NATIVE RHIZOBIA AND PHOSPHATE SOLUBILIZING BACTERIA AS POTENTIAL BIOFERTILIZERS FOR COMMON BEAN (*Phaseolus vulgaris* L.)

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DECLARATION AND RECOMMENDATION

Declaration

I declare that this thesis is my original work and has not been shared, presented or submitted wholly or in part for any award in any institution.

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Recommendation

This thesis has been submitted with our approval as supervisors.

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DEDICATION

This work is dedicated to my family.

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ABSTRACT

Soil fertility decline is one of the major factors that constrains the production of common bean. The use of plant growth promoting rhizobacteria (PGPR) including rhizobia and phosphate solubilizing bacteria (PSB) positively impact common bean production by enhancing nodulation, nutrient uptake, growth and grain yield. This study aimed to evaluate the ability of the native rhizobia and PSB to solubilize phosphates and produce indole acetic acid (IAA) and their potential to enhance common bean growth in the greenhouse. Additionally, the study investigated the suitability of sugarcane filter mud and liquid yeast extract mannitol broth as carrier materials for the survival of bacterial strains under refrigerated and room temperature storage conditions. Field experiments were set up to assess the response of common beans to selected rhizobia-PSB co-inoculation and the efficiency of different inoculant carrier materials. Results from the *in vitro* studies showed that the tested rhizobia strains have different IAA producing ability and phosphate solubilization efficiency with *Rhizobium pusense* (S5) and *R. phaseoli* (B3) produced higher levels of IAA with absorbance values of 1.33 and 1.14 respectively. Similarly, *R. pusense* (S5) had the highest solubilization efficiency (648), followed by *Bacillus megaterium* (HK2) (322.3). In the greenhouse study, specific rhizobia-PSB co-inoculation (*R. pusense* (B2) + *B. aryabhattai*, *R. pusense* + *B. megaterium* and *R. phaseoli* + *B. aryabhattai*) significantly increased the number of nodules compared to single rhizobia or PSB inoculation. The highest shoot biomass was observed when *R. phaseoli* was co-inoculated with *P. polymyxa* (4.3 g plant^{-1}) compared to the single *R. phaseoli* inoculation (1.1 g plant^{-1}). The inoculant stored under low temperatures (4℃) had a significantly higher (3.73 x 10^9 CFU per gram/ml of inoculant) survival than those stored at room temperature (16±2℃) with 2.87 x 10^9 CFU per gram/ml of inoculant. Storage under low temperature (4 ℃) sustained higher viable bacterial cells than at room temperature particularly for liquid inoculant, while filter mud sustained higher population under room temperature. Significantly higher yield (1.64 Mg ha^{-1}) was obtained with the filter mud as carrier materials for the bacterial strains while there was no significant difference in the yield of common bean between peat moss (1.56 Mg ha^{-1}) and YEMB (1.54 Mg ha^{-1}). From this study, it is concluded that co-inoculation of common beans with the specific rhizobia and PSB using sugarcane filter mud as a carrier material significantly enhances nodulation, nutrient uptake, shoot biomass, and grain yield of common bean. This study recommends adoption of co-inoculation of common bean with specific rhizobia and PSB using sugarcane filter mud as a carrier material to maximize common bean growth and yield.

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LIST OF ABREVIATIONS AND ACRONYMS

AEZ	Agro Ecological Zone
CESAAM	Centre of Excellence in Sustainable Agriculture and Agribusiness Management
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization Statistics
FNCA	Forum for Nuclear Cooperation in Asia
ICRISAT	International Centre for Research in Semi-Arid Tropics
IAA	Indole Acetic Acid
LH3	Lower Highland 3
LR	Long rains
KALRO	Kenya Agricultural and Livestock Research Organization
N_2	Nitrogen gas
PGPR	Plant Growth Promoting Rhizobacteria
PSB	Phosphate solubilizing bacteria
SSA	Sub Saharan Africa
SR	Short rains
UM1	Upper Midland 1
UM3	Upper Midland 3
YEM	Yeast Extract Mannitol
YEMA	Yeast Extract Mannitol Agar
YEMB	Yeast Extract Mannitol Broth
x g	Centrifugal Force

CHAPTER ONE
INTRODUCTION

1.1 Background information

Common bean (*Phaseolus vulgaris* L.) serves as a staple for over 300 million people globally, providing a vital source of protein, vitamins, minerals, and fiber (Uebersax *et al*., 2023). This crop is particularly significant in Africa, where it supplies up to 57% of the necessary dietary protein and 23% of energy (Mwanauta *et al*., 2015). Its low fat and cholesterol-free nature helps reduce the risk of diseases such as cancer, diabetes, and coronary conditions (Nchanji *et al*., 2023). Common beans are not only affordable compared to other protein sources but also contain flavonoids and isoflavonoids with medicinal properties that may inhibit cancer-related enzyme activities (Romero-Arenas *et al*., 2013). In Kenya, the leading bean producer in East Africa, approximately 300,000 to 500,000 hectares are cultivated, yielding 40,000 to 150,000 metric tons annually (Barkutwo *et al*., 2020).

The production of common bean in many SSA soils is hindered by poor soil fertility, posing a risk to food security. Nitrogen (N) and phosphorus (P) are the key nutrients limiting plant growth. Phosphorus is often scarce in certain soils because it is easily bound by iron and aluminum oxides (Bhattacharyya & Jha, 2012; Collavino *et al*., 2010). Nitrogen and P are usually replenished in the soil through the application of inorganic fertilizers. However, the costs of nitrogenous and phosphatic fertilizers have risen in many developing countries. Consequently, there is growing interest in utilizing plant growth-promoting rhizobacteria (PGPR). The PGPR are beneficial soil bacteria that colonize the rhizosphere and promote plant growth and development through various mechanisms, such as enhancing nutrient availability, stimulating root growth, and providing protection against pathogens (Ahemad & Khan, 2020; Bhattacharyya *et al*., 2021; Patel & Saraf, 2021). In terms of nutrient availability, some PGPR, particularly those belonging to genera such as *Azotobacter*, *Azospirillum*, and *Rhizobium*, have the ability to fix atmospheric nitrogen (N2) into ammonia (NH3), which can be directly utilized by plants (Abda-Alla *et al*., 2014; El-Akhal *et al*., 2013). A diverse number of PGPR possess enzymes such as phosphatases and organic acids that can solubilize insoluble forms of phosphorus (P) in the soil, making it more available to plants (Nawaz *et al*., 2021; Olanrewaju *et al*., 2016). Examples of phosphate-solubilizing bacteria include species of *Bacillus*, *Pseudomonas*, and *Rhizobium*.

Additionally, PGPR can synthesize and release phytohormones such as auxins (e.g., indole-3-acetic acid, IAA), cytokinins, and gibberellins, which regulate various aspects of plant

growth and development (Spaepen *et al*., 2007). Similarly, certain PGPR have antagonistic effects against plant pathogens through mechanisms such as competition for nutrients and niche exclusion, production of antimicrobial compounds such as antibiotics, siderophores, and induction of plant defence responses (Wang *et al*., 2021). Species of Bacillus, Pseudomonas, and Streptomyces, which are widely studied for their biocontrol potential against fungal, bacterial, and nematode pathogens (Lee *et al*., 2023; Yu *et al*., 2022). The PGPR have also been reported to facilitate nutrient uptake by plants through the release organic acids, enzymes, and siderophores that mobilize nutrients in the soil, chelate metal ions, and facilitate their uptake by plant roots (Ansari *et al*., 2023; Khoso *et al*., 2023; Singh *et al*., 2022).

Microorganisms play a crucial role in mineralizing organic phosphates and solubilizing inorganic phosphates, thereby enhancing the availability of phosphorus in the soil (Mardad *et al*., 2013). The use of native rhizobia as biofertilizers has been recommended because these bacteria adapt easily to the specific environmental conditions, which facilitates their survival and the successful nodulation of the host plant (Topre *et al*., 2011). As a result, biofertilizers can help address the challenge of feeding a growing global population, particularly as agriculture confronts various environmental stresses (Bhardwaj *et al*., 2014).

The co-inoculation of rhizobial bacteria and phosphate-solubilizing bacteria (PSB) has gained significant attention in agricultural research due to its potential to enhance the growth and yield of legume crops (Antune *et al*., 2020; Gopalakrishnan *et al*., 2021). This synergistic approach involves the simultaneous application of both types of beneficial bacteria to legume seeds or soil, aiming to improve nutrient uptake, promote plant growth, and increase crop productivity. The co-inoculation of rhizobial and PSB strains enhances nutrient uptake by legume plants, leading to improved growth, vigor, and biomass production (Anwar *et al*., 2021; Pishgar-Komleh *et al*., 2017; Xie *et al*., 2017). Additionally, increased phosphorus availability promotes root development, nutrient assimilation, and overall plant health, contributing to enhanced crop productivity and yield (Sivasakthivelan *et al*., 2021).

Despite numerous of PGPR being discovered, challenges of suitable biotechnological processing and shelf-life stability of the bacteria need to be overcome in order for their use as biofertilizers to be successful (Berger *et al*., 2018). To transfer viable bacteria in optimum population from the laboratory to the field, suitable carrier materials are an important factor (Brahmaprakash & Sahu, 2012) both in the biofertilizer production as well as in crop response (Maheswari & Kalaiyarasi, 2015). The carrier material serves as a medium for the bacteria to adhere to and provides protection against environmental stresses during storage, transportation, and application (Ahemad & Khan, 2021). Therefore, the carrier material used for biofertilizers

can significantly impact the survival and efficacy of bacterial strains. Carrier materials with good physico-chemical properties, are also a source of nutrients to the microbes which ensure their better survival (El-Fattah *et al*., 2013). Additionally, the right carrier material is extremely important as it must be able to hold a surplus of bacteria over the long term (Vishwakarma *et al*., 2018). The choice of carrier material plays a crucial role in determining the survival and effectiveness of bacterial strains in biofertilizers (Mohd Din & Hidayat, 2021; Pramanik *et al*., 2017; Sarkar *et al*., 2018). Selecting an appropriate carrier material based on factors such as moisture retention, aeration, nutrient availability, pH, stability, and compatibility with bacterial strains is essential for optimizing biofertilizer performance and promoting sustainable agriculture (Afzal *et al*., 2021; Khan *et al* 2020; Verma *et al*., 2021). Moreover, maintaining biofertilizers at the appropriate storage temperature is critical for preserving the viability and efficacy of bacterial strains. Proper storage conditions, including temperature control, moisture management, and protection from light and contaminants, are essential for maximizing the shelf life and performance of biofertilizers (Bhattarai & Pokhrel, 2021; Singh *et al*., 2019; Singh *et al*., 2020; Suman *et al*., 2019).

Biofertilizers keep the soil environment rich in a variety of micro- and macro-nutrients through nitrogen fixation, phosphate and potassium solubilization or mineralization and release of plant growth regulating substances in the soil (Sinha *et al*., 2014). Although the role of phosphorus fertilizers in nodulation, nitrogen fixation and growth of common bean has been reported, the role of phosphate-solubilizing bacteria in phosphorus availability, growth promotion and their interaction with N_2-fixing bacteria and their use under tropical conditions has received scant attention. Korir *et al*. (2017) identified seven Bacillus isolates found within the root environment of common bean in Nakuru County, Kenya. Two of these isolates were used to test their effect on growth of common bean in combination with rhizobia strains and found to work in synergy with the rhizobia. Bala *et al*. (2011) observed that despite the clear evidence of response to inoculation by legume crops, there is relatively little use of inoculants by smallholder farmers in sub-Saharan Africa. Furthermore, the use of highly effective native rhizobia in Kenyan soils has not been exploited since the current commercial inoculants used in the country contain mainly exotic strains (Mugabo *et al*., 2014). Therefore, there is need to isolate and identify more PSB and rhizobia in various agro-ecological zones in Kenya and test their efficacy in growth promotion of common beans.

1.2 Statement of the problem

Legume inoculation has not been widely adopted in SSA despite there being a huge potential to utilize it (Aloo *et al*., 2021). The benefits of indigenous soil microbes are hardly explored and when exotic commercial inoculants are used, they are usually not derived from microbes isolated locally and may therefore not be effective in stimulating responses in terms of growth and yield of the target crops. Efforts have been made in searching and investigating the rhizobia and other rhizosphere organisms and their mode of action, so that they can be exploited commercially as biofertilizers (Fahde *et al*., 2023; Nehra & Choudhary, 2015). Due to the various challenges faced in screening, formulation, and application, their potential as commercial inoculants has yet to be fully exploited (Ibáñez *et al*., 2023). Despite numerous studies on PSB and rhizobia effects on common bean in Kenya, no inoculant products based on the local isolates have been formulated. The current study aimed to address the limited information on the potential of indigenous soil microbes as biofertilizers for crop production. By conducting research on rhizobia and PSB, this study explored their modes of action and potential as biofertilizers. The objective was to overcome challenges in screening, formulating, and applying these beneficial organisms to exploit their full potential as commercial inoculants. Emphasis was placed on developing inoculant products based on local isolates to enhance growth and yield of common beans in Kenya, where despite numerous studies, no local isolate-based inoculant products have been formulated yet.

1.3 Objectives

1.3.1 Broad objective

To contribute to environmental sustainability and food security by increasing the grain yield of common bean in Kenya through utilization of effective PSB and rhizobia biofertilizer inoculations.

1.3.2 Specific objectives

i. To determine the indole acetic acid production and phosphate solubilization efficiency of selected native rhizobia and phosphate solubilizing bacteria isolates.
ii. To evaluate the effect of co-inoculation of selected rhizobial and phosphate solubilizing bacteria isolates on growth of common bean under greenhouse conditions.
iii. To assess the effect of inoculant formulation and storage conditions on the survival of bacterial inoculants.

iv. To determine the effect of different inoculant formulations on the effectiveness of the rhizobia and phosphate solubilizing bacteria strains on growth and yield of common bean under field conditions.

1.4 Hypotheses

i. The native rhizobia and phosphate solubilizing bacteria isolates do not produce indole acetic acid or solubilize phosphorous
ii. Co-inoculation of rhizobial and phosphate solubilizing bacteria isolates have no significant effect on the growth of common bean.
iii. The inoculant formulation and storage condition have no effect on the shelf life of bacterial inoculants.
iv. Inoculant formulation does not significantly affect the effectiveness of rhizobia and phosphate solubilizing bacteria strains.

1.5 Justification

Despite extensive research on the benefits of plant growth-promoting rhizobacteria (PGPR) and rhizobia, there is still a significant gap in the development of commercial inoculants based on local isolates. This study will contribute to closing this gap by providing scientific insights and practical solutions that can be translated into viable agricultural technologies. Inoculants from locally isolated strains are more likely to be effective, offering tailored solutions to the specific challenges faced by farmers in SSA. Thus, it is essential to isolate native rhizobia and PSB from common bean grown fields for multiple beneficial effects on the crops and for potential biofertilizers development. Therefore, there is need for more studies on plant-microbe interactions and their activities in different regions and ecologies in Kenya. Availability of more information will enable the development and widespread acceptance and use of inoculants, to improve soil ecology and plant growth. With the increasing use of microbes, numerous bacterial species have been isolated and their capacity to promote plant growth investigated. Further research and understanding of mechanisms of the beneficial organisms would allow for finding out more competent rhizobacterial strains which may work under diverse agro-ecological conditions. Exploitation of the benefits of the soil microbes in crop production will contribute in part to the fight against hunger. Finally, the findings of this study can inform agricultural policies and development programs aimed at improving farming practices in Kenya and aligns with the United Nations Sustainable Development Goals (SDGs), particularly those related to zero hunger and sustainable agriculture.

1.6 Scope and limitations

This study explored the mechanisms of action of native rhizobia and PSB isolated from selected agro-ecological zones of Kenya. Indole acetic acid production and phosphate solubilization efficiency of the isolates were carried out. Ideally, *in vitro* IAA determination requires the quantification of the levels produced by the isolates after running IAA standards to obtain a standard absorbance curve from which the levels can be determined. However, for this study, absorbance of levels of the IAA produced by the bacterial isolates have been reported as an indication of the IAA production by the tested isolates. The effectiveness of the native rhizobia applied singly or in combination with the *Bacillus* or the *Paenibacillus* strains was tested both in greenhouse and field conditions. Further, the native isolates were formulated into liquid and solid biofertilizers and the different formulations tested for their effectiveness. The ideal situation to determine the efficiency of the native rhizobia is testing them under many and varied agro-ecological zones and soil types. However, this study focussed only on three agro-ecological zones (Upper Midlands; UM1, UM3 and Lower Highlands; LH1) and three soils types namely Nitisol, Andosol and Ferralsol due not only to logistical and budgetary constraints, and limited time but more importantly zones that have the greatest potential for bean production.

1.7 Definition of terms

Biofertilizer: A substance that contains living microorganisms which, when applied to seeds, plant surfaces, or soil, promotes growth by increasing the supply or availability of primary nutrients to the host plant.

Plant growth promoting rhizobacteria: A diverse group of soil bacteria inhabiting around/on the root surface and are directly or indirectly exert beneficial effects on plant growth, health and development.

Rhizosphere: The zone of soil surrounding a plant root where the biology and chemistry of the soil are influenced by the root.

Carrier materials: Any substance that can be used to deliver viable microbial cultures from the laboratory to the field.

Formulation: Preparation of inoculants in the carrier materials

Indole acetic acid: A chemical that is synthesized by microbes and plants, and it plays a role in both root and shoot development.

Inoculants: Agricultural amendments that use beneficial rhizospheric or endophytic microbes to promote plant health.

Inoculation: The application of inoculants to the soil, seeds, or plant.

Co-inoculation: Inoculation of plants with a mixture of two or more plant growth- promoting microbiota.

Mechanisms of action: The biotic activities of the soil microbes in the soils that lead to the stimulation of plant growth through mobilizing nutrients in soils, production of numerous plant growth regulators, protection plants from phytopathogens etc.

Rhizobia: Bacteria found in soil that helps in fixing nitrogen in leguminous plants.

CHAPTER TWO
LITERATURE REVIEW

2.1 Common bean description and utilization

Common bean (*Phaseolus vulgaris* L.) is a herbaceous annual leguminous plant. There are two plant types: erect herbaceous bushes, up to 20-60 cm high; and twining, climbing vines up to 2-5 m long. All cultivars bear alternate, green or purple leaves, with three oval leaflets. Flowers are white, pink, or purple, about 1 cm long. Pods are 8–20 cm long, 1–1.5 cm wide, black, green, purple or yellow, each with four to six beans that are smooth, plump, kidney-shaped, up to 1.5 cm long, and differ in colour, often being mottled with two or more colours. The common bean is mainly self-pollinated. Common bean grows from sea level to an altitude of 2200-3000 m with an optimum annual rainfall of between 500 and 1500 mm, and where temperatures range between 15°C and 23°C. It can grow under higher temperatures (35°C) but this may hamper seed production (Wortman, 2006). Dry weather during the maturing stage benefits seed preservation. The common bean grows well on a large variety of soils with pH ranging from 4 to 9. However, it does better on well-drained, sandy loam, silt loam or clay loam soils, rich in organic content.

Common beans are an important source of proteins, minerals (iron and zinc) and vitamins for many human populations (Beebe *et al*., 2000). The main form of consumption is represented by dry seeds, however varieties suitable for other consumption forms, such as snap or shell beans, have been developed. The leaf is occasionally used as a vegetable, and the straw is used for fodder. Immature pods are eaten fresh and can be easily preserved by freezing, canning or dehydration. Mature pods and seeds are dried. Beans are eaten boiled, baked, fried, or ground into flour. Crop residues, such as dried pods and stems (straw) as well as processing by-products, can be used as fodder (Wortman, 2006).

2.2 Importance of common bean in Kenya

Common bean is the second most important food crop after maize in Kenya (One Acre Fund, 2016). Common bean is a major source of dietary protein, carbohydrates, and essential nutrients for millions of Kenyans (Celmeli *et al*, 2018). Common bean plays a crucial role in addressing food security and nutrition challenges, particularly in rural and low-income households who are the majority in the country (Beebe *et al*., 2013). The annual *per capita* consumption of common bean in Kenya is between 14-66 kg among low-income people who cannot afford to buy other food stuff, such as meats and fish (Beebe *et al*., 2013; Duku *et al*.,

2020). The common bean holds significant economic importance in Kenya, both as a cash crop for domestic consumption and export. Common bean provides a source of income through local sales in markets and to agro-processors (Muteti *et al*., 2022). Kenya exports common beans to international markets, contributing to foreign exchange earnings and trade balance. A Food and Agriculture Organization (FAO) report that in 2021 total bean exports from Kenya were estimated at 142,087 t with export value of US$ 119.6 million (FAOSTAT, 2021).

Common bean is widely cultivated in Eastern Africa, including Burundi, Ethiopia, Kenya, Malawi, Rwanda, Tanzania, and Uganda (Shiferaw *et al*., 2012). In Kenya, Common beans are grown across Kenya in diverse agro-ecological zones. In the highland areas, it is grown in Central Kenya (Nyeri, Murang'a), Western Kenya (Kisii, Kakamega), and Rift Valley (Nakuru, Bomet). In the mid-altitude areas common beans are grown in Eastern Kenya (Embu, Meru), Nyanza (Siaya, Kisumu), and parts of Rift Valley (Uasin Gishu, Trans Nzoia). In the lowland areas, it is grown in coastal regions (Kilifi, Kwale), and semi-arid regions of Kitui, and Machakos (Katungi *et al*., 2009).

Common bean production in Kenya is influenced by several factors, including climate variability (Kogo *et al*., 2021; Komo *et al*., 2022; Ochieng *et al*., 2020), poor soil fertility (Asibuo *et al*., 2022; Barkutwo *et al*., 2020), pests and diseases (Mangeni *et al*., 2020) and limited access to quality seed (Odendo *et al*., 2010). Market access challenges and fluctuating prices discourage investment, while post-harvest losses due to poor storage reduce yields (Katungi *et al*., 2009; Muteti *et al*., 2022).

2.3 Soil factors that affect common bean production

Common bean production is be influenced by a variety of soil factors. The type of soil, including its texture and structure, can significantly affect common bean production. Beans generally prefer well-drained soils with good aeration and organic matter content. Loamy soils, which have a balanced mix of sand, silt, and clay, are often ideal for common bean production as they provide good drainage and nutrient retention (Shabana *et al*., 2020). Well-structured soils with good aggregation allow for adequate root penetration, water infiltration, and aeration, which are important for common bean growth and development (Giuliani *et al*., 2024). Common beans prefer slightly acidic to neutral soil pH (around 6.0 to 7.0). Soil pH influences nutrient availability, and acidic soils may result in nutrient deficiencies or toxicities. Acidic soils may lead to nutrient deficiencies, while alkaline soils may result in nutrient imbalances and reduced bean yields (Gemada, 2021; Keba, 2021). Adequate soil moisture is crucial for common bean production, especially during germination, flowering, and pod development

stages. Excessively dry or waterlogged soils can adversely affect plant growth and yield (Alemu *et al*., 2023; Rao, 2021). Temperature and rainfall patterns, have an influence on common bean growth, development and yield (Burbano-Erazo *et al*., 2021; Karavidas *et al*., 2022; Suárez *et al*., 2020). Therefore, selecting bean varieties adapted to local climatic conditions can optimize production. Soil fertility, particularly nitrogen, phosphorus, and potassium level, directly impacts common bean growth and yield (El-Yazal *et al*., 2020; Mohamed *et al.,* 2021). Adequate levels of these nutrients are essential for plant development, flowering, and pod formation. Fertile soils with adequate nutrient levels support healthy plant growth and higher yields (Nigatie, 2021; Smith *et al*., 2022).

The most common constraints to bean production are low soil fertility, water stress and diseases (Mukankusi *et al*., 2018; Sinclair & Vadez, 2012). Soil degradation is a major global problem, with the effects strongly felt in developing countries where large proportions of the population reap their livelihoods directly from crop production (Tully *et al*., 2015). Poor soil management and removal of crop residues from the field also contribute for the substantial reduction in soil fertility (Turmel *et al*., 2015). Phosphorus is one of the most deficient nutrients for common bean cultivation (Beebe *et al*., 2011), largely due to the high phosphorus-fixing capacity of certain soils (Menezes-Blackburn *et al*., 2018). Since common beans need phosphorus to support energy production for their metabolic processes, they have high phosphorus requirements and are particularly sensitive to low levels of plant-available phosphorus in the soil (Lazali *et al*., 2017).

The low level of common bean production in SSA has been attributed partly to low levels of soil plant-available P and drought stress, caused by climate change variability (Beebe *et al*., 2011; Beebe *et al*., 2013). About 50% of the common bean producing areas in the world is affected by low P (Beebe, 2012) reducing common bean yield by over 60% (Acosta-Díaz *et al*., 2009). Bean yields are low due to low soil fertility, particularly phosphorus deficiency (Atemkeng *et al*., 2011). The declining soil phosphorus is due to continued nutrient mining without replenishment. It is estimated that beans remove 12.5kg $P_2O_5Ha^{-1}$ which is higher than additions in terms of phosphorus fertilization by resource-poor farmers (Kaihura *et al.,* 2011).

2.4 Plant growth promoting rhizobacteria (PGPR)

Plant growth-promoting rhizobacteria (PGPR) are naturally occurring soil bacteria that aggressively colonize plant roots and benefit plants by providing growth promotion. They include both the phosphate solubilizing bacteria (PSB) and rhizobia. Inoculations of crop plants with certain strains of PGPR at an early stage of development improve biomass production

through direct effects on root and shoot growth. PGPR are reported to influence the growth, yield and nutrient uptake by an array of mechanisms. There has been much research interest in PGPR and there is now an increasing number of PGPR being commercialized for various crops (Agrawal *et al*., 2014). Bacteria genera like *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Microbacterium*, *Pseudomonas*, *Rhizobium* and *Serratia* are recognized as the most significant PGPR (Korir *et al*., 2023; Saharan & Nehra, 2011). Among the PGPR, *Pseudomonas* and *Bacillus* are the most commonly described genera possessing plant growth promoting activities, but many other taxa are also included in PGPR group. Selected strains of PGPR are being used as seed inoculants (Ahemad & Khan, 2010; Rani *et al*., 2012). Bacteria residing around or within plant roots are more efficient at transforming, mobilizing, and solubilizing nutrients compared to those in bulk soils (Hayat *et al*., 2010). As a result, rhizobacteria play a crucial role in nutrient recycling and are essential for maintaining soil fertility (Glick, 2012). Most rhizobacteria in this group are Gram-negative rods, with a smaller proportion being Gram-positive rods, cocci, or pleomorphic (Bhattacharyya & Jha, 2012).

The PGPR belonging to various bacterial genera are known to participate in many important biological activities, such as the nutrient cycling and plant growth (Jha & Saraf, 2015). Phosphate-solubilizing bacteria (PSB) are regarded as promising biofertilizers because they can provide phosphorus to plants from otherwise inaccessible sources through various mechanisms (Gupta *et al*., 2015). Plant growth-promoting rhizobacteria (PGPR) have been shown to enhance the performance of rhizobia in nodulation and nitrogen fixation in common beans, resulting in improved plant growth. Greenhouse and field studies with PGPR strains have reported increased nodulation and nitrogen fixation in common bean (Korir *et al*., 2017; Sánchez *et al*., 2014).

2.5 Mechanisms of plant growth promotion by plant growth promoting rhizobacteria

The PGPR contribute to plant nutrition both by facilitating nutrient uptake and by increasing primary nutrient availability in the rhizosphere by different methods such as fixing atmospheric nitrogen, solubilizing mineral nutrients, mineralizing organic compounds and producing phytohormones (Arora, *et al.*, 2011; Bhardwaj *et al*., 2014; Hayat *et al*., 2010; Viveros *et al*., 2010). The use of rhizosphere-associated microorganisms as biofertilizers is now being considered as having potential for improving plant productivity. The PGPR promote plant growth by mobilizing nutrients in the soil, producing various plant growth regulators, protecting plants from phytopathogens by controlling or inhibiting them, enhancing soil structure, and bioremediating polluted soils (Ahemad & Malik, 2011; Hayat *et al*., 2010;

Rajkumar *et al*., 2010). Plant growth promoting rhizobacteria strains can enhance plant growth and development through both direct and indirect mechanisms. Direct stimulation includes processes such as biological nitrogen fixation, the production of phytohormones like auxins, cytokinins, and gibberellins, solubilization of minerals like phosphorus and iron, production of siderophores and enzymes, and induction of systemic resistance (Ortíz-Castro *et al*., 2009). The PGPR may use more than one of these mechanisms to enhance plant growth (Martinez-Viveros *et al*., 2010).

2.5.1 Biological nitrogen fixation

Nitrogen (N) is an essential macronutrient required for plant growth and development. Extensive research has investigated the bioavailability of nitrogen to plants, particularly in the context of plant growth-promoting rhizobacteria (PGPR). Among the various mechanisms, nitrogen fixation through the legume-rhizobia symbiosis is the most extensively studied. In this symbiotic relationship, rhizobia convert atmospheric nitrogen (N_2) into biologically accessible forms for plant utilization (Backer *et al*., 2018). Plant growth-promoting rhizobacteria can fix atmospheric nitrogen and make it available to plants through two mechanisms: symbiotic and non-symbiotic. Symbiotic nitrogen fixation involves a mutualistic relationship between a microbe and a plant. The symbiotic relationship between rhizobia and legume plants involves several steps. First, the legume roots release flavonoids into the soil, which act as signalling molecules. Rhizobia in the soil detect these flavonoids and respond by synthesizing *Nod* factors, which are key signalling molecules for initiating the symbiotic relationship (de Bruijn, 2015). The plant root hairs recognize the *Nod* factors, which causes the root hairs to curl around the rhizobia. Rhizobia induce the formation of an infection thread, that allows the bacteria to enter the root cells. The infection thread extends through the root hair into the cortical cells of the root (de Bruijn, 2015). Once inside the root cortical cells, rhizobia stimulate localized cell division, leading to the formation of root nodules. The nodules provide a specialized environment where rhizobia can fix atmospheric nitrogen. These nodules contain leghemoglobin, a protein that helps maintain a low-oxygen environment, crucial for the functioning of the nitrogenase enzyme. Inside the nodules, rhizobia differentiate into bacteroids, which are the active forms that carry out nitrogen fixation. The enzyme nitrogenase converts atmospheric nitrogen (N_2) into ammonia (NH_3), which the plant can use for growth and development (Ahemad & Kibret, 2014; de Bruijn, 2015).

Symbiotic bacteria which act as PGPR are *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, and *Mesorhizobium* with leguminous plants, *Frankia* with non-leguminous trees and shrubs

(Bhattacharyya & Jha, 2012). In addition to the symbiotic nitrogen fixation, free living nitrogen-fixing rhizospheric bacteria such as *Acetobacter*, *Alcaligenes*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Clostridium*, cyanobacteria, *Diazotrophicus*, *Enterobacter* and *Klebsiella* are able to convert N2 into plant usable forms (Ahmad *et al*., 2005; Bhattacharyya & Jha, 2012). Nitrogenase (*nif*) genes encompass structural genes, which are involved in the activation of the Fe protein, iron-molybdenum cofactor biosynthesis, electron donation, and regulatory genes necessary for the enzyme's synthesis and function (Glick, 2012). These *nif* genes are present in both symbiotic and non-symbiotic nitrogen fixation processes (Glick, 2012). The *nif* genes are found in both symbiotic and non-symbiotic nitrogen fixation activities (Reed *et al*., 2011). Inoculation by biological nitrogen fixing plant growth promoting rhizobacteria on crop provides an integrated approach for growth promotion and maintains the nitrogen level in agricultural soil. Several research also showed that inoculating selected common bean varieties with efficient strains of rhizobia can lead to improvements in nodulation, nitrogen fixation, and grain yield (Rahmani *et al*., 2011; Sánchez *et al.,* 2014; Stajković *et al*., 2011; Yadegari *et al.,* 2010;).

2.5.2 Solubilization of phosphates

Soil contains large quantities of phosphate in insoluble forms. Phosphate-solubilizing bacteria can convert this phosphate into soluble forms through processes such as acidification and chelation or through enzymatic action (Khan *et al*., 2010). Phosphate-solubilizing bacteria (PSB), particularly endophytes, when used as inoculants, can convert insoluble forms of phosphorus into a usable form, leading to increased plant yields (Walia *et al*., 2017). Microorganisms mineralize organic phosphorus in soil by solubilizing complex phosphates, such as tricalcium phosphate and rock phosphate, into inorganic forms that are accessible to plants. These PSB employ various mechanisms to convert insoluble phosphates into accessible forms. The primary mechanism involves the secretion of organic acids by microbes, a process driven by sugar metabolism. Microorganisms in the rhizosphere utilize sugars from root exudates, metabolizing them to produce organic acids (Goswami *et al*., 2014). These acids act as effective chelators for divalent Ca^{2+} cations, facilitating the release of phosphates from insoluble compounds. Many PSB lower the pH of the medium by secreting organic acids such as acetic, lactic, malic, succinic, oxalic, and citric acids (Patel *et al*., 2015). The solubilization of inorganic phosphorus typically results from the action of low molecular weight organic acids produced by various soil bacteria (Zaidi *et al*., 2009). This ability to convert insoluble

phosphates into plant-accessible forms is a key characteristic of PGPR that enhances plant yield (Sharma *et al*., 2013).

2.5.3 Potassium solubilization

A diverse array of soil microorganisms, including saprophytic bacteria, fungi, and actinomycetes, exhibit the potential to solubilize potassium (K), effectively converting soil K into plant usable forms (Etesami *et al*., 2017; Meena *et al*., 2016). Among these, potassium-solubilizing bacteria are particularly effective, as they can dissolve K-rich materials and convert insoluble potassium into soluble forms that plants can absorb both anaerobically and aerobically (Parmar *et al*., 2013). These potassium-solubilizing rhizobacteria employ various mechanisms to make potassium available to plants, including acidolysis, chelation, exchange reactions, complexolysis, and the production of organic acids (Meena *et al*., 2014; Meena *et al*., 2015; Uroz *et al*., 2009). Acidolysis, involving both organic and inorganic acids and the synthesis of protons, is the primary mechanism for K mineral solubilization (Meena *et al*., 2015). Potassium solubilizing bacteria produce a variety of organic acids such as citric acid, fumaric acid, gluconic acid, glycolic acid, 2-ketogluconic acid, lactic acid, malic acid, malonic acid, oxalic acid, propionic acid, succinic acid and tartaric acid that are effective in releasing potassium from potassium-bearing minerals (Keshavarz *et al*., 2013; Saiyad *et al*., 2015). Bacteria such as *Bacillus mucilaginosus*, *Bacillus edaphicus*, and *Bacillus circulans* are recognized as excellent potassium solubilizers in soil bacterial populations (Meena *et al*., 2015; Meena *et al*., 2016).

2.5.4 Bioavailability of micronutrients

Zinc is a vital component of various enzymes that catalyze numerous metabolic reactions in plants (Chauhan *et al*., 2021). Despite most of the soils being rich in zinc, they contain only a small amount of plant usable forms (Chauhan *et al*., 2021). Zinc-solubilizing rhizobacteria can serve as a sustainable approach to enhance the bioavailability of zinc in soil, which may help mitigate yield losses and address zinc malnutrition (Mumtaz *et al*., 2017). Khanghahi *et al*. (2018) reported that the use of *Agrobacterium tumefaciens* and *Rhizobium* sp. as bioinoculants improved zinc availability in soils. Zinc-solubilizing rhizobacteria produce various organic acids, including 2-ketogluconic acid and 5-ketogluconic acid, which aid in the mobilization of zinc. *Bacillus* and *Pseudomonas* spp. have the ability to solubilize these zinc sources in soil, making the essential nutrient more accessible to plants (Olanrewaju *et al*., 2017). Several strains of zinc-mobilizing bacteria have been demonstrated to enhance zinc uptake, thereby increasing yield in various crops, including rice, wheat, and soybean (Ramesh *et al*., 2014; Shakeel *et al*., 2015).

While iron is crucial for key plant processes such as chlorophyll biosynthesis, photosynthesis, and respiration, the soluble form of iron (Fe^{3+}) is not easily accessible to plants (Kenneth *et al*., 2019; Murugappan *et al*., 2012; Ojuederie *et al*., 2019; Rajkumar et al., 2010). Plant growth-promoting rhizobacteria (PGPR) help sequester iron by producing siderophores, including carboxylates, catecholates, and hydroxamates, in iron-deficient soils (Patel *et al*., 2018; Saharan & Nehra, 2011; Sun *et al*., 2022). Due to their high affinity for iron, siderophores can enhance plant growth and increase survival rates under climate change by sequestering iron from the plant rhizosphere (Backer *et al*., 2018; Vejan *et al*., 2016). Many bacterial strains enhance iron availability by producing organic acids or siderophores (Ahmed & Holmstrom, 2014).

2.5.5 Production of growth hormones

Plant growth-promoting rhizobacteria (PGPR) enhance plant growth through various mechanisms, including the production of growth regulators or promoters. These growth regulators include phytohormones such as indole-3-acetic acid (IAA), gibberellins, cytokinins (Kaur & Sharma, 2013; Perez-Montano *et al*., 2014), and other signalling molecules that influence plant development (Hassan *et al*., 2024). The production of these compounds by PGPR is a key aspect of their interaction with plants and contributes significantly to their plant growth-promoting activities.

Indole acetic acid IAA is the most well-known and studied auxin produced by PGPR. Auxins are critical for various plant growth processes, including cell division, root elongation, and differentiation (Nath *et al*., 2017). By influencing cell elongation, IAA contributes to overall shoot growth and plant biomass. Various PGPR species such as *Rhizobium*, *Azospirillum*, *Pseudomonas*, and *Bacillus* species, produce IAA as a direct mechanism to promote plant growth (Spaepen *et al*., 2007). In particular, plant growth promotion and root nodulation are influenced by IAA. Bacterial IAA enhances root surface area and length, which in turn provides plants with better access to soil nutrients. Indole acetic acid acts as a signalling molecule that initiates the nodulation process. The IAA levels increase in response to these signals, promoting cell division and the formation of nodule primordia (Ferguson & Mathesius, 2014; Spaepen & Vanderleyden, 2011). Most *Rhizobium* strains studied have been found to produce IAA, and several studies suggest that increased auxin levels in the host plant are essential for nodule formation (Glick, 2012).

Gibberellins are produced by PGPR, such as *Azospirillum*, *Azotobacter*, and *Bacillus*. Microorganisms produce gibberellic acid as secondary metabolites during their growth in the

soil. The production typically occurs through the terpenoid biosynthetic pathway, where enzymes catalyze the conversion of precursor molecules into gibberellins. Gibberellins are primarily known for their role in promoting stem elongation, seed germination, and flowering. They enhance cell elongation and division, leading to increased plant height (Jha & Saraf, 2015; Kumar *et al*., 2015). Cytokinins are another class of hormones produced by PGPR, such as *Bacillus* and *Pseudomonas* species. These hormones play a vital role in cell division in shoots and roots, shoot initiation, and leaf senescence (Stefen *et al*., 2022). Cytokinin produced by the PGPRs also increases abscisic acid, leading to the closure of the stomata during drought, thereby reducing water loss through the leaves (Arora *et al*., 2020).

The PGPRs have also been reported to produce exopolysaccharides that have water-holding and cementing properties (Grover *et al*. 2011; Tewari & Arora, 2014) and are therefore important in maintaining soil moisture and structure. Additionally, the PGPRs can induce drought tolerance through the synthesis of plant hormones that increase root growth, thereby leading to increased nutrient and water uptake, thereby helping plants overcome environmental stresses (Chibeba *et al*., 2015; de Andrade *et al*., 2023; Vurukonda *et al*., 2016). PGPRs produce exopolysaccharides that bind the Na+ cation and thereby decrease the Na+ content and alleviate salt stress in plants (Egamberdieva, 2011). Glick (2005) also reported that PGPRs lower the levels of the stress hormone, ethylene, making crops more tolerant to salt stress. Iron is an essential micronutrient that has recently gained attention for its role in promoting plant growth and alleviating various environmental stresses (Patel *et al*., 2018).

2.5.6 Alleviation of soil moisture and salinity stresses

Inadequate water supply to plants is a major challenge to crop production, especially in rain-fed agriculture. With the changing climate, crops need to adapt to conditions of drought. PGPMs residing in the rhizosphere of crops can confer tolerance/ resistance of the plant to drought/water stress conditions. PGPR have been reported to produce exopolysaccharides that have water-holding and cementing properties (Grover *et al*., 2011; Tewari & Arora, 2014) and are therefore important in maintaining soil moisture and structure. Additionally, the PGPMs can induce drought tolerance through the synthesis of plant hormones such as auxin (Vurukonda *et al*., 2016). These hormones increase root growth, thereby leading to increased nutrient and water uptake, thereby helping plants overcome environmental stresses (Chibeba *et al*., 2015; Vurukonda *et al*., 2016). Cytokinin produced by the PGPMs increases abscisic acid, leading to the closure of the stomata during drought, thereby reducing water loss through the leaves (Arora *et al*., 2020; Figueiredo *et al*., 2008). Rhizobium inoculation decreased

stomatal conductance under non-stressed conditions but notably increased it during drought stress (Bashir *et al*., 2020). Plant growth-promoting rhizobacteria (PGPRs) have the ability to regulate water content by modifying hydraulic conductivity and stomatal aperture (Zheng e*t al*., 2018). For instance, wheat plants treated with *Pseudomonas azotoformans* under drought stress exhibited a 16% increase in relative water content compared to untreated plants (Ansari *et al*., 2021). Furthermore, plant growth under water stress conditions was observed to improve in response to *Bradyrhizobium* inoculation (Musyoka *et al*., 2020). A study carried out in Ethiopia showed that PGPR inoculation significantly improved the growth of drought-susceptible common bean variety under water-deficit conditions than plants inoculated with rhizobia alone and the uninoculated plants (Eticha 2021). Results from this study showed that inoculation increased the root dry weight and leaf biomass under drought stress more than the control.

Soil salinity is an abiotic stress plants must deal with. Salinity has adverse effects on plant growth and development by reducing osmotic potential and creating an ionic imbalance, causing sodium and chloride toxicity. The PGPMs produce exopolysaccharides that bind the Na+ cation and thereby decrease the Na+ content and alleviate salt stress in plants (Egamberdieva, 2011). Glick (2005) also reported that PGPMs lower the levels of the stress hormone, ethylene, making crops more tolerant to salt stress. Inoculation of common beans with strains of *Rhizobium* PGPM led to a considerable yield despite being grown in a salt-stressed field (Yanni *et al*., 2016). Several studies have demonstrated that inoculation with certain bacteria can mitigate the detrimental effects of salinity stress on plants (Benidire *et al*., 2020; Jochum *et al*., 2019). In an earlier study, Sukweenadhi *et al*. (2015) discovered that thale cress (*Arabidopsis thaliana*) inoculated with *Bacillus* spp. and *Micrococcus* spp. exhibited greater tolerance to various abiotic stresses, particularly salt, drought, and heavy metal stress, compared to non-inoculated control plants. To date, a range of salt-tolerant rhizobacteria, including *Rhizobium*, *Pseudomonas*, and *Bacillus*, have been shown to interact beneficially with many plant species under stress conditions (Gopalakrishnan *et al*., 2015; Jochum *et al*., 2019). Findings by Abulfaraj and Jalal (2021) indicated that the growth of salt-stressed soybean plants improved in the presence of rhizobacterial isolates. Similar outcomes were reported by Metwali *et al*. (2015), who observed enhanced growth parameters in beans inoculated with *Pseudomonas fluorescens* and *Pseudomonas putida* under saline conditions.

2.5.7 Biological control against pathogens

Disease suppression is recognized as a microbial potential that indirectly contributes to plant protection. The PGPR protect crops by suppressing pathogens through various mechanisms, including competition for space and nutrients, antagonism via the production of antifungal antibiotics, the release of volatile organic compounds, and the synthesis of cell wall-degrading molecules (Araujo *et al*., 2005; Kai *et al*., 2009; Robin *et al*., 2008; Singh *et al*., 2008; Zhao *et al*., 2014). As biocontrol agents, strains of Rhizobium, Bacillus, and Pseudomonas, isolated from the root nodules and rhizosphere of common bean, have been effective against a wide range of plant pathogens such as *Sclerotinia sclerotiorum*, *Colletotrichum* sp., *Macrophomina phaseolina*, *Fusarium oxysporum*, *Fusarium solani*, and *Rhizoctonia solani* (Kumar & Dubey, 2012). As an effective biocontrol agent, *Bacillus subtilis* can produce volatile organic compounds that are crucial in stimulating plant defense responses by inducing systemic resistance and enhancing plant growth (Shafi *et al*., 2017). In common beans, PGPRs have been reported to reduce the incidence of diseases in crops. For instance, Sabaté *et al*. (2017) reported that after emergence, the control had 100% incidence of the pathogen, while the seedlings from the inoculated treatment had only 38%, implying that inoculation with the PGPRs significantly reduced the incidence of *M. phaseolina* by 62% compared to the nontreated seeds. Corrêa *et al*. (2014) reported that *B. cereus* DFs093 reduced the severity to 28% and progress (14%) of the *M. phaseolina* pathogen on common beans grown in soils infected with the pathogen. Results by Negi *et al*. (2021) showed that bio-priming French bean (*Phaseolus vulgaris* cv. Contender) seeds with *Rhizobium* (B1) and a PGPR strain reduced the occurrence of diseases like root rot and angular leaf spot in field conditions.

2.6 Role of rhizobia and phosphate solubilizing bacteria in legumes production

Direct interactions occurring between members of different microbial community often result in the promotion of key processes benefiting plant growth and health (Jha & Meenu, 2015). The inoculation of plants with PGPR can increase their native population and therefore improve plant nutrition (Sharma *et al*., 2013; Singh, 2014). A study by dos Santos Sousa *et al*. (2022) demonstrated that common bean plants inoculated with *Rhizobium tropici* exhibited significantly higher yields compared to non-inoculated controls. The inoculated plants showed increased pod number, seed weight, and overall biomass, indicating the substantial impact of effective rhizobia strains on bean productivity. A study by Aserse *et al*. (2020) explored the role of rhizobia inoculation in enhancing drought tolerance in common beans. The findings indicated that inoculated plants had better water retention, higher relative water content, and

improved osmotic adjustment under drought conditions. These physiological changes contributed to sustained growth and yield despite water stress. Furthermore, rhizobia inoculation leads to increased microbial diversity and the proliferation of beneficial microbes, such as those involved in nutrient cycling and pathogen suppression (Sharma *et al*., 2020). This positive shift in the soil microbiome enhances overall soil health and resilience.

Phosphate biofertilizer application has been reported to increase P availability for plants through the processes of acidification, chelation, exchange reactions and production of gluconic acid (Gulati *et al.*, 2010). Zafar *et al*. (2021) conducted an experiment on chickpeas, finding that the use of phosphorus-solubilizing bacteria (PSB) alongside synthetic fertilizer yielded the highest number of pods and grains per plant, with averages of 36.7 pods and 1.6 grains per plant, and a maximum of 4 grains per pod. Similarly, Hassan *et al*. (2017) found that inoculating mung beans with PSB increased the number of branches, with the highest count being 39 branches in plants treated with *Bacillus polymyxa* and *Pseudomonas*, compared to 19 branches per plant in the control. Hassan *et al*. (2017) reported that phosphorus-solubilizing bacteria (PSB) increased soil phosphorus content, with *Bacillus polymyxa* raising it to 13.50 mg/kg and Pseudomonas to 11.50 mg/kg, while the control showed the lowest phosphorus content at 3.5 mg/kg. Rafique *et al*. (2017) stated that inoculating maize seeds with PSB (*Lysinibacillus fusiformis*) and bagasse biochar increased the concentrations of nitrogen (N), phosphorus (P), and potassium (K). So, PGPR has huge potential in production of biofertilizers that can be harnessed to increase crop yields through their capacity in solubilizing fixed P in the soil (Hayat *et al*., 2012).

Use of PGPR mixtures whose functions are known and that could act synergistically is of interest as they offer multiple modes of action, and time or space variability. Understanding the interaction between group of microbial inoculants and plant systems will lead to more benefits from microbial inoculants for improving plant growth (Paul & Lade, 2014).

2.7 Co-inoculation of common bean with PGPR consortia

Co-inoculation is the combined application of PGPR from different genera and species to increase nodulation, growth, biomass, and plant tolerance to various biotic and abiotic conditions and yields (Shamseldin & Velázquez, 2020). The goal of co-inoculation is to harness the synergy of the consortia of microbiota for the benefit of the plant. Studies report varied responses, some showing synergy of co-inoculation, through improved growth and yields, others reporting similar results for single and co-inoculation (neutral), and while others reporting possible competition resulting in lower performance for consortia (Cardoso &

Ferreira, 2021; Gabre *et al*., 2020; Jesus *et al*., 2018; Korir *et al*. 2017; Steiner *et al*., 2020). Co-inoculation of *B. japonicum* and *P. putida* has a synergistic effect and improves soybean nodulation and growth and can be used to formulate a consortium of biofertilizers for sustainable production (Jabborova *et al*., 2021). Co-inoculation response is dependent on several factors, including abiotic conditions such as temperature and moisture and biotic factors including germplasm, pest, and diseases.

Co-inoculation of legumes with PGPR is a beneficial practice that enhances plant growth, improves soil health, increases yield, and supports sustainable and environmentally friendly agriculture**.** Common beans have been reported as a nonselective plant host and are able to perceive nodulation signals from different strains of rhizobia such as *R. tropici* (Cardoso & Ferreira, 2021; Gabre *et al*., 2020; Hungria *et al*., 2013; Jesus *et al*., 2018), *R. etli* bv. phaseoli (Shamseldin & Werner, 2007; Silva *et al*., 2003), *R. gallicum* bv. phaseoli, *R. giardinii* bv. giardinii, *R. leguminosarum* bv. phaseoli, and *R. leguminosarum* bv. viciae (Mhamdi *et al*., 2002), and this may promote nitrogen fixation and plant growth and development. Therefore, co-inoculation of common beans with an array of rhizobia strains and various PGPRs can be employed to improve the agronomic performance of common beans. This could be attributed to the multi-strain synergistic effect caused by the diverse strains of rhizobia and PGPRs applied to beans during inoculation. Co-inoculation results in a synergistic effect, which provides a greater stimulus compared to the effect of each mechanism of action alone (Hungria *et al*., 2013).

2.7.1 Influence of co-inoculation with PGPR on nodulation and growth of common bean

Co-inoculation of common beans with *Rhizobium* and *Trichoderma* has been shown to improve the nodulation of legumes, both in terms of the number of nodules and their effectiveness. A study in Malawi by Mweetwa *et al*. (2016) reported nodule effectiveness of up to 88% when common bean plants were co-inoculated with *Rhizobium* and *Trichoderma*. The authors reported that there was no significant difference between unamended controls and the single inoculations with either *Rhizobium* or *Trichoderma*. A similar study by Chilombo (2019) showed that single inoculation with *Trichoderma* or *Rhizobium* did not have a significant difference from the control. However, *Rhizobium* + *Trichoderma* co-inoculation showed a significant increase in the number of nodules per plant. The same trend was also reported in terms of nodule effectiveness, suggesting that *Trichoderma* increases the number of nodules by increasing infection sites through the extension of the root system of the plant.

A higher nodule numbers and nodule dry weights were reported when common beans were inoculated with a consortium of indigenous rhizobia strains compared to the inoculation with com- mercial rhizobia (CIAT 899) strain (Ouma *et al*., 2016). Additionally, the authors found that significantly highest shoot dry weight was achieved when the common bean was co-inoculated with a mixture of the indigenous rhizobia strains and the CIAT 899 than when CIAT 899 was singly applied. Studies on the mixed inoculation of native *Rhizobium* consortia and exotic/ commercial rhizobia strains have been carried out by other researchers in Kenya. A field study by Menge *et al*. (2018a) showed that the highest and significant nodule dry weight and shoot dry weight were achieved when the common bean was co-inoculated with a mix of native consortium + commercial rhizobia compared to inoculation with single commercial rhizobia strains. A greenhouse study by Menge *et al*. (2018b) showed that co-inoculation of common beans with a mixture of native and exotic rhizobia isolates significantly recorded the highest number of nodules and nodule dry weight. The study results showed that a significantly higher shoot dry weight was observed upon co-inoculation with a mixture of native consortium + exotic rhizobia when compared with single exotic and native rhizobia inoculations. A greenhouse and field study by Gicharu *et al*. (2013) showed that co-inoculation of climbing common bean varieties with a consortium of rhizobia strains produced significantly higher nodule numbers and yield than the control treatment. Babu *et al*. (2015) showed that co-inoculation of *Rhizobium* spp. and PGPR agent significantly improved nodulation, shoot and root dry matter, and grain yield in chickpea, and they further observed that that inoculation of isolates of PGPR and cyanobacteria improved growth of chickpea, pea, and lentil with an increase in hydrolytic enzyme activity. Sánchez *et al*. (2014) reported that co-inoculation of common bean variety DOR-364 with *R. pisi (R40982)* + *P. monteilii* (R43453) increased nodule number and nodule dry weight by 55% and 133%, respectively over single inoculation with *R. pisi (R40982)*.

Co-inoculation of *Rhizobium* and *Pseudomonas* strains have also been reported to increase nodulation, leaf chlorophyll content and other growth factors under greenhouse conditions (Samavat *et al*., 2012). Rajendran *et al*. (2012) reported an increased nodulation and root weight when common bean was co-inoculated with rhizobia together with other nodule associated bacteria. Similarly, Korir *et al*. (2017) demonstrated that the nodulation, growth and biological nitrogen fixation of common bean was enhanced as a result of co-inoculation of rhizobia with other nodule endophytic bacteria in the greenhouse. A study by Karanja *et al*. (2007) showed that the highest shoot dry weight was recorded in the K194 + USDA 2674 and K67 + CIAT 899 co-inoculations, while the least was recorded in the controls. All the isolates

of *Bacillus subtilis* and their combinations with *Rhizobium* strain USDA 2674 caused an increase in shoot dry weight. The total shoot dry matter of plants inoculated with rhizobia and other bacteria was significantly higher than the uninoculated and those inoculated with rhizobia alone. Similarly, no significant difference was reported between the shoot dry weight of common beans inoculated with rhizobia alone and the uninoculated ones as compared to co-inoculation (Wekesa *et al*., 2016). Research findings from pot and field experiments by Kumar *et al*. (2016) showed that three PGPR strains applied singly or in combination increased the germination, plant height and weight, and nodulation compared to the control. However, co-inoculation of the strains resulted in more efficient plant growth promotion than the single application.

A study by Pastor-Bueis *et al*. (2021) reported enhanced nodulation and nodule function of common bean due to the co-inoculation with *Rhizobium* and *Pseudomonas brassicacearum* subsp. *Neoaurantiaca* enhances nodulation and nodule functions as compared with *Rhizobium* alone. Similarly, a study by Ferreira *et al*. (2020) reported that co-inoculation of common bean significantly increased the shoot and root biomass, nodulation, and improved nutrient uptake of common bean compare to single-inoculated or control plants. Various other studies reports that co-inoculation of rhizobia with PGPR have allowed higher rates of nodulation, increased rhizobia survival, and higher presence of the inoculant in the nodules, which leads to an increase in nitrogen fixation effectiveness (Nascimento *et al*., 2012; Sarma & Saikia, 2014; Schwartz *et al*., 2013).

2.7.2 Influence of co-inoculation with PGPR on nitrogen and phosphorous concentration of common bean

Phosphorus nutrition is crucial in the nodule formation by rhizobia (Remans *et al*. 2007) and therefore, co-inoculation with PGPR possessing P solubilization in soils is vital. The PGPR belonging to the genus *Bacillus* have been reported to be an efficient phosphate solubilizer. A greenhouse study by Korir *et al*. (2017) showed that co-inoculation of rhizobia strains with PGPR enhanced the growth of common beans in phosphorous-deficient soil. There was an increased number of nodules and nodule weight due to co-inoculation of rhizobia strains with *Paenibacillus polymyxa* and *Bacillus megaterium* in respect to inoculation with *Rhizobium* alone in common beans. Furthermore, findings from the study showed that co-inoculation of common beans with *Rhizobium* and PGPR significantly increased shoot and root dry weights of plants in comparison to *Rhizobium* inoculation alone and the uninoculated control (Korir *et al*., 2017). Furthermore, a study carried out by Muthamia *et al*. (2013) to test the efficiency of

Rhizobium and *Azospirillum* co-inoculation in low and high phosphorous (P) soils, showed that there was an improvement in biomass and grain yield both at low P and high P soils in both bean varieties under study. The improvements were higher than any individual *Rhizobium* or *Azospirillum.* Elkoca *et al*. (2010) noted that triple inoculation with *Bacillus megaterium*, *Bacillus subtilis*, and *Rhizobium leguminosarum* significantly increased macro and micronutrients uptake and improved the yield of *P. vulgaris*. Menge *et al*. (2018a) reported that co-inoculation of common bean with multi-strain consortia of both native consortium and exotic rhizobia recorded the highest shoot nitrogen.

2.7.3 Influence of co-inoculation with PGPR on the synergy and effectiveness of soil microorganisms

Mixed PGPR strains survive through synergistic effects with other microbes (Enebe & Babalola, 2018; Maheshwari, 2012). A study by Kumar *et al*. (2016) reported a synergistic effect of *P. putida* and *B. amyloliquefaciens*, and there was no alteration of the microbial population with their application. The competition also enhances the survival of some PGPR strains in mixed biofertilizers. Jesus *et al*. (2018) showed that co-inoculation of *Bradyrhizobium* and *R. tropici* was competitive, with *R. tropici* competing with *Bradyrhizobium* for nodule infection. Dardanelli *et al*. (2008) observed a positive effect on co-inoculation of *Rhizobium tropici CIAT899* and *Rhizobium etli* ISP42 with *Azospirillum brasilense* on the expression of nod-gene and nodulation and benefited the plant by enhancing root branching and acetylene reduction activates.

2.7.4 Influence of co-inoculation with PGPR on yield and yield of legumes

Co-inoculation of common bean with rhizobium *leguminosarum* bv. *phaseoli* strains and PGPRs (*Pseudomonas brassicacearum* subsp. neoaurantiaca strain RVPB2-2 or the *Azotobacter chroococcum* type strain (ATCC 9043T) increased seed yield by 37% and 28%, respectively, compared to the control (Pastor-Bueis *et al*., 2021). Results by Abd El-Azeem (2022) showed that the co-inoculation of *Rhizobium* and PGPR significantly increased the yield and yield components of common bean compared with single inoculation and the uninoculated control. Horácio *et al*. (2020) reported grain yield increase ranging from 62-84% in common bean after double and triple co-inoculation of rhizobia with azospirilla and/or cyanobacteria. Yadegari *et al*. (2010) reported seed yield production from co-inoculation of *Rhizobium* + *P. fluorescens* P-93 with a significant increase of up to 73% over *Rhizobium* alone. Similarly, Hungria *et al*. (2013) also established that seed inoculation of common bean with *Rhizobium tropici* alone increased yield by 14.7%, while co-inoculation with *A. brasilense* boosted the

yield by 19.6%. Additionally, Yadegari (2014) showed that there were significant increases in seed yield of common bean as a result of treatment combination of *rhizobium* strains and PGPR strains. A study by Wekesa *et al*. (2016) carried out in Western Kenya showed that the number of pods per plant inoculated with a mixture of rhizobia and other nodule-associated bacteria was significantly higher than that inoculated with rhizobia alone, and no significant difference was noted on the number of pods per plant between the uninoculated control and single rhizobia inoculation. The authors reported similar trends with the number of seeds per pod and the weight of pods per plant with co-inoculation with rhizobia and other bacteria recording significantly higher values than single rhizobia inoculation and the control. Field trials have shown that common bean co-inoculated with rhizobia and PSB had better higher pod yield to single inoculation (Abd El *et al*., 2022).

2.8 Biofertilizers

Biofertilizers represent a promising alternative for sustainable crop production in the 21st century (Allouzi *et al*., 2022; Fasusi *et al*., 2021; Mahmud *et al*., 2021; Nosheen *et al*., 2021). Biofertilizers, are organic products containing specific microorganisms sourced from plant roots and root zones. These biofertilizers can increase plant growth and yield by 10-40% (Bhardwaj *et al*., 2014; Stewart & Roberts, 2012). When applied to the rhizosphere and the interior of plants, these bioinoculants colonize the environment and promote plant growth (Nosheen *et al*., 2021). They enhance soil fertility and crop yield by adding nutrients to the soil, while also protecting plants from pests and diseases. Additionally, after 3-4 years of continuous use, biofertilizers may no longer be necessary as the initial inocula become self-sustaining (Bumandalai & Tserennadmid, 2019).

The commonly used biofertilizers include nitrogen-fixing bacteria and cyanobacteria, phosphate-solubilizing bacteria, molds, and mushrooms (Umesha *et al*., 2018). Biofertilizers are economically viable, environment friendly, ecologically sound, biologically feasible and easily acceptable by small and marginal farmers, and these can be used to supplement chemical fertilizers (Atieno *et al*., 2020; Daniel *et al*., 2022; Kour *et al*., 2020; Lu *et al*., 2020; Mącik *et al*., 2020; Sreethu *et al*., 2024). Therefore, application of beneficial microbiomes as biofertilizers in sustainable agriculture practices has emerged as innovative and environment-friendly technology for improving soil fertility and plant growth (Fasusi *et al*., 2021; Korir *et al*., 2023). Biofertilizers come in solid, liquid, polymer entrapped formulations, and fluidized bed dry formulations (Mącik *et al*., 2020).

2.9 Biofertilizer formulation

Despite several benefits brought by inoculation technology, a major limitation is survival of inoculum in soil and the rhizosphere. Incorporation of inoculum in different carrier materials may increase the efficacy of bacterial inocula and permit long term storage. Formulation is a key aspect for producing inoculants and can determine the success or failure of an effective biological agent (Ruíz-Valdiviezo *et al*., 2015; Vishwakarma *et al*., 2018). The optimal activity of microorganisms in the biofertilizers could be guaranteed with choosing the appropriate carrier (Bargaz *et al*., 2018). The preparation of inoculants is a vital multi-step process that includes one or more strains of microorganisms, a suitable carrier, and additives that ensure a safe environment for their protection under challenging conditions during storage, strain survival, transportation, and establishment after introduction into soils. The proper formulation ensures effective delivery of microorganisms to the target area, enhancing their activity once introduced to the host (Namasivayam *et al*., 2014; Nehra & Choudhary, 2015). Moreover, the selection of carrier materials should take into account both cost-effectiveness and availability (Bashan *et al*., 2014). Additionally, some inoculants fail to produce the same beneficial effects in the field, often due to improper formulation (Biradar & Santhosh, 2018; Vassilev *et al*., 2015). Therefore, the formulation of inoculants is crucial and must ensure high survivability of PGPR from storage to application (Soumare *et al*., 2019; Amenaghawon *et al*., 2021). Formulations can be either solid-based or liquid, with solid forms being either dry or wet, depending on requirements (Berger *et al*., 2018; Berninger *et al*., 2017; Oliveira *et al*., 2017).

2.9.1 Characteristics of an effective biofertilizer carrier materials

A good carrier material should support and enhance the shelf life of inoculants (Bhattacharyya *et al*., 2020; Malusa *et al*., 2012; Sahu *et al*., 2018). The full benefits of inoculation technology are realized when the inoculant is properly produced, formulated, and applied. Inoculants may fail to perform their specific functions if there are issues with their production and formulation (Biradar & Santhosh, 2018; Vassilev *et al*., 2015). Thus, inoculants need to create an environment that prevents a rapid decline in the introduced bacteria (Berninger *et al*., 2016; Liffourrena & Lucchesi, 2018; Shahzad *et al*., 2017). The aim of inoculant formulations is to ensure higher survival rates of plant growth-promoting rhizobacteria (PGPR) during storage and at the application site, in both suitable and available forms.

The choice of carrier material can significantly influence the shelf life, efficacy, and overall success of biofertilizer formulations. Effective carrier materials provide suitable

microenvironments that protect and sustain microbial populations over time. The key characteristics of carriers are their ability to deliver the right number of viable cells at the appropriate time (Sahu & Brahmaprakash, 2016). The characteristics of the carrier used to store the inoculants are important for the survival and symbiotic potential of the rhizobia. This involves establishing the microorganisms in a suitable carrier together with additives that aid in the stabilization and protection of the microbial cells during storage, transport, and at the target site (Xavier *et al*., 2004). Additional desirable traits for a good carrier include high water holding capacity, cost-effectiveness, availability, uniformity, absence of lump-forming materials, non-toxicity, easy biodegradability, sterilizability, good buffering capacity, support for bacterial growth and survival, nutrient supplementation capability, and ease of mixing and packaging (Sahu & Brahmaprakash, 2016). The chosen carrier should provide a protective and suitable environment for the microorganisms, ensuring long-term effectiveness. Additionally, carriers must have a neutral or near-neutral pH and be environmentally friendly (Buntić *et al*., 2019). A high-grade carrier should have high water retention and stable pH, and it should be cheap, nontoxic for the strain or environment, and easy to sterilize (Swelim *et al*., 2010). The effectiveness of a carrier material depends on its nutrient content and moisture-holding capacity. Carriers with high moisture retention, a low carbon: nitrogen (C:N) ratio, and a pH close to 7 are considered optimal for extending the shelf life of bioformulations (Arora *et al*., 2014; Sohaib *et al*., 2020).

Various carriers, both organic and inorganic, are used in the formulation process, and the strengths and weaknesses of each affect the overall quality and effectiveness of biofertilizers (Bashan *et al*., 2014). Solid formulations consist of powder or granules made by mixing microbial culture with cornstarch, gum acacia, gluten, wheat granules, molasses, charcoal, farm manure, fly ash, or wheat flour (Fathi *et al*., 2021; Meftah Kadmiri *et al*., 2021). Currently, various types of carrier materials are available, but selecting an appropriate one is crucial because the carrier material supports the survival of bioagents. Peat and filter mud/bagasse, a waste product of sugarcane, which is surplus in large quantities, are widely used as carrier materials (Balume, 2013; Santos *et al*., 2019; Tabassam *et al*., 2015). Peat is the most commonly used carrier for biofertilizers because it effectively supports microorganism growth and survival. However, due to high sterilization costs, difficulty in large-scale application, and processing challenges.

Liquid-based biofertilizers serve as an alternative to carrier-based formulations (Mącik *et al*., 2020). They are produced using broth culture, mineral and organic oils, oil-in-water, and polymer-based suspensions (Bharti *et al*., 2017). These formulations should include specific

cell protectants to help develop cysts and dormant spores (Raimi *et al*., 2021). Liquid biofertilizers are preferred over solid inoculants due to their long shelf life of 1.5-2 years, lack of contamination, no need for sticky materials, compatibility with modern machinery, ability to endure high temperatures up to 45°C, and ease of handling and application (Mahanty *et al*., 2017). Additionally, their higher microbial densities mean lower dosages are needed to achieve the same effect as solid inoculants (El-Ramady *et al*., 2018; Mącik *et al*., 2020). Liquid carrier-based inoculants contain desirable strains along with protectants that improve shelf life up to 19-25 months under stressful conditions (Chandra *et al*., 2018). In several liquid formulations, PGPR maintained at least the minimal number of viable cells (around 10^7 CFU/mL or CFU/g) for six months at room or refrigeration temperature (Anith *et al*., 2016; He *et al*., 2015; Valetti *et al*., 2016). The main advantages of liquid inoculants are their easier processing and lower costs compared to solid-based formulations (Kumaresan and Reetha, 2011), making them a significant portion of the inoculant market (Lee *et al*., 2016). Despite their extended storage potential, microorganisms in liquid biofertilizers can face nutrient depletion, hypoxia, and environmental stresses, leading to a decline in microbial populations. Therefore, special storage conditions, such as cool temperatures, are required (Lee *et al*., 2016; Mącik *et al*., 2020). The primary challenge is improving formulations to maintain the high quality of liquid inoculants (Lee *et al*., 2016). However, microbes in these formulations are vulnerable to abiotic stresses, mainly due to nutrient limitations and thermal shock, which reduce the population of viable cells (Berger *et al*., 2018; Bernabeu *et al*., 2018; He *et al*., 2015).

2.9.2 Influence of storage condition on survival of strains in biofertilizer

Storage conditions should be optimized to support long-term cell survival (Berger *et al*., 2018; Berninger *et al*., 2018). The storage temperature significantly impacts the survival and longevity of inoculants (Kaljeet *et al*., 2011; Sohaib *et al*., 2020). Numerous studies have shown that biofertilizers have a better shelf-life under refrigeration compared to room temperature, due to reduced metabolic and physiological activities (El-Fattah *et al*., 2013; Gade *et al*., 2014; Phiromtam *et al*., 2013; Sandikar & Awasthi, 2010; Thirumal *et al*., 2017). However, some inoculants can maintain higher viable bacterial cell counts at room temperature. For instance, Arora *et al*. (2008) found that filter mud could maintain high numbers of *P. fluorescens* (2.0×10^9 CFU g^{-1}) and *R. leguminosarum* (7.9×10^8 CFU g^{-1}) after six months of storage at room temperature (25±2°C). Since refrigeration facilities are often unavailable in developing countries, including most of sub-Saharan Africa, good survival of inoculants at room temperature is a highly desirable trait (Paudyal *et al*., 2021). The ability of

microorganisms to remain inactive and resistant to environmental stresses at higher temperatures likely contributes to their higher viable cell counts and insensitivity to contamination (Zayed, 2016). Therefore, formulations that can extend microbial survival at higher temperatures are advantageous as they reduce maintenance costs by eliminating the need for refrigeration (Melin *et al*., 2016). Bashan *et al*. (2014) suggested that inoculants should retain their viability within a storage temperature range of -5°C to 30°C to be effective under typical storage conditions used by farmers and agro-dealers. Inoculants that remain viable at room temperature can be easily integrated into existing agricultural distribution systems that lack refrigeration (Aloo *et al*., 2022).

Microbial survival, carrier material qualities, biological effectiveness, and product storage life are all influenced by storage conditions such as temperature, humidity, and sunlight intensity (Krishnaprabu, 2020). The carrier must maintain the viability of microorganisms during storage in the farmer's warehouse and have a long shelf life and stability (Bashan *et al*., 2016). One major issue with biofertilizers is their shelf life. Live microbial cells used in biofertilizers typically have a limited shelf life of about 4-6 months at 20-25°C, necessitating careful storage and transportation, which increases the product's cost (Mitter *et al*., 2021). Proper storage, especially at the correct temperature, is crucial to prevent the number of viable bacterial cells in the biofertilizer from dropping below 10^8 $CFUmL^{-1}$ (Hoe *et al*., 2022). The optimal storage temperature for biofertilizers has been identified as 4°C for efficiency and extended shelf life (Raimi *et al*., 2021). For instance, *Azotobacter venelandi* NDD-CK-1 can be stored at 5°C for up to 90 days, suggesting that low-temperature storage prolongs shelf life (Raimi *et al*., 2021). The stability and quality of biofertilizers are highly dependent on storage conditions (Phiromtan *et al*., 2013).

2.10 Gaps in literature

In summary, there is a lack of information on the mechanisms of action of the rhizobia and phosphate-solubilizing bacteria (PSB) strains isolated from Kenya. Further research and field trials for locally isolated strains of plant growth-promoting rhizobacteria (PGPR) are needed to optimize co-inoculation strategies, identify effective microbial strains, and evaluate their performance under different agroecological conditions. Additionally, the choice of carrier material can significantly influence the shelf life, efficacy, and overall success of biofertilizer formulations. Understanding the properties and impacts of different carriers helps in developing more robust and effective biofertilizers for sustainable agricultural practices.

Moreover, the effects of formulating inoculants from these native bacterial strains have not been thoroughly explored or documented. This gap in knowledge has made it challenging to produce effective biofertilizers from native strains that could be readily made available to farmers to enhance bean production. Addressing these research gaps will be crucial for developing targeted biofertilizer solutions that can improve crop yields and contribute to sustainable agriculture in Kenya and similar regions.

CHAPTER THREE

INDOLE ACETIC ACID PRODUCING AND PHOSPHATE SOLUBILIZING BACTERIA ISOLATED FROM KENYAN SOILS PROMOTES GROWTH OF COMMON BEAN (*Phaseolus vulgaris* L.)

Abstract

The use of phosphate solubilizing bacteria (PSB) and nitrogen fixing rhizobia can have a positive effect on the growth of common bean. This study aimed at determining the mechanisms of action of native bacterial strains; and to determine their potential to enhance bean growth. The strains were screened for their ability to solubilize insoluble inorganic phosphates and production of indole acetic acid (IAA) *in vitro*. A greenhouse experiment was set up to evaluate the response of common bean to inoculation with selected bacterial strains. Six of the ten bacterial isolates namely *R. pusense* (S5), *R. phaseoli* (B3), *R. pusense* (B4), *R. pusense* (B5), *R leguminosarum* (B2) and *R. phaseoli* (S4) tested showed a positive result for IAA production. *Rhizobium pusense* (S5) showed the greatest solubilization efficiency of 648 followed by *Bacillus megaterium* - HK2 (322.3) and *R. phaseoli* - S4 (308.7). *Rhizobium pusense*, (S5) *B. megaterium (HK2)* and *R. phaseoli* (S4) were classified as high phosphate solubilizers (SI > 3.0). while *P. polymyxa* (HK1), *R. phaseoli* (B3), *B. aryabattai* (HK3) and *B. megaterium (HK4)* were considered medium solubilizers (SI = 2.0-3.0). Inoculation of common bean with rhizobia and PSB resulted in a significant increase on the number of nodules per plant. The highest shoot biomass was observed when *R. phaseoli* (B3) was co-inoculated with *P. polymyxa* - HK1 (4.3 g plant^{-1}) compared to the single *R. phaseoli* inoculation (1.1 g plant^{-1}). Shoot tissue nitrogen and phosphorous concentration increased by up to 32% and 75% respectively as a result of co-inoculation of PSB with rhizobia. Bacterial strains tested in this study have capacity to produce IAA, solubilize P and specific rhizobia-PSB co-inoculation resulted in higher bean biomass than in single inoculation. Therefore, these strains are recommended for being formulated and used as inoculants that can be used under varying field conditions.

3.1 Introduction

Over 400 million people in Sub-Saharan Africa (SSA) depend on common bean as a primary staple food (CIAT, 2018). The production of common bean (*Phaseolus vulgaris* L.) is, however, constrained by low soil fertility in many soils leading to a threat to food security. The low level of production in SSA has been attributed partly to low levels of soil plant-available

P and drought stress, caused by climate change variability (Beebe *et al*., 2013). Phosphorous is one of the most deficient nutrients for cultivation of common bean (Lazali *et al*., 2017) because of the high P fixing soils (Bhattacharyya & Jha, 2012; Collavino *et al*., 2010; Menezes-Blackburn *et al*., 2018). Phosphorous availability improves the growth productivity of common bean by enhancing the symbiotic efficiency of the common bean (Samago *et al*., 2018).

One area of increasing research interest is the use of microorganisms which act through a number of mechanisms such as nitrogen fixation, solubilization of phosphorous, production of indole acetic acid, cytokinins among others that facilitate nutrient acquisition by the plant (Shiri-Janagard *et al*., 2012; Uribe *et al*., 2012). Various soil microorganisms produce auxin, indole-3-acetic acid (IAA) as a direct mechanism to promote plant growth (Spaepen *et al*., 2007). In particular, plant growth promotion and root nodulation are both affected by IAA. Bacterial IAA increases root surface area and length, and thereby provides the plant with greater access to soil nutrients. In addition, bacterial IAA loosens plant cell walls facilitating increased amount of root exudations that provides additional nutrients to support the growth of beneficial rhizosphere bacteria (Glick, 2012). Most rhizobia strains produce IAA and several studies have suggested that increases in auxin levels in the host plant are necessary for nodule formation (Glick, 2012). Nodule bacteria including *Rhizobium leguminosarum, R. undicolam, R. etlii, Sinorhizobium meliloti, R. phaseoli, R. pusense* among others synthesizes IAA, thereby playing an important role in legume-rhizobia interaction (Chaundhary *et al*., 2021; Shoukry *et al*., 2018).

The soil holds large amounts of phosphate most of which are insoluble. Phosphate solubilizing bacteria (PSB) are reported to solubilize the phosphate in the soil through acidification, chelation, or enzymatically (Khan *et al*., 2010). These microorganisms mineralize organic phosphorus in soil by solubilizing complex-structured phosphates such as tricalcium phosphate to inorganic forms available to plants. Many of the PSB lower the pH of the medium by secretion of organic acids such as acetic, lactic, malic, succinic, oxalic and citric acids (Patel *et al*., 2015). The ability of some microorganisms to convert insoluble phosphates to plant-available forms is an important characteristic for increasing plant yields (Sharma *et al*., 2013). Research has reported that some species such as *Bacillus megaterium*, *Bacillus circulans*, *Bacillus coagulans*, *Bacillus subtilis*, *Paenibacilus polymyxa*, *Bacillus sircalmous*, and *Pseudomonas striata* are the most important strains (Anand *et al*., 2013; Goswami *et al*., 2013; Govindasamy *et al*., 2011; Muleta *et al*., 2013; Yegorenkova *et al*., 2016).

Although the role of phosphorus in nodulation, nitrogen fixation and growth of common bean has been reported, the role of phosphate-solubilizing bacteria in phosphorus availability,

growth promotion and also their interaction with N_2-fixing bacteria under tropical conditions requires thorough investigation. The indole acetic acid production and phosphate solubilization capacity of the native rhizobia and PSB need to be understood in order to select highly effective strains that can be exploited for inoculant production. The objective of this study was to assess IAA production and phosphate solubilization efficiency of locally isolated endophytic bacteria and to test their efficacy in growth promotion of common beans under greenhouse conditions.

3.2 Materials and methods

3.2.1 Bacterial isolates description

Seven native plant growth promoting rhizobacteria (PGPR) strains were obtained from Soil Microbial Ecology Laboratory, Egerton University from the isolation work done earlier by Korir *et al*. (2017). The PGPR were isolated from root nodules of common beans and molecularly identified as t belonging to the genera Bacillus and Paenibacillus. The strains used in this study were, *Paenibacillus polymyxa* HK1 (Accession number: NC_014483.1), *Bacillus megaterium* strain HK2 (Accession number: KF658192.1), *Bacillus aryabhattai* strain HK3 (Accession number; KJ009477.1), *Bacillus megaterium* HK4 (Accession number: JF496300.1), *Bacillus subtilis* HK5 (Accession number; KJ496376.1), *Bacillus megaterium* HK6 (Accession number; KC441754.1) and *Bacillus sp* HK7 (Accession number: AB508884.1). Ten rhizobacteria strains isolated from bean growing areas of Busia and Bungoma characterized by Wekesa *et al*. (2021) were also used in this study. The strains *Pseudomonas sp* (B1), *R. leguminosorum* (B2), *R. phaseoli* (B3), *R. pusense* (B4), *R. pusense* (B5), *Pseudomonas sp* (S1), *Paenibacilus sp* (S2), *R. leguminosorum* (S3), *R. phaseoli* (S4) and *R. pusense* (S5) were coded as S1-S5 for the different isolates from Busia and B1-B5 for the isolates from Bungoma Counties, respectively.

3.2.2 *In vitro* screening of indole-3 acetic acid (IAA) production

Indole-3 acetic acid (IAA) production was analysed using modified colorimetric method as described by Hung and Annapurna (2004). Ten bacterial isolates (*Pseudomonas sp* (B1), *R. leguminosorum* (B2), *R. phaseoli* (B3), *R. pusense* (B4), *R. pusense* (B5), *Pseudomonas sp* (S1), *Paenibacilus sp* (S2), *R. leguminosorum* (S3), *R. phaseoli* (S4) and *R. pusense* (S5) were grown in yeast extract mannitol broth supplemented with tryptophan (0.1%) and incubated at 28°C for 2 to 3 days in a shaking incubator replicated three times. Then, 3 ml of the log phase broth culture (10^9 cfu ml^{-1}) was centrifuged at 7826 x g for 15 minutes and 2 ml of the cell-free supernatant was transferred to a clean dry tube (15 ml capacity) to which 1

ml of 10 mM orthophosphoric acid and 4 ml of Salkowsky's reagent (1 ml of 0.5 M $FeCl_3$ in 49 ml of 35% Perchloric acid) was added and incubated in the dark at ambient temperature (25°C) for 25 minutes. The pink colour development was compared to the blank (sterile Luria Bertani broth with 0.1% of tryptophan and reagents) at wavelength of 530 ηm and the absorbance was used as an index of IAA production. Absorbance values of greater than 0.85 was considered high while those lesser than 0.85 considered low (Hung & Annapurna, 2004).

3.2.3 *In vitro* phosphate solubilization

The solubilization capacities of the ten rhizobia and seven PSB strains were tested on Pikovskaya's medium (Nautiyal, 1999). Pikovskaya's medium (containing l^{-1}: glucose, 10 g; $Ca_3(PO_4)_2$, 5 g; $(NH_4)_2SO_4$, 0.5 g; NaCl, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; KCl, 0.2 g; yeast extract, 0.5 g; $MnSO_4 \cdot H_2O$, 0.002 g; and $FeSO_4 \cdot 7H_2O$, 0.002 g) is commonly used to culture microorganisms, especially phosphate-solubilizing bacteria and fungi, from soil samples. It provides a suitable environment for these microorganisms to grow and express their phosphate solubilization activity (Nautiyal, 1999). The media preparation is outlined in Appendix 1. The the medium was mixed well and poured into sterile petri dishes under aseptic conditions to avoid contamination and let to solidify. Using a sterile inoculation loop, a single colony was picked from a pure culture of the bacterial strain and spot inoculated on the Pikovskaya's agar plates. The strains were grown in the medium in triplicate. The growth and solubilization diameter were determined after incubation at 28 ± 2°C for seven days. The growth diameter was measured using a vernier calliper. The size of the halo of solubilization was obtained by subtracting the value of the colony diameter from the total halo solubilization diameter. On the basis of diameter of clearing halo zones, solubilization efficiency (SE) and solubilization index (SI) were calculated as described by Edi-Premono *et al*. (1996) using the following formulae:

$$\mathrm{SE} = \left(\frac{\mathrm{SD}}{\mathrm{GD}}\right) \times 100 \quad \text{……Equation 1}$$

$$SI = \frac{CL+HD}{CD} \quad \text{……..Equation 2}$$

Where SE= solubilization efficiency; SI= solubilization index; SD = Solubilization diameter; GD = Growth diameter; CL = Colony Diameter; HD= Halozone Diameter

The solubilization capacity was evaluated according to the scale outlined by Matos *et al*. (2017), where SI values below 1.0 were categorized as very low solubilizers, values between

1.0 and 2.0 as low solubilizers, values from 2.0 to 3.0 as medium solubilizers, and values exceeding 3.0 as high solubilizers.

3.3 Greenhouse experiment

The plant growth promotion of the legume and stimulation of nodulation were tested for strains that showed capacity for phosphate solubilization and IAA production. Four rhizobia (*R. pusense* (B5), *R. pusense* (S5), *R. pusense* (B4), *R. phaseoli* (B3)), and three PSB (*P. polymyxa*, *B. megaterium* and *B. aryabhattai*) strains that exhibited high IAA production absorbance (>0.85) and phosphate solubilization efficiency > 2.0 respectively were used in the greenhouse study.

3.3.1 Inoculum preparation

Rhizobia inoculum was prepared in yeast extract mannitol (Appendix 1) medium and the PSB in Pikovskaya's medium (Nautiyal, 1999). Thus, bacterial cultures were inoculated in 500 mL conical flasks containing 150 ml of either the YEM or Pikovskaya's medium and incubated at 28 ±2^{o}C under shaking for three days to give an optical density of 0.5.

3.3.2 Treatment structure in the greenhouse

The experiment was conducted following guidelines by Figueiredo *et al*. (2008). Microbe-free vermiculite was obtained by autoclaving for 30 min at 121°C and 101 KPa, once a day for three consecutive days to ensure maximum sterilization. Pots of 5.3 L capacity (15.0 cm inner diameter and 30 cm length), were filled with 3 kg of cooled heat-treated vermiculite. Two bean seeds (*Chelalang* variety) were planted per pot and thinned to one seedling per pot one week later, after which it was inoculated as per treatment i.e. un-inoculated, un-inoculated + inorganic NP source, PSB, rhizobia and combination of PSB and rhizobia (1:1). For single inoculation, 1ml of broth culture containing rhizobia or PSB (10^{9}cfu ml^{-1}) was inoculated per plant. For the co-inoculation, 0.5 ml of YEM broth containing a rhizobia (10^{9}cfu ml^{-1}) plus 0.5 ml of nutrient broth containing the phosphobacteria (10^{9}cfu ml^{-1}) was applied per plant. Ten milliliters of nitrogen-free nutrient solution containing (KH_2PO_4, $FeC_6H_5O_7.H_20$, $MgSO_4$, $MnSO_4.H_2O$, H_3BO_3, $ZnSO_4.7H_2O$, $CuSO4.5H_2O$, $CoSO_4.7H_2O$, and $Na_2MoO_2.2H_2O$) was applied to the pots once a week until flowering started (Broughton and Dilworth, 1970). The treatment structure is shown in Table 3.1. The experiment was laid out in completely randomized design (CRD) with three replicates. The pots were watered regularly to maintain the substrate at field capacity. For the un-inoculated + inorganic NP source treatment, 0.25g of DAP per pot was mixed with the vermiculite at planting to supply 22.5 kg Nha^{-1}.

3.4 Data collection

At 50% flowering, the plants were uprooted to record number of nodules (NN). For evaluating the nodulation, the vermiculite was emptied from the pots gently and the whole plant carefully separated from adhering vermiculite. In the laboratory, the vermiculite from the roots were gently removed by washing under a tap water ensuring that the nodules were intact. Using a sharp scalpel the nodules were detached form the root hairs and cut cross-sectionally to check on the internal colouration. The nodules that were pinkish-red in colour were the counted and recorded. The nodule dry weight (NDW), shoot dry weight (SDW) and root dry weight (RDW) were determined by taking their weights on a weighing scale after drying at 65°C for 72 hours to a constant weight. The shoot samples were then ground using a grinder to a fine powder and analysed for total nitrogen and total phosphorous accumulation using the Kjeldahl and vanadomolybdophosphoric acid colorimetric methods, respectively, following procedure outlined by Okalebo *et al*. (2002).

Table 3.1: Treatment structure for the greenhouse co-inoculation study

Treatment ID	Description
TRT-1	*R. pusense* (B5)
TRT-2	*R. phaseoli* (B3)
TRT-3	*R. pusense* (B4)
TRT-4	*R. pusense* (S5)
TRT-5	*P. polymyxa* (HK1)
TRT-6	*B. aryabhattai* (HK3)
TRT-7	*B. megaterium* (HK4)
TRT-8	*R. pusense* (B5) + *P. polymyxa* (HK1)
TRT-9	*R. pusense* (B5) + *B. aryabhattai* (HK3)
TRT-10	*R. pusense* (B5) + *B. megaterium* (HK4)
TRT-11	*R. phaseoli* (B3) + *P. polymyxa* (HK1)
TRT-12	*R. phaseoli* (B3) + *B. Aryabhattai* (HK3)
TRT-13	*R. phaseoli* (B3) + *B. megaterium* (HK4)
TRT-14	*R. pusense* (B4) + *P. polymyxa* (HK1)
TRT-15	*R. pusense* (B4) + *B. aryabhattai* (HK3)
TRT-16	*R. pusense* (B4) + *B. megaterium* (HK4)
TRT-17	*R. pusense* (S5) + *P. polymyxa* (HK1)
TRT-18	*R. pusense* (S5) + *B. aryabhattai* (HK3)
TRT-19	*R. pusense* (S5) + *B. megaterium* (HK4)
TRT-20	Uninoculated + inorganic NP source (DAP)
TRT-21	Uninoculated control

3.5 Data analysis

Data on *in vitro* IAA production and phosphate solubilization (SE and SI) was subjected to analysis of variance (ANOVA) and the means separated using Tukey's honestly significant difference ($\alpha = 0.05$). For the greenhouse experiment, data was first tested for normal distribution and the count data on nodule number was log transformed (Log_{10} x+1) before analysis so as not to violate the assumptions of ANOVA (Payton *et al*., 2006). To determine the effects due to inoculation, analysis of variance at $p < 0.05$ was done and means separated using Tukey's test at $\alpha = 0.05$. Orthogonal contrast to test for significant effects between single and co-inoculation and between the inoculation and the controls (positive and negative) were done. Data was analysed using SAS Statistical Package Version 9.3 (SAS 2013).

Statistical model for the greenhouse study:

$\mathbf{Y_{ijk} = \mu + \alpha_i + \beta_j + \Sigma_{ijkl}}$ ……Equation 3

μ = overall mean

α_i = Effect due to the i^{th} replication

β_j = Effect due to the j^{th} treatment

Σ_{ijk} = Random experimental error

3.6 Results

3.6.1 Production of Indole-3 acetic acid (IAA) by *Rhizobium* species

There was significance difference on the concentration of IAA produced by the different Rhizobia isolates. Four out of the ten bacterial isolates tested were considered high IAA producers (>0.85) while the rest were low producers. From the four high IAA producers, *R. pusense* (Busia) and *R. phaseoli* (Bungoma) produced higher levels of IAA with absorbance values of 1.33 and 1.14 respectively compared to the other isolates (Figure 3.1). Some of the isolates produced negligible amount of IAA (Figure 3.1). The colour development showed that the Rhizobia isolates had different ability to produce IAA (Plate 3.1).

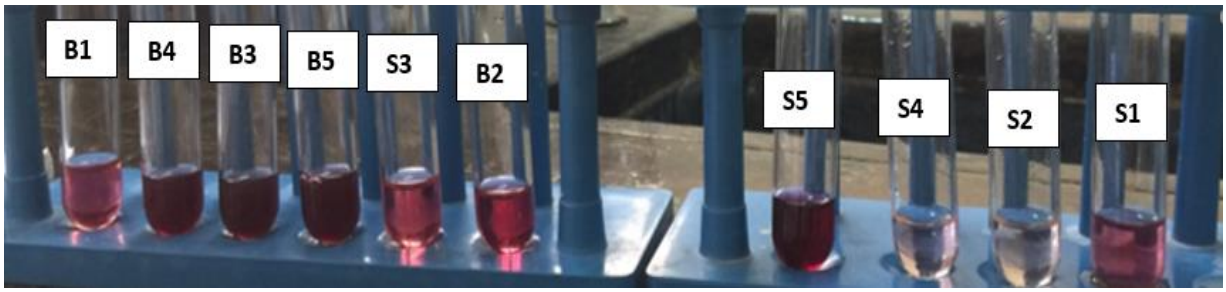

Plate 3.1: Colour development showing the intensity of IAA production by different Rhizobia isolates

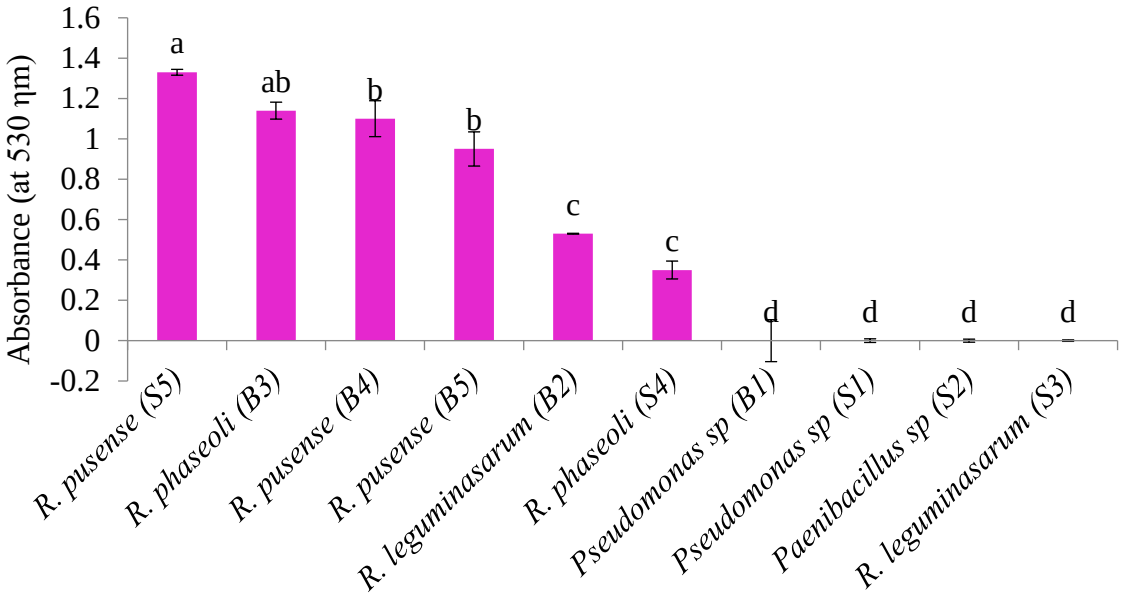

Figure 3.1: Absorbance (at 530 ηm) values for different rhizobia species using colorimetric method. Letters in brackets represent the location of isolation; S- Busia and B- Bungoma; and the numbers are codes given to the isolates. Means followed by different letters are significantly different from each other at $\alpha \leq 0.05$.

3.6.2 In vitro phosphate solubilization by the bacterial strains

Significant ($p<0.05$) differences in phosphate solubilization were observed among the different bacterial isolates (Table 3.2). *Rhizobium pusense* (Busia) showed the greatest solubilization efficiency of 648 and consequently the highest solubilization index of 7.3 (Table 3.2). It was followed by *B. megaterium* and *R. phaseoli* with an SE of 322.3 and 308.7 respectively. Based on the classification scale by Matos *et al*. (2017), *R. pusense*, *B. megaterium* and *R. phaseoli* (S5) were classified as high solubilizers (SI>3.0). *Paenibacillus polymyxa*, *R. phaseoli* (B3), *B. aryabattai* and *B. megaterium* were considered medium solubilizers (SI= 2.0-3.0); while the rest of the isolates were low solubilizers (Table 3.2). Plate 3. 2 shows the solubilization diameter of some of the isolates.

Table 3.2: Phosphate solubilization efficiency (SE) and index (SI) of the different bacterial isolates

Strain	SE	SI
R. pusense (S5)	648.0a	7.3a
B. megaterium (HK4)	322.3b	3.9bc
R. phaseoli (S4)	308.7b	4.2b
P. polymyxa (HK1)	171.7c	2.7bcd
Pseudomonas sp (B1)	145cd	2.4bcd
R. phaseoli (B3)	108.3cde	2.1cd
B. aryabhattai (HK3)	104.0cde	2.1cd
B. megaterium (HK2)	100.0cde	2d
R. leguminasarum (S3)	86.7de	1.9d
B. subtilis (HK5)	78.0def	1.8d
R. leguminasarum (B5)	74.7def	1.7d
R. pusense (B2)	64.7ef	1.6d
R. pusense (B4)	63.3ef	1.6d
Paenibacillus sp (S2)	50.0ef	1.5d
Bacillus sp (HK7)	49.3ef	1.5d
B. megaterium (HK6)	37.7ef	1.3d
Pseudomonas sp (S1)	0f	1d
Tukey's MSD ($\alpha \leq 0.05$)	79.2	1.8

Letters in brackets represent the location of isolation; S- Busia and B- Bungoma; and the numbers are codes given to the isolates. Means followed by different letters are significantly different at $\alpha \leq 0.05$ using. MSD- Minimum significant difference

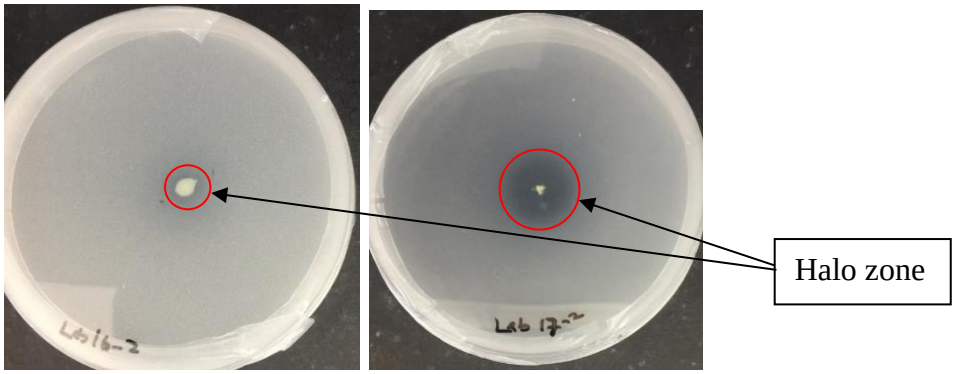

Plate 3.2: Zone of solubilization by some of the bacterial isolates showing the Halo zone (Lab 16-2 is *Rhizobium phaseoli* and Lab 17-2 is *Rhizobium pusense*).

3.6.3 Effect of co-inoculation with Rhizobia and PSB on the nodulation of common bean

Inoculation of common bean with Rhizobia and PSB had a significant ($p \leq 0.05$) effect on the number of nodules per plant. The control treatment did not contain any nodules (Figure 3.2). In terms of the effect of co-inoculation, the results were varied depending on the specific Rhizobia-PSB interaction. Some of the interactions led to a synergistic effect on the nodulation while in other treatments, single inoculation with rhizobia elicited a higher nodulation (Figure 3.2). For instance, the co-inoculation of *R. pusense* (B2) with *B. aryabhattai* and *B. megaterium* led to significantly higher number of nodules compared to the single *R. pusense* (B2); whereas when co-inoculated with *P. polymyxa*, the nodule numbers are significantly lower (Figure 3.2). The co-inoculation of *R. phaseoli* (B3) with *P. polymyxa* and *B. aryabhattai* led to significantly high number of nodules than the single *R. phaseoli* (B3) inoculation. However, there was depressed nodulation when it was co-inoculated with *B. megaterium* (Figure 3.2). Single inoculation with *R. pusense* (S5) had significantly higher number of nodules compared to the co-inoculation with the PSB strains (Figure 3.2).

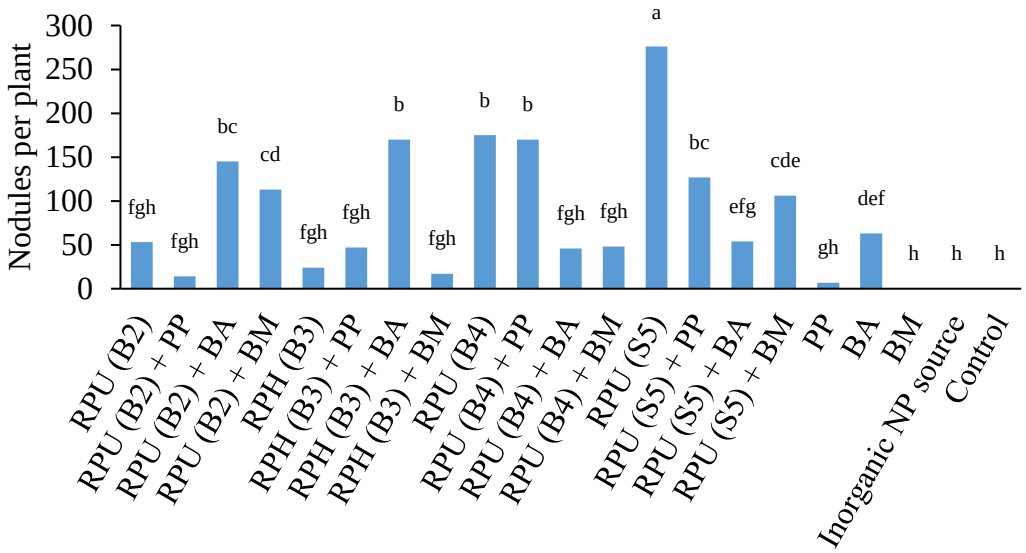

Figure 3.2: Effect of co-inoculation of common bean on number of nodules. Error bars represent the standard error of the means. RPU- *R. pusense*, RPH- *R. phaseoli*, BA- *B. aryabhattai*, BM- *B. megaterium*, PP- *P. polymyxa*

Similar to the number of nodules, inoculation of common bean with rhizobia and PSB had a significant ($p \leq 0.05$) effect on the nodules dry weight per plant (Table 3.3). In terms of the effect of co-inoculation, the results were varied depending on the specific rhizobia-PSB interaction. Some of the interactions led to a synergistic effect on the nodulation while in other treatments, single inoculation with Rhizobia elicited a higher nodulation (Table 3.3). For

instance, the co-inoculation of *R. pusense* (B2) with *B. aryabhattai* (HK3) and *B. megaterium* (HK4) led to significantly higher nodules dry weight compared to the single *R. pusense* (B2); whereas when co-inoculated with *P. polymyxa* (HK1) the nodule dry weights were significantly lower (Table 3.3). The co-inoculation of *R. phaseoli* (B3) with *P. polymyxa* (HK1) and *B. aryabhattai* (HK3) led to significantly high number of nodules than the single *R. phaseoli* (B3) inoculation. However, there the nodule dry weight was lower when it was co-inoculated with *B. megaterium*. Single inoculation with *R. pusense* (B2) and *R. pusense* (B4) had significantly higher nodule dry weight compared to the co-inoculation with the PSB (Table 3.3).

3.6.4 Effect of co-inoculation with Rhizobia and PSB on the shoot and root biomass of common bean

Inoculation of common bean with Rhizobia and PSB generally increased the shoot biomass compared to the control (Figure 3.3). Specific Rhizobia-PSB co-inoculation had varied influence on the shoot biomass of the common bean. Some of the interactions were synergistic while others led to a lower biomass compared to when singly applied (Figure 3.3). A positive interaction was observed in most of the interactions except for the *R. pusense* + *B. aryabhattai*, *R. pusense* + *B. megaterium*, *R. phaseoli* (B3) + *B. megaterium*; and co-inoculation of *R. pusense* with either *P. polymyxa* or *B. megaterium* (Figure 3.3). A noticeable positive co-inoculation effect was observed when *R. phaseoli* (B3) was co-inoculated with *P. polymyxa* (4.3g plant^{-1}) and with *B. aryabhattai* (3.4g plant^{-1}) compared to the single *R. phaseoli* inoculation with a biomass of 1.14 g plant^{-1} (Figure 3.3). Plate 3.3 shows a comparison on the above ground biomass of the bean crop between an inoculated and a control pot.

Inoculation of the common bean with the Rhizobia and PSB resulted in a significant ($p \leq 0.05$) increase in the root dry weight. The non-inoculated (control) treatment had significantly lower root mass (0.85 g plant^{-1}) compared to the inoculated treatment (Figure 3.4). In terms of the specific Rhizobia-PSB interactions, co-inoculation led to an increase in root biomass except for the *R. pusense* (S5) strain (Figure 3.4). Co-inoculation of *R. pusense* (B4) with *B. megaterium* resulted in significantly highest root biomass of 5.5g plant^{-1} (Figure 3.4).

Table 3.3: Effect of rhizobia and PSB strains inoculation on the nodule dry weight of common bean

Treatment	Nodule dry weight (mg/plant)
R. pusense (S5)	3.24a
R. pusense (B4)	1.83b
R. pusense (B4) + *P. polymyxa* (HK1)	1.80bc
R. phaseoli (B3) + *B. aryabattai* (HK3)	1.77bc
R. pusense (B2) + *B. aryabattai* (HK3)	1.50bcd
R. pusense (S5) + *P. polymyxa* (HK1)	1.32cd
R. pusense (S5) + *B. megaterium* (HK4)	1.18d
R. pusense (B2) + *B. megaterium* (HK4)	1.17d
B. aryabattai (HK3)	0.65e
R. pusense (S5) + *B. aryabattai* (HK3)	0.58ef
R. pusense (B2)	0.57ef
R. pusense (B4) + *B. megaterium* (HK4)	0.52ef
R. phaseoli (B3) + *P. polymyxa* (HK1)	0.48ef
R. pusense (B4) + *B. aryabattai* (HK3)	0.48ef
R. phaseoli (B3)	0.28efg
R. pusense (B2) + *P. polymyxa* (HK1)	0.18efg
R. phaseoli (B3) + *B. megaterium* (HK4)	0.18efg
P. polymyxa (HK1)	0.11fg
B. megaterium (HK4)	0.00g
Di-Ammonium Phosphate (DAP)	0.00g
Control	0.00g
MSD ($\alpha \leq 0.05$)	0.48

Letters in brackets represent the location of isolation; S- Busia and B- Bungoma; and the numbers are codes given to the isolates. Means followed by different letters are significantly different at $\alpha \leq 0.05$ using. MSD- Minimum significant difference

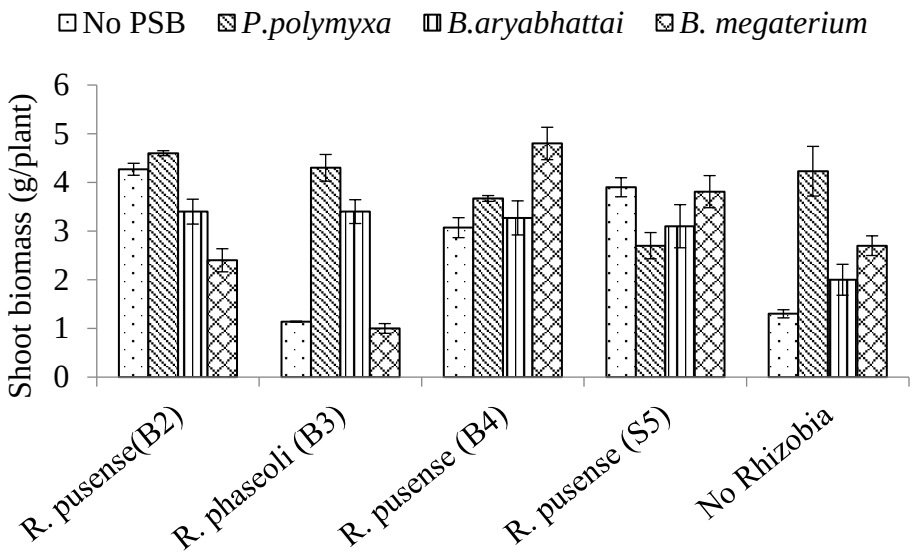

Figure 3.3: Effect of co-inoculation of common bean on the shoot biomass. Letters in brackets represent the location of isolation; S- Busia and B- Bungoma; and the numbers are codes given to the isolates. Error bars represent the standard error of the means

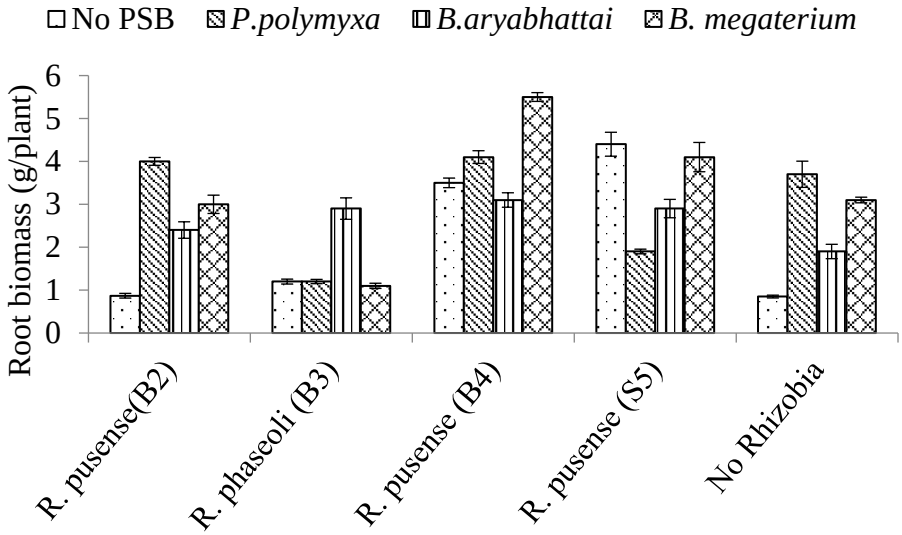

Figure 3.4: Effect of co-inoculation of common bean on the root biomass. Letters in brackets represent the location of isolation; S- Busia and B- Bungoma; and the numbers are codes given to the isolates. Error bars represent the standard error of the means

Plate 3.3: Growth of common bean as affected by inoculation *R. pusense* (B4) + *P. polymyxa* (Left) and the uninoculated and no-nitrogen (Right) at 35 days after inoculation.

3.6.5 Effect of co-inoculation on the tissue nitrogen (N) and phosphorous (P) concentration

Tissue N concentration was significantly affected by the inoculation of common bean with Rhizobia and PSB. Stimulatory effect was observed in specific Rhizobia-PSB interactions. For example, *R. phaseoli* + *B. aryabhattai* co-inoculation had significantly higher N concentration (2.38%) compared to the single *R. phaseoli* inoculation (2%) (Table 3.4). For the *R. pusense* co-inoculation, the highest N concentration was achieved when co-inoculated with *P. polymyxa* (2.38%). Similarly, co-inoculation of *R. pusense* (S5) with *P. polymyxa* led to significantly higher N concentration (2.73%) compared to the single *R. pusense* (S5) with 2.06% (Table 3.3). The highest tissue N concentration was observed in the *R. pusense* (B4) + *B. aryabhattai* co-inoculation (2.74%) that was significantly higher than the application of DAP with 2.48% N (Table 3.4).

For tissue P concentration, application of DAP resulted in the highest P accumulation of 0.98%. This was followed by co-inoculation of *R. pusense* (S5) + *P. polymyxa* (0.93%) despite the single *R. pusense* (S5) having the least %P concentration of 0.47% (Table 3.4). Co-inoculation of *R. phaseoli* (B3) with the PSB, *B. megaterium* led to significantly higher %P compared to the other two PSB and the single inoculation (Table 3.4). Similarly, *R. pusense* (B2) + *B. megaterium* had the highest %P concentration compared to the single inoculation and with the co-inoculation with *B. aryabhattai* and *P. polymyxa* (Table 3.4).

Table 3.4: Effect of rhizobia and PSB co-inoculation on the total tissue nitrogen and P concentrations in common bean

Treatment	Total N (%)	Total P (%)
	Singe inoculation	
Di-Ammonium phosphate (DAP)	2.48a	0.98a
R. pusense (B4)	2.36b	0.47b
B. megaterium	2.12c	0.59b
R. pusense (B2)	2.09cd	0.54b
R. pusense (S5)	2.06de	0.60b
B. aryabattai	2.03ef	0.48b
Negative control	2.02f	0.50b
P. polymyxa	2.00f	0.53b
R. phaseoli (B3)	2.00f	0.63b
Tukey's HSD ($\alpha \leq 0.05$)	0.04	0.35
	Co-inoculation	
R. pusense (B4) + *B. aryabattai*	2.74a	0.46g
R. pusense (S5) + *P. polymyxa*	2.73a	0.93b
R. pusense (B4) + *P. polymyxa*	2.49b	0.51f
Di-Ammonium phosphate (DAP)	2.48b	0.98a
R. phaseoli (B3) + *B. aryabattai*	2.38c	0.55e
R. pusense (B2) + *P. polymyxa*	2.38c	0.50fg
R. pusense (S5) + *B. aryabattai*	2.31d	0.65d
R. phaseoli (B3) + *B. megaterium*	2.10e	0.67d
R. pusense (B2) + *B. aryabattai*	2.09e	0.56e
R. pusense (S5) + *B. megaterium*	2.08e	0.72c
Negative control	2.02f	0.50fg
R. phaseoli (B3) + *P. polymyxa*	2.02f	0.49fg
R. pusense (B4) + *B. megaterium*	1.91g	0.51f
R. pusense (B2) + *B. megaterium*	1.55h	0.64d
Tukey's HSD ($\alpha \leq 0.05$)	0.05	0.02

Letters in brackets represent the location of isolation; S- Busia and B- Bungoma; and the numbers are codes given to the isolates. Means followed by different letters are significantly different from each other at $\alpha \leq 0.05$ for each of the single or co-inoculations analysis.

3.6.6 Selected orthogonal contrast

In terms of the number of nodules, there was significant contrast among all the selected orthogonal contrasts. There was a highly significant ($p<0.001$) contrast between the control versus the inoculations (single and co-inoculation) and between the co-inoculation and application of DAP (Table 3.5). In terms of the shoot dry weight, there were no significant contrasts between the single inoculation versus the co-inoculation; and between the co-inoculation versus DAP application. Compared to the control, there were significant contrasts with the inoculation (both the single and co-inoculation) (Table 3.4). For the root dry weight, all the selected contrasts were significant except for the single versus the co-inoculation (Table 3.5). The orthogonal contrast between all the selected contrasts were not significant except for the co-inoculation versus control on the concentration of tissue N (Table 3.5). There was a significant contrast between the co-inoculation versus the control, inoculation versus control, and the control versus the rest on the tissue P concentration. There was no significant contrast between the single inoculation versus the control; and the co-inoculation versus DAP application (Table 3.5).

Table 3.5: Mean square values for selected orthogonal contrasts

Contrast	NN	SDW	RDW	%N	%P
Single vs co-inoculation	1.49^{*}	0.41^{ns}	1.67^{ns}	0.17^{ns}	0.02^{ns}
Co-inoculation vs DAP	9.29^{***}	3.01^{ns}	12.32^{*}	0.01^{ns}	0.004^{ns}
Co-inoculation vs control	9.29^{***}	10.83^{*}	17.04^{**}	0.26^{*}	0.32^{***}
Single vs control	5.88^{***}	8.52^{*}	8.08^{*}	0.08^{ns}	0.02^{ns}
Inoculation vs control	10.45^{***}	14.74^{**}	26.49^{***}	0.11^{ns}	0.11^{**}
Control vs the rest	3.25^{**}	13.56^{**}	20.97^{**}	0.11^{ns}	0.08^{*}

NN- Number of nodules; SDW-Shoot dry weight; RDW-Root dry weight; +NP- inorganic nitrogen and phosphorous as Di-ammonium phosphate (DAP). Asterisks denotes the significance levels; *, **, *** significant at $p< 0.05$, 0.01 and 0.001 respectively. “The rest” include single, co-inoculation and DAP while “inoculation vs control” excludes DAP.

3.7 Discussion

3.7.1 IAA production and phosphate solubilization

The results from the present study showed that the tested bacterial strains were able to synthesize IAA *in vitro*. Six out of the eight of the bacterial isolates under study exhibited positive reaction by developing pink colour when reacted with Salkowski's reagent which indicates positive result for IAA production. Further, the results indicated that the microbes differed in their ability to produce IAA. This could be due to differences in the ability of the isolates to utilize the medium components for IAA production (Sridevi & Mallaiah, 2007). Earlier studies have shown that IAA production by microbes differed between different species or even within strains of the same species (Jasim *et al*., 2014; Özdal *et al*., 2016). From the eight bacterial species and strains tested in this study, the rhizobia produced higher levels of IAA compared to the *Paenibaccillus* species. This concurs with Tsavkelova *et al*. (2007) who reported that the strains of rhizobia are among the most active IAA producers.

The present study tested the *in vitro* phosphate solubilizing capacity of 17 bacterial isolates belonging to the genera *Rhizobium, Bacillus* and *Paenibacillus*. Fourteen of the 17 isolates were able to solubilize the insoluble tricalcium phosphate. The size of the solubilization varied among the bacteria isolates tested. The solubilization zone occurs due to the presence of some substances, such as organic acids, that are released by microorganisms into the medium that can form metal complexes with calcium, and thereby solubilize the P (Bashan *et al*., 2013). All the *Bacillus* strains tested in this study were able to solubilize the phosphate confirming what has been reported by previous studies indicating that they are efficient P solubilizers. For instance, Andrade *et al*. (2014), indicated that isolates of the genus *Bacillus* sp. were the most frequent P solubilizers and classified them as high efficiency solubilizers.

In addition to their beneficial nitrogen fixing activity with legumes, rhizobia can improve plant P nutrition by mobilizing inorganic and organic P. The present *in vitro* study showed that all the rhizobia species were able to form solubilization zones with the tricalcium phosphate. Notably, the present results showed that *Rhizobium pusense* (S5) recorded significantly highest solubilization efficiency and solubilization index compared to the *Bacillus* strains. Earlier studies have also shown rhizobia as efficient P solubilizers (Alikhani *et al*., 2006; Sridevi & Mallaiah, 2009). Yasmeen and Bano (2014) reported that the group of *Rhizobium leguminosarum* bv. *viciae* mobilized in liquid tri-calcium phosphate medium significantly released more P than other bacterial tested. This might be due to the production of acids in the growth media leading to the solubilization of the inorganic P (Alikhani *et al*., 2006).

3.7.2 Effect of co-inoculation of rhizobia and PSB on nodulation of common bean

Results from the present study showed that inoculation with rhizobia strains significantly affected the nodulation of common bean. This is in agreement with what has been previously reported by other authors (Horácio *et al*., 2024; Razafintsalama *et al*., 2022). The rhizobia strains used in this study were shown to have high IAA producing efficiency, thus resulting in enhanced nodule number and weights compared to the uninoculated control. Alemneh *et al*. (2020) reported that the number of effective nodules were increased when inoculated with IAA-producing rhizobia in pigeon pea. Inoculation of the four rhizobia strains and their co-inoculation with the three PSB generally increased nodules compared with the uninoculated control. Co-inoculation of common bean with rhizobia and PSB led to an increase in nodule number and nodule dry weight compared to the single rhizobia inoculation. This could be attributed to the multi-strain's ability to effectively nodulate and enhance solubilization of other essential soil minerals such as phosphorus (Koskey *et al*., 2017). Similar stimulatory effects on nodulation by co-inoculation of rhizobia and PSB has been reported by other authors (Benjelloun *et al*., 2021; Singh *et al*., 2011). On the other hand, the present study suggested that the co-inoculation of rhizobia and the PSB might not always increase nodules compared with the individual inoculation with one of the four rhizobia strains. While co-inoculation of PGPR typically results in positive effects on plant growth and health, there are several factors that could lead to a negative co-inoculation effect in plants. In some cases, the introduced PGPR strains may compete with each other for resources such as space, nutrients, or root colonization sites. This competition can limit the establishment and effectiveness of one or both PGPR strains, resulting in reduced plant growth promotion (Compant *et al*., 2019; Trivedi *et al*., 2020; Wan *et al*., 2020). Certain PGPR strains may produce allelopathic compounds or antimicrobial substances that inhibit the growth or activity of other PGPR strains present in the co-inoculant. These antagonistic interactions can negate the beneficial effects of co-inoculation on plant growth (Compant *et al*., 2019; Olanrewaju *et al*., 2017; Quin *et al*., 2015). Some PGPR strains may exhibit incompatible interactions with each other or with the host plant. Incompatibility can result in reduced nodulation, poor colonization of the rhizosphere, or even plant stress responses that negate the benefits of co-inoculation (Fitzpatrick *et al*., 2018; Kang *et al*., 2015; Vacheron *et al*., 2013). Environmental conditions such as soil pH, moisture levels, temperature, and nutrient availability can influence the success of PGPR co-inoculation. Suboptimal environmental conditions may limit the growth and activity of one or both PGPR strains, leading to a negative co-inoculation effect (Fierer, 2017; Mitter *et al*., 2021; Saleem *et al*., 2019).

These results have been reported previously in white clover by Matse *et al*. (2020), who showed that the co-inoculation of *Rhizobium* strains CHB1120 and *Azotobacter vinelandii* strain G31 significantly increased nodules of white clover compared with the individual inoculation of *Rhizobium* strains CHB1120, but the co-inoculation of *Rhizobium* strains CHB1121 and two PGPR significantly decreased nodules in comparison with the individual inoculation of *Rhizobium* strains CHB1121. This suggests that the compatibility between these two kinds of microorganisms should be evaluated before application. Earlier studies have been done to observe the interaction of mixed inoculation and the results showed that there was competition among the co-inoculated strains that may have resulted in improved expression of one or another depending on the superior competitor under conditions of the test (Pérez-Fernández *et al*., 2015; Pérez-Fernández & Valentine, 2017).

Results from this study showed that common bean in pots inoculated with either single *P. polymyxa* or *B. aryabhattai* developed nodules. Other than rhizobia, it was expected that the other bacterial strains will not elicit nodule formation. However, over the years, a vast number of bacteria other than rhizobia have been found in nodules (Korir *et al*., 2017; Leite *et al*., 2017; Pandya *et al*., 2013). A review by Martinez-Hidalgo (2017) highlighted that some of these non-rhizobial nodule endophytes have *nif* and *nod* genes and elicit nitrogen fixing on nodules just like rhizobia. A study by Gopalakrishnan *et al*. (2017; 2018) reported that all the three diazotrophic bacteria used in their study were found to have the nitrogen fixing genes that nodulated and enhanced nodulation in chickpea plants under greenhouse conditions. There is therefore a need to test more of the non-rhizobial nodule endophytes for their nitrogen fixing ability and presence of *nod* genes to further understand their mechanisms of plant growth promotion.

3.7.3 Effect co-inoculation of rhizobia and PSB on shoot and root weights of common bean

Results from the present study showed that inoculation of common bean with rhizobia generally increased the shoot biomass and root dry weight compared to the control. The improved growth of plants subjected to rhizobia inoculation is effectively attributed to its positive effect due to the symbiotic relationship between the rhizobia and the common bean (Bambara & Ndakidemi, 2010). Inoculation of seeds by phosphate solubilizing microorganisms is known to improve solubilization of insoluble phosphorus, which can therefore increase plant growth by enhancing the symbiotic efficiency of the common bean (Samago *et al*., 2018). In the present study, combined inoculation of the common bean with

rhizobia and PSB resulted in higher shoot and root dry weights compared to the single rhizobia inoculation. This can be attributed to better establishment of *Rhizobium*-legume symbiosis due to more secretion of plant growth promoting hormones, and improved nutrient availability especially P (Samago *et al*., 2018). Similarly, Khalifa and Almalki (2015) showed that co-inoculation of phosphate-solubilizing *B. megaterium* and *Sinorhizobium meliloti* had a positive effect on the growth of common bean.

Similar to the results on nodulation, some of the co-inoculation did not lead to improved growth as compared to the single inoculation. For instance, the shoot biomass of common bean co-inoculated with *R. pusense* and *B. megaterium* was significantly lower compared to their individual inoculations. This suggests that the two strains were not compatible. Other studies have shown the lack of positive effects of co-inoculation in respect to single rhizobial inoculation (Guiñazu *et al*., 2010; Rosas *et al*., 2006). Therefore, compatibility studies should be done before coming up with the right microbial consortia for formulations of biofertilizers to ensure enhanced crop growth and maximum benefits from the plant growth promotion of the introduced microorganisms.

3.7.4 Effect of co-inoculation of rhizobia and PSB on total N and P in shoots

Results from this study showed that inoculation of the common bean with Rhizobia strains increased the shoot N content compared to the uninoculated control. This could be attributed to the formation of nodules by rhizobia that stimulated biological nitrogen fixation by the crop. Similarly, de Souza *et al*. (2016) reported increased shoot N concentrations by rhizobia when common beans were inoculated with *R. leguminosarum* strains. Similarly, shoot P concentration was increased as a results of inoculation with the PSB and rhizobia. These findings are similar to those reported by Neila *et al*. (2014) who observed that native rhizobia increase shoot phosphorus in bean. The present study showed a stimulatory effect in specific Rhizobia-PSB interactions in the total N and P concentration in the plant tissues. For example, *R. phaseoli* + *B. aryabhattai* co-inoculation had significantly higher N concentration compared to the single *R. phaseoli* inoculation. The enhancement in total N and P content of shoot in present study might be due to increase of nitrogen and phosphorous acquisition due to altering root architecture and nodule formation in the crop (Egamberdieva *et al*., 2017). A study by Nimnoi *et al*. (2014) showed that the total N and P content of shoot was enhanced by co-inoculation of *Nocardia alba* strain S4301 with *Bradyrhizobium japonicum* USDA110 as compared to single inoculation of *Nocardia alba* strain S4301 and un-inoculated control treatments in soybean. The increased N content from the co-inoculation of Bacillus and

Rhizobia strains could also be attributed to the nitrogen fixing ability of Bacillus. A study on nitrogen fixing potential of diverse species of Bacillus has reported the presence of *nif* gene and hence the capability to fix atmospheric nitrogen (Ji *et al*., 2014; Yousuf *et al*., 2017).

3.8 Conclusions

This study showed that 40% of the locally isolated rhizobia strains have the capacity to synthesize IAA, an important auxin for plant growth promotion. Additionally, the study revealed that rhizobia species have different IAA producing ability. Similarly, the rhizobia and bacillus strains evaluated had the capacity to solubilize insoluble phosphate *in vitro* at varying degrees (SI ranging between 1.3-7.3). The greenhouse plant growth promotion assay showed that inoculation of common bean with the isolated bacteria led to increased bean growth and total shoot N and P. Therefore, phosphate solubilizing strains (*B. aryabhattai* and *P. polymyxa)* and nitrogen fixing bacterial strains (*R. phaseoli* (B3) and *R. pusense* (B4)) are recommended for being formulated and used as inoculants that can be used under varying field conditions to assess their efficacy in promoting crop growth, nutrient uptake, and contributing to sustainable agricultural practices and improved productivity in the region. Future research should also focus on optimizing inoculant formulations and exploring their potential for commercialization. Additionally, understanding the genetic and biochemical pathways underlying IAA production and phosphate solubilization in these strains could offer insights for genetic engineering to enhance these traits. Field trials over multiple growing seasons would be crucial to establish their practical benefits and economic viability. These findings provide a strong foundation for advancing agricultural biotechnology, with the potential to enhance plant growth, improve soil fertility, and promote sustainability.

CHAPTER FOUR

EFFECT OF CARRIER MATERIALS AND STORAGE TEMPERATURES ON THE VIABILITY OF BIOFERTILIZER INOCULANTS

Abstract

Development of appropriate carrier material is vital for successful field application of any biofertilizer. The present study investigated the suitability of sugarcane filter mud (from Chemilil Sugar factory) and liquid yeast extract mannitol broth in the formulation of rhizobia strains (*Rhizobium phaseoli* and *Rhizobium pusense*) and phosphate solubilizing bacteria (*Bacillus aryabhattai* and *Paenibacillus polymyxa*) refrigerated (4°C) and at room temperature (16.0±2°C) storage for a period of six months. Both experiments were evaluated at 14, 45, 75, 105, 135 and 165 days after injection of broth cultures. During each evaluation time, a ten-fold serial dilution series to 10^{-7} was prepared from the inoculants. The inoculant stored under room temperature had a steady decrease in survival of the bacterial strains and during the last sampling period at 165 days the bacterial population was below the recommended threshold for both carrier materials. However, for the inoculants stored at 4 °C conditions, the decrease of the bacterial population was gradual and had an extended shelf life compared to the room temperature storage. The inoculant stored in the fridge (4°C) had a significantly higher overall average (3.73± 0.33 x 10^9 CFU per gram/ml of inoculant) survival than those stored at room temperature (2.87 ± 0.38 x 10^9 CFU per gram/ml of inoculant across the period and carrier material. Under low temperature conditions (4°C), the broth carrier had a higher number of bacterial CFUs (3.83 ±0.14 x 10^9 CFU per ml) than in the filter mud carrier material (3.62 ± 0.17 x 10^9 CFU per gram) across the period. However, under room temperature, the filter mud as a carrier material for the bacterial strains contained higher CFUs (3.03 ±0.19 x 10^9 CFU per gram) than the broth (2.72±0.12 x 10^9 CFU per ml). Therefore, carrier material (filter mud) that enabled the bacterial strains to remain viable under room temperature storage can easily be integrated into existing agricultural distribution systems that lack refrigeration. Therefore, when using the filter mud as a carrier material, 6 months storage at low temperatures (4 ºC) is recommended, while 3-4 months is recommended for temperatures around 16ºC (shelf storage).

4.1 Introduction

Soil microbiota are key to the development of sustainable cropping systems (Bender *et al*., 2016). Advances in inoculant technology allows for the optimization and formulation of such beneficial microbiota into biofertilizers using various carrier materials (Bashan *et al*., 2014). The aim of legume inoculation is to provide high numbers of viable effective rhizobia to the rhizosphere. Various bacterial inoculant formulations have been developed using either liquids or solids as carrier materials. However, the shelf life and efficiency of inoculants in biofertilizer formulations greatly depend on the choice of carrier materials and storage temperatures (Sohaib *et al*., 2020). Therefore, to prolong the shelf life of the bacterial inoculant biofertilizers, there is need to design them with the appropriate formulation (Shaik & Sayyed, 2015). The quality of carrier materials determines the inoculants cell population and time-frame of realistic usability of biofertilizers (Somasegaran & Hoben, 2012). In addition, biofertilizer shelf life is affected by bactrerial strain used, unavailability of suitable carriers, poor storage and transportation conditions (Phiromtan *et al*., 2013). Therefore, biofertilizer short shelf life, unavailability of suitable carrier materials, vulnerability to high temperature during storage challenges are biofertilizer bottle necks that still need to be addressed to ensure their success (Sivasakthivelan & Saranraj, 2013).

The use of agricultural wastes as carriers for biofertilizer formulations is one commonly explored option because of their ready availability and cost-effectiveness (Shaikh & Sayyed, 2015). Filter mud is a by-product sugar processing and is high in organic matter content and has the potential to be used as inoculant carrier (Singh *et al*., 2011). Filter mud is locally available from the sugar manufacturing factories in Kenya. Balume *et al*. (2015) found that filter mud was a better carrier when compared to the inorganic locally available horticultural vermiculite. Sugarcane bagasse was shown to be the most appropriate carrier to store the rhizobial strains (2×10^9 cellsg^{-1}) and to maintain their survival. Arora *et al*. (2014) reported a significant difference in the survival of PGPR (*Rhizobium* and *Pseudomonas* strains) isolated from *Trigonella foenum graecum* at room temperature for 8 weeks stored in five different carrier materials.

Another factor influencing the survival of microorganisms in inoculants is the storage temperature and water activity, which directly affect the shelf life of PGPR inoculants (Goudar *et al*., 2017). A study by Pandey *et al*. (2021) examined the impact of different storage temperatures on the viability of rhizobium inoculants in biofertilizers and found that storage at 4°C significantly preserved cell viability compared to storage at 25°C and 37°C, where rapid declines were observed. Similarly, a study by Bhattacharyya *et al*. (2020) assessing the shelf

life of biofertilizers containing Bacillus strains stored at various temperatures reported that strains stored at 4°C retained over 80% viability after six months, whereas those stored at 30°C had reduced viability to below 50%. The Eastern Africa region experiences high temperatures, which negatively impact the survival of rhizobia in both packaged inoculants and inoculated seeds in the field (Deshmukh *et al*., 2014).

There is limited information on the survival of Kenyan native rhizobia and PGPR in different formulations and storage conditions that are suitable for the tropical climates and the storage practice of most of the agro-dealers and farming systems. The objective of the present study was to investigate the effects of two different inoculant formulations (liquid and solid carrier based) on the survivability over a storage period under two different temperature conditions of two locally isolated rhizobia and two PSB strains.

4.2 Materials and methods

4.2.1 Preparation of inoculant formulations

For the liquid carrier material, the *Rhizobium* isolates were grown to 10^9 cfu ml^{-1} in yeast extract mannitol (YEM) broth on a rotary shaker for 72 hours at 28±2°C. Likewise, the PSB isolates were grown in nutrient broth at 30°C for 48 hours to get a population density of about 10^9 cfu ml^{-1}. The liquid inoculum was stored in screw cap bottles kept at 4°C. For the solid formulation, filter mud was used as carrier materials. The filter mud is locally available and is a cheap carrier material for inoculants. The filter mud was sourced from Chemilil sugar factory in Kisumu County, Kenya. After drying and sieving (2 mm mesh) it was wetted to about 45 % moisture holding capacity and 20g of the filter mud was packed into 4 x 6-inch autoclavable polyethylene bags and sealed to maintain the moisture content (Balume *et al*., 2015). The bags were autoclaved for 60 minutes at 121ºC (Somasegaran and Hoben, 1994; FNCA, 2006). Sterilization was checked by placing a sample of the carrier material in petri plates containing nutrient agar, incubated at 28±2°C for 3-5 days. No microbial growth was expected. When microbial growth was detected, the carrier material was further sterilized as described above. In an aseptic condition, 1 ml of strain broth (turbidity: 1 x 10^9 cells ml^{-1}) was injected per 20 g of sterilized carrier using sterile syringes and mixed carefully. The syringe-punctured area was wiped with 70% alcohol, and an adhesive seal applied. The inoculant rhizobia bags were incubated at 28±2°C for 6 days for curing.

4.2.2 Assessment of the bacterial strain survival in storage

For assessing bacterial strains survival in the carrier materials, half of the inoculants (both liquid and solid (filter mud) were refrigerated at 4°C and the other half were stored at room temperature (22°C). Both experiments were evaluated at 14, 45, 75, 105, 135 and 165 days after injection of broth cultures. During each evaluation time, a ten-fold serial dilution series to 10^{-7} was prepared from the inoculants according to the procedures outlined by Somasegaran and Hoben (1994). Three drops of 20 µL from the last three dilutions was plated onto Yeast Extract Mannitol Agar and nutrient agar for the rhizobia and PSB respectively with three replicates for each dilution. All plates were incubated at 28±2°C for 3-7 days. The number of colonies that grew in the range of 30 to 300 colonies were counted and colony forming units g^{-1} were calculated. Each treatment was replicated three times in a completely randomized design (Herridge *et al*., 2002). Effects of strain, carrier, storage temperature and time, and their interactions were determined.

4.2.3 Statistical analyses

The Shapiro–Wilk test was used to test for normality of data and multiple comparisons of variances were performed using Analysis of Variance (ANOVA). Variables with significantly different means were subjected to posthoc analysis using Tukey's Honest Significant Difference (HSD) test to separate the means that were different. A t-test for paired samples was used to evaluate the viability of the biofertilizer formulations in the different carrier materials under room and refrigerated storage.

4.3 Results

Effect of formulation and storage condition on bacterial strain survival

As expected, there was an overall decrease in bacterial population in the inoculants over time (Figure 4.1). During the last sampling time (165 days after preparation) population for *P. polymyxa* and *B. aryabhattai* were below the threshold of 1.0×10^9 CFU per gram or ml of the inoculant (Figure 4.1). For the interaction between the storage and number of days, the inoculant stored under room temperature (16±2 °C) had a steady decrease in survival of the bacterial strains and during the last sampling time at 165 days the bacterial population was below the recommended threshold (Figure 4.2). However, for the inoculants stored at 4 °C conditions, the decrease of the bacterial population was gradual and had an extended shelf life compared to the room temperature storage (Figure 4.2). This trend was observed irrespective of the bacterial strain at both storage conditions (Figure 4.3).

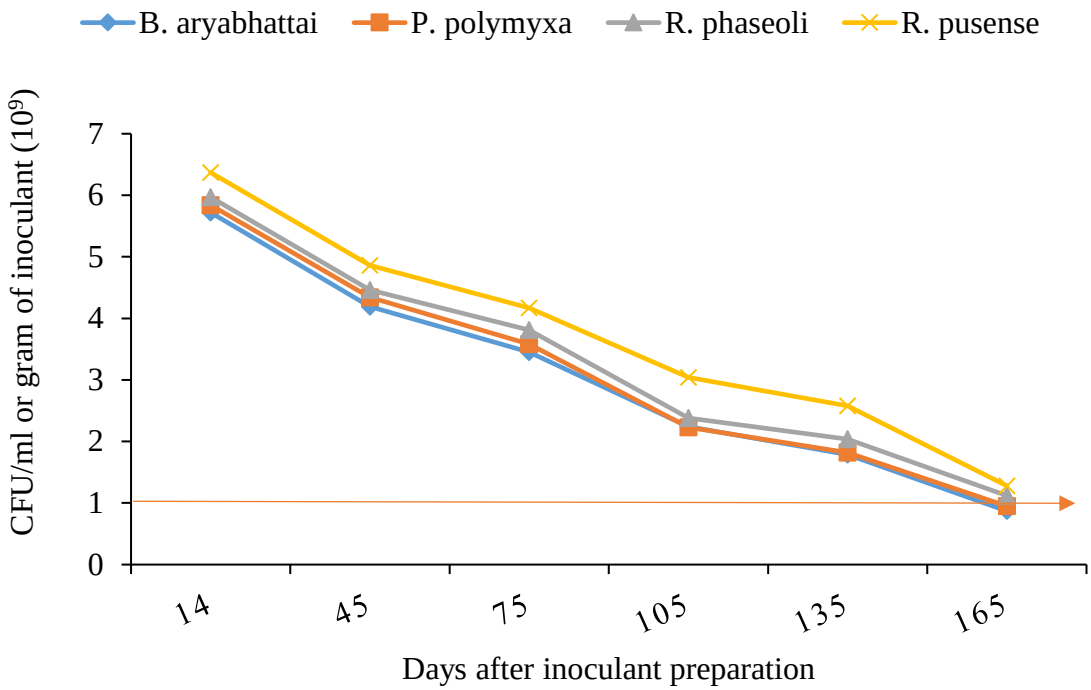

Figure 4.1: Overall survival of different bacterial strains during storage (combined for storage temperature and carrier material). The arrow shows the minimum threshold of the bacterial population CFUs per gram or millilitre of inoculant.

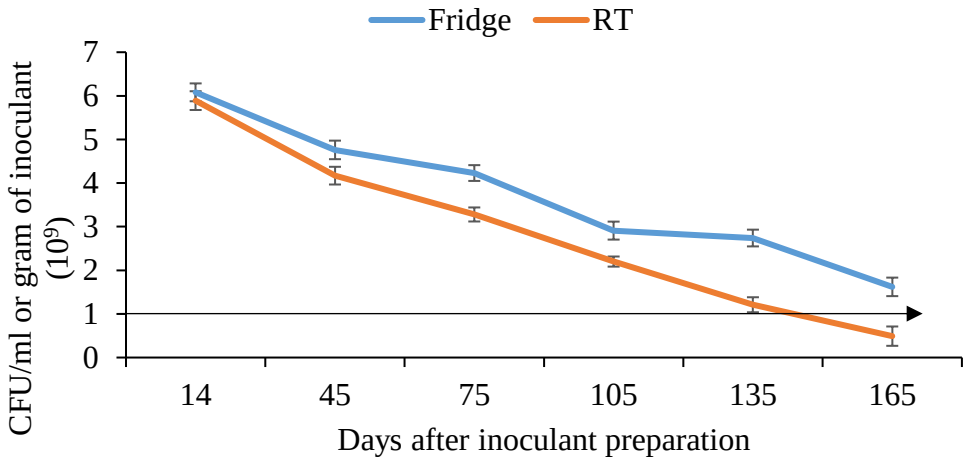

Figure 4.2: Effect of storage condition on the survival of bacterial strains in inoculant combined for 4 bacterial strains. Error bars represent the standard error of the means. RT- room temperature (16±2 °C). The arrow shows the minimum threshold of the bacterial population CFU per gram or millilitre of inoculant.

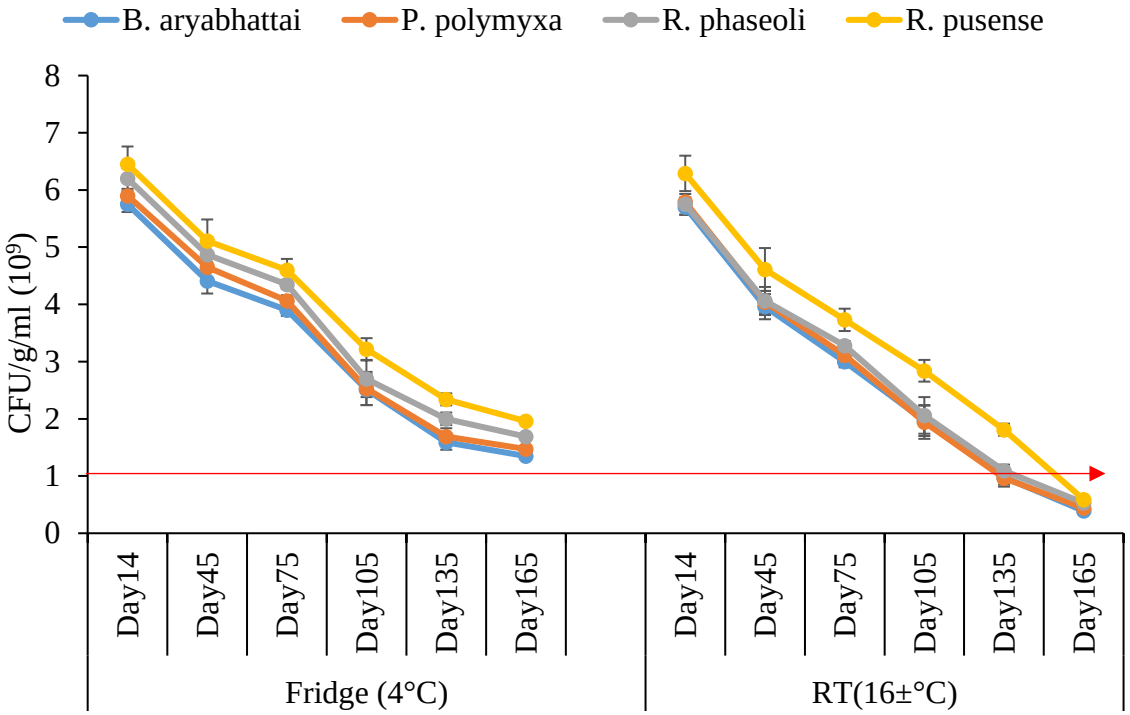

Figure 4.3: Interactive effect of storage condition and days after inoculant preparation on the survival of the four bacterial strains. The arrow shows the minimum threshold of the population of the CFU per gram or millilitre of inoculant.

In terms of the strains effect, the *R. pusense* showed the highest average survival (3.71 x 10^9 CFU/g/ml) during the 165 days of storage significantly higher than the other bacterial strain studied. This was followed by the *R. phaseoli* strain while the least survival was noted with the *B. aryabhattai* (Figure 4.4A). For the effect of the storage condition, the inoculant stored in the fridge (4°C) had a significantly higher (3.73 x 10^9 CFU per gram/ml of inoculant) survival than those stored at room temperature (16±2 °C) with 2.87 x 10^9 CFU per gram/ml of inoculant (Figure 4.4B). Under low temperature conditions (4°C), the broth carrier material had a higher number of bacterial CFU than in the filter mud carrier material (Figure 4.4B). However, under room temperature (16±2 °C), the filter mud contained higher CFU of the bacterial strains than the broth (Figure 4.4B).

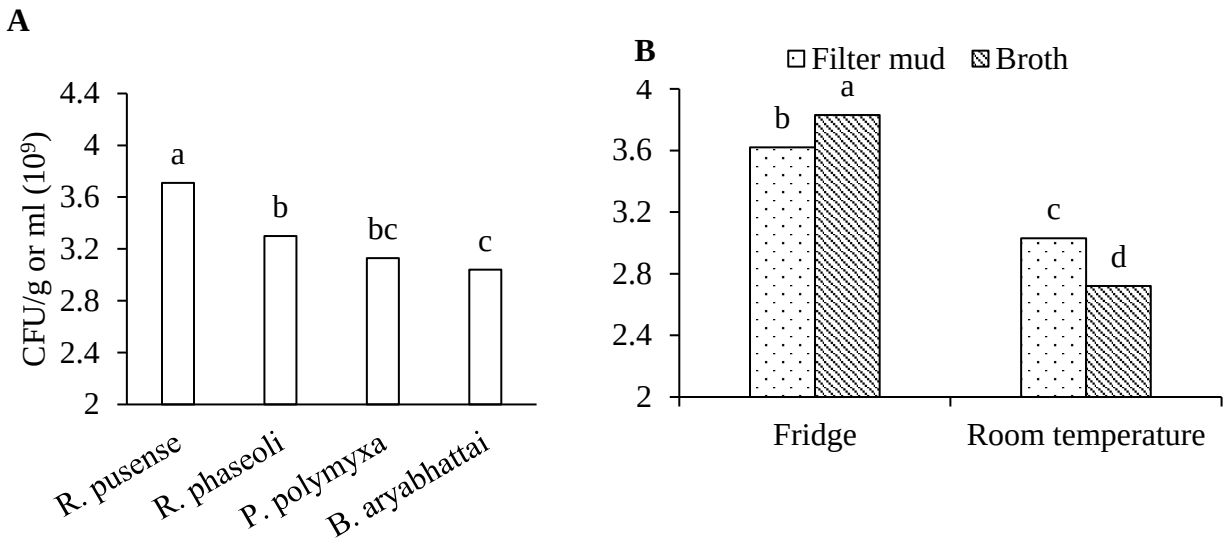

Figure 4.4: Survival of the different bacterial strains in inoculant (A; pooled data for both storage temperature and carrier materials) and the survival of the bacterial strains in different carrier materials at different storage temperature (B; pooled data for the bacterial strains). Bars with different letters in A are statistically different from each other at $\alpha \leq 0.05$. Room temperature = 16.0±2 ℃.

4.4 Discussion

The number of viable cells introduced into the soil greatly influences the effectiveness of biofertilizers in enhancing plant growth. Therefore, determining the survival of bacteria in the carrier material is vital in ensuring that there is the desired level of viable bacterial populations in the inoculants (Arora *et al*., 2014; Bashan *et al*., 2014). The carrier materials sustain the microbial inoculants and allow the product to be stored for a longer period before field application (Rashid *et al*., 2016). Studies have been done on the influence of type of carrier material, storage temperature and the bacterial strain used on the shelf life of bio-inoculants and their success (Lobo *et al*., 2019; Mohammadi & Sohrabi, 2012; Phiromtan *et al*., 2013; Sohaib *et al*., 2020; Sudjana *et al*., 2017). Consequently, to develop biofertilizers with a long shelf life and long-term survival of the bacterial populations, the effect of the carrier materials and storage conditions should be determined (Arora *et al*., 2008; Buntić *et al*., 2019; Shaik & Sayed, 2015).

In the present study, carrier material significantly affected the survival of the bacterial populations during storage. These results are similar to what was reported earlier (Aloo *et al*., 2021; Argal *et al*., 2015; Deaker *et al*., 2012). Patil *et al*. (2013) explained that different carrier materials contain specific characteristics that usually support or suppress the growth of bacterial strains thereby affecting the shelf-life. Results from the present study showed that

during storage, the liquid (broth) formulation had a higher survival when it was stored under low temperatures (4℃). However, at higher storage temperatures, the broth had significantly low a lower number of viable bacterial cells than in the filter mud formulation. Arora *et al*. (2014) reported a significant difference in the survival of *Rhizobium* and *Pseudomonas* strains isolated from *Trigonella foenumgraecum* at room temperature over a period of 8 weeks stored in five different carrier materials. Similarly, Ruíz-Valdiviez *et al*. (2015) reported that after a period of 8 months, the survival of rhizobia was higher in the solid carriers than in liquid carriers. The low survival in the liquid formulation might be attributed to the abiotic stress subjected to the microorganisms caused by depletion of nutrients, accumulation of toxic substances or thermal shock (Berger *et al*., 2018; Lopes *et al*., 2021). The higher number of viable cells in the filter mud carrier material under high temperature conditions is probably because the microorganisms can remain dormant and resistant to environmental stresses and withstand contaminations (Zayed, 2016). Therefore, solid formulations can extend microbial survival at higher temperatures, thereby reducing maintenance costs since refrigeration is not required (Melin *et al*., 2016). Herrmann and Lesueur (2013) pointed out that the use of liquid inoculants may be limited since they need extra and definite storage. However, the liquid inoculants shelf life can be improved by maintaining high quality production process (Lee *et al*., 2016) and addition of sufficient amount of nutrient and cell protectants (Brar *et al*., 2012).

The present study showed that the overall population (CFUs) for the respective bacterial strains declined over time from the initial population regardless of the carrier material and storage temperature. Similar observations were reported in earlier studies and the differences attributed to variations in microorganism content of the inoculant (Gade *et al*., 2014; Ma, 2019; Phiromtan *et al*., 2013; Sivparsad *et al*., 2016; Sohaib *et al*., 2020).

The storage temperature affects the survival and longevity of inoculants (Kaljeet *et al*., 2011; Sohaib *et al*., 2020). Results from this study showed a significant variation in the overall CFUs when the inoculants were stored under refrigeration and at room temperature. Storage under low temperature sustained higher viable bacterial cells than at room temperature. This has been attributed to the reduced metabolic and physiological activities (Gade *et al*., 2014; Phirontam *et al*., 2013). Similar results were reported by Abdallah *et al*. (2019) working on *Rhizobium* and PSB co-inoculants, who showed that the final populations were higher for co-inoculants kept at 4°C than at room temperature. Deaker *et al*. (2004) reported that storage of the inoculant at low temperatures to be more suitable but showed that this may not be practical in most tropical regions. Several other studies have reported that the shelf-life of biofertilizers is better under refrigeration than room conditions (El-Fattah *et al*., 2013; Gade *et al*., 2014;

Phiromtam *et al*., 2013; Priyadharshini *et al*., 2022; Sandikar & Awasthi, 2010; Thirumal *et al*., 2017). Other studies have also reported that some inoculants are able to maintain higher viable bacterial cells under room temperature. For instance, Arora *et al*. (2008) reported that filter mud was able to maintain high numbers of *P. fluorescens* (2.0 x 10^9 CFU g^{-1}) and *R. Leguminosarum* (7.9 x 10^8 CFU g^{-1}) after 6 months of room storage (25±2°C). Since refrigeration facilities are not easily available in developing countries including most of SSA, good survival of the inoculant strains at room temperature, therefore, constitute a desirable property (Paudyal *et al*., 2021). Bashan *et al*. (2014) suggested that inoculants should retain their viability over a storage temperature range of -5°C to 30°C within farmers' storage and agro-dealers' conditions for them to be effective. Results from the present study showed that the inoculants stored under room temperature maintained higher numbers of more than 1.0 x 10^9 CFU g^{-1} or ml^{-1} for up to four and half months (135 days). Formulations that remained viable under room storage can easily be integrated into existing agricultural distribution systems that lack refrigeration (Aloo *et al*., 2022).

Generally, the number of viable cells significantly declined in both the formulations and storage conditions. Similarly, previous studies have reported the decline in the number of viable cells over time from the initial population (Aloo *et al*., 2021; Balume *et al*., 2015; Kumar, 2014; Mwangi, 2019; Paudyal *et al*., 2021; Yakanto & Shutsrirung, 2020). The declines may be attributed to the diminishing nutrients and moisture, production of toxic substances by the actively growing bacterial cells leading to death (Phiromtam *et al*., 2013; Shaik & Sayyed, 2015; Sohaib *et al*., 2020).

4.6 Conclusion

The storage temperature of biofertilizers influences the survival, viability, and efficacy of the microbial strains they contain. Optimal low-temperature storage (around 4°C) helps maintain microbial viability by reducing metabolic rates, extending shelf life, and preventing thermal damage. High-temperature storage, on the other hand, accelerates microbial decline, reduces shelf life, and increases the risk of contamination. Storage under low temperature sustained higher viable bacterial cells than at room temperature particularly for liquid inoculant. Understanding the carrier materials and controlling the effects of storage temperature is crucial for the successful use and commercialization of biofertilizers in sustainable agriculture. Since refrigeration is not easily available in developing countries including Kenya, formulation of inoculants with filter mud under room temperature provides a viable solution for 3-4 months storage. Therefore, it is recommended that manufacturers and distributors of inoculants

implement proper storage practices, including refrigeration or cold storage facilities, to preserve the quality and efficacy of the inoculants during storage and distribution. Additionally, farmers and end-users should be educated about the importance of storing inoculants under appropriate conditions to ensure optimal performance when applied to agricultural fields.

CHAPTER FIVE

EFFECT OF INOCULANT FORMULATION AND CO-INOCULATION OF NATIVE RHIZOBIA AND PHOSPHATE SOLUBILIZING BACTERIA ON *Phaseolus vulgaris* L. GROWTH AND YIELD

Abstract

Incorporation of inoculum in different carrier materials may increase the efficacy of bacterial inocula and promote crop growth and productivity. Field experiments were conducted using two strains of rhizobia and phosphate solubilizing bacteria (PSB) and their promising respective combinations using different carrier material in common bean growing regions in three soil types (Nitisol, Ferralsol and Andosol). The field experiment was laid out in a split plot arrangement with the strain inoculations as the main plot while the sub plots were the carrier materials (filter mud, peat moss and Yeast extract mannitol broth-YEMB). Each main plot included two controls; uninoculated negative control and uninoculated positive control that received N and P fertilizer. Data was collected on the nodulation, shoot and root biomass, total shoot nitrogen and phosphorous, yield and rhizobia population build up. Co-inoculation of *R. phaseoli* + *B. aryabhattai* strains resulted in significantly higher number of nodules (55 nodules per plant) compared to single *R. phaseoli* inoculation (38 nodules). Inoculation and co-inoculation with the bacterial strains significantly increased the yield of common bean over the uninoculated control. The co-inoculation of rhizobia and PSB yielded at par with the application of DAP across the three soil types and seasons. Significantly higher yield was obtained with the filter mud (1.64 Mg ha^{-1}) while there was no significant difference in the yield of common bean between peat moss (1.56 Mg ha^{-1}) and YEMB (1.54 Mg ha^{-1}) as carrier materials for the bacterial strains. Co-inoculation of common bean with *R. phaseoli* + *B. aryabhattai* led to significantly higher rhizobia build up in the soil (1.37 x 10^8 CFU/g of soil) compared to the single *R. phaseoli* inoculation with 0.99 x10^8 CFU/g of soil. The solid carrier material, specifically filter mud, enhanced the efficiency of the inoculant strains resulting in significantly overall higher yield of common bean showing its potential for use in formulation of inoculants. Specific co-inoculation of rhizobia (*Rhizobium phaseoli* and *Rhizobium pusense* strains) and phosphate solubilizing bacteria (*Paenibacillus polymyxa* and *Bacillus aryabhattai*) increased the growth, nodulation, yield and N and P content of common bean more efficiently above the control. This study recommends exploring the use of filter mud as a carrier material, in formulating inoculants for common bean cultivation. Additionally, co-inoculation with

specific rhizobia and phosphate-solubilizing bacteria should be considered as a promising strategy to improve common bean growth and yield.

5.1 Introduction

Plant growth-promoting rhizobacteria have been demonstrated to enhance the performance of rhizobia in nodulation and nitrogen fixation in common beans, resulting in improved plant growth. The effectiveness of PGPR inoculation is significantly influenced by various soil properties, including soil texture, pH, organic matter content, moisture levels, and microbial diversity. For instance, a study by Chamizo *et al*. (2018) inoculated two cyanobacterial species, *Phormidium ambiguum* and *Scytonema javanicum* on different textured soils. Their results indicated that effects of inoculation differed among soil types with the highest improvement found in sandy and silty soils, which originally had lowest fertility. Soil pH interferes in plant metabolism, which can also disrupt biological activities, potentially inhibiting the microorganisms that live in the rhizosphere (Salwan *et al*., 2019) Soil nutritional condition can also affect the PGPM efficiency. In soils with high nutrient profile there can be absence of root colonization; this is due to microorganism displacement to regions more abundant in nutrients (Bhat *et al*., 2019; Egamberdiyeva, 2007). Inoculation with *Pseudomonas, Bacillus*, and *Mycobacterium* are often more effective in promoting plant growth in nutrient-deficient soils (Mathimaran *et al*., 2020). Soil in Kenya have variable soil properties that would affect not only the efficiency of the PGPR inoculation, but also on the overall plant growth. Andosols are from volcanic ash parent material and high in organic matter, highly erodible, and limited in phosphorus. Chemical fertility is variable, depending on degree of weathering. They have a high potential for agricultural production. The strong P fixation, however, is a problem. Ferralsols are the classic red soils of the tropics, because of high iron. They have low supply of plant nutrients and are not therefore impacted greatly by erosion; they have strong acidity and low levels of available phosphorus. They can suffer acidity and P-fixation, and when organic carbon decreases, they become very erodible (Jones *et al*., 2013). Nitisols are dominated by stable minerals such as aluminium oxides, iron oxides and kaolinite, giving this soil its red colour. These Al and Fe oxides often bind with P, making it unavailable for plant uptake (Jones *et al*., 2013).

The carrier material used in the formulation of biofertilizer is essential because it will determine the survival of PGPR strains in the field, in addition to protecting the PGPRs from stress and improving their shelf life (Mishra & Arora 2016). In the Eastern Africa region, different formulations and carrier materials have been used in the preparation of PGPR

biofertilizer. In Sudan, for example, charcoal was reported and used as the main carrier for *rhizobium*-based inoculants among the other locally available carrier materials (Elsalahi *et al*., 2016). Charcoal was reported to be readily available and in abundance in addition to its high water-holding capacity and low contamination levels (Elsalahi *et al*., 2016). In Uganda, the PGPR inoculants containing *Rhizobium* strains have been formulated using sterile peat as carrier material infused with yeast extract mannitol agar broth (Chianu *et al*., 2011). Other carrier materials used in the formulation of PGPR inoculants are filter mud (Bala *et al*. 2011), vermiculite (Balume *et al*., 2013). Strains *Sinorhizobium mexicanum* ITTGR7T, *R. calliandrae* LBP2-1T and *R. etli* CFN42T kept on perlite sugarcane bagasse induced the largest number of nodules in the common bean (Ruíz-Valdiviezo *et al*., 2015). Sugar waste was found to allow a higher growth of legume nodulating bacteria in media comparing to YEM standard media used for legume nodulating bacteria (Singh *et al*., 2011). Strains *Sinorhizobium mexicanum* ITTGR7T, *R. calliandrae* LBP2-1T and *R. etli* CFN42T kept on perlite sugarcane bagasse induced the largest number of nodules in the common bean (Ruíz-Valdiviezo *et al*., 2015). Sugar waste was found to allow a higher growth of legume nodulating bacteria in media comparing to YEM standard media used for legume nodulating bacteria (Singh *et al*., 2011).

Inocula containing different beneficial microorganisms affect in different ways the functional groups of soil microorganisms (Trabelsi & Mhamdi, 2013). Similarly, inoculant formulation technologies allow the use of two and more microorganisms. In general, co-cultivation was found to reduce the production costs and to solve problems related to the process parameters, use of nutrients, oxygen demand, between co-cultures (Hickert *et al*., 2014). While the co-culturing of different microorganisms within the same media seems to be a frequently used approach in some fermentation processes, it has not been widely used in formulation of plant-beneficial microbial inoculants (Vassilev *et al*., 2015). A co-cultivated microbial inoculant formulation can be easily adapted to field application and affect more efficiently plant growth and health. Co-cultivation of plant-beneficial compatible microorganisms is a highly promising strategy (Marmann *et al*., 2014; Vassilev *et al*., 2015).

The effects of carrier materials on inoculants made from native bacterial strains isolated in Kenya have not been explored or documented. This has made it difficult to obtain effective biofertilizers from the native bacterial strains that can be made available to farmers to boost bean production. This study therefore determined the effect of inoculant carrier material on the efficiency of inoculants and co-inoculation of rhizobia and phosphate solubilizing bacteria (PSB) on common bean growth and yield.

5.2 Materials and methods

5.2.1 Field experiments

The strains (*R. phaseoli* (B3), *R. pusense* (B4), *B. aryabhattai* and *P. polymyxa*) that enhanced plant growth in the greenhouse were tested in field trials using different formulations in common bean growing regions in three agro-ecological zones; Upper midland 1 (UM_1), 34°44.812'E, 0°16.489'N (Kakamega County, Lurambi Subcounty); lower highland 3 (LH_3), 36°5.562'E, 0°19.436'S (Nakuru County, Njoro Subcounty) and upper midland 3 (UM_3), 35°0.158'E, 1°1.145'N (Trans Nzoia, Saboti Subcounty) as shown in Figure 5.1. The field experiments were conducted using the two most promising strains of each *rhizobium* and PSB and their promising respective combinations. The experiment was conducted in two cropping seasons (2020 short rains (SR) and 2021 long rains (LR) seasons). During the second season (2021 LR), the same fields were used, and the specific treatments used in the experimental plots as in 2020 SR season. The soils in Kakamega, Nakuru and Trans Nzoia are characterized as eutric Nitisols (UM_1), mollic Andosols (LH_3) and rhodic Ferralsols (UM_3), respectively (Jaetzold *et al*., 2007).

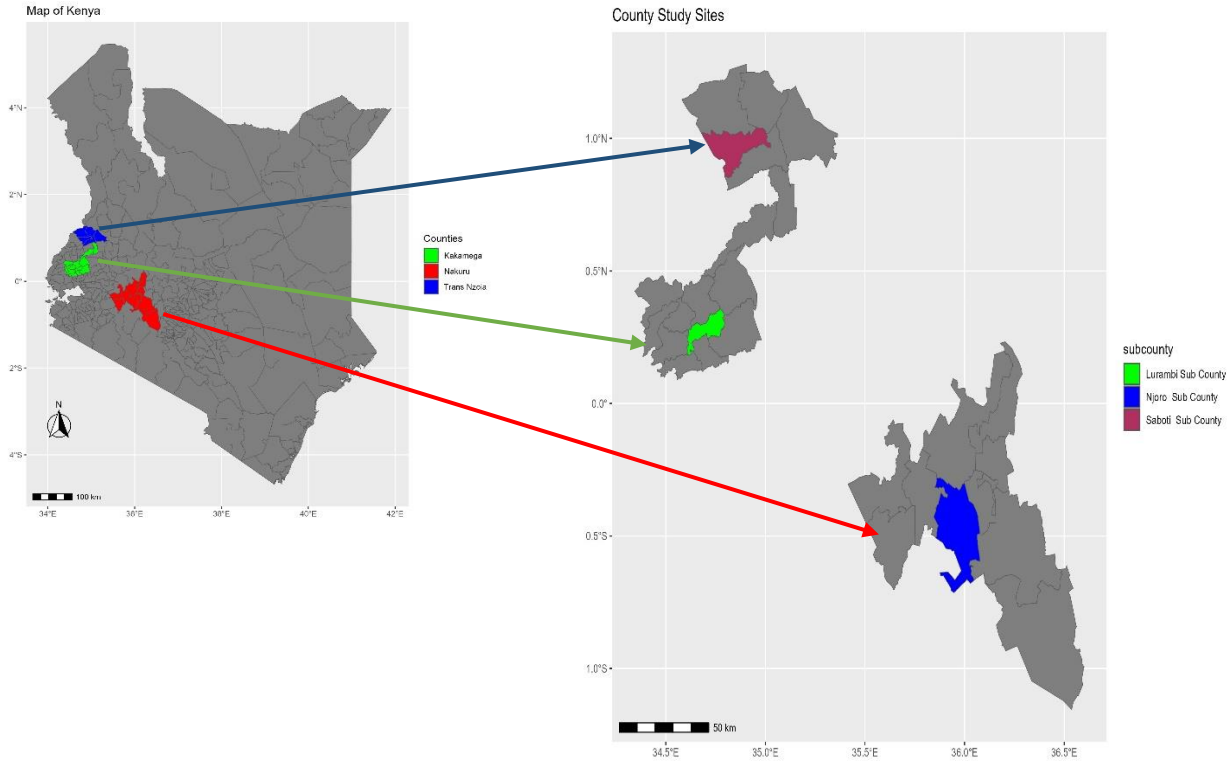

Figure 5.1: Map showing the study sites (Source: Own).

5.2.2 Soil sampling and analysis

Rhizosphere soil from the three study sites was dug out using a soil auger at a depth of 0-20 cm and collected in zip-lock bags. The samples were placed in plastic bags and stored at 4°C in the laboratory. The soils were air-dried, prepared and analysed using standard soil testing procedures as described by Okalebo *et al*. (2002). Soil pH was determined using a glass electrode pH meter at 1:2.5 soil/water ratio. Available P was extracted using the Olsen extractant and determined using the ammonium vanadate method on a spectrophotometer at 470 nm (Olsen, 1954). Organic carbon was determined by Walkley and Black sulfuric acid–dichromate digestion followed by back titration with ferrous ammonium sulfate, while nitrogen was determined using the Kjeldahl method (Sáez-Plaza *et al*., 2013). Soil texture was determined using the hydrometer method while the bulk density was determined using the oven-drying method after soil samples were collected using core rings (Walter *et al*., 2016). Exchangeable sodium, potassium, calcium, magnesium and selected micronutrients (iron, zinc and copper) were also determined using procedures as described by Okalebo *et al*. (2002).

5.2.3 Land preparation, inoculation and planting

The fields were ploughed, and ridged at a spacing of 40 cm apart. The plot size was 2 × 2 m and spacing of 40 cm between rows and 10 cm between plants. Bean seeds were surface sterilized with sodium hypochlorite (Commercial Jik) and moistened with a 20% (w/v) solution of sucrose and mixed with the appropriate inoculants at the rate of 10g per kg of bean seed under shade for immediate sowing (Yadegari *et al*., 2010). After drying under shade, the inoculated seeds were sown after the uninoculated seeds to avoid contamination.

5.2.4 Experimental design and treatments

The field experiment was laid out a randomized complete block design (RCBD) in a split plot arrangement with the strain inoculations as the main plot while the sub plots were the different inoculant formulations (Figure 5.2). Each main plot included two controls, uninoculated negative control and uninoculated positive control that received N and P fertilizer by application of Di-Ammonium Phosphate (DAP) at the rate of 125 $kgha^{-1}$ (22.5 $kgha^{-1}$ and 57.5$kgha^{-1}$ N and P respectively; 50g of DAP uniformly applied per plot). Sub plots were the carrier materials filter mud, peat moss, Yeast mannitol broth, and uninoculated negative control. The experiment was replicated three times. Treatments were randomly allocated per replication and per sub-plot at each site. Two seeds per hill were hand sown and later thinned to one seedling per hill two weeks after emergence.

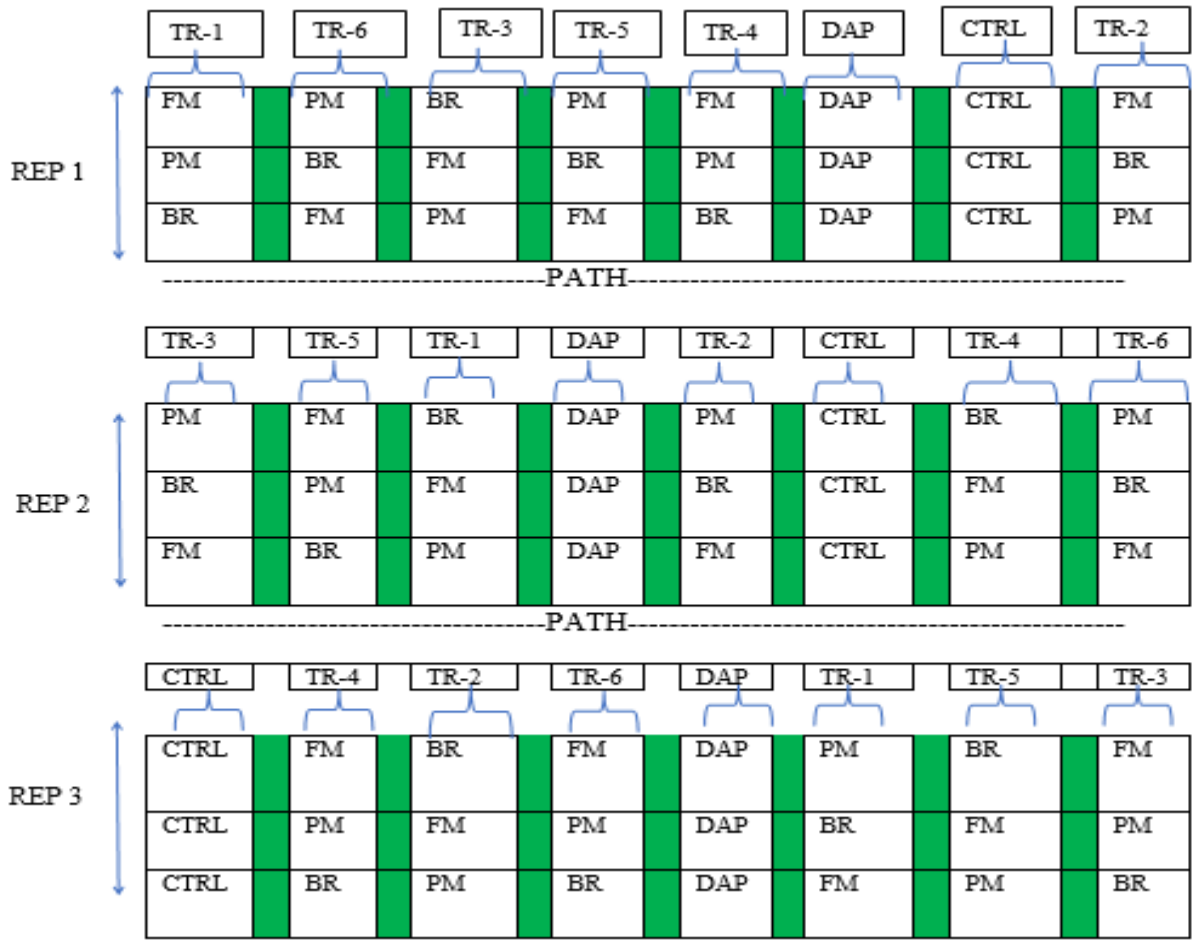

Figure 5.2: Field layout of strains treatments and carrier materials

Main plots= treatments i.e strain/strains combinations + controls (DAP and negative control)

TR-1= *R. pusense* (B4) + *B. aryabhattai*

TR-2= *R. pusense* (B4) + *P. polymyxa*

TR-3= *R. phaseoli* (B3) + *P. polymyxa*

TR-4= *R. phaseoli* (B3) + *B. aryabhattai*

TR-5= *R. pusense* (B4)

TR-6 = *R. phaseoli* (B3)

Sub plots: FM- filter mud, PM- peat moss, BR- broth, CTRL – uninoculated negative control

5.2.5 Plant sampling and data collection

Plant samples were taken at 50% flowering and harvest maturity growth stages. At 50% flowering, six plants were randomly collected per plot to evaluate nodulation (nodule number and dry weight per plant), shoot dry weight and total shoot nitrogen and phosphorous content.

For evaluating the nodulation, the six plants were collected by excavating the roots using a hand shovel ensuring that the root system was kept intact. In the laboratory, the soil or substrate from the roots were gently removed by washing under a tap water ensuring that the nodules were intact. Using a sharp scalpel the nodules were detached form the root hairs and cut cross-sectionally to check on the internal colouration. The nodules that were pinkish-red in colour were the counted and recorded (Santos *et al*., 2019). The shoots were similarly dried, and their dry weight recorded. During the second season, the dried shoots were ground into fine powder and the tissue nitrogen and phosphorous determined as described by Okalebo *et al*. (2002).

At harvest maturity, harvesting was done when plants and seeds were dry. All the plants in the three inner rows were counted (55-60 plants) and the pods collected for further processing. The pods were then threshed and the grains from the harvested area of each plot were cleaned and weighed at 11% moisture content to estimate the grain yield of a plot per hectare. Accordingly, seed weight of a plot divided by the number of plants harvested from that plot were multiplied by the number of plants calculated per hectare.

5.2.6 Effect of inoculation on rhizobia population in the soil

At the end of the second cropping season (2021LR), soil samples were collected from main plots to determine the build-up of rhizobia population in the soil. Within each main plot, rhizospere soil was sampled in each sub plot and mixed to make a composite sample of about 50 g. The samples were taken for each replicate from the three study sites and stored at 4℃ until the microbial analysis was done.

The soil samples were serially diluted by weighing 1 g of the soil into 9 ml of sterile physiological saline (9 g sodium chloride per litter of sterile water) up to 10^{-6}. Then, 0.1 ml of 10^{-4} to 10^{-6} serial dilution was poured and evenly spread on petri plates containing yeast extract mannitol agar (YEMA) culture media. The plates for each dilution were done in triplicate. The plates were incubated at an optimal temperature of 28°C ± 2. Colonies were counted individually using a colony counter, and the initial concentration of each was calculated. Only plates containing between 30 and 300 isolated colonies were selected. The bacterial concentration in the initial suspension was then calculated as follows (Olsen *et al.,* 1996):

$$M = \frac{n\ X\ V}{C} \quad \text{.....Equation 3}$$

M: concentration of the initial suspension (number of cells per ml)

n: number of isolated colonies counted on the selected plate(s)

V: volume of culture

C: dilution (sum of dilutions / number of selected plates)

5.3 Data analysis

Data was analyzed using SAS Statistical Package Version 9.3 (SAS 2013). The data was first tested for normal distribution and the data for nodule counts was normalized by logarithmic transformation (Log_{10} x+1) before analysis. To determine the effects due to inoculation and formulation, analysis of variance at 95% confidence limit was done and means separated using the Tukey's test at α=0.05. Pearson's correlation analysis was done to check for the relationships between the measured parameters.

Statistical model for the field study:

$$\mathbf{Y_{ijkl} = \mu + R_i + \alpha_j + R\alpha_{ij} + \beta_k + \alpha\beta_{jk} + \tau_k + \alpha\tau_{il} + \alpha\,\beta\tau_{ikl} + \Sigma_{ijkl}}$$

μ = overall mean

R_i = Effect due to the i^{th} replicate

α_j = Effect due to the j^{th} strain inoculation

$R\alpha_{ij}$ = Error (a) associated with the strain inoculation

β_k= effect due to the k^{th} formulation

$\alpha\beta_{jk}$= Interactive effect due to the j^{th} strain inoculation and the k^{th} formulation

Σ_{ijkl} = Random experimental error

5.4 Results

5.4.1 Soil physical chemical characteristics and weather condition of the study sites

The selected soil physico-chemical and biological properties were as presented in Table 5.1. The soil pH for the three study sites were medium alkaline. Andosol had low nitrogen (0.17%), while Ferralsols and Nitisols had adequate nitrogen levels, 0.25% and 0.28%, respectively. Phosphorous level was highest in the Ferralsol and least in the Nitisol although the levels were high in all the sites (>15 mg kg^{-1}). Organic matter was moderate in Andosols and adequate in both Ferralsol and Nitisol. The micronutrients were generally adequate except for copper in Ferralsol and Nitisol and zinc in the Nitisol that were low.

Table 5.1: Soil characteristics and weather of the study sites

Soil parameter	Andosols		Ferralsols		Nitisols	
	Value	Class	Value	Class	Value	Class
Soil pH	7.84	Medium alkaline	8.28	Medium alkaline	7.97	Medium alkaline
Total Nitrogen %	0.17	Low	0.25	Adequate	0.28	Adequate
Total Org. Carbon %	2.01	Moderate	2.79	Adequate	3.17	Adequate
Phosphorus (Olsen) (mg kg ^{1})	97	High	132	High	83	High
Potassium (Cmol/kg)	1.66	High	2.18	High	1.40	Adequate
Calcium (Cmol/kg)	25.2	High	40.0	High	61.4	High
Magnesium (Cmol/kg)	5.31	High	5.56	High	5.56	High
Manganese (Cmol/kg)	0.70	Adequate	0.29	Adequate	0.11	Adequate
Copper (mg kg ^{1})	3.06	Adequate	0.62	Low	0.36	Low
Iron (mg kg ^{1})	33.9	Adequate	12.1	Adequate	10.7	Adequate
Zinc (mg kg ^{1})	5.02	Adequate	13.3	Adequate	0.15	Low
Sodium (Cmol/kg)	0.81	Adequate	1.55	Adequate	0.47	Adequate
Elect. Cond. mS/cm	0.46	Adequate	0.81	Adequate	0.54	Adequate
Sand (%)	30		40		24	
Clay (%)	52		32		58	
Silt (%)	18		28		18	
Textural class		Clay		Clay loam		Clay
Rhizobia population (10^{8} CFU/g of soil)	0.51		0.52		0.07	
Total rainfall (mm)	2020SR - 492.2 mm 2021LR-299.2 mm		2020SR- 235.2 mm 2021LR-358.5 mm		2020SR-717.4 mm 2021LR - 452.4 mm	
Mean day temp. (°C)	22.0		24.5		26.0	
Mean night temp. (°C)	11.0		12.5		14.5	
Mean daily temp. (°C)	16.7		18.5		20.2	

Rhizobia population (x10^{8} CFU/g of soil)

Short rains (SR) = October-December; Long rains (March-May)

Weather data source (https://climateknowledgeportal.worldbank.org/country/kenya/trends-variability-historical)

5.4.2 Effect of co-inoculation and inoculant formulation on the number of nodules of common bean

Inoculation of common bean generally led to a significantly higher number of nodules compared to the uninoculated control and fertilizer only treatment (Figure 5.3). This was consistent across the three soil types. In the Andosol, for instance, the application of DAP suppressed the formation of nodules leading to the lowest nodule numbers compared to the uninoculated control. There was varied response to nodulation among the different rhizobia strains inoculation and their corresponding co-inoculation with the bacillus strains across the three soil types (Figure 5.3).

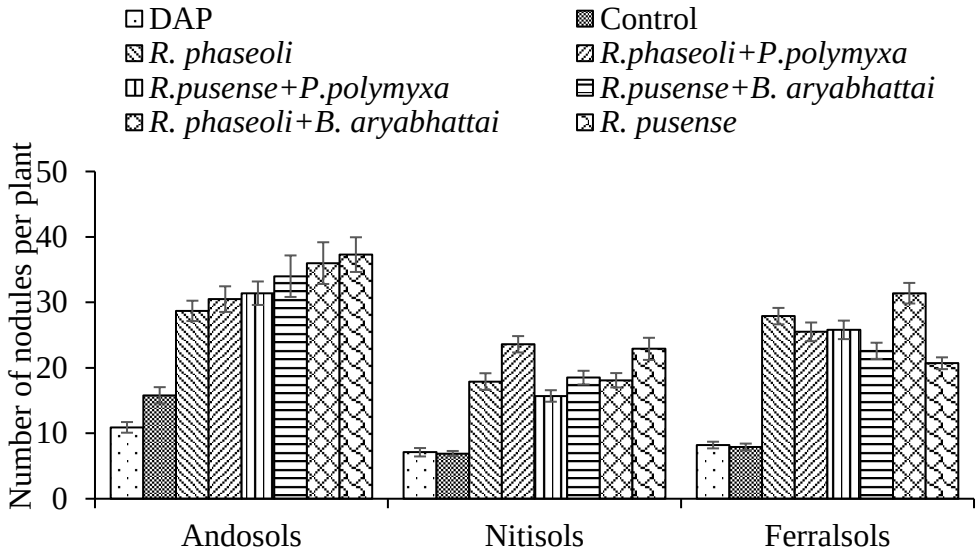

Figure 5.3: Effect of soil type on the number of nodules of common bean. Error bars represent the standard error of the means. The means are an average of the two cropping seasons.

Single inoculation of the common bean with the rhizobia and co-inoculation with the PSB led to significantly higher number of nodules than the DAP application and the uninoculated control. This was consistent across the three study sites and the two cropping seasons (Table 5.2). For instance, in the andosol, during the 2020SR season, the inoculated and co-inoculated treatments had significantly higher number of nodules than the DAP application and the uninoculated control (Table 5.2). The mixed inoculation of *R. phaseoli* + *B. aryabhattai* strains had significantly higher number of nodules (55 nodules) compared to single *R. phaseoli* inoculation (38 nodules). There was no significant ($p<0.05$) difference between the single *R. pusense* and *R. pusense* + *B. aryabhattai*. However, co-inoculation with *P. polymyxa* suppressed the number of nodules as compared to the single inoculation for both *R. pusense* and *R. phaseoli* (Table 5.2). During the 2021LR season, there was no significant differences in

the number of nodules between the inoculated treatments. However, there was significant differences compared to the DAP application and the uninoculated control (Table 5.2). In the nitisols, during the 2020SR season, inoculation of the common bean with the rhizobia and the PSB, led to formation of significantly more nodules per plant than the uninoculated control and the application of DAP. There was no significant difference among the inoculated treatments except for the co-inoculation of *R. phaseoli* + *B. aryabhattai.* During the 2021LR season, the co-inoculation of the common bean with *R. phaseoli* + *P. polymyxa* and single *R. pusense* inoculation led to significantly higher nodule numbers than the other inoculation and the controls. In the ferralsols, inoculation led to a greater number of nodules than the uninoclated control and DAP application during both seasons. In 2020SR season, co-inoculation with *R. phaseoli* + *B. aryabhattai* led to significantly highest number of nodules followed by *R. phaseoli* + *P. polymyxa;* the two co-inoculation treatments having more nodules than the single *R. phaseoli* inoculation (Table 5.2). In the 2021LR season, co-inoculation with *R. pusense* +*P. polymyxa* led to significantly higher nodule numbers than single *R. pusense* inoculation (Table 5.2). There was no significant difference between the single *R. phaseoli* inoculation with the respective co-inoculation with *B. aryabhattai* and *P. polymyxa* (Table 5.2).

Table 5.2: Effect of rhizobia and PSB co-inoculation on the number of nodules per plant in three soil types in 2020SR and 2021LR cropping seasons

	Andosol		Nitisol		Ferralsol	
Treatment	2020SR	2021LR	2020SR	2021LR	2020SR	2021LR
R. pusense+B. aryabhattai	51.4ab	16.5ab	20.9a	16.2b	16.6c	28.7a
R. pusense +P. polymyxa	42.9ab	20.0a	20.4a	11.5bcd	21.7bc	29.9a
R. phaseoli+ P. polymyxa	42.3ab	18.8a	20.8a	26.4a	29.9ab	21.1ab
R. phaseoli + B. aryabhattai	55.2a	16.7ab	19.8a	16.3b	38.6a	24.2ab
R. pusense	54.6a	20.1a	20.9a	24.8a	23.8bc	17.6bc
R. phaseoli	38.4b	19.0a	20.8a	15.0bc	29.0b	26.8ab
DAP	12.7c	6.9c	5.2b	8.4cd	6.9d	9.0c
Control	22.1c	9.5c	6.0b	7.3d	6.6d	9.0c
Tukey's MSD ($\alpha \leq 0.05$)	13.8	6.9	1.3	8.1	8.8	10.1

Means followed by different letters within a column are significantly different at $\alpha \leq 0.05$. MSD= Minimum Significant Difference, SR- short rains, LR = Long rains

The number of nodules was affected by the interaction between the bacterial strains and the carrier materials. However, the interaction was not consistent across soil types with the Ferralsols and the Nitisol showing interactions between the bacterial strain and carrier material (Figure 5.4A). However, in the Andosol, no significant carrier material by bacterial strains interaction was observed. In the Ferralsols, for instance, only the broth formulation of *R. phaseoli* + *P. polymyxa* elicited formation of more nodules compared to the filter mud and peat moss carrier materials (Figure 5.4A). In the case of filter mud carrier material, its formulation with *R. phaseoli* + *B. aryabhattai*, and *R. pusense* led to more nodule numbers, while peat moss formulation did not outperform either the broth or filter mud in any of the bacterial strains (Figure 5.4A). These results were consistent in the Nitisol, where the use of filter mud as a carrier material led to a higher number of nodules for most of the rhizobia strains inoculation and their respective co-inoculation with the bacillus strains (Figure 5.4B).

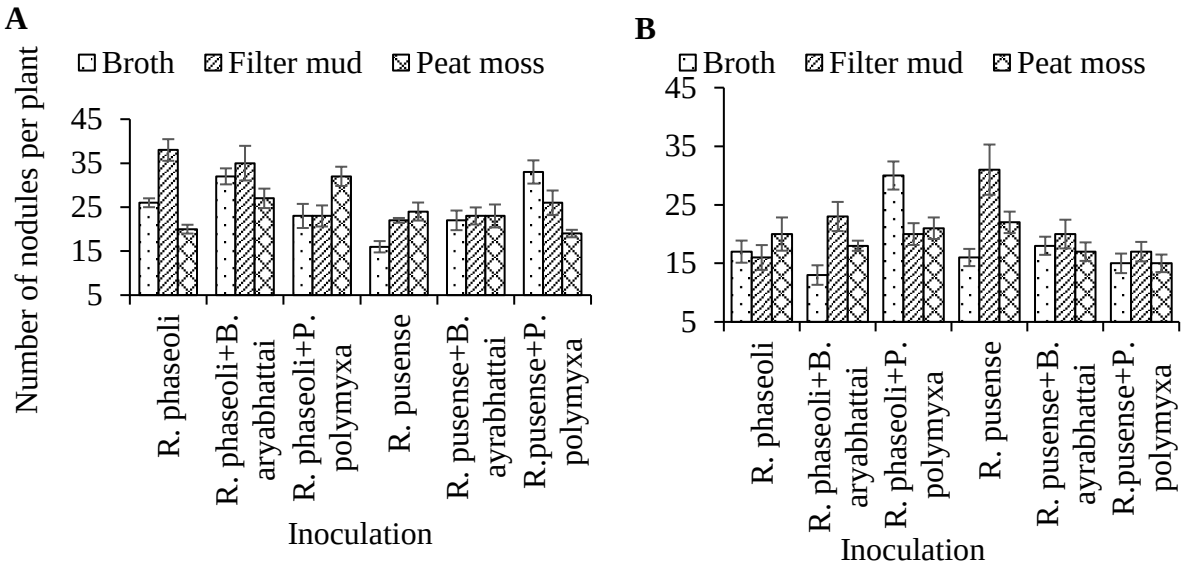

Figure 5.4: Interactive effect of rhizobia-PSB co-inoculation and carrier materials on the number of nodules of common bean in the Ferralsol (A) and Nitisol (B). Error bars represent standard error of the means. Data is combined for the 2020SR and 2021LR cropping seasons.

5.4.2 Effect of co-inoculation on root dry weight of common bean

Inoculation and co-inoculation of common bean with rhizobia and bacillus strains led to higher root dry weight compared to the uninoculated control, consistent across the three soil types and the two cropping seasons (Table 5.3). The application of DAP led to a higher root biomass while the uninoculated control had significantly lower root biomass (Table 5.3). The root biomass resulting from the inoculation with the other bacterial strains was significantly different from the uninoculated control (Table 5.3). In the 2021LR season co-inoculation with

R. phaseoli + B. polymyxa led to a higher root biomass among the inoculated treatments, however not significantly different from single *R. phaseoli* and *R. pusense* inoculation (Table 5.3). For Nitisol, during the 2020SR season, there were no significant difference among the inoculated plants, but they had significantly higher root biomass in comparison to the uninoculated control. In the 2021LR season, co-inoculation of the common bean with *R. phaseoli+ P. polymyxa* and *R. phaseoli + B. aryabhattai* performed at par with the DAP application. *R. phaseoli + P. polymyxa* co-inoculation led to a significantly higher root biomass than the single R. phaseoli inoculation (Table 5.3). For the Ferralsols, co-inoculation of the common bean *R. pusense + P. polymyxa* led to significantly higher root biomass compared to the other inoculated treatments in the 2020SR season. During the 2021LR, there was no significant difference between the various co-inoculation treatments and application of DAP (Table 5.3). However, the single inoculation of either the *R. phaseoli* or *R. pusense* had significantly lower root biomass than DAP application but higher than the uninoculated control (Table 5.3).

Table 5.3: Effect of co-inoculation with rhizobia and PSB on the root biomass of common bean in different soil types during the 2020SR and 2021LR cropping season

	Andosol (g/plant)		Nitisol(g/plant)		Ferralsol (g/plant)	
Treatment	2020SR	2021LR	2020SR	2021LR	2020SR	2021LR
R. pusense+B. aryabhattai	1.17b	0.53cd	0.70b	0.55c	0.80c	0.25abc
R. pusense +P. polymyxa	1.17b	0.50d	0.71b	0.58bc	0.87b	0.31a
R. phaseoli+ P. polymyxa	1.27b	0.63b	0.73b	0.65ab	0.77cd	0.23bc
R. phaseoli + B. aryabhattai	1.47b	0.53cd	0.69b	0.61abc	0.80c	0.25abc
R. pusense	1.30b	0.57bcd	0.71b	0.58c	0.73cd	0.22bc
R. phaseoli	1.30b	0.60bc	0.72b	0.57c	0.77c	0.27ab
DAP	1.57a	0.73a	0.83a	0.82a	1.19a	0.28a
Control	0.87c	0.40e	0.53c	0.50d	0.70d	0.20c
Tukey's MSD ($\alpha \leq 0.05$)	0.1	0.07	0.08	0.16	0.09	0.05

Means followed by different letters within a column are significantly different at $\alpha \leq 0.05$. MSD= Minimum Significant Difference, SR = Short rains, LR = Long rains

5.4.3 Effect of inoculation and formulation on the shoot biomass of common bean

Co-inoculation of common bean with the rhizobia and bacillus strains had a significant effect on the shoot biomass with all the inoculated plants having higher biomass than the

uninoculated control across all the soil types and cropping seasons (Table 5.4). In comparison to the application of DAP, most of the inoculated plants had similar shoot biomass, particularly in the second (2021LR) season. For instance, in Andosol, the *R. phaseoli + P. polymyxa* co-inoculated plants had the highest shoot biomass (Table 5.4).

Table 5.4: Effect of rhizobia and PSB co-inoculation on the shoot biomass of common bean in different soil types during the 2020SR and 2021LR cropping seasons

	Andosol(g/plant)		Nitisol (g/plant)		Ferralsol (g/plant)	
Treatment	2020SR	2021LR	2020SR	2021LR	2020SR	2021LR
R. pusense+B. aryabhattai	11.9d	5.5bc	5.8a	3.8b	6.2ab	6.8bcd
R. pusense +P. polymyxa	13.6c	5.6bc	5.6ab	3.7b	5.9ab	7.3a
R. phaseoli+ P. polymyxa	13.7c	6.2a	5.8a	4.2ab	6.1ab	7.0abc
R. phaseoli + B. aryabhattai	10.8d	5.4c	5.5b	4.0b	5.9ab	6.5d
R. pusense	13.8c	5.9ab	5.8a	4.6a	6.4ab	6.6cd
R. phaseoli	15.4b	5.7bc	5.8a	4.1b	6.2ab	6.8bcd
DAP	18.6a	5.8ab	6.1a	4.4ab	6.8a	7.1ab
Control	10.0e	4.5d	4.6c	3.2c	5.2c	5.8e
Tukey's MSD ($\alpha \leq 0.05$)	0.7	0.4	0.5	0.4	0.8	0.6

Means followed by different letters within a column are significantly different at $\alpha \leq 0.05$. MSD= Minimum Significant Difference, SR = Short rains, LR = Long rains

5.4.4 Effect of inoculation and inoculant formulation on the yield of common bean

Inoculation and co-inoculation with the bacterial strains significantly increased the yield of common bean over the uninoculated control. This was consistent across all the soil types and seasons except for the single rhizobia inoculation in Nitisol during the 2021LR season (Table 5.5). The co-inoculation of the rhizobia and the PSB yielded statistically at par with the application of DAP across the soil types and seasons (Table 5.5). For instance, the co-inoculation led to more yield than the DAP application in the Andosol (2020SR), Nitisol (2020SR) and in the Ferralsol (2021LR) though the difference was not significantly different (Table 5.5). Some specific rhizobia-PSB co-inoculation led to significantly higher yield than their respective single rhizobia inoculation. For example, in Ferralsol, *R. pusense + B. aryabhattai* co-inoculation led to significantly higher yield than the single *R. pusense* inoculation in the 2020SR season; while in the 2021LR, co-inoculation of either the rhizobia

strains with *P. polymyxa* led to significantly higher yield than their single inoculation (Table 5.5).

Table 5.5: Effect of rhizobia and PSB co-inoculation on the grain yield of common bean in different soil types during the 2020SR and 2021LR cropping seasons

	Andosol (Mg ha^{-1})		Nitisol Mg tha^{-1})		Ferralsol (Mg ha^{-1})	
Treatment	2020SR	2021LR	2020SR	2021LR	2020SR	2021LR
R. pusense+B. aryabhattai	2.49a	1.37b	1.38ab	1.41a	1.91ab	1.36abc
R. pusense +P. polymyxa	2.26ab	1.49ab	1.27ab	1.43a	1.69bc	1.76a
R. phaseoli+ P. polymyxa	2.28ab	1.51ab	1.42a	1.47a	1.67bc	1.64ab
R. phaseoli + B. aryabhattai	2.25ab	1.41ab	1.23ab	1.47a	1.91ab	1.25bc
R. pusense	2.30ab	1.38b	1.24ab	1.31ab	1.47c	1.15c
R. phaseoli	2.00b	1.49ab	1.18b	1.35ab	1.48c	1.18c
DAP	2.32a	1.64a	1.34ab	1.49a	2.18a	1.47abc
Control	1.43c	0.93c	0.82c	1.16b	1.09d	0.48d
Tukey's MSD $\alpha \leq 0.05$	0.37	0.25	0.22	0.31	0.35	0.42

Means followed by different letters within a column are significantly different at $\alpha \leq 0.05$. MSD= Minimum Significant Difference, SR = Short rains, LR = Long rains

On average inoculation and co-inoculation of common bean with the bacterial strains increased the bean grain yield compared to the uninoculated control (Figure 5.5). Co-inoculation of the rhizobia with the PSB yielded at par with the application of DAP, while DAP application yielded significantly more than the single rhizobia inoculation (Figure 5.4). No significant difference in yield of common bean was observed among the different bacterial strains' inoculation and co-inoculation (Figure 5.5). Application of DAP led to 77% increase in yield over the uninoculated control. Co-inoculation with the rhizobia and the PSB led to more than 60% increase in the yield of common bean compared to the uninoculated control while the single inoculation of rhizobia led to 49% and 47% for the *R. pusense* and *R. phaseoli* respectively (Figure 5.6). In terms of the overall effect of the carrier material, significantly higher yield was obtained when filter mud was used as the inoculant carrier material (1.64 Mg ha^{-1}). There was no significant difference in the yield of common bean between peat moss and broth (liquid) carrier materials (Figure 5.7).

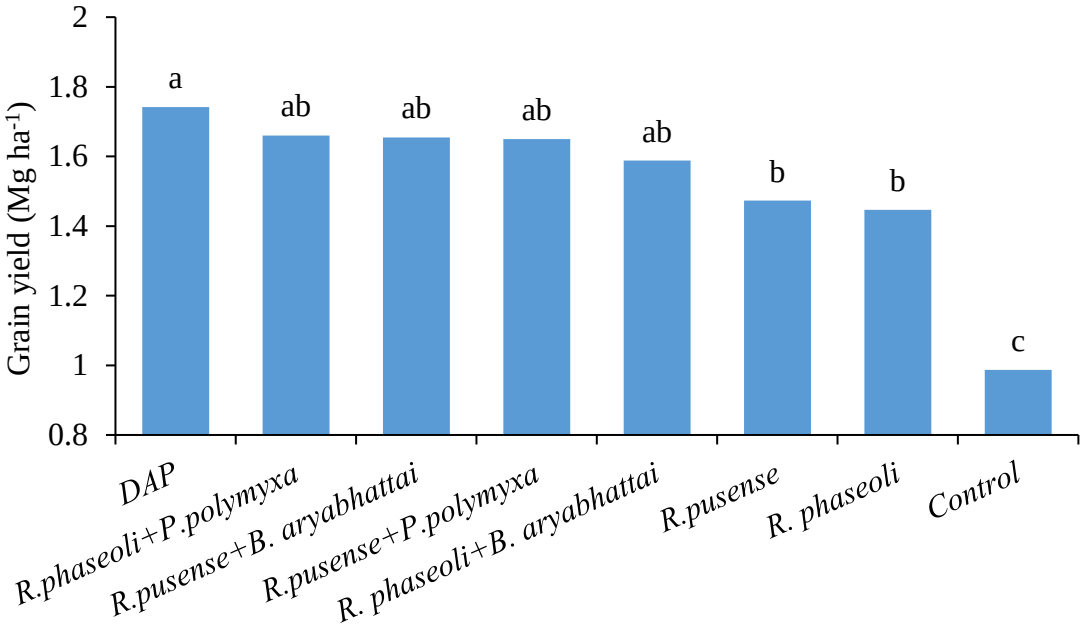

Figure 5.5: Effect of co-inoculation on the mean grain yield of common bean. Means followed by different letters are significantly different from each other at $\alpha \leq 0.05$. The means are from data across the three soil types (Andosol, Ferralsol and Nitisol) and the two cropping seasons (2020 short rains and 2021 long rains).

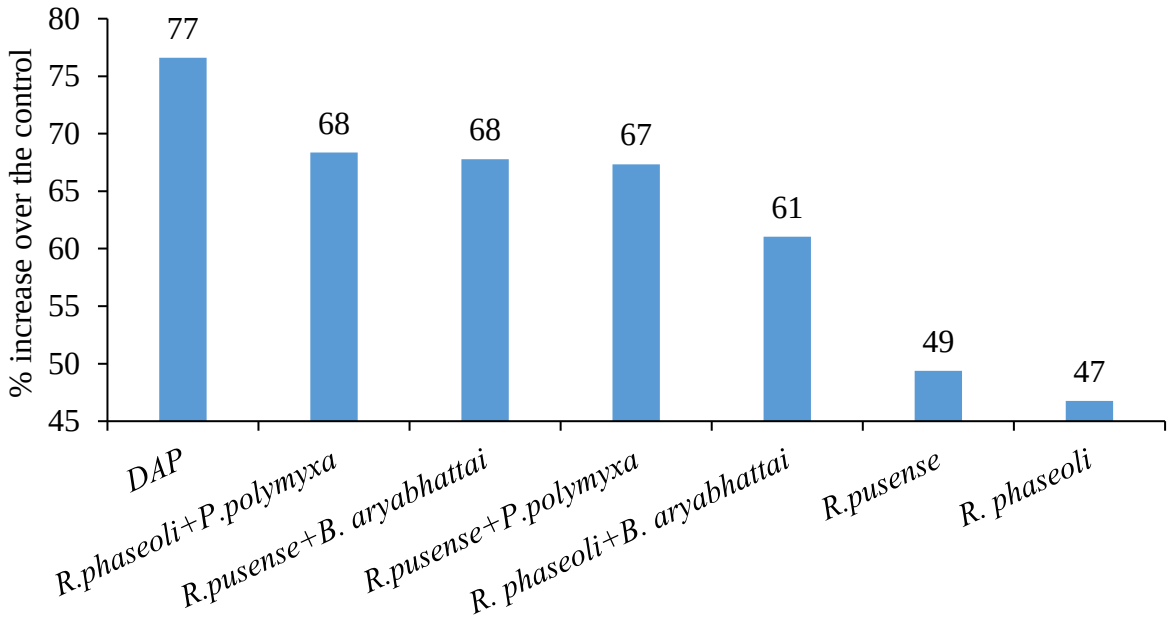

Figure 5.6: Percent (%) increase in common bean grain yield for inoculated and co-inoculate treatments over the negative control. Data are means pooled from the three soil types (Andosol, Ferralsol and Nitisol) and the two cropping seasons (2020 short rains and 2021 long rains).

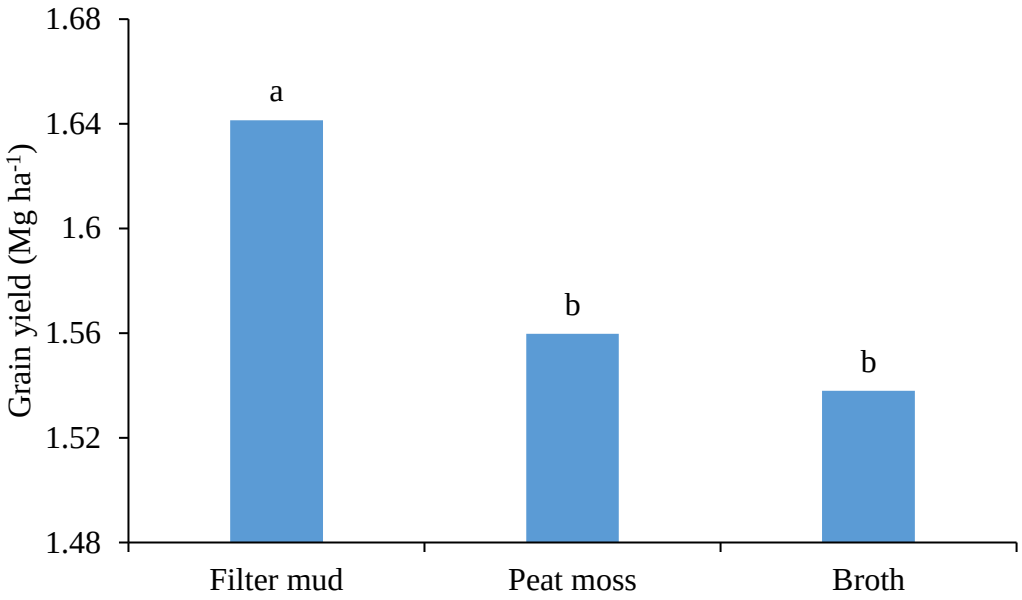

Figure 5.7: Effect of inoculant carrier material on the yield of common. Bars followed by different letters are significantly different from each other ($\alpha \leq 0.05$). The means are from data across the three soil types and the two cropping seasons.

The interaction between the bacterial strain and the carrier material on the yield of common bean showed that there was preference of strain to a carrier material. Inoculation involving *R. phaseoli* performed best when filter mud was used as the carrier material, although no significant difference was observed between the filter mud and peat moss in the *R. phaseoli* + *P. polymyxa* co-inoculation. For the *R. pusense* single inoculation, the broth (liquid) formulation performed significantly better than the solid-based (filter mud and peat moss) carrier materials. However, when co-inoculated with the *B. aryabhattai* and *P. polymyxa*, there were no significant difference in yield among the different carrier materials (Figure 5.8).

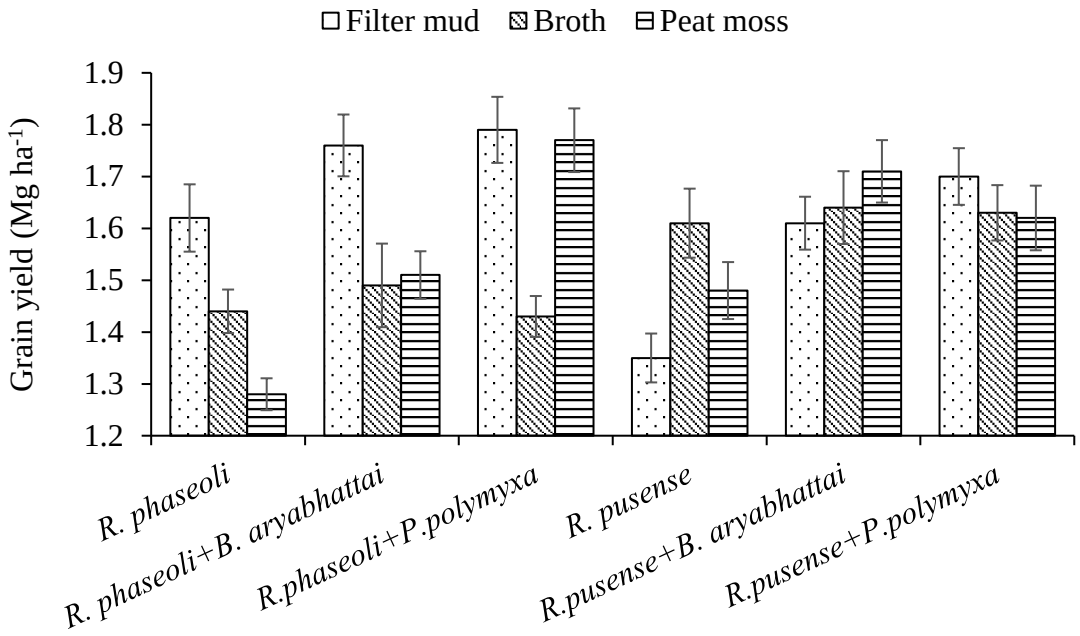

Figure 5.8: Interactive effect of strain and carrier material on the yield of common bean. Means are from the combined yield across the three soil types and the two seasons. Error bars represent the standard error of the means.

5.4.5 Effect of inoculation and formulation on the N and P tissue nutrient concentration of common bean

The application of DAP, inoculation and co-inoculation led to an increase in the N and P tissue concentration compared to the uninoculated control in all the soil types. The uninoculated plants had significantly lower N and P concentrations (Table 5.6). In the Andosol for example, there was no significant difference in both tissue N and P concentration between the inoculated and co-inoculated plants with the DAP application. In the Ferralsol, there was no significant difference in the tissue N between the DAP application and the co-inoculation of the common bean with *R. pusense +B. aryabhattai* and *R. pusense + P. polymyxa*. However, a significant difference as observed between the DAP application and the single application with *R. pusense*. A similar trend was observed with the P concentration except for the single *R. phaseoli* inoculation that was at par with the DAP application (Table 5.6). In the Nitisol, the respective co-inoculation of *R. phaseoli* with *either P. polymyxa* or *B. aryabhattai* and single *R. pusense* inoculation had a statistically similar N concentration as the DAP application. For the tissue P concentration, *R. pusense* inoculation led to the highest P value though not significantly different from the DAP application (Table 5.6). There was no significant effect of the carrier material in the overall tissue N and P concentration (Appendix 3).

Table 5.6: Effect of rhizobia and PSB co-inoculation on tissue N and P concentration of common bean in different soil types

	Andosol		Ferralsol		Nitisol	
Inoculation	N (%)	P (mg kg^{-1})	N (%)	P (mg kg^{-1})	N (%)	P (mg kg^{-1})
R. pusense +B. aryabhattai	2.36a	75.3a	1.99ab	63.8ab	1.72b	54.9a
R. pusense + P. polymyxa	2.35a	75.0a	2.10ab	67.1ab	1.55c	49.8b
R. phaseoli + P. polymyxa	2.37a	75.7a	1.85b	59.2b	1.84ab	55.6ab
R. phaseoli +B. aryabhattai	2.24a	72.4a	1.96b	60.3b	1.81ab	50.9b
R. pusense	2.37a	75.8a	1.86b	59.4b	1.82ab	58.4a
R. phaseoli	2.27a	72.9a	1.95b	62.4ab	1.75b	56.2ab
DAP	2.36a	79.2a	2.36a	71.9a	2.07a	57.9ab
Control	1.36b	36.8b	1.09c	31.4c	1.25d	36.2c
Tukey MSD ($\alpha \leq 0.05$)	0.19	8.4	0.37	11.5	0.31	6.7

Means followed by different letters within a column are significantly different at $\alpha \leq 0.05$. MSD= Minimum Significant Difference

5.4.6 Correlation among nodulation, growth, yield and nutrient uptake

The correlation analysis showed that there was significant correlation between the number of nodules and the root dry weight, shoot dry weight grain yield and the tissue N concentration. However, no significant relationship was observed between the number of nodules and the tissue P concentration (Table 5.7). The strongest positive correlation was observed between the tissue N and P concentrations ($r^2 = 0.93$). Similarly, the grain yield showed the strongest correlation ($r^2 = 0.69$) with the tissue P concentration followed by the tissue N concentration ($r^2 = 0.66$) (Table 5.7).

Table 5.7: Pearson's Correlation Coefficients for number of nodules, root and shoot dry weights, grain yield and tissue nutrient concentration (N & P)

	Nodules	RDW	SDW	GY	N	P
Nodules	1	0.44***	0.46***	0.42***	0.68**	0.13ns
RDW		1	0.75***	0.63***	0.26***	0.22**
SDW			1	0.61***	0.43***	0.50***
GY				1	0.66***	0.69***
N					1	0.93***
P						1

Asterisks denotes the significance levels; **, *** significant at $p < 0.01$ and $p < 0.001$ respectively; ns- not significant. RDW -root dry weight; SDW- shoot dry weight; GY – Grain yield; N- total nitrogen, P- total phosphorous.

5.4.7 Effect of inoculation on rhizobia population in the soil

The inoculation of common bean with the rhizobia singly or in combination with the PSB led to a significantly higher buildup of rhizobia population in the soil compared to the uninoculated control and the DAP application. Co-inoculation of common bean with *R. phaseoli + B. aryabhattai* led to significantly higher rhizobia build up in the soil (1.37×10^8 CFU/g of soil) compared to the single *R. phaseoli* inoculation with 0.99×10^8 CFU/g of soil (Figure 5.9). The buildup of the rhizobia populations following bacterial inoculation was consistent in all the study sites, but dependent on the specific rhizobia inoculation and its respective co-inoculations. For instance, in Andosol, co-inoculation of the common bean with *R. pusense + P. polymyxa* led to the highest rhizobia population while in Ferralsol, *R. phaseoli + P. polymyxa* inoculated plots had the largest rhizobia populations (Figure 5.10). In Nitisol, *R. pusense + P. polymyxa and R. pusense + B. aryabhattai* co-inoculations led to significantly larger rhizobia population than the other inoculation treatments (Figure 5.10).

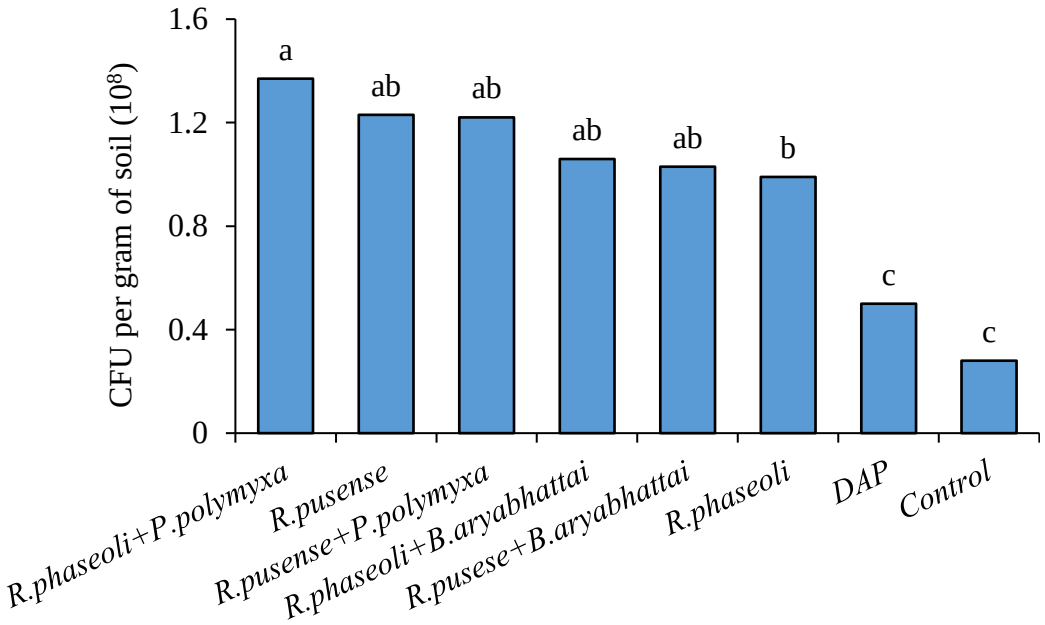

Figure 5.9: Effect of rhizobia and PSB co-inoculation on the population of rhizobia in the soil. Bars followed by different letters are significantly different from each other ($\alpha \leq 0.05$). The data is combined for three soil types (Andosol, Ferralsol and Nitisol).

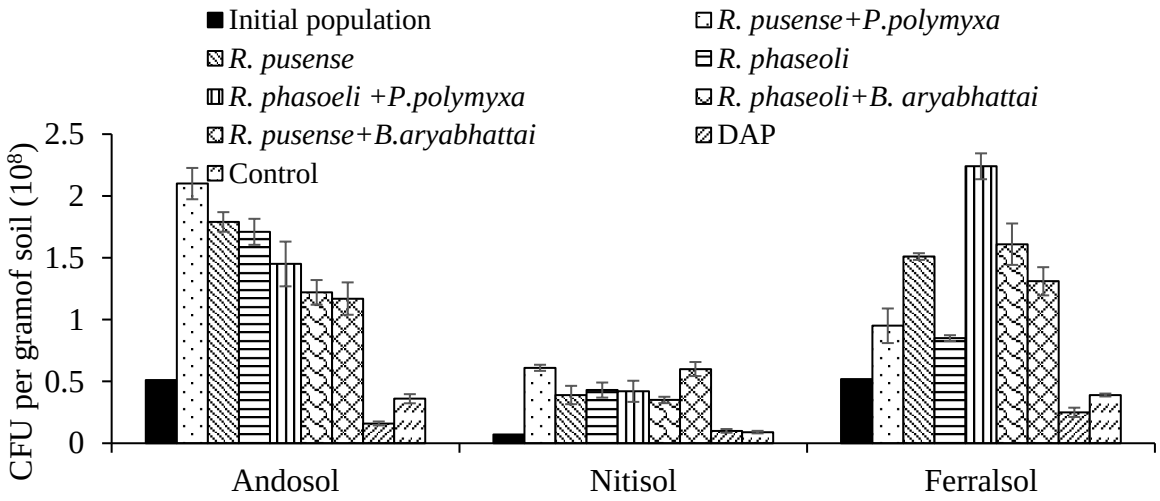

Figure 5.10: Effect of rhizobia and PSB co-inoculation on the population of rhizobia at the end of the two cropping seasons in the three soil types. Error bars represent the standard error of the means.

5.5 Discussion

5.5.1 Effect of inoculant formulation on common bean growth and yield

Results from this study revealed that, all three rhizobia and PSB formulations (peat, filter mud or liquid) generally increased nodulation, growth and grain yield, relative to uninoculated treatments. In terms of the effect of the carrier material, the results from the present study showed that generally, filter mud and peat-based inoculants performed better for enhancing nodulation, root and shoot growth, nutrient uptake and yield of common bean as compared to liquid inoculum. Previous authors have reported similar findings. For instance, Denton *et al*. (2009) reported that nodulation from liquid inoculants typically provided less nodule than peat inoculants. Similarly, Irfan *et al*. (2019) reported that filter mud and peat increased maize growth significantly when compared to un-inoculated control treatment and liquid inoculation. The improvement of crop growth and yield by the carrier-based inoculants over the liquid inoculants may be attributed to the ability of the carriers to increase bacterial survival rates by preventing them from being desiccated and drying (Berninger *et al*., 2018; Brahmaprakash & Sahu, 2012; Vassilev *et al*., 2020). Atieno *et al*. (2018) explained that cells grown in peat generally survive desiccation better than cells grown in liquid broth (Atieno *et al*., 2018). Ahmad *et al*. (2014) also reported that, carriers have better porosity, high water holding capacity and improved nutrient status, leading to better colonization and efficiency of inoculant. Moreover, application of liquid inoculum in natural conditions may not be successful due to different environmental constraints such as high soil temperatures, drought and salinity (Malusa *et al*., 2012).

Peat carrier material has been widely used and reported to be more superior in preserving inoculant population and improving crop performance. However, results from this study revealed that, the filter mud as a carrier material produced greater crop responses relative to the peat solid-based and the broth inoculants. A previous study by Ruíz-Valdiviezo *et al*. (2015) showed that strains *Sinorhizobium mexicanum* ITTGR7T, *R. calliandrae* LBP2-1T and *R. etli* CFN42T kept on sugarcane bagasse induced the largest number of nodules in the common bean. Similarly, Balume *et al*. (2015) found that filter mud was a better carrier when compared to the inorganic locally available horticultural vermiculite. Filter mud was reported to enhance almost all parameters significantly than un-inoculated treatment and liquid inoculation (Irfan *et al*., 2019). Sugarcane bagasse was shown to be the most appropriate carriers to store the rhizobial strains (2×10^9 cellsg^{-1}) and to maintain their survival. Sugar waste was found to allow a higher growth of legume nodulating bacteria in media comparing

to YEM standard media used for legume nodulating bacteria (Singh *et al*., 2011). These results are probably due to the differences in the physicochemical properties of the carrier materials (Ma, 2019; Roychowdhury *et al.*, 2015; Sohaib *et al*., 2020). One such feature is the moisture content and the water holding capacity. High moisture holding capacity of carrier material is desirable to support proper growth and multiplication of the bacteria (Mahdi *et al*., 2010; Sahu *et al*., 2016; Shahzad *et al*., 2017) thereby improving the survival and longevity of the inoculant (Sohaib *et al*., 2020; Zayed, 2016). Kumar *et al*. (2017) explained that high water holding capacity enhances the enzymatic processes involved in the breakdown of the organic materials thus providing essential nutrients for the bacteria. Contrary results have been reported by other authors who showed that liquid inoculant performed better than the solid-based carrier materials. For instance, Sahai and Chandra (2011) reported that liquid inoculants of *Mesorhizobium sp.* and PGPR led to significantly better nodulation and higher N and P uptake than their carrier-based inoculants. Use of liquid inoculant of PGPR resulted in higher N uptake by grain and straw by 6.7 and 19.4%, respectively, P uptake by grain by 7.1% and available N and P content in soil of 12.1 and 8.3%, respectively than its solid-based carrier inoculants (Sahai & Chandra, 2011). Arora *et al*. (2014) reported a significant difference in the survival of PGPR (*Rhizobium* and *Pseudomonas* strain) isolated from *Trigonella foenum graecum* at room temperature for 8 weeks stored in five different carrier materials. In addition, Brar *et al*. (2012) concluded that liquid biofertilizer formulation has a prolonged shelf life than carrier based biofertilizer.

5.5.2 Effect of co-inoculation on nodulation, growth and yield of common bean

Results from this study showed that inoculation of the common bean with rhizobia increased nodulation compared to the un-inoculated control and the mineral fertilizer application. Furthermore, upon co-inoculation of the common bean with the rhizobia strains and the PSB, there was a general increase in the nodulation compared to the single rhizobia inoculation. This is similar to previous results of studies carried out both in the greenhouse and under natural field conditions. For instance, Rajendran *et al*. (2012) reported an increased nodulation and root weight in greenhouse conditions when common bean was co-inoculated with rhizobia together with other nodule associated bacteria. Furthermore, Dumsane *et al*. (2020) reported that inoculation of white clover with *Rhizobium* in combination with two PGPRs, *Bacillus aryabhattai* strain Sb and *Azotobacter vinelandii* strain G31, enhanced significantly nodule number, as compared to single *rhizobium* inoculation. Similarly, Sánchez *et al*. (2014) reported that field co-inoculation of common bean with *R. pisi (R40982)* + *P.*

monteilii (R43453) increased nodule number and nodule dry weight by 55% and 133%, respectively over single inoculation with *R. pisi (R40982)*. However, significant increases in nodulation of co-inoculated treatments as compared to non-inoculated treatment have been observed (Souza & Ferreira, 2017). Zaheer *et al*. (2016) reported that co-inoculation of chickpea with Serratia enhanced successfully nodulation in a nutrient deficient soil. Furthermore, Raklami *et al*. (2019) reported the positive effect of co-inoculation with rhizobia and PGPRs on nodulation of faba been. Likewise, Sibponkrung *et al*. (2020) reported that co-inoculation of *Bacillus velezensis* S141 with *Bradyrhizobium* USDA110 into soybean resulted in enhanced nodulation and N2-fixing efficiency. A significantly higher increase was obtained by inoculating chickpea with Meso*rhizobium* sp. MA72 combined with E. aerogenes P1S6. This combination allowed an enhancement of more than 270% in nodulation (Benjelloun *et al*., 2021). Several other studies on the co-inoculation of PGPRs along with rhizobia has shown to enhance nodulation and symbiotic interaction of many legumes (Fatnassi *et al*., 2015; Kong *et al*., 2017; Morel *et al*., 2012; Samavat *et al*., 2012; Swarnalakshmi *et al*., 2020; Verma *et al*., 2010; Zafar *et al*., 2011).

The present results showed a varied response of the common bean to inoculation across the three study sites. This may be attributed to the soil and ecological factors during the study period such as the temperature, soil moisture, soil type, rhizobia population and their effectiveness (Mohammadi *et al*., 2012; Solomon *et al*., 2012). Previous studies have also reported site specific plant response to rhizobia and PGPR inoculants and this might be attributed to differences in effectiveness of *rhizobium* strains and native rhizobia strain population among locations (Kellman, 2008). The soil rhizobia population from the present study showed varying population of native rhizobia.

Results from this study showed that, generally there was an increase in the root and shoot dry weight of common bean due to inoculation with the rhizobia and PSB relative to the uninoculated control. Similar findings were reported by Abbas *et al*. (2018) whose study revealed that the inoculation of *Rhizobium, Bacillus* and *Pseudomonas* in faba bean showed a positive effect of on host plant's growth when compared to control. Furthermore, co-inoculation of rhizobia and the PSB led to higher root and shoot biomass of common bean compared to the single rhizobia inoculation for most of the specific rhizobia-PSB strain. The increase in shoot dry weight of common bean may be attributed to increased root proliferation induced by used rhizobia or PSB or both, mobilization of insoluble nutrients, thus promoting nutrient and water uptake by roots (Huang *et al*., 2015; Israr *et al*., 2016). Additionally, Talaat

et al. (2008) and Bello *et al*. (2018) suggested that growth hormones synthesized by *rhizobium* have been considered as the other probable means to promote plant growth.

Similar to the present findings, a previous study by Benidire *et al.* (2017) has reported that co-inoculation of rhizobial strains compared to single inoculation improved growth parameters and yield components of common bean under field conditions. Similarly, Zafar *et al*. (2011) reported that co-inoculation with rhizobia and PGPRs enhanced the shoot and root dry weight of common bean. Dumsane *et al*. (2020) demonstrated that both the individual inoculation of two *Rhizobium* strains and their co-inoculation with the two PGPR, *B. aryabhattai* strain Sb and *A. vinelandii* strain G31, significantly increased the dry weight of white clover. Furthermore, Raklami *et al*. (2019) reported the positive effect of co-inoculation with rhizobia and PGPRs on biomass and growth of faba been. Several authors have reported that co-inoculation stimulate plant growth of many legumes more than separate inoculation (Abbas *et al*., 2018; Benjelloun *et al*., 2021; Bulegon *et al*., 2017; Fatnassi *et al*., 2015; Fox *et al*., 2011; Hungria *et al*., 2013; Hungria *et al*., 2015; Kong *et al*., 2017; Morel *et al*., 2012; Singh *et al*., 2013; Verma *et al*., 2010; Zaheer *et al*., 2016).

Relative to the application of inorganic source of N and P in the present study, results showed that co-inoculation of the rhizobia and PSB led to a higher root and shoot dry weight that were statistically at par with the inorganic fertilizer application. Similar results were reported by Cardoso and Ferreira (2021) who found out that the co-inoculation of rhizobia and azospirilla treatment resulted in higher SDW, whereas the co-inoculation of rhizobia and azospirilla and the co-inoculation of rhizobia, azospirilla and trichoderma provided higher root and SDW, resulting in values similar to those of the N-fertilizer treatment. Comparison of the effect of co-inoculation on shoot and root dry weights across the three study sites and seasons showed that the observed increase in shoot and root dry weights resulting from specific rhizobia-PSB co-inoculation was not proportional. This may be explained by the fact that plants partition biomass between shoot and root to effect optimal utilization of available resources such as nutrients and water. Under nutrient and water stress conditions, more biomass is allocated mainly to the root system resulting in an increase in the absorbing plant organ. On the other hand, plants that have adequate nitrogen supply and soil moisture direct biomass predominately to the shoot. This explains the differences in the shoot and root weight of the common bean across the three study sites that had varying soil characteristics and variable rainfall amounts during the study period.

Findings from the present study showed that inoculation with rhizobia and rhizobia-PSB co-inoculation increased the grain yield of common bean compared to the uninoculated

control. Furthermore, rhizobia-PSB co-inoculation led to higher grain yield that was statistically at par with the application of inorganic N and P. However, the single rhizobia yields were significantly lower than the inorganic N and P application. Some studies have shown that inoculation of common bean with rhizobia results in equal and/or higher grain yield as compared to N-fertilizer treatment (Souza & Ferreira, 2017). These results suggests that inoculation with mixed bacteria inocula have a synergistic effect on the yield of the common bean. Bharti *et al*. (2014) explained that the applications of PGPR combinations are more in line with the natural law and allows for the interaction and synergy between various microorganisms in the natural ecological environment.

Benidere *et al*. (2017) reported that co-inoculation of rhizobial strains compared to single inoculation has advantages to improve growth parameters and yield components of common bean under field conditions. Hungaria *et al*. (2013) have also demonstrated that seed inoculation of common bean with *Rhizobium tropici* alone increased yield by 8.3%, while co-inoculation with *A. brasilense* boosted the yield to 19.6%. Additionally, Yadegari (2014) showed that there were significant increases in seed yield of common bean as a result of treatment combination of *rhizobium* strains and PGPR strains. Similarly, Yadegari *et al*. (2010) reported seed yield production from co-inoculation of *Rhizobium* + *P. fluorescens* P-93 with a significant increase of up to 73% over *Rhizobium* alone. Benjelloun *et al*. (2021) reported up to 242% increase in grain yield of chickpea as a result of co-inoculation with *Mesorhizobium* sp. MA72 combined with *E. aerogenes* P1S6. Several other studies have reported on the rhizobia PGPR co-inoculation advantage over single rhizobia inoculation in many legumes (Galindo *et al*., 2018; Hungria *et al*., 2015; Okon *et al*., 2015; Raklami *et al*., 2019; Singh *et al*., 2013; Souza & Ferreira, 2017; Verma *et al*., 2010; Zeffa *et al*., 2020).

Most of the tropical soils have low P availability and this has been shown to compromise the biological nitrogen fixation (BNF) of legumes. In the present study, the PSB *Bacillus aryabhattai* and *Paenibacillus polmyxa* were shown to increase the grain yield of common bean. This is probably as a result of their phosphate solubilizing potential availing P for the optimum activity of the rhizobia strains and BNF (Morel *et al*., 2012; Megu *et al*., 2024; Shiri-Janagard *et al*. 2012). A study by Samavat *et al*. (2012) indicated that legume plants inoculated with phosphate solubilizing rhizobacteria showed enhanced yield. These results were similar to findings of Elkoca *et al*. (2010) reporting that co-inoculation of chickpea with rhizobia and PSB such as *Bacillus subtilis* and *Bacillus megaterium* resulted in a higher seed yield.

5.5.3 Nutrient content in plant tissues

Co-inoculation of common bean with the two rhizobia strains and the respective PSB strains increased the concentration of N and P in the plant relative to the uninoculated control. Comparison of the inoculation with the inorganic N and P application revealed that in most of the study sites, specific rhizobia-PSB strain combination led to similar nutrient uptake level as the inorganic NP application. Zhang *et al*. (2014) and Damodharan *et al*. (2018) explained that inoculating PGPR can effectively make the invalid form of soil nutrients available and promote the efficient absorption and utilization of nutrients by plants. Abbas *et al*. (2015) reported that inoculation with PSB as biofertilizers enhances P accumulation and biomass production of plants. Lavakusha *et al*. (2014) demonstrated that naturally abundant rhizospheric microorganisms, phosphate solubilizing bacteria can dissolve insoluble P compounds in soil and help plants in accessing P required for growth. Increased P uptake have been reported for *Paenibacillus polymyxa* and *B. megaterium* in tomato (EI-Yazeid & Abou-Aly, 2011). Dumsane *et al.* (2020) also found out that the combination of *Rhizobium* and PGPR enhanced macronutrient contents in white clover under low P condition. Increase in shoot N and P in legumes under inoculation with rhizobia and PSB have been widely reported (Abbas *et al*., 2018; Souza *et al*., 2016).

5.5.4 Effect of rhizobia and PSB inoculation on the microbial build-up

The present study sought to determine the effect of field inoculation with rhizobia and PSB on rhizobia population. From the results obtained at the end of the two cropping seasons, it was evident that inoculation can alter the microbial community of a soil. The results showed that the inoculated plots had significantly higher number of rhizobia population compared to the uninoculated plots. Similar results have been reported by other authors. For instance, Fall *et al*. (2016) reported that *rhizobium* inoculation affected the soil microbiota in addition to enhancing crop performance. They observed an increase in soil microbial biomass as well as functional diversity. Similarly, Mawarda *et al*. (2020) reported a direct interaction between the soil microbial inoculants and the indigenous soil microbial community. Field inoculation showed significant effects on bacterial structure and diversity in the bulk soil of common bean (Trabelsi *et al*., 2011; Trabelsi *et al*., 2012). Furthermore, *rhizobium* inoculation has been documented to have significant influence on the bacterial community in the rhizosphere of soybean plants (Zhong *et al*., 2019). Other studies have demonstrated the increase in specific beneficial soil microbiota because of inoculation (Han *et al*., 2020; Numan *et al*., 2018; Peng

et al., 2021). The present findings shows that the introduced rhizobacteria strains affects the bacterial structure in the soil and these could lead to positive response to future inoculation.

5.6 Conclusions

In this study, it is concluded that the inoculant carrier material influences the effectiveness of the inoculants and plant performance. The solid carrier material, specifically filter mud, showed potential for use in formulation of inoculants since it enhanced the ability of the strain to survive/establish well enough. Further, specific co-inoculation of rhizobia (*Rhizobium phaseoli* and *Rhizobium pusense* strains) and phosphate solubilizing bacteria (*Paenibacillus polymyxa* and *Bacillus aryabhattai*) increased the yield of common bean. Inoculation with the bacterial strains significantly increased the yield of common bean over the uninoculated control. This was consistent across the soil types and seasons except for the single rhizobia inoculation in Nitisol during the 2021LR season. Furthermore, co-inoculation led to higher yield than the DAP application in the Andosol (2020SR), Nitisol (2020SR) and in the Ferralsol (2021LR). The two native rhizobia and two PSB used in this study might promote plant growth via one or a combination of their plant growth-promoting traits. Furthermore, this study has shown that inoculation led to a build-up of the rhizobia population in the soil. The increased build-up of rhizobia strains in soils could increase the competition between introduced rhizobia with the natural populations increasing the effectiveness of the inoculation practice. Based on these findings, it is recommended to explore the use of solid carrier materials, such as filter mud, in formulating inoculants for common bean cultivation. Additionally, co-inoculation with specific rhizobia and phosphate-solubilizing bacteria should be considered as a promising strategy to improve common bean yield, particularly in areas where chemical fertilizers are less accessible or environmentally undesirable. Further research could focus on optimizing inoculant formulations, assessing their performance under different environmental conditions, and evaluating their long-term effects on soil health and crop productivity.

CHAPTER SIX

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

6.1 General Discussions

6.1.1 In vitro IAA production and phosphate solubilization

The study revealed that rhizobia strains have different IAA producing ability. *R. pusense* (S5) and *R. phaseoli* (B3) produced higher levels of IAA with absorbance values of 1.33 and 1.14 respectively. This suggests that these particular strains possess a greater capacity to synthesize IAA, potentially offering enhanced benefits in promoting root elongation, cell division, and overall plant growth when used as bioinoculants (Singh *et al*., 2024). Further, the findings highlight significant variations in the performance of different rhizobia species, underscoring their potential applications in agricultural biotechnology. The current findings indicates that the tested rhizobia and bacillus strains possess the ability to solubilize insoluble phosphate *in vitro,* although to different extents. The solubilization of insoluble phosphate by soil microorganisms, including rhizobia, is a key mechanism to enhance phosphorus availability (Elhaissoufi *et al*., 2022). *Rhizobium pusense* (S5) showed the greatest solubilization efficiency of 648 and consequently the highest solubilization index of 7.3. This high solubilization efficiency suggests that *Rhizobium pusense* (S5) can convert insoluble phosphate into soluble forms more effectively than the other strains tested. The differential abilities of rhizobia and PSB strains to produce IAA and solubilize phosphate have significant implications for their use in sustainable agriculture. Strains with high IAA production and phosphate solubilization efficiency, can be strategically utilized to develop biofertilizers (Singh *et al*., 2024) reducing the need for synthetic chemicals and promoting eco-friendly farming practices.

6.1.2 Greenhouse plant growth promotion assay

The greenhouse plant growth promotion assay showed that inoculation of common bean with the isolated bacteria led to increased crop growth and uptake of both N and P. The highest shoot biomass was observed when *R. phaseoli* was co-inoculated with *P. polymyxa* (4.3 g plant^{-1}) compared to the single *R. phaseoli* inoculation (1.1 g plant^{-1}), underscoring the beneficial interactions between these microbial species. This is consistent with the findings of Ahmad *et al*. (2019), who demonstrated that co-inoculating mung bean plants with *Bacillus aryabhattai* S10 and *Bacillus subtilis* ZM63 led to increased nodulation, enhanced plant growth, and improved nutritional status of the plants. The improvement in nodulation parameters is likely

due to the enhanced formation of nodules, facilitated by the larger surface area available for rhizobial infection during nodulation. Similarly, Yasmeen and Bano (2014) observed that mixed inoculation of soybean plants with *Rhizobium* and PSB resulted in increased plant biomass, as well as better root nodulation parameters. This enhancement can be attributed to the increased production of auxin and gibberellin by the combined microbial activity, leading to greater cell division and elongation. Additionally, Kannapiran and Ramkumar (2011) found that the nitrogen status of black gram (*Phaseolus mungo*) was improved following inoculation with several bacteria, including two PSB strains (*Pseudomonas aeruginosa* and *Bacillus* sp). *Rhizobium phaseoli* is known for its nitrogen-fixing capabilities, converting atmospheric nitrogen into forms accessible to plants while *P. polymyxa*, on the other hand, is effective in solubilizing insoluble phosphate, thereby making phosphorus more available to plants (Mohd Din *et al*., 2020; Sanyal *et al*., 2020). The shoot tissue nitrogen and phosphorous concentration increased by up to 32% and 75% respectively as a result of co-inoculation of PSB with Rhizobia. These findings highlight the potential of microbial inoculants, particularly co-inoculation strategies, to enhance nutrient acquisition by common bean plants, thereby improving their overall growth and yield potential (Pastor-Bueis *et al*., 2021).

6.1.3 Survival of PGPR strains in carrier material and storage temperature

The storage temperature affects the survival and longevity of bacterial strains in inoculants. The inoculant stored in the fridge (4℃) had a significantly higher (3.73×10^9 CFU per gram/ml of inoculant) survival than those stored at room temperature (16±2℃) with 2.87×10^9 CFU per gram/ml of inoculant. Storage under low temperature (4 °C) sustained higher viable bacterial cells than at room temperature particularly for liquid inoculant, while filter mud sustained higher population under room temperature. This has been attributed to the ability of lower temperatures to slow down metabolic activities, reducing nutrient consumption and waste accumulation, thereby enhancing bacterial longevity (Thirumal *et al*., 2017). Bashan *et al*. (2014) noted that refrigerated storage is a common practice to maintain the viability of microbial inoculants, as it slows down cellular processes and minimizes stress factors that can lead to cell death.

This study also pointed out differential effects of storage temperature based on the form of the inoculant. While liquid inoculants sustained higher bacterial populations at low temperatures, filter mud inoculants maintained a higher population at room temperature. The high-water content in liquid inoculants can lead to more rapid bacterial metabolism and waste accumulation at room temperature, causing a decline in cell viability (Herrmann & Lesueur;

2013; Mahanty *et al*., 2017). Therefore, to sustain longer survival of strains liquid formulations, some additives are added to improve microbial strains survival under different environmental conditions (Mącik *et al*., 2020). For solid carriers like filter mud, room temperature storage might be adequate, reducing the need for refrigeration infrastructure, particularly in regions with limited access to cold storage facilities (Ariani & Simarmata, 2023).

Understanding the impact of storage temperature on bacterial survival has practical implications for the production, storage, and application of microbial inoculants (Berninger *et al*., 2018). Understanding the storage requirements can help in planning the distribution and application timing of inoculants to coincide with optimal planting periods, ensuring maximum benefits from microbial inoculants (Ali *et al*., 2019). This ensures that farmers receive inoculants with a high number of viable cells, enhancing their effectiveness.

6.1.4 Influence of carrier material and co-inoculation on the effectiveness of the rhizobia and phosphate solubilizing bacteria strain

The selection of inoculant carrier material is a critical factor influencing the effectiveness of microbial inoculants and overall plant performance. In terms of the overall effect of the carrier material, significantly higher yield was obtained when filter mud was used as the inoculant carrier material (1.64 Mg ha^{-1}) compared to peat moss (1.56 Mg ha^{-1}) and broth culture (1.54 Mg ha^{-1}). The effectiveness of different carrier materials can vary due to their physical and chemical properties, such as moisture retention, nutrient content, and pH buffering capacity. Filter mud, a byproduct of the sugarcane industry, is rich in organic matter and nutrients, providing a favourable environment for microbial survival and proliferation (Anwar *et al*., 2021). Additionally, filter mud has excellent moisture-holding capacity, which ensures that microbial inoculants remain hydrated and viable for longer periods, particularly under field conditions (Aloo *et al*., 2022; Alori & Babalola, 2018; Santos *et al*., 2019). Peat moss on the other hand, is known for its good pH buffering capacity and ability to retain moisture. Peat moss, while effective, has environmental drawbacks such as habitat destruction and CO_2 emissions. It also requires importation in regions without natural peat bogs, increasing costs (Santos *et al*., 2019). Broth cultures although effective, they might not provide the sustained release of nutrients and moisture that solid carriers like filter mud or peat moss can offer, potentially leading to lower yields in some cases (Arora *et al*., 2023). Bashan *et al*. (2014) reported that carriers with high organic matter content, such as filter mud, enhanced the shelf life and activity of microbial inoculants, resulting in higher crop yields compared to standard carriers like peat moss.

Results from the present study showed that co-inoculation with the rhizobia and the PSB led to more than 60% increase in the yield of common bean compared to the uninoculated control while the single inoculation of rhizobia led to 49% and 47% more than the uninoculated control for the *R. pusense* and *R. phaseoli* respectively. This highlights the potential of microbial inoculants to enhance crop productivity. The co-inoculation of rhizobia and PSB has been extensively studied for its potential to enhance nodulation and overall growth of legumes. The co-inoculation of rhizobia and phosphate-solubilizing bacteria significantly enhances nodulation, tissue N and P concentration, and overall plant growth in legumes (Matse *et al*., 2020; Shome *et al*., 2022). This synergistic interaction utilizes the nitrogen-fixing capability of rhizobia and the phosphorus-solubilizing ability of PSBs, leading to improved plant health and yield (Kumawat *et al*., 2021; Pandey *et al*., 2020). By improving nodulation, nitrogen fixation, and phosphorus availability, co-inoculation enhances nutrient uptake, root development, and overall plant growth (Abd El *et al*., 2022; Bechtaoui *et al*., 2020; Benjelloun *et al*., 2021). Several other studies have reported improved soil nutrient status, enhanced nutrient uptake and increase in nodulation, growth, yield component, yield, of common bean following co-inoculation of common with *rhizobium* strains with other PGPR strains such as *A. brasilense*, *Bacillus megaterium*, *Pseudomonas fluorescens* (de Almeida Leite *et al*., 2022; Filipini *et al*., 2021; Gharib *et al*., 2015; Messias *et al*., 2023; Steiner *et al*., 2019). Furthermore, the finding from this study underscore the potential of microbial inoculants in sustainable agriculture by reducing dependency on chemical fertilizers and improving crop productivity in a sustainable and environmentally friendly manner.

The response of plants to bacterial inoculant inoculation can vary significantly depending on the type of soil. Therefore, it is vital to consider soil type when applying bacterial inoculants in agriculture. Different soil textures and chemical compositions can influence the colonization and effectiveness of inoculants, thereby affecting plant growth and soil health outcomes (Bhat *et al*., 2019; Chamizo *et al*., 2018; Salwan *et al*., 2019). For practical applications, adapting inoculant strategies to specific soil types can optimize benefits and improve sustainable agricultural practices.

6.2 Conclusions

i. The study revealed significant variability in the ability of rhizobia strains to produce indole-3-acetic acid (IAA), a key plant growth regulator. *Rhizobium pusense* (Busia) and *Rhizobium phaseoli* (Bungoma) exhibited higher levels of IAA production compared to other strains. Additionally, both rhizobia and Bacillus strains demonstrated

varying degrees of phosphate solubilization capacity in vitro. *Rhizobium pusense* (Busia) showed the highest efficiency in phosphate solubilization, indicating its potential for enhancing plant phosphorus uptake.

ii. The greenhouse plant growth promotion assay demonstrated that inoculation of common bean with isolated bacteria leads to significant increases in crop growth and shoot N and P concentrations. Co-inoculation of *Rhizobium phaseoli* with *Paenibacillus polymyxa* resulted in the highest shoot biomass and increased shoot tissue nitrogen and phosphorus concentrations, indicating synergistic effects of co-inoculation on plant growth and nutrient uptake.

iii. The study found that storage temperature significantly affects the survival and longevity of bacterial strains in inoculants. Storage under low temperature (4 °C) sustains higher viable bacterial cells compared to room temperature (16±2°C), particularly for liquid inoculant. Inoculant carrier materials also influence bacterial survival, with filter mud sustaining higher populations under room temperature conditions.

iv. The effectiveness of inoculants and plant performance is influenced by the choice of inoculant carrier material. Filter mud is an effective carrier material, resulting in significantly higher yields compared to peat moss and broth culture carriers. Furthermore, co-inoculation with rhizobia and phosphate-solubilizing bacteria leads to substantial increases in common bean yield compared to uninoculated control plants.

6.3 Recommendations

i. Given the variability in indole-3-acetic acid (IAA) production and phosphate solubilization capacity among rhizobia and PSB strains, it is recommended to carefully select strains with high levels of these abilities for inoculant formulation.

ii. Using the filter mud carrier material, 6 months storage at low temperatures (4 ºC) is recommended, while 3-4 months is recommended for temperatures around 16ºC (shelf storage).

iii. The study recommends the use of filter mud as biofertilizer carrier materials since it enhanced rhizobial response and its efficacy should be further investigated under various soil types and environmental conditions.

iv. Co-inoculation of compatible rhizobia and PSB, such as *Rhizobium phaseoli* with *Paenibacillus polymyxa*, should be further explored as a strategy to enhance plant growth and nutrient uptake in common bean and potentially other leguminous crops.

6.4 Further Research

i. Studies exploring the synergistic or antagonistic interactions between rhizobia and PSB in co-inoculation practices can provide valuable insights into optimizing microbial inoculant formulations.

ii. For further studies, the effect of physicochemical properties of the carrier material to increase the inoculant shelf life for more than six months should be evaluated.

iii. More studies are necessary to evaluate efficacy of various inoculants stored under different storage conditions.

iv. Research focusing on the physical and chemical properties of carrier materials, their impact on microbial viability and plant-microbe interactions, and their compatibility with different soil types can help optimize inoculant formulations for maximum efficacy.

v. Studies addressing the scalability, cost-effectiveness, and practicality of large-scale production and distribution of microbial inoculants are needed to facilitate their widespread adoption by farmers.

vi. Collaboration between researchers, agricultural practitioners, and policymakers is essential to drive innovation and facilitate the development and adoption of effective microbial inoculants for sustainable agriculture.

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APPENDICES

Appendix A: Media preparation

Yeast Extract mannitol Agar

Reagents

1. Yeast extract - 5 g
2. Mannitol - 10 g
3. Dipotassium phosphate (K_2HPO_4) - 0.5 g
4. Magnesium sulphate ($MgSO_4.7H_2O$) - 0.2 g
5. Sodium chloride (NaCl) - 0.1 g
6. Agar - 15 g (for agar plates)
7. Distilled water - 1 liter

Procedure:

1. Dissolve yeast extract, mannitol, dipotassium phosphate, magnesium sulphate, and sodium chloride in distilled water in a suitable container or flask.
2. Mix the ingredients thoroughly until completely dissolved.
3. If making agar plates, add agar to the solution and mix well.
4. Autoclave the medium at 121°C for 15 minutes to sterilize it.
5. After autoclaving, allow the medium to cool down to around 45-50°C before pouring it into sterile Petri dishes for agar plates.
6. Let the agar plates solidify and then store them in a refrigerator at 4°C until ready to use.

Pikovskaya's medium

Reagents (gL^{-1}):

1. Dextrose (glucose) - 10 g
2. Calcium phosphate - 10 g
3. Potassium chloride - 0.2 g
4. Magnesium sulphate - 0.1 g
5. Manganese sulphate - 0.0001 g
6. Ferrous sulphate 0.0001 g
7. Yeast extract - 0.5 g
8. Agar - 15 g

Distilled water - 1 liter

Procedure:

1. Dissolve all the ingredients, except agar, in distilled water in a suitable container or flask.
2. Adjust the pH of the solution to around 7.0 using a pH meter or pH indicator strips.
3. If making agar plates, add agar to the solution and mix well.
4. Autoclave the medium at 121°C for 15 minutes to sterilize it.
5. After autoclaving, allow the medium to cool down to around 45-50°C before pouring it into sterile Petri dishes for agar plates.
6. Let the agar plates solidify and then store them in a refrigerator at 4°C until ready to use.

Preparation of Nitrogen-free Nutrient solution (Broughton and Dilworth, 1970)

Reagents

Reagent groups		Quantity (g/L)
1.	$CaCl_2.2H2O$	294.1
2.	KH_2PO_4	136.1
3.	$FeC_6H_5O_7.3H_20$	6.7
	$MgSO_4.7H_2O$	123.3
	K_2SO_4	87
	$MnSO_4.H_2O$	0.338
4.	H_3BO_3	0.247
	$ZnSO_4.7H_2O$	0.288
	$CuSO_4.5H_2O$	0.10
	$CoSO_4.7H_2O$	0.056
	$Na_2MoO_4.2H_2O$	0.048

Prepare stock solutions of reagent groups 1–4 using warm water to dissolve the ferric citrate. To make 10 L of full-strength solution, add 5 mL of each stock to 5 L DI water and mix. Dilute to 10 L by adding another 5 L of DI water. Adjust pH to 6.6–6.8 with 1 N NaOH.

Procedure

1. Dissolve the calcium chloride, magnesium sulphate, potassium dihydrogen phosphate, and potassium sulphate in distilled water in a suitable container or flask.
2. Add the trace element solution and iron (III) ethylenediaminetetraacetic acid (Fe-EDTA) to the solution and mix well.
3. Prepare a separate stock solution of boron, manganese, zinc, copper, and molybdenum by dissolving the respective salts in distilled water.
4. Add the appropriate volumes of the stock solutions of boron, manganese, zinc, copper, and molybdenum to the main solution and mix well.
5. Adjust the pH of the nutrient solution to the desired level (around 6.0) using dilute hydrochloric acid (HCl) or potassium hydroxide (KOH) as needed.

6. Top up the solution to 1 liter with distilled water and mix thoroughly.
7. Sterilize the nutrient solution by autoclaving at 121°C for 15 minutes.
8. After autoclaving, allow the solution to cool down before use.

Appendix B: ANOVA results for the factors affecting the survival of bacterial inoculant

Source of variation	d.f	Mean square
Replicate	2	0.41
Days	5	153.49***
Strain	3	6.34***
Days*strain	15	0.09ns
Carrier	1	0.17ns
Days*carrier	5	1.87***
Strain*carrier	3	0.18ns
Days*strain*carrier	15	0.02ns
Storage	1	52.04***
Days*storage	5	3.35***
Strain*storage	3	0.22ns
Days*strain*storage	15	0.04ns
Carrier*storage	1	4.89***
Days*carrier*storage	5	3.07***
Strain*carrier*storage	3	0.03ns
Days*strain*carrier*storage	15	0.06ns
Error	190	0.18
Total	287	
CV (%)		12.8
R^2		0.96

Appendix C: Mean square values for the factors affecting the nodulation, growth and yield of common bean

Source of variation	df	Nodules	Root weight	Shoot weight	Yield
Replicate	2	0.088	0.843	197.33	1.589
Soil type	2	1.407^{***}	27.40^{***}	5416.54^{***}	8.287^{***}
Inoculation	7	1.891^{***}	5.636^{***}	371.74^{***}	3.095^{***}
Site*inoculation	14	0.099^{*}	0.507^{ns}	77.67^{*}	0.216^{ns}
Season	1	3.324^{***}	147.117^{***}	13732.07^{***}	11.398^{***}
Season*inoculation	7	0.083^{ns}	0.545^{ns}	113.75^{**}	0.216^{ns}
Inoculation*Replicate (Error a)	14	0.117^{**}	0.161^{ns}	12.91^{ns}	0.119^{ns}
Formulation	2	0.047^{ns}	0.151^{ns}	23.03^{ns}	0.346^{*}
Inoculation*formulation	10	0.029^{ns}	0.198^{ns}	15.97^{ns}	0.348^{**}
Error	371	0.053	0.332	40.81	0.145
Total	431				
CV (%)		18.3	25.8	29.1	25.0
R^2		0.55	0.68	0.66	0.54

Asterisks denotes the significance levels; *, **, *** significant at $p< 0.05$, 0.01 and 0.001 respectively; ns –not significant

Appendix D: ANOVA means squares for the effect of inoculation on tissue N and P concentration

Source of variation	df	Nitrogen (N)	Phosphorous (P)
Replicate	2	0.35	1203.9
Soil type	2	4.32^{***}	5865.3^{***}
Inoculation	4	3.04^{*}	41.95^{*}
Soil type*inoculation	14	0.17^{*}	104.12^{**}
Inoculation*Replication (Error a)	14	0.28	177.77
Formulation	2	0.07^{ns}	157.96^{ns}
Inoculation*formulation	10	0.13^{ns}	214.18^{**}
Random Error	164	0.09	83.46
Total	215		
CV (%)		15.6	15.0
R^2		0.70	0.76

Appendix E: ANOVA mean square on the effect of inoculation on rhizobia population at harvest in the soil

Source of Variations	df	Mean square
Replicate	2	0.445
Soil type	2	2.302***
Inoculation	7	0.479***
Soil type*inoculation	14	0.086***
Error	46	0.022
Total	71	
CV (%)		1.9
R^2		0.90

Asterisks denotes the significance levels; *** significant at p< 0.001.

Appendix F: Research permit

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Appendix G: Abstract of first paper

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Co-inoculation of Rhizobacteria in Common Bean (*Phaseolus vulgaris*) Production in East Africa

Chapter | First Online: 21 March 2023
pp 207–224 | Cite this chapter

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Abstract

Plant growth-promoting microorganisms (PGPMs) have been shown as an important component of agricultural sustainability. The use of PGPMs is an environmentally friendly approach to increasing the yields of crops through their various direct and indirect mechanisms. It has been proven that the benefits of these PGPMs can be tapped by understanding the intrinsic crop–PGPM relationship and harnessing it for crop improvement. This review has examined work done on bean–rhizobia–PGPM globally, with specific examples from East Africa. The mode of action of PGPMs, their effect on nodulation, growth, and yield, considerations for the formulation of mixed consortia inoculations, and commercialization of such inoculants are discussed. Overall, many studies show a synergistic effect of rhizobia–PGPM in controlled and field environments. The response is influenced by abiotic factors including soil moisture stress, temperature, salinity, and biotic factors. Formulation of bacterial inoculants in East Africa is mostly peat-based, and none of the commercially available inoculants are made of rhizobia–PGPM consortia. The findings of this review indicate the opportunity for commercial exploitation of rhizobia–PGPM consortia for bean production in East Africa.

Appendix H: Abstract of second paper

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RESEARCH ARTICLE

Influence of native rhizobacteria co-inoculation and formulation of bacterial inoculants on the growth and yield of common bean (*Phaseolus vulgaris* L.)

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Abstract

Introduction: Incorporation of inoculum in different carrier materials may increase the efficacy of bacterial inocula.

Materials and Methods: Field experiments were conducted using two strains of rhizobium and phosphate-solubilizing bacteria (PSB) and their respective combinations using different carrier materials in common bean-growing regions in three soil types in Kenya. The field experiment was laid out in a split-plot arrangement with the strain inoculations as the main plot while the subplots consisted of the carrier materials (filter mud, peat moss and yeast extract mannitol broth [YEMB]). Each main plot included two controls: uninoculated negative control and uninoculated controls that received N and P fertilizer. The experiment was conducted for two cropping seasons. Data were collected on the nodulation, shoot and root biomass and yield.

Results: Co-inoculation of the common bean with *Rhizobium phaseoli* + *Bacillus aryabhattai* strains had significantly higher number of nodules (55 nodules per plant) compared to single *R. phaseoli* inoculation (38 nodules). The co-inoculation of the rhizobia and the PSB yielded statistically at par with the application of diammonium phosphate (18:46:0) across the soil types and seasons. The use of filter mud as a carrier material led to a higher number of nodules for most of the rhizobia strains inoculation and their respective co-inoculation with the bacillus strains. Significantly higher yield was obtained with the filter mud (1.64 Mg ha^{-1}) while there was no significant difference in the yield of common bean between peat moss and YEMB as carrier materials for the bacterial strains.

Conclusion: The solid carrier material, specifically filter mud, showed potential for use in the formulation of inoculants. Specific co-inoculation of rhizobia (*R. phaseoli* and *Rhizobium pusense* strains) and PSB (*Paenibacillus polymyxa* and *B. aryabhattai*) increased the growth, nodulation and yield of common bean more efficiently than the control.

KEYWORDS
carrier material, co-inoculation, common bean, formulation, rhizobia