EFFECTIVENESS OF *Pseudomonas fluorescens* AND NEEM EXTRACT IN MANAGEMENT OF *Fusarium oxysporum* ON FRENCH BEANS IN KIRINYAGA COUNTY, KENYA

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DECLARATION

I Muthoni Teresia Wanjiru declare this thesis is my original work and has not been presented for the award of a degree in any other university or any other award:

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DEDICATION

This thesis is dedicated to my beloved husband Patrick Maina and entire family of Mary Muthoni.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
CFU	Colony Forming Unit
DAI	Days after inoculation
DAT	Days after treatment application
EC	Emulsifiable concentrates
EU	European Union
$FeCl_3$	Iron (III) Chloride
Fop	*Fusarium oxysporum* f. sp. *phaseoli*
HCDA	Horticultural Crops Development Authority
ITIS	Integrated Taxonomic Information System
KHCP	Kenya Horticultural Competitiveness Project
KU	Kenyatta University
LSD	Least Significant Difference
NAFIS	National Farmers' Information Service
PABRA	Pan-Africa Bean Research Alliance
QTL	Qualitative trait linkage
USAID	United States Agency for International Development
VOCs	Volatile organic compounds
WP	Wettable powder

ABSTRACT

Fusarium wilt, *Fusarium oxysporum* f. sp *phaseoli*, is among the major diseases affecting French bean production in Kenya causing up to100% yield loss. Use of synthetic fungicides has caused environmental pollution, pesticide resistance and increased risk to human health. This study assessed use of *Azadirachta indica* and *Pseudomonas fluorescens* in managing Fusarium wilt disease on French beans. Twenty soil samples and diseased bean plant materials were randomly sampled from twenty farmer's field each in Kirinyaga County in November-December 2017. *In vitro* studies were conducted to isolate and identify effective *P. fluorescens* and determine their mode of action against *F. oxysporum*. Efficacy of neem against *F. oxysporum* was assessed using the poison food technique. Data on colony diameter was recorded in millimetres after 7 days of incubation at 28±2℃. Percentage growth inhibition of mycelia was used to select the most effective isolates for further studies. Greenhouse and field trials were carried out for two seasons to assess the efficacy of the most effective isolates on Fusarium wilt disease. The treatments included *Pseudomonas fluorescens*- Pf1, Pf2, Pf3, Pf4, Bio cure- B, neem extract, Pf1+neem and control. The bacterial isolates were multiplied on 100g of sterile rice bran and neem extract was prepared by blending plant material in 70% ethanol and concentrated by evaporation under vacuum. The treatments were applied twice by incorporating in the soil prior to planting and at vegetative growth stage**.** Data on disease incidence and severity, plant height (cm), shoot and root length, biomass, population dynamics and yield of French beans were collected. Data was subjected to analysis of variance using SAS version 9.2. Means were separated using Fischer's Least significant difference test ($P \leq 0.05$). A total of 12 *P. fluorescens* isolates were tested for antagonistic activity against *F. oxysporum* in dual culture technique. Isolate Pf1 was superior with 72.2% mycelial inhibition compared to the control. In addition, Pf1 isolate significantly ($P<0.001$) inhibited *F. oxysporum* mycelial growth through antibiosis, production of siderophores and metabolites. Among the metabolites, production of volatile compounds was the most effective mode of action and attained 80.9% mycelial inhibition. Under greenhouse condition, isolate Pf1 was significantly ($P<0.001$) superior than untreated control and recorded the least mean disease incidence and severity and promoted growth and yield of French bean. Results from the field study showed *P. fluorescens*- Pf1 and Pf2 recorded significantly ($P<0.001$) higher weight of marketable pods than other treatments and scored 4.5±0.56 and 4.9±0.45t/ha during the first and second season, respectively. The control plots recorded 1.5±0.33 and 1.5±0.18t/ha during the first and second season, respectively. In addition, Pf1 isolate had the highest rhizosphere population with $2.0\pm0.84\times10^9$ cfu/g during the second season. *Pseudomonas fluorescens* produced antimicrobial substances that inhibit the pathogen and indirectly promote growth and yields of beans. The study recommends isolates Pf1 and Pf2 to be formulated and commercialized for use as alternatives to fungicides in managing Fusarium wilt disease on French beans.

CHAPTER ONE: INTRODUCTION

1.1 Background information

French bean, *Phaseolus vulgaris* L. is a major horticultural crop grown in Kenya by both small and large scale farmers. Majority of farmers are small holders (USAID-KHCP, 2015) owing 0.5-5.0 acres of land (Ndegwa *et al*., 2010) and the produce is mainly meant for the export market (Mulanya *et al*., 2014). The European countries (EU) form the largest market for both fresh and processed French beans (Okello and Swinton, 2011). Production of this crop creates job opportunities for about 50,000 farmers involved in production and export chain (Marete *et al*., 2020). French beans accounts for 61% of total vegetable exports in Kenya (Fulano *et al*., 2021).

In Kenya, Kirinyaga, Embu, Murang'a and Nyeri Counties are the leading producers of French bean (Goro, 2013). The production is constrained by arthropod pests and diseases (Kaburu *et al*., 2012). Fusarium wilt caused by *F*. *oxysporum* f. sp. *phaseoli* is among the major diseases of French beans in Africa and causes up to 100% yield loss (Muriungi *et al*., 2013). Synthetic fungicides have been used in management of the disease. However, the fungicides had negative effect on microfauna and the environment (Kimani, 2014; Soliman *et al*., 2015). Therefore, there is need for more research on alternative strategies that are safe to use, ecofriendly and effective in management of the disease (Pereg and McMillan, 2015).

Use of plant extracts has also shown great potential in managing Fusarium wilt of beans (Obongoya *et al*., 2010). Neem *Azandiracta indica*

A. Juss has been reported to produce antifungal compounds that suppressed *F. oxysporum* (Ramaiah and Kumar, 2015). This study aimed at identifying potential *P. fluorescens* isolates and neem extract formulations that are effective against Fusarium wilt of French bean under greenhouse and field conditions.

1.2 Statement of the problem

Production of French bean is majorly constrained by pests and diseases (Keikotlhaile and Spanoghe, 2011). Fusarium wilt caused by *Fusarium oxysporum* Schlecht. f. sp. *phaseoli* Kendrick and Snyder is a major disease that infects more than a hundred species of gymnosperm and angiosperms (Liu *et al*., 2017). The pathogen causes premature death of French bean plants resulting to 100% yield loss (Muriungi *et al*., 2013). This has been as a result of lack of effective fungicide in management of the disease. Farmers have therefore adopted fungicides used in management of wilt in other crops of the same genre. These fungicides have led to emergence of resistant strain of *F. oxysporum* which cause severe crop damage and reduce yields (Sillero *et al*., 2010). This study addressed the use of *P. fluorescens* and neem extract as alternatives to synthetic fungicides for management of Fusarium wilt disease on French beans.

1.3 Justification of the study

French bean (*Phaseolus vulgaris* L.) is among the most important vegetable crops grown in Kenya in terms of production and income generated from its export (Mulanya *et al*., 2014). However, high yield losses have been reported due to pests and diseases (Kaburu *et al*., 2012) which lower the total yields and product quality. *Fusarium oxysporum* f. sp. *phaseoli* causes huge loss and this reduces income earned from the export of this crop (HCDA, 2013). To reduce the losses, chemical pesticides have been used in crop protection (Sumitra *et al*., 2012).

Lack of effective fungicide and their adverse effect on the environment and human health justifies a search for alternative methods that are effective and ecofriendly (Stadlinger *et al*., 2013). Biopesticides could be adopted as alternative strategies since they are effective, relatively affordable and safe to use (Quarles, 2013: Ouma *et al*., 2014). Use of beneficial microorganisms and botanical extracts has shown potential against *Fusarium* spp. (Pereg and McMillan, 2015).

Pseudomonas is among the major plant growth promoting bacteria in the French bean rhizosphere (Erdogan and Benlioglu, 2010). The bacteria colonize the root rhizosphere and produce microbial metabolites that act against soil borne phytopathogens (Erdogan and Benlioglu, 2010). The bacterium has beneficial effects on plants by direct stimulation of growth by improving nutrient availability and exerting antagonism towards soil borne pathogens (Deshwal *et al*., 2013). These characteristics make *Pseudomonas*

spp. effective in disease management thus suitable alternative to chemical control (Koche *et al.*, 2013). This study investigated the use of *P. fluorescens* and neem plant extract in control of Fusarium wilt on French bean. However, there is still need for more research and therefore this study aims at contributing to the ongoing research on alternatives methods to synthetic pesticides in management of *Fusarium oxysporum* f. sp *phaseoli*.

1.4 Research objectives

1.4.1 General objective

To determine the effectiveness of *Pseudomonas fluorescens* and Neem extract in the management of *Fusarium oxysporum* on French bean in Kirinyaga County, Kenya.

1.4.2 Specific objectives

i) To recover and identify *Pseudomonas fluorescens* isolates that are effective against *Fusarium oxysporum* f. sp. *phaseoli in vitro.*

ii) To evaluate the effectiveness of neem extract in management of *Fusarium oxysporum* f. sp. *phaseoli* under laboratory conditions.

iii) To determine the mechanisms of action of the *Pseudomonas fluorescens* isolates that are effective against *Fusarium oxysporum* f. sp *phaseoli*.

iv) To evaluate the efficacy of *Pseudomonas fluorescens* isolates and neem extract on growth and yield of French beans under greenhouse and field conditions.

1.5 Hypotheses

i) Agricultural farm habour *Pseudomonas fluorescens* that can significantly suppress *F. oxysporum* f. sp. *phaseoli* growth and infection on French beans.

ii) Neem extract can significantly suppress *F. oxysporum* f. sp. *phaseoli* growth and infection on French beans.

iii) *Pseudomonas fluorescens* exert different mechanisms of action against *F. oxysporum* f. sp. *phaseoli.*

iv) Application of *Pseudomonas fluorescens* and neem extract significantly control Fusarium wilt disease and increases growth and yield of French bean.

1.6 Conceptual framework

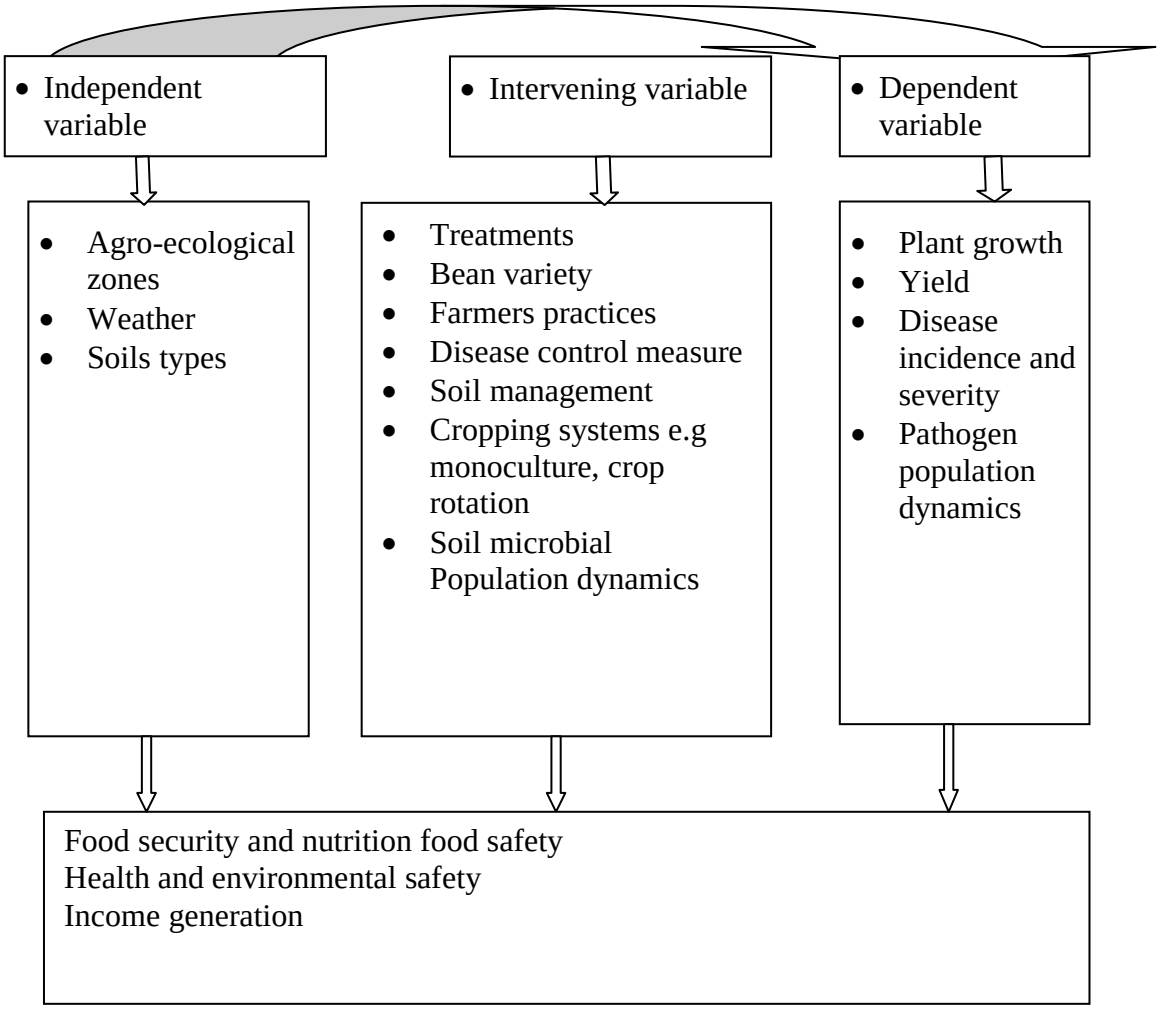


Figure 1.1: Conceptual framework

***** Arrow points towards direction of influence

CHAPTER TWO: LITERATURE REVIEW

2.1 Botanical classification of French bean

French bean (*Phaseolus vulgaris* L.) is also known as snap bean or green bean (ITIS, 2014). The wild *P. vulgaris* is native to the Americas and is believed to have been domesticated in Southern Andes region. *Phaseolus* species is a member of the legume family Fabaceae. The genus *Phaseolus* is comprised of both cultivated and wild species with *P. vulgaris* being the most cultivated species (Porch, 2013). French bean varies in species and is classified based on style of growth. The major *Phaseolus* species cultivated are the year bean (*P. dumosus*) and runner bean (*P. coccineus*) (Lioi and Piergiovanni, 2013: Bellucci *et al*., 2014).

2.2 Production of French bean in Kenya

Phaseolus vulgaris L. is among the major exotic vegetables cultivated in Kenya for export market. The crop is grown by both small and large scale farmers (Odero *et al*., 2013; USAID-KHCP, 2015). The area under French beans production has increased from 5,671 Ha in 2015 to 5,983 Ha in 2016. The leading counties are Kirinyaga, Machakos and Murang'a that account for 32, 20.7 and 13.5% respectively (Konda, 2019). The crop is grown for fresh consumption and canning (NAFIS, 2013).

The common varieties grown in Kenya include Amy, Gloria, Teresa, Morgan, Serengeti, Vanilla, Julia, Paulista, Claudia, Monal and Samantha (Fulano, 2016). Picking of the immature pods commence at 42 days after

planting, but the dates vary with variety, agro-ecological zone and management practices. The beans are then graded and packed according to the requirements of the export market. Fine and extra fine pods are sold in export market while rounded are canned.

2.3 Ecological requirements for French bean production

French beans do well in areas with optimum altitude of between 1,500-2,100 metres above sea level. The crop requires well-distributed rainfall throughout the growing season of between 900-1,200 mm with temperature that range between 20-25°C (Kamanu *et al*., 2012). The crop does well in a wide range of soils ranging from sandy, loam to heavy clay with pH of 6.5-7.5. Moisture content is essential in French bean production as it affects uniformity, yields and quality of beans. Lack of sufficient moisture causes flower abortion and curved pods which lower the total yields (Bonane, 2019).

2.4 Nutritional value of French beans

French bean is mainly grown for the export market but local consumption has increased over the years due to its nutritive value (Marete *et al*., 2020). French beans are rich in energy and amino acids content including vitamins A, B, D, ascorbic acid, calcium, phosphorus and starch (Menge *et al*., 2014). In addition, French bean is rich in zinc and iron, and hence can help to address iron deficiency problems especially in developing countries (PABRA,

2014). French beans reduce the risk of heart diseases due to their high levels of flavonoids and lack of cholesterol (Menge *et al*., 2014). High content of fiber, vitamin K and calcium promotes bowel movements, keeps bones intact and prevents bone deterioration and osteoporosis. French beans are a great source of folic acid which is vital for normal and healthy development of the foetus especially in preventing neural tube defects (PABRA, 2014).

2.5 Economic importance of French beans

French bean production has created job opportunities for the rural communities involved in the production and export chain of this crop (Wahome *et al*., 2013). Smallholder farmers in the rural areas form the biggest proportion of producers (USAID-KHCP, 2015) which has improved incomes and alleviated poverty. The sector has created job opportunities for actors in the production and marketing chain including growers, middlemen, exporters, logistic companies and the government that acts as the regulator of the industry through the Horticultural Crop Development Authority (HCDA, 2010).

The crop is among the major exported horticultural crops to the export market earning the country foreign exchange (Okello and Swinton, 2011; HCDA, 2014; USAID-KHCP, 2015). In 2016, the quantity of French beans exported was 62,000 tonnes from an area of 7,500 hectares earning the country Kshs. 5.8 Billion (HCDA, 2016). Local consumption of French beans has also

been increasing due to increased awareness on its nutritive and health value (Marete *et al*., 2020).

2.6 Constrains to French bean production in Kenya

Arthropod pests and fungal diseases have been among the major factors affecting French bean production (Keikotlhaile and Spanoghe, 2011). Insect pests cause damage to French bean crop through feeding and transmission of viruses leading to low productivity of beans. The major insect pests that attack French bean include; bean flies *Ophiomyia* spp. (Ojwang *et al*., 2009), thrips *Megalothrips* spp. (Nyasani *et al*., 2012), aphids *Aphis fabae* (Camella *et al*., 1978) and red spider mites (*Tetranychus telarius*) (Infonet-Biovision, 2015; Umass Amherst, 2015). These insect pests cause yellowing of leaves, wilting and stunted growth, when present in large numbers (Muvea, 2011).

The major disease affecting French beans includes; Fusarium wilt (*Fusarium oxysporum* f. sp. *phaseoli*), angular leaf spot (*Phaeoisariopsis griseola*), bean rust (*Uromyces appendiculatus*), common bacterial blight (*Xanthomonas campestris* pv. *phaseoli*) and Fusarium root rot (*Fusarium solani* f. sp. *phaseoli*) (Muthomi *et al.*, 2014; Infonet-Biovision, 2015). These diseases lower the pod quality and total yield of French beans (Kahuthia-Gathu, 2000, Wahome *et al*., 2013; Muthomi *et al*., 2014). *Fusarium oxysporum* cause major plant disease including leaf spots, vascular wilts, root

rots and rusts. Among the all these diseases, vascular wilts is the most important disease caused by *Fusarium oxysporum* (Beeba *et al*., 2012).

Root rots are economically important in French bean production e.g *Rhizoctonia* root rot caused by *Rhizoctonia solani, Fusarium* root rot (*F. solani* f. sp. *phaseoli*) and *Pythium* root rot (*Pythium* spp.) and they attack beans at different stages depending on the pathogen. *Rhizoctonia solani* attack beans at almost all stages (El-Mohamedy *et al*., 2015) while Fusarium attacks beans later after crop establishment. *Pythium* root rot affected French beans at the seedling stage leading to pre-emergence and damping off (Muthomi *et al*., 2014). These soilborne pathogens form disease complex which is a great threat to bean production. The fungal diseases cause stunted plant growth and lower the total yields (Beebe *et al*., 2012). The area under French beans production, volume and value has also been declining due to climate change. The prolonged drought experienced in Kenya between 2008 and 2010 led to a decrease of 45% in yield volume (Otieno *et al*., 2017).

Price fluctuation of French beans in the export market has also been identified as a marketing constraint. The beans are categorized into different grades for local and export market. Poor quality produce fetches low price in the market thus causing the farmers to incur huge losses. Low quality of the produce has been attributed to lack of knowledge and information on production. The bean value chain analysis Kenya (2012) reported that majority of farmers that are involved in the production add no value such as canning and freeing to their produce before marketing thus fetching low prices.

2.7 Fusarium wilt disease

2.7.1 Taxonomy of the pathogen

Fusarium oxysporum f. sp *phaseoli* is a soil borne plant pathogen in the phylum Ascomycota, class Sordariomycetes, order Hypocreales and family Nectriaceae (Kendrick and Syder, 1942). The pathogen is widely distributed in diverse environments, including soils and aquatic habitats (Palmero *et al*., 2009; Brandt and Park, 2013). The genre comprises of both pathogenic and non-pathogenic strains (Bao *et al*., 2002). *Fusarium oxysporum* f. sp *phaseoli* is the causal agent of Fusarium wilt disease of French beans.

Fusarium oxysporum attacks more than a hundred species of plants (Liu *et al*., 2017). The pathogen causes damage to commercially important crops including French beans *Phaseolus vulgaris* (Pastor-Corrales *et al.*, 1987), tomato *Solanum lycopersicum* (Fuchus *et al*., 1997) and watermelon *Citrullus lanatus* (Martyn and Gordon, 1996). The pathogen persists in the soil for long and cause huge losses especially under monoculture production system (Smith, 2007). The pathogen can cause 100% yield loss in French beans production (Muriungi *et al*., 2013).

2.7.2 Aetiology, symptomatology and epidemiology of *Fusarium oxysporum* f. sp *phaseoli*

The pathogen, *F. oxysporum* survives as an active saprophyte in soil, organic matter and infected plant debris in form of microconidia, macroconidia and chlamydospores. The fungus is favored by hot weather, soil

pH of 5–5.6 and infertile soils (Naseri, 2014). *Fusarium oxysporum* invades the roots of susceptible French beans using the mycelium. The pathogen moves from the root tip and advances through the cortex to the vascular tissues (Akrami and Yousefi, 2015). *Fusarium oxysporum* f. sp *phaseoli* secretes protein effectors that suppress plant defence and promotes invasion (Lo Presti *et al*., 2015). The fungus utilizes host metabolic pathways for its growth and development (Zeilinger *et al*., 2016). The microconidia produced, multiply and are carried upward in the xylem tissue clogging the vascular vessels. This prevents uptake of water and translocation of nutrients. The stomata close and the plant wilts and eventually dies. When the plant dies, the fungus attacks the neighboring tissues and later sporulates infecting other plants (Kutama *et al*., 2016).

Wilted plants show yellowing on leaves and wilting which progresses upwards into the younger leaves weakening the plants. The newly formed leaves turn yellow and wilt while older leaves droop (Dubey, 2014). The vascular system shows discoloration when a longitudinal cut is made on the lower stem and red-brown streaking is evidence of Fusarium species infection (Di *et al*., 2016). Severe infection causes premature death of plants which lead to 100% yield loss. *Fusarium oxysporum* is spread through soil, water, contaminated farm equipment and infected debris which act as alternate hosts (Dita *et al*., 2018). The fungus infects other field by wind, infected plant tissues and contaminated farm tools (FAO, 2015b).

2.8 Management of Fusarium wilt disease of beans

Management of wilt is important in maintaining the quality and quantity of French beans. This has been achieved through use of synthetic pesticides, integrated management which involves biological control measures and cultural practices like elimination of alternate hosts, managing irrigation and use of resistant varieties (Nyasetia, 2011). However, these strategies have not been fully effective due to emergence of new pathogenic strains.

2.8.1 Use of synthetic pesticides

Chemical pesticides have been used extensively in crop protection. Farmers use synthetic pesticides in management of fungal diseases since they are readily available in the market (Engindeniz and Ozrurk, 2013). Common synthetic pesticides used in control of wilt include; Megaprode Lock WP (Iprodione 17.5% and Carbendazin 35%), Osiris 65 EC (Metconazole 27.5% and Epoxiconazole 37.5%) and Sherrif WP (Carbendazin 12.25% W/w and Mancozeb 74%). Synthetic fungicides are effective when used in the very early stages of crop development before pathogen invasion (Amin *et al.*, 2014). However, some of these pesticides kill the non-target micro-organisms; pollute the soils thereby causing a decline in populations of micro-organism in the soil (Srijita, 2015).

2.8.2 Cultural practices

Cultural practices have been adopted in control of Fusarium wilt disease since they are safe and economically feasible. The practices include; use of resistant varieties, destruction of diseased plants and removal of volunteer plants that act as alternate hosts and use of certified seeds (Akila *et al*., 2011). These practices are integrated with fungicides to achieve disease threshold level. Crop rotation with non-host plants helps in cutting off the life cycle of the fungi. Use of compost was reported to be effective in management of *F. oxysporum* f. sp. *lycopersc*i (Reuveni *et al*., 2002; Kouki and Saidi, 2012).

Resistant varieties to Fusarium wilts have been developed and found effective in wilt control for instance cultivars for peas and common beans. Kannan *(*2019) reported that AD9.950 Maker linked with *Fusarium* RR resistance in dry bean improved resistance measured by increased plant vigor and biomass. Fall *et al.* (2001) identified major Qualitative Trait Linkage (QTL) in the small black bean breeding line and controlled 63.5% of variance of *F. oxysporum* of beans. Genetic markers for resistance to Fusarium wilt have also been studied in chickpea (*Cicer arietinum*) Sharma *et al.* (2005). Use of resistant varieties integrated with crop rotation has been reported as one way of managing *Fusarium oxysporum* of beans (Bonanomi *et al*., 2007).

2.8.3 Use of antagonists in control of Fusarium wilt disease

Fungal and bacterial antagonists have been used in crop protection (Kaur *et al*., 2010). Biocontrol agents have proved effective in disease control and are ecofriendly (Song *et al*., 2014). These antagonists have been found effective in management of fungal and bacterial diseases (Singh *et al*., 2002; Ahmed, 2011). This has shown the need to research on potential antagonist that can be commercialized for use in wilt management (Fravel *et al*., 2003).

2.8.3.1 Management of phytopathogens using fungal antagonists

Use of biological agents has proven successful and efficient against fungal pathogens (El-Mohamedy *et al*., 2014). Some of the fungal antagonist used in disease management include; *Azospirillum* spp., *Trichoderma* spp., *Rhizobia* spp. and *Mycorrhizae* spp. (Patale and Mukadam, 2011). El-Mohamedy and Abd-All (2013) reported that bio-priming seeds in biocontrol agents have been found effective against soil borne pathogens. Non-pathogenic *Fusarium* spp. significantly lowered the disease severity of tomato plants in soil infested with *F. oxysporum* under field conditions (Nawar, 2015). Kaur *et al*. (2011) reported that non-pathogenic *Fusarium* strains controlled Fusarium wilt disease of water melon and tomato under greenhouse condition. *Trichoderma viride* produced antibiotics that inhibited development of Fusarium wilt disease in tomato (Thangavelu and Mustaffa, 2010).

2.8.3.2 Management of phytopathogens using bacterial antagonists

Pseudomonas is among the rhizosphere colonizing bacteria used in management of most important diseases in plants. The bacteria colonize the plant rhizosphere and compete for nutrients thus suppress other pathogens (Erdogan and Benlioglu, 2010). Some of the *Pseudomonas* spp. used as biocontrol agents include *P. fluorescens* (Asha *et al*., 2011), *P. chlororaphis* and *P. putida* (De Freitas and Germida, 1991). These antagonists produce metabolites that suppress pathogens and promote growth through production of phytohormones (Erdogan and Benlioglu, 2010). In addition, *Pseudomonas* spp. produces enzymes that accelerate competition for nutrients in the soil rhizosphere making it unavailable for the pathogens (Heydari and Pessarakli, 2010).

Some *Pseudomonas fluorescens* - Pf1 isolates induce production of pathogenesis related proteins and deposits structural barriers in the plants for defense against pathogen attack. *Pseudomonas fluorescens* have also been reported to promote plant growth by stimulating uptake of phosphorous (Mustafa *et al*., 2019). *Pseudomonas fluorescens* suppressed Fusarium wilt in bananas (Karimi *et al*., 2012). *Bacillus subtilis* produce antimicrobial substances that suppress fungal pathogens by degrading their cell wall (Subramanian and Smith, 2015). Anitha and Rabeeth (2009) reported *Streptomyces griseus* suppressed *Fusarium oxysporum* f. sp. *lycopersci.*

2.9 Application of plant extracts in disease management

Natural products extracted from plants are a potential resource area of study in fungal disease management. Neem contains various elements which include; ascorbic acid, amino acids, and flavoniods which comprises of antibacterial and antifungal properties that make it effective in disease management (El-Ghany *et al*., 2015). Satish *et al*. (2009) reported that plant extracts inhibit germination and sporulation of fungal spores. Kagale *et al*. (2004) found that leaf extract of devil's trumpet *Datura metel* L. reduced disease incidence and severity of leaf spot and rust pathogens. Neem extract have been studied to contain hydrocarbons that make it effective in control of fungal and bacterial diseases in plants (Mishra, 2014). The hydrocarbon changes the plant pathogenic pathways and induces resistance against fungal diseases (Aboellil, 2007).

Globally, neem extract has been reported to have antifungal compounds that make it effective in management of fungal diseases (Dubey *et al*., 2011) with no harmful effects to the environment, human and animal health. Obongoya *et al*. (2010) reported that extracts from neem, Mexican marigold *Tagetes erecta* and tobacco *Nicotiana tabacum* showed potential in management of *Fusarium oxysporum* f. sp. *phaseoli*. Nahak and Sahu (2015) reported that neem extract reduced disease severity and promoted plant growth of tomatoes under greenhouse condition. Hassanien *et al.* (2010) and Al-Hazmi (2013) reported that neem inhibited growth *Fusarium oxysporum, Rhizoctonia solani* and *Alternaria solani in vitro*. Leaf extract of

neem inhibits germination and growth of *F. oxysporum* through production of antifungal compounds (Nahak and Sahu, 2015).

2.10 Mechanism of action of *Pseudomonas fluorescens* as an antagonist

The genus *Pseudomonas fluorescens* comprises of non-pathogenic bacteria and is the most efficient group of rhizobacteria in disease management (Mulet *et al*., 2010; Hesse *et al*., 2018). The bacterium is commonly used as a biocontrol agent against plant diseases (Haas and Défago, 2005; Pliego *et al*., 2011). The bacteria uses different mechanisms against fungal pathogens that include production of antibiotics (Koche *et al*., 2013), siderophores (Meera and Balabaskar, 2012), phytohormones (Bholay *et al*., 2012), hydrolytic enzymes and production of organic compounds (VOCs) (Rekha *et al*., 2010). *Pseudomonas fluorescens* protects the plants from pathogen attack by preventing pathogens from access of nutrients in the root rhizosphere (Faheem *et al*., 2015).

2.10.1 Production of organic compounds

Pseudomonas spp. produces organic compounds known as volatile organic compounds (Schulz and Dickschat, 2007) which are vital for bacterial communication processes (Kai *et al*., 2016). Lee *et al.* (2012) and Tahir *et al.* (2017) reported that volatile organic compounds (VOCs) promote plant growth and antimicrobial activity that induce resistance against diseases in plants. *Bacillus* spp., *Atrhrobacter* spp., *Serratia* spp. and *Pseudomonas* spp.

are examples of bacteria antagonist that produce volatile compounds. These bacteria have been reported to effective manage plant disease and promote growth in crops (Bailly and Weisskopf, 2012; Raza *et al*., 2016). *Pseudomonas fluorescens* UM270 produced VOCs that showed antifungal and plant growth promoting activity (Hernández-León *et al.*, 2015). The bacterium has been reported to produce antimicrobial agents, ammonia and hydrogen cyanide that induce systemic resistance in plants (Alemu and Alemu, 2015). *Pseudomonas fluorescens* SS101 was reported to have promoted plant growth through release of volatile organic compounds (Park *et al*., 2015).

2.10.2 Siderophores production

Bacteria secrete iron-chelating molecules called siderophores (Hider and Kong, 2010). Siderophores sequester iron available in natural environment that is required by microbes for survival and growth and make it unavailable to pathogens thus limiting their growth (Beneduzi *et al*., 2012). *Pseudomonas* spp. synthesis siderophores which supply the bacteria with iron when its availability is low in the environment thus promote plant-growth (Chen *et al*., 2017). Siderophores production is a competitive strategy used by *P. fluorescens* and has a significant agronomic importance (Beneduzi *et al*., 2013). It has been reported that some strains of *Pseudomonas putida* produce siderophores that increased yields and biosynthesis of the major essential oil components when they are inoculated to *Mentha piperita* (peppermint)

(Santoro *et al*., 2015). Siderophores have also shown ability to protect plants by triggering induced systemic resistance (ISR) (Trapet *et al*., 2016). Some crops that have benefited from siderophore-producing bacteria and include; potato *Solanum tuberosum* (Aloo *et al*., 2020) cucumber *Cucumis sativum* (Qi and Zhao, 2013) and pigeon pea *Cajanus cajan* (Dimkpa, 2016). Siderophores affects interactions and population dynamics between different microorganisms in the environment (Niehus *et al*., 2017).

2.10.3 Production of antibiotic substances

Antibiotics are organic compounds produced by antagonists that slow down growth and interfere with metabolic activities of phytopathogens. *Pseudomonas fluorescens* produce a wide range of antibiotic metabolites that suppress fungal pathogens *in vitro* (Ghirardi *et al*., 2012; Agaras *et al*., 2015). Many biocontrol agents produce multiple antibiotics such as phenazines, pyrrolnitrin, lipopeptides and hydrogen cyanide that suppress the pathogens (Park *et al*., 2011; Saraf *et al*., 2014; Meyer *et al*., 2016). *Pseudomonas* produces 2, 4-diacetylphlroglucinol (DAPG) that has been reported to be effective against *Pythium* spp. (De souza *et al*., 2003). These antibiotics form a protective layer on the root surface that inhibits growth of the pathogen before infection (Ramadan *et al.,* 2016). The diversity of these compounds has been an indirect way in which the compounds induce resistance and promote growth in crops (Glick, 2014).

Pseudomonas spp. show competitive interactions with phytopathogens through production of lipopeptides (Girard *et al*., 2020). In addition, *Pseudomonas* was reported to have produced phenazine which inhibited mycelia growth of *Fusarium oxysporum in vitro* (Chin-Awoeng *et al*., 2003). Some of these compounds e.g. phenazine mobilizes iron in soils and this has been experimentally proven with *Pseudomonas chlororaphis* PCL1391 (Haas and Defago, 2005). These antagonists produce antibiotics that colonize the rhizosphere and suppress soilborne root diseases thereby enhancing biological control (Ramadan *et al*., 2016).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study sites

Laboratory and greenhouse experiments were conducted at Kenyatta University (KU) located 15 km North of Nairobi at $1^0$11′S and $36^0$55′E at 1,600 metres above sea level. The area receives bimodal rainfall that range between 1,000-1,400 mm annually with average temperature of 18-24^0 C. The short rains are experienced between October-December and long rains between March-May. Laboratory bioassays involved isolation, screening and identification of potential *Pseudomonas fluorescens* isolates effective against *F. oxysporum* f. sp *phaseoli*. The greenhouse experiment was conducted at Kenyatta university agricultural greenhouses for two seasons between September – November 2018 and January- March 2019 respectively.

Field study on effectiveness of selected isolates was carried out at Kimbimbi in Mwea sub-county; Kirinyaga County for two cropping seasons between July-August 2018 (first season) and November 2018-January 2019 (second season. The experiment was carried out in farmers' field with known history of Fusarium wilt affecting French beans. Mwea is located at the lower midland zone 4(LM4) at an altitude of 1216 metres asl and coordinates 0°36'8′ S, 37º21′58′ E) (Fig 3.1). The area receives bimodal rainfall that ranges between 900-1200 mm with temperatures of 20-25°C annually which favour French beans production. The soils are silty-loam to heavy clay with pH range of 6.5-7.5 (Kamanu *et al*., 2012).

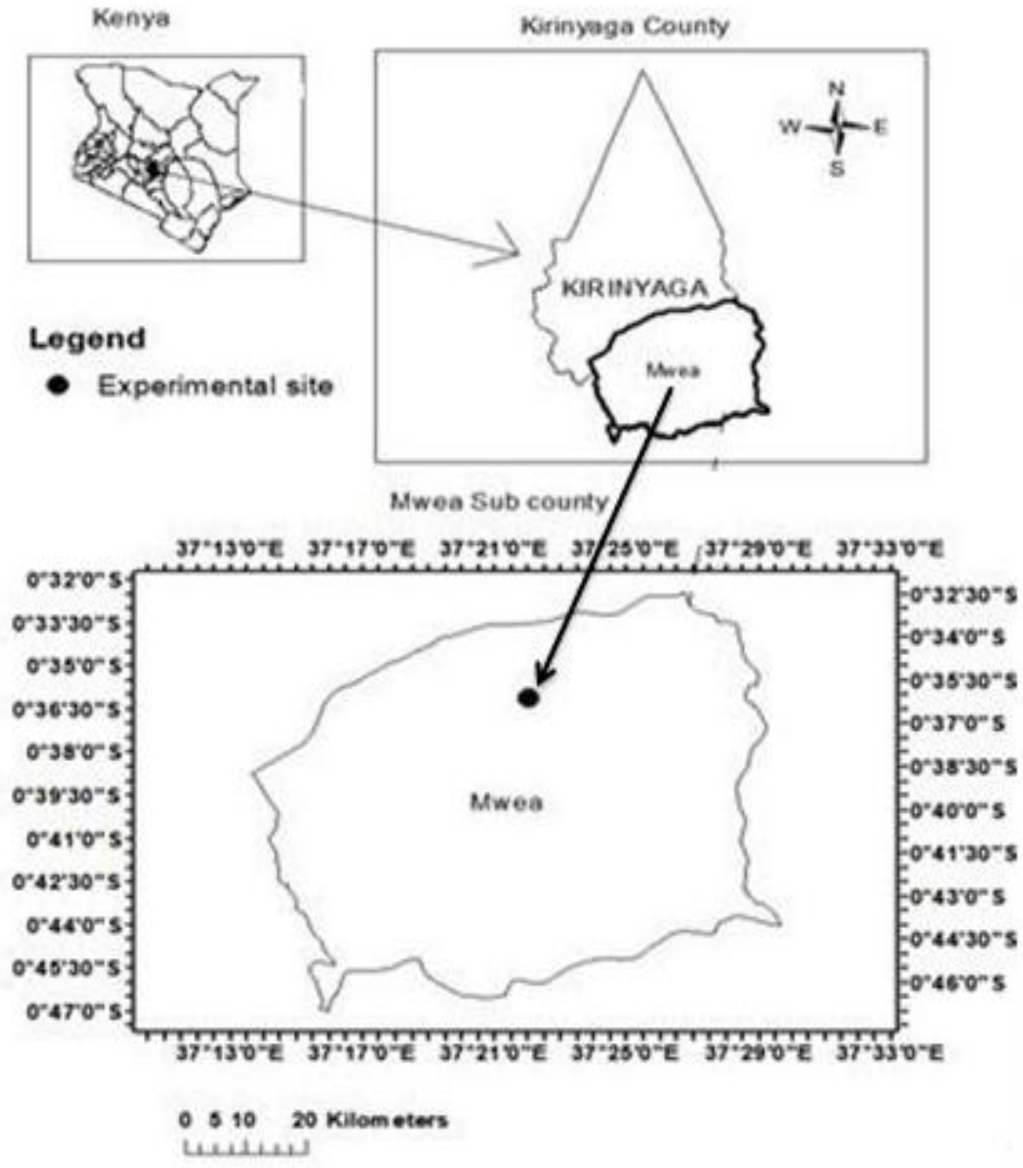


Figure 3.1: Map of Mwea indicating the location of Kimbimbi (Experimental site) in Kirinyaga County.

3.2 Source of the micro-organisms

Pseudomonas fluorescens was isolated from soil samples collected from French bean rhizosphere in agricultural fields in Kirinyaga County, between November and December 2017. The soil samples were sampled from twenty farmers field under French beans cultivation using a soil auger at a

depth of 10 cm after removing 2 cm top soil. The bacterium resides near the root zones where there is adequate moisture and food source from root exudates. The twenty soil samples collected from each field were mixed and a composite sample 50 grams was packed in sampling bag and labeled. These constituted to twenty soil samples which were further used for analysis. Each soil sample was air-dried on surface sterilized bench for one week. The samples were packed in khaki bags, labeled and stored at 4^{o}C in the refrigerator for subsequent use.

French bean plants showing Fusarium wilt symptoms were randomly collected in the twenty farmers' fields. The plants were uprooted and checked for reddish-brown streaking on the roots. A longitudinal cut on the stem was made using sterile scapel blade to check for dark-brownish vascular tissue. The soil samples and plant tissues were packed in khaki bags, sealed and carried in a cool box (105×66 cm) set at 4^{o}C to laboratory for analysis.

3.3 Preparation of culture medium

Isolation of *F. oxysporum* and *P. fluorescens* was done on potato dextrose agar (PDA) and King's B medium, respectively. Thirty nine grams of potato dextrose agar was suspended in 1000 ml of distilled water. *Pseudomonas fluorescens* was isolated on King's B medium by dissolving 42.23g and 15 ml of glycerol in 1000 ml of distilled water. The two media were stirred vigorously and autoclaved for 20 minutes at 121^{o}C and 15 psi (Killani *et al*., 2011). The media was cooled in a water bath (DK-8A) set at

45±2℃. Antibiotics (Tetracycline and Streptomycin sulfate 100mg/L each) were added to the PDA media using a micropipette to inhibit bacteria growth. Twenty milliliters of the media was dispensed into sterile plastic petri dishes (9 cm) in a laminar flow hood (BSS-H1100).

3.4 Isolation of microorganisms

Pseudomonas fluorescens strains were isolated following serial dilution technique (King *et al*., 1954). A composite soil sample of one gram was weighed on electronic weighing balance (BMLC-1-A2), added to nine milliliter of sterile water and agitated for five minutes to obtain standard soil suspension. The solution was serially diluted to 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}. From each dilution, a sterilized pipette was used to draw one milliliter of the suspension and aseptically spread on King's B medium. Three dishes were maintained per dilution, replicated three times, sealed and incubated at 25 ± 2^0C for 48 hrs. The dishes were inverted to prevent water droplets splashing the colonies. The dishes were checked daily for growth of bacterial colonies. After 48 hrs, the plates were checked for fluorescens under UV-trans illuminator (365 nm) for a few seconds. Colonies showing yellow-green pigments were sub-cultured on King's B medium petri dishes. The dishes were incubated for 48 hrs to obtain pure culture. Each bacterial isolate was stored at 4^0C in slants (200µl of sterile glycerol and 800 µl of the bacterial suspension). Pure colonies were used for characterization of *P. fluorescens* based on

Catalase test, siderophores production, production of metabolites (Reetha *et al*., 2014).

Diseased plant tissues were washed under running water for three minutes. The plant tissues were cut using a sterile blade from stems and roots of infected French beans. The pieces were soaked in 70% alcohol for 90 seconds. The tissues were later rinsed thrice with distilled water, dried on filter papers. Three pieces were transferred to each petri dish with PDA medium and incubated at 25±2°C for seven days. The fungal cultures were further sub-cultured to new PDA media (Siameto *et al*., 2010). One spore from each culture was transferred into new potato dextrose agar medium to obtain pure cultures (Choi *et al*., 1999).

3.5 Study of the morphological and biochemical properties of *Pseudomonas fluorescens*

The bacterial cultures were characterized based on morphological features such as colony color, shape, type and size and recorded following Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). Biochemical tests such as Gram staining, Catalase test, siderophores production, production of volatile metabolites and antibiosis were performed to confirm the identity of the bacterium (Reetha *et al*., 2014).

3.6 Identification of the causal agent of Fusarium wilt disease on French beans

All the fungal cultures from the diseased French beans were sub-cultured on sterile PDA media. The cultures were identified based on colony colour and shape of conidia and conidiophores. They were also examined under microscope where slide preparations were stained with lactophenol-cotton blue. *Fusarium oxysporum* cultures were identified by checking their morphological characteristics and confirmed using different identification keys (Killani *et al*., 2011). Pathogenecity test confirmed the identity of isolates to be *Fusarium oxysporum.* The isolated *F. oxysporum* were inoculated in susceptible French beans seedling var. Vanilla. Symptoms of chlorosis, necrosis and yellowing were observed. To complete Koch's postulates the plant tissues were surface sterilized and plated in sterile PDA medium to re-isolate the pathogen. The colonies formed were confirmed to be the same as the initially inoculated.

3.7 Evaluation of *Pseudomonas fluorescens* as a potential antagonist against *Fusarium oxysporum* under laboratory conditions

In vitro bioassays were done to assess antagonistic potential of *P. fluorescens* against *F. oxysporum* using dual culture (Kaur *et al*., 2007) and paper disc techniques (Bahraminejad *et al*., 2008). Culture discs of 5mm of *F. oxysporum* were inoculated separately at the center of petri dishes containing King's B agar medium in triplicates. The bacterial isolates were streaked on the opposite sides of petri dishes (90mm) equidistant from the pathogen at the

center. Control plates were inoculated with the pathogen and sterile water streaked in place of the antagonist. The dishes were arranged in a completely randomized design replicated three times. The growth of *F. oxysporum* was measured in all the petri dishes after seven days of incubation. Colony diameter of *F. oxysporum* was recorded in millimetres using a ruler. Colony diameter was expressed as the mean of two perpendicular diameters. This data was used to calculate the percentage inhibition which rated the level of antagonism as described by Whipps (1997).

The twelve isolates were further tested for their effectiveness against *F. oxysporum* using paper disc method. A loopful of the twelve isolates was inoculated separately in King's B broth agar and incubated at 27± 2^0C for 48 hrs. Fifteen sterile paper discs (5 mm) for each treatment were soaked in 5ml of the bacterial culture for thirty seconds. The fungal suspension initially prepared was spread over the King's B medium. The treated discs were air dried in a sterile laminar flow hood and plated on the dishes fifteen minutes after inoculation of *F. oxysporum*. The petri dishes were incubated at 25± 2^0C for 7 days. Data on the zone of inhibition was measured from the edge of the mycelium to the paper disc using a pair of caliper and ruler and recorded in millimeters (Shanmugam *et al.,* 2011). Percentage inhibition was worked out as described by Whipps (1997):

$$PI = \frac{M - N}{M} \times 100$$

Where, PI = Percentage Inhibition; M; Radial growth of pathogen in the control plates and N; Radial growth of the pathogen in the treated dishes

(Killani *et al*., 2011). Potential antagonistic bacterial isolates were selected for further evaluation *in vitro* and *in vivo* following a score of 1-4 as described by Bogumił *et al*. (2013) (Table 3.1).

Table 3.1: Scale showing different degree of antagonism

Level of antagonism	**Degree**	**Inhibition (%)**
Low	1	I < 51
Moderate	2	I = 50-59
High	3	I = 60-75
Very high	4	I > 75

3.8 Effects of neem extract on growth of *Fusarium oxysporum in vitro*

Fresh neem leaves were collected from Kenyatta University and identified following the identification keys. The leaves were air dried under shade and later crushed using an electric blender to powder. The powder was weighed to 200 g and soaked in 90% ethanol and acetone at the ratio of 1:1 for 24 hours (Wale and Adewunmi, 2020). The extract was evaporated on the rotary evaporator at 60℃ to obtain the supernatant. Twenty millilitres of crude extract was added separately to 8, 10, 12 and 14ml of sterile distilled water to constitute 60, 50, 40 and 30% concentrations respectively. *In vitro* evaluation of neem extract on radial growth of *F. oxysporum* was done following the poison food technique (Manmohan and Govindaiah, 2012). The different concentrations (30, 40, 50 and 60 ml) were mixed thoroughly with 70, 60, 50 and 40 ml of PDA medium, respectively (Aliero, 2003; Ali *et al.*, 2010).

The poisoned PDA was dispensed into each sterile petri dish receiving twenty milliliters of the medium. Three replications were prepared for each concentration. After solidification of poisoned media, 5mm diameter of vigorously growing pure culture of *F. oxysporum* was inoculated into the dishes. The control petri dishes for the three replications were maintained using only sterile water without any plant extract but with mycelial discs. All the petri dishes were incubated at 27 ± 2℃ for seven days. The mycelial diameter of *F. oxysporum* was measured using a ruler in treated and control petri dishes. The formula below was used to calculate the percentage inhibition (PI) as described by Soliman *et al.* (2015).

$$PI = \frac{B - C}{B} \times 100$$

Where: B= Mycelial growth inhibition in control; C= Mycelial growth in treatment.

3.9 Mechanism of action of antagonistic bacteria against *Fusarium oxysporum in vitro*

3.9.1 Production of volatile metabolites

One hundred microlitres of *P. fluorescens* (10^8 CFUml^{-1}) was placed on petri dishes containing King's B amended with 4.4 gl^{-1}glycine and maintained at 27± 2℃ for 24 hours. Subsequently, 5mm disc of 48 hours old *F. oxysporum* was placed on sterile King's B medium. The two half petri dishes were sealed face to face but without any physical contact between *F. oxysporum* and *P. fluorescens* suspension and incubated for three days. Data

on radial growth of *F. oxysporum* in control and treated dishes was measured using a ruler and recorded in millimetres. The percentage inhibition was calculated as described by Solliam *et al* (2015).

3.9.2 Production of non-volatile compounds by *Pseudomonas fluorescens*

The antagonistic efficiency of the bacterium strain was evaluated using the method described by Kraus and Loper (1990). Bacterial suspension (10^8 $CFUml^{-1}$) of *P. fluorescens* was spread on King's B medium treated with 1ml of Iron III chloride ($FeCl_3$) and incubated at 27± 1℃ for three days. The colonies formed were removed using chloroform. One hour later, a 5mm sterile cork borer was used to cut a mycelial plug from 48hrs old *F. oxysporum* culture. The plug was inoculated at the center of the dishes and radial growth of the pathogen was measured in treated and control.

3.9.3 Siderophores production

One hundred microlitres of *P. fluorescens* (10^8 $CFUml^{-1}$) was transferred to King's B medium in petri dishes with and without Iron III chloride ($FeCl_3$). Five mycelial discs of *F. oxysporum* were inoculated and incubated for 7 days at 27±2℃. In the control petri dishes, *P. fluorescens* was substituted with distilled water. Colony diameter was measured using a ruler and recorded in millimeters in treated petri dishes compared to control. The petri dishes, with and without Iron III chloride ($FeCl_3$) were arranged in a complete randomized design replicated thrice.

3.10 Plant growth promotion activity of *Pseudomonas fluorescens* strains

French bean var. Vanilla seeds were sterilized using 1.5 % sodium hypochlorite for 30 seconds. Two milliliters of *P. fluorescens* suspension with 2×10^8 cfu/ml was transferred to sterile dishes. Carboxyl methyl cellulose (1%) dissolved in one hundred millilitres of water was added and kept for 12 hours. The suspension was later drained off and the seeds were dried overnight. Data on germination percentage shoot and root length were recorded in centimeters. The plant vigour was calculated following the formulae below as described by Ramamoorthy (2002).

$$\text{PVI} = (\text{MSL} + \text{MRL}) \times \text{Germination percentage}$$

PVI= Plant vigor index; MSL= Mean shoot length; MRL= Mean root length

3.11 Mass production of the antagonist for evaluation under greenhouse and field conditions

The selected *P. fluorescens* isolates were thawed before mass multiplication. Rice bran was used as a substrate since it had shown good performance (Liu *et al.*, 2017) and was locally available. The substrate was sourced from Mwea rice processing units. It was cleaned to remove unwanted debris such as stones and foreign plant matter. One hundred grams of rice bran was soaked in water in 1000 ml beakers for eight hours. The excess water was drained off and the bran autoclaved at 121^0C (15 lb) for 15 mins. The autoclaved rice bran was transferred to sterile polythene bags after cooling prior to inoculation. *Pseudomonas fluorescens* suspension (1×10^8 CFU/ml)

was prepared through serial dilution technique. Two hundred milliliters of *P. fluorescens* (1×10^8 CFU/ml) was inoculated into each polythene bag containing 50g of the autoclaved rice bran.

The bag contents were then thoroughly mixed and incubated at 28± 2℃ for 7 days. For each *P. fluorescens* strain, three replicates of four polythene bags were maintained and the experiment was conducted three times with the bags arranged in a completely randomized design. One gram of rice bran from each bag was added to 9ml of sterile distilled water. The solution was serially diluted to 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}. From each dilution, a sterilized pipette was used to draw one milliliter of the suspension and aseptically spread sterile petri dishes in a laminar flow hood. Sterilized King's B medium was cooled to 45°C and 20 ml was poured into each plate and smoothly rotated to obtain uniformity. Three plates were maintained per treatment and incubated at 23±2℃ for 48hrs. The number of colonies were counted using colony counter and recorded as number of colony forming units (CFUs). The inoculated bran was later dried in plastic dishes measuring (24×24×6.5 cm), powdered using a mortar grinder and mixed with talc containing carboxyl methyl cellulose at the ratio of 2:1 to obtain final density of 2×10^8 CFU/g which was used in the greenhouse and field trials.

3.12 Greenhouse experiment

3.12.1 Experimental design, layout and treatment application

Sand and soil was oven dried and later thoroughly mixed with manure at a ratio of 2:1:1 and autoclaved. Plastic pots measuring (10 cm) in diameter were filled with soil, labelled and arranged in complete randomized design with three replicates. Plastic plates (12 cm diameter) were placed beneath every pot to avoid contamination among the different treatments. The treatments included: *Pseudomonas fluorescens* isolates Pf1, Pf2, Pf3 and Pf4, Bio cure -B (Positive control), Neem, Pf1+ Neem and untreated (control). Treatments were applied twice at planting and vegetative stage. *Pseudomonas fluorescens* isolates Pf1, Pf2, Pf3 and Pf4 were applied through soil incorporation at 4kg/ ha at a density of 2×10^8 CFU/g, Bio cure-B (3L/Ha) and neem was applied at 6 ml per litre of water. *Pseudomonas fluorescens* (Pf1) was selected as the best isolates since they showed higher percentage inhibition under *in vitro* studies. Three seeds of French bean var. Vanilla were planted per pot, and six pots constituted one treatment. The different application rates for *P. fluorescens*, Neem extract and Bio cure-B were arrived at preliminary trials carried out to test on the effectiveness of the isolates and their effects on plant growth.

3.12.2 Preparation of fungal inoculum for greenhouse experiment

Fungal inoculum was prepared from a seven day old culture of *F. oxysporum* f. sp. *phaseoli*. Five millimeter diameter discs of the culture was

cut and put in 10 ml sterile water and shaken to dislodge the spores. The number of spores were counted using hemocytometer. Sterile distilled water was added to adjust the spore density to10^6 conidia/ml. The formulae below were used to calculate number of spores per ml;

$$\text{No. of spores per ml} = \frac{N \times 1000}{X}$$

N= No. of spores counted/no. of squares, X = Volume of mounted solution

The fungal inoculum was prepared using sorghum grains sourced from local cereal shops around Kenyatta University. Fifty grams of the grains were weighed and washed, put in glass jars (1,000L) and soaked in tap water overnight. The grains were later autoclaved at 121^0C for 60 mins. The autoclaved grains were cooled in a water bath and emptied in sterile polythene bags. Three bags were inoculated each with five mycelial plug having a diameter 10 mm into the cooled sorghum under sterile condition in a laminar flow hood. The inoculated bags were then shaken thoroughly and incubated at 23± 2℃ for two weeks. The bags were shaken daily for about one minute to prevent aggregation of the inocula and improve aeration. The conidia were harvested by flooding the colonized grains by *F. oxysporum* with sterile distilled water. The fungal spores were obtained by filtering through two layer of cheesecloth. The conidia suspension was prepared by serial dilution with the aid of a hemocytometer to 1×10^6 conidia ml^{-1}. Five mililitres of the spore suspension was drenched near the root zone of French beans seedling under greenhouse condition as described by Patil *et al*. (2011).

3.12.3 Data collection

Disease incidence was assessed by counting the number of plants showing infection out of the total number per pot. Percentage incidence was calculated as described by Belete *et al*. (2021)

$$\text{Percentage disease incidence} = \frac{\text{Number of infected plants}}{\text{number of plants examined}} \times 100$$

Fifteen plants were randomly sampled and tagged from three inner rows in each plot and were also used to collect data on disease severity, plant height, shoot and root length. Data on disease severity was collected from the fifteen plants using the scale of 0-4 as described by Weitang *et al*. (2004) in Table 3.2. Data was scored from three leaves sampled at the bottom, middle and top of each French bean plant (Wahome *et al*., 2011). Data on root and shoot length were measured using a tape measure and recorded in centimeters.

Table 3.2: Scale for assessing Fusarium wilt severity on French beans

Scale	Disease severity
0	No symptoms
1	Slight yellow on the lower leaves (At least 2 leaves).
2	Moderate yellow plant (2-3 leaves infected)
3	Wilted plant (All plant leaves are yellow and wilted)
4	Complete infection (Plant severely wilted and destroyed)

The percent disease index was calculated using McKinney (1923) formulae as described below;

$$PDI = \frac{\text{Sum of numeric rating}}{\text{No. of leaves observed}} \times \frac{100}{\text{Maximum disease rating}}$$

3.13 Evaluation of the survival of *Pseudomonas fluorescens in vitro*

The population dynamics of *Pseudomonas fluorescens* in the soil under greenhouse condition was determined at two week intervals until termination of the experiment. From each treatment, 5 grams of soil was collected and used to assess the density of *P. fluorescens*. One gram of the soil was added to 9 ml of sterile distilled water in a 100 ml conical flask and placed on a shaker for 10 min and likewise dilutions of up to 10^{-8} were made. From the 10^{-6}, 10^{-7}, 10^{-8} dilutions, 1ml of the suspension was transferred to the centre of sterilized dishes in a laminar flow hood. Sterilized King's B medium was cooled to 45°C and 20 ml was poured into each plate and smoothly rotated to obtain uniformity. The plates were maintained at 27± 2℃ for 2 days (King *et al*., 1954). The population of *P. fluorescens* was expressed as cfu/ gram of soil as described by Aneja *et al*. (2003).

$$\text{CFU per gram of carrier} = \frac{\text{No. of colonies}}{\text{Amount plated}} \times \text{Dilution factor}$$

3.14 Assessment of French bean yields and yield attributes

Harvesting commenced six weeks after planting and this was done thrice per week in all treatment and this continued for three weeks. The pods were graded into two groups marketable and non- marketable. Marketable pods comprised of extra fine and fine pods. The category of non-marketable pods included disease damaged, deformed, overgrown, thrips damage and

other damages and their weight taken. The weight of pods was recorded in grams at every harvest. The total pod yield was later converted into tonnes per hectare Wahome *et al*. (2013).

Data on root length and shoot length (cm) was collected from 15 plants initially tagged at termination of the experiment. The plants were uprooted and washed to remove soil particles and a tape measure was used to take the length. The materials were transported to the laboratory and oven dried at 70^0C for three days. The materials were later weight and the biomass recorded (Bakker *et al*., 2002).

3.15 Field experiment

3.15.1 Experiment layout, design and treatment application in the field trial

The experimental field was ploughed to fine tilth. The experiment was laid out in a randomized complete block design (RCBD) with eight treatments replicated three times in plots measuring 3 M by 3 M. A spacing of 50 cm was left between the plots and 1M between replicates. French bean var. Vanilla, were used since they are highly susceptible to *Fusarium oxysporum* f. sp. *phaseoli*. Treatments included *Pseudomonas* isolates Pf1, Pf2, Pf3 and Pf4, Bio cure –B, neem extract and Pf1+neem and control. *Pseudomonas fluorescens* isolates were applied in the soil at the rate of 4 kg/ha with 1000kg of organic manure equivalent to 3.9g/900g of organic manure per plot, Bio cure –B and neem were drenched at 3L/ha equivalent to 2.7ml/L of water and

6ml/litre/plot, respectively, at planting. French bean seeds were planted in single rows with spacing of 15 cm x 30 cm in each plot. Weeding was done once per month for the two seasons. Insect pests were managed using confidor (Imidacloprid 700g/kg) which was applied at the rate of 5g in 20 L of water at 7 day intervals until harvesting. The sprays commenced at 50% flowering. The crops were irrigated twice a week in absence of rains.

3.15.2 Data collection

Data on disease incidence and severity, plant height, yield and dry weight of shoots and roots and population dynamics of *P. fluorescens* was assessed and recorded as described in section 3.13.

3.16 Data analysis

Data on colony diameter, zone of inhibition, disease incidence and severity, height of beans (cm), shoot and root length of French beans, yield (weight of pods in grams), dry weight of shoots and roots (g), population dynamics of *P. fluorescens.* The data was transformed to test for homogeneity and arranged in Microsoft Excel Spreadsheet. All the data was subjected to one way analysis of variance (ANOVA) using SAS version 9.2. Treatment means were compared using Fisher's protected least significant difference (LSD) at $P \leq 0.05$. The results were presented in tables and bar graphs.

CHAPTER FOUR: RESULTS

4.1 Morphological and biochemical characteristics of *Pseudomonas fluorescens* isolates

During the study, 34 isolates of *P. fluorescens* were obtained from rhizosphere soil of French beans collected from Kirinyaga County. Twelve isolates produced fluorescent pigment under UV lamp at 365nm in King's B media while the remaining twenty two isolates had no pigmentation. The colonies were small, round shaped, yellowish white, with regular margin, 2-3 mm in diameter (Plate 4.1). Biochemical tests showed that the isolates produced volatile and non-volatile compounds and siderophores were negative to starch hydrolysis test and Gram staining and positive to catalase test (Table 4.1).

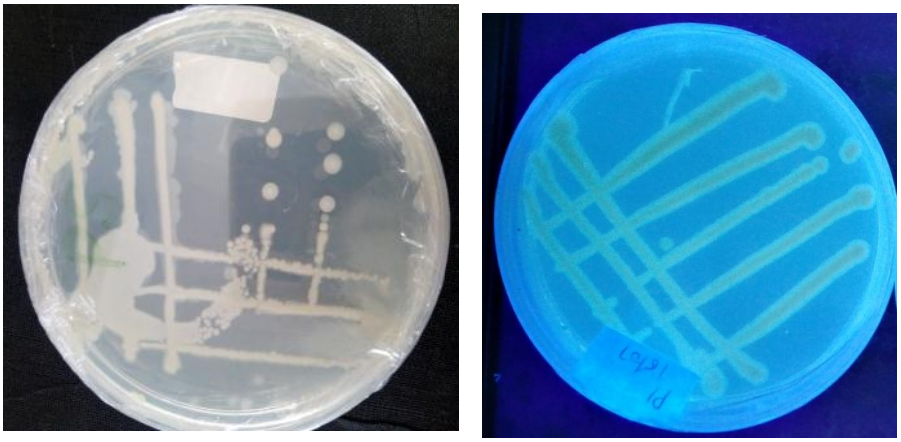


Plate 4.1: Pure cultures of *P. fluorescens*

Table 4.1: Morphological characteristics of *Pseudomonas fluorescens*

Isolates	Shape	Colony	Colour	Fluorescence emissions under UV light
PF1, PF10, PF12	Rod	Round	Yellowish	Bright
PF2, Pf3, PF4, PF6, PF8, PF7, PF9, PF12	Rod	Round	Yellowish White	Bright
PF5	Rod	Round	White	Bright

4.2 Cultural characteristics of *Fusarium oxysporum* f. sp. *phaseoli*

Eight cultures were isolated from French beans infected with *F. oxysporum*. Out of the eight isolates, five were identified as *Fusarium solani* while the remaining three *Fop* 1, *Fop* 2 and *Fop* 3 were identified and confirmed to be *F. oxysporum* f. sp. *phaseoli* using morphological characteristics. The three *F. oxysporum* f. sp. *phaseoli* showed luxuriant mycelial growth and moderate sporulation. The cultures produced both micro and macroconidia. The isolates differed in size and shape of the septa. The colony diameter was 84mm after 7 day of incubation at 27± 2℃. The pigmentation of the isolate was pink on reverse side on PDA medium (Plate 4.2).

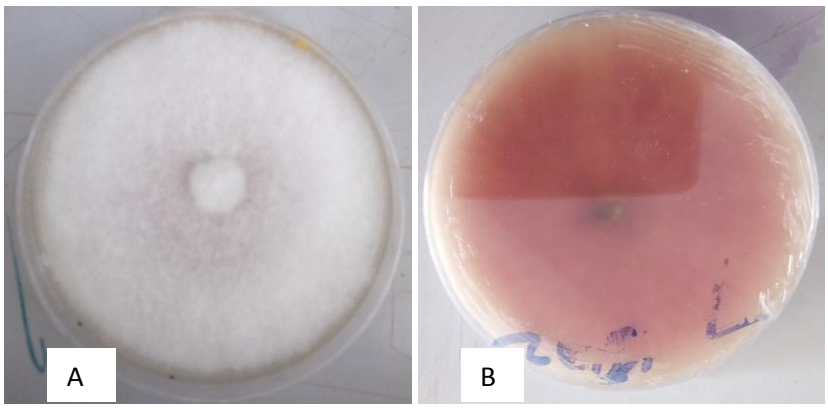


Plate 4.2: Cultures of *F. oxysporum* f. sp. *phaseoli* A (front), B (reverse)

4.3 Antagonism of *Pseudomonas fluorescens* against *Fusarium oxysporum* f. sp. *phaseoli in vitro*

Twelve isolates of *P. fluorescens* were tested against *F. oxysporum* f. sp. *phaseoli* isolates using dual culture technique on King's B medium (Plate 4.3). The colony diameter of the isolates differed significantly (F=22.75; df=11, 38; P=0.001) compared to the control. Seven isolates recorded above 50% inhibition of mycelial growth. *Pseudomonas fluorescens* isolate- Pf1 scored 72.2% which was the highest reduction. The highest mean mycelial growth was recorded in the control with 71.7±0.88 mm followed by Pf12 which had 66.7±3.53 mm. Under the paper disc method, Pf1 scored the highest zone of inhibition of 20.0±1.54 mm and this was significantly different (P=0.001) from the other isolates (Table 4.2).

Table 4.2: Mean growth inhibition by *Pseudomonas fluorescens* isolates in dual culture with *Fusarium oxysporum* f. sp *phaseoli*

P. fluorescens isolates	Dual culture technique		Paper disc method
	Mycelia diameter (mm)	% Inhibition	Zone of inhibition (mm)
Pf1	19.7±0.22^{e}	72.2	20.0±1.54^{a}
Pf2	23.5±0.57 ^{de}	67.6	12.3±1.45^{b}
Pf3	29.0±1.76^{cde}	59.2	11.0±0.58^{bc}
Pf4	29.5±1.26^{cde}	59.2	9.0±0.58^{c}
Pf5	32.2±0.87^{bcde}	54.9	5.3±0.88^{d}
Pf6	35.0±6.51^{bcd}	50.7	4.0±0.58^{de}
Pf7	35.3±1.76^{cde}	50.7	2.7±0.33^{ef}
Pf8	40.0 ±1.50^{bc}	43.7	2.3±0.33^{ef}
Pf9	40.4±1.94^{bc}	43.7	2.0±0.58^{efg}
Pf10	43.0±1.73^{b}	39.4	1.7±0.33^{gf}
Pf11	44.5±3.42^{b}	38.0	1.0±0.58^{gf}
Pf12	66.7±3.53^{a}	7.0	0.7±0.33^{fg}
Control	71.7±0.88^{a}	0	0.0±0.00^{g}
LSD	13.08	-	2.02
P-value	0.001	-	0.001

Means followed by the same letter within a column are not significantly different according to Fisher's LSD test at P≤ 0.05

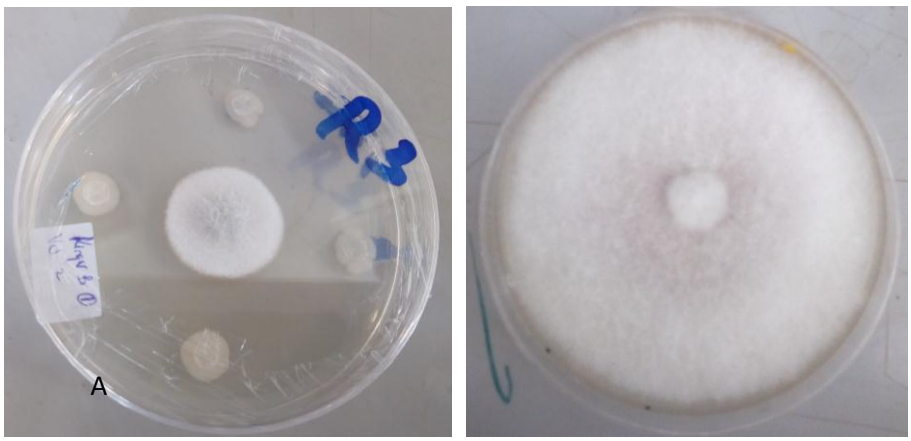


Plate 4.3: Dual culture plates of *Pseudomonas fluorescens* (A) and *Fusarium oxysporum* on King's B medium (seven-day-old) compared to *Fusarium oxysporum* alone (B).

4.4 Antifungal activity of neem extracts using the food poison technique

Leaf extract of *A. indica* at different concentrations significantly (F=53.41; df =4, 14; P=0.001) suppressed radial growth of the test pathogen. Increasing the concentration from 30 to 60% of neem leaf extract showed varied degree of inhibition. The highest colony diameter of 82.7±1.45 mm was recorded in the control. Neem extract (60%) recorded the lowest colony diameter of 27.7±2.03 mm followed by 50% neem extract concentration with 48.0±1.15 mm (Table 4.3).

Table 4.3: Mean diameter (mm) of *Fusarium oxysporum* mycelial growth treated with neem extract at different concentrations

Neem extract Concentration (%)	Mycelia growth of *Fusarium oxysporum* (mm)	Percentage inhibition
60	27.7±2.03^{c}	67.1
50	48.0±1.15^{b}	41.5
40	50.0±0.29^{b}	39.0
30	52.3±0.88^{b}	36.6
Control	82.7±1.45^{a}	0.0
LSD	8.46	-
P –value	0.001	-

Means followed by the same letter within a column are not significantly different according to Fisher's LSD test at P≤ 0.05

4.5 Mechanisms of action of *Pseudomonas fluorescens* against *Fusarium oxysporum in vitro*

4.5.1 Siderophores production

There was significant difference (*F*= 62.70; df=12, 38; *P*=0.001) on the level of inhibition among the isolates as compared to the control (Table 4.4). The percentage inhibition of *F. oxysporum* by *P. fluorescens* isolates ranged between 4.1-64.5% in presence of $FeCl_3$ and 1.7- 69.8 % in the absence of $FeCl_3$. The lowest mean inhibition of mycelia was recorded in control plates with 77.3±4.06, 80.7±3.53 mm followed by Pf11 with 76.0±4.51, 77.3±2.40 mm and Pf12 with 74.7±1.33, 75.7±2.33 mm in presence and absence of $FeCl_3$, respectively. With regard to production of siderophores, a higher inhibition of *F. oxysporum* mycelia growth was observed in media without $FeCl_3$ compared to $FeCl_3$ fortified media.

Table 4.4: Mean *Fusarium oxysporum* f. sp *phaseoli* mycelial growth inhibition by siderophores produced by *Pseudomonas fluorescens*

Isolate	Siderophores Without iron	Percentage inhibition	Siderophores with iron	Percentage Inhibition
PF1	23.3±1.76^{f}	69.8	28.7±0.33^{f}	64.5
Pf2	31.7±0.88^{ef}	59.1	35.3±2.03^{ef}	56.2
Pf3	41.3±3.53^{cd}	46.6	46.3±2.60^{de}	42.6
Pf4	49.0±2.08^{bcd}	36.6	52.7±2.40^{cd}	34.7
Pf5	50.0±4.62^{bc}	35.3	54.0±3.46^{cd}	33.1
PF6	56.7±2.40^{bcd}	26.7	61.3±3.84^{c}	24.0
PF7	51.3±5.81^{b}	33.6	56.3±5.90^{cd}	30.2
PF8	61.7±5.78^{bc}	20.3	63.7±6.12^{bc}	21.1
PF9	57.0±2.65^{bc}	26.3	58.3±2.96^{cd}	27.7
PF10	59.3±7.31^{bc}	23.3	63.3±9.39^{bc}	21.5
PF11	76.0±4.51^{a}	1.7	77.3±2.40^{a}	4.1
PF12	74.7±1.33^{a}	3.4	75.7±2.33^{ab}	6.2
Control	77.3±4.06^{a}	0	80.7±3.53^{a}	0
LSD	11.79	-	12.40	-
P-value	0.001	-	0.001	-

Means followed by the same letter within a column are not significantly different according to Fisher's LSD test at P≤ 0.05

4.5.2 Production of volatile and non-volatile compounds

All *Pseudomonas* isolates significantly (*F*=56.48; df=12, 38; *P*=0.001) inhibited the mycelial growth of *Fusarium oxysporum* via production of volatile metabolites (Plate 4.4) compared to the control. *Pseudomonas fluorescens* isolate Pf1 had the lowest mean mycelia growth of 16.3±0.88 mm

and this was significantly different from the control (85.7±2.33 mm) (Table 4.5). All the isolates significantly inhibited mycelia growth of *F. oxysporum* but this varied with production of metabolites by the different isolates. All the *P. fluorescens* isolates significantly (F=41.88; df=12, 38; P=0.001) inhibited mycelial growth of *F. oxysporum* through production of non-volatile metabolites compared to control. Among the isolates, Pf2 recorded the highest inhibition of 68.8% and Pf8 the lowest inhibition of 9.1%. Production of volatile compounds had the highest percentage inhibition of the pathogen compared to non-volatile compounds *in vitro*.

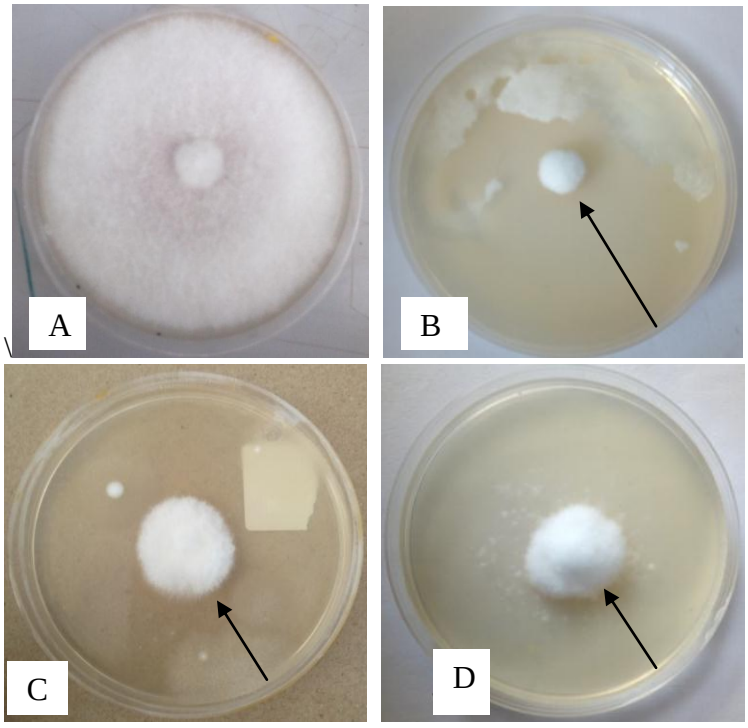


Plate 4.4: Inhibition of *Fusarium oxysporum* by volatile metabolite of *P. fluorescens* isolates (A) Control, (B) *P. fluorescens* Pf1 (C) PF3 (D) Pf4

*Arrow indicates the *Fusarium* colony growth

Table 4.5: Mean *Fusarium oxysporum* f. sp. *phaseoli* radial growth inhibition by compounds produced by *Pseudomonas fluorescens*

Isolates	Mycelia growth (mm)			
	Growth with volatile compounds (mm)	% inhibition	Growth with non-volatile compounds(mm)	% inhibition
Pf1	16.3±0.88^{i}	80.9	37.3±2.19^{de}	51.5
Pf2	21.7±0.88^{hi}	74.7	24.0±1.15^{e}	68.8
Pf3	41.7±2.03^{efg}	51.4	50.0±1.15^{cd}	35.1
Pf4	32.7±1.76^{gh}	61.9	48.7±4.06^{cd}	36.8
Pf5	44.3±3.84^{efg}	48.3	52.0±6.35^{cd}	32.5
Pf6	46.7±5.33^{def}	45.5	60.3±0.88^{abc}	21.7
Pf7	38.3±6.89^{fg}	55.3	56.0±10.69^{bc}	27.3
Pf8	54.0±3.46^{cde}	37.0	70.0±7.57^{ab}	9.1
Pf9	58.0±3.6^{cd}	32.3	61.3±3.71^{abc}	20.4
Pf10	59.3±7.45^{cd}	30.8	59.7±10.49^{bc}	22.5
Pf11	66.0±5.29^{bc}	23.0	61.7±5.04^{abc}	19.9
Pf12	77.3±6.36^{ab}	9.7	64.7±7.97^{abc}	16.0
Control	85.7±2.33^{a}	0	77.0±2.08^{a}	0
LSD	12.84	-	17.18	-
P-value	0.001	-	0.001	-

Means followed by the same letter within a column are not significantly different according to Fisher's LSD test at P≤ 0.05

4.5.3 Inhibition of *Fusarium oxysporum* growth by *Pseudomonas fluorescens* through production of antibiotics

In vitro study revealed that *Pseudomonas* strains had a significant (*F*= 15.58; df= 12, 38; *P*= 0.001) effect on *F. oxysporum.* f. sp *phaseoli* by antibiosis (Table 4.6). All isolates controlled the fungus growth zone by 11-65

% as compared to control except for strain Pf11. *Pseudomonas* Pf1 recorded the highest inhibition of 65.9% and Pf12 had the lowest inhibition of 11.9%. The untreated had the highest mycelia growth of 71.3±7.06 mm and this was significantly (*P*= 0.001) different from what was recorded in Pf11 (71.3±7.06 mm) and Pf12 (63.0±3.21mm) at seven days after incubation.

Table 4.6: Mean inhibition of Fusarium growth by *Pseudomonas fluorescens* through production of antibiotics

Isolates	Radial growth of *F. oxysporum* f. sp *phaseoli* (mm)	Percentage inhibition
Pf1	24.3 ± 0.88^{e}	65.9
Pf2	30.3 ± 3.18^{de}	57.5
Pf3	32.3 ± 3.67^{de}	54.7
Pf4	32.0 ± 2.31^{de}	55.1
Pf5	41.7 ± 1.76^{cd}	41.6
Pf6	44.7 ± 2.40^{cd}	37.4
Pf7	36.3 ± 6.64^{cde}	49.1
Pf8	35.0 ± 5.03^{cde}	50.9
Pf9	41.0 ± 11.37^{cde}	42.5
Pf10	49.7 ± 11.78^{bc}	30.4
Pf11	71.3 ± 7.06^{a}	0.0
Pf12	63.0 ± 3.21^{ab}	11.7
Control	71.3 ± 7.06^{a}	-
LSD	17.19	-
P-value	0.001	-

Means followed by the same letter within a column are not significantly different according to Fisher's LSD test at P≤ 0.05

4.5.4 Evaluation of *Pseudomonas fluorescens* as a plant growth promoting agent *in vitro*

All the *Pseudomonas* isolates differed in their ability to promote growth *in vitro*. *Pseudomonas fluorescens* -Pf1 showed significantly longer shoot length (F=9.56; df= 5, 23; P= 0.001) and root length (F=7.48; df=5, 23; P=0.006) compared to control. Among the *P. fluorescens* strains, Pf1 had the highest plant vigor index of 794.1±40.70 followed by Bio cure- B with 696.1±29.46 and the lowest was in Pf4 with 468.4±63.78 (Table 4.7). The germination percentage was higher in treated seeds compared to untreated control. *Pseudomonas fluorescens* -Pf4 scored the lowest plant vigour index when compared to all the *P. fluorescens* isolates (Table 4.7).

Table 4.7: Mean germination percentage, shoot and root length of French beans var. Vanilla treated with *Pseudomonas fluorescens* isolates using Roll Towel Method

Treatments	Shoot length(cm)	Root length (cm)	Germination percentage	Plant growth vigor
P. fluorescens (Pf1)	0.5±0.04^{a}	9.3±0.48^{a}	82.5±1.50^{a}	794.1±40.70^{a}
P. fluorescens (Pf2)	0.4±0.04^{ab}	8.0±0.71^{ab}	81.5±1.26^{ab}	671.9±62.05^{a}
P. fluorescens (Pf3)	0.3±0.03^{cd}	6.5±0.65^{bc}	78.0±0.82^{cd}	525.5±46.49^{b}
P. fluorescens (Pf4)	0.2±0.05^{d}	6.0±0.71^{c}	75.5±1.26^{cd}	468.4±63.78^{b}
Bio cure –B	0.3±0.04^{bc}	8.5±0.29^{a}	79.5±1.26^{abc}	696.1±29.46^{a}
Control	0.2±0.04^{d}	5.5±0.29^{c}	74.0±1.83^{d}	419.8±24.08^{b}
LSD	0.12	1.63	4.02	139.3
P-value	0.001	0.006	0.001	0.007

Means followed by the same letter within a column are not significantly different according to Fisher's LSD test at P≤ 0.05

4.6 Evaluation of treatments in the greenhouse

4.6.1 Effects of treatments on disease incidence under greenhouse condition

All isolates significantly (F=78.25; df= 7,23; P=0.005) reduced the disease incidence during the first season with Pf1 recording the lowest mean disease incidence of 37.8±0.33% at 42 days after inoculation compared to control. Among the treatments, neem recorded the highest mean disease incidence and this was significantly different from Pf3, Pf4, Bio cure- B and Pf1+neem. French bean plants from plots treated with Pf1+neem recorded significantly (F=80.92; df= 7, 23; P=0.001) lower disease incidence during the second season compared to control. However, this was significantly different from all the other treatments except Pf1 and Bio cure- B which scored 35.2±0.98 and 35.6±1.70%, respectively (Table 4.8).

Table 4.8 Mean disease incidence (%) of Fusarium wilt disease after inoculation in the greenhouse

Treatments	September-November 2018 (SN1)			January –March 2019 (SN2)		
	Mean disease incidence			Mean disease incidence		
	14 DAI	28 DAI	42 DAI	14 DAI	28 DAI	42 DAI
P. fluorescens (Pf 1)	9.2±1.52^{c}	23.7±2.96^{c}	37.8±0.33^{c}	25.2± 1.62^{c}	31.5±1.33^{c}	35.2±0.98^{de}
P. fluorescens (Pf 2)	15.8±2.20^{bc}	33.3±2.91^{bc}	39.2±0.83^{c}	29.3±0.98^{bc}	33.3±2.22^{c}	39.6±1.33^{cd}
P. fluorescens (Pf 3)	21.3±1.76^{ab}	35.7±1.7^{bc}	48.1±5.16^{bc}	28.9±2.57^{bc}	33.7±2.59^{c}	41.9±2.06^{c}
P. fluorescens (Pf 4)	23.0±4.04^{ab}	36.7±4.47^{b}	46.7±3.84^{bc}	33.0±2.06^{bc}	36.7±1.70^{bc}	42.2±1.70^{c}
Bio Cure B	20.2±4.79^{ab}	35.4±2.28^{bc}	44.6±3.70^{bc}	27.4±1.96^{bc}	32.6±0.74^{c}	35.6±1.70^{de}
Neem	25.7±4.70^{ab}	52.0±5.17^{a}	56.2±3.35^{ab}	34.8±2.06^{b}	42.2±1.70^{b}	48.5±0.74^{b}
Pf1+ Neem	17.0±3.00^{bc}	36.3±1.96^{bc}	45.4±4.05^{bc}	27.4±1.48^{bc}	33.0±2.06^{c}	34.8±1.48^{e}
Control	30.1±2.58^{a}	56.4±8.29^{a}	63.9±6.92^{a}	49.3±6.92^{a}	62.2±2.22^{a}	75.2±1.48^{a}
LSD	9.91	12.75	12.15	9.03	5.70	4.46
P-value	0.013	0.001	0.005	0.001	0.001	0.001

Means followed by the same letter within a column are not significantly different according to Fisher's LSD test at P≤ 0.05
SN1-Season one; SN 2- season two; DAI- Days after inoculation

4.6.2 Effects of *Pseudomonas* and neem extract on severity of Fusarium wilt

Application of the treatments at planting had a significant effect on disease severity of French beans at 14, 28 and 42 days after inoculation compared to control (Plate 4.5). All the treated plants had significantly (F=8.77; df=7,23; P=0.001: F=4.21; df= 7,23; P=0.008) lower severity compared to the control during the first and second season, respectively. French beans treated with Pf1+neem recorded a lower mean disease severity compared to those treated with neem alone and this was significantly different (P=0.001) at 42 DAI. The highest mean disease severity was recorded on the control plots and this differed significantly from all the other treatments. During the first and second season, French beans from plots treated with Pf1 isolate had the lowest mean disease severity with 40.7±1.32, 48.0±4.07 followed by Bio cure – B (commercial product) with 43.3±0.63, 52.5±8.43, respectively.

Plate 4.5: French beans treated with *P. fluorescens* (A) and untreated (B) under greenhouse condition

Table 4.9: Mean (%) disease severity of Fusarium wilt of French bean plants treated with *Pseudomonas fluorescens* isolates

Treatments	September-November 2018(SN1)			January –March 2019(SN2)		
	Mean disease severity			Mean disease severity		
	14 DAI	28 DAI	42 DAI	14 DAI	28 DAI	42 DAI
P. fluorescens (Pf 1)	23.3 ±0.63^{c}	27.0±1.33^{c}	40.7±1.32^{d}	12.9± 1.85^{b}	32.9±2.82^{b}	48.0±4.07^{d}
P. fluorescens (Pf 2)	27.1±0.97^{bc}	28.5±1.59^{c}	44.8±1.00^{bcd}	16.7±3.21^{b}	44.7±1.49^{ab}	54.7±3.50^{cd}
P. fluorescens (Pf 3)	27.0±2.25^{bc}	30.4±2.41^{bc}	45.9±2.67^{bcd}	31.4±2.17^{a}	43.9±4.27^{ab}	73.1±3.85^{ab}
P. fluorescens (Pf 4)	30.4±1.94^{bc}	33.7±1.00^{bc}	49.6±4.86^{bc}	37.2±4.96^{a}	44.9±8.84^{ab}	69.5±6.46^{abc}
Bio cure B	24.8±1.34^{c}	27.8±0.63^{c}	43.3±0.63^{cd}	18.9±4.85^{b}	40.2±6.55^{ab}	52.5±8.43^{cd}
Neem	32.9±3.00^{b}	35.5±3.39^{b}	51.5±2.40^{b}	36.4±1.95^{a}	50.8±8.95^{ab}	76.7±5.09^{ab}
Pf1 + Neem	24.8±1.35^{c}	28.1±1.97^{c}	44.0±0.36^{bcd}	15.5±1.67^{b}	44.6±8.93^{ab}	59.4±8.41^{bcd}
Control	44.1±4.87^{a}	46.3±3.70^{a}	80.7±4.11^{a}	37.8±7.22^{a}	56.3±11.87^{a}	80.0±5.00^{a}
LSD	7.24	6.76	7.99	11.87	22.49	17.67
P- value	0.003	0.003	0.001	0.004	0.001	0.008

Means followed by the same letter within a column are not significantly different according to Fisher's LSD test at P≤ 0.05

SN1-Season one; SN 2- season two; DAI- Days after inoculation

4.6.3 Effects of neem extract and *Pseudomonas fluorescens* on plant height of French bean under greenhouse condition

A marked increase in plant height was scored in *Pseudomonas* treated plants compared to the untreated control (Fig. 4.1). During the first season, plants treated with *P. fluorescens* isolates, Bio cure-B and neem had significantly (F=20.94; df=7, 23; P=0.001) higher plant height with *P. fluorescens* Pf1 scoring 24.3±0.88 cm compared to control. The results were consistent whereby a significant (F=8.94; df= 7, 23; P=0.002) increase in height was recorded during the second season and Pf1 had the highest mean height of 22.6±0.33 cm compared to control. The lowest plant height was recorded in control plants with 6.3±0.88 cm and 5±1.53 cm followed by neem 13.7±3.18 and 11±1.53 during the first and second season, respectively.

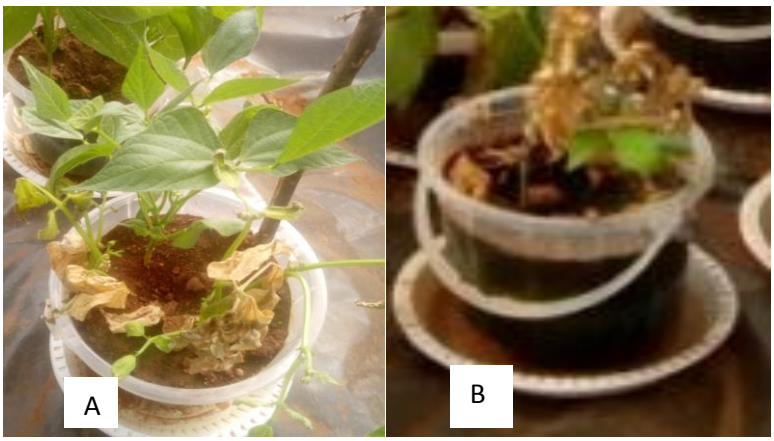
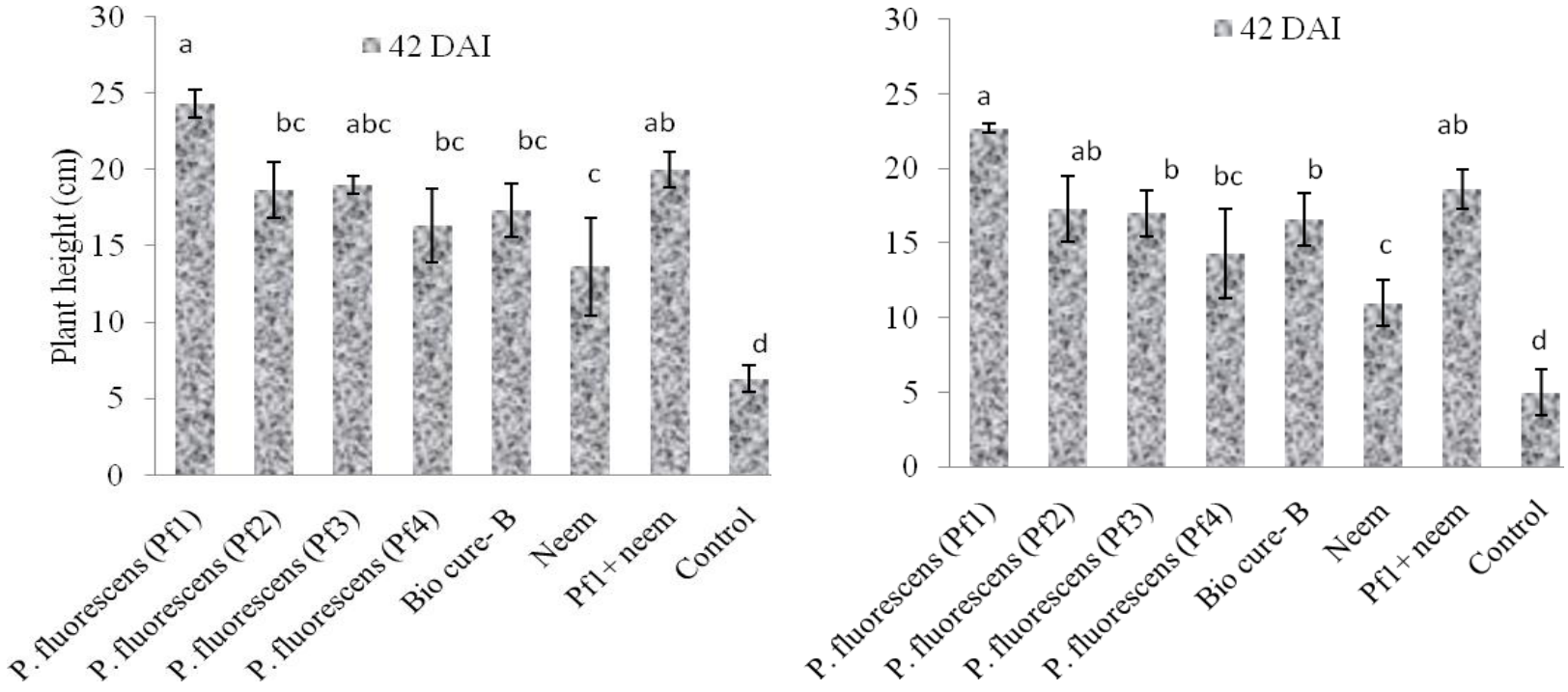


Plate 4.6: French beans in greenhouse showing difference in height between healthy(A) and infected plants (B)



Season one

Season two

Figure 4.1: Mean plant height of French beans treated with *P. fluorescens* under greenhouse condition during the two seasons DAI - Days after inoculation

* Error bars indicate standard error comparing means for each treatment

4.6.4 Effects of treatment on shoot length of French beans in the greenhouse

Application of treatments had significant effect on shoot length compared to the untreated control (Table 4.10). A significantly (F=3.45; df=7, 23; P=0.002) higher shoot length was recorded in all treatments compared to control during the first season. Among the treatments, plants treated with isolate Pf1 had the highest shoot length of 22.0±2.30 cm and the lowest was recorded in plants treated with neem 14.7±0.33 cm at 42 DAI. There was significant (F=2.08; df= 7, 23; P=0.001) difference in shoot length in treated plants compared to untreated control during the second season. Among the *P. fluorescens* isolates, plants treated with Pf4 recorded the lowest shoot length of 13.7±1.20 cm and the highest was those treated with Pf1 with 20.0±1.15 cm (Table 4.10).

Table 4.10: Mean shoot length of French beans at 60 days after treatment application in the greenhouse

Treatments	September-November 2018 (SN1)	January –March 2019 (SN 2)
	Mean shoot length	Mean shoot length
P. fluorescens (Pf1)	22.0±2.30[a]	20.0±1.15[a]
P. fluorescens (Pf2)	20.7±1.76[ab]	18.0±1.15[ab]
P. fluorescens (Pf3)	17.3±3.38[ab]	14.3±1.45[bc]
P. fluorescens (Pf4)	16.3±0.88[abc]	13.7±1.20[bc]
Bio cure- B	18.3±2.02[ab]	16.3±0.88[bc]
Neem	14.7±0.33[bc]	10.7±1.76[c]
Pf1+ neem	19.7±2.60[ab]	17.0±2.51[ab]
Control	10.3±1.45[c]	4.0±0.58[d]
LSD	6.16	4.34
P-value	0.023	0.001

Means followed by the same letter within a column are not significantly different according to Fisher's LSD test at P≤ 0.05

SN1-Season one; SN 2- season two

4.6.5 Effects of *Pseudomonas fluorescens* and neem extract on root length of French beans inoculated with *Fusarium oxysporum* under greenhouse condition

Plants treated with *P. fluorescens* isolates, Bio cure-B and neem had significantly (F=10.85; df=7, 23; P=0.003) longer root length compared to untreated control during the first season. French beans plants treated with Pf1 isolate had the highest root length of 14.7±0.33 cm while neem had the least with 7.7±0.88 cm. During the second season, a significantly shorter root

length was recorded in all treatments compared to untreated control: Plants treated with *P. fluorescens* isolates had a significantly shorter root length during the first season as compared to second season with Pf1 recording the longest with 10.7±1.76 cm (Table 4.11).

Table 4.11: Mean root length of French beans at 60 days after treatment application in the greenhouse

Treatments	September-November 2018 (SN1)	January –March 2019 (SN 2)
	Mean root length (cm)	Mean root length (cm)
P. fluorescens (Pf1)	14.7 ± 0.33^{a}	10.7 ± 1.76^{a}
P. fluorescens (Pf2)	10.7 ± 1.76^{bc}	8.0 ± 1.15^{ab}
P. fluorescens (Pf3)	10.0 ± 1.52^{bc}	7.7 ± 1.45^{ab}
P. fluorescens (Pf4)	8.7 ± 1.45^{bc}	6.3 ± 0.88^{bc}
Bio cure- B	8.7 ± 1.85^{bc}	7.7 ± 1.45^{ab}
Neem	7.7 ± 0.88^{c}	5.3 ± 0.88^{bc}
Pf1+ neem	11.7 ± 0.88^{ab}	9.3 ± 1.20^{ab}
Control	7.3 ± 0.88^{c}	3.3 ± 2.02^{c}
LSD	3.88	4.21
P-value	0.003	0.049

Means followed by the same letter within a column are not significantly different according to Fisher's LSD test at $P\leq 0.05$

SN1-Season one; SN 2- season two

4.6.6 Effects of treatments on biomass of French beans inoculated with *Fusarium oxysporum*

Application of treatments before inoculation of *F. oxysporum* had a significant effect on the biomass of French beans (F=12.37; df=7, 23; P=0.002) during the first season (Table 4.12). Among the isolates, plants treated with Pf1 and Pf2 separately had the highest biomass with 72.3±3.66 g and 71.7±9.27 g, respectively. A higher biomass of 45.7±12.25g was attained in French beans treated with Pf1+neem compared to application of neem solely which had 29.3±10.34g. The results were consistent during the second season whereby a significant F=1.51; df= 7, 23; P=0.001) higher biomass was recorded in treated plants compared to untreated control. French beans treated with Pf1 scored a higher biomass compared to Bio cure- B with 44.1±1.96g, 32.2.7±2.69g, respectively (Table 4.12).

Table 4.12: Mean biomass of French beans at 60 days after treatment application under greenhouse condition

Treatments	September-November 2018 (SN1)	January –March 2019 (SN2)
	Mean biomass (g)	Mean biomass (g)
P. fluorescens (Pf1)	72.3±3.66[a]	44.1±1.96[a]
P. fluorescens (Pf2)	54.0±10.21[ab]	34.4±3.48[bc]
P. fluorescens (Pf3)	71.7±9.27[a]	31.4±5.14[bc]
P. fluorescens (Pf4)	42.7±6.96[bc]	25.7±3.25[cd]
Bio cure- B	30.3±2.60[bc]	32.2±2.69[bc]
Neem	29.3±10.34[c]	19.0±2.78[de]
Pf1+ neem	45.7±12.25[bc]	35.0±2.52[ab]
Control	26.3±2.33[c]	12.2±0.83[e]
LSD	24.21	9.17
P-value	0.002	0.001

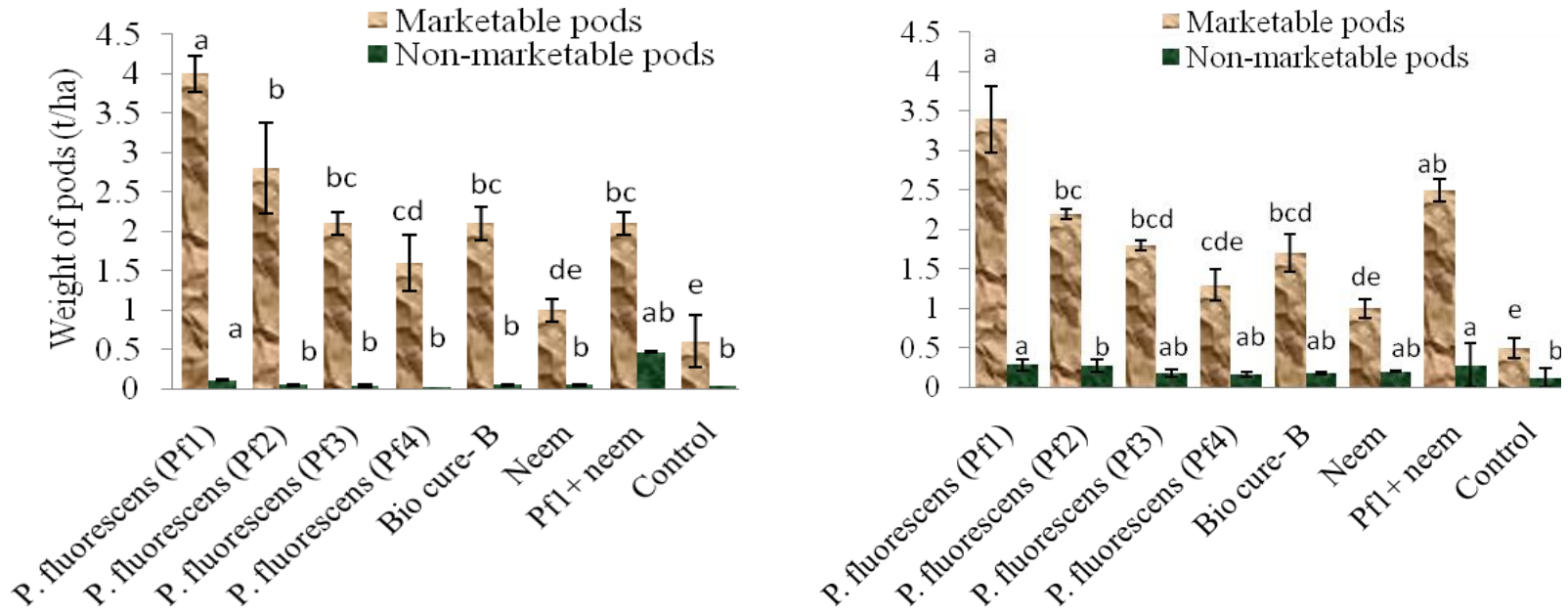
Means followed by the same letter within a column are not significantly different according to Fisher's LSD test at P≤ 0.05

SN1-Season one; SN 2- season two

4.6.7 Effects of treatments on yield of French bean under greenhouse condition

There was a marked increase in the weight of marketable pods in French bean plants treated with neem extract, Bio cure-B and *P. fluorescens* compared to untreated control (Fig.4.2). A significantly (F=12.01; df= 7, 23; P= 0.001) higher weight of marketable pods was recorded for all treatments compared to control. *Pseudomonas fluorescens* - Pf1 attained the highest weight of 4.0±0.11t/ha and the least was recorded in control with 0.6±0.04t/ha.

French beans treated with Pf1 isolate had the highest weight of non-marketable pods compared to control. During the second season, a higher weight of marketable pods was scored in French bean plots treated with Pf1+neem with 2.5±0.28 t/ha compared to Bio cure-B (commercial product) with 1.7±0.18t/ha (Fig.4.2).



Season one

Season two

Figure 4.2: Mean weight of marketable and non-marketable pods from French beans treated with *Pseudomonas fluorescens* under greenhouse condition.

*Error bars represent standard errors of the means per treatment

4.6.8 Assessment of survival rate of *Pseudomonas fluorescens* in the greenhouse

Pseudomonas fluorescens strains Pf1, Pf2, Pf3 and Pf4 differed markedly in their population size in the soil over time (Table 4.13). At 42 days after inoculation of *Fusarium oxysporum*, the population of Pf1 was consistently higher with $3.5\pm0.05\times10^{8}$ compared to other isolates during the first season (Table 4.13). A decline in the population was recorded for all the treatments at 60 DAI. There was significant (F=87.61; df=7, 23; P=0.001) difference in rhizosphere populations of *P. fluorescens* in treated plots compared to control during the second season. There was no population of *P. fluorescens* recorded for both neem and control since no application was done. Among *P. fluorescens* isolates, Pf3 isolate had the least population of $2.0\pm0.12\times10^{8}$ cfu/g during the second season.

Table 4.13: Mean population dynamics of *Pseudomonas fluorescens* in French bean rhizosphere soil under greenhouse condition

Treatments	Sep-Oct 2018 (SN1)				Jan –March 2019 (SN2)			
	Population of *P. fluorescens* ($\times 10^8$ CFU /g of soil)				Population of *P. fluorescens* ($\times 10^8$ CFU /g of soil)			
	14 DAI	28 DAI	42 DAI	60 DAI	14 DAI	28 DAI	42 DAI	60 DAI
P. fluorescens (Pf1)	2.4 ± 0.16^{a}	3.1 ± 0.09^{a}	3.5 ± 0.05^{a}	3.3 ± 0.06^{a}	2.4 ± 0.06^{a}	2.9 ± 0.06^{a}	3.3 ± 0.09^{a}	3.0 ± 0.11^{a}
P. fluorescens (Pf2)	2.2 ± 0.56^{ab}	2.8 ± 0.06^{ab}	3.2 ± 0.08^{ab}	3.0 ± 0.06^{b}	2.3 ± 0.06^{ab}	2.6 ± 0.09^{b}	3.1 ± 0.10^{ab}	2.7 ± 0.09^{ab}
P .fluorescens (Pf3)	2.1 ± 0.03^{b}	2.2 ± 0.11^{d}	2.4 ± 0.03^{c}	2.1 ± 0.06^{e}	2.0 ± 0.06^{c}	2.2 ± 0.03^{cd}	2.3 ± 0.03^{d}	2.0 ± 0.12^{b}
P. fluorescens (Pf4)	2.0 ± 0.15^{b}	2.2 ± 0.12^{d}	2.3 ± 0.09^{c}	2.2 ± 0.06^{e}	2.0 ± 0.12^{c}	2.1 ± 0.15^{d}	2.2 ± 0.12^{d}	2.0 ± 0.15^{c}
Bio cure B	2.1 ± 0.06^{b}	2.6 ± 0.12^{bc}	2.9 ± 0.29^{b}	2.8 ± 0.06^{c}	2.1 ± 0.10^{bc}	2.4 ± 0.12^{bc}	2.7 ± 0.29^{bc}	2.5 ± 0.23^{b}
Neem	0.0 ± 0.00^{c}	0.0 ± 0.00^{e}	0.0 ± 0.00^{d}	0.0 ± 0.00^{f}	0.0 ± 0.00^{d}	0.0 ± 0.00^{e}	0.0 ± 0.00^{e}	0.0 ± 0.00^{d}
Pf1 + Neem	2.1 ± 0.12^{b}	2.4 ± 0.09^{cd}	2.6 ± 0.04^{c}	2.4 ± 0.06^{d}	2.1 ± 0.02^{bc}	2.4 ± 0.10^{bc}	2.5 ± 0.09^{cd}	2.3 ± 0.12^{bc}
Control	0.0 ± 0.00^{c}	0.0 ± 0.00^{e}	0.0 ± 0.00^{d}	0.0 ± 0.00^{f}	0.00 ± 0.00^{d}	0.0 ± 0.00^{e}	0.0 ± 0.00^{e}	0.0 ± 0.00^{d}
LSD	0.24	0.25	0.34	0.15	0.25	0.25	0.34	0.37
P- value	0.001	0.011	0.002	0.002	0.001	0.002	0.002	0.019

Means followed by the same letter within a column are not significantly different according to Fisher's LSD test at $P \leq 0.05$
SN1-Season one; SN 2- season two; DAI- Days after inoculation; CFU – Colony forming unit

4.7 Evaluation of treatments in the field experiment

4.7.1 Effect of bacterium isolates and neem extract on disease incidence of French beans

All *Pseudomonas fluorescens* isolates, neem and Bio Cure -B reduced the incidence of Fusarium wilt on French beans (Table 4.14). French beans from control plots had significantly (F=4.22; df=7,23; P= 0.008: F=17.61; df=7,31; P=0.001) higher number of infected plants compared to treated plants at 42 days after treatment application (DAT) during the first and second season, respectively. Among the treatments, plants treated with neem extract scored the highest disease incidence of 41.9±3.66% during the first season. French beans treated with *Pseudomonas fluorescens*- Pf1 was the most effective treatment with significantly lower disease incidence compared to Pf1+neem for both seasons (Table 4.14).

Table 4.14: Mean disease incidence (%) of Fusarium wilt on French bean plants treated with *Pseudomonas fluorescens* isolates

Treatments	July - September 2018 (SN1)			November 2018 - January 2019 (SN2)		
	Mean disease incidence			Mean disease incidence		
	14 DAT	28 DAT	42 DAT	14 DAT	28 DAT	42 DAT
P. fluorescens (Pf 1)	9.0±1.77^{d}	12.3±2.56^{b}	26.7±1.67^{d}	4.7±086^{c}	12.7±1.28^{c}	14.5±1.87^{e}
P. fluorescens (Pf 2)	5.9±0.87^{cd}	13.3±1.21^{b}	29.3±2.34^{cd}	5.4±0.59^{c}	12.5±1.77^{c}	20.5±1.81^{de}
P. fluorescens (Pf 3)	10.1±2.74^{bcd}	18.4±2.89^{b}	37.4±6.58^{bcd}	10.1±1.18^{b}	16.9±1.22^{bc}	23.8±1.08^{cd}
P. fluorescens (Pf 4)	14.7±1.52^{ab}	15.1±4.60^{b}	38.6±2.60^{abc}	10.2±2.41^{b}	18.0±0.46^{bc}	29.6±4.03^{c}
Bio Cure B	10.3±1.31^{bcd}	16.5±2.76^{b}	32.9±1.41^{bcd}	7.1±1.53^{bc}	14.9±3.09^{c}	23.9±2.15^{cd}
Neem	17.0±2.64^{a}	32.2±1.76^{a}	41.9±3.66^{ab}	15.6±0.61^{a}	22.5±1.66^{ab}	38.5±3.25^{b}
Pf1+ Neem	13.6±0.73^{abc}	17.3±0.67^{b}	30.9±2.37^{cd}	7.4±1.43^{bc}	14.1±3.75^{c}	26.3±3.63^{cd}
Control	14.9±1.70^{ab}	35.3±7.19^{a}	49.1±4.83^{a}	17.8±1.62^{a}	28.7±3.66^{a}	54.6±4.26^{a}
LSD	5.40	10.62	10.75	4.09	7.03	8.68
P-value	0.008	0.014	0.008	0.001	0.009	0.001

Means followed by the same letter within a column are not significantly different according to Fisher's LSD test at P≤ 0.05

SN1-Season one; SN 2- season two; DAT- Days after treatment application

4.7.2 Effects of treatments on Fusarium wilt severity on French beans under field condition

The severity of Fusarium wilt was significantly (F=15.03; df=7, 23; P=0.001) reduced by application of different treatments compared to untreated control during the first season. A progressive increase in diseases severity was recorded from 14, 28 and 42 days after treatment application (Table 4.15). Plants treated with *Pseudomonas fluorescens* (Pf1) recorded the lowest disease severity of 29.3±2.03% and 19.5±0.96% during the first and second season, respectively. The results were consistent during the second season whereby a significant (F=13.64; df= 7, 31; P=0.001) difference was recorded in treated plants compared to control. Maximum disease severity was recorded in the control plots with 48.8±6.57% followed by Pf4 with 29.3±1.25% during the second season at 42 DAT (Table 4.15).

Plate 4.7: French beans treated (A) and untreated (B) with *P. fluorescens* at 28 day under field condition.

Table 4.15: Mean disease severity (%) of Fusarium wilt on bean plants treated with *Pseudomonas fluorescens* isolates

Treatments	July-September 2018(SN1)			November-January 2019(SN 2)		
	Mean disease severity			Mean disease severity		
	14 DAT	28 DAT	42 DAT	14 DAT	28 DAT	42 DAT
P. fluorescens (Pf 1)	23.0±1.67^{d}	26.7±2.03^{c}	29.3±2.03^{e}	10.8±1.11^{d}	15.3±0.48^{de}	19.5±0.96^{c}
P. fluorescens (Pf 2)	28.5±2.34^{cd}	28.9±2.35^{c}	29.8±1.93^{de}	11.8±0.85^{d}	14.5±1.32^{e}	20.3±1.93^{c}
P. fluorescens (Pf 3)	27.6±1.11^{cd}	28.9±2.11^{c}	29.8±1.93^{de}	18.5±1.85^{bc}	21.3±1.03^{bc}	24.8±1.25^{bc}
P. fluorescens (Pf 4)	32.9±1.24^{bc}	33.3±4.02^{bc}	37.8±1.93^{bc}	23.3±1.80^{ab}	17.5±1.89^{cd}	29.3±1.25^{b}
Bio cure B	34.7±1.41^{bc}	35.6±4.64^{bc}	37.3±3.62^{bcd}	16.3±4.01^{cd}	18.8±1.19^{cde}	19.5±1.85^{c}
Neem	36.9±3.66^{b}	38.2±0.89^{b}	43.6±3.89^{b}	22.3±2.17^{abc}	25.3±1.70^{b}	28.5±1.26^{b}
Pf1 + Neem	27.6±2.37^{cd}	29.8±2.47^{bc}	32.1±1.44^{cde}	12.0±2.16^{d}	18.3±1.18^{cde}	19.5±1.50^{c}
Control	51.8±3.89^{a}	55.3±4.83^{a}	59.1±3.11^{a}	28.0±1.41^{a}	35.0±2.04^{a}	48.8±6.57^{a}
LSD	7.43	9.02	7.83	6.19	4.18	7.87
P- value	0.001	0.004	0.001	0.001	0.002	0.013

Means followed by the same letter within a column are not significantly different according to Fisher's LSD test at P≤ 0.05

SN1-Season one; SN 2- season two; DAT- Days after treatment application

4.7.3 Effects of treatments with *Pseudomonas fluorescens* on plant height of French beans under field conditions

Application of *P. fluorescens* isolates promoted growth of French beans compared to the untreated control (Plate 4.8). The plant height progressively increased with treatment application. There was significant difference (F=2.49; df =7, 23; P=0.001: F=23.66; df= 7, 31; P=0.001) in height in the treated French bean plants compared to the control during the first and second season, respectively. The highest plant height (28.7±1.45 cm) was scored in plants treated with *Pseudomonas fluorescens* - Pf1 and this was significantly different from the control. During the second season, French beans plants treated with Pf1 recorded a higher mean plant height of 32.5±1.17 followed by Pf2 (29.5±0.65.) and Pf1+neem (28.5±0.87cm). French beans in control plots recorded the lowest plant height with 10.3±2 33 in the first and 17.8±1.03 cm during the second season (Fig 4.3).

Plate 4.8: French beans treated with *P. fluorescens* (A) and control plots (B) at 28th day under field condition

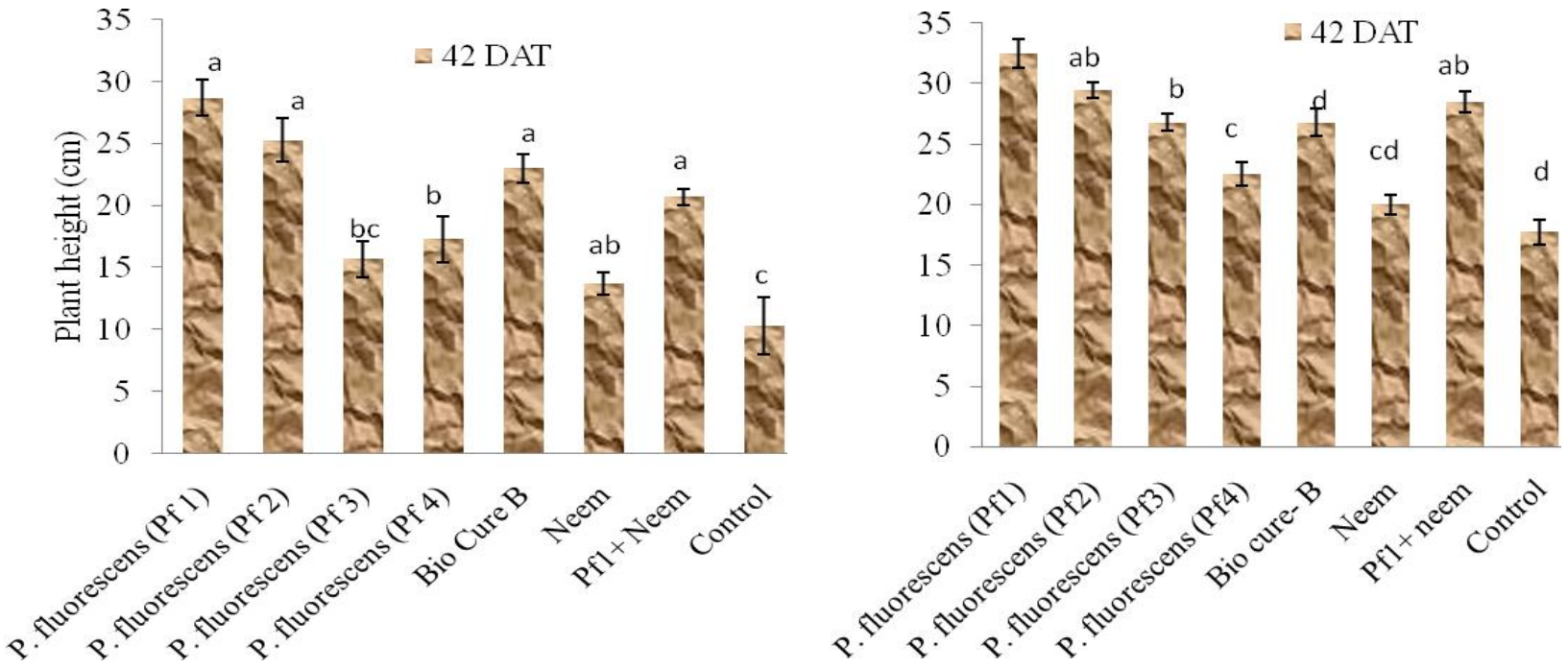


Figure 4.3: Mean plant height of French beans under field condition

SN1-Season one; SN 2- season two; DAT- Days after treatment application

*Error bars represent the standard error of means of treatments

4.7.4 Effects of bacterial isolates and neem on shoot length of French beans under field condition

Application of treatments significantly (*P*= 0.001) increased the shoot length of French beans during the first and second seasons compared to the untreated control. French beans treated with *Pseudomonas* isolate Pf1 and Pf1+neem were at par and had the highest shoot length of 32.7±2.52 cm and 32.7±2.08 cm, respectively. French bean plants treated with Pf1 isolate performed better and attained 38.8±1.88 cm and this was significantly different from all the treatments except Pf3, Pf4 and neem compared to untreated control. Plants in the control plots had the lowest shoot length of 21.0±1.73 cm and 22.8±0.63 during the first and second season, respectively (Table 4.16).

Table 4.16: Mean shoot length of French beans at 60 days after treatment application

Treatments	July - September 2018 (SN1)	November 2018- January 2019 (SN 2)
	Mean shoot length	Mean shoot length
P. fluorescens (Pf1)	32.7±2.52[a]	38.8±1.88[a]
P. fluorescens (Pf2)	34.0±3.61[a]	37.3±2.50[a]
P. fluorescens (Pf3)	29.0±2.64[ab]	32.0±0.91[b]
P. fluorescens (Pf4)	31.0±2.00[a]	30.3±1.44[c]
Bio cure- B	30.3±5.03[ab]	36.3±2.78[ab]
Neem	25.3±2.10[bc]	27.8±1.55[c]
Pf1+ neem	32.7±2.08[a]	35.3±0.48[ab]
Control	21.0±1.73[c]	22.8±0.63[d]
LSD	5.09	4.99
P-value	0.009	0.001

Means followed by the same letter within a column are not significantly different according to Fisher's LSD test at P≤ 0.05

SN1-Season one; SN 2- Season two

4.7.5 Effects of treatments on root length of French beans at 60 DAT under field condition

The data revealed that application of *P. fluorescens,* Bio cure -B and neem extract at planting significantly increased root length compared to untreated control (F=6.88; df=7, 23; P=0.007) during the first season. The highest root length was attained in plants treated with *P. fluorescens* - Pf1 with 47.0±3.61 cm followed by Pf2 which recorded 44.0±1.00 cm in the season. The results were consistent in the second season whereby a significantly (F=14.07; 7, 31; P=0.001) higher root length was recorded in Pf1 treated

plants compared to control (Plate 4.9). French beans treated with Pf1+neem recorded higher root length of 43.3±2.14 cm compared to use of neem extract solely which scored 31.8±1.81 cm during the second season (Table.4.17).

Plate 4.9: French bean root length untreated (A) and treated (B) with *P. fluorescens* under field condition

Table 4.17: Mean root length of French beans at 60 days after treatment application

Treatments	July - September 2018 (SN 1)	November 2018-January 2019 (SN 2)
	Mean root length	Mean root length
P. fluorescens (Pf1)	47.0±3.61[a]	50.3±1.75[a]
P. fluorescens (Pf2)	44.0±1.00[ab]	45.5±2.25[ab]
P. fluorescens (Pf3)	37.7±4.93[bcd]	39.3±2.29[cd]
P. fluorescens (Pf4)	35.7±5.51[cd]	37.5±1.26[d]
Bio cure- B	38.7±4.16[bc]	45.5±1.26[ab]
Neem	31.7±3.17[de]	31.8±1.81[e]
Pf1+ neem	40.0±1.73[bc]	43.3±2.14[bc]
Control	28.7±5.03[e]	29.3±2.69[e]
LSD	6.88	5.62
P-value	0.007	0.001

Means followed by the same letter within a column are not significantly different at P≤ 0.05 (Fisher's LSD test)

SN1-Season one; SN 2- season two

4.7.6 Effect of *Pseudomonas fluorescens* and neem on biomass of shoots and roots under field conditions

Plants treated with *P. fluorescens* isolates, neem and Bio cure –B recorded a higher biomass of shoots and roots of French beans compared to untreated control (Table 4.18). A significant difference (*P*=0.001) in biomass was observed in all treatments compared to the control during the seasons. French beans treated with Pf1 isolate recorded the highest mean biomass of 365.9±27.47g followed by Pf2 which had 345.5±25.19g during the first

season. French beans in plots treated with neem extract had the lowest mean biomass of 418.5±28.05 g followed by control with 223.5±31.40 g. (Table 4.18).

Table 4.18: Mean biomass (g) of French beans at 60 days after treatment application

Treatments	July - September 2018 (SN1)	November 2018-January 2019 (SN 2)
	Biomass (g)	Biomass (g)
P. fluorescens (Pf1)	365.9±27.47[a]	742.9±59.09[a]
P. fluorescens (Pf2)	345.5±25.19[a]	708.6±81.92[a]
P. fluorescens (Pf3)	263.5±25.21[b]	502.6±38.67[bc]
P. fluorescens (Pf4)	208.40±16.64[c]	487.1±22.67[c]
Bio cure- B	264.9±21.19[b]	636.1±85.84[ab]
Neem	194.2 ±31.27[c]	418.5±28.05[c]
Pf1+ neem	225.7±30.70[bc]	604.7±34.12[ab]
Control	127.4±23.01[d]	223.5±31.40[d]
LSD	44.26	154.85
P-value	0.001	0.001

Means followed by the same letter within a column are not significantly different according to Fisher's LSD test at P≤ 0.05

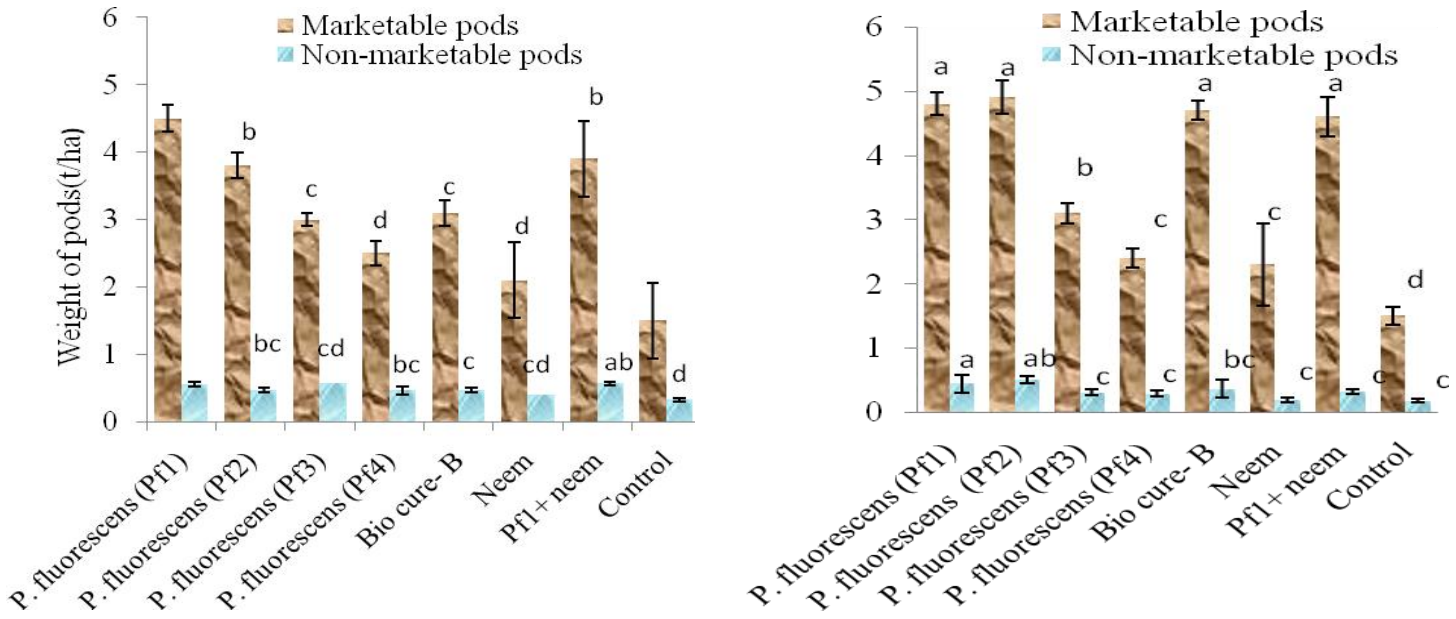
SN1-Season one; SN 2- Season two

4.7.7 Effects of treatments on yield of French beans under field condition

The harvested green pods from each plot were grouped into marketable and non-marketable and thereafter weighed (Plate 4.9). Results indicated that all treatments significantly increased the weight of marketable pods (F=51.32; df=7, 23; P=0.001) compared to the untreated control (F=53.18; df=7, 31;

P=0.001) during the first and second season, respectively. *Pseudomonas fluorescens* -Pf1 had 4.4±0.2 t/ha which was the highest pod weight in the first season. However, *Pseudomonas fluorescens* - Pf2 produced the highest weight of pods with 4.8±0.18 t/ha during the second season (Fig 4.4). The untreated control attained 1.5±0.56 t/ha which was the least weight of marketable pods during the seasons.

Plate 4.10: Marketable (K) and non- marketable pods (L) of French beans.



Season one **Season two**

Figure 4.4: Mean pod weight of marketable and non-marketable French beans under field condition

Error bars indicate the standard error of the means per treatment

4.7.8 Population dynamics of *Pseudomonas fluorescens* at different intervals

Initial population of *P. fluorescens* was determined before application of the treatments. There was a significant (F=89.42; df= 7,31; P= 0.001) increase in the population of *P. fluorescens* isolates in the soil from 14th to 42nd day after treatment application (DAT) during the first season. However, a significant (F=43.38; df=7,31; P=0.001) decrease in *P. fluorescens* was recorded for all the treatments at 60 DAT (Table 4.19). Among the treatments, *P. fluorescens* Pf1 treated plots supported the highest mean population of 11.4±0.65×10^{8}cfu/g and this was significantly different from Pf2 which had 10.2±0.37×10^{8} and Bio cure-B with 10.9±0.24 ×10^{8}cfu/g at 60 DAT. The results obtained during the second season showed there was a significant (F=70.39; df=7,31; P=0.001) increase in population of *P. fluorescens* upto 60 day after treatment application in all the treatments except for neem and untreated control.

Table 4.19: Mean population of *Pseudomonas fluorescens* in rhizosphere soil at different days after treatment application

Treatments	Population of *P. fluorescens* ($\times 10^8$ CFU /g of soil)(SN 1)					Population of *P. fluorescens* ($\times 10^8$ CFU /g of soil)(SN 2)				
	Initial	14 DAT	28 DAT	42 DAT	60 DAT	Initial	14 DAT	28DAT	42 DAT	60 DAT
P. fluorescens (Pf 1)	4.4 ± 0.18^{a}	8.8 ± 0.15^{a}	10.8 ± 0.82^{a}	15.3 ± 0.43^{a}	11.4 ± 0.65^{a}	3.6 ± 0.05^{a}	9.4 ± 0.18^{a}	12.2 ± 0.33^{a}	17.9 ± 0.57^{a}	20.2 ± 0.84^{a}
P. fluorescens (Pf 2)	4.3 ± 0.06^{ab}	8.8 ± 0.33^{a}	9.8 ± 0.16^{a}	13.1 ± 0.27^{b}	10.2 ± 0.37^{ab}	3.0 ± 0.24^{bc}	8.7 ± 0.16^{ab}	11.1 ± 0.51^{b}	16.1 ± 0.18^{a}	17.1 ± 0.84^{b}
P .fluorescens (Pf 3)	3.4 ± 0.11^{c}	7.0 ± 0.17^{b}	8.0 ± 0.21^{b}	9.3 ± 0.13^{cd}	8.7 ± 0.21^{c}	2.6 ± 0.17^{c}	9.2 ± 0.95^{ab}	10.3 ± 0.37^{bc}	11.3 ± 0.33^{bc}	12.7 ± 0.61^{d}
P. fluorescens (Pf 4)	4.3 ± 0.12^{ab}	6.2 ± 0.28^{c}	7.1 ± 0.26^{b}	8.9 ± 0.31^{d}	8.1 ± 0.67^{c}	2.7 ± 0.20^{c}	8.1 ± 0.51^{b}	9.2 ± 0.26^{d}	10.4 ± 0.84^{bc}	11.4 ± 0.43^{d}
Bio cure B	4.4 ± 0.15^{ab}	8.8 ± 0.18^{a}	9.9 ± 0.12^{a}	12.7 ± 0.19^{b}	10.9 ± 0.24^{ab}	3.1 ± 0.22^{abc}	9.8 ± 0.14^{a}	10.5 ± 0.33^{bc}	12.0 ± 1.41^{b}	15.2 ± 0.46^{c}
Neem	3.6 ± 0.12^{c}	3.5 ± 0.16^{d}	3.6 ± 0.31^{c}	4.1 ± 0.54^{e}	4.8 ± 0.24^{d}	2.8 ± 0.22^{c}	3.2 ± 0.11^{c}	3.5 ± 0.20^{e}	2.9 ± 0.24^{d}	2.9 ± 0.16^{e}
Pf1 + Neem	4.0 ± 0.20^{b}	6.6 ± 0.34^{bc}	7.7 ± 0.47^{b}	10.5 ± 0.86^{c}	10.0 ± 0.49^{b}	3.4 ± 0.06^{ab}	9.1 ± 0.28^{ab}	9.8 ± 0.14^{cd}	10.0 ± 0.12^{c}	12.7 ± 0.46^{d}
Control	3.6 ± 0.15^{c}	3.5 ± 0.24^{d}	3.8 ± 0.13^{c}	4.4 ± 0.08^{e}	3.8 ± 0.22^{d}	2.9 ± 0.26^{bc}	3.1 ± 0.23^{c}	3.2 ± 0.13^{e}	2.4 ± 0.43^{d}	2.4 ± 0.46^{e}
LSD	0.41	0.71	1.11	1.23	1.24	0.56	1.21	0.90	1.91	1.69
P- value	0.001	0.001	0.001	0.001	0.001	0.005	0.001	0.001	0.001	0.001

Means followed by the same letter within a column are not significantly different according to Fisher's LSD test at $P \leq 0.05$

SN1 –Season one, SN 2-Season two DAT- Days after treatment application; CFU- Colony forming unit

CHAPTER FIVE: DISCUSSION

5.1 Discussion

Pseudomonas fluorescens strains were more abundant in the French bean rhizosphere around the plant roots. Thirty four *Pseudomonas* spp. were isolated and twelve were identified to be *P. fluorescens* strains through morphological and biochemical tests. The isolates were rod shaped, round, yellowish and produced yellow-green pigments when observed under ultraviolet light at 365 nm. Kim (2002) reported that *P. fluorescens* are the most predominant genera isolated from French beans rhizosphere. Nehra and Saharan (2011) reported that *P. fluorescens* are in high densities in areas around the roots compared to bulk soils Todar (2004) and Sivasankari and Anandharaj (2013) reported that *P. fluorescens* produces a soluble and greenish fluorescent pigment under UV light at 365 nm. Biochemical tests i.e., hydrogen cyanide production, starch hydrolysis, catalase test and siderophores production further confirmed the isolates to be *P. fluorescens* as reported by Reddy *et al.*(2007); Prasanna-kumar *et al.* (2009) and Nathan *et al*. (2011).

Fusarium oxysporum f. sp. *phaseoli* was isolated on potato dextrose agar. Three isolates were confirmed to be *F. oxysporum*. f. sp. *phaseoli* and showed luxuriant, scanty aerial mycelial growth and formed pink colour on the reverse side on the PDA medium. The isolates had profuse sporulation and formed macro- and micro conidia. The results were in line with Li *et al.*

(2000) who reported that *F. oxysporum* f. sp. *phaseoli* form macro-conidia and micro-conidia.

Results of bioassays in dual culture demonstrated that *P. fluorescens* isolates inhibited *F. oxysporum* growth on King's B medium. The inhibition zone suggested the production of metabolites secreted by the bacteria. The isolates varied in the production of antibiotics, siderophores, volatile and non-volatile compounds and this correlated to their ability to inhibit *F. oxysporum* f. sp. *phaseoli in vitro. Pseudomonas fluorescens* Pf1 and *Pseudomonas fluorescens* Pf2 recorded the highest percentage inhibition in management of the *Fusarium* spp. through antibiosis. These results are in agreement with Deshwal *et al*. (2013) who reported that *P. fluorescens* produce antibiotics and antimicrobial metabolites that inhibit mycelial growth of *F. oxysporum* under laboratory conditions.

The *P. fluorescens* strains tested also demonstrated the ability to produce volatile and non-volatile compounds. These compounds showed to have antifungal activity which acted against *F. oxysporum* under laboratory conditions. The proximity of the pathogen to the antagonist may have also contributed to the effectiveness of the isolates. Kumar *et al*. (2002) and Karkachi *et al*. (2010) in their study indicated that *Pseudomonas fluorescens* produce metabolites that inhibited growth of *Fusarium oxysporum.* Audrain *et al.* (2015) reported that *P. fluorescens* produce volatile compounds that exhibit antimicrobial and nematicidal activity *in vitro*. *Pseudomonas fluorescens* UM270 was reported to produce volatile organic compounds that showed

antifungal and plant growth promoting activity (Hernández-León *et al.*, 2015). Athukorala *et al*. (2010) also reported that *Pseudomonas* species produce volatile metabolites that inhibit growth of fungal pathogens. Other studies have illustrated that *Pseudomonas* species including *P. fluorescens* and *P. auerofaciens* produce non-volatile metabolites including pyrrolnitrin, phenazines and antibiotics (Athukorala *et al*., 2010; Ramarathnam *et al*., 2011). Akila *et al.* (2011) reported that *P. fluorescens* strains Pf1 and TRC 54 produced non-volatile metabolites that reduced the mycelia growth of *F. oxysporum*.

Pseudomonas fluorescens isolates produced siderophores which sequestered iron limiting its availability to *F. oxysporum* f. sp *phaseoli*. This inhibited germination and growth of the fungal pathogen. *Pseudomonas* strain Pf1 and Pf2 recorded the highest inhibition against *F. oxysporum* in King's B medium compared to medium amended with iron chloride. Increased siderophores under limiting iron concentration suggested they have a role in inhibiting growth of *F. oxysporum*. Siderophores proved to be one of the major strategies the bacterium utilizes to suppress fungal pathogen (Bhatia and Singh, 2021). Neem extract inhibited growth of *F. oxysporum* through the food poison technique and the level of inhibition differed with the concentration of antifungal compounds. It has been reported that neem extracts reduced the mycelial growth of *F. oxysporum*, *Alternaria solani*, *Rhizoctonia solani* and *Sclerotinia Sclerotium in vitro* (Hassanien *et al*., 2010).

Obongoya (2010) also reported that neem extract controlled *F. oxysporum* f. sp. *phaseoli* of common beans *in vivo*.

Reduced disease incidence and severity was recorded in *Pseudomonas fluorescens* Pf1 and Pf2 isolates under greenhouse condition. This was attributed to application of *P. fluorescens* isolates before inoculation of the fungus thus allowing establishment of *Pseudomonas* in the rhizosphere. The bacterium therefore faced less competition with other soil micro-organisms since sterilized soil was used as compared to the field experiment. In addition, inoculation of *F. oxysporum* at two- leaf stage of French beans gave time for *Pseudomonas* isolates to colonize the root rhizosphere therefore reduced the infection sites for the test pathogen. Other studies showed that *P. fluorescens* BE8 controlled *Fusarium oxysporum* on cucumber *Cucumis sativus* under greenhouse condition by colonizing the root zone of the plant (Szentes *et al*., 2013).

Poor vegetative growth of French bean was observed under greenhouse as compared to field trial. This may have been attributed to high inoculum load of the *F. oxysporum* in the pot compared to the naturally infested field. This led to high disease incidence and severity and this translating to poor growth. During the second season high temperatures (28±2℃) were recorded which may have favoured root infection by *F. oxysporum* (Mui-Yun, 2003). The optimum temperature for *F. oxysporum* root infection has been reported to be 30℃ under greenhouse condition. This favoured growth of *F. oxysporum* f. sp *phaseoli* thus led to poor growth of both roots and shoots and the total yield

of French beans. In addition, French beans performed well under field conditions compared to greenhouse condition because the beans do well in open field conditions. Hanaa *et al.* (2011) reported that high *F. oxysporum* inoculum load and high temperatures led to poor growth of tomato *Solanum lycopersicum* grown under greenhouse condition.

Quantification of *P. fluorescens* indicated that high population densities were found in French beans rhizosphere under field condition as compared to greenhouse experiment. *Pseudomonas fluorescens* is soilborne therefore thrive well under natural habitat compared to pot experiment. This may have favoured the survival and multiplication rates leading to the high numbers of the population dynamics recorded in the field trials compared to greenhouse experiment. During the seasons, high temperatures in the greenhouse were (28±2℃) which might have affected the survival and reproduction of the antagonist. A decrease in the CFU counts at 60 days after inoculation may be linked to change in the root morphology and decrease in the number of fine roots of French beans. Chowdhury *et al*. (2013) reported that a higher population of *Pseudomonas* RU47 was recorded in lettuce (*Lactuca sativa*) rhizosphere initially inoculated at field scale compared to greenhouse experiment.

Results obtained from the field showed that *P. fluorescens* Pf1 and Pf2 reduced the number of infected plants and disease severity of Fusarium wilt on French beans in the field. Reduction of the disease may have been as a result of the antimicrobial compounds produced by *P. fluorescens* that inhibited the

growth of the phytopathogens in the soil. According to the results, management of the disease by *P. fluorescens* isolates may have also been attributed to production of siderophores which competed for the available iron in the soil thus limited growth and establishment of the pathogen (Sivasakathi *et al*., 2014). Koche *et al*. (2013) reported that *P. fluorescens* reduce severity of many diseases through production of secondary metabolites. Similarly, Hol *et al*. (2013) investigated that *P. fluorescens* strains maintain soil health and protect crops from soil borne pathogens. Report by Gopalakrishnan *et al*. (2011) showed the potential use of *P. fluorescens* against Fusarium wilts. Production of siderophores and secondary metabolites *P. fluorescens* of strains were effective in managing *R. solani* and *F. oxysporum* (O'Sullivan *et al* 1992; Nagarajkumar *et al*., 2004).

Plants treated with neem alone recorded significantly higher mean disease severity, shorter shoot and root length. This translated to lower yields both in greenhouse and field conditions. This may have been attributed to the lower efficacy of the extract which may have been caused due to method of extraction, application methods and rates. The results from this study are not in line with Nahak and Sahu (2015) who reported that neem extract reduced the disease severity and promoted growth of tomatoes under greenhouse condition. However, plants treated with *P. fluorescens* performed better and controlled the disease. High efficacy of the isolates may have been attributed to the ability of *P. fluorescens* to produce diverse metabolites that inhibited growth of *F. oxysporum* (Hernández-León *et al.,* 2015).

Combination of *Pseudomonas fluorescens* – Pf1 +neem recorded lower disease incidence and severity as compared to application of neem alone. This showed a synergistic relationship between the bacterium and the neem extract thus a better integrated method for wilt control on French beans. Naseby *et al*. (2001) indicated that combined application of *P. fluorescens* and neemzol reduced Fusarium wilt disease in chickpea (*Cicer arietinum*). Application of *P. fluorescens* isolates and neem extract showed increased plant growth and total yield of French beans. The bacterium has been reported to regulate carbon cycles thus enhance nutrient uptake including nitrogen and phosphorous which promoted growth of French beans (Adesemoye and Egamberdieve, 2013; Sivasakthi *et al*., 2014).

Increase in population of the bacterium in the soil has also been reported to increase availability of iron and lower soil pH thus promote nutrient uptake leading to high yields. Ahmed *et al*. (2008) and Sindhu *et al*. (2009) reported that *P. fluorescens* promotes growth through production of phytohormones. Singh *et al*. (2011) and Das *et al*. (2014) indicated an increase in growth of lentils (*Lens culinaris*) and mungbeans (*Vigna radiate*) through application of *P. fluorescens*. Similarly, Koley and Pal (2011) reported that use of *Azotobacter* and *P. fluorescens* under field condition increased plant growth and yields of sword lily (*Gladiolus oppositiflorus*) through early colonization of rhizosphere. Increase in plant height therefore may have been attributed to availability of soluble phosphorous to the plants (Nihorimbere *et al*., 2011). These results were confirmed by Afza *et al*. (2010) who found out

that *Pseudomonas* spp. increased plant growth of faba beans (*Vicia faba*) through production of antimicrobial substances. In addition, *P. fluorescens* produce phytohormones e.g. cytokinins and gibberellins which increase the absorptive surface for uptake of water and nutrients by the plants (Nihorimbere *et al*., 2011). *Pseudomonas fluorescens* Pf1 and Pf2 had the longest shoot and root length. Increase in the length may have been enhanced by production of secondary metabolites involved in root development (Chadha *et al*., 2014). Gibberellin was reported to cause an increase in the root surface area and number of root tips in plots treated with *P. fluorescens* isolates (Bhattacharyya and Jha, 2012).

French beans treated with *P. fluorescens* isolates had a higher dry weight of shoots and roots compared to control. Accumulation of carbohydrates in French bean tissues was as a result of nutrient uptake from the soil which may have resulted to the increase in dry weight of beans in the plots treated with *P. fluorescens* isolates. The results are in line with those of Son *et al*. (2006) who reported that *Pseudomonas* increased the biomass of nodes in soya bean (*Glycine max*). *Pseudomonas* solubilize phosphorous by producing organic acids which decrease the environmental acidity thus enhance release of insoluble phosphorous from the soil in form of organic phosphoric acid, thus enhance nutrients uptake (Chung *et al*., 2005; Gulati *et al*., 2010).

Increase in total weight of marketable pods was recorded in plants treated with *P. fluorescens* strain Pf1 and *P. fluorescens* Pf2 and combination

of *P. fluorescens* strain Pf1 and neem. Production of siderophores inhibited *F. oxysporum* thus promoted growth of French beans. Siderophores are reported to produce Pseudobacin which induce systemic resistance by strengthening cell wall in the infection site thus reduce number of infected plants and indirectly promoted growth and total yield of beans. The bacteria compete and utilize the available nutrients by suppressing the fungal pathogen. *Pseudomonas fluorescens* may have also enhanced release of nutrients e.g. phosphorous which helped in pod development thus increase in the total yields. This confirmed the results of the previous work by El-Mohamedy *et al.* (2015) who reported that *Pseudomonas fluorescens* improves plant growth and yield of French beans. In addition, Asha *et al*. (2011) reported the bacterium causes increase in plant growth and yield through enhanced uptake of nutrients from the rhizosphere. Saharan *et al*. (2010) indicated that application of *Pseudomonas* R81 increased the yield of cowpea (*Vigna unguiculata*) by 46% through uptake of nutrients and phosphorous from the soil rhizosphere.

CHAPTER SIX: CONCLUSIONS AND RECOMMEDATIONS

6.1 Conclusion

Laboratory studies showed that local environment habour *P. fluorescens* that are effective against *F. oxysporum* f. sp. *phaseoli*. These *P. fluorescens* isolates were found effective in reducing *in vitro* the mycelial growth of *F. oxysporum* f. sp *phaseoli* the causal agent of Fusarium wilt disease of French beans.

Leaf extracts of *Azadirachta indica* inhibited the growth of *Fusarium oxysporum in vitro* with the higher concentration being the most effective.

Pseudomonas fluorescens- Pf1 and Pf2 were the most promising isolates in managing the test pathogen. The isolates produced siderophores, antibiotics and antimicrobial compounds that reduced mycelium growth of the test pathogen.

Application of *P. fluorescens* and neem extract reduced the disease development and improved growth of French beans in the greenhouse and field condition. The results showed *P. fluorescens* to be a potential biocontrol agent in management of fungal diseases. *Pseudomonas* - Pf1 and Pf2 reduced the disease and performed better than Bio cure- B (positive check) and much better than application of neem alone. This translated to increased growth and high quality yields with greater volumes of marketable pods. Increase in the volume of marketable pods will increase the income from sale of the produce

thus improve the livelihood of farmers and widen the employment opportunities in the sector.

6.2 Recommendation

i. *Pseudomonas fluorescens* Pf1 and Pf2 to be formulated and commercialized for use by farmers in management of Fusarium wilt disease and promoting plant growth.

ii. Neem extract to be formulated for use in management of *F. oxysporum* f. sp. *phaseoli*.

iii. *Pseudomonas fluorescens* produce antimicrobial compounds that inhibit growth of fungal pathogen thus further research can be done on the exact compounds produced.

iv. Further research on the antifungal compounds produced by *Pseudomonas fluorescens* to characterise and determine their specificity and activity against Fusarium and other soil borne pathogens.

v. Further research to investigate the persistence of *P. fluorescens* in the soil and understand success of the bacterium in different ecological zones.

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APPENDICES

Appendix I: Soil analysis methods

Soil samples were collected from Mwea (Kimbimbi) field site where the trial was carried out for Physiochemical analysis. The soil were analysed for available nutrient (P, K, Na, Ca, Mg and Mn) using Mehlich Double Acid method (1962), availability of trace elements (Fe, Zn and Cu): Extraction with 0.1 M HCl, presence of organic carbon was determined using Calorimetric method, Total nitrogen was determined using Kjeldahl method, Total nitrogen was determined using Kjeldahl method, Soil pH– water- pH – meter. The results are presented in the table below.

Table 6.1: Physical and chemical results of soil samples from Mwea

Soil pH	4.18	Strong acidity
Exchangeable acidity me%	0.5	Adequate
Nitrogen %	0.18	Low
Organic Carbon %	1.92	moderate
Phosphorus (Mehlich) ppm	120	High
Potassium me (%)	1.03	adequate
Calcium me (%)	3.4	adequate
Magnesium me (%)	4.07	High
Manganese me(%)	1.42	adequate
Copper ppm	8.87	adequate
Iron ppm	47.8	adequate
Zinc ppm	9.73	adequate
Sodium me%	0.22	adequate

Appendix II: NACOSTI Research authorization

NACOSTI

NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY AND INNOVATION

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NACOSTI, Upper Kabete
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P.O. Box 30623-00100
NAIROBI-KENYA

Ref: No. **NACOSTI/P/19/24756/28047**

Date: **12th February, 2019**

Muthoni Teresia Wanjiru
Kenyatta University
P.O Box 43844-00100
NAIROBI.

RE: RESEARCH AUTHORIZATION

Following your application for authority to carry out research on ***"Effectiveness of pseudomonas fluorescens and neem in management of fusarium oxysporum on french beans in Kirinyaga County, Kenya"*** I am pleased to inform you that you have been authorized to undertake research in **Kirinyaga County** for the period ending **12th February, 2020.**

You are advised to report to **the County Commissioner and the County Director of Education, Kirinyaga County** before embarking on the research project.

Kindly note that, as an applicant who has been licensed under the Science, Technology and Innovation Act, 2013 to conduct research in Kenya, you shall deposit **a copy** of the final research report to the Commission within **one year** of completion. The soft copy of the same should be submitted through the Online Research Information System.

GODFREY P. KALERWA MSc., MBA, MKIM
FOR: DIRECTOR-GENERAL/CEO

Copy to:

The County Commissioner
Kirinyaga County.

The County Director of Education
Kirinyaga County.

National Commission for Science, Technology and Innovation is ISO/IEC 2008 Certified

Appendix III: Published paper

Muthoni, W., Kahuthia–Gathu, R., Mwangi, M., Waceke, J.W. (2021). Mechanisms of action of *Pseudomonas fluorescens* against *Fusarium oxysporum* f. sp *phaseoli in vitro. www.researchjournali.com/pdf/5701.pdf*