *Bradyrhizobium* sp. CB756 on the carbon assimilation and eco-physiological parameters (root and shoot biomass, chlorophyll content, and photosynthetic efficiency) of local cowpea genotypes irrigated with mine water. We intend to shed light on sustainable farming methods that use microbial bio-inoculants to improve crop resilience by investigating these genotypes' physiological reactions to stress.

6.2 MATERIALS AND METHODS

6.2.1 Experimental Conditions

As outlined in section 3.2.1 of chapter 3.

6.2.2 Bacterial Strains

As outlined in section 3.1.2 of chapter 3.

6.2.3 Cowpea Co-Inoculation

As outlined in section 3.2.2 of chapter 3.

6.2.4 Acid Mine Drainage

As outlined in section 3.2.3 of chapter 3.

6.2.5 Plant Growth Media

As outlined in section 3.2.4 of chapter 3.

6.2.6 Experimental Design

As outlined in section 4.5 of chapter 4.

6.3 DATA COLLECTION

Data was collected during the vegetative, flowering, and pod filling growth stages, and at harvest. Plant shoot biomass was sampled at physiological maturity (dry biomass). Photosynthetic rates (A), stomatal conductance (gs), intercellular CO_2 concentration (Ci), and transpiration rate (E) data was collected during flowering stage (heavy metal stress exposure). Carbon assimilation parameters ($\delta^{13}C$ (‰)**,** C concentration, C/N ratio, and Shoot C content) were also determined at physiological maturity.

6.3.1 Gas-exchange measurements

While sampling cowpea shoots for ^{13}C isotopic analysis, gas-exchange studies were done on cowpea leaves. Photosynthetic rates, stomatal conductance, internal CO_2, water use efficiency and transpiration rate were measured in four young leaves (flag leaves) per plant at 30 days after planting, using Infrared Red gas Analyzer (portable photosynthesis system) (LI-6400XT - LI-COR Bioscientific Ltd., UK). Measurements and conditions were as described by Makoi et al. (2010). Photosynthetic water-use efficiency and carboxylation efficiency were derived by dividing A by E and A by Ci, respectively. Leaves were allowed to acclimate to the light environment in the chamber for 4-5 min. Without any trouble shooting, each measurement took approximately 2 min, which was the minimum time allowed for the readings to stabilize before they were recorded. Measurements were taken with the following conditions in the leaf chamber: photosynthetic photon flux density (PPFD) = 1100 µmol (quantum) m–2 s–1, relative humidity = 44%, leaf vapour pressure deficit = 1.83 kPa, flow rate = 400 µmol s–1, reference CO_2 = 400 ppm, and leaf temperature = 25°C. Water-use efficiency was calculated as described by Hamid et al. (1990).

6.3.2 Carbon isotope analysis was used to determine the water-use efficiency of Cowpea

The $^{13}C/^{12}C$ ratio for each sample was used to calculate the ^{13}C natural abundance (^{13}C) as described by Stout and Rafter (1978):

$$\delta^{13}C = \frac{\left(^{13}C/^{12}C\right)_{sample} - \left(^{13}C/^{12}C\right)_{standard}}{\left(^{13}C/^{12}C\right)_{standard}} * 1000$$

Where $\left(^{13}C/^{12}C\right)_{sample}$ is the isotopic ratio of the sample, and $\left(^{13}C/^{12}C\right)_{standard}$ is the isotopic ratio of PDB, a universally accepted standard from Belemnite Pee Dee limestone formation (Craig, 1957). Shoot C content per plant was calculated as:

Shoot C content = %C x shoot dry matter per plant.

6.3.3 Analysis of Data

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

6.4 RESULTS AND DISCUSSION

6.4.1 Effect of co-inoculation of *B. subtilis* strain BD233 and *Bradyrhizobium* sp. CB756 on the carbon assimilation in cowpea

The results presented in Table 6.1 reveals that the carbon assimilation physiology of cowpea was remarkably resilient to both mine water irrigation and microbial inoculation treatments. The two significant effects observed were isolated, with no significant interaction between the factors, indicating that the microbial treatments performed consistently across all mine water dilution levels.

Regardless of the treatment, the intercellular CO_2 content of cowpea was significantly ($p<0.05$) affected by the dilution of mine water. In comparison to the lowest value in the 0% control treatment (0.80 ± 0.07 mol m^{-2} s^{-1}), the highest intercellular CO_2 value was found at the 25% mine water level (1.06 ± 0.10 mol m^{-2} s^{-1}) (Table 6.1). This pattern implies that a low concentration of mine water caused mild, temporary stress, which may have resulted in a partial limitation in mesophyll conductance that prevented CO_2 from diffusing to the chloroplasts and caused it to build up in the intercellular gaps. Literature (Flexas et al., 2004; Farquhar et al., 2010; Nouri et al., 2015) showing that mild abiotic stress can interfere with internal gas exchange systems prior to showing up as a decrease

in photosynthetic rate supports this conclusion. However, according to results obtained in this study, mine water irrigation had no significant effect on the photosynthetic rate, stomatal conductance, transpiration rate and water use efficiency, regardless of treatment.

Similarly, regardless of mine water dilution, the treatments had no significant effect on the photosynthetic rate, stomatal conductance, and water use efficiency. Importantly, the rates of photosynthesis varied only slightly between 23.53 and 23.81 µmol (CO_2) m^{-2} s^{-1} during these treatments, indicating the resilience of the photosynthetic apparatus. This resilience was reflected in the slight variations in stomatal conductance (ranging from 0.03 to 0.06 mol (H_2O) m^{-2} s^{-1}) and internal CO_2 (ranging from 0.79 to 1.13 mol (H_2O) m^{-2} s^{-1}) across the treatments. In particular, the Sudan+ *Bradyrhizobium* sp. CB756 treatment had an exceptionally high transpiration rate (401.07 mol (H_2O) m^{-2} s^{-1}), which was the only significant effect among the treatments. The lowest transpiration rates found in the Sudan and Sudan+ *B. subtilis* BD233 + *Bradyrhizobium* sp. CB756 treatments (~247 mol (H_2O) m^{-2} s^{-1}) were about 62% lower than this value. Since other treatments inoculated with *Bradyrhizobium* sp. CB756 did not show this effect, the physiological basis for this outlier is unknown. It might be connected to a particular, non-replicated interaction with the Sudan cultivar.

The most important finding is that water usage efficiency (WUE) and photosynthetic rate consistently show no significance in response to the treatments. With the highest value (24.95 µmol (CO_2) m^{-2} s^{-1} in 'Sudan+ *B. subtilis* BD233 + *Bradyrhizobium* sp. CB756) just 14% higher than the lowest (21.87 µmol (CO_2) m^{-2} s^{-1} in Glenda+ *B. subtilis* (BD233) + *Bradyrhizobium* sp. CB756), photosynthetic rates varied little across all treatments. In a similar vein, despite a wide numerical range, WUE—a crucial measure of plant performance under stress, showed no statistically significant variations. The Glenda+ *Bradyrhizobium* sp. CB756 treatment had the highest WUE (956.24 mmolCO_2.mol$^{-1}H_2O$), whereas the Glenda control had the lowest (561.51 mmolCO_2.mol$^{-1}H_2O$). Despite significant numerical differences, this lack of statistical significance highlights the high variability in WUE responses and is consistent with research indicating that, although microbial inoculants can occasionally improve WUE, a consistent and significant effect is not always guaranteed, particularly in the absence of severe, sustained water deficit (Riva

et al., 2021). In addition, the mine water and treatment interaction did not significantly affected all measured carbon assimilation parameters. In summary, when cowpea was irrigated with mine water, co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 did not negatively impact core photosynthetic metrics. The potential to use these microbial partnerships to enhance cowpea agriculture in marginal locations with poor water quality is highlighted by the stability of photosynthesis and the lack of a widespread negative physiological response.

Table 6.1 Carbon accumulation parameters of cowpea co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 and irrigated with mine water

FACTOR	Photosynthetic rate µmol (CO_2) m^{-2} s^{-1}	Stomatal Conductance mol (H_2O) m^{-2} s^{-1}	Intercellular CO_2 mol m^{-2} s^{-1}	Transpiration rate mol (H_2O) ms^{-2} s^{-1}	Water Use Efficiency mmolCO_2.m^{-1}.H_2O
Mine Water (%)					
0	23.57±0.44a	0.04±0.00a	0.80±0.07b	261.78±12.42	821.41±55.34a
25	23.53±0.61a	0.04±0.00a	1.06±0.10a	259.48±22.24a	793.27±72.90a
75	23.81±0.63a	0.04±0.00a	0.90±0.08ab	274.46±24.81a	838.23±63.49a
Treatment					
Glenda	23.68±0.99a	0.06±0.01a	1.13±0.23a	251.71±5.74b	561.51±78.63a
Sudan	23.64±0.97a	0.06±0.01a	0.89±0.17a	247.22±4.76b	603.92±117.95a
Pan 311	22.62±1.13a	0.04±0.00a	0.79±0.15a	257.23±4.59b	873.81±119.11a
Glenda+*B. subtilis*	22.16±1.24a	0.04±0.01a	0.84±0.14a	255.02±4.44b	875.32±117.58a
Glenda+ *Bradyrhizobium sp.*	24.80±1.28a	0.04±0.01a	0.85±0.14a	259.51±4.52b	956.24±148.01a
Glenda+*B.stubtilis*+ *Bradyrhizobium*	21.87±1.00a	0.04±0.01a	0.94±0.18a	248.67±5.22b	755.66±109.18a
Sudan+*B. subtilis*	23.33±0.93a	0.04±0.01a	1.03±0.21a	254.06±4.05b	931.70±145.69a
Sudan+ *Bradyrhizobium* sp.	24.63±1.05a	0.04±0.00a	0.85±0.16a	401.07±40.35a	782.03±119.09a
Sudan+*B.stubtilis*+ *Bradyrhizobium*	24.95±1.26a	0.04±0.00a	1.06±0.18a	248.06±5.57b	837.19±148.48a
Pan 311+*B. subtilis*	23.70±1.21a	0.04±0.00a	0.94±0.18a	255.96±4.63b	824.30±118.91a
Pan 311+ *Bradyrhizobium sp.*	24.92±1.32a	0.03±0.00a	0.82±0.14a	250.76±5.26b	901.82±89.74a
Pan 311+*B.stubtilis*+ *Bradyrhizobium*	23.34±1.09a	0.04±0.00a	0.94±0.17a	253.59±4.43b	908.14±89.51a
F-Statistics					
Mine Water	0.068^{ns}	1.499^{ns}	2.130*	0.254^{ns}	0.126^{ns}
Treatment	0.802^{ns}	1.800^{ns}	0.344^{ns}	2.135*	0.949^{ns}
Mine Water X Treatment	0.362^{ns}	1.070^{ns}	0.333^{ns}	0.682^{ns}	1.132^{ns}

6.4.2 Effect of co-inoculation of B. subtilis BD233 and Bradyrhizobium sp. CB756 on the carbon accumulation in cowpea

According to the results in Table 6.2, the carbon accumulation in cowpea irrigated with mine water stress and microbial inoculation reveals a complex interplay in which the particular microbial treatment and cultivar have a greater impact on plant growth and carbon balance than the mine water level alone, with a crucial interaction seen in nutrient partitioning. The microbial treatment's major impact on shoot dry matter (DM) and the carbon/nitrogen (C/N) ratio is a crucial discovery. The main indicator of growth, shoot DM, varied greatly, with Pan 311+ *B. subtilis* (BD233) having the largest biomass (2.05 g plant^{-1}), while Glenda+ *B. subtilis* BD233 had the lowest (1.28 g plant^{-1}). This 60% increase in Pan 311 shoot DM shows that the same *B. subtilis* (BD233) has a substantial cultivar-specific response to inoculation. In one genetic context (Pan 311), the *B. subtilis* BD233 was quite helpful; nevertheless, in another (Glenda), it was harmful. This emphasizes how the genotype of the host plant is crucial to the effectiveness of microbial inoculation, regardless of mine water stress. In support of this claim, a recent study by Manley et al. (2025) found significant ($p < 0.001$) differences in $\delta^{13}C$ among genotypes of Guar (*Cyamopsis tetragronoloba* (L.) Taub.) subjected to water stress conditions. In the current study, the stable carbon isotope signature ($\delta^{13}C$) and shoot carbon content were significantly impacted by mine water stress alone. When compared to the 0% control (-26.07 ‰), the $\delta^{13}C$ value of cowpea treated with 25% mine water (-26.72 ‰) was noticeably more negative. Lower long-term water-use efficiency (WUE) is generally indicated by more negative $\delta^{13}C$ values, indicating that the plants' water-use economy was hampered by mild mine water stress. This is consistent with the significantly lower shoot carbon content at 25% mine water (396.55 mg plant^{-1}) compared to the control (403.13 mg plant^{-1}), indicating a mild but measurable stress effect. Taken together, this finding is aligned with reports by Wang et al. (2022), when they reported use of $\delta^{13}C$ as an indicator of WUE at different alternate wetting and drying irrigation regimes on the growth of rice under field conditions.

For the C/N ratio, the significant mine water x treatment interaction is the most subtle result. This suggests that the inoculants' impact on the equilibrium between nitrogen

assimilation and carbon buildup was contingent upon the mine water level. This crucial metabolic balance is shown in the C/N ratio, which peaked in the Sudan+ *B. subtilis* BD233 treatment (22.83 g g^{-1}) and was lowest in the Glenda+ *Bradyrhizobium* sp. CB756 treatment (14.47 g g^{-1}). A high C/N ratio indicates that nitrogen uptake is constrained in comparison to carbon fixation. The remarkably high ratio in Sudan+ *B. subtilis* BD233 (37% difference) suggests that in the current conditions, symbiotic nitrogen fixation or absorption may have been compromised by this particular inoculation, resulting in nitrogen restriction. On the other hand, the low C/N ratio in Glenda+ *Bradyrhizobium* sp. CB756 suggests that *Bradyrhizobium* sp. CB756 was more successful in encouraging nitrogen assimilation in the Glenda genotype, guaranteeing a nutritional balance more conducive to growth. Nonetheless, current physiological and microbiological research provides strong support for these conclusions (Egamberdieva et al., 2018; Foster et al 2025). A fundamental aspect of plant-microbe interaction study is the cultivar-specific response to inoculation. The significant variation in *B. subtilis* BD233 performance between the Pan 311 and Glenda genotypes can be explained by studies like those conducted by Marasco et al. (2012), which consistently demonstrate that the result of plant growth-promoting rhizobacteria (PGPB) inoculation is not universal but is determined by specific genotype-by-genotype interactions. Research on how plants react to abiotic stress is consistent with the change in δ13C under the stress of mine water. According to Farooq et al. (2012), stomatal limits brought on by drought and ionic stress might result in greater negative δ13C values because of discrimination against the heavier ^{13}C isotope when stomata are open, which reflects a decrease in overall water-use efficiency. Work like that of Sulieman and Tran (2015), who showed that environmental stress can specifically affect nitrogen metabolism in legumes, supports the substantial interaction for C/N ratio noted in this current study. An increased C/N ratio denotes a decoupling of nitrogen and carbon assimilation, which frequently results from stress-induced inhibition of nitrate reductase or nitrogenase activity, causing a build-up of carbon without a corresponding rise in nitrogen. In summary, the result demonstrates that while mine water stress imposes a physiological constraint on cowpea water-use efficiency, the ultimate impact on plant growth and nutritional balance is predominantly dictated by the specific compatibility between the cowpea genotype and the inoculated

microbes. This highlights the imperative for a precision-based approach, selecting tailored host plant-microbe partnerships, to enhance resilience and productivity in marginal soils affected by acid mine drainage.

Table 6.2 Carbon accumulation parameters of cowpea co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 and irrigated with AMD

Factor	Shoot DM (g plant $^{-1}$)	$\delta^{13}C$ (‰)	C concentration (%)	C/N-ratio (g g^{-1})	Shoot C content (mg plant $^{-1}$)
Mine Water (%)					
0	1.83±0.12a	-26.07±0.12a	0.40±0.00a	18.16±0.48a	403.13±1.87a
25	1.65±0.09a	-26.72±0.16b	0.44±0.05a	17.58±0.81a	396.55±2.29b
75	1.62±0.14a	-26.40±0.17ab	0.39±0.00a	17.18±0.38a	400.02±1.82ab
Treatment					
Glenda	1.76±0.16ab	-25.88±0.27a	0.40±0.00a	15.70±0.81ab	395.77±3.59ab
Sudan	1.57±0.34ab	-26.81±0.26ab	0.40±0.00a	19.44±1.17ab	403.54±3.99ab
Pan 311	1.86±0.18ab	-26.29±0.30ab	0.60±0.21a	19.08±1.57ab	398.95±5.89ab
Glenda+*B. subtilis*	1.28±0.18b	-26.24±0.28ab	0.39±0.00a	17.72±1.09ab	396.69±3.24ab
Glenda+ *Bradyrhizobium sp.*	1.39±0.17ab	-26.24±0.27ab	0.40±0.00a	14.47±0.54b	401.82±2.78ab
Glenda+*B.stubtilis*+ *Bradyrhizobium sp.*	1.53±0.15ab	-25.97±0.26ab	0.40±0.00a	15.09±0.52b	405.88±5.12a
Sudan+*B. subtilis*	1.58±0.16ab	-26.88±0.21b	0.38±0.00a	22.83±1.97a	391.04±4.51b
Sudan+ *Bradyrhizobium sp.*	1.96±0.21ab	-26.21±0.31ab	0.40±0.00a	18.59±1.33ab	401.56±2.84ab
Sudan+*B.stubtilis*+ *Bradyrhizobium sp.*	1.99±0.16ab	-26.03±0.29ab	0.40±0.00a	17.57±0.78ab	400.99±3.15ab
Pan 311+*B. subtilis*	2.05±0.19a	-26.50±0.32ab	0.40±0.00a	18.27±0.73ab	405.98±2.95a
Pan 311+ *Bradyrhizobium sp.*	1.88±0.41ab	-26.84±0.34b	0.39±0.00a	16.68±0.57ab	397.68±3.89ab
Pan 311+*B.stubtilis*+ *Bradyrhizobium sp.*	1.59±0.25ab	-26.87±0.45b	0.39±0.00a	16.26±0.80ab	398.94±4.93ab
F-Statistics					
AMD	0.991^{ns}	4.792**	0.863^{ns}	0.822^{ns}	2.717*
Genotype	1.188*	1.559*	0.975^{ns}	4.425**	1.176*
AMD x Treatment	1.346^{ns}	1.388^{ns}	0.978^{ns}	1.640*	0.956^{ns}

6.4.3 Effect of co-inoculation of *B. subtilis* BD233 and *Bradyrhizobium* sp. CB756 on nutrient partitioning in cowpea

The three-way interaction between cowpea genotype, microbial co-inoculation, and mine water irrigation, as illustrated in Figure 6.1, had a significant influence on the Carbon/Nitrogen (C/N) ratio of cowpea shoots. This finding underscores that the nutritional balance and nitrogen metabolism of cowpea under metalliferous stress are not governed by any single factor, but by the complex interplay between the plant's genetic background and its associated microbiome.

The C/N ratio serves as a sensitive indicator of the equilibrium between carbon assimilation through photosynthesis and nitrogen acquisition via symbiotic fixation. The significant interaction effect reveals that the ability of microbial inoculants to support nitrogen fixation was highly dependent on both the cowpea genotype and the level of mine water stress. For instance, with 25% mine water irrigation, Pan 311 exhibited higher C/N ratio (24.24) compared to Glenda (14.70) under the mild stress condition. It is clear that certain genotypes, such as Pan 311, likely maintained a more stable and favorable C/N ratio across mine water levels when co-inoculated with both *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756, (at 0% (17.33), at 25 (15.33) and at 75% (17.39)), indicating a resilient symbiotic partnership that effectively supplied nitrogen even under stress. In contrast, other genotypes, potentially Sudan, may have exhibited a sharp increase in C/N ratio under mine water stress, particularly with single-strain inoculations, suggesting a breakdown in symbiotic efficiency and the onset of nitrogen limitation.

Bacillus subtilis (BD233) appears to play a context-dependent co-inoculant role. Its presence may have synergistically increased *Bradyrhizobium* sp. CB756 activity in some genotype-stress combinations, resulting in a reduced C/N ratio by encouraging nodulation, nitrogen fixation, or nitrogen absorption. For example, at 25% mine water, Sudan + *B. subtilis* (BD233) had the highest C/N ratio (27.04) compared to when Sudan was co-inoculated with both *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 (15.78) (Fig 6.1). This finding is consistent with the known function of plant growth-promoting rhizobacteria (PGPB), such as *Bacillus* species, in boosting plant stress tolerance and improving nutrient uptake through processes like phytohormone synthesis and biofilm formation (Mohammed et al., 2022). The presence of *B. subtilis* (BD233) may, however, have given the rhizobia no advantage or maybe a competitive disadvantage in other less suitable combinations, leading to a greater C/N ratio and indicating a failed tripartite alliance.

This genotype-specific response is a cornerstone of plant-microbe interaction research. The findings confirm that the success of a microbial inoculant is not universal but is contingent upon a compatible dialogue with the host plant's genetics. The work of Marasco et al. (2012) and others (Marasco et al., 2012; Bamba et al., 2024) has

consistently shown that the outcome of inoculation is a product of specific genotype-by-genotype interactions, which can either unlock synergistic benefits or lead to neutral or antagonistic outcomes.

In conclusion, the significant interaction effect on the C/N ratio demonstrates that optimizing nitrogen nutrition and carbon balance in cowpea subjected to mine water stress requires a tailored approach. A "one-size-fits-all" inoculation strategy is insufficient. Instead, the selection of microbial partners must be matched to specific cowpea genotypes to build effective, stress-resilient holobionts capable of maintaining efficient nitrogen fixation and supporting growth in challenging environments.

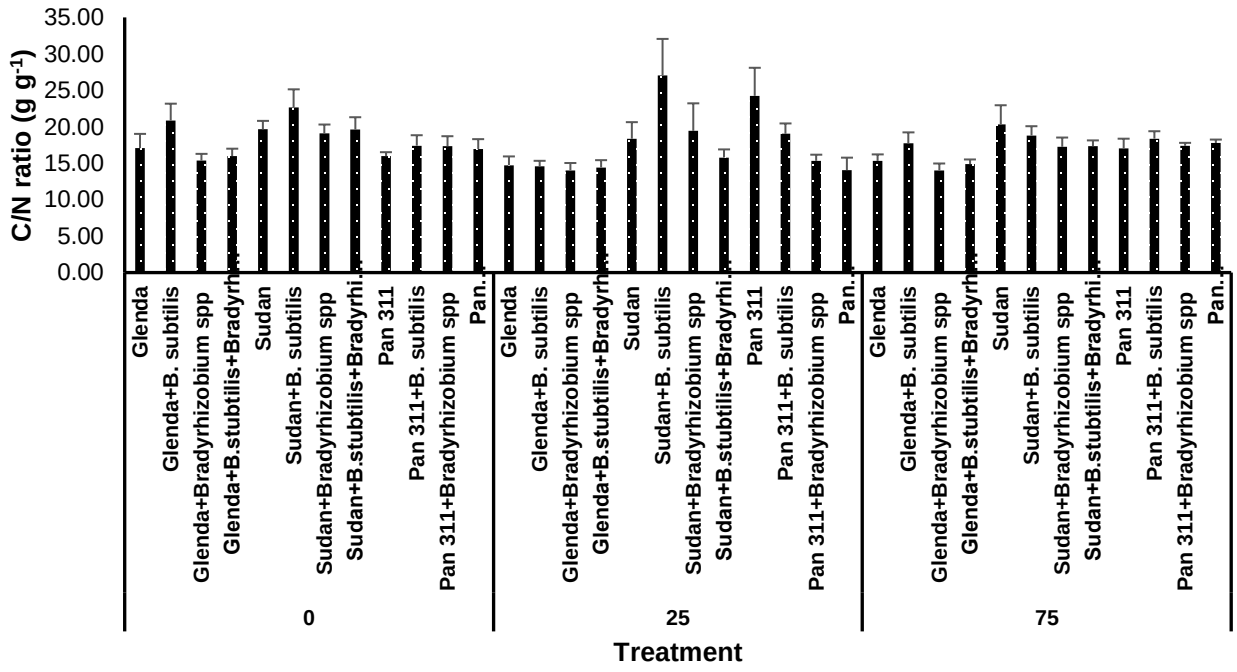

Figure 6.1 Interaction effect of co-inoculation with *B. subtilis* (BD233) x *Bradyrhizobium* sp. CB756 x mine water irrigation on C/N ration of cowpea genotypes.

6.5 SUMMARY

South Africa is characterised by limited water resources and has a longstanding history r of gold and coal mining, and AMD has polluted most surface waters including lakes, streams and rivers in provinces where there is need for agricultural water use. Elemental

analysis of the mine water used to irrigate cowpea revealed the presence of heavy metals (Al, Cd, Co, Cu, Cr, Fe, Ni, Pb, Sr, & Zn) and their concentrations were below the South African standards for drinking water and agricultural water use limit. Cowpea irrigated with mine water and co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 showed resistance to heavy metal stress with respect to their symbiotic performance and genotypic differences were also noted. Pan 311 with the lowest $\delta^{15}N$ and highest amount of N-fixed was found to be more dependent on nitrogen fixation for N nutrition compared to Glenda and Sudan, and it also had highest soil N uptake with the heaviest dry shoot biomas. Similarly, cowpea co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 had more nodules per plant compared to control (no inoculation) or with single inoculation. Genotypic response was also significantly affected with respect to nodulation, Glenda exhibited the highest number of nodules per plant compared to Pan 311 with the least number of nodules. Similarly, there were significant differences noted among the treatments, cowpea with co-inoculation (*B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756) had lower C/N values compared to those inoculated with single or without inoculation. According to these findings, co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 is a promising technology for the bioremediation of AMD polluted water bodies and soils in mining communities in South Africa, especially for the cultivation of leguminous crops such as the local cowpea genotypes. However, more studies using more local genotypes under field conditions are required to verify the findings in the current study.

CHAPTER SEVEN: Effect of co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 on the nutritional composition of local cowpea genotypes subjected to mine water.

7.1 INTRODUCTION

South Africa has a long mining history, and most surface waters and soils are polluted with heavy metals due to acid mine water. In areas impacted by mining operations, where soil and water quality are frequently degraded, the search for sustainable agriculture practices has grown in importance. A staple legume in many tropical and subtropical areas, cowpeas are valued for their nutritional content and capacity to enhance soil health by fixing atmospheric nitrogen. However, cowpea development and nutritional quality are severely hampered when crops are irrigated with mine water, which often contains salt and heavy metals (Zhao et al., 2018). In this regard, using beneficial bacteria like *Bacillus subtilis* and *Bradyrhizobium* sp. presents a viable way to improve the nutritional makeup and resilience of cowpea genotypes subjected to such stresses.

According to Kloepper et al. (2004) and Raaijmakers et al. (2009), *B. subtilis* is a well-known plant growth-promoting rhizobacterium (PGPR) that can improve nutrient uptake, promote root development, and increase plant stress tolerance. By generating phytohormones and antimicrobial chemicals that can lessen the negative impacts of environmental stresses, the strain *B. subtilis* BD233 has been particularly noted for its potential to support plant health (Huang et al., 2018). Additionally, co-inoculating *B. subtilis* with nitrogen-fixing bacteria like *Bradyrhizobium* sp. can have synergistic effects that improve nodulation and overall plant growth including nutrient accumulation (Rudrappa et al., 2012).

According to Sanginga et al. (2003), *Bradyrhizobium* sp., is essential for developing symbiotic partnerships with legumes, promoting nitrogen fixation, and enhancing soil fertility. Environmental elements, such as water quality, can affect these bacteria's symbiotic effectiveness. Research has shown that heavy metals in irrigation water can negatively affect rhizobia's nodulation and nitrogen-fixing capacity, which in turn affects legumes' nutritional profile (Zhao et al., 2018). When compared to uninoculated plants during mine water stress, research by Rahman et al. (2022) showed that the combination

of *B. subtilis* and *Bradyrhizobium* sp. boosted critical amino acid contents and protein content by 25–30% in cowpea grains. According to research by Liu et al. (2023), co-inoculated cowpea plants retained 40–50% higher zinc concentrations and 35-45% higher iron content in their tissues when compared to control plants. Furthermore, a study by Musa et al. (2023) found that grains of cowpea co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. (CB756) had higher levels of important micronutrients like iron and zinc, with increases of 15-20% for iron content and 10-15% for zinc content when compared to controls. The majority of these investigations were carried out in the field with native cowpea genotypes and limited literature that exist under glasshouse conditions.

This research seeks to evaluate the combined effects of co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. (CB756) on the nutritional composition of local cowpea genotypes subjected to mine water irrigation under glasshouse conditions. By examining key nutritional parameters such as protein content and mineral composition, this research seeks to elucidate the potential of microbial co-inoculation as a strategy for enhancing the quality and productivity of cowpea in challenging environments such as mine water polluted soils.

7.2 MATERIALS AND METHODS

7.2.1 Experimental Conditions

As outlined in section 3.2.1 of chapter 3.

7.2.2 Bacterial Strains

As outlined in section 3.1.2 of chapter 3.

7.2.3 Cowpea Co-Inoculation

As outlined in section 3.2.2 of chapter 3.

7.2.4 Acid Mine Drainage

As outlined in section 3.2.3 of chapter 3.

7.2.5 Plant Growth Media

As A outlined in section 3.2.4 of chapter 3.

7.2.6 Experimental Design

As outlined in section 4.5 of chapter 4.

7.2.7 Determination of Mineral Content in Plant Organ

Samples were analysed using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) to quantify elemental concentrations. For the determination of elements in mine water, an aliquot of a thoroughly mixed, homogeneous aqueous sample was carefully measured for processing. In cases where an aqueous sample contained undissolved material, total recoverable analysis required solubilization of analytes by gentle reflux with nitric and hydrochloric acids. After cooling, the sample volume was adjusted, mixed, then either centrifuged or allowed to settle overnight prior to analysis.

For elemental analysis in plant organs, three plants per treatment were harvested at 90 days after planting (DAP), and the shoots and grains separated. Each component was oven-dried at 60°C, weighed, and ground to a fine powder (0.85 mm mesh size) before mineral quantification. To measure mineral elements (P, Ca, Cu, Zn, Mn, Fe, and Al) in cowpea shoot and grain, 1 g of ground material was ashed overnight in a porcelain crucible at 500°C. The ash was dissolved in 5 mL of analytical-grade 6 M HCl, incubated at 50°C for 30 minutes, and diluted with 35 mL of deionized water. Filtration was performed using Whatman No. 1 filter paper. Mineral concentrations in plant extracts were determined from three replicates using ICP-MS (Ataro et al., 2008). Data quality assurance was conducted utilizing certified standard solutions, and for each element, a known sample standard was analysed every ten samples.

Sulphur was quantified via wet digestion using 65% high-purity nitric acid. Specifically, 1 g of milled plant material was digested overnight with 20 mL of 65% nitric acid in a 250 mL glass beaker. The digest was gently boiled on a sand bath until near dryness (approximately 1 mL remaining), followed by addition of 10 mL of 4 M high-purity nitric

acid and boiling for 10 minutes. The extract was cooled, washed into a 100 mL volumetric flask, and filtered through Whatman No. 2 filter paper. Sulphur content was subsequently determined by direct aspiration using calibrated simultaneous ICP-MS.

7.2.8 Data Collection and Analysis of Data

For elemental analysis in plant organs, three plants per treatment were harvested at 90 days after planting (DAP), and the shoots and grains separated. Each component was oven-dried at 60°C, weighed, and ground to a fine powder (0.85 mm mesh size) before mineral quantification. Data was analysed as describe in section 6.3.3 above.

7.3 RESULTS AND DISCUSSION

7.3.1 Effect of co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 on nutritional composition in shoot of local cowpea genotypes

The analysis of variance indicated statistically significant differences in the elemental composition and concentrations within cowpea shoots co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 and irrigated with mine water under glasshouse conditions. According to Table 7.1, regardless of cultivar and inoculation, mine water affected the accumulation of nutrients in cowpea shoot. Cowpea irrigated with 75% mine water had higher K (1.42%), Mg (0.54 mg/kg), Na (1865.63 mg/kg), Zn (39.37 mg/kg), Mn (617.18 mg/kg) and Al (61.98 mg/kg) compared to control (0% mine water) with lower values (1.05%, 0.40 mg/kg, 1577.03 mg/kg, 27.72 mg/kg, 196.15 mg/kg and 52.65 mg/kg, respectively). In contrast, the shoot Fe content was significantly higher in control (303.04 mg/kg) compared to shoot Fe content of cowpea irrigated with 75% mine water (198.89 mg/kg). Cowpea irrigated with 25% mine water had more Ca in shoot (1.64±0.05 mg/kg) compared those irrigated with 75% mine water (1.46 mg/kg). However, there were no significant differences in P, Cu and B in shoots of cowpea exposed to microbial inoculation (treatments) (Table 7.1). Taken together, these results demonstrate that long-term cowpea irrigation with mine water probably results in cumulative heavy metal (Zn and Al) and salts (Na) deposition in the soil, which could change soil chemistry, hinder soil biological activity, and increase the likelihood of metal transfer into crops. The results are in agreement with reports by Lianman et al. (2022), who indicated that irrigating ryegrass

with mine water increased elements (such as K, Na, Ca and Mg) in soils and the increases dependent on level of mine water. Nevhulaudzi et al. (2020) also reported that increase in elemental composition was dependent of the dilution factor of the mine water used to irrigate cowpea under greenhouse conditions.

Similarly, the microbial inoculation treatment employed in this study had a significant effect on the nutritional composition in shoot of cowpea, regardless of mine water regime. Glenda inoculated with *Bradyrhizobium* sp. CB756 had higher amounts of P (0.35%), K (1.58%), Ca (1.83 mg/kg), Mg (0.57 mg/kg), Fe (313.15 mg/kg), and Zn (49.21 mg/kg) compared to when not inoculated (0.29%, 1.20%, 1.55 mg/kg, 0.49 mg/kg, 277.10 mg/kg, and 40.75 mg/kg, respectively). In general, inoculation (single or dual) enhanced the nutritional composition in shoot of cowpea compared to un-inoculated cowpea. Pan 311 co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 exhibited higher amounts of Ca (1.81 mg/kg), Mg (0.57 mg/kg), Mn (517.98 mg/kg), B (55.86 mg/kg) and Al (70.45 mg/kg) compared to un-inoculated Pan 311 (1.35 mg/kg, 0.48 mg/kg, 395.44 mg/kg, 46.32 mg/kg and 55.99 mg/kg, respectively) (Table 7.1). Co-inoculating Pan 311 with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 improved the shoot nutrient contents by 24% (Ca), 16% (Mg), 24% (Mn), 17% (B) and 21% (Al) compared to when not inoculated. These findings are aligned with those of other researchers (Liu et al., 2023; Rahman et al., 2022; Musa et al., 2023), who indicated enhanced nutrient accumulation is cowpea co-inoculated with bacteria and rhizobia compared to control. A similar study conducted by Diop et al. (2023) revealed that co-inoculation with *B. subtilis* and *Bradyrhizobium* sp. Under mine water stress conditions resulted in increased concentrations of essential minerals such as iron, zinc, and calcium in three local cowpea genotypes and genotypic differences were noted.

In the current study, the interaction of mine water regime and microbial inoculation had a significant effect on the elemental composition and concentration in shoot of cowpea under glasshouse conditions. According to Figure 7.1, shoots of Pan 311 co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 and irrigated with 75% mine water exhibited higher amount of P (0.21%) compared to control (0.17%) and when irrigated with 25% mine water (0.19%).

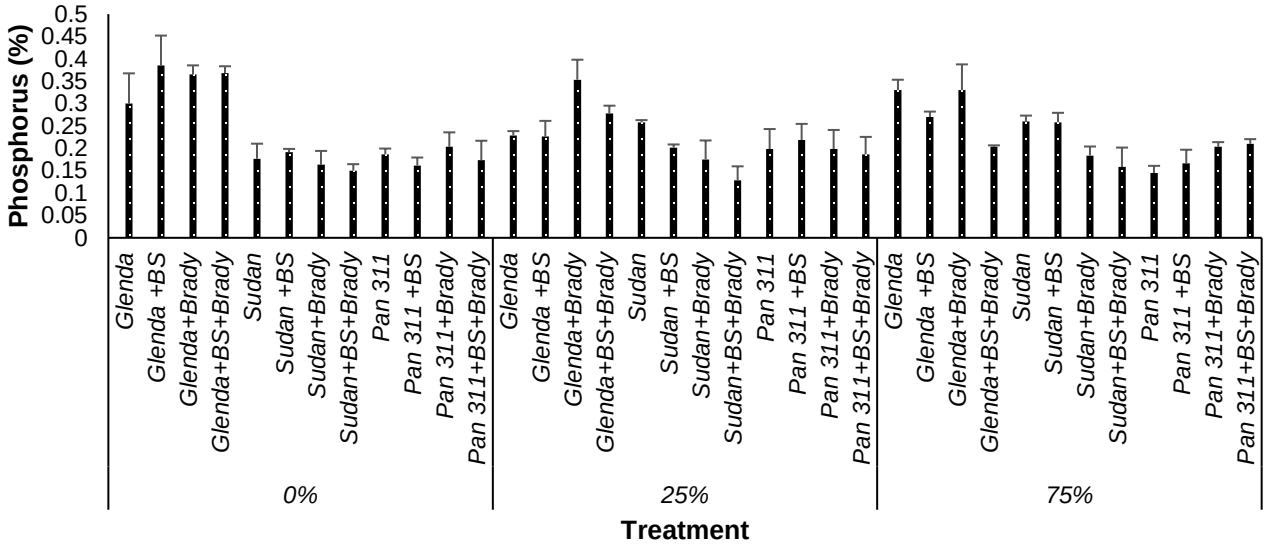

Figure 7.1 Interactive effect of co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 and mine water regimes on P (%) in cowpea shoot.

Similarly, the interaction of mine water regime and microbial inoculation had a significant effect on shoot K (%). Shoot of Pan 311 co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 and irrigated with 75% mine water exhibited higher amount of K (1.18%) compared to control (0.95%) and when irrigated with 25% mine water (1.02%) (Fig. 7.2).

The interaction of mine water regime and microbial inoculation also had a significant effect on shoot Mg (mg/kg). Shoot of Pan 311 co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 and irrigated with 75% mine water exhibited higher amount of Mg (818.28 mg/kg) compared to control (376.32 mg/kg) and when irrigated with 25% mine water (459.34 mg/kg) (Fig. 7.3).

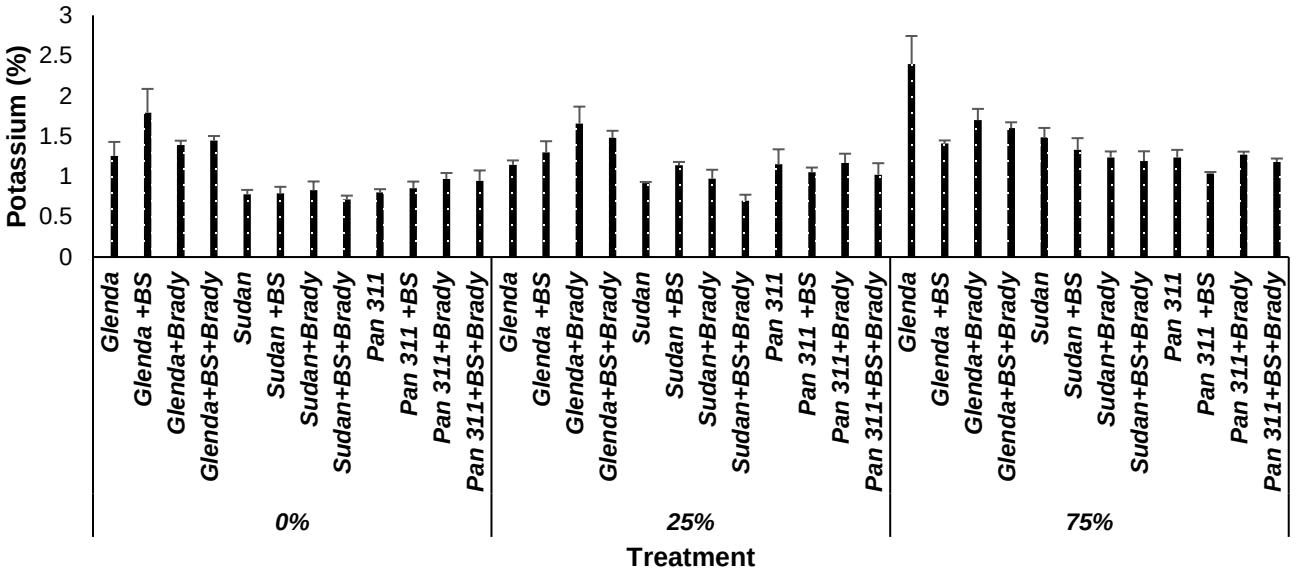

Figure 7.2 Interactive effect of co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 and mine water regimes on Potassium (%) cowpea shoot.

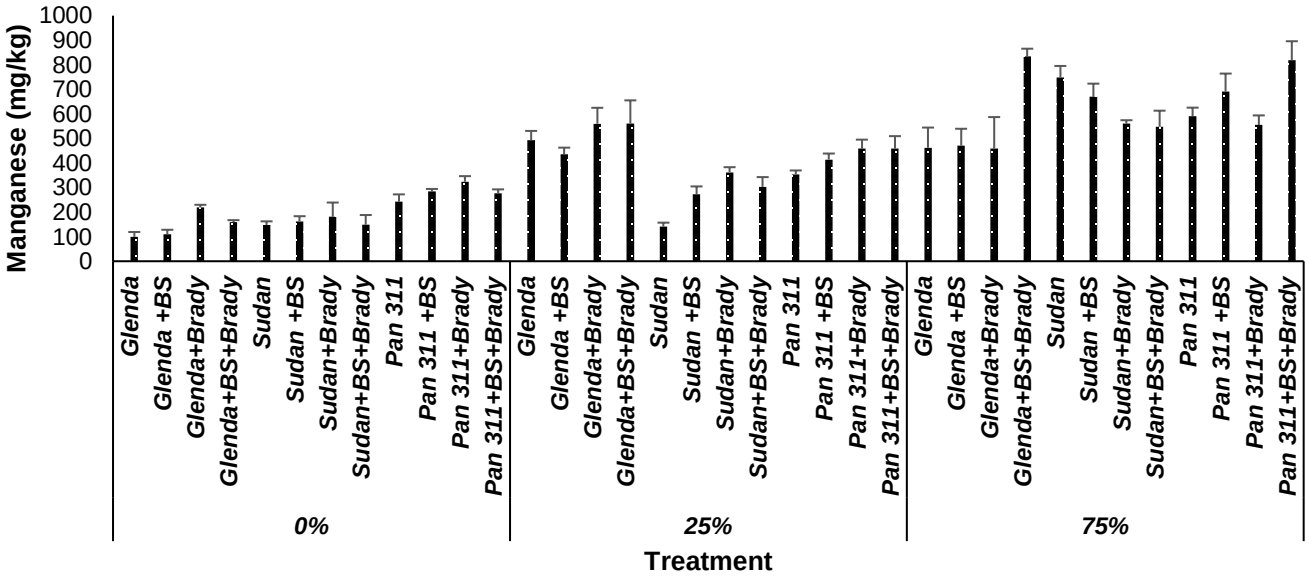

Figure 7.3 Interactive effect of co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 and mine water regimes on Mg (mg/kg) in cowpea shoot.

Similarly, the interaction of mine water regime and microbial inoculation had a significant effect on shoot Al (mg/kg). Shoot of Pan 311 co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 and irrigated with 75% mine water exhibited higher amount of

Al (88.52 mg/kg) compared to control (63.04 mg/kg) and when irrigated with 25% mine water (59.81 mg/kg) (Fig. 7.4).

Collectively, these interaction results indicate significant differences in nutrients accumulation among cowpea genotypes when co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 under heavy metal stress conditions. Gyogluu Wardjomto et al. (2023) reported similar findings, when they indicated that the synergistic interactions between rhizobia and seaweed extract enhanced nutrient accumulation in cowpea leaves and genotypic differences were noted. In addition, results obtained in the current study are in agreement with reports by Gerrano et al. (2022) on the elemental concentrations in shoots in different cowpea genotypes in different regions in South Africa. Furthermore, our results are confirmation of the mitigating effect of bacteria and rhizobia, especially when combined, on nutrients accumulation in shoot of plants under stressful conditions such as mine water stress.

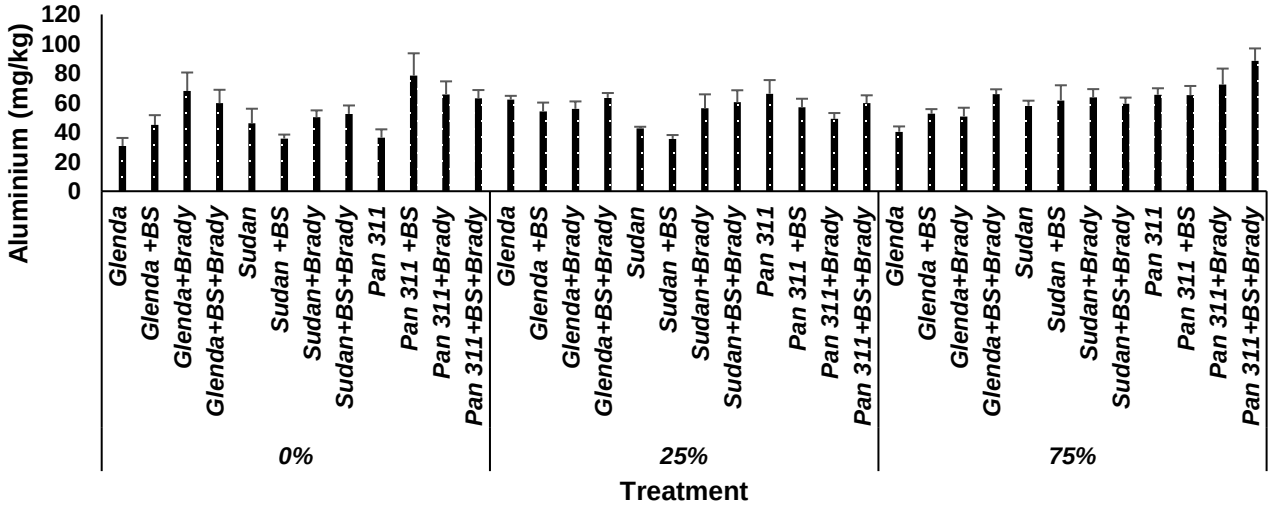

Figure 7.4 Interactive effect of co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 and mine water regimes on Aluminium (mg/kg) in cowpea shoot.

Table 7.1 Effect of co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 on nutritional composition shoot of local cowpea genotypes

	Mineral composition in shoot (DM)										
			mg/kg								
	P (%)	**K (%)**	**Ca**	**Mg**	**Na**	**Fe**	**Cu**	**Zn**	**Mn**	**B**	**Al**
Mine water (%)											
0	0.24±0.01a	1.05±0.05b	1.49±0.05b	0.40±0.01c	1577.03±111.50b	303.04±32.65a	9.80±1.41a	27.72±1.23b	196.15±10.75c	47.65±1.18a	52.65±2.78b
25	0.22±0.01a	1.14±0.04b	1.64±0.05a	0.49±0.01b	1548.26±91.83b	251.59±11.00ab	9.70±0.57a	38.30±1.61a	401.02±18.17b	45.91±1.10a	55.23±1.85b
75	0.23±0.01a	1.42±0.05a	1.46±0.05b	0.54±0.01a	1865.63±128.83a	198.89±7.09b	9.99±2.80a	39.37±1.65a	617.18±23.20a	49.07±1.09a	61.98±2.16a
Inoculation											
Glenda	0.29±0.02b	1.20±0.18b	1.45±0.10bc	0.49±0.02b	1152.61±157.37cd	277.10±41.43b	9.60±0.80b	40.75±4.19b	351.42±52.13d	46.55±2.03cde	44.40±3.88e
Glenda +BS	0.29±0.03b	1.50±0.11a	1.75±0.08a	0.52±0.02ab	1105.22±159.29d	245.35±23.48b	8.70±0.79b	40.34±2.18bc	338.75±46.01d	48.42±0.86bcd	50.61±3.12cde
Glenda+Brady	0.35±0.02a	1.58±0.09a	1.83±0.11a	0.57±0.02a	1063.44±159.98d	313.15±66.97a	12.33±1.01ab	49.21±4.37a	412.56±56.88bcd	50.49±1.88abc	58.24±4.95abcd
Glenda+BS+Brady	0.28±0.02b	1.51±0.04a	1.70±0.08a	0.53±0.02ab	1769.61±170.44bc	258.81±34.25b	8.54±0.90b	39.87±2.45bc	518.20±74.07a	52.89±1.36ab	63.06±3.24abc
Sudan	0.23±0.01c	1.06±0.08bc	1.36±0.07bcd	0.36±0.02c	1462.78±126.34bcd	182.52±15.07b	6.48±0.47b	27.69±2.67e	345.23±70.96d	40.15±1.39e	48.98±3.63de
Sudan +BS	0.22±0.01c	1.09±0.08bc	1.30±0.10cd	0.37±0.02c	1906.83±141.55b	209.24±19.07b	8.23±0.75b	30.15±3.07de	368.27±56.69cd	39.45±2.27e	44.31±4.54e
Sudan+Brady	0.17±0.02d	1.01±0.07bc	1.32±0.08bcd	0.40±0.02c	2068.33±246.65b	332.99±98.40a	9.21±1.95b	29.17±1.45de	367.77±42.45cd	41.89±1.68ef	56.73±3.99bcde
Sudan+BS+Brady	0.15±0.02d	0.87±0.07c	1.26±0.08d	0.38±0.02c	2672.11±336.94a	203.52±22.19b	6.12±0.94b	28.55±2.74e	333.14±48.11d	42.79±1.61def	57.39±3.50bcd
Pan 311	0.18±0.02d	1.06±0.08bc	1.35±0.07bcd	0.48±0.03b	1607.89±248.60bcd	223.31±17.94b	7.30±0.92b	31.17±2.23de	395.44±38.39cd	46.32±2.21cde	55.99±4.99bcde
Pan 311 +BS	0.18±0.02d	0.98±0.04	1.58±0.09ab	0.50±0.02b	1497.17±169.42bcd	266.69±31.65b	13.35±5.13ab	32.16±2.47cde	462.83±47.86ab	55.02±2.53a	66.89±5.86ab
Pan 311+Brady	0.20±0.02cd	1.14±0.05b	1.58±0.08ab	0.54±0.02ab	1679.00±194.03bcd	258.32±23.15b	20.74±1.06a	37.58±3.39bcd	445.79±29.28abc	50.74±2.14abc	62.38±5.16abc
Pan 311+BS+Brady	0.19±0.02d	1.05±0.07bc	1.81±0.11a	0.57±0.03a	1978.67±241.90b	243.04±12.31b	7.35±0.92b	34.93±3.12bcde	517.98±61.97a	55.86±2.27a	70.45±4.75a
F-Statistics											
Mine Water (MW	0.619^{ns}	**29.347*** **	**4.968*** **	**53.768*** **	**3.113***	**6.747*** *	0.006^{ns}	**22.836*** **	**227.710*** **	2.796^{ns}	**5.773*** *
Inoculation	**11.194*** *	**13.366*** **	**5.715*** **	**18.053*** **	**5.409*** **	1.198^{ns}	1.236^{ns}	**5.871*** *	**5.775*** **	**8.910*** **	**4.348*** **
MW x Inoculation	**1.939***	**3.291*** **	0.841^{ns}	1.137^{ns}	1.331^{ns}	1.156^{ns}	1.034^{ns}	1.014^{ns}	**5.241*** **	1.022^{ns}	**2.336*** *

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.

7.3.2 Effect of co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 on crude carbon and nitrogen content in shoot of local cowpea genotypes

Co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 notably improved the local cowpea shoot nutrition when irrigated with mine water under glasshouse conditions. According to Table 7.2, regardless of treatment, cowpea not irrigated with mine water (control) exhibited lower crude N% content (2.37%) and higher C% content (39.23%) and C/N partitioning (17.41) compared to those treated with mine water (25% and 75%), which showed no significant differences between them with respect to crude nitrogen and carbon contents. However, significant difference was noted in cowpea shoot with respect to C/N ratio when plants treated with 25% and 75% mine waters are compared.

Similarly, microbial inoculation had a significant effect on nutritional quality of shoots of local cowpea genotypes. Generally, inoculation (single or dual) enhanced the nutritional quality of cowpea shoot compared to un-inoculated cowpea. Pan 311 with either single (*B. subtilis* (BD233) (3.51% (N), 39.87% (C) and 11.59 (C/N)) or *Bradyrhizobium* sp. CB756 (2.87%, 39.60% and 22.64) or dual (*B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756) (2.80%, 36.64% and 13.12) inoculation exhibited more shoot N%, C% and C/N ratio compared to when not inoculated (1.64%, 35.77% and 12.90, respectively) (Table 7.2). Co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 enhanced crude nitrogen by 44% (N%), carbon content by 3% (C%) and C/N partitioning by 2%. According to a study by Singh et al. (2021), co-inoculation of cowpea genotypes with *B. subtilis* and *Bradyrhizobium* sp. under mine water conditions led to a 15-20% increase in nitrogen content and a 10-15% enhancement in mineral composition (including phosphorus, potassium, and iron) compared to uninoculated controls. These improvements can be attributed to the combined effects of *B. subtilis* and *Bradyrhizobium* sp. on the nutritional composition of local cowpea genotypes under acid mine drainage stress, and their findings are consistent with this idea. Diop et al. (2023) reported the effect of co-inoculation with *B. subtilis* and *Bradyrhizobium* sp. on the nutritional composition of local cowpea genotypes subjected to acid mine drainage stress and their findings is in agreement with this notion. Furthermore, recently, Fontes et al. (2025)

ascertained this claim when they examined the effect of co-inoculation with *Bradyrhizobium japonicum* and *Azospirillum brasilense* on the growth and nutritional contents of cowpea. Together, these results demonstrate the potential of inoculation (single or dual) in enhancing nutrient accumulation in shoots of cowpea compared non-inoculated cowpea.

Table 7.2 Crude Carbon and Nitrogen composition in cowpea shoot

	Mineral composition		
	N%	**C%**	**C/N**
Mine water (%)			
0	2.37±0.09b	39.23±0.18a	17.41±0.65a
25	2.59±0.14a	37.66±0.33b	16.19±0.89b
75	2.72±0.13a	37.96±0.36b	14.93±0.64c
Treatment			
Glenda	2.04±0.19d	38.88±0.14ab	20.27±1.71b
Glenda +*B. subtilis*	1.94±0.06de	39.07±0.25ab	20.28±0.58b
Glenda+ *Bradyrhizobium* sp.	2.07±0.09d	38.13±0.45bc	18.73±1.01bc
Glenda+ *B. subtilis* + *Bradyrhizobium* sp.	3.12±0.07b	37.25±0.94cd	12.01±0.50fg
Sudan	2.15±0.07d	35.55±0.81e	16.67±0.55cd
Sudan + *B. subtilis*	2.14±0.10d	39.87±0.10a	18.98±1.01bc
Sudan+ *Bradyrhizobium* sp.	3.72±0.26a	39.40±0.44ab	11.14±0.97g
Sudan+ *B. subtilis* + *Bradyrhizobium* sp.	2.69±0.11c	39.28±0.11ab	14.80±0.56de
Pan 311	1.64±0.06e	35.77±0.32e	12.90±0.37fg
Pan 311 + *B. subtilis*	3.51±0.24a	38.95±0.17ab	11.59±0.92fg
Pan 311+ *Bradyrhizobium* sp.	2.87±0.09bc	39.60±0.31a	22.64±0.78a
Pan 311+ *B. subtilis* + *Bradyrhizobium* sp.	2.80±0.06bc	36.64±0.73de	13.12±0.22efg
F-Statistics			
Mine water	8.029***	15.531***	10.046***
Treatment	27.149***	10.884***	25.143***

7.3.3 Effect of co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 on nutritional composition in grains of local cowpea genotypes

The results revealed significant differences in the elemental composition and concentrations in cowpea's grains co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 and irrigated with mine water under glasshouse conditions. According to Table 7.3, mine water affected nutrient accumulation in cowpea grains, irrespective of the cultivar or treatment applied. Grains of cowpea not irrigated with mine water (control (0%) had lower amounts of Ca (0.08 mg/kg), Cu (2.91 mg/kg), Zn (23.68

mg/kg), Mn (19.74 mg/kg) and B (10.16 mg/kg) compared to those treated with mine water (25% and 75%). Compared to grains of control plants, cowpea irrigated with 25% mine water had higher amounts of Fe (94.64 mg/kg), Cu (3.61 mg/kg), and Zn (39.47 mg/kg) and those irrigated with 75% had more Mn (42.08 mg/kg) and B (11.43 mg/kg). However, there were no significant differences in grains of cowpea irrigated with mine water (25% and 75%) in comparison to those treated with 0% mine water (control) with respect to elements such as P, K, Mg and Al contents (Table 7.3).

Furthermore, inoculation (single or dual) affected the nutritional composition in grains of cowpea compared to those not inoculated under glasshouse conditions. Regardless of mine water regime, grains of Glenda co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 had lower content of P (0.49%), K (1.46%), Ca (0.09 mg/kg), Mg (0.22 mg/kg), Fe (52.42 mg/kg), Cu (2.85 mg/kg), Zn (28.39 mg/kg), and Al (1.61 mg/kg) compared to when it was inoculated with *Bradyrhizobium* sp. CB756 alone (0.63%,1.98%, 0.15 mg/kg, 0.26 mg/kg, 174.84 mg/kg, 3.71 mg/kg, 74.80 mg/kg, and 12.82 mg/kg, respectively). In contrast, grains of Pan 311 co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 had higher amount of Cu (4.20 mg/kg) compared to when it was inoculated with *Bradyrhizobium* sp. CB756 alone (3.38 mg/kg). In addition, grains of Sudan inoculated with *B. subtilis* (BD233) had higher amount of Na (232.00 mg/kg) compared to grains of Glenda inoculated with same bacteria (157.33 mg/kg), highlighting genotypic differences in response to *B. subtilis* (BD233) inoculation (Table 7.3). Together, these results are in line with reports by other authors indicating that co-inoculation with bacteria and rhizobia can enhance the growth, yield and nutritional contents in cowpea grains. Diop et al. (2023), for instance, conducted a greenhouse study to examine the impact of co-inoculation with *B. subtilis* and *Bradyrhizobium* sp. on three cowpea genotypes exposed to two mine water regimes. The co-inoculated plants had higher protein, fat, and carbohydrate contents than the non-inoculated plants, as well as higher concentrations of vital minerals like iron, zinc, and calcium in the cowpea grains. According to the authors, a significant improvement in the nutritional quality of the cowpea genotypes was noted including genotypic differences in response to co-inoculation.

Table 7.3 Effect of co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 on nutritional composition grain of local cowpea genotypes

	Mineral composition in grain (DM)										
			mg/kg								
	P (%)	K (%)	Ca	Mg	Na	Fe	Cu	Zn	Mn	B	Al
Mine water											
0	0.47±0.02a	1.41±0.04a	0.08±0.01b	0.21±0.01a	200.25±11.70a	63.61±2.11b	2.91±0.09b	23.68±1.23c	19.74±1.56c	10.16±0.36b	4.57±1.12a
25	0.48±0.02a	1.55±0.10a	0.10±0.01a	0.22±0.01a	191.58±8.99ab	94.64±7.84a	3.61±0.21a	39.47±7.69a	29.91±1.62b	10.92±0.31ab	5.98±1.78a
75	0.48±0.02a	1.52±0.05a	0.10±0.01a	0.21±0.01a	169.25±10.47b	55.20±2.61c	3.30±0.13a	31.01±0.63ab	42.08±4.05a	11.43±0.45a	3.38±0.46a
Treatment											
Glenda	0.62±0.05a	1.74±0.10ab	0.15±0.03a	0.26±0.01a	157.00±10.56bc	59.81±4.54b	3.24±0.23bcd	33.84±1.84b	50.68±1.62a	13.77±0.90a	5.23±0.48b
Glenda +BS	0.47±0.03b	1.44±0.08bc	0.10±0.01b	0.23±0.01bc	157.33±18.91bc	49.16±2.72b	2.75±0.18d	28.89±1.51b	31.67±7.00bc	12.99±0.67a	2.05±0.12b
Glenda+Brady	0.63±0.05a	1.98±0.33a	0.15±0.02a	0.26±0.02a	186.67±30.23abc	174.84±78.89a	3.71±0.44abc	74.80±7.15a	39.20±0.69ab	11.15±1.21b	12.82±6.49a
Glenda+BS+Brady	0.49±0.03b	1.46±0.05bc	0.09±0.01b	0.22±0.01cd	142.67±15.35c	52.42±2.79b	2.85±0.13d	28.39±0.45b	27.41±3.29bc	11.35±0.15b	1.61±0.15b
Sudan	0.48±0.03b	1.35±0.06bc	0.08±0.01b	0.22±0.01cd	211.33±15.85ab	67.93±3.79b	2.93±0.14cd	23.83±1.00b	29.89±1.52bc	10.39±0.52b	3.14±0.29b
Sudan +BS	0.41±0.04b	1.38±0.15bc	0.08±0.01b	0.21±0.02cd	232.00±19.60a	73.86±6.24b	2.79±0.36d	23.75±3.36b	25.28±5.71bc	11.13±0.25b	11.69±3.01a
Sudan+Brady	0.47±0.01b	1.58±0.04bc	0.08±0.01b	0.20±0.01cd	205.33±18.13abc	66.72±5.63b	3.43±0.10abcd	28.42±1.33b	28.32±4.05bc	9.68±0.90b	2.64±0.20b
Sudan+BS+Brady	0.43±0.03b	1.41±0.04bc	0.07±0.01b	0.20±0.01cd	197.00±34.20abc	70.47±6.54b	2.82±0.21d	22.01±3.05b	26.78±2.68bc	9.71±0.19b	1.98±0.22b
Pan 311	0.40±0.02b	1.32±0.05c	0.07±0.01b	0.17±0.01e	186.33±19.35abc	58.81±2.60b	3.83±0.45ab	26.96±2.42b	22.73±5.54c	10.22±0.46b	1.68±0.22b
Pan 311 +BS	0.45±0.03b	1.37±0.06bc	0.07±0.01b	0.19±0.01de	184.00±26.48abc	60.24±3.89b	3.34±0.10bcd	27.36±0.97b	25.69±2.13bc	9.70±0.20b	4.49±1.64b
Pan 311+Brady	0.44±0.01b	1.49±0.05bc	0.09±0.01b	0.19±0.01de	195.00±19.01abc	59.73±3.03b	3.38±0.16bcd	29.36±1.63b	30.07±4.17bc	10.02±0.23b	4.96±1.44b
Pan 311+BS+Brady	0.46±0.05b	1.38±0.09bc	0.08±0.01b	0.19±0.01de	189.67±19.45abc	59.79±3.28b	4.20±0.45a	29.04±3.67b	29.21±4.72bc	9.91±0.52b	3.42±0.55b
F-Statistics											
Mine Water	0.126^{ns}	1.494^{ns}	5.882***	0.513^{ns}	2.553*	3.490*	7.635**	4.203**	21.513***	4.847*	1.447^{ns}
Treatment	4.595***	2.574**	6.257***	6.410***	1.561*	2.257*	3.398**	3.311*	2.443*	5.376**	3.057*

7.4 SUMMARY

In summary, local cowpea genotypes are better able to withstand stress caused by mine water because of the synergistic interaction between *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756, which improves their nutritional content and makes them more appropriate for consumption and marketing. This co-inoculation strategy can help create cowpea production systems that are more economically viable and sustainable, especially in places affected by mine water.

CHAPTER EIGHT: General Conclusions and Recommendations

8.1 CONCLUSIONS

This study examined the interactive effects of co-inoculating *B. subtilis* (BD233) with *Bradyrhizobium sp.* CB756 on the germination, growth, and yield of local cowpea genotypes (Glenda, Pan 311, and Sudan) under exposure to mine water irrigation. The study found that co-inoculating *B. subtilis* (BD233) and *Bradyrhizobium sp.* CB756 under varying mine water stresses significantly influenced the germination, growth (plant height, stem diameter, plant biomass), nodulation and yield components of all three tested local cowpea genotypes. The results implies that cowpea seeds treated with 25% mine water had the fastest germination rate that resulted to a slightly higher germination percentage (24.44%) compared to those treated with 75% mine water with the slowest germination rate. *B. subtilis* (BD 233) and *Bradyrhizobium* sp. CB756 co-inoculation significantly affected the germination indices of cowpea genotypes at seven days of incubation (Table 4.3). Additionally, the rate at which seeds germinated, was significantly influenced by co-inoculation as Pan 311 with *B. subtilis* (BD 233) and *Bradyrhizobium* sp. CB756 co-inoculation exhibited the lowest GRI value (0.00) (fastest rate) that resulted in highest germination percentage (G% (36.11%) compared to the other treatments. The results also revealed that *B. subtilis* (BD 233) and *Bradyrhizobium* sp. CB756 co-inoculation enhanced the growth, nodulation and yield of local cowpea genotypes under controlled glasshouse conditions (Table 4.4), Sudan genotype that were co-inoculated with *B. subtilis* (BD 233) and *Bradyrhizobium* sp. CB756 reached a maximum plant height (18.43 cm) and produced the greatest pod yield (3.11) per plant. This study clearly shows that co-inoculating with *B. subtilis* and *Bradyrhizobium* can enhance the growth and yield of all three tested local cowpea genotypes, even under different mine water concentrations (0, 25, and 75%). Similarly, co-inoculation with *B. subtilis* (BD 233) and *Bradyrhizobium* sp. CB756 significantly affected the symbiotic performance of local cowpea genotypes exposed to mine water stress. The study demonstrated significant differences in measured symbiotic parameters ($\delta^{15}N$ (‰), %N, N-Fixed, %Ndfa, and Soil N uptake (mg $Plant^{-1}$)) as a result of the synergistic effect observed with co-inoculation of *B. subtilis* (BD233) and *Bradyrhizobium sp.* CB756 across various genotypes, mine water

concentrations, and experimental treatments. According to Table 4.1, nitrogen fixation levels was notably influenced by genotypic response when cowpea plants were subjected to dual inoculation with *B. subtilis* (BD233) and *Bradyrhizobium sp.* CB756, alongside mine water treatments. For example, Pan 311 recorded the highest quantity of N-fixed (49.20 mg Plant^{-1}), whereas Sudan exhibited the lowest value (37.28 mg Plant^{-1}). Similarly, Glenda showed the greatest %Ndfa (90.07%), followed by Pan 311 (87.01%). Additionally, the study revealed that co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 significantly affected the eco-physiological parameters and carbon assimilation of local cowpea genotypes irrigated with mine water under glasshouse conditions (Tables 6.1 & 6.2). Cowpea with co-inoculation (*B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756) had lower C/N values compared to those inoculated with single or without inoculation. The results indicate that co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 represents a promising strategy for the bioremediation of AMD-contaminated water bodies and soils within South African mining communities, particularly in the cultivation of leguminous crops such as local cowpea genotypes. According to the results obtained, cowpea's carbon assimilation remained resilient under both mine water irrigation and microbial co-inoculation. The findings of this study further indicate that mine water irrigation did not produce any significant effects on photosynthetic rate, stomatal conductance, transpiration rate, or water use efficiency across all treatment groups. Further, microbial inoculation (single or dual) enhanced the nutritional composition in shoot of cowpea compared to un-inoculated cowpea. Pan 311 co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 exhibited higher amounts of Ca (1.81 mg/kg), Mg (0.57 mg/kg), Mn (517.98 mg/kg), B (55.86 mg/kg) and Al (70.45 mg/kg) compared to un-inoculated Pan 311 (1.35 mg/kg, 0.48 mg/kg, 395.44 mg/kg, 46.32 mg/kg and 55.99 mg/kg, respectively) (Table 7.1). Additionally, co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 was found to further improve shoot nutrient contents in Pan 311. Similarly, co-inoculation with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 had a substantial impact on the quality of local cowpea shoot's nutrition even when irrigated with mine water under glasshouse conditions. As shown in Table 7.2, cowpea not irrigated with mine water (control) had lower crude N% content (2.37%) and higher C% content (39.23%) and C/N partitioning

(17.41) compared to those treated with mine water (25% and 75%). Additionally, no significant differences were observed in crude nitrogen and carbon content between the 25% and 75% mine water treatments. Co-inoculation affected the nutritional composition in grains of cowpea compared to those not inoculated under glasshouse conditions. For example, Pan 311 grains co-inoculated with *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 contained more Cu (4.20 mg/kg) than those inoculated only with *Bradyrhizobium* sp. CB756 (3.38 mg/kg) (Table 7.3).

In conclusion, this study found that using both *B. subtilis* and *B. japonicum* together, along with mine water supply; can influence cowpea plants' growth, nodulation, yield, physiology, and nutritional value. Notably, the combined inoculation of these bacteria may improve cowpea seed germination, enhance plant growth, and improve nutritional value, even when watered with acidic mine water face extreme temperature changes due to climate shifts. The synergistic effect of these microbes seems to mitigate heavy metal stress on cowpea's growth. In summary, the results indicate that *B. subtilis* (BD233) and *Bradyrhizobium* sp. CB756 may serve as bacteria resistant to heavy metals, making them particularly valuable in soils contaminated by mine water. This technology has the potential to make cowpea farming more economically viable and sustainable, particularly in regions impacted by mine water pollution.

8.2 RECOMMENDATIONS

- Co-inoculation with *Bacillus subtilis* (BD233) and *Bradyrhizobium* sp. CB756 can reduce stress effects at moderate mine water levels (25%) thereby improving cowpea shoot biomass, root growth, pod yield, and grain nutrients.
- Tailored microbial applications can boost crop yields in soil affected by mine water.
- These study results could be important for farmers with limited resources who want to grow cowpeas in today's changing climate to improve livelihoods, food security, and resilience of smallholder farming communities.
- Further research is needed to clarify how mine water levels, genotypes, and inoculation treatments affect cowpea dry shoot biomass, as no significant differences were observed.

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