BIOCONTROL POTENTIAL OF CHITIN AND CHITOSAN EXTRACTED FROM BLACK SOLDIER FLY PUPAL EXUVIAE AGAINST BACTERIAL WILT OF TOMATO

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Biocontrol Potential of Chitin and Chitosan Extracted from Black Soldier Fly Pupal Exuviae against Bacterial Wilt of Tomato

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

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This thesis has been submitted for examination with our approval as university supervisors.

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DEDICATION

I dedicate this work to my beloved parents Mr. and Mrs. John Kemboi, siblings and friends for their moral support and guidance throughout my academic life. This work is also dedicated to the Almighty God for sustaining me strong and in good health in the entire period of research and thesis preparation.

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ABBREVIATION AND ACRONYMS

α	Alpha
ANOVA	Analysis of Variance
β	Beta
BSF	Black Soldier Fly
cfu/ ml	Colony forming units per milliliter
CHT	Chitosan
CRD	Completely Randomized Design
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization Statistics
γ	Gamma
ICIPE	International Centre of Insect Physiology and Ecology
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KOH	Potassium Hydroxide
TZC	Triphenyl Tetrazolium Chloride

ABSTRACT

Globally, *Ralstonia solanacearum* (Smith) is ranked one of the most destructive bacterial pathogens inducing rapidly and fatal wilting symptoms on tomato. Yield losses on tomato vary from 0 to 91% and most control measures are unaffordable to resource poor farmers and ineffective. This study investigated the biocontrol potential of chitin and chitosan extracted from black soldier fly (BSF) pupal exuviae by chemical methods against *R. solanacearum*. *Ralstonia solanacearum* was isolated from soil samples collected from JKUAT tomato growing fields. Morphological, biochemical, and molecular techniques were used to characterize isolated *R. solanacearum* for *in vitro* and *in vivo* experiments. All experiments were done in triplicates and all data were expressed as means ± standard error. One-way analysis of variance (ANOVA) was used to test difference in means and means were separated using the Bonferroni range test. Results revealed that at higher concentration (5%w/v) BSF chitosan significantly inhibited *in vitro* growth of *R. solanacearum* by 19.83 ± 1.17 mm when compared to 1% acetic acid (11.67±2.35 mm). However, there was no significant difference in the antibacterial activities between BSF pupal exuviae chitosan and commercial chitosan against *R. solanacearum* ($p>0.05$). Cocopeat amended with 20 g BSF-chitin and 20 g chitosan demonstrated a reduction in bacterial wilt disease incidence by 30.31% and 34.95%, respectively. On the other hand, disease severity was reduced by 22.57 and 23.66 % when inoculated tomato plants were subjected to cocopeat amended with BSF chitin and chitosan, respectively. These findings show that BSF pupal shells are an attractive renewable raw material for the recovery of valuable products (chitin and chitosan) with promising eco-friendly management option for bacterial wilt *R. solanacearum*. Further studies should explore integrated pest management options that involve multiple components including insect-based chitin and chitosan to manage bacterial wilt disease and contribute to increased tomato production.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Cultivated tomato (*Solanum lycopersicon* L.*)* is considered one of the most important and highly consumed vegetable crops. It belongs to the family Solanaceae which comprises other economically important crops such as potatoes, pepper and eggplants. Tomato is grown for export or for local consumption due to its economical and nutritional values. According to (FAOSTAT, 2017), approximately 60% of tomato world production is from Asia, 13.3% from Europe, 11.3% from Africa, 8.7% from America and 6.6% from Central and South America. Kenya is among the leading countries in tomato production in sub-Saharan Africa (FAO, 2018). Despite this, its production in Kenya has been declining owing to various biotic and abiotic factors that cause losses in yield and income (Nuwamanya *et al*., 2023)

Bacterial wilt, caused by *Ralstonia solanacearum* is one of the most devastating diseases limiting tomato production (Azeem *et al*., 2019). *Ralstonia solanacearum* is a soil borne pathogen found commonly in tropical, subtropical and warm temperate regions (Akintokun *et al*., 2019). The bacterium infects a wide range of plant species including economically important crops in the solanaceae family such as tomatoes, potatoes, pepper, tobacco and eggplants (Manda *et al*., 2020**).** It is famous for its persistence in soil, being able to survive in deeper layers of soil, deeper parts of plant tissues, weeds and water (Azeem *et al*., 2019). *Ralstonia solanacearum* pathogen do vary in its genetic composition hence considered as a species complex (Paudel *et al*., 2020) thus difficult to manage.

Despite the many research trials carried out by researchers worldwide, the efficacy of the management strategies for controlling bacterial wilt such as soil fumigation, cultural practices such as crop rotation and use of resistant cultivars are still limited (Pandey *et al*., 2020). Several chemical pesticides and bactericides have been used as a strategy in managing *R. solanacearum* in the recent days. However, they have

shown low efficacy due to the pathogen species complexity and long persistence in soil (Gatahi *et al*., 2017). Chemical controls also cause loss of microbial flora, development of resistant pathogens and environmental pollution (Lengai *et al*., 2020). Therefore, environment friendly approaches such as use of biocontrol agents have been adopted as alternatives to chemical pesticides. Biological control of *R. solanacearum* has been achieved using beneficial microorganisms such as *Bacillus* and *Pseudomonas* species, avirulent mutants of *R. solanacearum*, bacteriophages and chitin derivatives with combination of other chemical agents and antagonistic microbes (Balamurugan *et al*., 2020).

Chitin has been proposed to be a possible biocontrol agent. It is a linear polysaccharide occurring naturally as the second most abundant biopolymer on earth after cellulose. It occurs primarily as structural component of various organisms such as exoskeleton of arthropods, shells of crustaceans, mollusks and cell walls of some fungi and algae (Morin *et al*., 2019). On the other hand, chitosan is a chitin derived cationic polysaccharide that is produced through deacetylation of chitin (Jayanegara *et al*., 2020). Insects possess a chitinous exoskeleton therefore can be a promising source of chitin. Recently studies have shown that insects such as black soldier flies are potential sources of commercial chitin and chitosan (Smets *et al*., 2020).

Black soldier fly (BSF), *Hermetia illucens*, is a true fly (Diptera) in the family Stratiomyidae that are mainly found in decaying organic matter (Armul *et al*., 2022). It constitutes an economical way of converting organic waste into valuable sources of proteins, lipids and fertilizers (Caligiani *et al*., 2018). Usually, adult forms are used for reproduction while the larvae are utilized as source of proteins for animal feeds (Kim *et al*., 2021). Similarly, black soldier fly can also serve as a source of chitin and chitosan (Hahn *et al*., 2022). Wa *et al*., 2016 found that chitin extracted from BSF had some new physicochemical properties in comparison to other described chitin hence opens additional possibilities of using it as a new source of chitin for different applications. In agriculture, they are applied as biopesticides for protection of plants from pests and diseases and enhancing antagonist microorganisms' action and biocontrol agents (Fan *et al*., 2023). They are also being used as biofertilizers in seed treatment and soil amendment (Malerba & Carena

2019). This is because they are able to enhance the beneficial symbiotic plant microorganisms' interactions (Pusztahelyi, 2018) and regulate plant growth and development (Li *et al*., 2021). Several studies have shown that chitin and chitosan from different sources have antimicrobial activities against *R. solanacearum* and able to reduce bacterial wilt disease incidence and severity in tomato (Rkhaila *et al*., 2021).

1.2 Statement of the problem

In the recent past, occurrences of many plant diseases have been a great challenge facing agriculture globally. Plant pathogens such as early blight (*Alternaria solani*), late blight (*Pytophthora infestans*), bacterial wilt (*Ralstonia solanacearum*) cause broad economic losses to crop plants of economic importance (Yang *et al*., 2016). *Ralstonia solanacearum* is a causal agent of bacterial wilt in most solanaceous crops. It is the most destructive pathogen which in severe infections can cause up to 100% crop loss (Chamedjeu *et al*., 2018).

Cultural, chemical, physical and biological methods have been adopted as control strategies of this pathogen however none of this have been successful. *Ralstonia solanacearum* exhibit genetic variations grouped together as *Ralstonia solanacearum* species complex consisting of four different phylotypes Phylotype I, II, III and IV. Each of these phylotypes infect different hosts and is found in different regions thus allows this pathogen have broad host range and worldwide distribution. This makes use of crop rotation practices and resistant cultivars as its management strategy unsuccessful (Mamphogoro *et al*., 2020). Studies have revealed that *R. solanacearum* can survive for up to 8 years in soil and can live saprophytically in plant debris and weeds until it encounters a suitable host thus using crop rotation and soil fumigation may not be effective (Mamphogoro *et al*., 2020). The long persistence of the pathogen in soil cause prolonged use of chemicals which cause adverse effects such as environmental pollution, loss of microbial flora and residual effects (Oluoch *et al*., 2022). Furthermore, application of pesticides after appearance of wilt symptoms can cause losses since *R. solanacearum* is highly fastidious making it hard to control after infection (Gatahi, 2017). The broad-spectrum activities of pesticides interfere

with non-target and beneficial organisms hence can cause loss of biodiversity (Fan *et al*., 2019). Likewise, the indiscriminate use of synthetic pesticides has led to development of resistance in most plant pathogens (Malerba & Cerana, 2019). Biological control agents such as bacteriophages, antagonist microorganisms (Balamurugan *et al.,* 2020), plants extracts (Azeem *et al*., 2019) and biopolymers such as chitin derivatives (Gatahi, 2017) have been studied as alternatives for controlling *R. solanacearum*. Most of studies have been done using chitin and chitosan extracted from other sources such as shrimps and lobsters (Younes & Rinaudo, 2015a) however chitin and chitosan extracted from BSF has not been studied in controlling *R. solanacearum*. Hence this study sought to investigate biocontrol potential of chitin and chitosan extracted from black soldier fly pupal exuviae against bacterial wilt of tomato.

1.3 Justification of this study

In order to mitigate the problems caused by chemical formulations, research has been conducted for development of alternatives environmentally friendly measures for controlling *R. solanacearum*. Despite chitin and chitosan having useful biological activities and agrochemical applications, they have not been fully utilized. Chitin and chitosan are biodegradable and non-toxic hence environmentally friendly (Uddin *et al*., 2021). They have a potential of controlling soil-borne plant pathogens such as fungi, bacteria, viruses, nematodes, improving soil quality, promoting plant growth and defense (Riseh *et al*., 2022). They also have additional benefits over other biological control agents since they also induce resistance to diseases in the host plants and enhance biodiversity in the rhizosphere (Shamshina *et al*., 2019). In addition, chitin and its derivatives are specific in their action and with broad-spectrum of activities (Orzali *et al*., 2017). The uses of chitin and chitosan extracted from black soldier fly have not been fully exploited especially in Kenya. Therefore, this research is important to generate knowledge on potentiality of chitin and chitosan obtained from BSF in controlling *Ralstonia solanacearum* on tomato plants.

1.4 Hypothesis

BSF-derived chitin and chitosan have no effects on *R. solanacearum* and tomato plant survival in *R. solanacearum* infected soil.

1.5 Objectives

1.5.1 General objective

To determine the biocontrol potential of chitin and chitosan extracted from black soldier fly pupal exuviae against bacterial wilt of tomato

1.5.2 Specific objectives

i. To characterize *R. solanacearum* isolated from soil sampled from JKUAT farm
ii. To investigate *in vitro* antimicrobial activities of BSF pupal exuviae chitosan on *R. solanacearum* and its *in vivo* effects with chitin on *R. solanacearum* population in soil
iii. To investigate the effects of BSF chitin and chitosan in bacterial wilt disease incidence and severity in tomato plants

CHAPTER TWO

LITERATURE REVIEW

2.1 Taxonomy and Botanical description of Tomato plant

Tomato (*Lycopersicon esculentum* L.) belongs to the order Solanales and family Solanaceae. It is perennial in its native habitat however it is grown as an annual crop in temperate climates (Gatahi, 2017). The growth habit of the plant varies and may grow up to 3metres in height. The leaves are covered with glandular, hairy trichomes and alternately arranged on the weakly woody stem. The leaves range in shape from lobed to compound with segments arranged pinnately. All leaves are covered with glandular and hairy trichomes (Shvachko *et al*., 2020). The stem is angular and covered by hairy and glandular trichomes that confer a characteristic smell. The fruit is an edible, brightly colored (usually red), globular or ovoid with enclosed seeds on the pulp. Though it is botanically a berry the fruit is nutritionally categorized as a vegetable (Ranjole, 2023).

2.2 Economic importance of tomato

Tomato is among the most commonly cultivated and consumed vegetable in most parts of the world due to its nutritive and economical values. It contains bioactive compounds such as vitamins, phenolic compounds and carotenoids which promote health and can be integrated as a nutritious part of balanced diet (Meng *et al*., 2022). These compounds have a broad range of physiological properties such as antimicrobial, antioxidant, anti-inflammatory, cardio-protective and anti-allergic (Quinet *et al*., 2019). Tomatoes contains high contents of carotenoids making them main source of lycopene in human's diet (Ochilo *et al*., 2019). Additionally, their carotenoids and phenolic compounds enhance their sensory and functional qualities including aroma, taste and texture. Tomatoes also contain Vitamins A, C and E which serves as antioxidants and high amounts of metabolites including ascorbic acids, sucrose, citrate and malate (Li *et al*., 2018). Tomato production also play important role in creation of employment, income generation and earning of foreign exchange ultimately alleviating poverty levels (Karuku *et al*., 2017).

2.3 Tomato production in Kenya

Tomato is among the most cultivated vegetable crop in Kenya accounting for about 7% of horticulture and 14% of vegetable production (Mwangi, 2020). It is mainly grown in open fields and greenhouse production systems. The country produces on average 410, 033 tons annually with an area under cultivation being 20,111 hectares making Kenya among the top producers in sub-Saharan Africa (Ochilo *et al*., 2019). In Kenya, tomato is mainly grown in places with altitudes ranging from 1150 and 1800m above the sea level. It grows well in warm temperatures between 20-27˚C with annual rainfall of over 600mm (Kambura, 2020). Tomato plants requires well drained clay or sandy loamy soil containing organic matter content. Additionally, it grows suitably at soil pH ranging from 5.0-7.5 (Hazman *et al*., 2022). Since tomato grows well in warm temperatures it is mainly grown in frost free in regions. The leading counties producing tomato in Kenya includes Kirinyaga (14%), Kajiado (9%), Taita Taveta (7%), Meru (6%), Bungoma and Kiambu (5%), Migori and Makueni (4%), Homa Bay and Nakuru (3%) and Machakos (2%) (Matumwabirhi, 2020).

2.4 Challenges in tomato production

Over many years tomato production has faced many challenges. The biotic factors include bacterial, fungal and viral diseases, nematodes, pest and arthropods. The major diseases affecting tomato include bacterial wilt (*Ralstonia solanacearum*), early blight (*Alternaria solani*), late blight (*Phytophthora infestans),* Leaf spot (*Septoria lycopersici*), yellow leaf curl virus, tomato spotted wilt virus and root-knot (*Meloidogyne spp*) (Kambura, 2020). The pests include African bollworms (*Helianthis armigera*), leaf miner (*Tuta absoluta*), red spider mites (*Tetranychus spp*), aphids, leaf hoppers (*Empoasca spp*) and tobacco white fly (Karuku *et al*., 2017).

Besides biotic factors, there are a number of abiotic factors affecting tomato production. They include poor weather conditions, poor soils fertility**,** unavailability

of quality seeds, unsuitable cultural practices and utilization of unadapted varieties (Zhou *et al*., 2019).

2.5 Bacterial wilt of tomato

Bacterial wilt disease is a major biotic factor affecting tomato production and other solanaceous plants. It is caused by a soil borne *Ralstonia solanacearum* bacterium. The disease was first identified and described by E.F. smith in tomato production in 1869 with its origin being unclear. The disease is found worldwide mainly in tropical and subtropical regions (Jibat & Alo, 2020). It causes huge losses in crop production with 0-90% yield loss in tomato, 33-90% in potato, 80-100% in banana, 10-30% in tobacco, and 0- 20% in groundnuts (Khan *et al*., 2019). The pathogen is spread through use of contaminated planting materials, contaminated surface water and irrigation water and contaminated farm tools and equipment (Manda *et al*., 2020). Plant to plant spread can also occur through movement of the bacterial wilt disease causing bacteria from roots of infected plants to uninfected plants. The pathogen can also be spread by crops residues left in fields infested with *R. solanacearum.* Additionally, insects have also been considered as natural spread of the pathogens (Alelign, 2020).

Ralstonia solanacearum is highly favored by high temperatures ranging from 24 to 35^{o}C, moisture of between 155.6mm and 495.2 mm and acidic soil of pH less than 7.0 (Jibat & Alo, 2020). It causes high infection in areas with altitude ranging from 1520m to 2120m above the sea level. In Kenya, these areas include Eastern Region, Rift Valley and Central (Kambura, 2020). Unhealthy or weak plants are highly destroyed by the pathogen as compared to healthy plants with high resistance mechanism towards the pathogen (Ireri, 2020).

2.6 Characteristics of *Ralstonia solanacearum*

Ralstonia solanacearum is an aerobic, non-spore forming, non-capsulate, Gram negative, monotrichous short rod-shaped beta-Proteobacterium (Khasabulli *et al*., 2017). It grows well at an optimum temperature range from 28^{0}C-37^{0}C on a selective media triphenyl tetrazolium chloride media and semi selective media of South Africa

(SMSA) with the colonies being visible after 24-48 hours (Ibrahim *et al*., 2019). On these media, the virulent strains appear as smooth, irregular, fluidal white with pink centers while avirulent strains appear as round, dry and uniformly red-pink colonies (Kambura *et al*., 2020). *R. solanacearum* bacteria are gram negative on Potassium hydroxide (KOH) test, catalase-positive, nitrate reducing, ammonia- forming, cannot hydrolyze starch and can liquefy gelatin at slow rate or not at all (Jibat *et al*., 2020).

2.7 Classification of *Ralstonia solanacearum*

Ralstonia solanacearum is grouped into five races based on host specificity. Race I infect a wide host range most in *Solanaceae* Family and is common in Asia, United States, South America and Africa. Race II infect *Musaceae* Family principally the banana and is commonly found is Southeast Asia and Central America. Race III infects predominantly the potatoes and is globally distributed mainly in the temperate climate (Balamurugan *et al*., 2020). Race IV affects *Zingiberaceae* family mainly ginger and cardamon and is found in Asia and Hawaii. Race V attacks *Moraceae* family particularly mulberry and is common in China (Ibrahim *et al*., 2019).

The bacterium is classified into six biovars based on its ability to acidify basal media containing three hexose alcohols (dulcitol, mannitol, and sorbitol) and three disaccharides (maltose, sucrose and lactose) (Paudel *et al*., 2020). They include biovar 1, 2, 2-T (T refers to tropical), 3, 4 and 5. The biovars 3, 4,1 and 2 are associated with Race I, the *Solanaceae* family. Biovar, 2-T, 1, 2 are linked with Race II, the *Musaceae* Family, biovar 2,1, 2-T are associated with Race III specifically the potato. Biovar 3,4 are associated with the *Zingiberaceae* family (ginger and cardamon). Biovar 5 are associated with *Moraceae* family particularly mulberry (Balamurugan *et al*., 2020).

Using multiplex polymerase chain reaction (PCR), *R. solanacearum* strains are classified into four phylotypes based on the sequence variation on internal transcribed spacer (ITS) region. Phylotype I produces an amplicon size of 144bp, phylotype II produces an amplicon size of 372bp, phylotype III produces an

amplicon size of 91bp and phylotype IV produces an amplicon size of 213bp (Fegan & Prior, 2005).

Based on intraspecific variations in whole genome sequence, the phylotypes are classified into species; *Ralstonia pseudosolanacearum* corresponds to Phylotype I and III strains, *Ralstonia solanacearum* corresponds to Phylotype II and *Ralstonia syzygii* corresponds to Phylotype IV (Etminani *et al*., 2020). The phylotype can be further classified to 20 sequevars based on highly conserved partial sequence regions of endoglucanase (*egl*) gene (Sharma *et al*., 2022).

2.8 Economic importance of *Ralstonia solanacearum*

Ralstonia solanacearum is an economically important soilborne plant pathogenic bacteria. It has a worldwide distribution and wide host range comprising of over 400 plant species in 54 different plant families with the highest being solanaceous crops (Garcia *et al*., 2018). It infects many commercially important plants such as tomato, potato, banana, eggplants, tobacco, pepper, chilli, groundnuts, ginger and other ornamental plants (Lowe power *et al*., 2022). It is the causal agent of brown rot of potato and bacterial wilt of tomato, eggplant, Moko diseases of banana and other ornamentals (Kambura *et al*., 2020).. The bacterium is considered the world most damaging plant pathogenic bacteria. It can cause 100% loss in crop yield thus posing threat to food security. The pathogen can also cause low-income generation from crop farming as a result of reduced crop productivity (Okinda, 2022).

2.9 Bacteria wilt disease cycle

Ralstonia solanacearum infection is initiated by entry through the roots via wounds created by soil borne pathogens, nematodes, during transplanting, or natural openings formed by borne organisms such lateral root emergence (Caldwell *et al*., 2017). Once it gets into the roots or stems, the bacterium being aided by its flagella spreads systematically through to the root extension regions. The virulent strain secretes enzymes such as exoglucanases and endoglucanases which degrade plant cell walls enabling it to live and feed in the intercellular regions (Okinda *et al*., 2022). Once the pathogen is in the root cortex it forms intracellular pockets (Alvarez *et al*., 2021). On

permeating the endodermis, it invades the xylem vessels in the vascular bundles. The pathogen then damages the parenchymal cells and spreads over to the apex of the plant. As the bacterium spreads it multiplies and blocks movement of water through the xylem leading to wilting and eventual death of the infected plants (Lowe-Power *et al*., 2018). Similarly, after colonizing the vascular system, the virulent strains produce large amounts of extracellular polysaccharides that are implicated to cause wilting and death by blocking the xylem tissue in the infected plants (Caldwell *et al*., 2017). After the death of the infected plant, the bacterium lives saprophytically in soil until it gets into contact with roots of another host plant (Okinda *et al*., 2022).

2.10 Signs and symptoms of *Ralstonia solanacearum*

Symptoms specific to *R. solanacearum* do vary among the wide range of susceptible hosts. The common symptoms shared among the susceptible hosts include wilting and yellowing of younger leaves (Garcia *et al*., 2019). The first symptom is wilting of young leaves usually during the day hottest times while they remain green. Wilting is usually on one side of the stem and recover during late evening when the temperatures are low. Wilting progresses as the pathogen infestation increases at the cortex leading to yellowing of the leaves, stunted growth and eventual death of the plant (Kambura *et al*., 2020). Another visible symptom is development of adventitious roots near the infected roots (Okinda, 2022). Vascular discoloration on cross section of the infected stem of mature plants as a result of rotting from inside is also observed (Jibat *et al*., 2020).

A common sign of *R. solanacearum* pathogen is a milky white bacterial ooze from a freshly cut infected stem, tubers or rhizomes of infected plant (Garcia *et al*., 2019). In potato plants, bacterial wilt symptoms are observed in two phases: wilting and rotting phase. Rotting phase symptoms include ring brown discoloration on cut tubers and the infected exude creamy-white bacterial ooze (Balamurugan *et al*., 2020).

2.11 Management strategies of bacterial wilt (*Ralstonia solanacearum*)

Ralstonia solanacearum have proven difficult to manage once it infects the soil and plants however, a number of its management strategies have been proposed. They include cultural practices, chemical methods, biological methods and integrated disease management strategies.

2.11.1 Cultural method

The cultural method includes crop rotation, soil amendments, field sanitation, use of disease-free seeds and use of resistant varieties.

2.11.1.1 Crop rotation

Crop rotation has been commonly used as a management strategy for *R. solanacearum*. This involves planting crops of different family in the same land where tomato have been grown for sometimes (Li *et al*., 2019). Crop rotation prevents the establishment of the pathogen population in soil. When practiced for a period of more than two years, crop rotation can reduce the disease incidence however the pathogen still persists in the soil for long period (Okinda *et al*., 2020). Studies have revealed that growing potato in rotation with sorghum, millet and carrots reduces bacterial wilt disease occurrence (Ahmed *et al*., 2022).

2.11.1.2 Soil amendments

Soil amendments involve the use of organic matter to suppress bacterial wilt disease in plants. Soil amended with compost manure, farmyard manure and sugarcane bagasse has been reported to significantly decrease bacterial wilt incidence (Mamphogoro *et al*., 2020). The breakdown of organic matter in soil amendments cause release of inhibitory substances which may limit growth of some pathogens in favor of some microbial communities which cause competition and antagonistic effects (Ahmed *et al*., 2022). Additionally, soil amendments increase nutritional levels in the soil which improves plant growth and vigor thus enhancing its resistance to bacterial wilt disease (He *et al*., 2020). Studies have also reported reduction in

bacterial wilt incidence through application of inorganic fertilizers such as calcium and Nitrogen + phosphorus + potassium (NPK) fertilizers (Ireri *et al*., 2020). Organic soil amendments can also be used with inorganic fertilizers to enhance their activities against bacterial wilt disease. For instance, utilization of silicon fertilizers with sugarcane bagasse suppresses bacterial wilt disease incidence while increasing tomato yield (Manda *et al*., 2020).

2.11.1.3 Field sanitation

Field sanitation involves the adoption of measures taken to prevent the spread of bacterial wilt pathogen in the fields and inside the greenhouses. The measure involves use of sterilized tools and equipment, removal of plants residues removing disease asymptomatic plants and uprooting infected plants (Belete *et al*., 2022). It also involves using disinfectants such as chlorine bleach, hydrogen peroxide, chloride dioxide ethanol can be are used for field sanitation (Kambura, 2020).

2.11.1.4 Resistant cultivars

Growing cultivars that are resistant to *R. solanacearum* has been considered one of the strategies to managing bacterial wilt disease in tomato. Breeding for resistant varieties have been done for tomato by introduction of Arabidopsis nonexpressor of pathogenesis related (*NPR1*) gene. The *NPR*1 gene contributes to general plant health when the plants' immune response is increased (Kashyap *et al*., 2022). Research have revealed that introduction of the *NPR1*gene in to tomato plant cause reduction in bacterial wilt disease incidence by approximately 70% after 28 days of inoculation (Manda *et al*., 2020). Despite proving good in managing bacterial wilt, resistant cultivars affect the crop quality and yield hence not widely accepted by the farmers (Ireri *et al*., 2020).

2.11.2 Chemical methods

The use of chemicals in controlling bacterial wilt have been intensified in the recent years. These chemicals include carbenazim, benomyl, flubendazole and propiconazole. Pesticides such as 1,3-dichloropropene, metam sodium, chloroprin,

silicon, algicide (3,3-indolyl butanoic acid), validoxylamine, validamycin A, and methy bromide have been used to manage bacterial wilt disease (Mamphogoro *et al*., 2020). Some bactericides, streptomycin and bleaching powder have demonstrated success in managing *R. solanacearum.* Application of a combination of acibenzolar-S-methyl and thymol has showed reduction in bacterial wilt disease incidence in fields (Ganiyu *et al*., 2020). Additionally, Di-Bromo Di-nitro propane 1,3-diol produced by Osho chemical industries limited in Kenya have been used in managing *R. solanacearum* (Kariuki *et al*., 2020). Soaking tomato seeds in a solution of sodium chloride increases plant resistance to bacterial wilt disease (Kambura, 2020). However proven success in management of bacterial wilt, chemicals cause adverse health effects and environmental degradation thus the use of chemicals have been greatly criticized.

2.11.3 Biological methods

Biological methods involving use of living organisms or their constituents have been widely used in managing *R. solanacearum*. Some microorganisms which are antagonist to *R. solanacearum* have proven effective in its management. These microorganisms produce effects such as food competition, production of toxic substances which are detrimental to the pathogens, lytic enzymes and parasitism which causes the pathogen suppression (Kambura, 2020). Several research have recommended a number of bacterial species as biocontrol agents of *R. solanacearum*. They include *Bacillus, Pseudomonas. Paenabacillus, Serratia and Actinomyces* species. *Bacillus subtilis* showed a significant reduction (86%) in bacterial wilt of chilli (Dowarah *et al*., 2020) and in tomato by 40.5 % (Sood *et al*., 2021). In a study by Mohammed *et al*., (2020), *Pseudomonas aeruginosa* and *Pseudomonas syringae* managed bacterial wilt in tomato through antibiosis. Similarly, avirulent mutants of *Pseudomonas solanacearum* have also demonstrated antagonistic activities against *R. solanacearum* (Akintokun *et al*., 2019). Other than bacterial special some fungal species have been found to have antibacterial effects against *R. solanacearum. Trichoderma viridae*, *Trichoderma harzianum* and *Glomus* species have been reported to be potential biocontrol agents against bacterial wilt disease (Ahmed *et al*., 2022). Additionally, bacteriophages have been reported for their biocontrol effects

against bacterial wilt disease. Research done by Ramirez *et al*., (2020) revealed that lytic bacteriophages act against bacterial wilt disease in banana.

2.11.4 Integrated disease management strategies

Integrated disease management approach involves use of various management techniques to get the highest possible level of controlling bacterial wilt pathogen. The aim of this is mainly to reduce the initial inoculum and its effectiveness, postpone disease development and boost host resistance. Due to the pathogen complexity and wide host range, using one management approach might not be effective in controlling the pathogen (Ahmed *et al*., 2022). Research have demonstrated that combination of several approaches can enhance the control of the disease up to 100%. Wu *et al*., (2022) reported that using in combination cultural, chemical and biological methods can reduce bacterial wilt disease by 30-90%. Similarly, studies by Ganiyu *et al*., (2019) showed that application of grafting, actigard (acibenzolar-S-methyl) and thymol significantly reduced bacterial wilt disease incidence.

2.12 Structural, chemical and biological properties of chitin and chitosan

Chitin is a linear polysaccharide that was first discovered by Professor Henri Braconnot in 1811 and its name 'chitin' came about after its isolation in an insect in 1830s (Khayrova *et al*., 2019). Structurally, chitin is linear polysaccharide consisting of N- acetyl-D-glucosamine (GlcNAc, A) and D- glucosamine units (GlcN, D) (Harmsen *et al*., 2019). It exists in three crystalline forms; α, β and γ-chitin with α-chitin being the most abundant form (Fernado *et al*., 2022). Chitin occurs as a white, rigid and hard nitrogenous polysaccharide (Pakizeh *et al*., 2021). It is insoluble in water and most organic solvents but highly soluble in highly toxic solvents such as lithium chloride and dimethylacetamide (Rkhaila *et al*., 2021). Its solubility in dilute acids can be improved through its conversion to chitosan via enzymatic or chemical processes (Mutreja *et al*., 2018).

Chitosan is a linear polysaccharide consisting of D-glucosamine (deacetylated unit) and N- acetyl-D- glucosamine (acetylated unit) in (Hao *et al*., 2021). It is a white

powder with randomly distributed beta-(1-4) linked D- glucosamine and N-acetyl-d-glucosamine (Iber *et al*., 2022). Chitosan is a natural cationic polysaccharide due to the presence of positively charged amino groups on the glucosamine monomer. It is insoluble in water but soluble in organic acids such as acetic acid (Rkhaila *et al*., 2021) . The positive charge on its amino group makes it soluble in water at acidic pH (Ardean *et al*., 2021). Both chitin and chitosan have important biological properties such as biodegradability, biocompatibility, non-antigenicity, non-toxicity, antimicrobial activity and adsorption *(Sethi et al*., 2022). These biological properties make them useful for different applications.

2.13 Antimicrobial activities of chitin and chitosan

Antimicrobial activities of chitin and chitosan against a wide variety of microorganisms have been widely studied. However, chitin has been showed to have no substantial antimicrobial activities *in vitro* because it is insoluble in nature (Gumgumjee *et al*., 2018). On the other hand, chitosan has been proved to have antifungal, antibacterial and antiviral activities (Stasińska-Jakubas *et al*., 2022). It has been found to have antibacterial activities against human pathogenic bacteria such as *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa*, and *B. subtilis* (Confederat *et al*., 2021). In the recent research, chitosan has been shown to have both *in vitro* and *in vivo* antibacterial activities against plant pathogenic bacteria such as *Pseudomonas* species, *Ralstonia solanacearum*, *Xanthomonas* species, *Burkholderia Seminalis* (Chakraborty *et al*., 2020).

Various mechanisms of chitosan antimicrobial activities of chitosan and its derivatives have been studied and discussed. The main mechanism involved is the electrostatic interactions between chitosan and the cell wall of the bacteria (Kumar *et al*., 2023). Being cationic in nature the positive charges in chitosan structure interact with the negative charges on bacterial surfaces. This results in degradation of cell wall, alteration and increased in cell membrane permeability (Riseh *et al*., 2022). Studies have shown that chitosan and its products have higher effects on Gram negative bacteria than the gram positive (Varma & Vasudevan 2020). This could be due to the differences in their surface structures and cell wall composition.

Another proposed mode of action of chitosan involves the alteration of cell permeability. This includes its deposition on the pathogen cell surface resulting in creation of impermeable polymeric layer that inhibits the uptake of nutrients in the cell and changes of the metabolite secretion in the extracellular matrix (Matica *et al*., 2019). Chitosan has a capacity of chelating some essential nutrients, trace elements and metals that are necessary for microbial growth thus inhibiting their growth and toxin production (Chakraborty *et al*., 2020).

Chitosan also inhibits *in vitro* and *in vivo* fungal growth of many plant-pathogenic fungi such as *Rhizoctonia solani* and *Aspergillus niger* (Kanawi *et al*., 2021), *Aspergillus Fumigatus, Aspergillus oryzae, Penicillium* species*, Alternaria solani, Alternaria alternata* (Al- Zahrani *et al*., 2021) *Rhizopus stolonifera* and *Botrytis cinerea* (Lopez- Moya *et al*., 2019). Recent studies have also shown that chitosan has antifungal activities against various human fungal pathogens including *Candida albicans* (Kritchenkov *et al*., 2020) and *Cryptococcus gatti* (Lam *et al*., 2019). Chitosan prevents development of phytopathogenic fungi at different stages including mycelial growth, germ-tube elongation, sporulation, spore viability, germination and at the production of fungal virulence factors (Riseh *et al*., 2022). The inhibition of fungal pathogens in soil can be postulated as; addition of chitosan to soil activates soil borne chitinolytic microbes which produce chinolytic enzymes to degrade and use chitosan. The chinolytic enzymes attack chitin contained in the cell walls of plant pathogenic fungi hence impairing growth of fungi in the soil (Malerba & Carena 2020).

Besides antifungal and antibacterial activities, chitosan is able to inactivate replication of viroid and viruses hence inhibiting their spread (Struszczyk & Pospieszny 2020).

2.14 Chitin and chitosan as soil amendments

Chitin and its derivatives as soil amendment produces effects on soil microbial community. Their addition to soil alters the environmental conditions in soil in favor of beneficial microorganisms and to disadvantage plant pathogens (Dave *et al*.,

2021). In the recent studies, soil amendment with chitin and chitosan showed suppression of soil borne pathogenic fungi such as *Fusarium solani* (Ghule, *et al*., 2020), *Rhizoctonia solani* and *Verticillium dahliae* (Rkhaila, *et al*., 2021).

One of the proposed mechanisms for the reduction of pathogen population in soil is that chitin application to soil increases the activities of chinolytic microorganisms such as *Bacillus subtilis* and *Pseudomonas* species which have antagonistic effects to *R. solanacearum (*Shahrajabian *et al.,* 2021). Addition of chitinous materials in soil avails carbon and nitrogen nutrients promoting growth and increase in population of chinolytic bacteria (Winkler *et al*., 2017). Increase in population of chinolytic microorganism stimulates competition for space and nutrients which may limit growth of *R. solanacearum* and other soil phytopathogens (Musheer *et al*., 2020). Additionally, amendment of soil with chitin and its products causes suppression of soil pathogen indirectly by its products such as ammonia formed during its decomposition by chitin degrading microorganisms (Jayaraman *et al*., 2020).

2.15 Application of chitin and chitosan on biocontrol of plant diseases

Chitin and its derivatives have been widely applied as biocontrol agents against different plant pathogens in various crops (Kumar *et al*., 2018). Research have demonstrated that 0.5% chitosan can reduce *Fusarium* wilt disease severity by 59.4% in bananas (Widodo *et al*., 2020) and *Fusarium* blight in rice (Ma *et al*., 2019). Similarly, 50 mg/L chitin and 100mg/L chitosan extracted from shrimp shells of *Prapenaeus longirostris* showed reduction in *R. solanacearum* wilt disease severity in tomato plants by 117.02% and 142.86% respectively (Rkhaila *et al*., 2021). Additionally, 200µg/ml chitosan nanoparticles extracted from commercial chitosan have been found to reduce bacterial wilt incidence and severity by 81.64% and 77.63% in tomato and 78.93% and 71.85% in potato plants (Khairy *et al*., 2022).

Despite this, the mechanisms of action in which chitin and chitosan enhance growth and protection against diseases in plants are not clear. Some modes of action of chitin and chitosan in protection against disease in plants have been proposed. They are able to trigger plant defense responses and to activate different pathways which

increase the plant resistance to diseases (Zheng *et al*., 2021). Chitin and chitosan treatment in plants cause formation of mechanical and chemical barriers and synthesis of new enzymes and molecules which protect against pathogens (Singh *et al*., 2018). In certain instances, they are able to stimulate hypersensitive response in plants primarily around the infection site that results to programmed cell death (Mouniga *et al*., 2022). The hypersensitive response can be followed by systemic responses that include the synthesis and buildup of secondary metabolites such as pathogenesis- related proteins, phytoalexins and phenolic compounds (Sravani *et al*., 2022). Systemic response can also modulate the activity of key enzymes such as chitinase (El-Garhy *et al*., 2022) and peroxides that are involved in metabolic pathways in the defense response (Czékus *et al*., 2021). Chitosan is able to produce direct antimicrobial activities against the pathogen through disruption of cell membrane and inhibition of biofilm formation (Beatrice *et al*., 2017).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site and source of black soldier fly

This study was conducted at Jomo Kenyatta University of Agriculture and Technology (JKUAT), Juja Kiambu County. *In vitro* and field tests were done at the Department of Botany laboratory and Department of Horticulture and Food Security greenhouse, respectively. The pupal exuviae from which both chitin and chitosan were extracted was obtained from the BSF mass production facility at the Animal Rearing and Containment Unit (ARCU) at the International Centre of Insect Physiology and Ecology (*icipe*), Nairobi, Kenya.

3.2 Soil Sample Collection

Soil samples were randomly collected from tomato growing fields that had tomato plants that were dead or presenting wilting symptoms at Jomo Kenyatta University of Agriculture and Technology (JKUAT). Blocks were demarcated for the trial then divided into plots. Three independent plots were selected then subdivided into numbered units. Three units per plot were randomly selected and soils collected by digging around the plant roots at 30 cm depth using a sterile hoe. This was done at three different sites in each selected unit. The soil samples were collected and transported to the laboratory using a clean bucket.

3.3 Isolation of *Ralstonia solanacearum*

The collected soil samples were allowed to dry in greenhouse conditions and then used to isolate *R. solanacearum*. The isolation and purification of *R. solanacearum* was carried out based on protocol by Mutimawurugo *et al*., (2019). Triphenyl tetrazolium chloride (TZC) media (10g peptone, 2.5g glucose, 1g casamino-acid, 18g agar, 50 mg TZC in 1 liter of distilled water) was used to isolate and differentiate the virulent and avirulent types of *R. solanacearum.* Ten (10) grams of the dried sampled soil was suspended in 100 mL of sterile distilled water then agitated for 10 minutes

on a mechanical shaker. Serial dilution was performed by transferring 1ml of the suspension into 9ml of sterile distilled water in test tubes to dilution factor 10^{-6}. Plating was done by pipetting and spreading 0.1 ml of the suspension from each dilution tube onto sterile TZC media agar plates. The plates were then incubated at 37 $^{\circ}$C for 24 h.

3.4 Characterization of isolated *Ralstonia solanacearum*

3.4.1 Morphological characterization

Typical cultural characteristics of *R. solanacearum* were determined using 24 h old cultures grown at 37 $^{\circ}$C on TZC gar according to the procedure used by Sharma & Singh, (2019). The fluidal, mucoid, irregularly round colonies with white margins and light pink centers were considered to be virulent *R. solanacearum* colonies and were sub-cultured to obtain pure cultures for subsequent use.

Gram staining of pure cultures of the *R. solanacearum* was done following the procedure described by Mutimawurugo *et al.*, (2019). This involved spreading a loopful of the colonies on a glass slide and heat- fixing on a very low flame. Aqueous crystal violet solution was spread over the smear and left for 30s then washed with running tap water for one minute. It was then flooded with iodine and left for one minute, rinsed with tap water, then decolorized with 95% ethanol until clear runoff. After washing, the smear was counter-stained with safranin for 30 s, washed with tap water, dried, and observed at a magnification of x100 using oil immersion. Pink short rod-shaped cells were considered a positive test for *R. solanacearum* (Razia *et al*., 2021).

3.4.2 Biochemical Characterization

3.4.2.1 Potassium Hydroxide solubility Test

The bacterial solubility in 3% KOH was examined to eliminate any possible confusion of the bacterial pathogens that cause wilting in tomatoes as described by Khasabulli *et al*. (2017). The pure culture of the pathogen was picked using a

sterile wire loop and placed on the glass slide containing a drop of 3% KOH solution. It was stirred for about 10s then raised for a few centimeters from the slide while observing for mucoid/slime threads. The formation of a viscous solution or slime thread indicated a positive test (KOH soluble).

3.4.2.2 Catalase Oxidase Test

A catalase test was done as described by Khasabulli, 2017, whereby a loopful of fresh bacterial culture of *R. solanacearum* was mixed with a drop of 3% hydrogen peroxide (H_2O_2) on a glass slide and observed for the production of gas bubbles. An observation of effervescence suggested a positive test.

3.4.2.3 Gas Production Test

A gas production test for the pure cultures of isolated *R. solanacearum* was conducted according to the procedure done by Pawaskar *et al*. (2014). Sterile nutrient broth with 2% glucose in test tubes containing inverted Durham tubes was used. The test tubes were inoculated with 0.5ml of bacterial suspension and incubated at 37 ˚C for 24 h. The presence of air bubbles in the inverted Durham tube was an indication of gas production hence considered a positive test.

3.4.2.4 Starch Hydrolysis Test

The ability of *R. solanacearum* isolate to hydrolyze starch in nutrient agar was tested according to the procedure done by Pawaskar *et al*., (2014).The bacterial cultures of *R. solanacearum* were inoculated and spread on the center of sterile nutrient agar plates containing 0.02% starch then incubated at 37 ˚C for 24 h. After incubating, the plates were flooded with Lugol's iodine. A clear zone around bacterial culture suggested a positive test (Kones *et al*., 2020).

3.4.3 Molecular Characterization

Polymerase chain reaction (PCR) was used to confirm the identity of the virulent isolates using primer pair (Nmult: 21: 2F/ Nmult: 22: RR) specific to *R.*

solanacearum species (Fegan & Prior 2005). The genomic DNA was extracted using Quick-DNA^{TM} Fungal/Bacterial Miniprep kit (Zymo Research Corp, Irvine, CA, USA) based on the manufacturer's instructions. A pure culture of *R. solanacearum* was sub-cultured on Casamino acids Peptone Glucose (CPGA) plates and incubated overnight then used for extraction of DNA.

The PCR reaction was carried out in a total volume of 25 µL reaction containing: One Taq 2X Master mix, *R. solanacearum* species specific primers; Nmult: 21: 2F primer sequence; AAGTTATGGACGGTGGAAGTC and Nmult: 22: RR primer sequence TCGCTTGACCCTATAACGAGTA, DNA template, and Taq DNA polymerase. Amplifications were done in an Eppendorf AG, 22331 Hamburg, Germany thermocycler using the following conditions: initial denaturation at 94 ˚C for 3 min, 35 cycles of denaturation at 94 ˚C for 30 s, annealing at 55 ˚C for 30 s, extension at 72 ˚C for 30 s, and a final extension step at 72 ˚C for 7 min. The amplified PCR products were run through electrophoresis on 1% (*w/v*) agarose gel and visualized on a UV transilluminator (UVT-20 SML model).

3.4.4 Pathogenicity Test

A pathogenicity test was conducted on one-month-old healthy tomato seedlings of Prostar F1 cultivar, a highly tolerant variety to bacterial wilt. This test was done using 24- hour old fresh cultures of *R. solanacearum* isolated from JKUAT fields and those obtained from the International Centre of Potato Research (CIP) as control. The soil drench method was used for inoculation of the plants with the pathogen according to the procedure by Kambura (2020).The seedlings were transplanted onto plastic pots containing autoclaved cocopeat mixed with Hoagland's solution. The plants were left in the medium for three days to let them stabilize. After three days of stabilization in the medium, the seedlings were injured on the stems just above the growth media surface and the roots using a sterile scalpel blade. Turbidity of bacterial suspension was adjusted to 0.5 McFarland equivalents then 10 mL drenched on the injured stems and roots using a sterile syringe. A set of transplanted seedlings drenched with sterile distilled water served as the control.

Each treatment consisted of 10 plants (one plant per pot) and the experiments done in triplicate. The plants were then monitored for bacterial wilt symptoms in the greenhouse under natural sunlight conditions for 30 days. The plants were watered other standard agronomic practices were observed to ensure that plants were free from any form of stress. Plants with at wilted leaf and brown discoloration of vascular bundles were considered diseased. After the development and appearance of wilting symptoms, the pathogen was reisolated on TZC media and re-identified using cultural, morphological, biochemical, and molecular methods to confirm causal organism (Kones *et al*., 2020).

3.5 Extraction of Chitin and Chitosan

Chitin and chitosan were extracted from the BSF pupal exuviae using chemical methods as described by Kaya *et al*., 2015. The pupal exuviae were sorted, washed thoroughly with tap water, and rinsed twice with distilled water, sun-dried, and ground into a fine powder using a blender. The obtained pupal exuviae powder was first demineralized by treating 100 g of the powder with 1000 ml of 1 M HCl in Erlenmeyer flask then allowed to boil on hot plate for two hours to remove all the minerals. The demineralized material was then washed with distilled water until a neutral pH was achieved then filtered and dried in an oven at 60 ˚C for 6 h. The dried demineralized powder weighing 100 g was then treated with 1000 ml of 1 M sodium hydroxide (NaOH) in Erlenmeyer flask then allowed to boil on hot plate with continuous stirring for 4 h to remove proteins. The extracted chitin material was washed thoroughly with distilled water until it attained a neutral pH and dried in an oven at 60 ˚C for 6 h. Thereafter, the chitin was further processed to produce chitosan as described by Kaya *et al*. 2015 but with slight variations. One hundred grams of dried chitin was refluxed in 1000 ml of 40% NaOH then boiled for 8 h with continuous stirring. The obtained product was washed thoroughly with distilled water to a neutral pH then dried in an oven at 60 ˚C.

3.6 Characterization of extracted chitin and chitosan

The extracted chitin and chitosan samples were characterized using Fourier transform infrared spectroscopy (FT-IR). The dried samples were mixed with potassium bromide (KBr) and ground into a fine powder. The dried mixture was then pressed under a vacuum in a mold to form a KBr disc containing the sample. Fourier transform infrared spectroscopic (Bruker Ever, Belgium) measurements were then performed at a scanning range of 4000 to 400 cm-1. A standard commercially obtained chitosan was used as control. Chitosan (CHT) extracts weighing 0.5, 1, 2.5, and 5 g were dissolved in 100 ml of 1% (*v/v*) acetic acid solution separately to obtain different concentrations of CHT (*w/v*). Following the dissolution of chitosan in acetic acid, it was sterilized by autoclaving at 121 ˚C for 15 min and stored at 4 ˚C for later use.

3.7 Antimicrobial Activities of BSF Pupal Exuviae Chitin and Chitosan

3.7.1 In vitro Antimicrobial activities of BSF Pupal Exuviae Chitosan against Ralstonia solanacearum

Antimicrobial activities of BSF chitosan against *R. solanacearum* were tested using disc diffusion method (Spirescu *et al*., 2021). Pure cultures of *R. solanacearum* were sub-cultured overnight at 37 ˚C on casamino peptone glucose (CPG) agar media. Bacterial colonies from the plates were suspended in sterile normal saline and adjusted to obtain an equivalent of 0.5 McFarland then pipetted onto 20 mL of Mueller Hilton Agar plates. L-form glass spreader was sterilized by passing over a flame and used to spread the bacterial suspension over the agar plates. Sterile Whatman's filter paper discs of 6 mm diameter were soaked in 50µl BSF chitosan of different concentrations (0, 0.5, 1, 2.5, and 5%). Chitosan-soaked discs were air-dried at room temperature in safety cabinet then gently picked using sterile forceps and placed on the surface of inoculated agar plates at equidistant positions. Filter paper discs soaked in sterile distilled water were used as the negative control. The plates were incubated at 37 ˚C for 24 h. The experiment was done in three replicates. The diameter of zones of inhibition were measured in millimeters and the average

diameter calculated for the three replicates. The positive "control" consisted of different concentrations of commercially obtained chitosan (0, 0.5, 1, 2.5, and 5%). Eight conventional antibiotic discs: Ampicillin (25 µg), Tetracycline (100 µg), Nitrofurantoin (200 mcg), Nalidixic acid (30 µg), Streptomycin (25 µg), Sulphamethoxazole (200 µg), and Cotrimoxazole (25 µg) were used for comparative study of antimicrobial activities produced by them, BSF chitosan and commercially obtained chitosan against *R. solanacearum*.

3.7.2 Determination of the effects of BSF- derived chitin and chitosan on population of Ralstonia solanacearum

The effects of BSF chitin and chitosan on *R. solanacearum* was done based on procedure used by Kambura (2020) with minor modifications. The population of *solanacearum* population was determined thrice during the experiment. The first experiment was done before any treatment was applied to the sampled soil collected from the farm. Ten (10) grams of soil samples were placed into 250 ml conical flasks containing 90 ml sterile distilled water. The suspension was then vigorously shaken in a rotary for 15 minutes. The suspension was serially diluted up to 6-fold dilution factor. One milliliter from each of the 5^{th} and 6^{th} dilutions was plated on Kelman's Tetrazolium chloride media using the spread plate method then incubated at 28^{o}C for 48 hours. The plates were then observed for *R. solanacearum* growth and its typical colonies were counted. Different weights of chitin and chitosan (1g, 2.5g and 5g) were added to the sampled soil separately. Non-chitin/chitosan amended soil samples served as controls. This was carried out in triplicates. The changes in *R. solanacearum* population were monitored in an interval of 30 and 60 days.

Changes in the pathogen population by the treatment was calculated based on XU *et al*., 2012 with slight modifications and expressed as percentage;

$$\text{Changes in the pathogen population} = \frac{\text{Initial population - Population at 30/60}}{\text{Initial population}} \times 100 \quad (1)$$

3.8 Effects of BSF-based pupal exuviae chitin and chitosan on bacterial wilt disease incidence and severity on tomato

Tomato seeds cv. Prostar F1 (Simlaw Seeds Company, Nairobi, Kenya) were grown in seed propagating trays for 30 days. The seedlings were then transplanted onto plastic pots containing a mixture of 200 g of sterile cocopeat and 20 g of BSF-derived chitin and chitosan separately. Three days after transplanting 50 ml of Hoagland's solution was added to the transplanted plants. Fourteen days after transplanting, the plants were inoculated by drenching 10 ml of 0.5 McFarland suspension of *R. solanacearum*. The plants were injured using sterile scalpel around roots and on stem slightly above the growth media surface. The pathogen was drenched around the injured surface using a sterile syringe. Seedlings transplanted into sterile growth media devoid of chitin or chitosan extracts and inoculated with *R. solanacearum* served as the positive control. Other seedlings transplanted onto growth media without chitin or chitosan and not inoculated with *R. solanacearum* bacterial suspension served as the negative control. The experiments were replicated three times and arranged in a completely randomized design in the greenhouse under natural light conditions. The plants were irrigated after every 12 h and other standard agronomic practices were observed to ensure that plants were free from any form of stress.

Bacterial wilt disease incidence (DI) was monitored daily for one month after pathogen inoculation. Observations were made on wilting symptom expression on leaves and on the stem (vascular tissues). Plants with wilted leaves were recorded as diseased plants. The percent disease incidence was calculated using the formula by (Kempe & Sequira 1983).

$$\%DI = \frac{n}{N} \times 100 \quad (2)$$

where n = Number of wilted leaves per plant, N = total number of leaves.

Bacterial wilt disease severity was evaluated based on the Kempe & Sequira 1983

scale (0–5) of where 0 = no symptoms; 1 = 1–25% leaves wilted; 2 = 26–50% leaves wilted; 3 = 51–75% leaves wilted; 4 = more than 75% but less than 100% of leaves wilted; 5 = all leaves wilted and plant death.

The percent disease severity (DS) was calculated using the formula Kempe & Sequira 1983:

$$\%DS = \frac{\sum ni \times vi}{V \times N} \text{ X } 100 \qquad (3)$$

where ni = number of plants with the respective disease rating; vi = disease rating; V = the highest disease rating; and N = the number of plants observed

3.9 Data Collection and Analysis

Data on cultural, morphological, biochemical, and molecular characterization were collected by observation and the results were recorded on excel sheets. The data on antimicrobial activity was collected by measuring diameter of clear zones of inhibition around each disc on each plate of each test organism. All data were expressed as means ± standard error. One-way analysis of variance (ANOVA) was performed to test difference in mean inhibition zone using Stata SE-64 2011 statistics software. The difference was considered significant at $p \leq 0.05$. Means were separated using the Bonferroni range test.

CHAPTER FOUR

RESULTS

4.1 Isolation of *Ralstonia solanacearum*

The isolated R. solanacearum bacteria from the sampled soils presented as typical virulent colonies which appeared as fluidal, irregularly round, elevated and white with pink-red centers in Kelzman's Tetrazolium agar plates as shown in Figure 4.1.

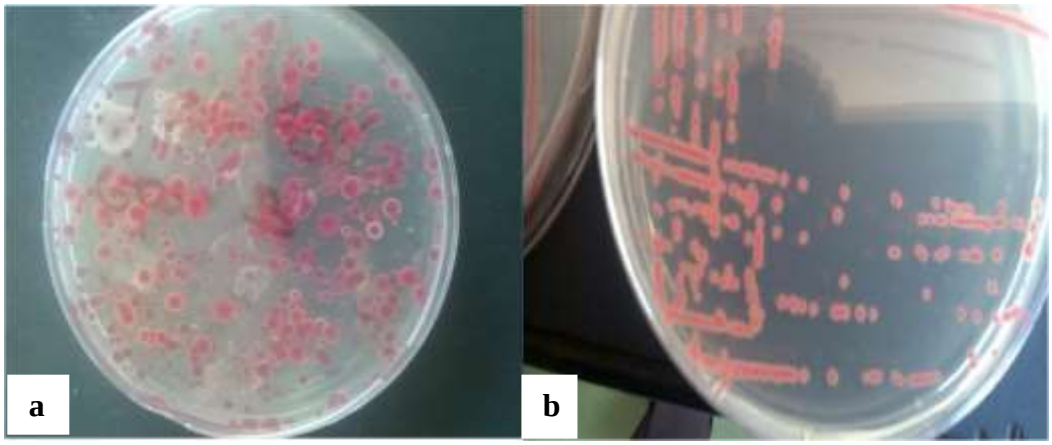

Figure 4.1: (a) Mixed colonies of virulent and avirulent *R. solanacearum*; (b) pure colonies of virulent *R solanacearum*

4.2 Characterization of *Ralstonia solanacearum*

4.2.1 Gram Staining Tests

The observation of Gram staining reaction done on the colonies of *R. solanacearum* isolates showed pink small rod-shaped cells under the microscope at ×100 magnification (Figure 4.2).

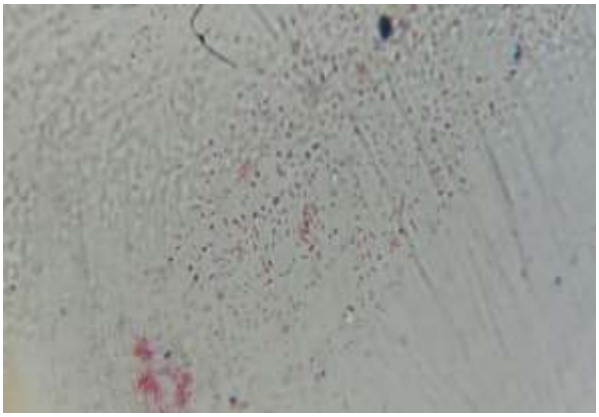

Figure 4.2: Gram stain reaction of *R. solanacearum* colonies under the microscope at ×100 magnification power

4.2.2 Biochemical tests

All the isolates tested positive for catalase test. The isolates produced gas from glucose within 24 hours of incubation as indicated by presence of gas bubbles in Durham tubes. Additionally, the tested isolates were not able to hydrolyze starch as indicated by absence of clear zone around the bacterial growth after being flooded with Lugol's iodine solution *(*Table 4.1).

Table 4.1: Gram staining and biochemical reaction tests for the isolated *R. solanacearum*

Test	Observation
Gram stain reaction Test	-
Potassium Hydroxide solubility test	+
Catalase Test	+
Gas production Test	+
Starch hydrolysis Test	-

(-) Negative), + (positive).

4.2.3 Molecular characteristics of *R. solanacearum*

Polymerase chain reaction (PCR) based on internal transcribed Spacer (ITS) region produced an amplicon band size of 372 bp, confirming that the sample was *R. solanacearum* (Figure 4.3).

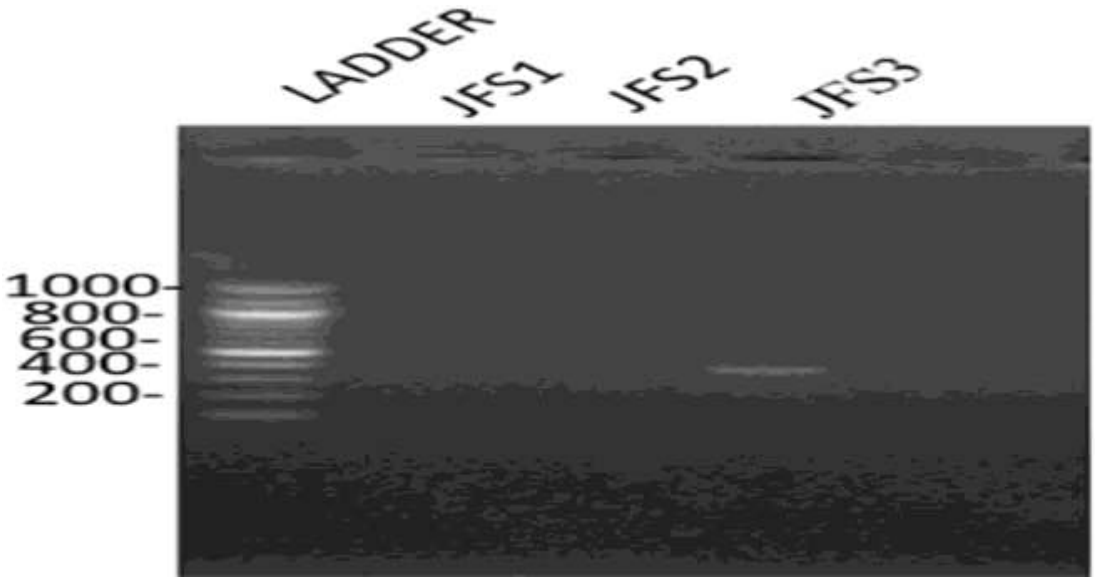

Figure 4.3: DNA bands for *R. solanacearum* based on internal transcribed Spacer (ITS) as observed under UV-transilluminator: JFSI - isolate from unit 1, JFS2- isolate from unit 2 and JFS3- isolate from unit 3

4.2.4 Pathogenicity tests

The inoculated tomato plants showed bacterial wilt symptoms including yellowing of the younger lower leaves, dropping of the leaves, and wilting of the plants' leaves and stems during daytime. These were observed two weeks after transplanting and inoculation (Figure 4.4). One month later after inoculation with the pathogen, there was the formation of brown discoloration of vascular bundles in the stem above the soil layer.

Figure 4.4: Tomato plants showing bacterial wilting symptoms after two weeks of inoculation: (a) a tomato plant inoculated with *R. solanacearum* isolated from JKUAT farm, (b) a tomato plant inoculated with *R. solanacearum* obtained from International Potato Centre (CIP) and (c) a tomato plant not inoculated with *R. solanacearum*

4.3 Antimicrobial activities of BSF-based pupal exuviae chitin and chitosan against *Ralstonia solanacearum*

4.3.1 *In vitro* antimicrobial activities of BSF-based pupal exuviae chitosan test on *Ralstonia solanacearum*

Chitosan extracted from the pupal exuviae of black soldier fly demonstrated antimicrobial effects against *R. solanacearum* as indicated by clear zones on the inoculated MHA agar plates (Figure 4.5). Larger zones of inhibition were observed at 5 g/ml concentrations of chitosan. The negative control did not produce inhibition zones.

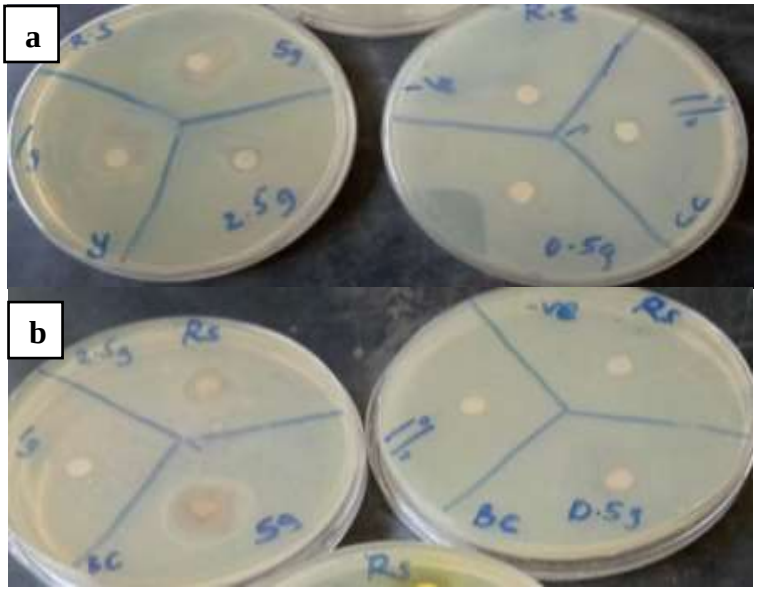

Figure 4.5: MHA plates showing antimicrobial activities of; (a) commercially obtained chitosan, (b) BSF- derived chitosan against *R. solanacearum*.

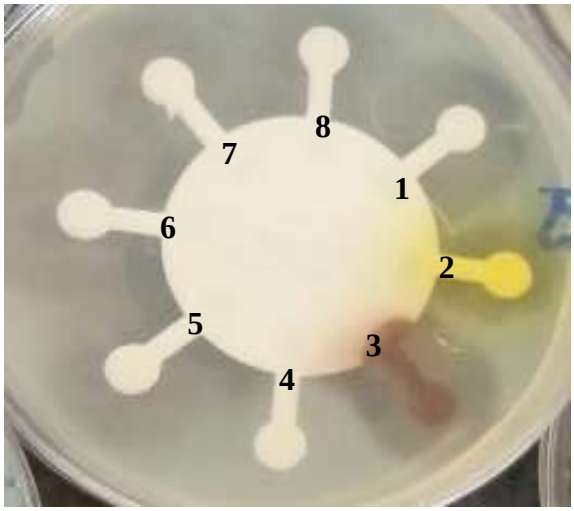

Figure 4.6: MHA plates showing antimicrobial activities of Conventional antibiotic discs, (1) nalidixic acid (2) nitrofurantoin (3) tetracycline (4) ampicillin (5) gentamycin (6) Cotrimoxazole (7) streptomycin (8) sulphamethoxazole against *R. solanacearum*

Based on comparative study, no significant difference was observed in diameters of zones of inhibitions produced by BSF-based pupal exuviae chitosan and commercial

obtained chitosan at $p \leq 0.05$. However significant differences were observed in antibacterial activities of BSF chitosan, commercial chitosan and tested antibiotics at $p \leq 0.05$. Highest zones of inhibitions were observed in nalidixic acid, cotrimoxazole, gentamycin and tetracycline while ampicillin did not show any inhibitions (Figure 4.6 & Table 4. 2).

Table 4.2: Antimicrobial effects of BSF, commercially obtained chitosan, and conventional antibiotic discs against *R. solanacearum.*

Treatment	Inhibition zone(mm ± standard error of the mean)	p-value
Nalidixic acid (30 µg)	26.00 ± 2.35^{a}	0.002
Cotrimoxazole (25 µg)	21.00 ± 2.35^{b}	0.021
Gentamycin (10 µg)	20.00 ± 2.35^{b}	<0.001
Tetracycline (100 µg)	20.00 ± 2.34^{b}	0.002
BSF Chitosan (5g/ml)	19.83 ± 1.17^{c}	0.004
Commercial chitosan(5g/ml)	18.50 ± 1.17^{c}	<0.001
Sulphamethoxazole (200 µg)	16.67 ± 2.35^{d}	0.021
Nitrofurantoin (200 µg)	15.67 ± 2.34^{d}	0.002
1% acetic acid without chitosan	11.67 ± 2.35^{e}	0.002
Streptomycin (25 µg)	10.67 ± 2.35^{e}	0.041
Ampicillin (25 µg)	0.00 ± 0.00^{f}	<0.001
Sterile distilled water	0.00 ± 0.00^{f}	<0.001

Data shown are means of three replications. Means with different letter(s) (superscript) are significantly different at $p \leq 0.05$

4.3.2 Effects of BSF-based pupal exuviae chitin and chitosan on *Ralstonia solanacearum* population in soil

Before chitin and chitosan were applied to the soil, average *R. solanacearum* population in soil were 8.9×10^{6} cfu/g. After 30 days the pathogen population

increased by 14.6% in non-amended soil (control). On the other hand, decrease in population was observed in the soil with different concentration of BSF pupal exuviae chitin and chitosan (Figure 4.7). The pathogen populations were reduced by 20.2% and 21.3% in BSF pupal exuviae chitin and chitosan treated soil respectively. Chitin and chitosan showed no significant difference in the reduction of the pathogen population at $p \leq 0.05$.

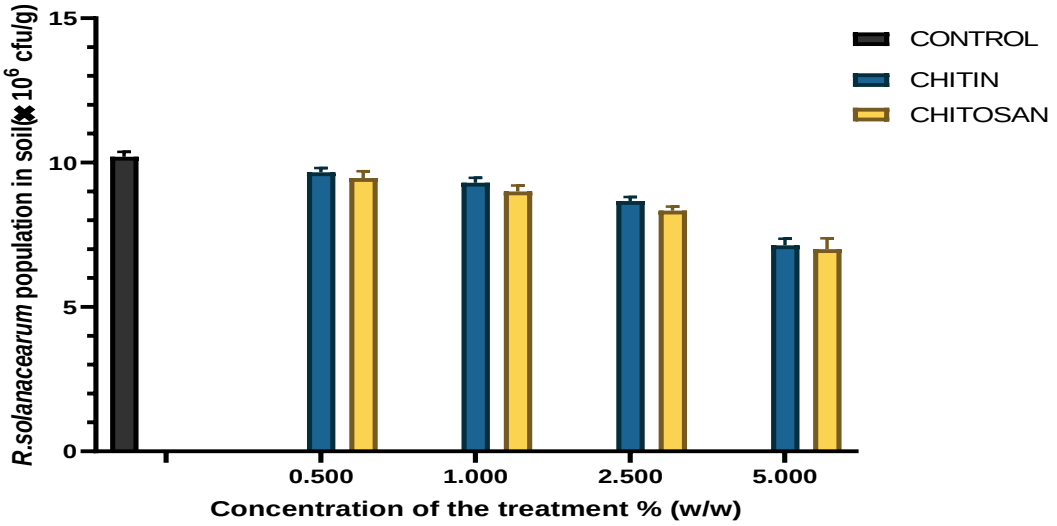

Figure 4.7. Effects of BSF-based pupal exuviae chitin and chitosan on *R. solanacearum* population in soil (cfu/g) after 30 days of treatment application

Sixty days after treatment application, the population of the pathogen increased by 5.6% in the control where treatments were not applied. However, there was a reduction in the population of the pathogen in soils with different concentrations of BSF pupal exuviae chitin and chitosan. The reduction in populations of the pathogen increased with increase in concentration with significant reduction recorded at 5% w/w for both treatments (Figure 4.8). Five percent chitin and chitosan reduced *R. solanacearum* population by 57.8% and 58.2% respectively. There was no significant difference in the reduction of the pathogen population by chitin and chitosan at $p \leq 0.05$.

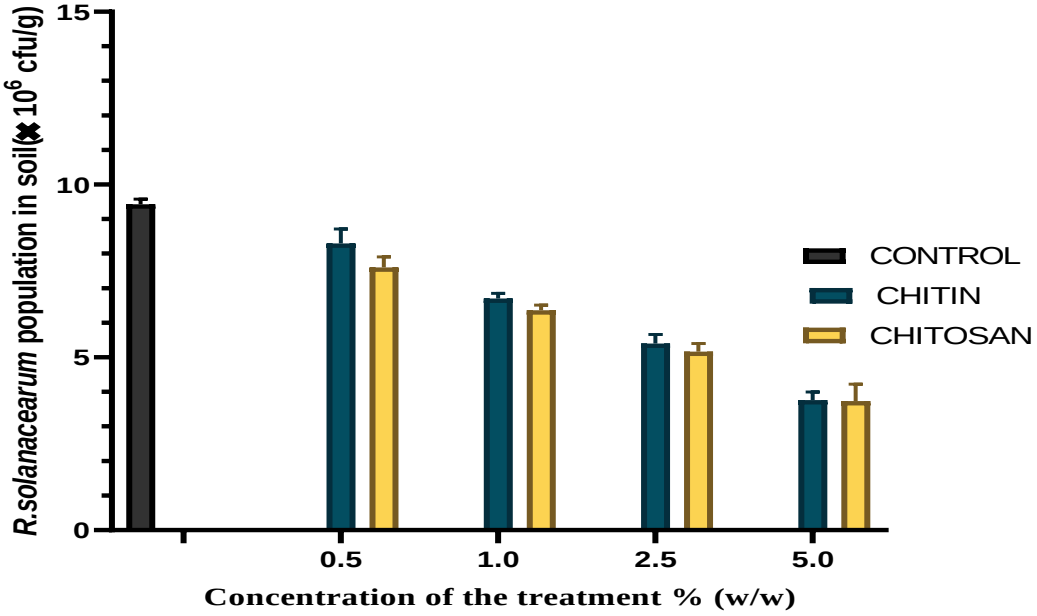

Figure 4.8: Effects of BSF-based pupal exuviae chitin and chitosan on *R. solanacearum* population in soil (cfu/g) after 60 days of treatment application

4.4 Effects of BSF-based pupal exuviae chitin and chitosan on bacterial wilt disease incidence and severity on tomato

There was a reduction of disease incidence in tomato plants treated with BSF-based pupal exuviae chitin and chitosan at 30.31% and 34.95% respectively. The disease severity was also reduced by 22.57% and 23.66%, respectively, compared to the control. There was no significant difference in reduction in disease incidence and severity between BSF-based pupal exuviae chitin and chitosan. However, the treatments showed a significant difference ($p<0.001$) with control. (Table 4.3).

Table 4.3: Bacterial wilt disease incidence (DI) and disease severity (DS) after 30days of inoculation.

Treatment (20g in 200g cocopeat)	Mean % disease Incidence ± Standard error of the mean	Mean % disease severity ± Standard error of mean
BSF-based pupal exuviae chitin	30.31 ±1.36[a]	22.57±2.01[a]
BSF-based pupal exuviae chitosan	34.95 ± 0.60[a]	23.66±1.15[a]
Control	44.78±1.56[b]	36.95±1.49[b]
p – values	<.001	<.001

Data shown are means of three replications. Means in a column followed by the different letter are significantly different at $p \leq 0.05$.

Wilting symptoms were more in non-treated plant than BSF-based pupal exuviae chitin and chitosan treated plant (Figure 4.9).

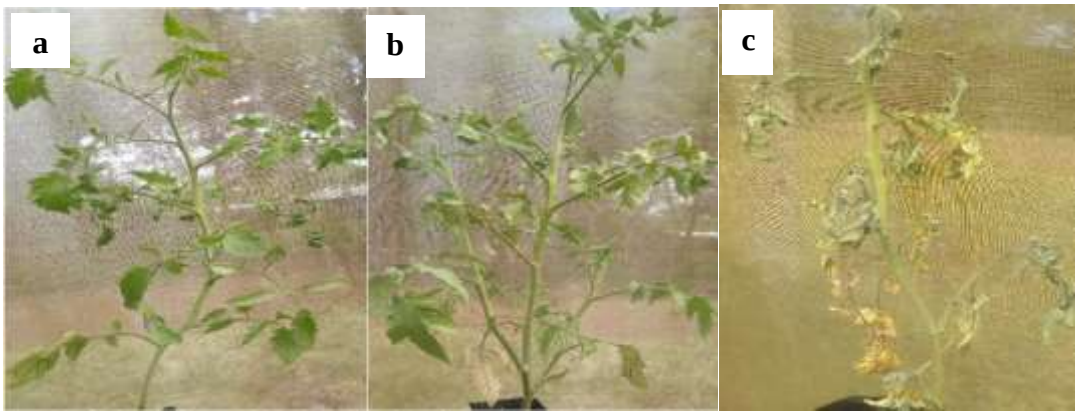

Figure 4.9: Wilting symptoms in (a) BSF-based pupal exuviae chitosan treated plant, (b) BSF-based pupal exuviae chitin treated plant, and (c) non-treated plant.

CHAPTER FIVE

DISCUSSION

5.1 Isolation and Characterization of *Ralstonia solanacearum*

Kelzman's tetrazolium chloride (TZC) media-enabled successful isolation and differentiation of virulent and avirulent strains of *R. solanacearum* from the soil samples collected from the JKUAT farm. Most colonies on the observed plates appeared as fluidal, irregularly round white with a pale red to the pink center on TZC media and were considered virulent isolates as per Balabel, (2018) previous description.

Gram stain reactions and biochemical tests conducted confirmed the *R. solanacerum* isolates. The isolates which appeared as pink short rods were similar to observations reported by Mutimawurugo *et al*., (2019) where all isolated *R. solanacearum* from three regions in Rwanda were Gram-negative short rods. This is because *R. solanacearum* is a Gram-negative rod-shaped bacterium and that all plant pathogenic bacteria are usually Gram-negative except *Streptomyces* and *Clavibacter* (Razia *et al*., 2021).

Potassium Hydroxide (KOH) solubility tests conducted on all the isolates in this study resulted to the formation of viscous thread similar to findings that were reported by Teli *et al*. (2018) where the formation of mucoid or slime thread was observed when the bacterial suspension of *R. solanacearum* was raised from a glass slide containing 3% KOH. The formation of viscous, mucoid, or slime thread is due to the outer membrane of Gram-negative bacteria being readily disrupted when exposed to 3% KOH releasing the viscous DNA (Khasabulli *et al*., 2017b).

The catalase test showed that all the isolates from this study were able to produce gas bubbles when they were mixed with a drop of 3% hydrogen peroxide hence were considered as catalase positive. These findings conform to those found by Hossain *et al*., (2021) , who reported that *R. solanacearum* isolated from papaya in Bangladesh

were catalase-positive. The production of gas bubbles reveals that *R. solanacearum* is able to produce catalase enzyme (Tondo *et al*., 2020).

All isolates of *R. solanacearum* in this study were able to produce gas from glucose after 24 h of incubation hence was considered positive for the gas production test. This was also observed by Khasabulli *et al*. (2017), who found that *R. solanacearum* was able to produce gas within 18 h of incubation. The production of gas can be ascribed to the presence of enzyme systems in the bacteria that are necessary for oxidation and utilization of simple sugars (Borines *et al*., 2019).

The isolates investigated were not able to hydrolyze starch. This was indicated by the absence of clear zones on the agar after incubation, confirming that the isolates were *R. solanacearum*. The findings were similar to the results of the studies conducted by Sharma and Singh, (2019); Bawari and Narendrappa, (2019) who found that *R. solanacearum* were unable to hydrolyze starch. These observations means that *R. solanacearum* are unable to produce exoenzymes particularly amylases enzymes that hydrolyze starch.

Based on the molecular characterization of the samples, the bands produced were in the range of 372 bp, confirming that the isolate was *R. solanacearum.* These findings concurred with that of Paudel *et al*. (2020) who observed a band size of 372 bp generated for primers Nmult21:2F/Nmult2: RR.

The isolates of *R. solanacearum* were able to cause infection and disease in tomato plants. These observations are similar to those reported by Kambura, (2020) where *R. solanacearum* isolated from diseased tomato plants were able to cause wilting symptoms in tested tomato plants. The causing of wilting in tomato plants can be explained by various pathogenicity and virulent traits associated with *R. solanacearum*.

5.2 Antimicrobial Activities of BSF-based pupal exuviae chitin and chitosan against *Ralstonia solanacearum*

5.2.1 In vitro Antimicrobial Activities of BSF-based pupal exuviae chitosan test on *Ralstonia solanacearum*

BSF-based pupal exuviae chitosan demonstrated antibacterial activities against *R. solanacearum* as indicated by zones of inhibitions on Petri dishes in this study. The findings are in line with those of Gatahi (2017) who reported that chitosan obtained from commercial chitin had antibacterial activities against *R. solanacearum*. In another study by Leke-Aladekoba (2018), chitosan extracted from black soldier fly meal demonstrated antimicrobial activities against various human pathogens including *E. coli*, *S. aureus*, *P. aeruginosa* and *C. albicans*. Lucia *et al*. (2019) also reported that chitosan extracted from shells of *Prapenaeus longirostris* showed growth inhibition against *R. solanacearum*.

The exact mechanisms of antimicrobial activities of BSF-based pupal exuviae chitosan are not well known. However, there are some proposed modes of action of chitosan reported in previous studies. The most known mechanism involved in antimicrobial activities is attributed to the presence of amino groups on the chitosan structure that cause electrostatic interaction between their positive charges and negative charges of lipopolysaccharide on the bacterial cell membrane (Morin-Crini *et al*., 2019). The electrostatic interaction has been known to disrupt the cell wall and cell membrane thus interfering with its permeability. Another postulate is that chitosan anionic charges (amino group) bind to the negatively charged phosphate groups on DNA and amino acids of the proteins thus inactivating or inhibiting messenger RNA (mRNA) and protein synthesis (Kumaraswamy *et al*., 2018). Chitosan can also inhibit bacterial growth by direct toxicity and chelating essential nutrients and metals (Razia *et al*., 2021). Chitosan is able to prevent biofilm formation by pathogenic bacteria such as *R. solanacearum*, *P. aeruginosa* and *Listeria monocytogens* (Kim *et al*., 2018).

5.2.2 Effects of BSF-based pupal exuviae chitin and chitosan on *Ralstonia solanacearum* population in soil

BSF pupal exuviae chitin and chitosan showed reduction in the population of *R. solanacearum* in soil after 30 and 60 days of application compared to the non-treated soil where the pathogen population increased. There were significant differences in the population changes after 30 and 60 days whereby after 60 days the population in the control increased at lower percentage while the population decreased significantly in the treated soil. There was no significant difference in reduction of *R. solanacearum* population between chitin and chitosan. This reduction in the *R. solanacearum* population is in consistent with the findings of in Andreo-Jimenez *et al*., (2021) who reported that soil treatment with chitin and other organic amendments suppressed *Rhizoctonia solani* population in soil. Another study by Randall *et al*., (2020) reported similar findings where soil amended with chitin significantly suppressed the levels of *Fusarium oxysporum* f.sp. *lactucae.*

This observation could be ascribed to the fact that chitin and its derivatives application to soil increases the activities of chinolytic microorganisms such as *Bacillus subtilis*, *Trichoderma* and *Pseudomonas* species which have antagonistic effects to *R. solanacearum* (Shahrajabian *et al*., 2021). Increase in population of chinolytic microorganism stimulates competition for space and nutrients which may limit growth of *R. solanacearum* and other soil phytopathogens. Similarly, addition of chitinous materials in soil avails carbon and nitrogen nutrients promoting growth of antagonistic bacteria (Wieczorek *et al*., 2019). Additionally, amendment of soil with chitin and its products causes suppression of soil pathogen indirectly by its products such as ammonia formed during its decomposition by chitin degrading microorganisms (Shimoi *et al*., 2020).

5.3 Effects of BSF-based pupal exuviae chitin and chitosan on bacterial wilt disease incidence and severity on tomato

Chitin and Chitosan from BSF pupal exuviae were observed to reduce bacterial wilt disease incidence and disease severity on tomato test plants in this study. These

findings are in line with *in vitro* studies conducted whereby BSF-based pupal exuviae chitosan produced antimicrobial activities against *R. solanacearum* and also both BSF-based pupal exuviae chitin and chitosan reduced population of *R. solanacearum* in cocopeat. Additionally, these results conform to those reported by Gatahi, 2017 who found that commercial chitosan was able to reduce bacterial wilt caused by *R. solanacearum* in soil.

These findings can be explained by the fact that chitosan is able to produce direct antimicrobial activities against the pathogen through disruption of cell membrane, chelation of essential nutrients and inhibition of biofilm formation (Khairy *et al*., 2021). Additionally, chitin and its derivatives are able to boost plant immune against pathogens by increasing phenolic contents, inducing activities of defense enzymes and maximizing expression of pathogen related proteins (Kumaraswamy *et al*., 2018). These characteristics make chitin and chitosan play an important role in protection against various pathogens in plants.

5.4 Conclusions

From the findings of this study, the following were deduced;

i. Virulent *R. solanacearum* strains were isolated from soil collected from tomato growing fields at Jomo Kenyatta University of Agriculture and Technology farm. The isolates were confirmed to be able to cause bacterial wilt in tomato plants.
ii. This study demonstrated that BSF-based pupal exuviae chitosan has *in vitro* antibacterial activities of BSF-based pupal exuviae chitosan against *R. solanacearum*.
iii. This study found out that BSF-based pupal exuviae chitin and chitosan have the ability to reduce in *R. solanacearum* population in soil.
iv. BSF-based pupal exuviae chitin and chitosan showed capacity to reduce bacterial wilt disease incidence and severity.
v. Chitosan showed better results than chitin hence can be concluded that use of chitosan is the best approach.

5.5 Recommendations

From the findings of this study, the following should be taken and research further for establishment of proper bacterial wilt control strategy and increase tomato production;

i. BSF-based chitin and chitosan can be applied as integrated disease management strategies for bacterial wilt diseases in tomato and other commercially important crops.
ii. BSF pupal exuviae being a good source of chitin and chitosan can be used as soil amendment thus helping in suppressing *R. solanacearum* in soil.
iii. Further studies should explore the effects of direct application of the BSF pupal exuviae on *R. solanacearum* in soil.
iv. Further studies should explore the integration of other insect-based chitin and chitosan to manage bacterial wilt disease and contribute to increased tomato production.

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APPENDICES

Appendix I: Publication from peer reviewed journal

microorganisms

MDPI

Article

Biocontrol Potential of Chitin and Chitosan Extracted from Black Soldier Fly Pupal Exuviae against Bacterial Wilt of Tomato

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Abstract: Globally, *Ralstonia solanacearum* (Smith) is ranked one of the most destructive bacterial pathogens inducing rapid and fatal wilting symptoms on tomatoes. Yield losses on tomatoes vary from 0 to 91% and most control measures are unaffordable to resource-poor farmers. This study investigated the antimicrobial activities of chitin and chitosan extracted from black soldier fly (BSF) pupal exuviae against *R. solanacearum*. Morphological, biochemical, and molecular techniques were used to isolate and characterize *R. solanacearum* for in vitro pathogenicity test using disc diffusion technique. Our results revealed that BSF chitosan significantly inhibited the growth of *R. solanacearum* when compared to treatments without chitosan. However, there was no significant difference in the antibacterial activities between BSF and commercial chitosan against *R. solanacearum*. Soil amended with BSF-chitin and chitosan demonstrated a reduction in bacterial wilt disease incidence by 30.31%

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