BACTERIAL BIOLOGICAL CONTROL AGENTS IN MANAGEMENT OF BACTERIAL WILT (*CURTOBACTERIUM FLACCUMFACIENS* PV. *FLACCUMFACIENS*) IN THE COMMON BEAN

LILIAN WANGUI MUNENE

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DECLARATION

This thesis is my original work and has not been presented elsewhere for a degree or any other award.

Signature…… Date……

Munene Lilian Wangui
Department of Biological Sciences,
B526/1275/2019

This thesis has been submitted for examination with our approval as the University Supervisors

Signature……. Date……

Prof. Romano Mwirichia,
Department of Biological Sciences,
University of Embu.

Signature……. Date……

Dr. Julius Mugweru,
Department of Biological Sciences,
University of Embu

DEDICATION

I dedicate this work to everyone who supported me throughout my studies. A special dedication to my family, whose unending support will have a special place in my heart forever. They have provided me with both physical and emotional support throughout the journey. I also dedicate this work to the residents of Muhotetu for their unwavering support throughout my academic pursuit.

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LIST OF ABBREVIATIONS/ACRONYMS

ANOVA	Analysis of variance
BCAs	Biological control agents
Cff	*Curtobacterium flaccumfaciens* pv. *flaccumfaciens*
CFFSM	*Curtobacterium flaccumfaciens* pv. *flaccumfaciens* semi-selective medium
CFU	Colony forming unit
CMC	Carboxymethylcellulose
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid
NaCL	Sodium chloride
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulphate
TSA	Trypticase soy agar
TSB	Trypticase soy broth
BLAST	Basic local alignment search tool
NCBI	National center for biotechnology information

ABSTRACT

Common beans (*Phaseolus vulgaris*) are widely grown throughout the world and provide valuable protein, vitamins and other nutrients. If the challenges related to its production are resolved, food security can be guaranteed for the growing population. Disease-causing pathogens are a major constraint in bean production. *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* causes bacterial wilt of common bean disease. This pathogen causes high economic losses due to seedling death, yield loss and seed discoloration. Copper fungicides can reduce secondary spread but are expensive, harmful to the environment and lead to resistance of the pathogen. Bacterial control agents are effective and environmentally friendly alternatives to chemicals, as they provide protection throughout the crop period and are specific to plant diseases. However, less has been done in managing bacterial wilt using bacterial bio-controls. The objectives of this study were; to isolate wilt-causing pathogen in dry beans, to isolate bacterial antagonists against the pathogen from the rhizosphere of *P. vulgaris* and to control the pathogen *in vitro* and *in vivo* using the recovered bacterial bio-controls. The study was done in an experimental bean field located in Kimbimbi, Kirinyaga County. From infected leaves and seeds of bean plants, *Curtobacterium* species were isolated and characterized morphologically, biochemically, molecularly and by pathogenicity tests. Biological control agents were identified in the soil samples obtained from the rhizospheres of bean plants and screened against *Curtobacterium flaccumfaciens* using soft agar overlay assay. Primer pair 8F and 1492R were used for amplification of the 16S rRNA gene and sequencing. Analysis of the 16S rRNA gene was conducted to identify the *Cff* isolates and bacterial antagonists. Both a foliar spray and a seed treatment were used to apply bio-controls. A completely randomized design was employed for the experiment. Data on disease incidence and disease severity were analyzed using analysis of variance at a 95% confidence level. Phylogenetic analysis was done using the neighbor-joining method and phylogenetic tree constructed with MEGA software. Eleven *Curtobacterium* spp. were identified. Nine bacterial control agents showed antagonistic activities against *Cff*. A partial 16S rRNA gene sequence of these isolates identified species belonging to the genera *Bacillus*, *Paenibacillus* and *Pseudomonas*. Seed treatment coupled with foliar application of the antagonistic bacteria significantly reduced disease incidence and disease severity ranging from 16-29% and 4-10% respectively, compared to the control. *Pseudomonas fluorescens* was most successful in controlling the *Cff* pathogen with an efficacy of 81%, while *B. cereus* and *P. polymyxa* reduced the disease development by 79% and 68%, respectively. The use of rhizobacterial species provides a safe, effective and sustainable alternative to control bacterial wilt in the common bean. Herein, the rhizobacteria *P. fluorescens*, *B. cereus* and *P. polymyxa* species seed treatment coupled with foliar application significantly reduced the disease incidence and disease severity of bean bacterial wilt. The study recommends further research on distribution of bacterial wilt of common bean disease in bean-producing regions of Kenya and to determine if the isolated bacterial control agents are effective against other plant diseases.

CHAPTER ONE

INTRODUCTION

1.1 Background

The common bean (*Phaseolus vulgaris* L.), is an annual crop of the *Fabaceae* family and accounts for more than 85% of bean cultivation worldwide (Gupta *et al*., 2019). It is the second-largest food crop in Kenya after maize, primarily produced by small-scale farmers. On the basis of use, common beans are primarily divided into snap beans and dry beans. According to Kenya Agricultural and Livestock Research Institute (KARLO), the dry bean varieties produced in Kenya include Mwitemania (GLP X92), Wairimu, Mwezi moja (GLP 1004), Canadian wonder (GLP 24), Rose coco (GLP2), Nyayo (GLP 1124), Kenya mavuno (Mac64), Kenya tamu (Mac34) and Kenya safi (Mac13). Among the well-known Snap bean varieties cultivated in Kenya are Lomami, Samantha, Amy, Boston, Serengeti, Belcampo, Rexas, Julia, Paulista, Hawaii, Pekera, Morgan, Picasso, Tokai, Buffalo, Teresa, Cupvert, Gloria, Espadia, as well as Seagull.

Kenya ranked 87th out of 116 countries in the 2021 Global Hunger Index with a score of 23.0 (Von *et al*., 2021), indicating that hunger is a serious problem in the country. Kenya's economy relies heavily on agriculture. However, the country continues to struggle with severe food insecurity issues. Its population faces chronic malnutrition due to a lack of adequate food quality and variety in its daily diets (Ogada *et al*., 2020). Several problems affect food production, such as soil degradation, a shortage of available land, poor integration into the market, climate change as well as increased disease pressures (Andersson and Gabrielsson, 2012).

In Kenya, beans are mostly consumed as green and dry grains that are used in stews and "Githeri" dishes. The common bean has various nutritional and health benefits (Celmeli *et al*., 2018) due to its high protein, fiber and carbohydrate content. This cholesterol-free protein source lowers the likelihood of developing medical conditions such as diabetes, cancer, as well as coronary heart disease (Nosworthy *et al*., 2023). It is rich in iron, phosphorus calcium, copper, potassium, magnesium and Vitamins (A, B, C, D and K)

which are essential to body health. Flavonoids and antioxidants in common beans prevent the body from aging by nullifying free radical attacks. Green leaves and pods are also commonly eaten as vegetables in Kenya (Katungi *et al*., 2011). Besides serving as food for humans, legumes are also used as feedstock for livestock.

Different types of legumes are used as feedstock for livestock, including beans, peas, lentils and alfalfa. These plants are often grown specifically for use as animal feed and they can be fed to a wide range of livestock, including cattle, pigs, sheep, goats as well as chickens (Vasconcelos *et al*., 2020). One of the main advantages of using legumes as feedstock for livestock is that they are highly nutritious (Karlsson *et al*., 2015). Protein, which is vital for animal growth and development, is rich in legumes. They also contain high levels of fiber, which helps to improve digestive health and can reduce the risk of digestive disorders in livestock. Legumes are not only a nutritious source of feed for animals, but they are also sustainable and environmentally friendly. Unlike many other animal feeds, which are derived from corn and other grain crops, legumes require less water and fewer inputs to grow. This makes them a more sustainable option, particularly in areas where water is scarce (Shiferaw *et al*., 2014).

Different groups of rhizobacteria fix atmospheric nitrogen form symbiotic relationships with legumes through nodulation maintaining and improving soil fertility. Through a symbiotic relationship, in exchange for fixed nitrogen, bacteria receive a niche from plants and fixed carbon (Liu *et al*., 2018; Sindhu *et al*., 2019). In agricultural systems, the process is primarily limited to legumes (Mus *et al*., 2016). Through crop rotation and intercropping, legumes can facilitate the cultivation of non-legumes, like cereals, due to their N-fixing activity. No synthetic nitrogenous fertilizer is required and hence, it ensures a sustainable environment (Ntatsi *et al*., 2020). Legumes improve potassium and phosphorus availability within the soil, reducing the need for potassium and phosphorus fertilizers (Williams *et al*., 2017).

Common beans are cultivated on a small, intermediate and large scale in Kenya. In terms of national production, dry beans are primarily produced in the Lake Victoria zone, Rift Valley, Central, Eastern and Western regions. Bean cultivation has not kept up with the

trend of increase in population as a result of biotic as well as abiotic constraints. Common bean is vulnerable to different kinds of viral, bacterial and fungal diseases. Common diseases include; bacterial blight, anthracnose, halo blight, angular leaf spot, rust as well as bean common mosaic virus (Masheti, 2019). These diseases affect yields, reduce bean storability and marketability.

Bacterial wilt in beans is amongst the important diseases that are threatening the production of legumes. The common bean bacterial wilt was initially found in South Dakota in the United States in 1920 (Hedges, 1922). It is caused by *Curtobacterium flaccumfaciens* pv. *flaccumfaciens (Cff)* which is a quarantine pathogen in many countries (EPPO, 2022). In bean-producing regions, including Kenya, the disease results in high economic losses (Harveson *et al*., 2015; Osdaghi *et al*., 2020). The pathogen causes reduction of common bean production up to 90% (Nascimento *et al*., 2021). Infected beans display low emergence, wilting and seed discoloration (Harveson and Schwartz, 2007). By invading vascular tissues, *Cff* prevents water and nutrients from reaching foliage (Harveson *et al*., 2015). Five variants of *Cff* have been identified and include the yellow variant, orange variant, red variant, purple variant and pink variant (Harveson and Vidaver 2008; Osdaghi *et al*., 2020).

There are no chemicals effective against the bacterial wilt of beans (Hsieh *et al*., 2003a). However, secondary spread can be reduced through spraying copper-based fungicides like copper oxychloride, copper sulphate as well as copper hydroxide (Martins *et al*., 2013). Streptomycin seed treatment has been proven to be highly efficient against surface contamination, however, it does not achieve complete control (Schwartz, 2007). Chemicals harm both the environment and the well-being of humans, cause resistance to pathogen and disrupt natural biodiversity due to their non-target effect. Use of pathogen-free seeds that have been certified for planting and crop rotation with non-host crops are the recommended control methods for bacterial wilt of beans (Gonçalves *et al*., 2021). A management system that is environmentally friendly and effective in combating common bean bacterial wilt is necessary. Biological control is one of the remedies in addressing the high prevalence of food insecurity, poverty and malnutrition. It has the potential to combat common bean bacterial wilt. Under greenhouse conditions, application of

Pantaoea agglomerans as seed treatment and soil drench effectively suppressed bacterial wilt disease (Hsieh *et al*., 2005). *Rhizobium leguminosarum*-treated bean seeds reduced the severity of bacterial wilt in a greenhouse environment *(*Huang *et al*., 2007). When bacterial wilt infects crops early in the growing season, crop losses are significant. Therefore, control measures implemented early in the growth of crops may be more efficient, especially for diseases transmitted by seeds like *Cff* (Martins *et al*., 2013).

Rhizospheres are unique habitats where plant roots and soil microbes interact intricately. Organic carbon is released into the soil by plants through cell lysates, mucilage, border cells intact, as well as root exudates (Jones *et al*., 2009). Plant species excrete various types and amounts of exudates depending on their genotype, growth stage, soil conditions (toxicity, nutrient availability and soil moisture) and rhizosphere organisms. Plant exudates contain organic compounds that have low molecular weight, such as fatty acids, sugars, sterols, amino acids, growth hormones and vitamins. Specific bacteria can be enriched in a plant's rhizosphere by carbon sources excreted by the plant. For instance, the dicarboxylates released by tomato roots promote *Pseudomonas* growth whereas, *Rhizobium leguminosarum* is selected by pea plants by excreting homoserine into the rhizosphere (Mus *et al*., 2016). The plant roots exudations attract beneficial rhizobacteria that carryout important biological activities (Bhattacharyya *et al*., 2016; Mhlongo *et al*., 2018). These associative bacteria use chemotaxis to colonize the rhizosphere of many plants in response to their root exudates (Compant *et al*., 2010; Santi *et al*., 2013). The rhizobacterial strains colonize the plant roots either externally or endophytically. The majority of endophytic rhizobacterial species may multiply and spread within plant tissues without harming them and trigger powerful defense responses (Pedraza, 2008; Nair and Padmavathy, 2014). The rhizobacterial strains contribute to plant development, yield and resource acquisition of phosphorus, nitrogen and other essential minerals due to their proximity to the root (Ahemad and Khan, 2011).

The biocontrol potential of rhizobacterial strains in managing seed-borne and also soil-borne pathogens has previously been reported (Liu *et al*., 2017; Araújo *et al*., 2018). The rhizobacterial species belong to the genera *Pseudomonas*, *Bacillus*, *Enterobacter*, *Serratia*, *Rhizobium* and *Azospirillum* (Saharan and Nehra, 2011). The *Bacilli* and

Pseudomonas rhizobacterial species are the most studied due to their biocontrol activities. They have the potential of colonizing the plant tissues and spreading on their own thus providing long-term protection throughout the growing period. The mechanisms employed in disease suppression include induced resistance, nutrients and space competition and secretion of the lytic enzymes, siderophores and antibiotics (Alabouvette *et al*., 2006; Saharan and Nehra, 2011). Rhizobacterial strains of healthy *Phaseolus vulgaris* may have potential in plant protection. Combining application method is one of the strategies for enhancing biocontrol effectiveness (Marian and Shimizu, 2019). In majority of biocontrol research, the bacterial biocontrol agent is delivered by seed treatment (Corrêa *et al*., 2014). Combining seed treatment with foliar application could significantly improve the management of bacterial bean wilt. The objective of this study was to identify wilt-causing pathogen from infected bean plants and seeds and to control the pathogen using bacterial bio-controls isolated from the rhizosphere of *P*. *vulgaris* both *in vitro* and *in vivo*.

1.2 Statement of the problem

Curtobacterium flaccumfaciens pv. *flaccumfaciens* is a serious plant pathogen of beans that reduces germination ability or kills the infected plants lowering the yields. It also causes discoloration (yellow, orange, purple or pink) and shriveling of seeds affecting common beans' quality and thus marketability. Seedlings arising from infected seeds are characterized by high disease severity and plant mortality. Under severe conditions, flowers are blighted causing a reduction in seed set. The majority of farmers in Kenya that cultivate common beans use seeds saved from the previous harvest or purchased from nearby markets for planting which may be infected by seed-borne pathogens such as *Cff*. This contributes to the progression of the bacterial wilt disease. Phytosanitary inspection of imported bean seeds involves visual examination while most of *Cff*- infected seeds are asymptomatic leading to dissemination of the disease in short and long distances (Tegli *et al*., 2020). Controlling disease-causing pathogens in plants with chemicals has drawbacks such as disruption of natural biodiversity. Non-selective pesticides are the most harmful since they kill both useful and harmless species. Pesticide residues that remain on the food chain have negative health effects such as cancer. However, there is no effective chemical

against bacterial wilt in the common beans (Tegli *et al*., 2020). Hence, effective and environmentally friendly disease prevention strategies are urgently needed. Use of bacterial biological control agents are one such strategy. However, currently there is no effective bacterial control agents that has been investigated against bacterial wilt.

1.3 Justification

Identifying the specific bacterial pathogen responsible for the disease is the first step towards developing effective control strategies. This knowledge will aid in designing targeted interventions or disease management practices, to mitigate the impact of the disease on bean crops. Bacterial control agents are safer and environmentally friendly and thus a potential alternative for managing plant diseases (Martins *et al*., 2013). They are specific to the pathogen and non-target species are not harmed. These agents prevent the establishment or infection of the pathogen in the plant. Utilizing locally sourced biocontrol agents offers several advantages, including better adaptation to the local environment, reduced environmental impact, and cost-effectiveness. By assessing the biocontrol efficacy, researchers can identify the most promising agents that demonstrate strong inhibitory activity against *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*. These findings will inform the development of practical applications for biocontrol agents, potentially leading to the formulation of effective biopesticides or inoculants that farmers can use to manage the wilt disease. After identification of a suitable bacterial control agent, it can be produced in large amounts in liquid culture and used as a soil drench, seed dressing, or foliar spray (Arwiyanto and Mada, 2014). Moreover, a successful biocontrol approach can contribute to sustainable agriculture by reducing the reliance on chemical pesticides, promoting environmental health, and preserving beneficial soil microorganisms. Apart from disease control, bacteria such as rhizobacteria also stimulate plant development and growth by producing plant growth-promoting molecules and hence, contribute to high crop yield (Manoj *et al*., 2020).

1.4 Research questions

1. Which pathogen causes bacterial wilt disease in *Phaseolus vulgaris* in Kirinyaga County?

2. Are there soil bacterial antagonists that have the potential to inhibit the growth of *Cff* from common bean growing soils in Kirinyaga County?
3. Can pure soil isolates of native biocontrol agents be used to control *Cff* growth *in vitro* and *in vivo*?

1.5 Objectives of the study

1.5.1 General objective

To screen rhizobacteria with biocontrol potential as biological control agents against *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* responsible for bacterial wilt of common beans.

1.5.2 Specific objective

1. To isolate and characterize wilt-causing bacterial pathogen from infected beans in Kirinyaga county.
2. To isolate and characterize bacterial control agents against *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* from soils in Kirinyaga county.
3. To assess the *in vitro* and *in vivo* biocontrol efficacy of the isolated biocontrol agents against *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*.

1.6 Significance of the study

By identifying the specific pathogen responsible for bacterial wilt in dry beans, farmers can implement targeted disease management strategies. This knowledge will result in reduced crop losses and improved yields, leading to enhanced food production and increased income for farmers. The study contributes to the understanding of the etiology and epidemiology of bacterial wilt in common beans, adding to the scientific knowledge of plant pathology and microbial interactions. The study aligns with government policies promoting sustainable agriculture and environmentally friendly practices. The adoption of biological control methods supports the government's commitment to reducing the use of chemical pesticides and promoting sustainable farming systems. By enhancing common bean production and reducing crop losses, the findings contribute to the government's efforts to achieve food and nutrition security for the population. Biological

control methods offer an environmentally friendly alternative to chemical pesticides, reducing the ecological impact of disease management practices in agriculture. Successful biocontrol strategies can provide a sustainable, long-term approach to managing bacterial wilt, helping farmers maintain crop productivity without compromising soil health or biodiversity. Effective biocontrol agents could be developed into commercial biopesticides, supporting the growth of the biocontrol industry in Kenya and beyond.

CHAPTER TWO

LITERATURE REVIEW

2.1 Common bean production in Kenya and its economic importance

Kenya is ranked seventh in the production of beans worldwide while in East Africa it ranks second. The common bean ranks second after maize in importance as a food crop. It is widely grown in regions with high and medium rainfall by about 1.5 million small-scale subsistence farmers with yields of around 0.6 MT/ha. The major bean-producing regions are; Western, Rift valley, Central and Eastern regions (Duku *et al*., 2020). National consumption is about 755,000 metric tons annually and production is assessed to be about 600,000 metric tons a year. Total production of common bean was 774000 tons in 2020, which improved by 1% from the previous year's record of 747000 tons (FAO, 2022). There are two growing seasons for the common bean which include, the March-May season and the October-December season (Duku *et al*., 2016). It matures fast with growth cycle of about 70 days which allows production even during irregular rains. Common bean is mainly cultivated by women for consumption and markets. Leguminous crops are staple foods that ensure food security and help fight hunger in developing countries (Pingali, 2015; Muthamilarasan and Prasad, 2021).

The nitrogen cycle is the mechanism through which nitrogen is converted between different forms and moved through the environment. Nitrogen plays a crucial role in life and it is a key component of proteins, DNA and other biomolecules. The nitrogen cycle is essential for maintaining the sustainability of the environment and ensuring that plants and animals have the nitrogen they need to survive and thrive (Moreau *et al*., 2019). The nitrogen cycle begins with nitrogen gas, that comprises 78% of the Earth's atmosphere. Nitrogen gas is relatively unreactive and it is not readily used by plants as well as animals. To be transformed into a form that can be utilized, nitrogen gas must be "fixed" by certain bacteria and other microorganisms. One of the main ways that nitrogen is fixed is through the process called nitrogen fixation. Bacteria that reside in soil, the roots of certain plants and the oceans carry out this process (Santi *et al*., 2013). These bacteria convert nitrogen gas into ammonia, which is a more reactive form of nitrogen that can be used by plants

and animals. Once nitrogen has been fixed, it can be converted into other forms through a process called nitrogen cycling. Ammonia is transformed into nitrite and ultimately nitrate during this process. The most easily absorbed form of nitrogen by plants is nitrate, which is crucial for plant development and growth (Dixon and Kahn, 2004).

Legumes are crucial for sustainable agriculture because they enhance soil health and reduces the usage of artificial nitrogen fertilizers (Karavidas *et al*., 2022). One of the main benefits of legumes is that they can improve soil fertility by fixing nitrogen in soil (Yuvaraj *et al*., 2020). Nitrogen is a crucial nutrient for the plants, but it is also a highly reactive gas that is not readily available to plants. Legumes, however, are symbiotic with bacteria known as rhizobia, which live in root nodules of the plant. These bacteria transform atmospheric nitrogen into a form that plants can utilize and they also release some of this nitrogen into the soil, where it can be used by other plants (Wang *et al*., 2012). In addition to fixing nitrogen, legumes also have other benefits for soil fertility. They have extensive root systems that enhance soil quality and boost the soil's capacity to hold water. They also produce large amounts of organic matter when they are grown and harvested, which can contribute to enhancing the soil's fertility and structure (Oldroyd *et al*., 2011). Legumes are also a valuable component of crop rotation, which is a technique that involves growing various crops in a field in a sequential manner. By rotating legumes with other crops, farmers can help to improve soil fertility and reduce the need for synthetic fertilizers. This can save farmers money and also reduce the environmental impact of agriculture (Jimenez-Lopez *et al*., 2020).

Millions of people throughout the world depend on common beans as a staple diet as beans provide an inexpensive source of protein, fiber and other necessary components *(*Misganaw *et al*., 2019). One of the key nutritional benefits of the common bean is its high protein content. Protein is an essential nutrient that helps build and repair tissues, produce enzymes and hormones and support the immune system. The common bean is a plant-based source of protein, making it a valuable food for vegetarians and vegans. In fact, a cup of cooked common beans contains about 15 grams of protein, which is roughly the same amount found in two ounces of meat (Szparaga *et al*., 2019). Common beans are a good source of fiber as well as protein. The body cannot digest fiber, a form of

carbohydrate, which is crucial for maintaining a healthy digestive system. Consuming a diet high in fiber helps improve weight management, lowers the risk of heart disease and prevent constipation. Practically, 15 grams of fiber are present in a cup of cooked common beans, which is approximately half of the adult daily fiber recommended (Khan *et al*., 2007). Iron, zinc and magnesium are among the other vital nutrients that are abundant in common beans. Iron is an important mineral that helps transport oxygen throughout the body, while zinc and magnesium play a role in maintaining a healthy immune system (Celmeli *et al*., 2018). Common beans additionally contain folates, a B vitamin necessary for the formation of red blood cells and the healthy operation of the nervous system (Ramírez *et al*., 2016). Aside from their nutritional benefits, common beans are also an important crop for farmers around the world. Common beans are a major food supply and a source of income in many developing nations, making them an essential part of the local economy. Additionally, common beans may be produced in a range of regions and are comparatively simple to grow making them a valuable crop for small-scale farmers (Medendorp *et al*., 2022).

2.2 Constraints to common bean production in Kenya

One of Africa's top producers of common beans is Kenya, but the crop faces several constraints that limit its production. These constraints include diseases, pests, lack of improved varieties, as well as inadequate access to inputs such as seeds and fertilizers (Mangeni *et al*., 2014). Pests and diseases are among the main factors limiting the production of common beans in Kenya. The main bean pests are pod borer (*Maruca vitrata*), common bean bruchids (*Acanthoscelides obtectus*) and bean fly (*Ophimyia phaseoli*). Insect pests such as bean bruchids, cutworms and aphids (*Aphis fabae*) cause damage to seed in the field and storage, some transmit the pathogens in field while others attack the seedlings causing significant reduction in yields (Buruchara *et al*., 2010). On the other hand, common bacterial blight (*Xanthomonas phaseoli*), anthracnose (*Colletotrichum lindemuthianum*), halo blight (*Pseudomonas syringae* pv. *phaseolica*), root rots (*Rhizoctonia solani*, *Fusarium solani*, *Sclerotium rolfsii* and *Pythium* spp.), bean leaf rust (*Uromyces appendiculatus*), bean scab (*Elsinoe phaseoli*), angular leaf spot (*Pseudocercospora griseola*), as well as bean common mosaic virus diseases are the major

diseases limiting bean production. Bean mosaic virus (BMV) is a viral disease that is spread by aphids and other insects. It causes the leaves of the plant to become mottled and distorted and can also cause stunting and yield reduction.

Bean rust is a fungal disease that causes reddish-brown pustules to develop on a plant's leaves, stems and pods. It can cause significant yield loss if left untreated. Another fungal disease that damages the plant's leaves, stems and pods is anthracnose. It causes dark, sunken lesions to form on the plant tissue, resulting to premature defoliation and yield loss. Common bacterial blight causes dark, water-soaked lesions to form on the plant tissue, leading to premature defoliation and yield loss. Halo blight is a serious disease that affects beans, causing significant crop loss in many regions in the world. The disease attacks the plant's leaves, stems and pods. The symptoms of halo blight include the development of small water-soaked lesions appearing on the leaves, which may grow into large, necrotic areas. The disease can also cause the seeds to become discolored and misshapen, making them unmarketable. Most of these pathogens are seed borne that result in poor germination, low yields and poor-quality seeds making it difficult for farmers to produce enough beans that will meet the demand (Schwartz *et al*., 2005b; Singh and Schwartz, 2010; Meziadi *et al*., 2016).

Kenya's production of common beans is further hampered by the lack of improved varieties. Many farmers in Kenya still rely on traditional varieties of common beans, which tend to be less productive and less resistant to pests as well as diseases. Improved varieties of common beans, on the other hand, are more productive, have higher yields and greater pest and disease resistance. However, these improved varieties are not widely available in Kenya, which limits the ability of farmers to increase their yields. Inadequate access to inputs such as seeds and fertilizers is also a constraint to Kenyan production of beans (Katungi *et al*., 2009; Enid *et al*., 2011). Many small-scale farmers in Kenya lack access to high-quality seeds and fertilizers, which may reduce their crops' production. In addition, the cost of these inputs can be prohibitively high for many farmers, making it difficult for them to invest in their crops. As a result, many farmers are unable to produce enough common beans to meet the demand, which can lead to food insecurity and lower incomes (Ochieng *et al*., 2016; Acevedo *et al*., 2020). Low yield in beans can also be

attributed to nitrogen deficiency in soils which is an essential plant nutrient. Farmers rarely use nitrogenous fertilizers because they rely on the beans' capacity to fix nitrogen (Kawaka *et al*., 2018).

2.3 Economic importance of genus *Curtobacterium*

Curtobacterium belongs to *Microbacteriaciae* family within *Actinobacteria* phylum (Evtushenko and Takeuchi, 2006). This genus has been found in every designated environment, including the terrestrial, aquatic and human microbiome ecosystems. However it is most abundant in terrestrial ecosystem, mainly associated with plants (Chase *et al*., 2016). Research on *Curtobacterium* focuses mainly on its function as a plant pathogen of economic importance (Huang *et al*., 2010). *Curtobacterium* species perform other ecological functions such as eliciting plant defense responses (Bulgari *et al*., 2011), controlling plant pathogens (Lacava *et al*., 2007; Horuz and Aysan, 2018) and promotion of plant growth (Mayer *et al*., 2019; Vimal *et al*., 2019). *Curtobacterium flaccumfaciens* is the sole species associated with pathogenesis of agricultural important plants. *Curtobacterium flaccumfaciens* is divided into different pathovars which include; *Curtobacterium flaccumfaciens* pv. *poinsettiae* which causes stem rots, leaf spots, defoliation, cankers and vascular discoloration on poinsettia (*Euphorbia pulcherrima*), Malabar spinach *(Basella rubra)* is affected by the bacterial leaf spot disease caused by *C. flaccumfaciens pv. basellae.* Red beet (*Beta vulgaris* var. *rubra*) silvering disease is caused by *C. flaccumfaciens* pv. *betae. Curtobacterium flaccumfaciens* pv. *oorti* causes bacterial wilt and spot in genus *Tulipa*. *Curtobacterium flaccumfaciens* pv. *ilicis* causes bacterial blight of American holly. *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* causes bacterial wilt of dry beans and can infect many other species of Fabaceae (Osdaghi *et al*., 2018). *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*) is seed-borne pathogen that causes bacterial wilt of common beans. It also causes diseases in soybeans, peas, lentils, and alfalfa. The pathogen causes high economic losses in common bean production. It reduces the productivity of common bean up to 90%. It causes stunting as well as yellowing of the leaves, which reduces the plant's ability to photosynthesize and produce food which further reduces its yield. In severe cases, *C. flaccumfaciens* pv.

flaccumfaciens can even kill the plant. Seedlings emerging from infected seeds exhibit severe symptoms.

2.4 Common bean bacterial wilt disease

Curtobacterium flaccumfaciens pv. *flaccumfaciens* is a bacterium that causes bacterial wilt, a disease that affects edible legumes like common beans, soybeans, mung beans and cowpeas. The pathogen is considered economically important owing to the significant crop losses it can cause. The disease is particularly prevalent in regions with hot and dry summers, as well as extended periods of drought. The European and Mediterranean Plant Protection Organization (EPPO) has classified it as a high-risk disease that requires tight quarantine measures and zero tolerance in the dry bean industry in various nations (EPPO, 2022). Common bean bacterial wilt was first reported in South Dakota in 1922, but it has since spread to South America, Canada, Australia, Europe, western Asia (Iran) and Africa (Osdaghi *et al*., 2020). Observations of this pathogen infecting plants have previously been made in Africa, particularly in countries like Kenya, Tunisia, Mauritius and South Africa. But it was not until 2019 that it was found on soybean plants in Zambia and it was formally recognized as existing in Africa (Pawlowski and Hartman, 2019).

2.4.1 Pathogen biology and pathogenicity mechanism

Curtobacterium flaccumfaciens pv. *flaccumfaciens* is identified as a Gram-positive, aerobic non-sporing bacterium that produces yellow, orange, red and pink pigments on agar media. The main host are the Fabaceae crops which are the common bean (*Phaseolus vulgaris*), hyacinth bean (*Lablab purpureus*), runner bean (*Phaseolus coccineus*), adzuki bean (*Vigna angularis*), lima bean (*Phaseolus lunatus*), mung bean (*Vigna radiata*), black gram (*Vigna mungo*), soybean (*Glycine max*), cowpea (*Vigna unguiculata*), yardlong bean (*Vigna unguiculata* subsp. *sesquipedalis*), pea (*Pisum sativum*) as well as *Zornia glabra* (Osdaghi, 2014; Osdaghi and Lak, 2015; EPPO, 2022). In soybean, it causes bacterial tan spot disease and it was first recorded in Iowa, USA in 1975 (Dunleavy, 1983).

Infected seeds serve as the main inoculum source, a way of dissemination and a means of survival. It has been proven that the root-knot nematode (*Meloidogyne incognita*) acts as

a vector for the disease by mechanically harming host plants' roots (Harveson *et al*., 2015). In addition to injuries on roots and above-ground plant parts, bacteria can also enter through stomata, albeit much less frequently than other prevalent bacterial diseases. Infections spread through wounds following hail and rainstorms. *C. flaccumfaciens* pv. *flaccumfaciens* can be transmitted through the seeds of wheat. The pathogen can survive in the seeds of wheat and grow endophytically and systemically in wheat plants that are not its primary hosts. In a wheat-bean rotation system, sowing *Cff*-contaminated wheat seeds will generally result in an increase in the incidence of bean bacterial wilt disease during the subsequent years if favorable environmental conditions are present (Afkhamifar *et al*., 2022). The chemical, physical and climatic characteristics of the soil affect *C. flaccumfaciens* pv. *flaccumfaciens'* ability to survive in the soil. According to Gonçalves *et al*., (2018), the pathogen survived in intact soil in Brazil for 34 - 80 days under field conditions; however in Nebraska, USA, it thrived without crop debris for up to two years (Nascimento *et al*., 2022). Under laboratory conditions, it remains viable on and within the seeds for up to 24 years (Sammer and Reiher, 2012). The pathogen can persist on the debris of infected plants or weeds (Harveson *et al*., 2015). Straw and debris from infected common beans are crucial for *C. flaccumfaciens* pv. *flaccumfaciens* to survive. Wheat (*Triticum aestivum*), sunflowers (*Helianthus annuus*), corn (*Zea mays*), alfalfa (*Medicago sativa*), as well as barley (*Hordeum vulgare*) crops act as survival sources of *Cff* without symptoms making management of the disease difficult (Gonçalves *et al*., 2021). Phytopathogenic bacteria often have a limited host range, meaning that they can only infect certain types of plants. However, many phytopathogenic bacteria are able to survive between crop seasons by infecting alternative hosts, which are plants that are not typically targeted by the bacteria but can still provide a suitable environment for their survival (Schulze-Lefert and Panstruga, 2011). The ability of phytopathogenic bacteria to survive between crop seasons depends on finding alternate hosts. They provide an opportunity for the bacteria to persist in the environment even when their primary host plants are not present. This can make it more difficult to control the spread of these bacteria, as they may be able to infect new crops when they become available (Gonçalves *et al*., 2017). Temperatures of above 30°C favors *Cff* and moisture stress causes wilting in common beans (Puia *et al*., 2021).

There is lack of pathogenicity determinants in the bacterium (Gonçalves *et al*., 2021). The pathogen multiplies in the vascular (xylem) system causing wilting of plants during moisture stress. There is formation of a biofilm in the plant tissues that causes water deficiency through absorption of liquids from the cells. Biofilm play a role in aggressiveness and pathogenicity of *Cff* by resulting in total blockage and plant death (Harding *et al*., 2022). The pathogen can also spread systematically into seed embryos causing seed discoloration. *Curtobacteriun flaccumfaciens* pv. *flaccumfaciens* use lytic enzymes (esterase, peptidase, lipase and beta-glucosidase), toxins (bacteriocins) and hormones in disruption of the cell wall to assimilate nutrients (Osdaghi *et al*., 2020).

2.4.2 Symptoms of common bean bacterial wilt

Seedlings that have developed from infected seeds exhibit the primary symptoms at the stage of the second trifoliate leaf or the cotyledon. The disease can also spread during the vegetative, flowering and fruiting stages of plant growth (Osdaghi *et al*., 2020). Interveinal chlorotic and necrotic regions on leaves are among the field symptoms of bacterial wilt, which also causes systemic plant wilt and plant mortality (Harveson *et al*., 2015). Chlorotic lesions are the first symptoms, which progress to foliar wilting and necrosis on leaves and are typically encircled by a yellow halo. During hot, dry weather or times of moisture stress, the pathogen's presence in the vascular system prevents normal water passage from the roots into the leaves, resulting in leaf wilting (Huang *et al*., 2009; Harveson *et al*., 2015). *C. flaccumfaciens* pv. *flaccumfaciens* is mainly a vascular invader and results in vascular browning (Agarkova *et al*., 2012). In Zornia spp., wilting of individual plant parts or entire plants as well as seedling death are common. There is no wilting in soybean, cowpea, or mung bean, but there is leaf chlorosis with spotting and occasionally flower blighting (Soares *et al*., 2013). Early shield leaves or, more frequently, trifoliate leaves may develop necrotic lesions. Spots gradually get dry, turn tan from bleaching and shred in the wind, causing the leaf to look tattered. Young plants that develop the infection become stunted and chlorotic. Moisture stress causes symptoms severe (Huang *et al*., 2009). Seed discoloration is a typical symptom of bacterial wilt disease in common bean (Schwartz *et al*., 2005b). When infected bean crops produce

mature seeds, the seeds are typically discolored as a consequence of bacterial colonization and infection, especially in cultivars that yield white seeds (Ishimaru *et al*., 2005).

The symptoms of bacterial wilt and common bacterial blight (*Xanthomonas axonopodis* pv. *phaseoli*) diseases may be confused for one another in areas where both attack the same field (Harveson *et al*., 2011). Lesions from bacterial wilt develop between veins, frequently followed by wilting and plant death in plants that are severely infected. While neither of these pathogens is systemic, common bacterial blight and other bacterial diseases, such as brown spot and halo blight are less likely to cause wilting and plant death (Schwartz *et al*., 2005a; Harveson and Schwartz, 2007). Laboratory isolation would be necessary to distinguish these pathogens.

2.4.3 Identification methods of bacterial wilt pathogen

The diagnosis of symptomatic plants for *Cff* requires visual inspection followed by laboratory tests. An *in vitro* semi-selective isolation media for *Cff* were developed by Tegli *et al*. (1998); Maringoni and Camara, (2006). Colonies of *Cff* on non-selective media appear circular, smooth, and measure 2-4 mm in diameter. They are mostly convex and translucent, but some may also be flat and semi-opaque. The pigmentation of the colonies varies, ranging from creamy to bright yellow or red/orange, influenced by temperature and pH. Incubation for 48-72 hours at 25-30°C is required (EPPO, 2022). Two polymerase chain reaction (PCR) tests with different specific primer sets have been designed for the identification of *Cff* isolated colonies and detection in bean seed extracts; one according to Messenberg *et al*. (2001) and the other according to Tegli *et al*. (2002). The pathogenicity tests involve inoculating susceptible bean plantlets with suspected *Cff* isolates. Hsieh *et al*. (2003a) demonstrated a simple and effective hilum injury/seed inoculation method for testing the pathogenicity of *Cff*. The observation period for symptom development is up to 21 days in the injection method and up to 15 days in the seed inoculation method. Proper controls are crucial for accurate interpretation of results. Overall, these methodologies are essential for accurate isolation and characterization of *Cff* in common beans.

2.5 Disease management

Managing bacterial wilt disease has been a challenge since 1922 when it was first reported. This is because the pathogen is seed borne and there is little information on its pathogenicity and virulence determinates. It is recommended to control the disease through rotating crops with non-host plants and using disease-free seeds for planting (Sammer and Reiher, 2012). It is necessary to get rid of weeds and plant debris that act as infection reservoirs. There is no existing chemical control that is reliable against bean bacterial wilt disease. According to Harding *et al*., (2022), the potential of *Cff* pathogen to produce biofilms causes resistance to chemicals. The effectiveness of recently developed antimicrobial compounds for managing bean bacterial wilt disease was examined in a 7-year (2010-2016) field trial according to a report by Harveson, (2019). In Nebraska, USA, two popular copper-based products, that is copper sulfate and copper hydroxide, were compared with the antimicrobial compounds and they showed considerable potentials for lowering losses caused by bacterial wilt. When compared to the controls, treatments using peroxyacetic acid, hydrogen peroxide and ecoAgra 300 (plant-based fatty acids) significantly improved yields while had no effect on disease incidence. Although these products do not seem to control diseases, they serve as protectors by preventing or slowing the spread of the pathogen (Harveson, 2019). Treatment of seeds with antibiotics can reduce surface contamination. For example, Streptomycin Agri-Strep 500 slurry seed treatments have been demonstrated to be successful in preventing infection of common bean seeds with *C. flaccumfaciens* pv. *flaccumfaciens* (Schwartz, 2007). The bacterium was eliminated from naturally infected seeds through soaking them in Agrimycin 500 solution, which contained copper sulfate and oxytetracycline; however, it was ineffective on seeds that had been artificially inoculated with 10^8 cfu/ml suspension of the pathogen (Estefani *et al*., 2007).

Multiple industries have made attempts to develop cultivars that are bacterial wilt resistant. In Canada, the cultivars include; AC Litekid, Redkanner and chinook 2000. Emerson is a wilt resistant cultivar produced in central high plains of USA (Osdaghi *et al*., 2015). Breeding for resistance is a lengthy and resource intensive approach.

2.6 Use of bacterial biocontrol agents (BCAs) in management of common bean bacterial wilt

Biological control, also known as biocontrol, is the management of pathogen populations using living organisms. For bacterial biological control case, bacteria are used to control the growth as well as the spread of the pathogens. Besides their ability for suppressing plant pathogens, bacterial biocontrol agents are also safe (Kharwar *et al*., 2014). This approach has several benefits which include; their self-sustaining potential, ability to spread on their own after the initial establishment, reduction in the input of non-renewable resources and their ability to suppress a disease for a long time in an environmentally friendly way. Bacterial biocontrol agents grow rapidly *in vitro* and can be produced in large volumes at relatively low cost (Nion and Toyota, 2015). There are no toxic residues left, no harmful effect on soil microbes and neither affects the natural biocontrol population (Pertot *et al*., 2019). They are simple to genetically manipulate and quite simple to cultivate for use in commerce (Irtwange, 2006). They also have multiplicity in mode of action. These include antibiosis which is a common antagonism utilized by BCAs by producing secondary metabolites like bacteriocins, antibiotics and enzymes. Another mode of action is competition of nutrients which occurs at the leaf surface and in soil. Induced resistance is a form of antagonism where the host plant defense pathways is triggered by the BCA (Alabouvette *et al*., 2006). Due to these characteristics, biocontrol offers an alternative approach in controlling diseases of plants and increasing production without harming the environment.

Most of bacterial biological control agents comprises of rhizobacterial, endophytic and epiphytic bacteria species (Mamphogoro *et al*., 2020). *Bacillus subtilis* is one of the most prevalent bacterial biocontrol agents. It is a naturally occurring soil bacterium that is used extensively to eradicate a variety of plant diseases. It is effective against several bacterial diseases which include bacterial wilt, black rot and crown gall. Another example of a bacterial biocontrol agent is *Pseudomonas fluorescens*, which controls a variety of plant diseases caused by nematodes, bacteria, as well as fungi. It is often applied as a seed treatment or sprayed onto the leaves of plants to prevent and control infections. In addition to their effectiveness, bacterial biocontrol agents have the added benefit of being easy and

inexpensive to produce. They can be mass-produced in laboratories using fermentation techniques and can be applied by spraying or drenching methods.

The potential of biocontrol methods in managing bean bacterial wilt has been reported. Hsieh *et al*. (2005) demonstrated that application of *Pantaoea agglomerans* as seed treatment or soil drench effectively suppressed bacterial wilt in greenhouse conditions. Huang *et al*. (2007) showed that treatment of bean seeds with *Rhizobium leguminosarum* reduced bacterial wilt severity under greenhouse conditions. Bacterial wilt reduction using biological control agents has ranged from 42% to 76% (Osdaghi *et al*., 2020). In most studies in bacterial wilt biocontrol, the bacterial biocontrol agent is applied by seed treatment. Combined seed treatment and foliar application could improve biocontrol performance in managing bacterial wilt disease in beans as *Cff* also affects plant photosynthesis (Martins *et al*., 2015).

2.7. Identification and efficacy screening techniques of bacterial antagonists from soil rhizosphere

Isolating bacterial antagonists from the soil rhizosphere is essential for harnessing biocontrol agents' potential in sustainable agriculture (Berendsen *et al*., 2012). Bioassays such as plant assays or *in vivo* experiments, evaluate antagonistic efficacy under natural conditions, confirming their ability to suppress pathogens and promote plant growth. Antibiosis assays, including agar overlay, dual culture, and well-diffusion methods, screen and isolate bacterial antagonists with potent inhibitory activity against plant pathogens (Compant *et al*., 2010). These assays focus on assessing antimicrobial compounds or metabolites produced by the antagonistic bacteria. In the agar overlay assay, cell-free supernatant of the antagonist containing antimicrobial compounds, is poured onto agar plates inoculated with the pathogen, leading to zones of inhibition around the antagonist colony. The dual culture assay enables direct antagonistic interactions between the bacteria and pathogen on the same plate, resulting in inhibition of pathogen growth. In the well-diffusion assay, antagonistic bacterial extracts create zones of inhibition when added to wells on agar plates containing the pathogen (Backer *et al*., 2018). Visual evidence of inhibition confirms the antagonistic activity and promising bacterial isolates can be further

investigated, including the identification using molecular techniques like PCR and DNA sequencing (Raaijmakers *et al*., 2009).

In vivo bioassays include; seed treatment, soil drenching and foliar spray. For seed treatment method, antagonistic bacteria are applied as seed treatments to assess their ability to protect common bean plants from *Cff* infection during germination and early seedling stages. Soil drenching involves applying the antagonistic bacteria to the rhizosphere soil of common bean plants, evaluating their efficacy in suppressing *Cff* infection in a more natural setting (Hsieh *et al*., 2005; Huang *et al*., 2007). For foliar spray method, antagonistic bacterial suspensions are sprayed onto common bean leaves to investigate their ability to protect plants from infection. However, this method has not been applied previously. Among the strategies for improving biocontrol performance is combining methods of application (Marian and Shimizu, 2019)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site description

This study was conducted in an experimental field where beans are continuously grown located in Kimbimbi, Kirinyaga county. Kimbimbi lies between a latitude of 0º 37' S and a longitude of 37º 22' E with altitude of 1202 m above sea level. It experiences two rainfall seasons with annual mean of 1057mm; long rain occurring March-May and short rain October-December with a mean of 71mm and 50mm respectively. The mean temperatures are 27.8°C during the long rainy season and 15.6°C during the short rainy season. The high temperatures favor the development of bacterial wilt disease.

3.2 Collection of infected bean leaves

A field of ongoing yield and disease trial experiments of common beans was targeted. The experimental design was a randomized complete block design, comprising 9 blocks. The blocks were 45 cm between each other and 15 cm between plants. Using a random sampling technique, ten infected GLP X92 bean plants with well developed symptoms were collected from the guard rows. The plants that displayed wilting and interveinal necrotic lesions with yellowing on the leaves were put in different bags and transported in cooler box to the research laboratory of the University of Embu for analysis.

3.3 Isolation of the *Cff* pathogen

Three leaves were used per sample and 2-4 diseased spots were excised from each leave using a sterile scalpel blade and surface sterilized as follows; 70% ethanol for 30 sec, 3% hypochlorite amended with Tween 20 for 10 Sec followed by a thorough rinse using sterile water. The sterilized leaf sections were crushed in a mortar and using a pestle and 1ml aliquot transferred into 10ml of enrichment medium (peptone, sucrose, beef extract, skim milk, Congo red, nalidixic acid, chlorothalonil, nitrofurantoin, oxacillin and thiophanate methyl). The inoculated medium was incubated at 30°C for 2 days in a shaker, 115 rpm. Upon growth, the cultures were streaked onto CFFSM (Maringoni and Camara, 2006)

semi-selective medium (5g peptone, 5g sucrose, 3g beef extract, 5g Skim milk and 15g of agar per one liter) that was amended with 0.05g Congo red, 0.001g Nalidixic acid, 0.001g Chlorothalonil, 0.001g Nitrofurantoin, 0.001g Oxacillin and 0.001g Thiophanate methyl. A 48-hour incubation was performed at 30°C. Additionally, remnant bean seeds of the experimented seed were used for the isolation of the pathogen. Colonies with morphological characteristics resembling *Cff* bacteria (circular shape, hydrolyzed casein and Congo red fading around the colonies) were sub-cultured on TSA medium.

3.4 Characterization of the putative isolates of *Cff* recovered from leaves of bean plants

3.4.1 Morphological characterization of the isolates

Morphological characteristics such as pigmentation, consistency, Gram reaction and shape were examined using microbiological techniques that are standard (Cappuccino and Sherman, 2011) and presented in a table.

3.4.2 Characterization of the isolates biochemically

The ability of the bacterial isolates to produce several extracellular enzymes, like cellulases, xylanases, amylases, xanthanases and pectinases, was tested. For each test, medium containing $25gL^{-1}$ of Lennox broth, $15gL^{-1}$ of agar and 2g of the respective substrate was prepared by autoclaving the media at 121°C for 15min. After allowing the media to cool, it was aseptically poured into sterile plates and allowed to set.

3.4.2.1 Determination of amylolytic activity

Using a sterile inoculating loop, each bacterial isolate was inoculated by spotting on starch agar plates and incubated for 24 hours at 30°C. Upon growth, the cultures were overlaid with Iodine solution and the excess reagent was poured off after one minute. The appearance of distinct zones around the bacterial growth implied amylase production.

3.4.2.2 Determination of pectinolytic activity

Using a sterile inoculating loop, the bacteria were inoculated by spotting into pectin agar plates and incubated for 24 hrs at 30°C. Upon growth, the cultures were overlaid with iodine and clear zones around the colony indicated pectinase production.

3.4.2.3 Determination of cellulolytic activity

Screening of cellulase producing bacteria was done on CMC and cellulose agar plates. Bacterial isolates were inoculated by spotting using a sterile wire loop and incubated at 30°C for 24 hrs. Clear zones around the colony indicated positive outcomes after the cultures had grown and were flooded with Lugol's iodine solution.

3.4.2.4 Determination of xylanolytic activity

The isolates' xylanolytic activity was evaluated on xylan agar plates. Bacterial isolates were inoculated by spotting using a sterile wire loop and incubated for 24 hrs at 30°C. Upon growth, the cultures were flooded with iodine and clear zones around the colony were a sign of positive results.

3.4.2.5 Determination of xanthanases production

Using a sterilized wire loop, bacterial isolates were inoculated by spotting onto xanthan agar plates and incubated for 24 hrs at 30°C. Upon growth, the cultures were flooded with iodine and clear zones surrounding the colony indicated positive outcomes.

3.4.3 Molecular characterization of the isolates

3.4.3.1 DNA extraction

The phenol-chloroform protocol was used to extract the total genomic DNA as described by Sambrook *et al*., (1989). Pure isolates were grown in TSB medium for 2 days at 30°C. One milliliter of the cultures was aliquoted into sterile 2 ml Eppendorf tube, centrifuged at 8000 rpm for five minutes and the supernatant discarded. The resulting pellets were re-

suspended in 100 µl of solution A which contained 100 mM EDTA (pH 8.0) and 100 mM Tris-HCL (pH 8.0). Ten microliters of lysozyme (20 mg/ml solution) were added and incubated at 37°C for 15 minutes in a water bath. After incubation, 400 µl of the lysis buffer (60 mM EDTA (pH 8.0), 400 mM Tris-HCL (pH 8.0), 1% sodium dodecyl sulfate and 150 mM NaCl) was added and the tubes were incubated for 10 minutes at room temperature. Ten microliters of proteinase K (20 mg/ml) was added and the mixture was incubated in a water bath for 15 minutes at 65°C. An equal volume of chloroform was added and then spinning at 13200 rpm for 5 min at 4°C. The aqueous solution was transferred into a fresh Eppendorf tube. One hundred and fifty microliters of sodium acetate (pH 5.2) and an equal volume (supernatant + sodium acetate) of isopropyl alcohol was added. The tubes were mixed by inverting them gently and centrifuged at 13,200 rpm for ten minutes and the supernatant decanted. The DNA pellet was washed using 300 µl of 70% ethanol, centrifuged at 10,000 rpm for one minute and the supernatant decanted. The resulting DNA pellet was air dried and dissolved in 30 µl of PCR water. The quality of the DNA was confirmed using 1% agarose gel (1g of agarose/ 100ml of TAE buffer) stained with sybr green fluorescent dye and visualized under ultraviolet (UV) light.

3.4.3.2 PCR amplification

Total genomic DNA for each 23 bacterial isolates was used for amplification of 16S rRNA gene. Bacterial primers 8F (5'-AG (A/G) GTTTGATCCTGGCT-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used to amplify the 16S rRNA gene. Amplification was performed in a reaction volume of 30 µl containing the following reagents; 6 µl Taq reaction buffer, 0.6 µl of each primer (10 µM), 0.6 µl Taq polymerase, 22.8 µl PCR water and 0.5 µl DNA sample. The control contained all the afore-mentioned reagents except the DNA template. The reaction mixture was subjected to the following cycling conditions: initial denaturation at 95°C for 5min followed by 35 cycles of 45s at 95°C for denaturation step, 30s at 51°C for annealing step and 1 minute at 72°C for elongation step; followed by a final extension step of 5min at 72°C. The amplification products were checked on 1% agarose gel and visualized under UV light.

3.4.3.3 Purification of PCR products and Sequencing

The amplified PCR products were purified by mixing 2.5 µl of ExoSap-IT^{TM} (Thermo Fisher Scientific) with 12.5 µl of PCR product and incubated for 30 min at 37°C followed by heating the mixtures for 5 min at 95°C to stop the reaction. The reaction mixture was then held at 4°C for 2 min. The PCR products were then sent for sequencing at Inqaba Biotech in South Africa and sequenced using the same primers (8F and 1492R).

3.5 Pathogenicity tests

GLP X92 seeds (108 seeds) were sterilized as follows; 70% ethanol for 30 seconds, 3% hypochlorite for 5 minutes and then a thorough rinsing using sterile water. Eleven *Curtobacterium* isolates were grown in TSB medium and the concentration was adjusted to OD 0.5 at 600 nm wavelength using spectrophotometer. Seeds were soaked in the bacterial suspension for 1hr while the untreated controls were soaked in sterile water. They were planted in sterilized cocopeat in seedling trays (200-hole tray) with one seed per cell. Three seeds replicated three times were planted per treatment under greenhouse conditions at 24 - 28°C. The experiment was arranged in a complete randomized design and plants were watered as needed. Fourteen days after inoculation, seedlings were evaluated for wilt symptoms. Disease severity (DS) and disease incidence (DI) percentages were calculated.

$$\mathrm{DI} = \frac{\text{number of seedlings displaying wilt symptoms}}{\text{number of seedlings germinated}} \times 100$$

A scale of 0-5 (Hsieh *et al*., 2003b) where 0=healthy seedling, 1=wilt on one of the primary leaves, 2=wilt on both primary leaves, 3=wilt on first trifoliate, 4=death of seedling after the development of primary leaves and 5= un-emerged seedling or death of seedling before the development of primary leaves, was used for disease severity. Disease severity was calculated according to McKinney (1923) formula.

$$\mathrm{Ds} = \frac{\text{sum of all numerical ratings}}{\text{total number of inoculated plants} \times \text{maximum grade}} \times 100$$

In addition to seed inoculation method, pathogenicity test was also conducted using foliar spray and detached leaf assay in the laboratory. Ten days old seedlings were transplanted in a sterile 1L sampling container. They were supported with a cotton wool that was moistened with hydroponic water. The seedlings were inoculated by spraying the pathogen on the abaxial as well as adaxial sides of the leaves to run off using a hand sprayer. For detached leaf assay, fully developed middle leaflet of the first trifoliate leaf was detached from bean plants. The leaves were placed in Petri dishes with cotton that had been moistened with sterile water and the pathogen was inoculated by spotting. Disease symptoms was evaluated after 21 days when the disease score was expected to be maximum. To fulfill Koch's postulates, bacterial isolates were re-isolated from the wilted seedlings to confirm that the infection of plants was caused by the wilt-causing pathogen.

3.6 Isolation of antagonists

Ten soil samples were obtained using a random sampling technique from the bean growing experimental field and used for isolation of bacterial control agents. Soft-agar overlay assay was used for isolation of antagonists as described by Hockett and Baltrus, (2017) with slight modifications. One gram of the soil sample was mixed in 1ml of phosphate-buffered solution (pH 8) and the solution was diluted to a twentyfold dilution. Trypticase soya agar was prepared and upon cooling, an aliquot of 100 µl of the soil solution was added. Cycloheximide was also added to prevent fungal growth. The medium was dispensed into sterile Petri dishes and incubated at 30°C for 24 hrs. Upon growth, the cultures were overlaid with prepared TSA media inoculated with 50 µl of *Curtobacterium* spp. which were 24 hrs old grown in trypticase soya broth (TSB). Bacterial isolates that indicated antagonistic activity were purified on a TSA medium.

3.7 *In vitro* antibiosis screening of bacterial isolates against *Cff*

The potential for putative antagonistic bacteria to prevent *Cff* growth was examined. They were tested for their efficacy to inhibit *Cff* growth on petri dishes using the soft agar method described by Hockett and Baltrus, (2017) with slight modifications. The putative antagonists were grown in TSB medium for 24 hrs. The isolates were then spotted onto the TSA medium and the plates incubated for 24 hrs at 30°C. Each isolate had three

replicates. Trypticase soy agar medium was prepared and upon cooling, an aliquot of 100 µl of *Curtobacterium* spp. was pipetted into the medium and mixed thoroughly. The medium was overlaid on antagonists and the plates were further incubated at 30°C for 48 hrs. The formation of halos indicated inhibition of *Cff* growth and they were measured. The inhibition ability of antagonists was rated using a 0-6 scale (0= no zone, 1= 1-4 mm zone, 2= 5-8 mm zone, 3= 9-12 mm zone, 4= 13-16 mm zone, 5= 17-20 mm zone and 6= ≥ 21 mm zone). Biochemical and molecular characterization, as described in sections 3.4.2 and 3.4.3, were used to identify the antagonistic bacteria.

3.8 Efficacy of bacterial antagonists in common bean plants

At the University of Embu, a greenhouse experiment was done to evaluate three bacterial antagonists' efficacy; BC6 (*Bacillus cereus*), BC24 (*Pseudomonas fluorescens*) and BC14 (*Paenibacillus polymyxa*) in managing bacterial wilt of common bean. They were chosen based on their antagonistic activity (≥10 mm inhibition zone) against all the *Curtobacterium* spp. The inoculum of the bacterial antagonists was prepared as illustrated by Islam *et al*., (2016) with few modifications. The bacterial species were grown in 200ml Trypticase soy broth in 250ml of conical flasks for 48 hrs at 30°C on an orbital shaker at 150rpm. Centrifugation was used to harvest bacterial cells at 8000rpm for 5min at 4°C. The pellets were resuspended by vortexing in 20ml of sterile distilled water and the concentration adjusted to 1x 10^8 CFU/ml and used for seed treatment. GLP X92 *P. vulgaris* seeds were obtained from the market and surface sterilized as follows 70% ethanol for 30s, 3% hypochlorite amended with Tween 20 for 10s followed by a thorough rinse using sterile water. The seeds were then dried overnight. Twenty-four seeds were soaked in each bacterial suspension for one hour and the mixture was frequently stirred. The treated seeds were air-dried overnight at room temperature. The treated seeds were sown in sterile soil. The control was soaked in sterilized distilled water in place of the bacterial suspension. Four seeds were sown per pot that contained a sterilized mixture of soil and sand in a ratio of 3:1.

After 15 days of germination, the plants were foliar sprayed with the antagonists using a hand sprayer till run-off. After 24 hrs, they were inoculated with *Cff* at a concentration of

A_{600}= OD 0.2. A sterile needle was used for the inoculation, which was performed by dipping it into the bacterial suspension and being inserted into each plant's internode as described by Urrea and Harveson (2014). Thereafter, the plants were sprayed once per week (day 7, 14 and 21 after pathogen inoculation) with bacterial antagonist to maintain the population density and watered regularly. Four treatments were replicated six times in the completely randomized experimental design. The treatments consisted of: *Bacillus cereus* + *Cff*, *Pseudomonas fluorescens* + *Cff*, *Paenibacillus polymyxa* + *Cff* and *Cff* alone. The experiment was performed twice.

The plants were assessed for signs of wilt six weeks after germination and disease incidence was recorded. A score of 0-9 (Maringoni, 2002) was used for disease severity where 0= no symptoms, 1= mosaic on the leaves, 3= some wilted leaves(1-3 leaves less than 10% of the leaves), 5= approximately 25% of the leaves wilted and yellowing, 7= approximately 50% of the leaves wilted, yellowing and with necrosis of the leaflets, plants with dwarfism 9= approximately 75% or more of the leaves wilted, severe dwarfism and/or plant death. Data on disease severity and biocontrol efficacy (BE) was calculated. The percentage efficacy of the bacterial antagonists was calculated according to Abbott's Formula (Abbott, 1987) as follows;

$$\mathrm{BE} = \frac{\text{disease incidence in control treatment} - \text{disease incidence in bacterial antagonist treatment}}{\text{disease incidence in control treatment}} \times 100$$

3.8.1 Screening for colonization of common bean plants by bacterial bio-controls

Six weeks after the experiment was over, six plants per treatment were used to determine colonization of bean plants by the bio-controls. Leaves were excised, surface sterilized and homogenized in 10 ml phosphate buffer solution in a sterile mortar and a pestle. Using the aforementioned standard microbiological techniques, the bio-controls were re-isolated and identified by 16S rRNA sequence analysis.

3.9 Data analysis

3.9.1 Phylogenetic analysis

Sequences obtained were edited using Chromas Lite software. Basic Local Alignment Search Tool (BLAST) in the national center for biotechnology information (NCBI) website was used to compare the 16S rRNA gene sequences with those in the GenBank database and aligned by CLUSTAL W program. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) and the evolutionary distances were computed using the Tamura-Nei method (Tamura and Nei, 1993). Evolutionary analyses were conducted in MEGA11 (Tamura *et al*., 2021).

3.9.2 Statistical analysis

Analysis of variance (ANOVA) in GenStat software was used to analyze data on disease incidence and disease severity from pathogenicity and biocontrol assays and Duncan's multiple range test at $P \leq 0.05$ was used to separate means.

CHAPTER FOUR

RESULTS

4.1 Isolation of *Cff* from bean leaves and morphological characterization

Ten symptomatic plants were collected from the field as shown in Figure 4.1.

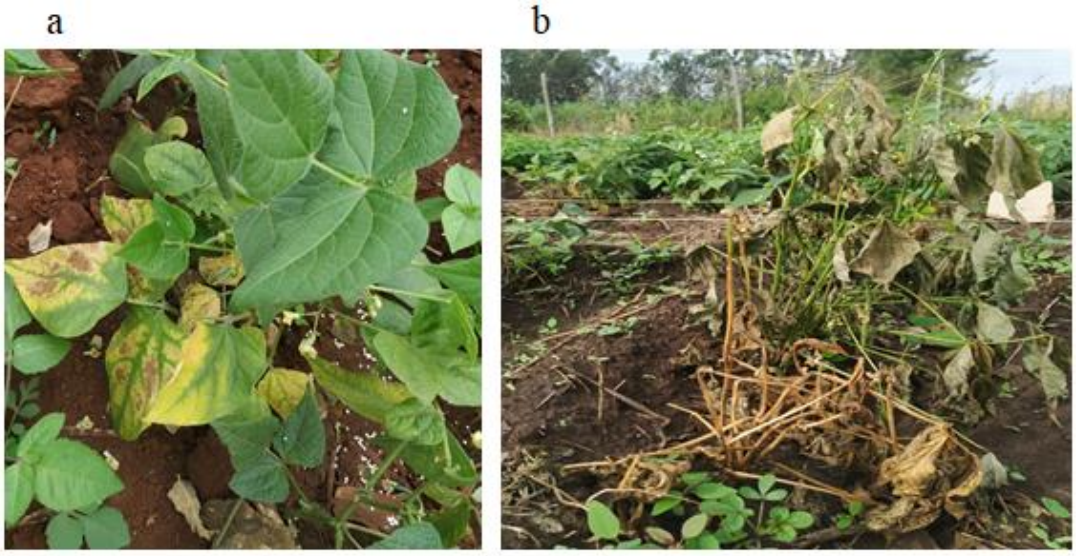

Figure 4.1 Symptomatic GLP X92 bean cultivars in a field. **a**; Necrosis symptoms on leaves with chlorotic borders, **b**; wilt symptoms.

A collection of 23 bacterial isolates were successfully recovered from 30 infected plant leaves. Ten isolates had typical characteristics of *Cff* that is, hydrolyzed skim milk and Congo red dye fading around the bacterial growth. Putative *Cff* isolates were Gram positive, produced yellow and orange pigment and were mucoidal on TSA medium (Figure 4.2).

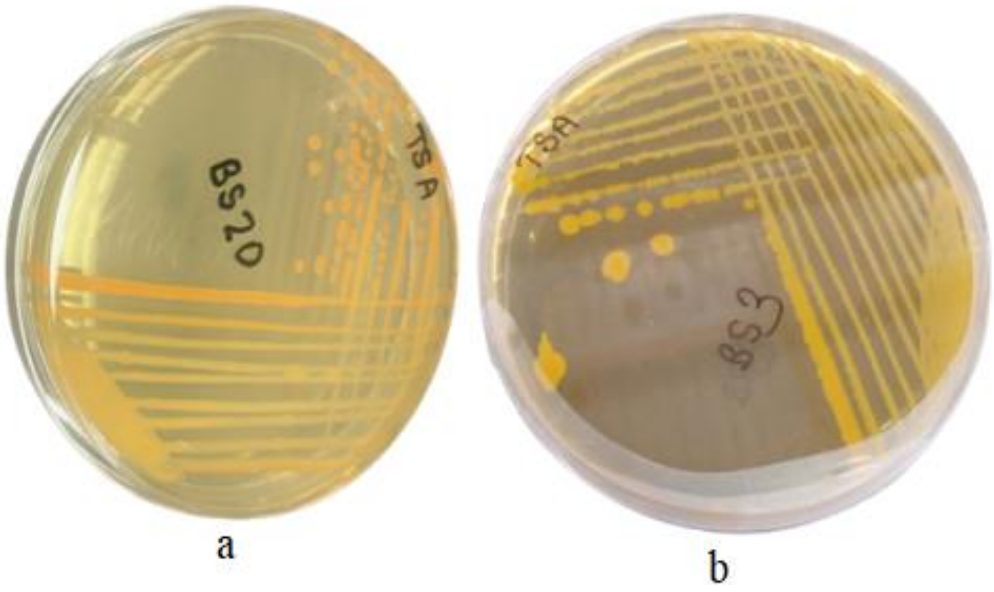

Figure 4.2: Colony characteristics of putative *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* isolates on Trypticase soy agar medium. a: Isolate BS20, orange pigmented and mucoid, b: Isolate BS3, yellow pigmented and mucoid.

Alongside the putative *Cff* isolates, other endophytic bacterial strains were isolated. Seven isolates were cream (BS4, BS5, BS6, BS12, BS14, BS18 and BS19) while others produced pigments such as orange (BS1 and BS2), red (BS8), yellow (BS7 and BS11) and pink (BS17). They were separated into two groups; mucoid, whose sticky nature is related to the formation of polysaccharides and dry, whose production of polysaccharides was minimal or absent. The cell morphology of the isolates was rod and the Gram reaction showed that 3 isolates were Gram Positive while 10 were Gram negative. The morphological characteristics of the bacterial isolates were described based on the microscopic and macroscopic appearances and recorded as shown in Table 4.1.

Table 4.1: Morphological characteristics of the bacterial isolates recovered from bean leaves.

Isolate	Pigmentation	Consistency	Gram stain	Shape
BS1	Orange	Mucoid	Positive	Rod
BS2	Orange	Mucoid	Positive	Rod
BS3	Orange	Mucoid	Positive	Rod
BS4	Cream	Mucoid	Negative	Rod
BS5	Cream	Mucoid	Negative	Rod
BS6	Cream	Mucoid	Negative	Rod
BS7	Yellow	Mucoid	Positive	Rod
BS8	Red	Mucoid	Negative	Rod
BS9	Yellow	Mucoid	Positive	Rod
BS10	Orange	Mucoid	Positive	Rod
BS11	Yellow	Dry	Negative	Rod
BS12	Cream	Dry	Negative	Rod
BS13	Yellow	Mucoid	Positive	Rod
BS14	Cream	Dry	Negative	Rod
BS15	Yellow	Fluidal	Positive	Rod
BS16	Yellow	Mucoid	Positive	Rod
BS17	Pink	Dry	Negative	Rod
BS18	Cream	Mucoid	Negative	Rod
BS19	Cream	Mucoid	Negative	Rod
BS20	Orange	Mucoid	Positive	Rod
BS21	Orange	Mucoid	Positive	Rod
BS22	Orange	Mucoid	Positive	Rod
BS23	Orange	Mucoid	Positive	Rod

4.2 Screening for enzymatic activity of the isolates

Out of the 23 bacterial isolates, 19 hydrolyzed cellulose, 16 hydrolyzed carboxymethylcellulose and xanthan, 17 hydrolyzed starch, 20 hydrolyzed xylan and 11

hydrolyzed pectin. Formation of halos around the bacterial growth, as shown in Figure 4.3, indicated hydrolysis.

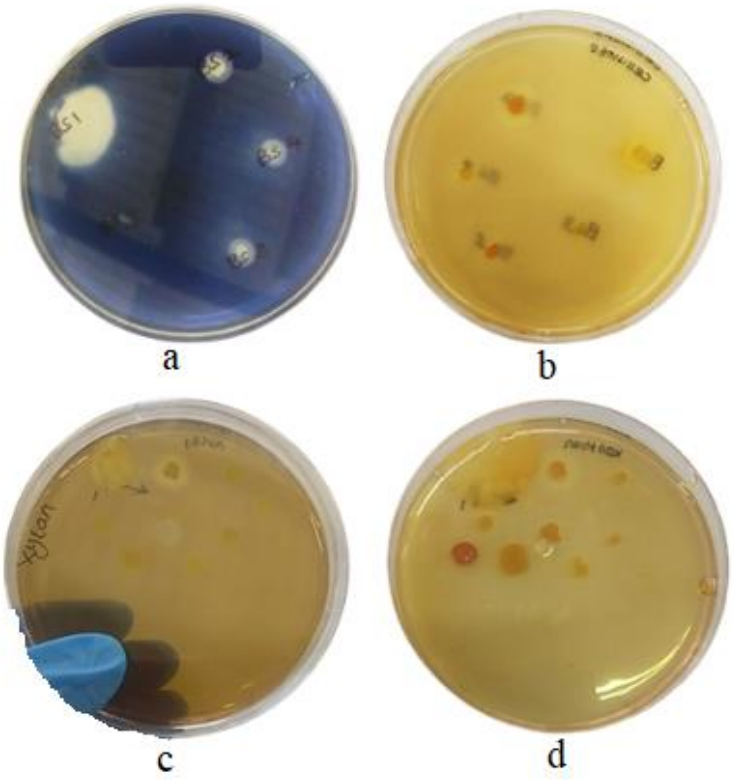

Figure 4.3: Hydrolase activity of the bacterial isolates recovered from bean leaves. a: Starch hydrolysis, b: Cellulose hydrolysis, c: Xylan hydrolysis, d: Xanthan hydrolysis

All the putative *Cff* isolates (BS3, BS9, BS10, BS13, BS15, BS16, BS20, BS21, BS22 and BS23) hydrolyzed casein, 10 isolates hydrolyzed cellulose, carboxymethylcellulose, xanthan and xylan and nine isolates hydrolyzed starch. They were all negative for pectin hydrolysis (Table 4.2).

Table 4.2: Extracellular enzyme activities of the bacterial isolates recovered from bean leaves.

Isolate	Cellulose	CMC	Starch	Xanthan	Xylan	Pectin
BS1	+++	-	+++	-	+++	+
BS2	+++	++	++	-	+++	-
BS3	++	+	+	-	++	-
BS4	++	++	+++	++	++	+
BS5	++	++	+++	-	++	+
BS6	+++	-	++	+	++	++
BS7	-	+++	+	++	-	+
BS8	-	+	-	-	+	-
BS9	+	+	-	+	+	-
BS10	+	+	-	+	+	-
BS11	+	-	+	++	+	+
BS12	+	-	-	-	++	+
BS13	+	+	+	++	+	-
BS14	+	-	-	+	++	+
BS15	-	+	+	++	-	-
BS16	+++	++	++	++	+	-
BS17	-	+	-	-	-	-
BS18	+	+	++	+	++	++
BS19	+	-	+++	-	++	+++
BS20	+	-	+	+	+	-
BS21	++	++	++	++	+	-
BS22	++	+	++	++	+	-
BS23	++	++	++	++	+	-

Keys: + indicates low activity (0-3mm), ++ indicates moderate activity (3.1-6mm), +++ indicates high activity (>6mm), - indicates no enzyme activity, CMC-carboxymethylcellulose.

4.3 Molecular characterization of isolates recovered from the leaves of common bean plants

Total genomic DNA of all the isolates was successfully extracted using phenol-chloroform protocol as described by Sambrook *et al*., (1989) as shown in Figure 4.4.

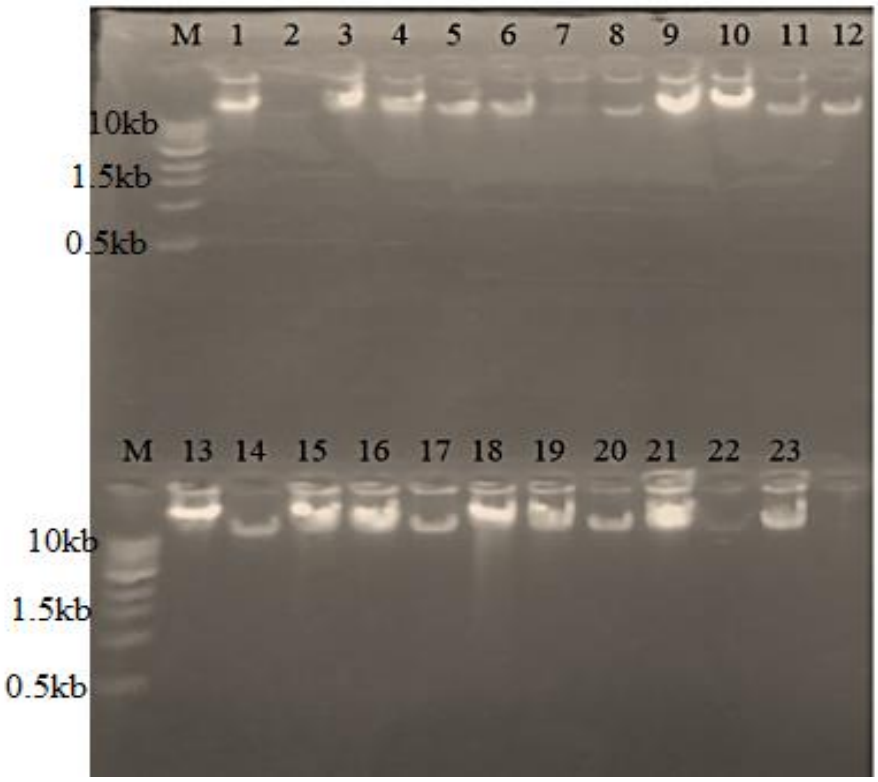

Figure 4.4: Gel electrophoresis stained with sybr green fluorescent dye showing successful DNA extraction from bacterial isolates recovered from bean leaves. Lane M: 10kb DNA ladder; Lane 1-23; isolates BS1, BS2 BS3, BS4, BS5, BS6, BS7, BS8, BS9, BS10, BS11, BS12, BS13, BS14, BS15, BS16, BS17, BS18, BS19, BS20, BS21, BS22 and BS23.

4.3.1 PCR amplification of the 16S rRNA gene

The universal bacterial primers 8F (forward) and 1492R (reverse) were used to amplify the 16S rRNA gene. The PCR products were separated on 1% agarose (1g/100ml) gel in

1× TAE buffer stained with sybr green fluorescent dye and then visualized under UV light as shown in Figure 4.5.

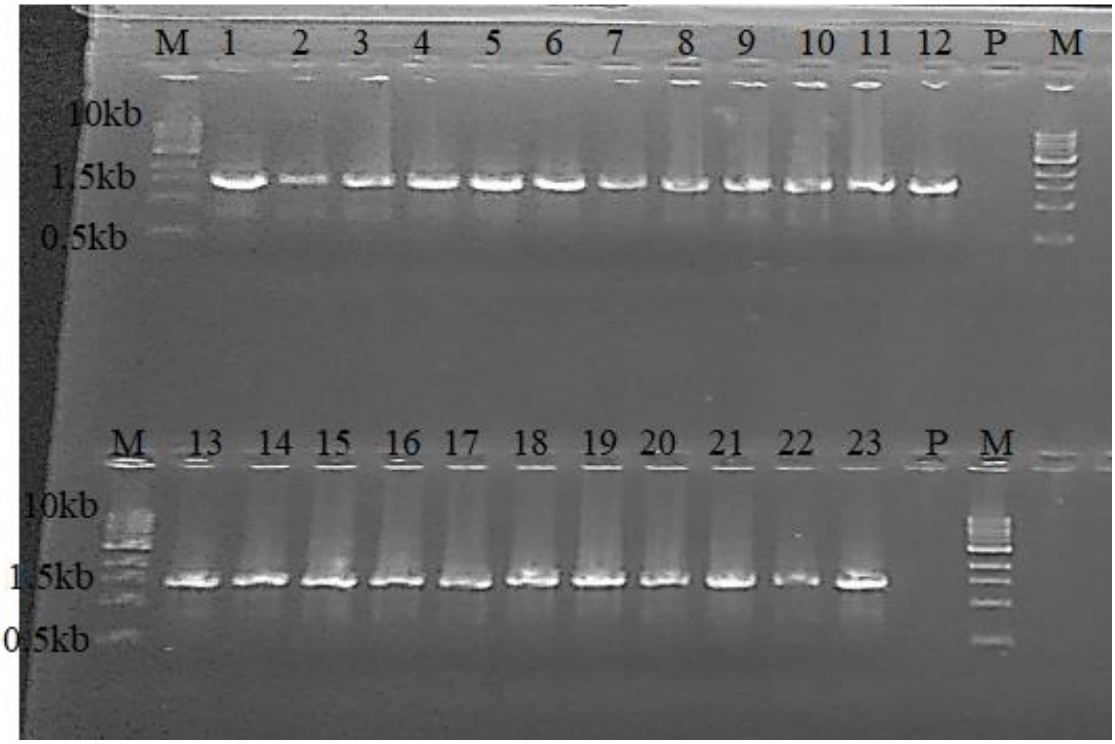

Figure 4.5: Gel electrophoresis showing amplicons of the 16S rRNA gene (1.6kb) in the bacterial isolates recovered from bean leaves. Lane M: 10kb DNA ladder; Lane 1-23: PCR products from isolates BS1, BS2, BS3, BS4, BS5, BS6, BS7, BS8, BS9, BS10, BS11, BS12, BS13, BS14, BS15, BS16, BS17, BS18, BS19, BS20, BS21, BS22 and BS23; Lane P: a negative control.

4.3.2 Phylogenetic analysis of the sequences

The resulting sequences were analyzed using BLASTN program in the NCBI website and their closest relatives were determined based on percentage identity. The isolates were affiliated to the genera *Curtobacterium* (10 isolates), *Pseudomonas* (8 isolates), *Bacillus* (2 isolates) and *Methylorubrum* (2 isolates) as shown in Table 4.3. Isolate BS2 was mixed with background noise therefore, its sequences were not blasted.

Table 4.3: Phylogenetic analysis and identification of the *Curtobacterium* spp. isolated from bean leaves.

Isolate code	Closest Neighbor	Accession Numbers	Identity (%)
BS1	*Bacillus velezensis* strain WLYS23	CP055160.1	99.53
BS3	*Curtobacterium citreum* strain 51A	KC329830.1	99.88
BS4	*Pseudomonas fluorescens* strain CFBP 11386	FN666563.1	100
BS5	*Pseudomonas fluorescens* strain CFBP 11386	FN666563.1	100
BS6	*Pseudomonas putida* strain OsEnb_HZB_G20	MN889396.1	99.74
BS7	*Bacillus velezensis* strain KKLW	CP054714.1	99.65
BS8	*Methylorubrum extorquens* strain BF19-02M	MT346348.1	91.91
BS9	*Curtobacterium sp*. strain MRDDc	MK578274.1	97.75
BS10	*Curtobacterium citreum* strain PD7-1	AB506119.1	100
BS11	*Pseudomonas oryzihabitans* strain CPO 4.233	MN733061.1	99.76
BS12	*Pseudomonas sp*. strain NNC7	MT507062.1	99.76
BS13	*Curtobacterium citreum* strain CPO 4.218	MN733046.1	100
BS14	*Pseudomonas sp*. strain NNC7	MT507062.1	99.65
BS15	*Curtobacterium citreum* strain CPO 4.218	MN733046.1	100
BS16	*Curtobacterium sp*. NJ-10	AM396911.1	100
BS17	*Methylobacteriaceae bacterium* KVD-unk-12	DQ490343.1	99.88
BS18	*Pseudomonas aeruginosa* strain Codelsu	MK875780.1	100
BS19	*Pseudomonas aeruginosa* strain SWA	MH760804.1	100
BS20	*Curtobacterium citreum* strain PD7-1	AB506119.1	100
BS21	*Curtobacterium citreum* strain CPO 4.218	MN733046.1	99.88
BS22	*Curtobacterium sp*. MR MD2014	KU740254.1	99.52
BS23	*Curtobacterium oceanosedimentum* strain P120	MT487608.1	98.54

The 16S rRNA gene sequences were compared with those in the GenBank database using the Basic Local Alignment Search Tool (BLAST) in the national center for biotechnology information (NCBI) GenBank database. Accession numbers provided are from the GenBank.

The phylogenetic tree showed that the putative *Cff* isolates belonged to the genus *Curtobacterium* (Figure 4.6).

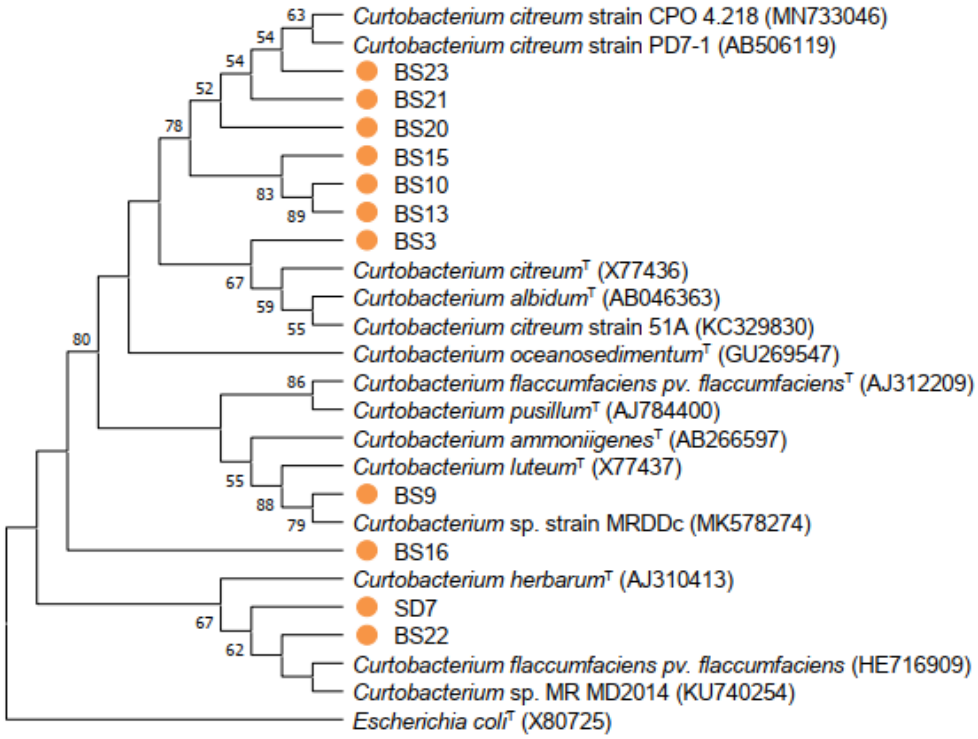

Figure 4.6: The phylogenetic relationship of the putative *Cff* isolates recovered from bean leaves and seeds and their closest relatives. Phylogenetic tree was generated using the neighbor-joining method based on comparison of the 16S rRNA (1.6kb) sequences of the putative *Cff* isolates (highlighted) and their closest phylogenetic relatives. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Tamura-Nei method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA11. GenBank accession number is given in parentheses. The 16S rRNA sequence of *Escherichia coli* was used as an outgroup.

4.4 Molecular characterization of bacterial isolates from seeds

The phenol-chloroform method was used to extract the bacterial genomic DNA of isolates recovered from seeds. The universal bacterial primers 8F (forward) and 1492R (reverse) were used to amplify the 16S rRNA gene. The PCR products were separated on a 1% agarose gel in a 1×TAE buffer stained with a fluorescent dye and then visualized under UV light, as shown in Figure 4.7.

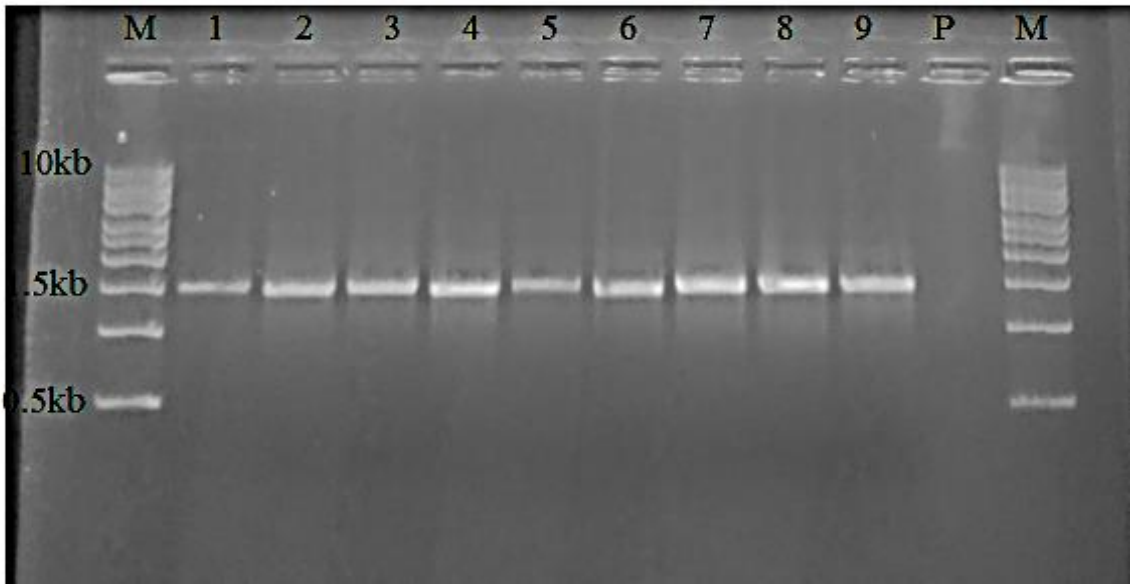

Figure 4.7: Gel electrophoresis showing PCR amplification of 16S rRNA gene (1.6kb) of bacterial isolates recovered from bean seeds. Lane M: 10kb DNA ladder; Lane 1-9: PCR products from isolates SD1, SD2, SD3, SD4, SD5, SD6, SD7, SD8 and SD9. Lane P: a negative control.

4.4.1 Phylogenetic analysis of the isolates

Blasting of the 16S rRNA gene revealed isolates were from the genera *Micrococcus* (2 strains), *Bacillus* (2 strains), *Microbacterium* (2 strains), *Pseudarthrobacter* (1 strain) and *Curtobacterium* (1 strain) as shown in Table 4.4.

Table 4.4: Phylogenetic analysis of bacterial isolates from seeds

Isolate code	Closest Neighbor	Accession Numbers	Identity (%)
SD1	*Microbacterium* sp. LPPA 946	HE650842.1	99.28
SD2	*Micrococcus luteus* strain INBI-1-16	MK503663.1	99.88
SD3	*Micrococcus luteus* strain MA3	MT072186.1	99.28
SD4	Bacillus thuringiensis strain Bt57	MT292096.1	99.53
SD6	*Pseudarthrobacter* sp. strain 18H5P11	MK129293.1	98.8
SD7	*Curtobacterium flaccumfaciens pv. flaccumfaciens* strain YJM-09	HE716909.1	99.88
SD8	*Microbacterium* sp. NA11006	AB921258.1	99.28
SD9	*Bacillus* sp. strain LA9	MT279342.1	98.06

The 16S rRNA gene sequences were compared with those in the GenBank database using the BLAST program found in the NCBI GenBank database. Accession numbers provided are from the GenBank. The isolates were highly related to their respective closest neighbor with similarity percentage ranging between 98-99%.

4.5 Pathogenicity of *Curtobacterium* strains on common bean

In the experimental setting, the *Curtobacterium* spp. isolated from common bean leaves and seeds caused pathogenesis on common beans. The symptoms that appeared in plants were identical to those observed in the field. Symptoms developed in 5-7 days after planting. Wilting of plants, death of seedlings and un-emerged seedlings were observed (Figure 4.8).

Figure 4.8: Wilted seedlings from infected GLP X92 *P. vulgaris* seeds.

The bacteria were re-isolated from symptomatic plants and molecularly identified as *Curtobacterium* species (Table 4.5).

Table 4.5: Identification of the re-isolated pathogens and bio-controls

Isolate code	Closest neighbor	Accession Numbers	Identity (%)
BS3	*Curtobacterium citreum* strain 51A	KC329830.1	99.88
BS9	*Curtobacterium* sp. strain MRDDc	MK578274.1	97.75
BS10	*Curtobacterium citreum* strain PD7-1	AB506119.1	100
BS13	*Curtobacterium citreum* strain CPO 4.218	MN733046.1	100
BS15	*Curtobacterium citreum* strain CPO 4.218	MN733046.1	100
BS16	*Curtobacterium* sp. NJ-10	AM396911.1	100
BS20	*Curtobacterium citreum* strain PD7-1	AB506119.1	100
BS21	*Curtobacterium citreum* strain CPO 4.218	MN733046.1	99.88
BS22	*Curtobacterium* sp. MR MD2014	KU740254.1	99.52
SD7	*Curtobacterium flaccumfaciens pv. flaccumfaciens* strain YJM-09	HE716909.1	99.88
BC6	*Bacillus cereus strain S8*	MT611946.1	99.77
BC14	*Paenibacillus polymyxa strain ZJTZ-4*	MT605508.1	98.58
BC24	*Pseudomonas fluorescens strain 90F12-2*	KT695840.1	99.64

The 16S rRNA gene sequences were compared with those in the GenBank database using the BLAST program found in the NCBI GenBank database. The isolates were highly related to their respective closest neighbor with percentage similarity ranging between 99-100%.

The *Curtobacterium* isolates were pathogenic to GLP X92 *P. vulgaris* plants with high disease severity of up to 82% in *Cff* (SD7) that was obtained from infected seeds (Figure 4.9). *Curtobacterium citreum* isolates (BS3, BS10, BS13, BS15, BS20 and BS21) were pathogenic to the GLP X92 *P. vulgaris* with mean disease incidence and severity of 32%-55% and 8%-24% respectively (Appendix 1).

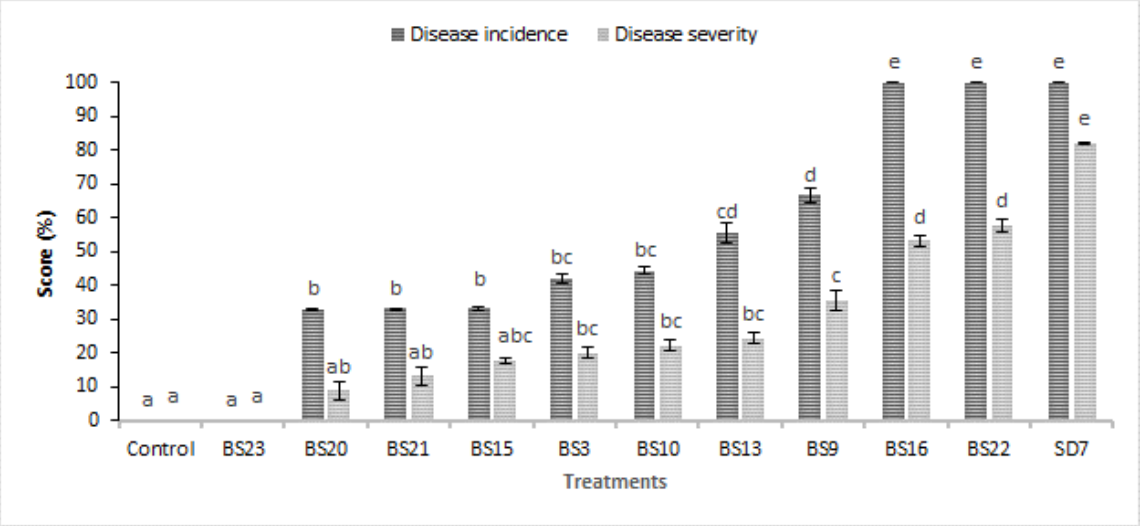

Figure 4.9: Pathogenicity of *Curtobacterium* spp. to GLP X92 *P. vulgaris* assessed using seed inoculation method. Bars headed with the same letters are not significantly different ($P \leq 0.001$) according to DMRT. The line on each bar represents the ± SE of means of two experiments of three seedlings replicated three times per 11 treatments. Data were collected 14 days after planting.

Leaves inoculated by foliar spray in detached leaf assay developed necrotic lesions surrounded by yellow borders as shown in Figure 4.10.

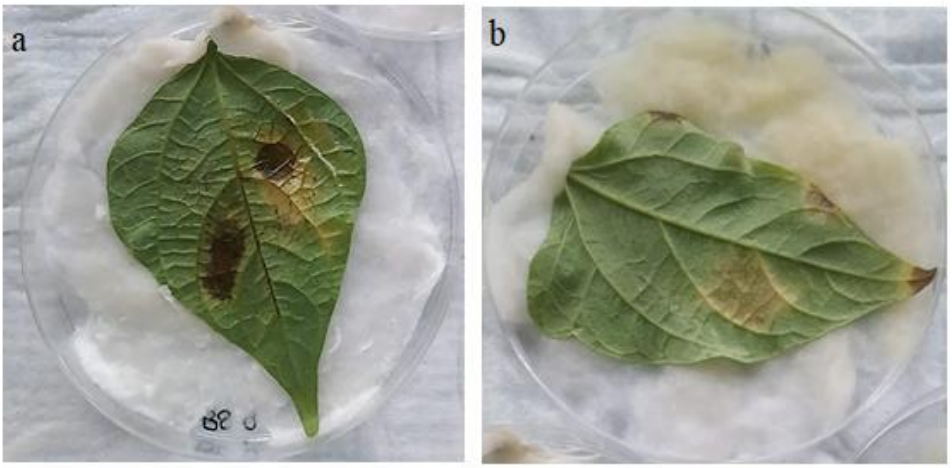

Figure 4.10: Bean leaves showing interveinal necrotic lesions surrounded by yellow borders in a detached leaf assay. **a**; isolate BS9 and **b**; isolate BS21

Plants inoculated by foliar spray developed symptoms of bacterial wilt under room temperature (20°C) and greenhouse temperature (24-28°C). The plants wilted and leaves displayed interveinal necrotic lesions surrounded by yellow borders (Figure 4.11). Under greenhouse conditions, the disease severity was higher in comparison to laboratory conditions.

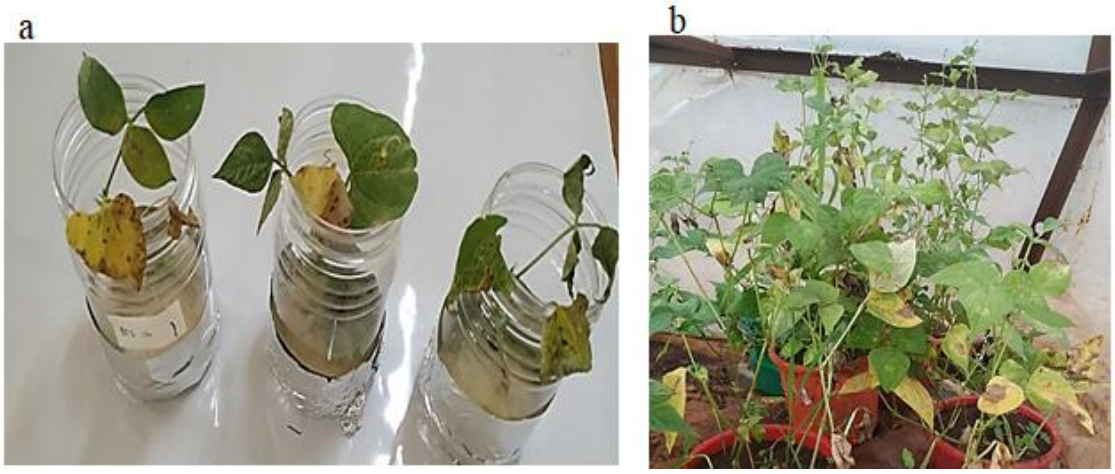

Figure 4.11: Bean leaves showing symptoms of interveinal necrotic lesions with yellowing and wilting. **a**: under laboratory conditions, **b**: under greenhouse conditions. Plants were inoculated by foliar spray.

Weeds that grew alongside the bean plants also had symptoms of yellow borders surrounding interveinal necrotic lesions as shown in Figure 4.12. They were collected and used for isolation of *Cff*. Colonies produced on CFFSM medium were morphologically related to putative *Cff* isolates. Sequence analysis of the 16S rRNA gene identified the isolates as *Curtobacterium spp.* similar to the re-isolated species (BS3, BS9 and BS16).

Figure 4.12: Weeds showing necrotic lesions with yellowing

4.6 Isolation of bacterial antagonists and *in vitro* antibiosis screening of the isolates against *Cff*

From the *Phaseolus vulgaris* rhizosphere soil, a total of 26 bacterial isolates were successfully obtained. The bacterial isolates exhibited varied antagonistic activity (Appendix 2). *In vitro* screening results revealed that 12 of the 26 isolates tested on TSA medium inhibited *Cff* growth, which was indicated by the formation of clear zones in the dishes as shown in Plate 1.

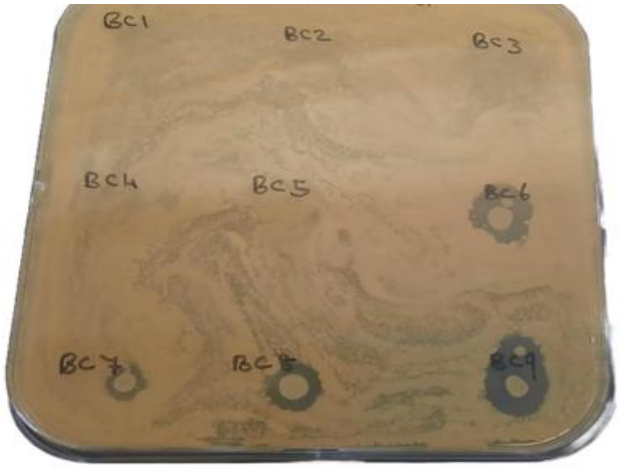

Plate 1: Growth inhibition of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* using different antagonists on TSA medium. Bacterial antagonists BC6, BC7, BC8 and BC9 (strains of *Bacillus cereus*) inhibited the growth of the *Curtobacterium* sp. whereas isolates BC1, BC2, BC3, BC4 and BC5 did not have inhibition activity.

4.7 Molecular identification of the antagonistic bacteria from the rhizosphere soil

Genomic DNA for the 12 antagonistic bacterial isolates was confirmed on agarose gel electrophoresis and visualized under ultraviolet light as shown in Figure 4.13.

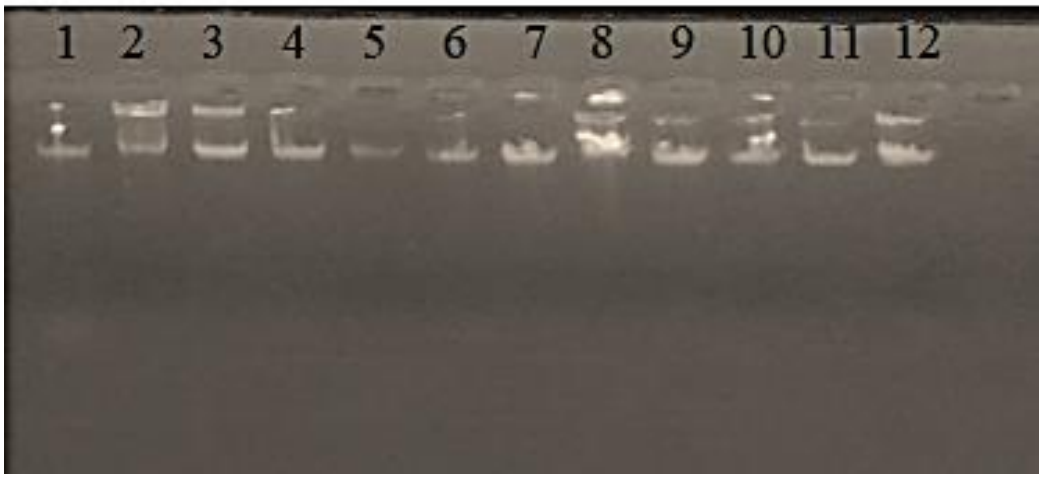

Figure 4.13: Gel electrophoresis showing extracted DNA of antagonistic bacteria. Lane 1-12; DNA from isolates BC6, BC7, BC8, BC9, BC10, BC14, BC15, BC18, BC21, BC23, BC24 and BC25.

4.7.1 PCR amplification of the antagonistic bacteria

The amplification of the conserved region in 16S rRNA gene using bacterial universal primers 8F and 1492R using PCR resulted to amplicons of about 1.6kb (Figure 4.14).

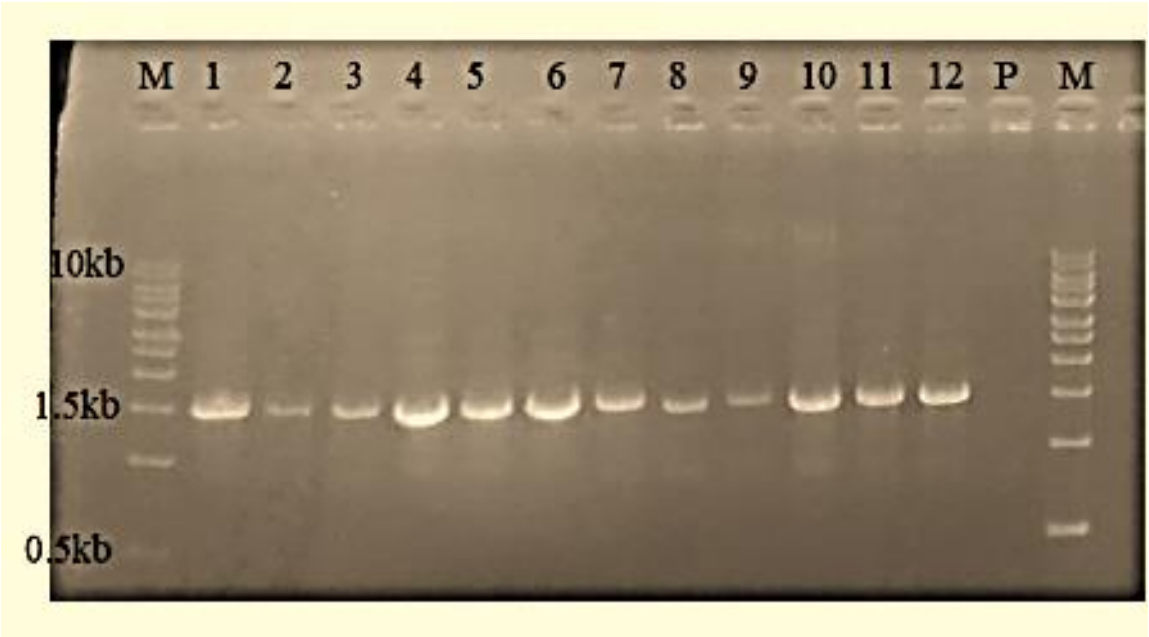

Figure 4.14: A 1% agarose gel showing amplification of the 16S rRNA gene of the bacterial antagonists. Lane M: 10kb DNA ladder; Lane 1-12: amplicons from isolates BC6, BC7, BC8, BC9, BC10, BC14, BC15, BC18, BC21, BC23, BC24 and BC25. Lane P is a negative control.

4.7.2 Identification of the antagonistic bacteria, 16S rRNA gene sequencing and phylogenetic analysis

The bacterial antagonists were characterized according to molecular aspects. Sequence analysis of the 16S rRNA gene revealed that 7 isolates were *Bacillus cereus* with a sequence identity of 99% and the rest of the strains showed similarity with *Paenibacillus polymyxa* (98%) and Pseudomonas (99%) as shown in Table 4.6. Isolate BC18, BC21 and BC25 were not sequenced since their inhibition activity was low.

Table 4.6: Phylogenetic analysis and identification of antagonistic bacteria isolated from rhizosphere soil

Isolate code	Closest neighbor	Accession Numbers	Identity (%)
BC6	*Bacillus cereus* strain S8	MT611946.1	99.77
BC7	*Bacillus cereus* strain S8	MT611946.1	99.77
BC8	*Bacillus cereus* strain S8	MT611946.1	99.77
BC9	*Bacillus cereus* strain S8	MT611946.1	99.77
BC10	*Bacillus cereus* strain NIBSM OsR5	KY930705.1	99.77
BC14	*Paenibacillus polymyxa* strain ZJTZ-4	MT605508.1	98.58
BC15	*Bacillus cereus* strain NIBSM OsR5	KY930705.1	99.65
BC23	*Bacillus cereus* strain S8	MT611946.1	99.88
BC24	*Pseudomonas fluorescens* strain 90F12-2	MT507071.1	99.64

The 16S rRNA gene sequences were compared with those in the GenBank database using the BLAST program found in the NCBI GenBank database. Accession numbers provided are from the GenBank. The isolates were highly related to their respective closest neighbor with similarity percentage ranging between 98-99%.

Based on comparisons of 16S rRNA sequences and their closest phylogenetic relatives, a phylogenetic tree was derived using neighbor-joining. The numbers next to the branches indicate the percentages of bootstrap sampling derived from 1000 replications (Figure 4.15).

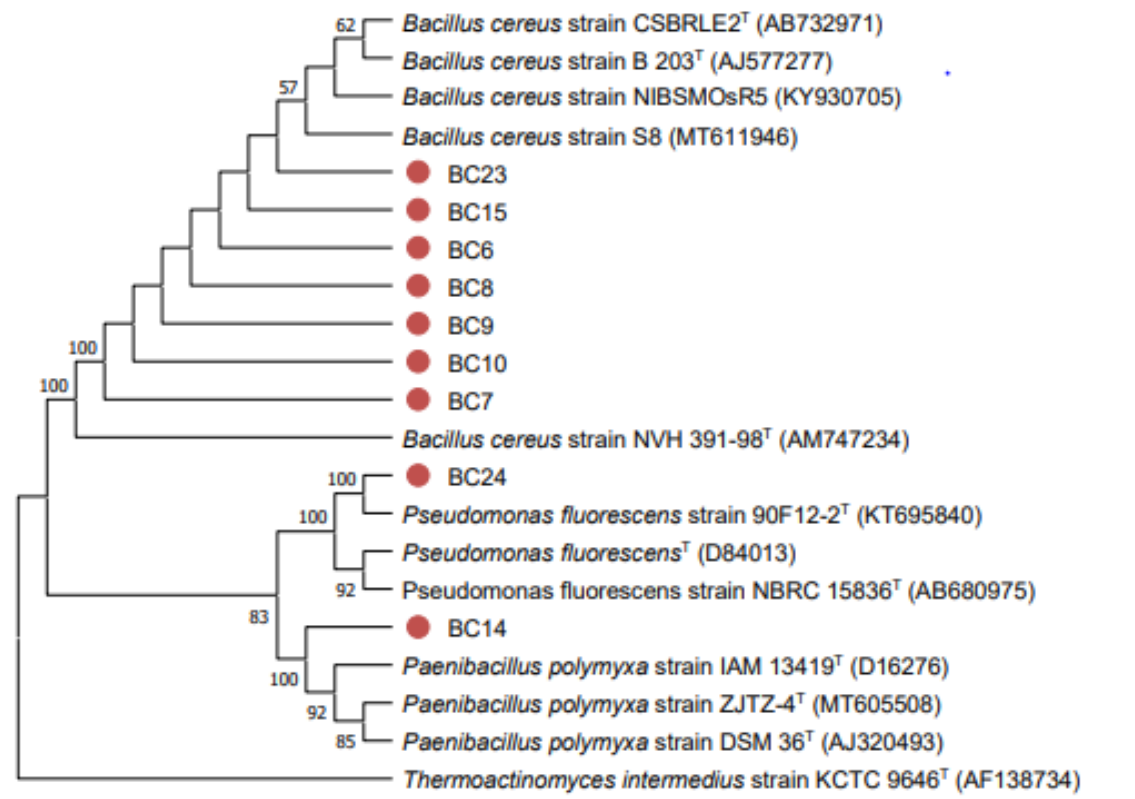

Figure 4.15: Phylogenetic analysis of antagonistic bacteria recovered from rhizosphere soil. Phylogenetic tree was generated using the neighbor-joining method based on comparison of the 16S rRNA sequences of bio-controls (highlighted) and their closest phylogenetic relatives. Percentages of bootstrap sampling derived from 1000 replications are shown by the numbers next to the branches. GenBank accession number for the type strains is shown in parentheses. The 16S rRNA sequence of *Thermoactinomyces intermedius* was used as an outgroup.

4.8 Screening the antagonistic bacteria for production of enzymes

The bacterial isolates were assessed for their potential to secrete extracellular enzymes by spotting them onto basal medium enriched with respective substrate. The production of cellulases, xanthanases, amylases, carboxymethylcellulases, xylanases and pectinases were indicated by formation of haloes on the specific media (Figure 4.16).

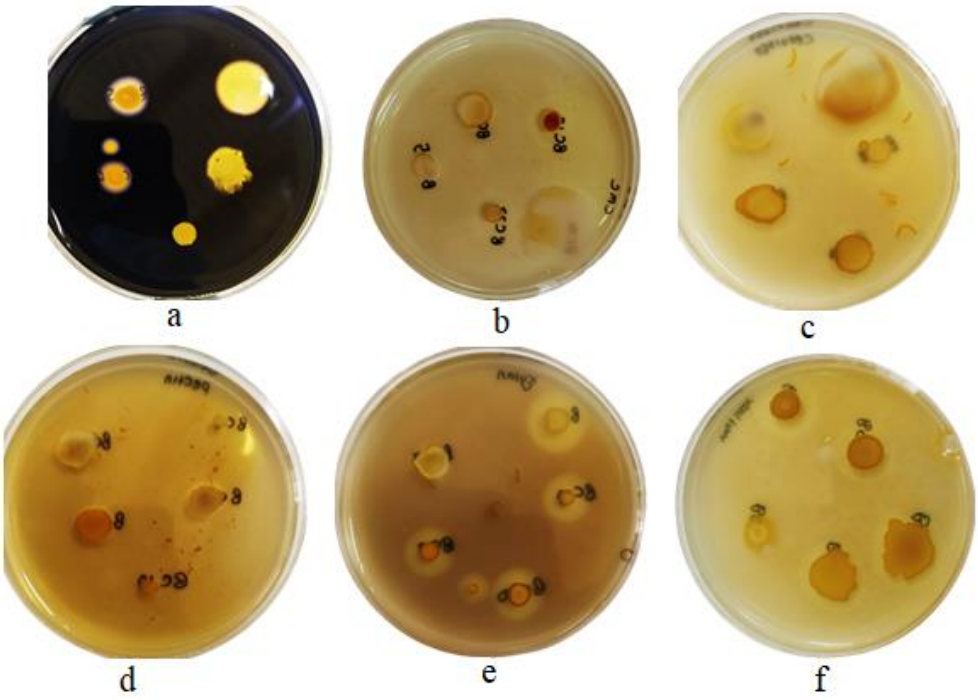

Figure 4.16: Extracellular hydrolase activities of the antagonistic bacterial isolates. a**:** Starch hydrolysis, b: Carborxymethylcellulose hydrolysis, c**:** Cellulose hydrolysis, d: Pectin hydrolysis, e: Xylan hydrolysis, f: Xanthan hydrolysis

Eight bacteria bio-controls hydrolyzed xanthan and xylan while six bacterial isolates hydrolyzed pectin. Starch, cellulose and carboxymethylcellulose were hydrolyzed by all the antagonistic bacteria (Table 4.7).

Table 4.7: Enzymatic activity of the bacterial antagonist

Isolate	Starch	Cellulose	CMC	Xanthan	Xylan	Pectin
BC6	+++	+++	+++	+++	++	+
BC7	++	++	++	+	+++	-
BC8	+++	+++	+++	+++	+++	+
BC9	+++	+++	+++	+++	+++	+
BC10	+++	+++	+++	+++	+++	+
BC14	++	++	+++	+++	+++	++
BC15	++++	+++	+++	+++	+++	-
BC23	+	+++	+++	+++	+++	-
BC24	+++	++	++	-	-	++

Key: - no activity, + (0-3mm), ++ (3.1-6mm), +++(>6mm), CMC-carboxymethylcellulose

4.9 Efficacy of bacterial antagonists against bacterial wilt in common bean plants

Pathogen inoculated plants had symptoms of chlorosis, interveinal necrotic lesions and wilting with disease incidence of 91.67% and disease severity of 85.19%. In biological control treatment, the disease incidence and disease severity ranged between 16-29% and 4-10% respectively (Appendix 3). All of the tested bacterial antagonists significantly ($p \leq 0.001$) reduced the disease incidence and disease severity. In comparison with untreated controls, *B. cereus, P. fluorescens and P. polymyxa* greatly reduced disease incidence and severity. *P. fluorescens* was the most effective in controlling the *Cff* pathogen with efficacy of 81%, while *B. cereus* and *P. polymyxa* reduced the disease development by 79% and 68%, respectively (Figure 4.17).

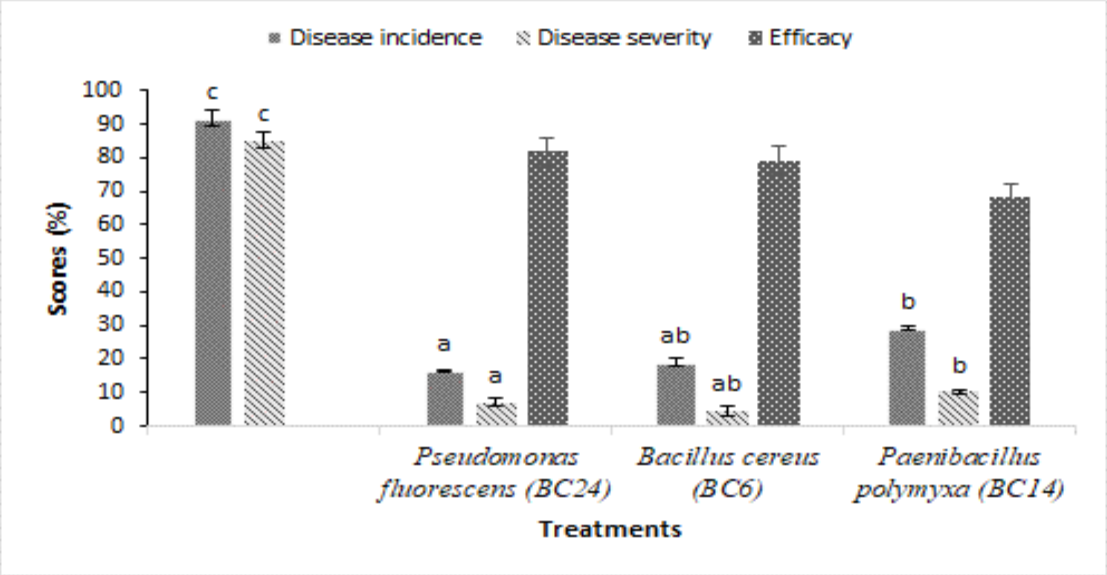

Figure 4.17: Efficacy of different bacterial antagonists as combined seed treatment and foliar application in GLP X92 *P. vulgaris*. Bars headed with the same letters are not significantly different ($p \leq 0.05$) according to DMRT. The line on each bar represents the ± SE of means of two experiments with six replicates of four seedlings per three treatments. Data was collected 6 weeks after planting.

The phylogenetic analysis from the partial sequence of the 16S rRNA gene showed that the re-isolated bacteria biocontrol isolates were affiliated to *B. cereus, P. fluorescens and P. polymyxa.*

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

The present study aimed at isolating wilt-causing pathogen and controlling the pathogen using bacteria isolated from the rhizosphere of *Phaseolus vulgaris*. Detecting and controlling bacterial wilt disease is important to avoid losses in production of common beans. This study highlight that the plant rhizosphere is a valuable source of potent rhizobacteria that may serve as an eco-friendly solution for the control of plant diseases. Common beans that were grown in Kimbimbi, Kirinyaga County for field trials had disease symptoms that appeared to be bacterial wilt of bean. The research plots were under irrigation systems. Previous studies have reported that this bacterium is often widespread in irrigated areas because it thrives in moist conditions and can be easily transmitted through water (Harveson and Vidaver, 2008; Osdaghi, 2018). One of the key factors that contribute to the widespread nature of *C. flaccumfaciens* pv. *flaccumfaciens* is the fact that it can last in the soil for a very long time. It can also survive on plant debris, which means that it can persist in an area even after a crop has been harvested. This enables the bacterium to remain a threat to new crops that are planted in the same area.

Isolation and morphological characterization of the pathogen

A wide range of media have been previously used for isolation of *Cff*. In the present study, CFFSM medium (Maringoni and Camara, 2006) was used. The media contains carbon sources that allowed selection of *Cff* isolates and antimicrobial products that inhibited the growth of other saprophytes. A yellow strain of *C. flaccumfaciens* pv. *flaccumfaciens* was isolated from bean seeds in the present study. According to EPPO (2011), infected seeds are the main source of inoculum for transferring the pathogen to new locations with no history of the disease. Many small-scale farmers in Kenya use their own seeds for planting or seeds bought from the nearby market. This may result to the pathogen establishing and spreading within the region or other nearby regions. Strict detection measures should be used for certification in order to prevent contaminated seeds. The *Curtobacterium* spp. in

the present study showed variable morphological characteristics. Use of non-selective medium is required for the characterization of pigment synthesis (Harveson *et al*., 2015). Therefore, the excretion of different pigments was evaluated on Trypticase soy agar medium. The isolates produced yellow and orange pigmented colonies which were either mucoidal or fluidal. Five different color variants on culture media have previously been reported and include; yellow, orange, pink, red and purple (Tegli, 2011; Osdaghi *et al*., 2020). The cell morphology was Gram positive and rod shaped. This results were consistent with previous findings (Huang *et al*., 2010; Osdaghi *et al*., 2015; Tegli *et al*., 2020). Excretion of different pigments is a survival mechanism microbe use under unfavorable conditions. In plant-associated microorganisms, pigmentation offers protection from ultraviolet radiation. The capacity of *Cff* to create a high quantity of colony pigment makes this bacterium very effective in terms of fitness as well as survival, including unfavorable environmental conditions.

The phylogenetic analysis of the 16S rRNA sequence clustered the isolated bacteria into *Curtobacterium* species. The phylogeny distinguished the clades of *Curtobacterium* strains reliably and the bootstrap supporting values were significantly higher. The bacterial sequences were clustered into two lineages mainly the gram-positive bacteria (*Curtobacterium* spp.) and gram-negative (*Escherichia coli* which was an outgroup). The putative *Cff* isolates were clustered into orange pigmented strains (BS23, BS21, BS20, BS15 and BS10) versus yellow pigmented strains (BS9, BS16, BS22 and SD7). These results were consistent with previous findings by Osdaghi *et al*. (2018) showed that there are two lineages within *Cff* strains which include one with yellow-colored colonies and another with orange- or red-colored colonies.

Pathogenicity of the pathogen

Pathogenicity tests were conducted on GLP X92 seeds (pinto class market) which is susceptible to *Cff*. The results obtained in pathogenicity confirmed susceptibility of common beans (*Phaseolus vulgaris*) to these *Curtobacterium* isolates with high disease severity of up to 82% (Figure 4.9). Seedlings were stunted, wilted and eventually died. These findings corroborate with previous reports of Hsieh *et al*. (2003b) and Osdaghi *et*

al. (2015) that bacterial wilt of common bean is a significant seed-borne disease and causes stunted seedlings, wilting and plant mortality. A previous study by Osdaghi *et al*. (2018) showed that there are two lineages within *Cff* strains which include one with yellow-colored colonies and another with orange- or red-colored colonies. In the present study, a yellow pigmented variant was isolated. Isolates of *Curtobacterium citreum* were pathogenic with disease incidence and severity of 32%-55% and 8%-24% respectively. *Curtobacterium citreum* has not been previously reported to be pathogenic to *P. vulgaris* and further experiments on pathogenicity could be carried out. The development of the pathogen within the xylem tissues blocks the movement of water to the foliage causing wilting. Symptoms of bacterial wilt of common beans are highly severe in seedlings emerging from infected seeds. Usually the young plants die.

Weeds that grew together with the beans in the greenhouse experiment were colonized by the pathogen (Figure 4.12). These results concur with those from Harveson *et al*. (2015) that the pathogen can persist on non-host plant species. The pathogen was isolated from wheat and maize. Studies by Júnior *et al*. (2012) and Gonçalves *et al*. (2017) showed that *Cff* colonized barley, black oat, canola, white oat, rye grass and wheat without causing symptoms when grown in rotation with bean. *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* pathogenic to dry bean was isolated from symptomless pepper, eggplant as well as tomato plants by Osdaghi *et al*., (2018) in Iran. The potential of phytopathogenic bacteria to survive between crop seasons depends on their ability to find alternate hosts.

Curtobacterium flaccumfaciens pv. *flaccumfaciens* was highly pathogenic to GLP X92 *P. vulgaris* seedlings under greenhouse conditions in comparison to laboratory conditions (Figure 4.11). This supports earlier research on how temperature affects the severity of bacterial wilt (Krause *et al*., 2009). Martins *et al*. (2014) showed that at temperatures of 30°C a 2.4-fold increase in disease severity was seen compared to common bean plants kept at 20°C. Nascimento *et al*. (2021) demonstrated that *Cff* was able to persist in the rhizosphere and phylosphere of beans at temperatures above 30°C.

Production of enzymes by the pathogen

Biochemical characterization of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* showed that it produced various hydrolytic enzymes. These lytic enzymes disrupt the host plant's cell walls and absorb nutrients (Chen *et al*., 2021). These findings are consistence with the previous report (Osdaghi *et al*., 2020) that *Cff* contains lytic enzymes such as beta galactosidase, lipases esterase and peptidases. Full genome sequencing revealed that the pathogen contains pectate lyase, 1-4, beta xylanase and serine protease genes which confirms secretion of cell wall degrading enzymes (Chen *et al*., 2021). According to earlier studies by Bulgari *et al*. (2014); Dimkić *et al*. (2021); Lopes *et al*. (2016), some *Curtobacterium* species have characteristics that are beneficial to plants in terms of mineral nutrition through phosphate solubilization. They also boost plant resistance to abiotic as well as biotic stressors through the secretion of 1-amino-cyclopropane-1-carboxylate (ACC) deaminase. They stimulate plant development by synthesis of indoleacetic acid (IAA), enzymes, as well as siderophore. Herein, *in vitro* assays showed that the *Curtobacterium* species produced various extracellular enzymes (Table 4.2). By secreting these enzymes, *Cff* can effectively colonize and infect common bean plants, leading to the development of bacterial wilt disease. The breakdown of plant cell walls and tissues, coupled with the acquisition of nutrients from the host, allows *Cff* to establish and proliferate within the plant, causing the characteristic wilting symptoms. Moreover, the ability to form biofilms protects *Cff* from the host's immune responses, enabling its persistence and further spread within the plant (Krimi *et al*., 2023).

Isolation of the bacterial antagonists

An eco-friendly method of controlling plant diseases is through biological control using microorganisms. The main screening technique applied to *in vitro* conditions relies on the antagonistic activity as the initial step in finding prospective biological control agents. In the current study, a collection of 26 rhizobacteria were isolated from *P. vulgaris* rhizosphere and tested for their inhibition activity against *Cff in vitro*. Nine isolates showed strong antagonistic activity against *Cff*. These bacterial isolates were identified using 16S rRNA gene partial sequencing which revealed species belonging to *Bacillus*,

Paenibacillus and *Pseudomonas* genus. The results are consistence with previous findings (Martins *et al*., 2013) on isolation of *Paenibacillus* and *Bacillus* species from the rhizosphere to control bacterial wilt of bean. The association of different microbial species with the plant rhizosphere is associated to the root exudates that attracts and favors the growth of these species (Huang *et al*., 2014). According to earlier studies, bean roots produce substantial amounts of amino acids and sugars that meet the rhizosphere microbes' requirements for carbon and nitrogen (Mendes *et al*., 2019). These microbes support plant development and growth, nutrition and plant protection against soil-borne pathogens (Philippot *et al*., 2013).

Production of enzymes by antagonistic bacteria

Production of cell wall degrading enzymes was evaluated since it is an important mechanism of pathogen inhibition. Most of these bacterial isolates produced cell wall degrading enzymes. One of the primary strategies used by biocontrol agents to control plant diseases is the production of lytic enzymes (Jadhav *et al*., 2017; Ezrari *et al*., 2021). The results showed that the bacterial antagonists produced pectinase, cellulase, amylase, xylanases and xanthanases. Budi *et al*., (2000) showed that the structural integrity of the walls of *Fusarium oxysporum* can be affected by the chitinolytic and cellulolytic activity of *Paenibacillus* sp. strain B2. According to Bibi *et al*., (2018), it is possible for rhizo- and endophytic bacteria to colonize roots intracellularly because of the hydrolytic enzymes they produce.

Biological control of *Cff* by rhizobacteria in common bean plants

In the present study, the efficacy of the bacterial isolates (*Bacillus cereus*, *Paenibacillus polymyxa* and *Pseudomonas*) to control bacterial wilt disease in bean plants was evaluated. The *Pseudomonas fluorescens* was most effective in controlling bacterial wilt of beans up to 81%. *Bacillus cereus* and *Paenibacillus polymya* reduced the disease development up to 79% and 68% respectively. These results collaborate earlier findings that showed the effectiveness of *Bacillus cereus* and *Pseudomonas fluorescens* in controlling bacterial wilt disease in beans (Corrêa *et al*., 2014). Proteolytic, chitinolytic and lipolytic activity and production of ammonia reduced the bacterial wilt severity (Corrêa *et al*., 2014). This is

the first report indicating that *Paenibacillus polymyxa* have high potential in controlling bacterial wilt of beans. An integrated approach of combining foliar application with seed application was used. This improved the efficacy of the biocontrol agents. In most research on biocontrol of *Cff*, the bacterial control agents have been delivered through seed treatment however, combining seed treatment and foliar application could improve control of bacterial wilt of beans. Similar research on the management of several plant diseases showed that combining application techniques can be more effective than using only one kind of treatment. This approach overcome inconsistence performance of biocontrol agents. Seed treatment in combination with foliar application of *P. fluorescens* was shown to be most efficient in controlling onion leaf blight disease caused by *Alternaria palandui* (Karthikeyan *et al*., 2008) and late leaf spot disease in groundnut caused by *Cercosporidium personatum* (Meena and Marimuthu, 2012). After application of the biocontrol agent, its survival is of critical importance for successful disease suppression. *Bacillus* and *Paenibacillus* species form endospores that are more stable and enables biocontrol agents to survive under unfavorable conditions. Studies by Cochard *et al*. (2022) have demonstrated that *P. fluorescens* can migrate from seeds treated with the bacteria to aerial parts of plants. *Pseudomonas fluorescens* is able to survive and multiply within the rhizosphere and phyllosphere when applied as seed treatment and foliar spray. This explains its effectiveness in controlling bacterial wilt of beans observed in this study.

5.2 Conclusion

- *Curtobacterium* species isolated from common bean leaves and seeds were pathogenic to GLP X92 *P. vulgaris* plants. *C. flaccumfaciens* pv. *flaccumfaciens* isolated from bean seeds had high disease severity of up to 82%. Seedlings emerging from infected seeds exhibited severe symptoms.
- Rhizosphere of *P. vulgaris* harbors bacteria that have antagonistic activity against *C. flaccumfaciens* pv. *flaccumfaciens*. They produced different enzymes that are known to lyse the cell wall of the pathogen.
- This study demonstrated application of *Pseudomonas fluorescens, Bacillus cereus* and *Paenibacillus polymyxa* to seed and foliage effectively controlled bacterial wilt of beans. *Pseudomonas fluorescens* was the most effective in controlling the

Cff pathogen with efficacy of 81%, while *Bacillus cereus* and *Pseudomonas polymyxa* reduced the disease development by 79% and 68%, respectively. Therefore, this approach can be exploited as it is safe, effective and durable alternative to use of chemicals in control of bacterial wilt disease of beans.

5.3 Recommendations

This study achieved its objective by controlling *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* with rhizobacteria isolated from rhizosphere of *Phaseolus vulgaris*. The following recommendations are important for future research.

1. Further research on the distribution of bacterial wilt of common bean disease in bean producing regions in Kenya.
2. Further studies to determine mechanisms employed by these biocontrol agents in management of common bean bacterial wilt and field trials to determine their efficacy.
3. Further investigation to determine if the isolated biocontrol agents are effective in controlling other plant diseases.

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APPENDICES

Appendix 1: Data on disease incidence and disease severity of *Curtobacterium* spp. on GLP X92 *P. vulgaris* plants

	Disease Incidence				Disease Severity			
Isolate Code	**Rep 1**	**Rep 2**	**Rep 3**	**Mean**	**Rep 1**	**Rep 2**	**Rep 3**	**Mean**
BS3	41.6	42.1	42.6	**42.1**	18.54	21.78	19.68	**20**
BS9	65.25	68.72	66.05	**66.67**	36.21	34.5	35.95	**35.56**
BS10	42.92	45.57	44.86	**44.45**	23.8	21.63	21.23	**22.22**
BS13	54.7	57.76	54.2	**55.56**	23.78	26.4	23.14	**24.44**
BS15	32.5	33.78	33.71	**33.33**	16.96	18.76	17.62	**17.78**
BS16	100	100	100	**100**	52.62	53.85	53.52	**53.33**
BS20	33.41	30.82	34.71	**32.98**	8.5	9.23	8.94	**8.89**
BS21	33.16	31.69	34.63	**33.16**	14.08	13.41	12.5	**13.33**
BS22	100	100	100	**100**	55.92	59.85	57.57	**57.78**
BS23	0.0	0.0	0.0	**0.0**	0.0	0.0	0.0	**0.0**
SD7	100	100	100	**100**	81.69	81.37	83.6	**82.22**
Control	0.0	0.0	0.0	**0.0**	0.0	0.0	0.0	**0.0**

Appendix 2: Measurements of zone of inhibition of *Curtobacterium* spp. using different bacterial antagonists

Isolate Code	BS3	BS9	BS10	BS13	BS15	BS16	BS20	BS21	BS22	SD7
BC1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BC2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BC3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BC4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BC5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BC6	15.0	18.0	10.0	16.0	15.0	14.0	14.0	19.0	16.0	15.0
BC7	10.0	15.0	12.0	11.0	16.0	10.0	15.0	13.0	16.0	16.0
BC8	11.0	15.0	11.0	12.0	10.0	11.0	12.0	16.0	15.0	15.0
BC9	14.0	15.0	14.0	15.0	16.0	14.0	13.0	15.0	16.0	14.0
BC10	15.0	19.0	14.0	0.0	8.0	15.0	11.0	15.0	10.0	11.0
BC11	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BC12	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BC13	3.0	0.0	0.0	0.0	2.0	0.0	3.0	3.0	0.0	0.0
BC14	15.0	12.0	11.0	7.0	10.0	10.0	15.0	15.0	8.0	8.0
BC15	11.0	18.0	16.0	14.0	11.0	8.0	14.0	14.0	11.0	12.0
BC16	0.0	0.0	0.0	0.0	0.0	6.0	0.0	0.0	0.0	0.0
BC17	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BC18	0.0	2.0	0.0	5.0	2.0	0.0	9.0	9.0	6.0	7.0
BC19	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BC20	2.0	0.0	0.0	0.0	3.0	0.0	0.0	0.0	0.0	0.0
BC21	0.0	0.0	0.0	0.0	3.0	0.0	9.0	6.0	9.0	11.0
BC22	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BC23	0.0	0.0	0.0	0.0	15.0	16.0	7.0	7.0	0.0	15.0
BC24	0.0	8.0	0.0	0.0	12.0	0.0	0.0	0.0	0.0	12.0
BC25	0.0	0.0	0.0	0.0	7.0	6.0	0.0	0.0	8.0	8.0
BC26	0.0	0.0	0.0	0.0	6.0	0.0	0.0	0.0	0.0	0.0

Appendix 3: Efficacy data of different bacterial antagonists as combined seed treatment and foliar application evaluated by disease incidence and disease severity in GLP X92 *P. Vulgaris*

	Disease Incidence							Disease Severity							
Isolate code	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 4**	**Rep 5**	**Rep 6**	**Mean**	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 4**	**Rep 5**	**Rep 6**	**Mean**	**Efficacy**
BC6	19.3	17.56	20.16	20.6	18.24	18.62	**18.08**	5.51	4.93	5.51	3.78	5.81	3.29	**4.49**	79.19
BC14	29.72	27.63	28.06	27.3	31.5	30.81	**29.17**	10.85	10.12	9.81	10.66	10.47	10.91	**10.33**	68.18
BC24	16.22	16.87	17.38	16.74	17.32	15.49	**16.67**	6.9	6.8	7.2	8.3	6.2	7.9	**7.1**	81.82
Control	92.63	91.29	93.25	91.32	90.39	91.14	**91.67**	87.54	86.17	82.84	83.44	86.54	88.54	**85.19**	-