INDUCTION OF SYSTEMIC ACQUIRED RESISTANCE AGAINST *Ralstonia solanacearum* IN IRISH POTATO

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A Thesis Submitted to the Graduate School in Partial Fulfilment of the Requirements for the Award of the Degree of Master of Science in Microbiology and Biotechnology of Chuka University

CHUKA UNIVERSITY

OCTOBER 2023

DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has not been presented for an award of a diploma or conferment of degree in any institution.

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Recommendation

The thesis has been examined, passed and submitted with our approval as University Supervisors.

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DEDICATION

This thesis is dedicated to my family members and friends. A special feeling of appreciation to my loving parents, Maurice Ooga and Seline Otieno whose love, support and words of encouragement towards my academic journey have been immense.

ACKNOWLEDGEMENT

I sincerely thank the almighty God for his grace, faithfulness, sustenance and love from the beginning of my academic life. He has given me strength during the challenging moments towards completing this thesis and I am grateful for His endless love.

I would like to express my deepest gratitude to my supervisors, Prof. Moses M. Muraya and Dr. Eric K. Kuria for their guidance, encouragement and support during this entire process. Their continual supervision, insightful comments and valuable feedback were very substantial in conceptualization of this work, proposal writing, conducting experiments and writing of the thesis. Through your assistance I have learned to think critically, identify problems and solve them. Once again, I thank you for accepting to read through the thesis from its very first draft and helping in its improvement.

Special credit to Chuka University management for the award of internal research grant to conduct my research effectively. I am grateful to Prof. Eunice W. Githae, the Chairperson of the Department of Biological Sciences, Chuka University, for your support especially on those crucial occasions when some documents needed to be signed urgently. To all the staff in microbiology, biotechnology, zoology, chemistry and biochemistry laboratories, I am indebted to you for your continued support, guidance and analysis of Ultra-Violet spectrophotometric data during laboratory experiments. To Chuka University farm management and plant science department, thank you so much for allowing me to use the greenhouse to carry out my controlled experiments without any interruptions.

I wish to express my genuine gratitude to my parents and siblings for their financial support, exceptional love, words of encouragement and prayers. Their constant support and prayers for me and my progress during this entire journey has allowed me to successfully complete this project. Lastly, I would like appreciate Ruth, Irene, Stephen and Kennedy for their continued support during this period. Everyone who had been there for me emotionally, spiritually and intellectually throughout my academic years, I thank you all.

ABSTRACT

Irish potato (*Solanum tubesorum*) is an economically important crop throughout the world with large-scale production and consumption. Irish potato is rich in carbohydrates, vitamins and minerals, especially iron, magnesium and potassium. Irish potato production has been plagued by constraints such as lack of certified seeds, pests and diseases. Bacterial wilt caused by *Ralstonia solanacearum* is the major bacterial disease of potato resulting to 50 - 100% crop losses. Management of *R. solanacearum* is difficult since it has a wide host range and the fact that it is a soil borne pathogen. Several methods including biocontrol, chemical control, cultural practices, host resistance and phytosanitation have been used to manage *R. solanacearum*. However, there is no method that has been found to effectively control the disease but a combination of different methods have been reported to reduce the impact of the disease. Inducing systemic acquired resistance is another alternative to control bacterial wilt. Systemic acquired resistance is the response of a plant to a pathogen attack and confers long-lasting protection against a broad spectrum of microorganisms. Thus, this study determined the induction of systemic acquired resistance against bacterial wilt in Irish potato through injection of attenuated pathogen and non-pathogens. The ability of *Escherichia coli*, *Saccharomyces cerevisiae* and attenuated *R. solanacearum* to induce systemic acquired resistance against bacterial wilt of Irish potato was tested in the greenhouse. Attenuation assay was conducted using four levels of formaldehyde concentrations and incubation duration for attenuation of *R.solanacearum* using randomized complete block design. Randomised complete block design was also used to evaluate *in-vivo* effect of different treatments and varieties on *R. solanacearum* in the greenhouse. Bacterial wilt symptoms were observed and a rating scale of 0 - 6 was used to score for disease severity. Leaves of the treated plants were collected for salicylic acid quantification in the laboratory. Disease severity, colony count, amount of salicylic acid and tuber yield data were analysed using statistical analysis software version 9.4 to determine induction of systemic acquired resistance. Significant means were separated using Least Significant Difference test at 5% probability level. Result from this study showed that there was statistically significant effect ($p < 0.05$) of formaldehyde concentrations and incubation duration for attenuation of *R. solanacearum*. The results from attenuation of *R. solanacearum* showed that colony count ranged from 0.67 colony forming units (in 0.6% formaldehyde concentration and incubation duration of attenuation of two hours) to 233 colony forming units (in 0% formaldehyde concentration and incubation duration of attenuation of two hours). There was significant ($p < 0.05$) treatment effect on disease severity, salicylic acid levels and tuber yield of Irish potato. Higher disease severity was recorded in *S. cerevisiae* treatments (80%) followed by *E. coli* treatments (40%) while least disease severity was recorded in attenuated *R. solanacearum* treatments. Attenuated *R. solanacearum* induced the highest level of salicylic acid (3409.64 mg/kg of Irish potato leaves) while *R. solanacearum* induced the lowest (1222.74 mg/kg of Irish potato leaves). Irish potato plants with attenuated *R. solanacearum* treatment had the highest yield of tubers (0.5772 kg/plant) while those treated with *S. cerevisiae* treatments had the lowest (0.4840 kg/plant). This study concluded that attenuated *R. solanacearum* and *E. coli* triggered systemic acquired resistance in Irish potato plants thereby increasing the levels of salicylic acid, which reduced the negative impact of the disease in the plants. Induction of systemic acquired resistance is a viable method for induction of resistance against bacterial wilt in Irish potato. The study recommends the identification of the specific molecules involved in induction of resistance from attenuated *R. solanacearum* and *E. coli* and their utilization in the control of bacterial wilt in order to mitigate the huge loses that are caused by this disease.

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ACRONYMS

ANOVA:	Analysis of variance
ASM:	Acibenzolar-S-Methyl
CBP:	Calmodulin Binding Protein
EDS1:	Enhanced Disease Susceptibility 1
EDS5:	Enhanced Disease Susceptibility 5
HIR:	Herbivore Induced Resistance
ICS:	Isochorismate synthase
ISR:	Induced Systematic Resistance
JA:	Jasmonic Acid
MATE:	Multidrug and Toxin Extrusion
MeSA:	Methyl Salicylic Acid
NDR1:	Non-race specific Disease Resistant 1
PAD4:	Phytoalexin Deficient 4
PAL:	Phenylalanine ammonia-lyase
PAMPs:	Pathogen Associated Molecular Patterns
PCD:	Programmed Cell Death
PCN:	Potato Cyst Nematode
PRIs:	Plant Resistance Inducers
PRRs:	Pathogen Recognition Receptors
PTI:	PAMP Triggered Immunity
SA:	Salicylic Acid
SAR:	Systemic Acquired Resistance
SARD1:	SAR Deficient 1
UV:	Ultra violet

CHAPTER ONE

INTRODUCTION

1.1 Background to the Study

Irish potato (*Solanum tuberosum*) is cultivated as an annual crop. Globally Irish potato is ranked the fourth most important food crop after rice, wheat and maize while in Kenya, it is the second most important food crop after maize (Ndegwa *et al.,* 2020, FAOSTAT 2021). Therefore, Irish potato is an important crop for food security. Moreover, the human population growth and diversification of crops has led to an increase in potato production (Mutunga, 2014; NCPK, 2016). This makes Irish potato a target crop to improve food security and incomes for many farmers. The global Irish potato production has increased over time from 333.6 million metric tonnes in 2010 to 370 million metric tonnes in 2019 (Shahbandeh, 2021). Irish potato productivity in Africa stands at 20 tonnes ha^{-1} (Chilipa, et al., 2021) while in East Africa it is at 7.8 tonnes ha^{-1}. In Kenya, potato is an important crop grown by about 800,000 small scale farmers and employing almost 2.5 million people.

Irish potato is used in a variety of ways, which includes vegetables for cooking, processed into food products and some are consumed fresh. Irish potato is also utilized in non-food products like potato starch that is widely used by textile, pharmaceutical, wood and paper industries as an adhesive, texture agent and a binder. The crop is grown for its tuber and is rich in starch (7 - 29%), minerals and iron (Hameed *et al*., 2018; Robertson *et al*., 2018). Freshly harvested potato contains 80% water and 20% dry matter with 60 - 80% of the dry matter having starch. It is low in fat and rich in macronutrients majorly vitamin C when eaten with its skin. It is also a source of iron and minerals such as potassium, phosphorous and magnesium (FAO, 2008).

Irish potato grows over wide ecological conditions, with respect to rainfall, altitude, temperature and soils. It can grow in areas with rainfall ranging from 850 – 1200 mm, altitude of 1400 – 3000 m above sea level, temperatures between 12 – 20 ^{0} C and soil pH of 5.0 - 7.5 (KENFAP, 2013; Mwakidoshi *et al*., 2021). Altitudes lower than 2000 m are associated with higher disease pressures due to higher pest populations and incidences of bacterial wilt. Irish potatoes grow well in a wide variety of soils, with best production occurring between 5.5 and 6.8. However, the crop is less susceptible to scab when soil pH is between 5.0 and 5.5 (KENFAP, 2013).

Irish potato production has many constraints such as lack of high yielding cultivars, post-harvest losses, lack of access to credit by the farmers, insufficient clean seeds, pests and diseases. Most farmers prefer varieties, which are high yielding, disease tolerant and high dry matter content in tubers. Irish potato breeding has led to development of improved varieties that are tolerant to biotic and abiotic factors in order to mitigate sub-optimal growing environments. Plant breeding has led to development of many tolerant varieties that are high yielding under multiple stresses (Handayani *et al*., 2019). Some of the common varieties grown in Kenya include; Tigoni, Asante, Shangi, Kenya Mpya, Sherekea, Purple gold, Kerr's pink also (mew pink), Dutch robijn and Nyayo (Hardenburg, 1950; Kaguora *et al*., 2017).

The quantity and quality of potato produced have reduced due to pre- and post-harvest pests and diseases. One of the major diseases of potato is bacterial wilt which is also referred to as brown rot, southern wilt or sore eye. This disease is caused by *Ralstonia solanacearum* which was previously known as *Pseudomonas solanacearum* (Jiang *et al*., 2017). The disease leads to yield losses in the range of 50 - 100% (Muthoni *et al*., 2012). *Ralstonia solanacearum* is an aerobic, non-spore forming and gram- negative bacteria (Khairy *et al*., 2021). The bacterium is a soil borne pathogen, found in tropics and areas with warm climate that favours its development (Wang *et al*., 2023). Bacterial wilt attacks many species of plants for example crops such as tobacco, potato, tomato, pepper, banana and beans. The disease also attacks weeds such as thorn apple (Buyela, 2017). The bacterium penetrates the plants through wounds on the roots and base of stems and is spread by infected tubers, contaminated tools and soil, crop residues and water. It can survive in the soil for more than four years. Some of the symptoms of this disease include rapid wilting, brown rot in the vascular ring and slimy whitish discharge emanating from the eyes of the affected tubers (Zinnat *et al*., 2021).

There are several methods of controlling crop diseases, which includes use of chemicals, phytosanitation, use of certified seeds and resistant varieties, soil sterilization and crop rotation (Finch and Lane 2014; Marburger *et al*., 2015; Olowe, 2018). However, biological and cultural methods are of limited value to subsistence farmers. Diminishing acreage available for crop production makes crop rotation unrealistic. Moreover, the disease has a wide range of host plants. For example, *Ralstonia solanacearum* has over 400 plant hosts belonging to over 50 families, hence, impractical to control it using cultural practices. Pesticide application has negative environmental effects, risks to human health and can also weaken ecosystem stability. Chemicals have a side-effects on the abundance and the species diversity of the natural populations (Delpuech & Allemand, 2011). The use of resistant cultivars can be an alternative in the bacterial wilt management but introgression of R genes is considered a 'species complex' and has significant variation with ecological conditions. For example, it has been shown that stress conditions greatly affect gene expression (Arbona *et al*., 2013). Therefore, there is need to seek alternative methods to control this versatile pathogen.

Resistance breeding is one of the strategies that have been used to combat crop pathogens in modern agriculture without compromising the population dynamics of beneficial microbes (Zuccaro & Langen, 2020). Traditional breeding has been used to introduce resistance genes from wild relatives but this is time-consuming since many pathogens adapt rapidly (Fry, 2008). Innate immunity of plants can be enhanced by plant resistance inducers (PRIs) since they work indirectly on the pathogen through their innate immunity. Plant resistance inducers are more environmentally sustainable with less impact on human health as compared to chemicals (Kromann *et al*., 2012).

There are two types of systemic resistance in plants i.e. systemic acquired resistance (SAR) and induced systemic resistance (ISR). Systemic acquired resistance is the induction of local responses associated with the transport of defense signals throughout the plant resulting in a broad-spectrum disease resistance against secondary infections (Gao *et al*., 2015). Systemic acquired resistance is a type of induced resistance that is activated in a plant after exposure to elicitors from virulent, avirulent or artificial chemical stimuli such as chitosan and salicylic acid. It is a whole plant resistance that occurs when a plant has been exposed to a pathogen or simply as the plants response to a pathogen attack (Winter *et al*., 2014). Resistance in ISR is activated by infection and depends on increasing physical or chemical barrier of the host plant. In both SAR and

ISR, plants are preconditioned prior infection or treatment that results in resistance against a pathogen or a parasite attack. Such type of resistance can be induced locally or systematically by application of a variety of biotic and abiotic agents that leads to a broad-spectrum and long-lasting resistance. Biological inducers include necrotizing pathogens, non-pathogens and root colonizing bacteria. Most of the inducing agents reduce disease by 20-85% (Ons *et al*., 2020). The expression of induced resistance is highly influenced by environment, genotype and crop nutrition since it is a host dependent response (Walters *et al*., 2013). Several chemicals have been studied and found to activate networks and mimic all or parts of the biological activation resistance. The chemicals include acidbenzolar-5-methyl (BION) that activates resistance in many crops against a broad spectrum of diseases and is translocated systemically in the plants. The other chemical is probenzole (ORYZEMATE) that is mostly used in rice against blast and bacterial leaf blight (Oostendorp *et al*., 2001).

Bacterial wilt can be controlled by inducing systemic acquired resistance (SAR) into the plants (Patil *et al*., 2012). Once acquired, SAR can confer long-lasting protection against a broad spectrum of microorganisms (Durrant and Dong, 2004). Establishment of SAR involves generation and transport of signals through the phloem to the uninfected tissues. Salicylic acid (SA) and jasmonic acid are phytohormones that play an important role in transducing the activation of plant defense systems against pathogen attack (Tamaoki *et al*., 2013). Systemic acquired resistance requires the signal molecule SA, which is associated with accumulation of pathogenesis-related proteins that contribute to resistance (Durrant & Dong, 2004). Systemic acquired resistance can be characterized by the increased expression of a large number of pathogenesis-related genes, in both local and systemic tissue. However, SA can serve as a signal for the induction of SAR. Salicylic acid can be measured to determine its levels in the plants. There are several methods that can be used to quantify the level of salicylic acid in plants. They include use of solid-phase extraction and isocratic high-performance anion-exchange chromatography, High Performance Liquid Chromatography (HPLC) system, biosensor- based method and use of spectrophotometry (Rozhon *et al.,* 2005; Defraia *et al.,* 2008).

Chemical and biotic inducers have been used to induce SAR against *R. solanacearum*. Pradhanang *et al*. (2005) used a chemical elicitor, acibenzolar-S-methyl (ASM) to induce SAR against *R. solanacearum* in tomato which significantly reduced bacterial wilt incidence in moderately resistant cultivars. Acibenzolar-S-methyl has been shown to increase host resistance to bacterial wilt of tomato from moderate to a significantly higher level (Pradhanang *et al*., 2005). Hassan *et al*., (2009) used plant extracts of *Hibsicus sabdariffa, Punica granatum* and *Eucalyptus globulus* to protect potato plants against bacterial wilt under greenhouse and field conditions. The treatments of these extracts reduced disease severity with their applications resulting to similar reductions of disease severity. All the tested plant extracts were able to inhibit the growth of bacterial pathogen by inducing systemic resistance in potato plants. Fungal and bacterial elicitors have also been used to induce SAR against *R. solanacearum*.

Previous studies have shown that human enteric pathogens can induce defense responses upon colonization. Although human pathogens are able to colonize plants and induce plant defense responses, certain enteric pathogen mechanisms differ from those of phytopathogens. PeaT1, a protein from *Alternaria tenuissima* produced in *E. coli* was used to treat tobacco leading systemic resistance and significant reduction in both number and size of TMV lesions in the leaves (Zhang *et al*., 2011). Another PRI, chitosan produced by crustaceans improves resistance against *R. solanacearum*, *F. oxysporum* and *P. capsici* in tomato by inducing SAR and stress-related pathways (Kiirika *et al*., 2013). This study induced SAR against *R. solanacearum* in potato by treating plants with attenuated pathogens and microorganisms that are non-pathogenic to plants and screening resultant plants for resistance under greenhouse conditions.

1.2 Statement of the Problem

Irish potato plays a major role in food security in Kenya, as they are high in carbohydrates, dietary fibre, vitamins (A and C) and minerals like potassium, calcium, iron and magnesium. Despite their importance, the production of Irish potatoes has been constrained by many abiotic and biotic factors with bacterial wilt being the most prominent. Bacterial wilt is caused by *Ralstonia solanacearum*, which is a soil-borne pathogenic bacterium. The disease causes loss in yields in the range of 50 - 100%. Some of the methods that have been used to control bacterial wilt in potatoes include cultural practices, use of chemicals, phytosanitation and use of resistant varieties. These methods have not been able to effectively curb the disease besides some being expensive, impractical and ineffective since the bacterium has an unusually wide host range with over 400 hosts distributed over 50 families. There is, therefore, need to explore alternative methods for controlling the disease such as induction of SAR using biotic inducers. Most of the inducers that have been used to induce SAR in various plants are chemicals. Previously, acibenzolar-S-Methyl (ASM) has been used as an elicitor in inducing SAR against *R. solanacearum* in tomatoes and the results were positive. Several biotic inducers such as *E. coli* proteins and fungal elicitor chitosan have been shown to induce systemic resistance leading to significant reduction in disease severity in the plants. However, information on their application and effectiveness in inducing SAR in Irish potato is lacking. Therefore, there is need to come up with ways of developing resistance in potato plants against *R. solanacearum*.

1.3 Objectives of the Study

1.3.1 General Objective

To induce systemic acquired resistance against *Ralstonia solanacearum* in Irish potato.

1.3.2 Specific Objectives

i. To determine the effect of formaldehyde concentration and duration of incubation on the number of colonies of attenuated *Ralstonia solanacearum.*
ii. To evaluate the effect of *Escherichia coli*, *Saccharomyces cerevisiae* and attenuated *Ralstonia solanacearum* on severity of bacterial wilt in selected Irish potato varieties.
iii. To determine the effect of *Escherichia coli*, *Saccharomyces cerevisiae* and attenuated *Ralstonia solanacearum* on the level of salicylic acid in selected Irish potato varieties.
iv. To determine the effect of induced systemic acquired resistance (*Escherichia coli*, *Saccharomyces cerevisiae* and attenuated *Ralstonia solanacearum* treatment) on yield of selected Irish potato varieties infected with bacterial wilt.

1.4 Hypotheses

H_{01}: There is no significant effect of formaldehyde concentration and duration of incubation on the number of colonies of attenuated *Ralstonia solanacearum*

H_{02}: There is no significant effect of *Escherichia coli*, *Saccharomyces cerevisiae* and attenuated *Ralstonia solanacearum* on severity of bacterial wilt in selected Irish potato varieties

H_{03}: There is no significant effect of *Escherichia coli*, *Saccharomyces cerevisiae* and attenuated *Ralstonia solanacearum* treatment on the level of salicylic acid in selected Irish potato varieties infected with bacterial wilt.

H_{04}: There is no significant effect of induced systemic acquired resistance (*Escherichia coli*, *Saccharomyces cerevisiae* and attenuated *Ralstonia solanacearum*) on yield of selected Irish potato varieties infected with bacterial wilt.

1.5 Justification of the Study

Bacterial diseases are a major problem faced by Irish potato farmers in Kenya (Kwambai *et al*., 2022). Bacterial wilt is a major disease that has spread to all potato growing areas in Kenya attacking over 70% of potato farms resulting to yield losses of between 50% - 100% (Muthoni *et al*., 2012). This has been attributed to use of contaminated crop residues (manure used for soil enrichment) and infected tubers by the farmers. *Ralstonia solanacearum*, the causative agent for bacterial wilt, has a wide host range including the weeds (Yuliar *et al*, 2015). The bacterium survives in the soil for a long period making it difficult to control even with the use of certified seeds and uncontaminated tubers (Muthoni *et al*., 2014). Therefore, there is need to come up with alternative methods of controlling bacterial wilt, especially those that are less reliant on chemicals.

Inducing systemic acquired resistance in potato plants is an alternative method that can be used to control bacterial wilt. The findings of this study will provide an avenue for development of a control strategy for bacterial wilt disease. Consequently, benefiting the farmers who majorly depend on tubers from previous harvest as planting materials. The findings of this study will be beneficial to all the Irish potato growers and the agricultural organizations in Kenya and elsewhere as information on use the of microbes will be availed to organizations that will use it to come with a control strategy for bacterial wilt providing an alternative method of controlling the disease. Induction of SAR by attenuated *Ralstonia solanacearum*, *Escherichia coli* and *Saccharomyces cerevisiae* will lead to development of control strategy that is efficient, environment friendly and cost effective eventually leading to increased and sustainable production of Irish potatoes.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview Production and Utilisation

2.1.1 Potato Production

Potato (*Solanum tuberosum)* belongs to *Solanaceae* or nightshade family that includes tobacco, tomato, eggplant and mandrake. It originated from Peru in South America and has been cultivated there for over 8000 years (Randall *et al*., 2010). Potato is currently cultivated throughout the world and ranks fourth in food crop production (FAO, 2019). It is an herbaceous perennial plant, which is grown for its edible tubers. The potato plant has a branched stem and alternately arranged leaves consisting of leaflets which are both of unequal size and shape.

Potato has five growth stages (Liu *et al*., 2020; Kacheyo *et al*., 2020). During the growth stage I, the seed tuber is the only source of energy. It begins with sprouts developing from the eyes and emerging from the soil. The stems of the potato also sprout from the eyes. At growth stage II the leaves, branches, roots and stolon are formed. This stage begins at emergence from the soil and lasts until tubers starts to develop. The first two growth stages takes a period of 30 - 70 days depending on environmental factors (KENFAP, 2013).

In growth stage III, tubers start forming at the stolon tips and this occurs when the plant produces more carbohydrate. Initially the plant produces about 20 - 30 tubers but only 5 - 15 reaches maturity. The number of tubers that reach maturity is dependent on available moisture and nutrition. This stage takes about two weeks (Liu *et al*., 2020). In growth stage IV, tuber cells expand with the accumulation of water, nutrients and carbohydrates. It is the stage that takes the longest duration lasting up to three months but mostly lasts 45 - 60 days. In the last growth stage, photosynthesis decreases with leaves turning yellow and becoming lose, tuber growth rate slows and the vines die. The stage is also characterized by increase in dry matter content (Kacheyo *et al*., 2020). The plant matures and tubers are ready for harvest (Alam & Rustgi, 2020).

2.1.2 Utilization of Potato

Potato ranks fourth in world food production and second most important food and cash crop after maize in Kenya (Chapman, 2012; NCPK, 2016). It is grown by approximately 800,000 small scale farmers thus generating employment to people in Kenya and playing an important role in food security (NCPK, 2016). Potato production contributes about 50 billion shillings to the economy of Kenya (Janssens *et al*., 2014). It is a source of livelihood for millions of Kenyans. Irish potato farming addresses the issue of low farm incomes in Kenya due to its high productivity per unit area and its versality in utilization. Therefore, potato crop plays a key role in curbing poverty and lack of employment. Potato production is successful in areas with rainfall between 850 mm-1200 mm and at altitudes of 1400 mm – 3000 mm above the sea level. The ideal soil should be well drained loamy soils with a pH of 5.5 - 7.5 and daily temperatures of 12 – 20 ^{0}C (Hardenburg, 1950; KENFAP, 2013). The above ecological conditions allows growth of potato to be carried out in the rift valley, central and eastern regions of Kenya (KENFAP, 2013).

Despite being an important crop, potato production potential has not been achieved due to a number of constraints. The problems plaguing the potato sector include soil infertility, lack of clean and certified seeds, low yielding varieties, poor marketing system, diseases and pests (Riungu, 2011; Muthoni *et al*,. 2013). A research carried out by Muthoni *et al*., (2013) revealed that over 75% of potato farmers cited diseases as the main constraint in potato production. The diseases and lack of certified seeds has led to poor quality produce and low yields (GIZ-PSDA, 2011; Riungu, 2011). Due to lack of certified seeds farmers use seeds from previous harvests or from the local markets which speeds the spread of diseases. Viruses, viroids, fungi, nematodes and bacteria are the most important causative agent of potato diseases affecting 77% of potato farms (Kaguongo *et al*., 2010; Muthoni *et al*., 2012). In Kenya, bacterial wilt has spread to all areas where potato is grown leading to loss in yields in the range of 50-100% affecting over 70% of potato farms in the country (Muthoni *et al*., 2014).

Many potato varieties are grown in Kenya and these includes Tigoni, Asante, Shangi, Kaumbere, Kenya mpya, Sherekea, Purple gold, Kerr’s pink also (mew pink), Dutch robin and Nyayo (Hardenburg, 1950). Different potato varieties are used for making different types of food for example; the Dutch robjin variety is used for making potato crisps (Randall *et al*., 2010). Tigoni variety is used for making frozen chips, stew and mashed food (Muthoni *et al*., 2014) because it is easy to cook.

2.2 Development and Spread of Bacterial Disease in Plants

A bacterial disease is a type of malfunctioning and destruction of plants caused by bacteria (Vivader *et al*., 2004). Bacteria depend on natural openings or wound for its entry and penetration into the plants. The wounds can be as a result of damage by insects, tools during field operations or even by other pathogens (Melotto *et al*., 2008). Once inside the plant, they only become active if there is a conducive environment for them to multiply and survive. The pathogen is spread through water splash or contaminated equipment and can survive in soil, debris and seeds (Tripathi, 2017).

Bacterial diseases that affect potatoes include potato ring rot caused by *Corynebacterium michiganese pv sepedonicus,* potato scab caused by *Streptomyces scabies*, bacterial blackleg or tuber soft rot caused by *Pectobacterium* and bacterial wilt caused by *Ralstonia solanacearum* (Charkowski *et al*., 2020). Bacterial wilt in potato is also referred to as jammy eyes or sore eyes because of the soil that stick on the bacterial ooze from the eyes as shown in plate 3. Infection of potato plants and tubers is via the soil into the plant through weak points or through the infected seed tubers. In Kenya, it was first reported in Embu County in the year 1945 and then later spread to other potato growing areas in the country (Muthoni *et al*., 2014). The disease was introduced by bacterial wilt infected tuber seeds that were imported from Europe (Plate 1).

Plate 1: Bacterial wilt infected potato tuber with soil stuck on the bacterial ooze from the eye of the tuber

Biosecurity, 2020

2.2.1 Bacterial Wilt Development and Spread in Solanaceae Family

Bacterial wilt is a bacterial disease that attacks more than 200 species including weeds such as nightshade and thorn apple. It is common worldwide and in Africa it has been reported in many countries (EPPO, 2014). Examples of solanaceous plants attacked by bacterial wilt include tomato, tobacco, potato, brinjal, chilli and capsicum which are used as food thus playing a common role in the human diet (Genin & Denny, 2012; Namisy *et al*., 2019) The disease is caused by *Ralstonia solanacearum*. In tobacco, it is referred to as Granville wilt and causes losses varying from 25-100%. Infected tobacco leaves, show unilateral wilt and yellowing with scorch- like symptoms on the edge of the leaves (plate 2). This is then followed by death of the plant. Tobacco plants that have not collapsed may have stunted and distorted leaves while the roots when dug out will clearly have rotting symptoms. The vascular system at an early stage has a yellow to brown colour but darkens as most parts of the tissue become infected leading to a hollow stem as the pith decays (Lucas, 1958; Rodriguez & Thiessen, 2019).

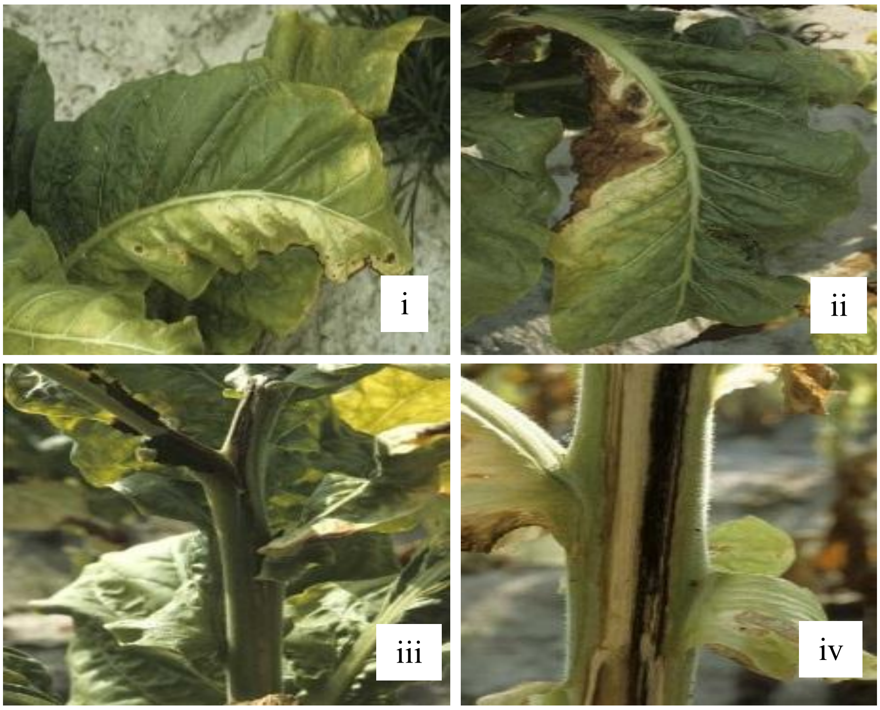

Plate 2: Development of bacterial wilt in tobacco. (i) Wilting and yellowing of tobacco leaf, (ii) Infected area turns light green then yellow and finally becomes necrotic and dries up, (iii) Longitudinal, dark brown to black streak in the stem and (iv) infected cortex and pith.

Rodriguez & Thiessen, 2019

In tomatoes, the first symptoms of the disease are observed on the foliage leaves which is wilting of the younger leaves (plate 3) at the time of the day when the temperatures are too high. The wilting only occurs in some parts of the leaf and the plant recovers when the temperature cools down. As the disease develops, the whole plant may wilt leading to general wilting, yellowing and stunted growth followed by collapse of the plant. In some circumstances the vascular bundles will be long, narrow and with dark brown streaks eventually leading to collapse of the stem. Cross-sectional cut of the stems shows brown discoloration of the infected tissues in a well-established infection (Ali Khan *et al*., 2020; Le *et al*., 2020)

Plate 3: Young tomato plant infected by bacterial wilt having wilted leaves drooping to the ground during the day

2.2.2 Bacterial Wilt Symptoms and Spread in Potatoes

The bacterium *R. solanacearum* causing bacterial wilt penetrates potato plants at the roots hence infection starts at the roots. They penetrate the plant through natural openings or wounds. Once inside the plant, the bacteria enters the vascular system and secrete enzymes which dissolve the cell walls thus creating cavities that are filled with slimy masses of bacteria. These substances then block the vascular system by inducing the formation of tyloses. The enzyme secreted by the bacterium degrades the host tissues (Kinyua *et al*., 2001).

Wilting is a visible symptom in severely infected plants during high temperatures while some plants may fail to wilt because of having some resistance against the disease or cold weather. This is then followed by permanent wilting which extends up to the stems and yellowing of the leaves. Starting at the base of the stem the leaves finally turn brown and fall off. The roots and tubers rot and disintegrate making them to turn brown and a cross-section oozes bacterial exudates. The disease development is favoured by conducive environmental conditions such as temperature range of 25-30 ℃ and limited temperature below 10 ℃. The bacterium is also favored by soil moisture since dry soil conditions limits and to some extent stops its growth (Osdaghi, 2020).

The bacterium is spread by water, contaminated equipment, plant debris and insect pests. Dissemination both locally and long distance by infected seeds is another way through which the disease is spread from one place to another. *Ralstonia solanacearum* overwinters in volunteer potatoes, diseased plant debris, seeds, soil and related species such as tomatoes, tobacco, nightshade and thorn apple (Muthoni *et al*., 2014; Biosecurity, 2020).

2.2.3 Life Cycle of *Ralstonia solanacearum*

Ralstonia solanacearum is a gram negative, non- spore forming and aerobic bacteria which is 0.5-0.7×1.5-2.0 micrometres in size (Johnson, 2003). It is a soil borne pathogen that causes bacterial wilt in solanaceous plants and survives in hot and temperate parts of the world. The bacterium survives on infected plant debris, volunteer plants, contaminated equipment and soil. It then penetrates the plant through natural openings or wounds created by field operations, nematodes or insect pests (Waleed & Mousa, 2016). Infection starts at the roots and spreads to the other part of the plant through the vascular bundles where bacteria multiply. Once in the xylem vessels, the bacterium is able to enter the intracellular spaces in the cortex and pith dissolving the cell walls. This results in cavities that are filled with slimy masses of bacteria and cell debris which clogs the vascular system thus wilting in the plants and eventual death.

The pathogen remains in the debris, seeds and volunteer plants after plant death. From the decaying plant, the pathogen returns to the soil where it survives until it gets a susceptible and suitable host to attack. The pathogen in the soil can be spread to healthy plants through running water and water splash *(*Alvarez *et al., 2010;* Alam & Rustgi, *2020)*. Once it has entered the host the cycle continues as illustrated in figure 4 below (Alam & Rustgi, 2020).

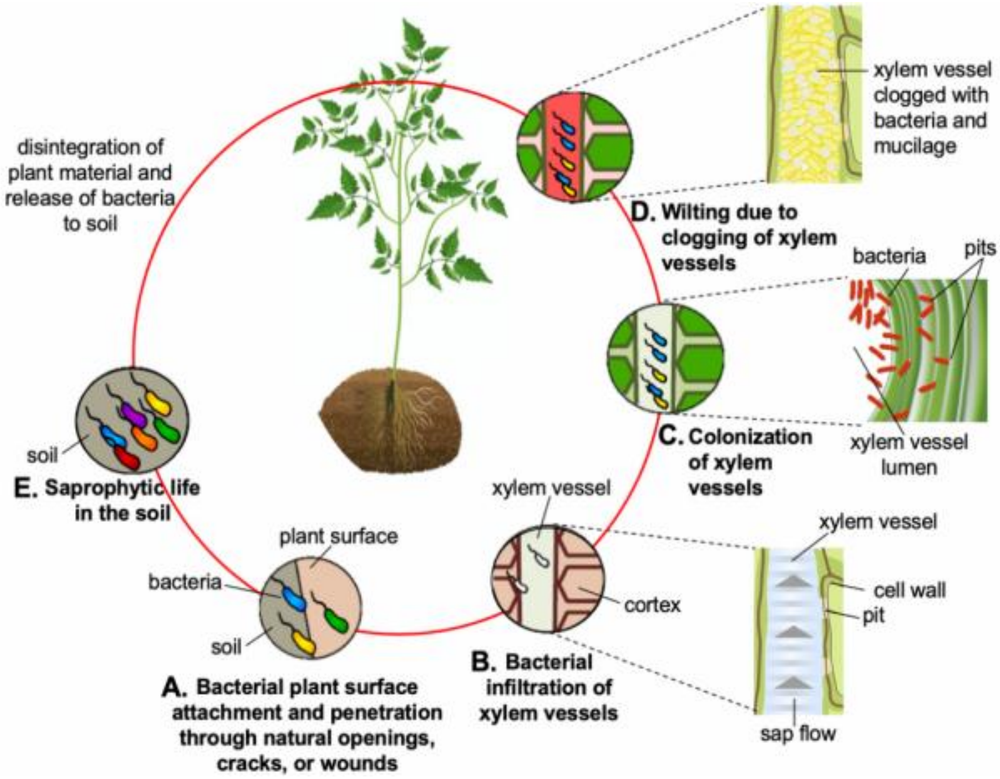

Figure 1: Phases of the life cycle of *Ralstonia solanacearum*.
Alam & Rustgi, 2020

The bacterium survives on soil or volunteer plants. A) Penetration through natural openings and wounds. B) Bacteria make their way through cortex to the xylem vessels. C) Bacteria colonize the vascular system. D) Bacteria enter the vessels, multiply, and fill them with cells debris and mucilage blocking the xylem vessel thus wilting. E) Wilting of the whole plant eventually leading to plant death. Bacteria released into the soil or survives on other reservoirs until the availability of a suitable and susceptible host.

2.2.3.1 Overview of *Ralstonia solanacearum*

The strains of this bacterium are subdivided into 5 races and 5 biovars based on the host range and catabolic properties (utilization and oxidization of disaccharides and hexose alcohols) respectively (Buddenhagen *et al*., 1962; Denny, 2006). Biovar 1 strains do not metabolize disaccharide and hexose alcohols, biovar 2 strains metabolize disaccharides, biovar 3 strains metabolize both disaccharides and hexose alcohols, biovar 4 strains metabolize hexose alcohols and biovar 5 strains metabolize all apart from dulcitol and sorbitol (He *et al*., 1983; Razia *et al*., 2021). Race 1 strains attack tobacco and other solanaceous plants, race 2 strains attacks musaceous species and triploid banana, race 3 strains attack potato, race 4 strains infect ginger plants and lastly race 5 strains cause disease on mulberry tree (He *et al*., 1983; Razia *et al*., 2021). The bacterium is classified into the following taxonomic units:

Table 1: Classification of *Ralstonia solanacearum*

Rank	Name
Domain	Bacteria
Phylum	Proteobacteria
Class	Betaproteobacteria
Order	Burkholderiales
Family	Ralstoniaceae
Genus	Ralstonia
species	*R. solanacearum, R. insidiosa, R. mannitolilytica, R. pickettii, R. detusculanense, R. syzygii*

2.2.4 Management of Bacterial Wilt caused by *Ralstonia solanacearum*

Management of *R. solanacearum* is cumbersome since the pathogen has a wide host range and is a soil borne pathogen that survives in the soil for long periods of time. There is no method that has been found to control bacterial wilt effectively but combining some of the methods has been able to contain the disease to some extent (Champoiseau, *et al*., 2010; Njau *et al*., 2021). Such methods include biocontrol, chemical control, cultural practices and host resistance (Champoiseau, et al., 2010). Cultural practices can be effective in areas where bacterial wilt is endemic or where it is not yet established. They include crop rotation, delayed planting, intercropping, positive and negative selection and soil amendments (Kinyua *et al*., 2001; EPPO, 2004). In order to achieve better results, a range of 5-7 years (EPPO, 2004) with an exception of host plants should be considered. Delayed planting reduces the disease incidence but this may not be practical in Kenya since the rains are short and erratic. Generally, cultural practices are not effective in controlling bacterial wilt due to various reasons (Kaguongo *et al*., 2008). Practices like intercropping depends on the type of crop used and the distance between plants respectively (Kirigo, 2019). Soil amendments to increase soil fertility and raising soil pH may not be practical in Kenya since many farmers grow potato in small scale thus lack the capital to carry out such practices. Crop rotation though cheap and practical may not be practiced by the farmers in Kenya due to small pieces of land hence the growers only major on essential food crops (Champoiseau *et al*., 2010; Kirigo, 2019).

In chemical control, methyl bromide was the chemical that was commonly used in fumigating contaminated soils but was withdrawn. Besides the price of chemicals that are used for control of bacterial wilt are very expensive and cannot be afforded by small scale farmers who grow potato for subsistence purposes (Kaguongo *et al*., 2008; Champoiseau *et al*., 2010; Kirigo, 2019). Most of the biological control agents that have been used have been found to be largely ineffective. The only cheap and practical way to control bacterial wilt is by use of resistant varieties and cultural practices. Use of chemicals and biocontrol are ineffective, tedious to apply and some biocontrol agents are not commercially available (Muthoni, Shimeli *et al*., 2012; Yuliar *et al*., 2015).

Non-toxic alternatives in plant protection such as the use of plant resistance inducers (PRIs) activating the plants' own defense could therefore come to play an even more important role in developing countries. Furthermore, many PRIs used in SAR give a broad-spectrum resistance, which in turn lessens the likelihood of the development of pathogen pesticide resistance (Oostendorp *et al*., 2001). For example, probenzole has been used against *Magnaporthe grisea*, the rice blast fungus, and *Xanthomonas* which causes bacterial leaf blight in rice, for more than 30 years and resistance in the pathogen has not been reported (Bektas & Eulgem, 2014). Likewise, potassium phosphate has been used against late blight in potato for many years in some tropical countries

2.3 Attenuation of Bacteria

Attenuation inactivates the bacterial cells while preserving their surface structures there by making them harmless or less virulent. It is carried out to inactivate bacterial cells and preserve their surface structures. Inactivation by chemical agents (Urbaniak *et al*., 2014), heat (Cantwell & Kelso, 1981), sonication (Chandrasekaran *et al*., 2006) and UV radiation (Wang *et al*., 2016) are some of the fixation methods that have been established in attenuation of different microorganisms. The most commonly used chemical fixatives include 2.5% glutaraldehyde, 10% formalin, acetone and acetic acid solution. Aldehyde based solutions are preferred to alcohol ones since alcohol existence results in detachment of the surface ultrastructures. Thermal inactivation is one of the important preservation methods which involves wet heat and dry heat inactivation. Wet heat inactivation damages the membrane while dry heat inactivation denaturizes the protein (Cantwell & Kelso, 1981). Bacterial growth is influenced by have the population density and switch from a unicellular to multicellular lifestyle once they obtain a cell number threshold making it possible to be attenuated through quorum sensing.

2.3.1 Bacterial Quorum Sensing

Quorum sensing (QS) is a regulatory mechanism of bacteria that involves cell to cell communication to coordinate gene expression. Quorum sensing inhibition has been used (Stevens *et al*., 2011; Li & Tian, 2012) to attenuate various pathogenic microorganisms. It allows individual bacteria within colonies to coordinate and carry out functions such as sporulation, biofilm formation, virulence conjugation and bioluminescence (Diggle *et al*., 2007; Li & Tian, 2012). In bacterial QS, inhibition controls the virulence of the pathogen strains by regulating the production of extracellular polysaccharide (EPS) and secondary metabolites, cellular motility and biofilm production (Yoshihara *et al*., 2020). Quorum sensing is regulated by production of auto inducers (AI molecule signals) and detected by cognate receptor proteins (Joint *et al*., 2007). The most common auto inducer in Gram negative bacteria is the N-acylated L- homoserine lactones (AHLs) (Welch *et al*., 2005; Stevens *et al*., 2011). Quorum sensing involves production, detection and response to AIs that accumulate in the environment as the bacterial density increases and monitors the information in order to track changes and alter gene expression. Most QS systems depend on AI production, detection and response despite their differences in regulatory systems and molecular mechanisms.

Gram-negative and Gram-positive bacteria have different QS system types. Gram-positive use AI peptides (AIPs) as signal molecules. After production of AIPs, the molecules are processed and secreted (Li & Tian, 2012). Auto inducer Peptides when at its highest concentration binds to cognate membrane-bound histidine kinase receptor thereby activating its activity and passes a phosphate to a cognate cytoplasmic response regulator. This response regulator then activates transcription of the genes in the QS regulon. Autoinducer peptides can also be transported back into the cytoplasm where they interact with transcription factors to modulate their activity and in turn modulate gene expression changes. Gram-negative bacterium uses small molecules such as acyl-homoserine lactones (AHLs) and those whose production depend on S-adenosylmethionine (SAM) (Wei et al., 2011). Production of AIs occurs within the cell and freely diffuse across the membranes. At high AIs concentration, they bind to cytoplasmic receptors which later regulate expression of the genes in the QS regulon. In some cases, Gram-negative AIs are detected by two- component histidine kinase receptors that function analogously as compared to those in Gram-positive QS bacteria.

2.3.2 Inactivation of Bacteria by Heat Method

Thermal inactivation is a technology that is applied in food processing and agricultural industries to prevent food spoilage and inactivate pathogenic microorganisms respectively. An understanding of this mechanism is essential for optimizing heat treatments to eradicate spoilage associated with different strains of microbes, foodborne and agricultural diseases. This process is associated with irreversible denaturation of nucleic acids, ribosomes and membranes but there is inadequate information on the patterns of macromolecular changes that induce cell death during heat treatment. There are thermal analysis techniques that have been used to detect, monitor and characterize heat induced conformational and phase transitions. A good example of these techniques is differential scanning calorimetry (DSC), where a number of overlapping transitions are observed in the process of microbial heat inactivation (Kaletunç, 2001). The observed transition peaks correspond to the denaturation of cellular components. The individual peaks detected in thermograms of whole cells are assigned to cell components by comparing transition temperatures of isolated cell components with corresponding transition temperatures in whole cells. There is an existing relationship between thermal death of microbes and the first major peak in DSC thermograms that is a temperature range of 60 ℃ to 80 ℃ attributed to ribosomal melting. Irreversible denaturation of nucleic acids require temperatures above those of cell inactivation since at ribosome denaturation temperatures, the DNA transition is reversible (Mohacsi-Farkas, Farkas *et al*., 1999). Differential scanning calorimetry of microbes to high temperatures (≥ 100℃) result in inactivation of the microbes but also demonstrate that most rescans do not display any peaks except for an endothermic transition attributed to DNA.

2.3.3 Formaldehyde Treatment on Pathogenic Microorganisms

Formaldehyde ($H_2C{=}O$), is the simplest of all aldehydes and is highly cytotoxic (Tang *et al*., 2009). The use of $H_2C{=}O$ and associated dangers from environmental exposure have been well documented (Pullen *et al*., 2021). Detoxification systems for $H_2C{=}O$ are found throughout the biological world and they are especially important in methylotrophic bacteria, which generate this compound as part of their metabolism of methanol (Tang *et al*., 2009). Formaldehyde has been used widely as a disinfectant for sterilization and is highly toxic to microbes but an important cellular metabolite. Cadirci, (2009) described fumigation of hatching eggs with formaldehyde as one of the most effective methods of reducing bacterial count on the eggs because it kills most of the microbes including their spores (Salthammer *et al*., 2010). Formaldehyde can be used to kill pathogenic bacteria and reduce bacterial count. It is therefore, imperative to find the optimum formaldehyde concentration and incubation time that significantly reduces microbial count and kills them. In this study, the effect of different formaldehyde concentration and incubation time on *Ralstonia solanacearum* was assesed.

2.3.3.1 Mechanisms of Formaldehyde Toxicity

Formaldehyde reactivity as an electrophile (reaction with free thiol and amine groups) on protein and DNA gives rise to its toxicity in cells. Nucleophilic addition of an amine to formaldehyde produces N-methylol adduct that might consequently condense to an imine carbon. The imimne carbon is susceptible to extra nucleophilic addition by a second amine forming an irreversible cross-link composed of a methylene bridge. The reaction of formaldehyde and thiols leads to nucleophilic addition of sulfur atom to the aldehyde forms that gives rise to hemithioacetal which cyclize quickly and irreversibly with neighbouring amine to generate a thiazolidine adduct (Higgins & Giedroc, 2014). Certainly, the exposure of formaldehyde has been revealed to result in formation of irreversible formaldehyde adducts, formaldehyde-catalyzed DNA-DNA, proteins and DNA damage in bacterial pathogens (yu *et al*, 2015; lu *et al* 2010). There are three major pathways for formaldehyde detoxification and assimilation; thiol-dependent, riblose monophosphate (RuMP)- dependent and pterin-dependent. The transformation of these pathways progress with primary capture of formaldehyde as a less reactive derivative which is assimilated subsequently into the usual pathways for carbon metabolism or detoxified to formate. The oxidation of formaldehyde to formate in bacterial species occurs in a single step which independent of pterin, RuMP or thiol pathways (Liao *et al*, 2013). This process is catalyzed by zinc-independent formaldehyde hydrogenase using an electron acceptor.

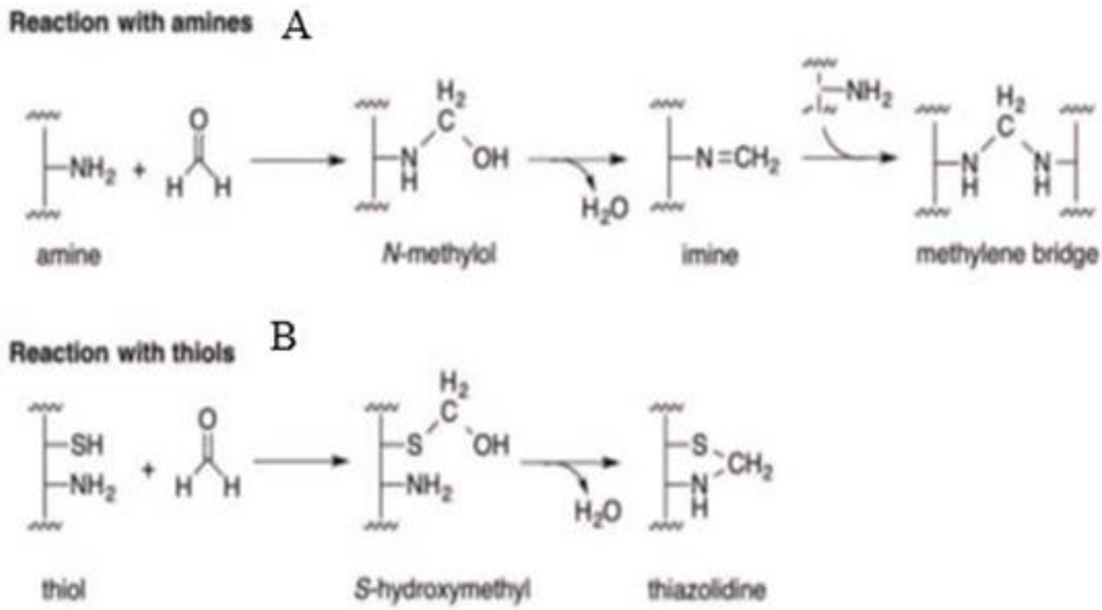

Figure 2: Mechanisms of formaldehyde toxicity. (A) The reaction of formaldehyde where amines form an imine adduct via an N-methylol intermediate and react further with other amines to form methylene bridges between protein and DNA molecules. (B) The reaction between formaldehyde and thiol forms S-hydroxymethyl and thiazolidine adducts.

The best characterized pathway for formaldehyde detoxification and assimilation by bacteria uses thiols as the initial formaldehyde acceptor. The thiol found in many microbes is tripeptide glutathione (GSH). Necleophic GSH is first added to GSH to form S-hydroxymethylglutathione (HMGS) which occurs spontaneously but only in specific bacterial species. The process is catalyzed by formaldehyde-activating emzyme (Goencrich *et al*., 2002; Wilson *et al*., 2008). S-hydroxymethylglutathione is oxidized by zinc containing NAD-dependent alcohol dehydrogenase to to generate thioester S-formylglutathion (SFG). From this process, formate is produced and GSH is generated finally upon hydrolysis of SFG. GSH-dependent systems for formaldehyde toxicity are not only found in environmental bacteria but also in microbes that do not oxidize methanol such as *E. coli* and *Bacillus subtilis* (Huyen *et al*., 2009). Bacteria uses thiol mycothiol (MSH) or bacilithiol (BSH) as the formaldehyde carrier instead of glutathione. These two contain glycoside linkages between N-acetylated cysteine, D-glucosamine and myo-inositol moieties. Although an S-formylmycothione hydrolase has not been identified in the organisms, formate and MSH have been detected as the final products of formaldehyde oxidation. Similarly, a BSH-dependent homolog of AdhC has been identified in *B. subtilis* (Huyen *et al*., 2009) but the corresponding *S*-formylbacillithione hydrolase has not been identified. Whether formate is generated as the final oxidation product is yet to be determined.

The RuMP pathway comprises of 3-hexulose-6-phosphate synthase and 6-phospho-3-hexuloisomerase enzymes. It was first described in methylotrophic bacteria but has now been identified in non-methylotrophic bacteria (Huyen *et al*., 2009). The process is initiated by initial capture of formaldehyde by Hps-catalyzed condensation with C1 carbon of ribulose-5-phosphate to form D-arabino-3-hexulose-6-phosphate. This product is then isomerized by Phi to generate fructose-6-phosphate, which is shuttled into the glycolytic pathway. Formaldehyde acceptor ribulose-5-phosphate is regenerated from fructose-6-phosphate and glyceraldehyde-3-phosphate via a series of transketolase, transaldolase, and isomerization reactions (Kato *et al*., 2006). The use of sugar phosphates in formaldehyde detoxification has also been identified in eukaryotic microbes such as *Candida* sp. However, xylylose-5-phosphate is used as the initial formaldehyde acceptor.

In pterin-dependent pathway, spontaneous condensation between formaldehyde and a secondary amine in the pterin forms N^5, N^{10}-methylene-THF, which is oxidized to N^5, N^{10}-methenyl-THF. The latter is catalyzed by a dehydrogenase using $NADP^+$ as the electron acceptor. A N^5, N^{10}-methenyl-THF is hydrolyzed further to N^{10}-formyl-THF by a cyclohydrolase. In the final step, N^{10}-formyl-THF is hydrolyzed by formate THF ligase to generate formate as the final product and regenerate THF (Vorholt, 2002). Enzymes that constitute the central pathway for methyl transfer is required for synthesis of purines and amino acids and initiation of protein translation. In species that already possess the thiol- or RuMP-linked pathways, the THF-dependent pathway for methyl transfer may still act as a secondary or auxiliary system for the removal of formaldehyde. The THF-linked pathway is regulated in the presence of methane or methanol, presumably to cope with the production of formaldehyde during methane or methanol oxidation in methontrophs and methylotrophs (Vorholt, 2002). The two pterins are structurally related and molecular details of the THF- and THMP-linked pathways are analogous.

2.4 Plant Resistance Mechanism

Plant disease resistance is the reduction of a pathogen reproduction on or inside the plant that denotes much less disease development in a genotype than that within the susceptible range. Plants have developed a complex defense system against pests and pathogens. Plant immune systems rely on the ability of receptors to recognize enemy molecules, carry out signal transduction and respond defensively through pathways (Andersen *et al*., 2018). Different form and mechanisms of resistance exist (Table 2). Plant disease resistance can be grouped on the basis of the number of host genes involved and effectiveness against different races of the pathogen. Polygenic resistance is effective against all strains of a pathogen and is usually controlled by many resistance genes (Andersen *et al*., 2018). This type of resistance majors on slowing down the rate of disease development and spread but it is more durable thereby it is impossible for new strains of the pathogen to develop and overcome the resistance. Monogenic or race-specific resistance on the other hand is controlled by one or a few resistance gens and can more effective against some races of the pathogen and less effective on others (Burdon *et al*., 2014).

Resistance genes work by allowing the plant to detect effectors that is produced by the pathogen. After detection of the effectors, the plant metabolic defense mechanism are activated leading to suppression of infection by the pathogen. These effector molecules are products of avirulence genes in the pathogen and are detected by plants containing corresponding R genes. This mechanism fits a gene for gene resistance model where correct R gene and the pathogen have the corresponding avirulence gene enhancing the pant to detect the pathogen (Dong & Ronald, 2019). In cases where the pathogen lacks corresponding avirulence or R genes, the plant fails to detect the pathogen leading to disease occurrence and development. The gene for gene model describes how a new pathogen race can develop. For instance, if the avirulence gene in the pathogen mutates to the point where R gene products no longer recognize the resulting molecule, then pathogen presence will not trigger defense hence ineffectiveness of resistance (Jia *et al*., 2000). Majority of these gene for gene resistance results in hypersensitive reaction in plants. Hypersensitive reaction enables plant cells near the point of infection to undergo programmed cell death to disrupt disease development. Plants cell may therefore release substances that inactivate toxins produced by the pathogen or even have antimicrobial effect on the pathogen. Some of these same defense systems are activated in susceptible varieties of plants, but the activation is delayed allowing infection of the pathogen. Race specific resistance can convey immunity to a disease or reduce the level of disease incidence and severity. But with race non-specific resistance, some symptoms may develop with an R gene resistance reaction. Race specific and non -race specific forms of resistance can be combined in the same variety to provide benefits of both types of resistance (Dong & Ronald, 2019).

Table 2: Overview of forms of plant resistance

Resistance phenomenon	Mechanism
Innate resistance	
Non-specific, general resistance	
Non-host resistance	HR, ROS, BAX inhibitor-1, PEN genes
Basal resistance against bacteria	Flagellin/FLS2 interaction, ROS, antimicrobial compounds
Race non-specific mlo resistance and quantitative resistance to fungi	Cell wall thickening Antimicrobial compounds ROS
Resistance to necrosis-inducing stresses	High antioxidant capacity
specific resistance (cultivar/pathogenic race specificity)	
Extreme resistance – symptomless gene-for-gene resistance	
Rx-resistance against viruses without HR	
Symptomless reaction to rust pathogens, no visible HR	
Gene-for-gene resistance	ROS
R-gene « Avr-gene interaction associated with the hypersensitive response (HR)	Phytoalexins Phenol oxidation Stress proteins
Resistance to pathogen toxins	Enzymatic detoxification Lack of toxin receptors
Gene silencing	Recognition and decomposition of foreign RNAs with ribonucleases
Acquired resistance	
After a primary infection an acquired resistance develops against a second infection _Stress memory	Accumulation of SA, SAR Stimulated antioxidants Gene silencing Rhizobacterial induction

Source: Kira´ly *et al*., 2007

There are two forms of systemic resistance in plants: Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR). Induced systematic resistance is naturally present in plants but enhanced by plant-associated non-pathogenic rhizobacteria and is independent of Salicylic Acid (SA) (Yu *et al*., 2022). This type of resistance is plant specific and dependent on genotype. Jasmonic acid and ethylene play an important role in ISR as they regulate plants' responses to biotic and abiotic stresses including pathogen attack (Wang *et al*., 2021). Ethylene production is stimulated by wounds or pathogen attack which thereafter onsets the defense responses. Systemic acquired resistance unlike ISR, is activated by exposure to a pathogen. This resistance is similar to human being immune response after vaccination. SAR develops primarily from tissue necrosis caused by a pathogen. Necrosis is followed by release and accumulation of SA in the phloem which triggers hypersensitive response and induction of SAR in the plants (Klessig *et al*., 2018).

2.4.1 Breeding for Resistance

Breeding crops for resistance involves identification of resistant breeding sources which includes ancient known plant varieties, wild relatives, cultivated varieties, land races, mutations, somaclonal variation and unrelated species (Kozjak & Megli, 2012). Identification of disease resistance genes is achieved with the help of traditional genetic evaluation using easy inheritance studies and tests of allelism. Breeding methods include crossing of a desirable plant variety to another variety that is a source of resistance in order to generate plant populations that segregate for the traits of the parents (Anand et al., 2023). Selection, introduction, marker assisted selection, genetic engineering, gene pyramiding and hybridization are some of the methods in breeding for resistance (Dormatey et al., 2020). The most common technique of breeding for resistance is pedigree choice where crosses are made among parents and the individual plant life is settled on for resistance from F2 generation. The choices are allowed to supply seed for the following generation. The choice manner is repeated in every technology, and a better percentage of resistant plant life is acquired in every successive technology till homozygosity is acquired. During backcross breeding, the hybrid resulting from a cross among the donor plant and a recurrent parent is crossed again to the recurrent parent and the progeny are screened for the disease resistance trait by inoculation with the pathogen. The selected individuals are crossed once more to the recurrent parent and the process is repeated. The advent of molecular markers enables to overcome main boundaries of backcross breeding (Maurya et al, 2021). Breeding techniques for major gene disease resistance contain identity of resistance genes and incorporating them into high yielding, however susceptible types through pedigree/backcross breeding.

2.4.2 Systemic Acquired Resistance

Systemic acquired resistance (SAR) is the induction of local responses associated with the transport of defense signals throughout the plant resulting in a broad- spectrum disease resistance against secondary infections. The activation of defensive responses of plants is a good tool for controlling pathogens that cause diseases (Llorens *et al*., 2017). Systemic acquired resistance can be induced by inoculating plants with a pathogen rendering it more resistant to subsequent pathogen infection (Mishina & Zeier, 2007). Resistance in plants can also be triggered by the presence of non-pathogenic microorganisms (Dey *et al*., 2014; Martínez-Hidalgo *et al*., 2015; Singh *et al*., 2016) and application of certain chemicals such as jasmonic acid (JA) and salicylic acid (SA) (Luo *et al*., 2011; Yuan *et al*., 2019). Inducing SAR in one part of the plant can result in resistance developing in the other parts of the plant (Hammerschmidt, 1999). This type of resistance is expressed against a broad spectrum of pathogens that include fungi, bacteria and viruses lasting for some period of time thereby hampering any invasion by a virulent pathogen.

Resistance triggered by pathogens can either be divided into SAR, herbivore induced resistance (HIR) and induced systemic resistance (ISR) depending on the type of pathogen (Pieterse, et al., 2014). Plants respond to the presence of pathogens by recognizing pathogen-associated molecular patterns (PAMPs) that leads to the activation of PAMP triggered immunity (PTI) (Jones & Dangl, 2006). Pathogen-associated molecular patterns are recognized by pattern recognition receptors (PRRs) (Schwessinger & Ronald, 2012) and PTI can be suppressed by avirulence factors (Bozkurt *et al*., 2011; Caillaud *et al*., 2012; Cheong *et al*., 2013; Cui *et al*., 2013; Dangl *et al*., 2013). After the pathogen is recognized, the speed with which the plant cell can mobilize its defences usually determines its level of resistance to the pathogen attack (Pastor *et al*., 2014).

2.4.2.1 Salicylic Acid as Systemic Acquired Resistance Regulator

Establishment of SAR involves generation and transport of signals through the phloem to the uninfected tissues. Salicylic acid is one of the signals contributing to SAR thus playing an important role in disease resistance in plants i.e. Salicylic Acid (SA) levels increase in many pathosystems upon infection by the pathogenic microorganisms. Salicylic acid (SA) is involved in both local and systemic defense responses in a plant making SAR a SA dependent response (Gao *et al*., 2015). Salicylic acid plays important physiological roles in a plant such as increasing the response of plants to both biotic and abiotic conditions by stimulating the internal paper dissection endogenous signalling to withstand a number of stresses. Salicylic acid serves as a stimulant or transmitter of the cell to tolerate environmental stress conditions including dryness, coldness, heat, stress of heavy elements, and conditions of ammonia tension. It also boosts the resistance of plants to salt stress, especially sodium chloride. Salicylic acid is derived from isochorismate synthase (ICS) and phenylalanine ammonia-lyase (PAL) pathways. Endogenous levels of SA are well established in plants and its exogenous application induces both local and SAR in plants against different types of pathogens (Kundu *et al*., 2011; Jendoubi *et al*., 2017; Le Thanh *et al*., 2017). The molecules of SA spread throughout the plant signalling the need for defense and this results in a resistance that can be effective for weeks.

2.4.2.2 Salicylic Acid Biosynthesis

Salicylic acid synthesis occurs via the shikmic acid pathway that forms two sub-branches, the ICS and PAL pathways (figure 2) (Dempsey *et al*., 2011; Singh, *et al*., 2013). In PAL pathway, phenylalanine is first converted to trans-cinamic acid and the reaction catalysed by PAL enzyme that is induced by pathogen infection. The ICS pathway involves the conversion of chorismate to isochorismate by ICS followed by conversion of isochorismate to SA by isochorismate pyruvate lyase (IPL). Salicylic acid that is synthesized in the chloroplast is exported to the cytosol via EDS5, a member of the MATE transporter family that is located in the chloroplast envelope. Triphosphate tunnel metalloenzyme 2 is a negative regulator that functions in defense signal amplification (Ung *et al*., 2014). The expression of ICS1 requires the binding of calmodulin binding protein CBP60g and non-calmodulin binding SARD1 (SAR deficiency 1) to the ICS1 promoter. Calmodulin binding protein 60 and SARD1 bind the GAAATTTTGG sequence in the ICS1 promoter (Truman 2012). Systemic acquired resistance can be induced via SA-independent factors and SA accumulation in distal tissues can result in activation of stronger defense response upon secondary infection (Návarová *et al*., 2012; Gruner *et al*., 2013)

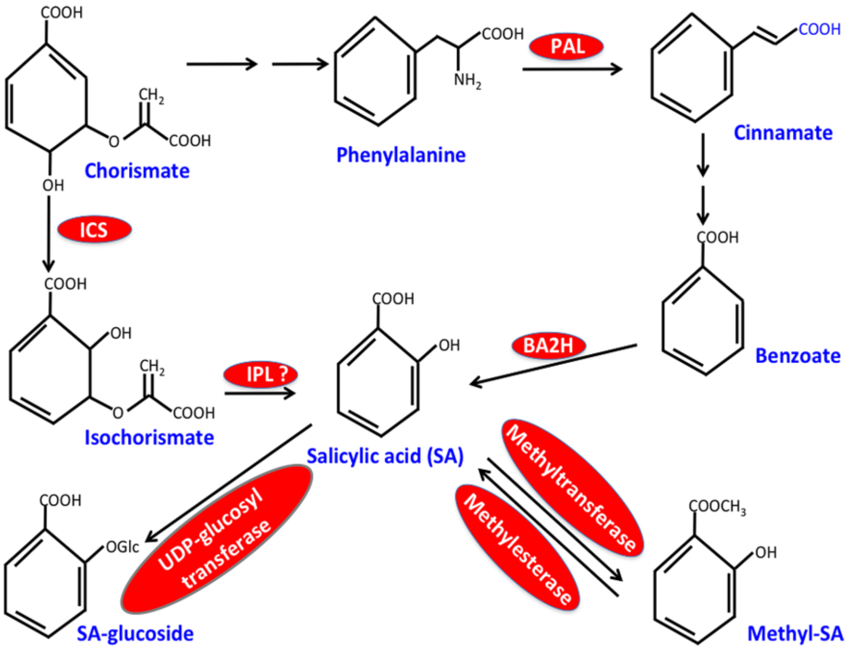

Figure 3: Scheme for salicylic acid biosynthesis in plants. Critical enzymes are shown in red; IPL, isochorismatepyruvatelyase; PAL, phenylalanineammonia-lyase; BA2H, benzoicacid2-hydroxylase.

2.4.2.3 Derivatives of Salicylic Acid in Systemic Acquired Resistance

Most of the synthesized SA is converted and stored as biologically inactivated derivatives through glucosylation, methylation and amino acid conjugation (Heidel *et al*., 2004). The derivatives include; SA2-*O*-β- D-glucose (SAG), SA glucose ester (SGE), methyl SA (MeSA), and SA-amino acid conjugates (Vlot *et al*., 2009; Dempsey *et al*., 2011). A study by (Zhang *et al*., 2013) shown that SA was derivatized to 2,3-dihydroxybenzoic acid (2,3-DHBA) catalysed by SA 3-hydroxylase (S3H). The storage derivatives of SA can be converted back to free SA (Kachroo & Kachroo, 2012). Methyl SA is a volatile and mobile derivative that accumulates in infected and distal tissues in response to pathogen infection. Methyl SA must be converted back to SA for systemic acquired resistance. Methyl salicylic acid is converted back to SA by methyl esterase and the reaction catalysed SA methyltransferases enzyme.

2.4.2.4 Regulation of Salicylic Acid Accumulation

Apart from ICS, PAL and EDS5, there are other proteins that have been identified to participate in pathogen induced SA accumulation. The proteins include; enhanced disease susceptibility 1 (EDS1), phytoalexin deficient 4 (PAD4) and non-race specific disease resistance 1(NDR1) (Cacas *et al*., 2011; Knepper *et al*., 2011; Lu *et al*., 2013). Mutations in EDS1, PAD4 and NDR1 cause partial reductions in SA level unlike in ICS1 and EDS5 mutations. Lipase-like protein such as EDS1 and PAD4 bind together with other lipase-like proteins to form binary and ternary complex (Feys *et al*., 2005; Zhu *et al*., 2011). Enhanced Disease Susceptibility 1 interacts with senescence associate gene 101 (SAG101) in the nucleus while with PAD4 in both cytosol and nucleus. For example, all the three proteins are required for R-mediated resistance in turnip crinkle virus (TCV) but only PAD4 is required for R gene which confers HR against TCV (Chandra-Shekara *et al*., 2007). Both nuclear and extra nuclear localization is required for defense (García *et al*., 2010).

2.5 Overview of Potato Production Yield

A total of 376 million tonnes of potatoes were produced worldwide in the year 2021 with China producing 94 million tonne followed by India at 54 million tonnes. This data indicated that the total area harvested globally was 18, 132, 694 hectares (FAO, 2021). Potato production in Africa has been on the rise with 70% of the growth concentrated in Eastern Africa. In the continent, the crop is grown under a wide range of conditions from irrigated commercial farms to small scale farms. Statistics from FAOSTAT 2021 revealed that Egypt is the leading potato producing country in Africa with 69 metric tonnes (MT) followed by Algeria (4.3 MT), South Africa (2.5MT), Kenya (2.1 MT) and Morocco (1.6 MT). Greater involvement by private sector in seed potato value chains places Africa in a good position to produce more potatoes for the global market. In Eastern Africa, Kenya is leading in potato production with 2.1 metric tonnes followed by Ethiopia (1.1 MT) and Tanzania (1.0 MT) (FAO, 2021).

Kenya is ranked at position 30 in the world and fourth largest Irish potato producer in Africa with an output of 2,107,824 metric tonnes in 2021 (FAO, 2021). In Eastern Africa, it is the best Irish potato producing country. This crop is the second most important food crop after maize and plays a crucial role in national and nutrition security. It is produced by small-scale farmers on highland areas of the country and the number of cropping season ranges from three to four per year depending on the variety planted and weather conditions. This crop addresses food insecurity, unemployment and low income in rural areas due to its high productivity and versatility in utilization (MoALF, 2016). Major potato producing counties in Kenya are from Mt. Kenya and Rift valley regions due to the favourable climatic conditions. Despite the importance of this crop, its production has been declining in the recent years due to lack of quality tuber seeds, variations in climatic conditions, pests and diseases.

Despite the increase in Irish potato in the country, the amount of potato produced is reduced due to infestation by pests such as Potato Cyst Nematode and diseases like bacterial wilt. The low yield in potato production areas is also attributed to poor agronomic practices, low soil fertility and limited access to quality tubers (Muthoni and Nyamongo, 2009). Bacterial wilt imposes significant losses in Irish potato production globally. Potato production is associated with use of chemicals that pollute the environment to a great extent and food produced in such envronment may not be safe or of good quality (Liljeroth *et al*., 2016).

2.5.1 Potato Bacterial Wilt Incidence and Severity

Potato production is affected by numerous diseases with the second most paramount being bacterial wilt. (Uwamahoro *et al*., 2018) Bacterial wilt is considered to be more challenging than late blight which is the most important potato disease (Damtew *et al*., 2018). This is because it cannot be controlled by chemicals or agronomic practices since there is no known chemical that can be used to control the disease once the plant has been affected. The persistence, wide host range, broad geographic distribution and disastrousness makes it difficult to control (Huet, 2014; Uwamahoro et al., 2018). The disease affects 3.75 million acres in almost 80 countries thereby leading to yield losses that is approximated to 75 % (Khairy *et al*., 2021). In Kenya, the potato industry is threatened by this disease with high prevalence in Mount Kenya and North rift region of the country (Kwambia *et al*., 2011). Nyangeri, (2011) showed that 58.7 % of the potato farms assessed were infested with *Ralstonia solanacearum*. Infected seed and soils are the main modes of bacterial wilt spread and that majority of farmers controlled the disease by uprooting the wilted plants (Kaguongo *et al*. (2009).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

The study was conducted at the Department of Biological Sciences Laboratory and Horticultural Demonstration Field, at Chuka University main campus in Tharaka Nithi County, Kenya. The study site is located on latitude 0°19'13'' South and longitude 37° 9' 30'' East at about 1400 m above sea level. The area receives adequate annual rainfall of 1375 mm bimodally distributed with short rains from October to December and long rains from March to June (CGoK, 2018). Temperatures range from 18.2 °C to 20.6°C. The area has volcanic foot ridge fertile soils, which are classified as humic nitsoils (Jaetzold *et al*., 2006). The area is suitable for growing Irish potato and other crops such as tea, bananas, sweet potato, beans and maize.

3.2 Experimental Design

For attenuation assay, a Randomized Complete Block Design (RCBD) was used with formaldehyde concentrations at four levels (0%, 0.2%, 0.4% and 0.6%), incubation durations for attenuation at four levels (30, 60, 90 & 120 minutes). These treatments were replicated three times. In SA quantification, disease severity and yield of Irish potato experiments, a 4 x 5 factorial experiment laid out in a RCBD with three replicates. There were two factors, i.e. Irish potato varieties (at 4 levels: Kenya karibu, Kenya mpya, Tigoni and Unica) and SAR inducing factors (at 5 levels: inoculation with attenuated *R. solanacearum*, inoculation with *E. coli*, inoculation with *Saccharomyces cerevisiae*, sterilized distilled water and *R. solanacearum*. This constituted 20 treatments resulting from all possible combination of factor levels.

3.3 Experiments

3.3.1 Laboratory Experiments

3.3.1.1 Media Preparation

Nutrient broth was prepared by suspending 13 g of nutrient broth powder in one litre of distilled water and then swirled to dissolve the components. The dissolved components were autoclaved at 121℃ for 15 minutes and then 20 ml of the broth filled in 15 sterilized bijou bottles inside a laminar flow to be used later in culturing *E. coli* and *R. solanacearum*. Nutrient agar was prepared by suspending 28 grams of the powder in one litre of distilled water. The media was heated to boil to completely dissolve the medium followed by sterilization in an autoclave at 15 lbs for 15 minutes. After autoclaving the media was cooled to 45 - 50 ℃ and 15 ml of the media dispensed into petri dishes inside the cabinet flow. These were preserved in a refrigerator for later utilization in culturing *E. coli* and *R. solanacearum*.

Potato dextrose agar (PDA) was prepared by suspending 19.5 grams of PDA powder in 500 ml distilled water and then heated to boiling while swirling to dissolve the medium completely. The media was then sterilized in an autoclave at 15 lbs pressure (121 ℃) for 15 minutes, cooled to 45 - 50 ℃ and mixed well before dispensing 15 ml into petri dishes inside a laminar flow cabinet. This was preserved for culturing *Saccharomyces cerevisiae*.

3.3.1.2 Culture of *Ralstonia solanacearum*

Ralstonia solanacearum was obtained from stocks at the Department of Biological Sciences Microbiology laboratory, Chuka University. Bijou bottles were sterilized by washing with jik and autoclaving at 121 ℃ for 15 minutes and then filled with 20 ml of the prepared broth. The inoculum was streaked in a plate containing nutrient agar, cultured in an incubator at 30 ℃ for 24 hours and later sub cultured in nutrient broth media at 30 ℃ for 48 hours. After media preparation, the inoculum was cultured in bijou bottles. A sterilized loop was used to streak Ralstonia culture from the plate and the dipped in the bottles. The loop was flame sterilized and the procedure repeated for all the bottles and incubated at 30 ℃ for 48 hours.

3.3.2 Attenuation of *Ralstonia solanacearum*

Cultures of *Ralstonia solanacearum* were grown overnight in broth (20 ml) in 12 bijou bottles at 30 ℃. Pure cultures in each of the bijou bottles was divided into four bottles containing 5 ml broth constituting 48 samples. This was followed by centrifugation of the broth at 4000 xg for 10 minutes to get the cells. Phosphate-Buffered Saline (PBS) of pH 7.2 was prepared by adding 1000 ml of distilled water to 10× PBS liquid then sterilized in an autoclave at 121 ℃ for 15 minutes. Different concentrations of formaldehyde (0%, 0.2%, 0.4% and 0.6%) in PBS were then prepared. The concentration was prepared by calculating the percentages of the different concentrations of formaldehyde in the total volume. For each concentration five millilitres of the formaldehyde solution were added to cells and incubated at 37 ℃ for 30, 60, 90 and 120 minutes. The tubes were then incubated at 4 ℃ for 18 hours. The samples were then centrifuged at 4000 xg for 10 minutes and the inactivated cells were washed in sterilized PBS solution, centrifuged and resuspended again in 5 ml of PBS solution. The inactivated cells were plated on nutrient agar and incubated at 30 ℃ for 72 hours to test the efficacy of formaldehyde at different concentration and incubation durations (Figure 4).

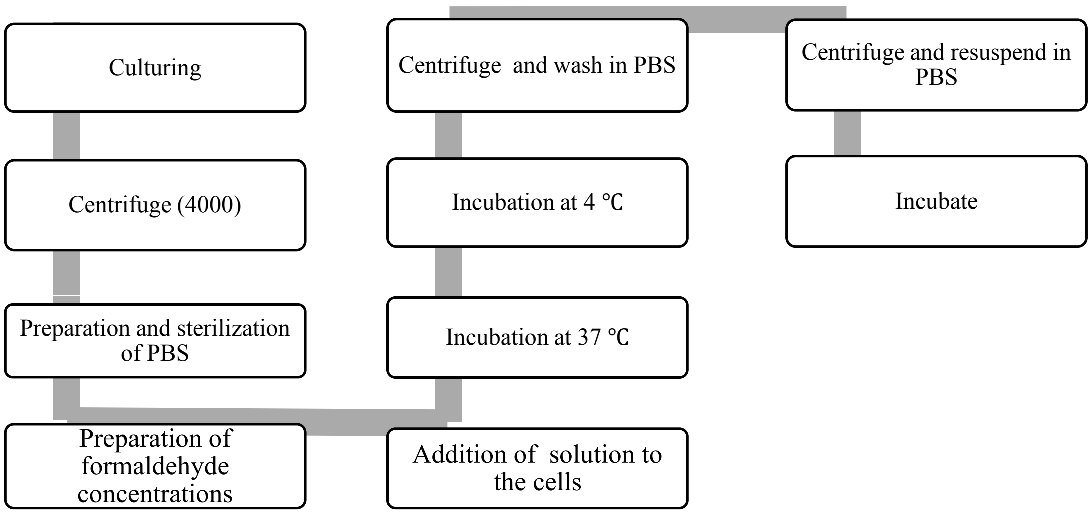

Figure 4: Attenuation of *Ralstonia solanacearum* process

3.3.2.1 Culture of *Escherichia coli* and *Saccharomyces cerevisiae*

To determine the induction of SAR against *R. solanacearum* by *E. coli*, the bacterium was obtained from Chuka University Microbiology laboratory and cultured on nutrient broth. The inoculating loop was heat sterilized by passing half of it over a flame until it glowed red. The loop was cooled, streaked into the *E. coli* culture, removed and gently dipped into the bottles having the broth. The bottles containing nutrient broth were placed in the incubator for 48 hours at 37℃ after which turbidity was observed in the bottles. The cultures were used for induction of SAR against *R. solanacearum* in potted potato plants under greenhouse.

Saccharomyces cerevisiae (saf-instant baker's dry yeast) was purchased locally from the shop as baker's yeast and cultured on PDA media to obtain pure colonies. Yeast broth was prepared by adding 1 gram of yeast powder into 50 ml of warm water. The mixture was then swirled continuously to dissolve the components. This was followed by streak culturing in PDA medium. Pure colonies were then used for induction of SAR in Irish potato.

3.3.3 Induction of Systemic Acquired Resistance against *Ralstonia solanacearum*

Certified seeds of the four Irish potato varieties; Unica, Kenya mpya, Kenya karibu and Tigoni were purchased from Kenya Agricultural and Livestock Research Organization (KALRO) in Tigoni, Limuru Town. The mode of collection was based on the size of the potato seeds and small sized seeds (28 - 45 mm in diameter) were used in the project. Small sized seeds produce fewer sprouts as compared to large or middle sized ones that produce many sprouts and have to be cut into halves or the sprouts scooped. The greenhouse was prepared by levelling using a rake and then water was sprinkled to remove dust. A black polythene bag was then laid down to prevent contact of the potting bags with the soil and contamination from the treated plants from spreading to the ground. Substrate (sub soil) was sterilized in an autoclave at 121 °C for 20 - 30 minutes followed by addition of manure (livestock manure from Kiriani university farm) to have a blended soil mixture. The potting bags 5 litres in size were placed in the greenhouse at fixed positions with a spacing of 35 cm by 31 cm. The Irish potato seeds were then planted in the bags.

Seeds were placed one in every bag with the eye buds facing up and then covered with four inches of the blended soil. Planting was done in 3 blocks with each block having 60 plants constituting a total of 180 plants. Each treatment was replicated 3 times and blocked into three. Finally, the newly planted seeds were watered regularly and evenly to maintain the soil moisture. After 30 days, the potting bags were labelled using plastic tags for easy identification during inoculation. The plants were also sprayed with Dynamec 018 EC to control red spider mites and leaf minors. Ten millitres of the pesticide was mixed with 20 litres of clean water in a clean knapsack sprayer.

After a period of 35 days, the potato plants were inoculated with 0.5 ml of attenuated *Ralstonia solanacearum*, *E. coli*, *Saccharomyces cerevisiae*, water (negative control) and *R. solanacearum* (positive control) treatments by injection. The seedlings that were not treated were used as positive control treatment. The plants were again inoculated with *Ralstonia solanacearum* by injection after a period of seven days (Figure 5).

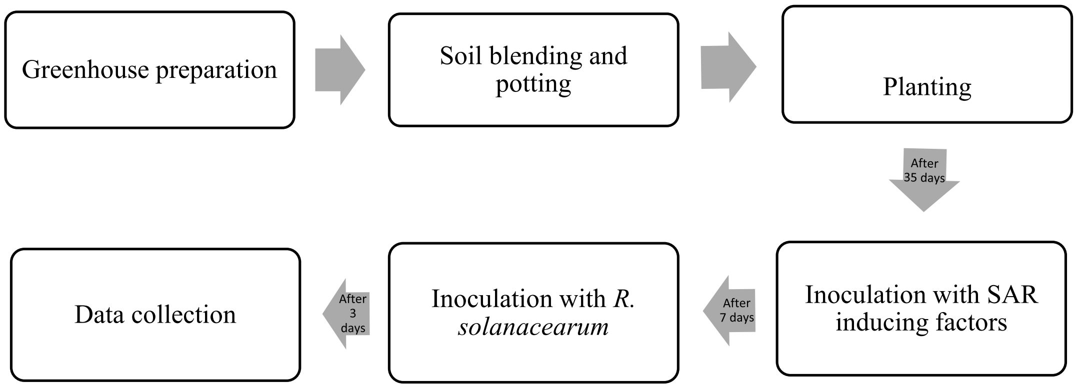

Figure 5: Greenhouse experiment

3.4 Data Collection

3.4.1 Determination of the Induction of Systemic Acquired Resistance against *Ralstonia solanacearum*

Three days after inoculating the potted Irish potato plants with *R. solanacearum*, changes occurring in the plants were recorded to assess whether attenuated *Ralstonia solanacearum*, *E. coli* and yeast triggered systemic acquired resistance against bacterial wilt in Irish potato. Health of the plants with respect to wilting, wilted branches, chlorosis and death were some of the factors that were assessed in the greenhouse. The plants were rated for disease severity which is the severity percentage of disease damage and yield loss on the four potato varieties. Severity symptoms on individual plants were rated on a scale of 0-6 dependent on branches affected by the wilt and subsequent chlorosis and death. The scale is as follows; 1 = 0%, 2 = 20%, 3 = 40%, 4 = 60%, 5 = 80% and 6 = 100% which was a modification of Trapero-Casas & Jimenez-Diaz, (1985). Disease symptoms on individual plants were categorically rated as follows; 1 = healthy, 2 = wilting, 3 = wilted, 4 = partial chlorosis, 5 = complete chlorosis and 6 = dead plant (Appendix I). Disease severity was assessed 3, 6, 9, 12, 15 and 18 days after inoculating the plants with *R. solanacearum*.

3.4.2 Determination of Salicylic Acid Levels in the Leaves of Induced Potted Potato Plants

Potato leaf samples were collected on the 60th day after sowing (DAS) by plucking two leaves from each treatment and replicates. The same process of leaf collection was repeated to the other two remaining blocks. The samples were placed in specimen bags and then preserved in the freezer (at -80℃) for use in quantification of SA. Sixty (leaves per treatment combined) treated potato leaf samples were frozen in liquid nitrogen and then ground to powder. The samples were then left at room temperature to thaw. An amount of 0.3 grams of leaf samples were weighed and added in a centrifuge tube. Two millimetres of ethanol were added to the samples in the centrifuge tube and vortex for I minute. The sample was then centrifuged for 10 minutes at 10,000 ×g. Ethanol was used for the extraction of SA by swirling the solvent and centrifuging at 10,000 ×g for 10 minutes. The supernatant was stored in a freezer (at 4℃) for SA measurement. Ferric chloride and SA standards were prepared in water.

Salicylic acid standard (1000 ppm) was prepared by weighing 0.1 g of SA (purchased from science lab limited, Nairobi) in 100 ml volumetric flask, followed by dissolving in 100 ml sterile distilled water. The solution was then dissolved in a sonicator to have a uniform distribution. One hundred ppm of the standard was prepared from the stock solution and topped to the mark (100 ml) for use in SA measurement. Ferric chloride standard was prepared using the same protocol of SA standard as discussed above. Working standards used to formulate the calibration curve were 0.5 ppm, 1 ppm, 2 ppm, 4 ppm and 8 ppm. Two hundred microliters of the sample (plant extract) was pipetted into a 50 ml volumetric flask, water added and then complexed with 2 ml of freshly prepared ferric chloride. The volume of the reaction mixture was made to 50 ml by adding water, SA extract and ferric chloride. Complex formed between Fe^{3+} ion and SA was determined by UV-spectrophotometry, measuring the absorbance of the complex in the visible region (at 535 nm; Figure 7). The absorbance values were plotted against the known concentrations of the standard solutions (Figure 6).

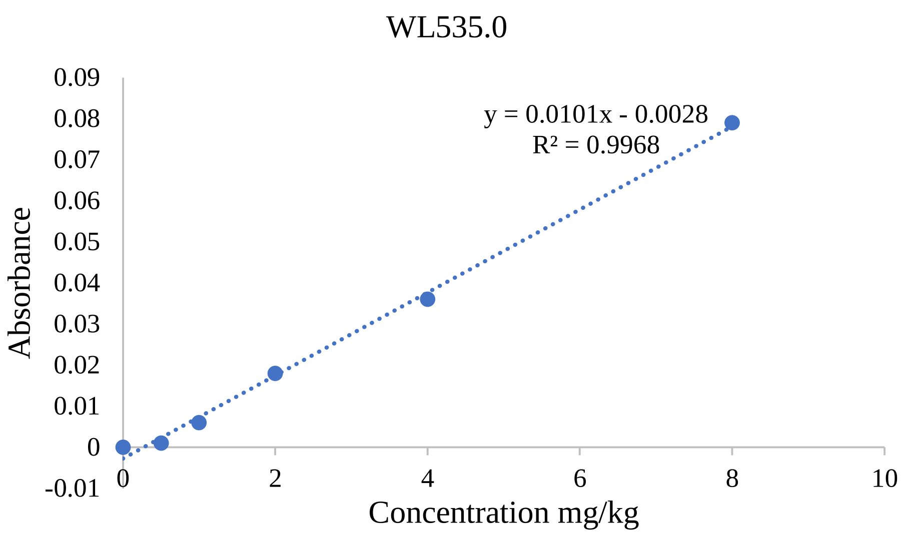

Figure 6: Calibration curve for salicylic acid with absorbance plotted against concentration mg/kg ground Irish potato leaves

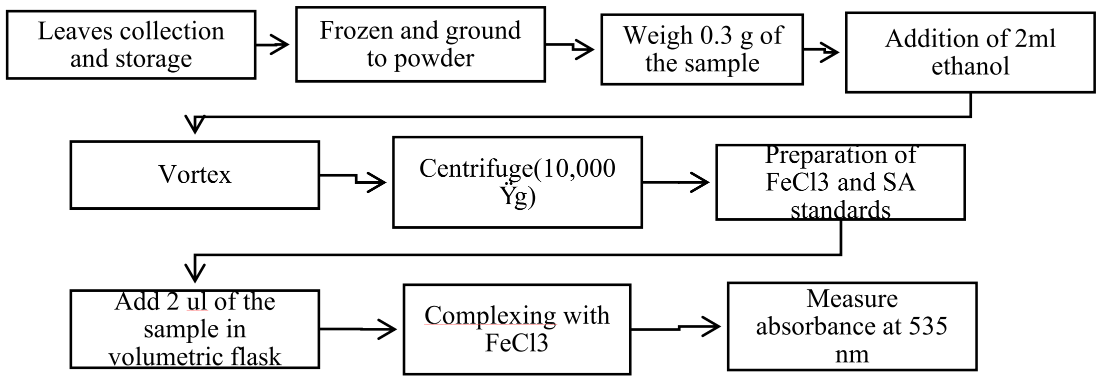

Figure 7: Flowchart of preparation and quantification of salicylic acid in inoculated Irish potato leaves

3.5 Data Analysis

3.5.1 Determination of the Induction of Systemic Acquired Resistance against *Ralstonia solanacearum*

Data obtained from attenuation of *R. solanacearum*, salicylic acid quantification and potato tuber yield were tested for normality using Levene's test before subjecting it to ANOVA and found to be normally distributed. Data on colony counts, SA and potato tuber yields was subjected analysis of variance (ANOVA) using statistical analysis software (SAS) version 9.4 (SAS, 2013) to determine the effect of treatments on response variable. Significant means were separated using Least Significance Difference (LSD) at 5% probability level. Data on disease severity was analysed using Kruskal- Wallis test to determine the effect of various treatments on severity of bacterial wilt on selected Irish potato varieties.

3.5.2 Statistical Model

To determine the effect of formaldehyde concentration and duration of incubation on the number of colonies of attenuated *Ralstonia solanacearum*, the following statistical model was used,

$$Y_{ijk} = \mu + I_i + V_j + (IV)_{ij} + R_k + e_{ijk}$$

where, Y_{ijk} = response, μ = overall mean, I_i = effect of formaldehyde concentrations, V_j = Effect of incubation duration, $(IV)_{ij}$ = interaction between formaldehyde concentrations and incubation durations, $(R)_k$ = effect of replicates, e_{ijkl} = error effect.

To determine the effect of SAR inducing factors and Irish potato varieties on salicylic acid levels, disease severity and yield of Irish potato infected with bacterial wilt, the following model was used,

$$Y_{ijk} = \mu + I_i + V_j + (IV)_{ij} + R_k + e_{ijk}$$

where, Y_{ijk} = response, μ = overall mean, I_i = effect of the induction factors, V_j = Effect of variety, $(IV)_{ij}$ = interaction between induction factors and variety, $(R)_k$ = effect of replicates, e_{ijkl} = error effect.

3.6 Ethical Consideration

After clearance by Chuka University Ethics Committee, another permit was obtained from the National Committee of Science, Technology and Innovation (NACOSTI; Appendix II). To prevent the microorganisms used from spreading into the environment, the following were done; the experiment was set up in the greenhouse which was kept clean and tools were sanitized by watering and drying after use. Adequate watering, mulching, and weeding to prevent its spread. Care was also taken to prevent *E. coli* from causing infections to human beings close to the study site environment. All the samples and cultures were aseptically handled in biosafety cabinet and autoclaved at the end of the study. All the plant samples grown in the greenhouse were collected in a pit hole, drenched with 10% formalin for two weeks then burned down. During data collection, care was taken to prevent pathogen escape to contain the spread. Plagiarism was avoided for the integrity of the research study and works of others were appropriately cited and referenced.

CHAPTER FOUR

RESULTS

4.1 Effect of Formaldehyde Concentration and Duration of Incubation on the Number of Colonies of Attenuated *Ralstonia solanacearum*

4.1.1 Culture of *Ralstonia solanacearum*

Cultural evaluation of *Ralstonia solanacearum* revealed that colonies on nutrient agar were smooth circular, raised and cream white with optimal feature being opaque (Plate 4). After 24 hours of growth, the cells were raised and spreading sidewise from line of inoculation (Plate 4(ii)).

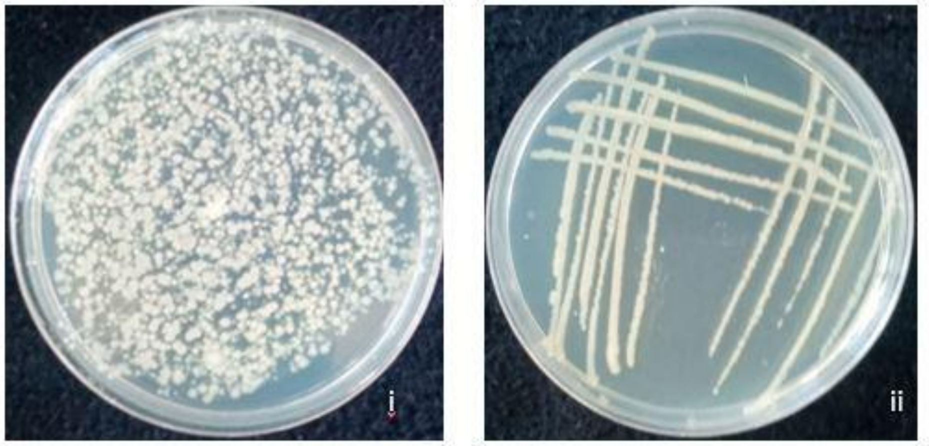

Plate 4: *Ralstonia solanacearum* sub culture on nutrient agar. (i) *R. solanacearum* pour plate on nutrient agar. (ii) *R. solanacearum* streak plate on nutrient agar sub culture

On broth, colonies of *R. solanacearum* were visible in the form of thin pellicle on the surface of nutrient broth after a period of 24 hours, which later became thick and the medium became turbid with a putrefactive odour (plate 5). The growth of the bacterium after five days was yellowish.

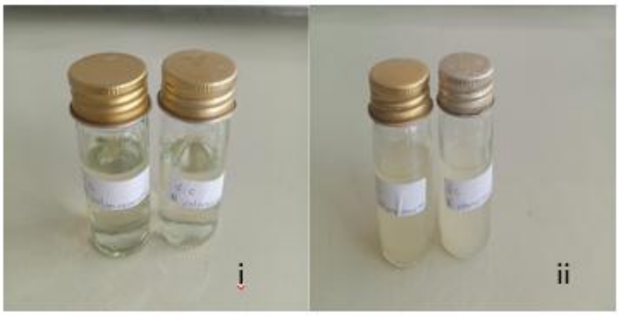

Plate 5: *Ralstonia solanacearum* on nutrient broth. (i) *R. solanacearum* before incubation, the broth was homogeneous, free-flowing and beige in colour (ii) *R. solanacearum* after 48 hours of incubation, broth became cloudy due to increased number of microbes.

4.1.2 Effect of Formaldehyde Concentration and Incubation Duration for Attenuation on the Number of Colonies of Attenuated *Ralstonia solanacearum*

The cells were counted after 72 hours of incubation using a colony counter. The level of attenuation was measured in terms of colony forming units (CFUs). Visible colonies were observed on the plates at different concentrations of formaldehyde and incubation time for attenuation of *R. solanacearum*. Colony forming units varied with different incubation time for attenuation of *R. solanacearum* (Plate 6).

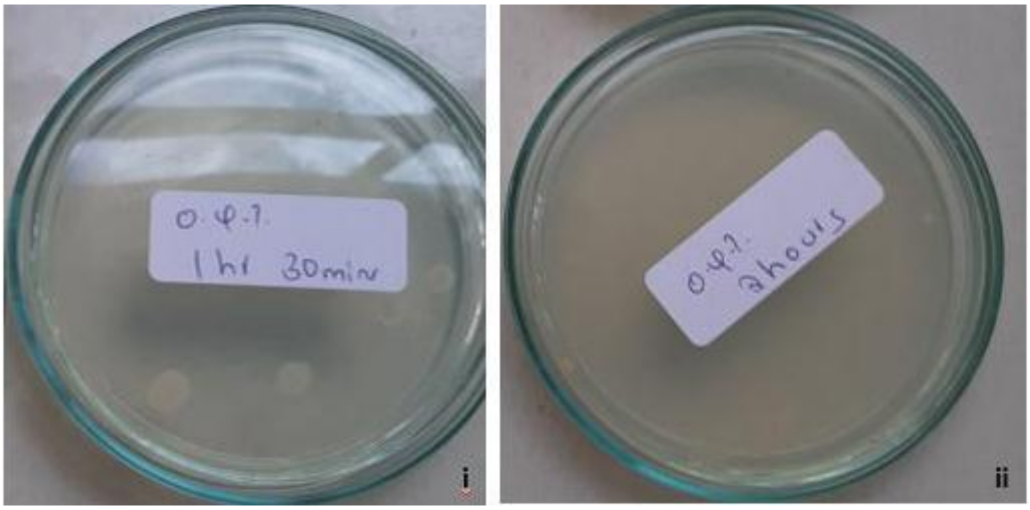

Plate 6: Colony forming units on plates with an interval of 30 minutes of incubation for attenuation of *Ralstonia solanacearum*.

The test of fitted model indicated that it was adequate ($p < 0.05$) in describing the linear relationship between treatments (formaldehyde concentration and incubation duration) on attenuation of *Ralstonia solanacearum* (Appendix III). Tests on the effect of each factor and their interaction indicated that there was a significant effect ($p < 0.05$) of each factor and their interaction on colony count of *R. solanacearum* during attenuation (Appendix IV). The blocking of the treatments was found to be effective ($p < 0.05$). The analysis of effect of formaldehyde concentration on colony count of *R. solanacearum* showed that the number of colony counts ranged from 2.08 CFU/ml (in 0.6% formaldehyde concentration) to 179.25 CFU/ml (in 0% formaldehyde concentration; Table 3). However, formaldehyde treatment at a concentration of 0.4% and 0.6% did not give significantly different number of colony counts of *R. solanacearum*. The analysis of effect of incubation duration of attenuation on colony count of *R. solanacearum* revealed that the number of colony counts ranged from 51.75

CFU/ml (for 30 minutes) to 66.50 CFU/ml (for 120 minutes; Table 3). However, duration of attenuation for 90 minutes and for 60 minutes did not give significantly different number of colony counts of *R. solanacearum*.

Table 3: The effect of formaldehyde concentrations and incubation time for attenuation on the number of colonies of *Ralstonia solanacearum*

Formaldehyde concentration (%)	No. of colonies (CFUs/ml)	Incubation duration (minutes)	No. of colonies (CFUs/ml)
0	1179.25a	120	66.50a
0.2	45.92b	90	57.83b
0.4	5.00c	60	56.17b
0.6	2.08c	30	51.75c
LSD	4.1331	LSD	4.1331

The coefficient of variation was 8.53% indicating that the data collected was reliable with R-squared of 0.997 (Table 4). The analysis of the effect of the interaction of the factors (combined effect of formaldehyde concentration and duration of attenuation effect) on colony count of *R. solanacearum* showed that the number of colony counts ranged from 0.67 CFU/ml (in 0.6% formaldehyde concentration and incubation duration of attenuation of 120 minutes) to 233 CFU/ml (in 0% formaldehyde concentration and incubation duration of attenuation of 120 minutes; Table 4). Generally, 0% formaldehyde concentration at any given incubation duration of attenuation gave higher number of colony count while 0.6% formaldehyde concentration at any given incubation duration of attenuation gave lower number of colony count of *R. solanacearum*.

Table 4: Number of *Ralstonia solanacearum* colonies in different formaldehyde concentrations and incubation duration for attenuation of *Ralstonia solanacearum*.

Formaldehyde concentration	Incubation duration (minutes)	Number of colonies (CFUs/ml)
0%	120	233a
0%	90	192.67b
0%	60	155.33c
0%	30	136.00d
0.2%	30	75.67e
0.2%	60	44.33f
0.2%	90	34.33g
0.2%	120	29.33g
0.4%	30	8.67h
0.4%	60	5.00h
0.4%	90	4.33h
0.4%	120	3.33h
0.6%	30	3.00h
0.6%	60	2.33h
0.6%	90	1.00h
0.6%	120	0.67h
Mean	58.06	
LSD	8.2661	
CV%	8.537658	
R-squared	0.997276	

[a]Means with the same letters are not significantly different at 5% significance level

4.2 Effects of Escherichia coli, Saccharomyces cerevisiae and Attenuated Ralstonia solanacearum on Severity of Bacterial Wilt of Inoculated Plants

4.2.1 Culture of Escherichia coli and Saccharomyces cerevisiae

Escherichia coli grown on nutrient agar had colonies that were circular, thick, greyish white, moist, smooth and translucent disc colonies (Plate 7). The colonies of the bacterium in fresh cultures were smooth which were emulsifiable in saline while subsequent sub cultures had capsulated strains and rough forms of colonies. This variation is due to repeated sub culturing that is associated with loss of surface antigens.

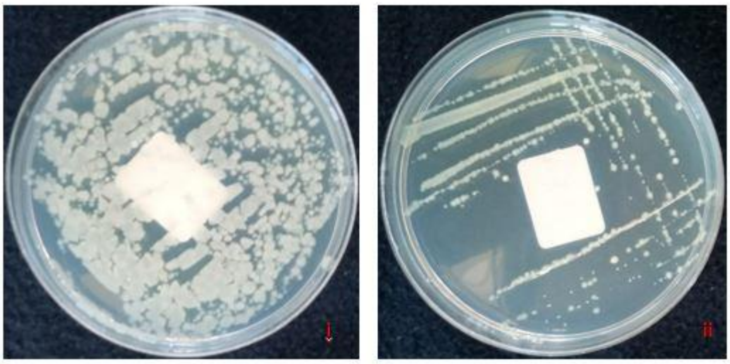

Plate 7: *Escherichia coli* on nutrient agar. (i) *E. coli* pour plate on nutrient agar is smooth and greyish, (ii) streak plate of *E. coli* showing smooth regular colonies.

On nutrient broth, there was a homogeneous murky development of the broth due to the increment in the quantity of *E. coli* cells in the broth. Pellicles were visible on the surface of the liquid after 24 hours of incubation with heavy deposits of colonies that dispersed when the broth was shaken. Turbidity increased after 48 hours of incubation (Plate 8).

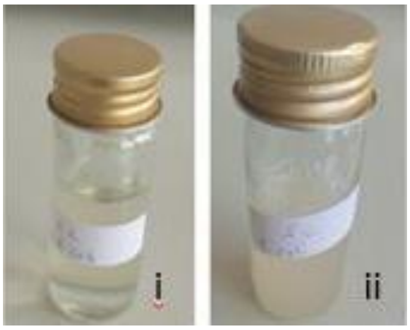

Plate 8: *Escherichia coli* on nutrient broth. (i) *E. coli* on nutrient broth before incubation, broth is transparent. (ii) *E. coli* on broth after 48 hours of incubation shows turbidity.

Colonies of *S. cerevisiae* that were grown on PDA media had a round shape, raised margin, smooth surface and cream white in colour with a characteristic smell of yeast (Plate 9). The size of colonies varied from small to large.

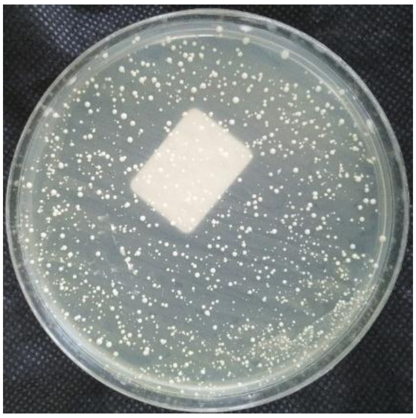

Plate 9: *Saccharomyces cerevisiae* is cream white in colour on PDA

4.2.2 Effect of *Escherichia coli, Saccharomyces cerevisiae* and Attenuated *Ralstonia solanacearum* on Disease Severity of Bacterial Wilt in Selected Irish Potato Varieties

Data on disease severity were scored using a disease score criteria (Appendix I). The findings of the study showed that treatments (SAR inducing factors and Irish potato varieties) had significant ($p < 0.05$) effect on disease severity across different stages of plant growth and development (Appendix V; Appendix VI). However, there was no significant difference in all the treatments on the 18th day after infection (Appendix VI). In case of treatment with *S. cerevisiae*, all the treatments showed different rates of disease severity with the controls (distilled water and *R. solanacearum*) having high disease severity rates. *Escherichia coli* and attenuated *R. solanacearum* treatments recorded low disease severity in all the varieties (Appendix VII). Inoculating plants with *R. solanacearum* had impact in all the treatments, with all the varieties used in the experiment showing symptoms after inoculation with *R. solanacearum*. Both *R. solanacearum* treatment and distilled water treatments displayed symptoms of the late bacterial wilt with *R. solanacearum* being highly susceptible (Figure 5). The plants showed initial symptoms of the disease on the third day after infestation. Plants inoculated with distilled water and *R. solanacearum* treatments were severely affected by the pathogen with most of them dying on the 12th days after infection. Priming of SAR with *Saccharomyces cerevisiae* generated severe adverse symptoms in all the varieties (Figure 5; Appendix VII). Three days after inoculating the plants with the pathogen, some of the plants showed up to 60% disease severity with significant difference ($p < 0.05$) between different treatments and varieties (Appendix VII). Disease severity at 3 days after infection (DAI) was ≤ 60%, 6 DAI (0 - 60%), 9 DAI (0

- 80%), 12 DAI (0 - 100%), 15 DAI (0 - 100%) and 18 DAI (40% - 100%). Plants that were inoculated with attenuated *R. solanacearum* recorded the lowest disease severity ≤ 40% while those injected with *S. cerevisiae* recorded ≥ 40% disease severity.

Disease severity scores revealed that treatments with *E. coli* and attenuated *R. solanacearum* were resistant and highly resistant (Table 5). One treatment of *S. cerevisiae* was moderately resistant while the remaining three were susceptible. All sterilized distilled water and *R. solanacearum* treatments were susceptible and highly susceptible (Table 5). Tigoni had the highest susceptibility while Kenya Mpya was highly resistant to the pathogen particularly for *E. coli* and attenuated *R. solanacearum* treatments, which induced resistant against bacterial wilt (Table 5). On the basis of varieties, Kenya Karibu had the lowest disease severity followed by Unica, Kenya Mpya and lastly Tigoni (Appendix VII; Figure 8). Generally, Kenya Karibu is a resistant variety while Unica is moderately resistant variety.

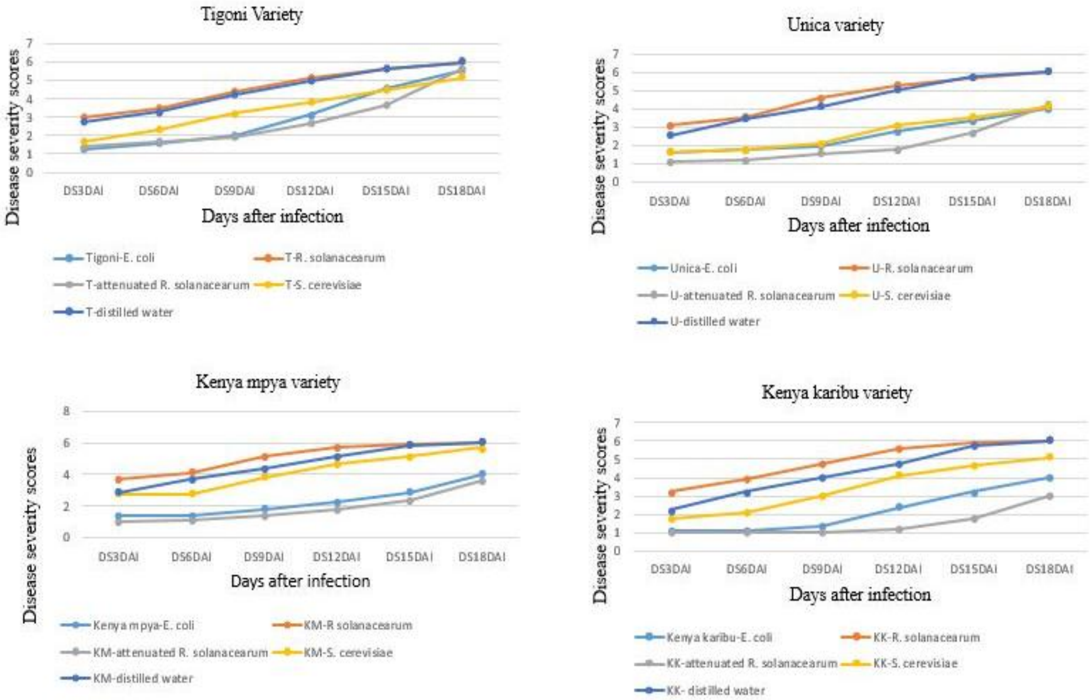

Figure 8: Medians of disease severity in inoculated Irish potato varieties. Kenya karibu variety having the lowest disease severity followed by Unica, Kenya mpya and lastly Tigoni variety

Table 5: Disease scores showing reactions of different treatments on the last day of disease severity assessment

Treatment	Disease reaction
KKE	Highly resistant
KKO	Susceptible
KKR	Highly resistant
KKS	Susceptible
KKW	Susceptible
KME	Highly resistant
KMO	Highly susceptible
KMR	Highly resistant
KMS	Moderately resistant
KMW	Highly susceptible
TE	Moderately resistant
TO	Susceptible
TR	Resistant
TS	Susceptible
TW	Susceptible
UE	resistant
UO	Highly susceptible
UR	Resistant
US	Susceptible
UW	Highly susceptible

Where, KK = Kenya Karibu, KM = Kenya Mpya, U = Unica and T = Tigoni. R = attenuated *Ralstonia solanacearum*, S = *Saccharomyces cerevisiae*, E = *Escherichia coli*, W = sterilized distilled water and O = *Ralstonia solanacearum*.

Cross-section cut of the stems revealed a brown discoloration of the infected tissues. As the disease developed plants (*S. cerevisiae*, *R. solanacearum* and distilled water treatments) were observed to be having complete wilting, that is, wilting of the whole plant. This was then followed by chlorosis which was also progressive; partial and complete chlorosis (Plate 10). Plants with *Saccharomyces cerevisiae* treatments and controls had partial to complete chlorosis followed by drying of the leaves (brown in colour) and eventually death. Bacterial wilt symptoms were also present in the tubers at later stages of the disease. Potato tubers with *Saccharomyces cerevisiae* and controls produced bacterial ooze in the eyes of the tuber (Plate 10(A-C)). Cross-section cut of the infected potato tubers revealed a grey-brown discoloration of vascular ring (Plate 9(A)). A milky white exudate was observed in freshly cut sections of the yeast and controls plant tubers.

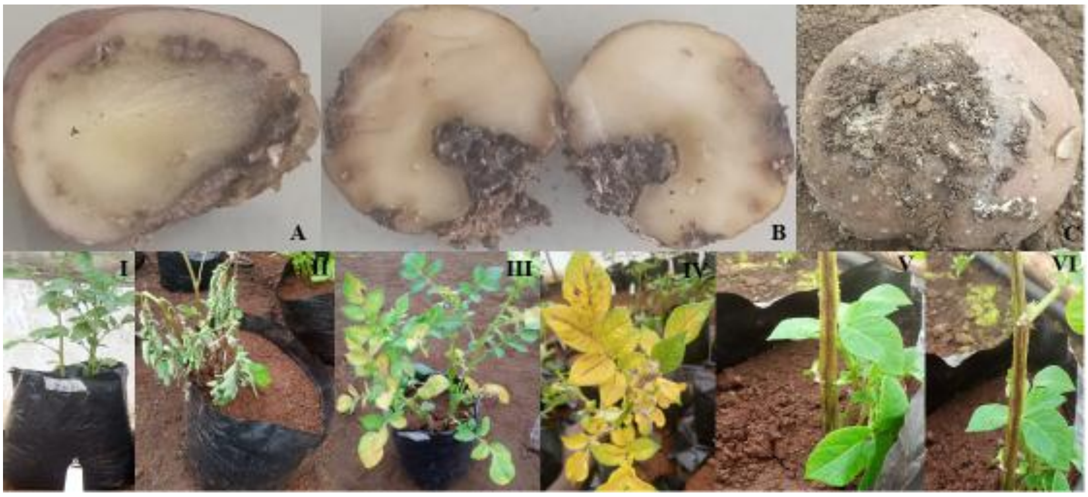

Plate 10: Bacterial wilt progressive symptoms in Irish potato. (A) Cross-section showing brown discoloration of the vascular ring, (B) cross-section showing bacterial ooze, (C) bacterial ooze on the infected tuber with soil clamped on it. (I) healthy plant, (II) wilting leaves at the end of branches, (III) partial chlorosis, (IV) complete chlorosis, (V &VI) brown discoloration of the vascular bundle visible on the stem.

4.3 Effect of *Escherichia coli*, *Saccharomyces cerevisiae* and Attenuated *Ralstonia solanacearum* on Salicylic Acid Levels in the Leaves of Induced Potted Potato Plants

Ultra violet (UV) spectrophotometric method was used for the quantitative determination of the amount of SA in leaf extract of the Irish potato. The levels of SA were quantified by comparing the area of the corresponding peaks with the standard curve created using different concentrations (0.5, 1, 2, 4 and 8 ppm; Figure 4). The test of the fitted model revealed that it was adequate ($p < 0.05$) in explaining the relationship between treatments (SAR inducing factors and varieties) and the level of SA (Appendix VIII). Test on the effect of each factor and their combined effect indicated that there was a significant effect ($p < 0.05$) of each factor and their interactions on the levels of SA (Appendix IX). The highest SA level was recorded in samples that were primed with attenuated *Ralstonia solanacearum* (3409.64 mg/kg of leaves), while *Ralstonia solanacearum* recorded the lowest SA level (1222.74 mg/kg of leaves). Two inducing factors, *Escherichia coli* and *S. cerevisiae* recorded SA level above the overall mean (1890.43 mg/kg of leaves) while the remaining two factors (*R. solanacearum* and distilled water) recorded SA levels below the overall mean. The analysis of the effect of varieties on the level of salicylic acid revealed that the mean ranged from 1928.31 mg/kg (in Kenya Mpya) to 2320.33 mg/kg of leaves (in Kenya Karibu; Table 6).

However, Kenya karibu and Unica varieties did not give statistically significant different amount of salicylic acid levels.

Table 6: Means of salicylic acid in different induction factors and varieties

SAR inducing factors	Level of Salicylic acid (mg/kg of leaves)	Variety	Level of Salicylic acid (mg/kg of leaves)
Attenuated *Ralstonia solanacearum*	3409.64a	Kenya Karibu	2320.33a
Escherichia coli	2619.80b	Unica	2310.23a
Saccharomyces cerevisiae	2021.31c	Tigoni	2218.22b
Sterilized distilled water	1697.88d	Kenya Mpya	1928.31c
Ralstonia solanacearum	1222.74e		
LSD	59.239	LSD	52.985

The analysis of the combined factors effect (interaction between SAR inducing factors and Irish potato varieties) on level of salicylic acid revealed that the mean ranged from 813.11 mg/kg of leaves (in *R. solanacearum* and Kenya Mpya variety) to 3826.13 mg/kg of leaves (in attenuated *R. solanacearum* and Kenya Karibu variety; Table 7). The overall mean of salicylic acid in all the treatments was 2194.27 mg/kg (Table 7). Generally, Irish potato plants that were primed to induce SAR with attenuated *R. solanacearum* gave high amount of salicylic acid in all the varieties while *R. solanacearum* treated plants recorded very low amount of the phenolic pheromone in all the four varieties (Table 7).

Table 7: The combined of systemic acquired resistance inducing factors and Irish varieties on the levels of salicylic acid in Irish potato leaves infected with *Ralstonia solanacearum*

Treatment	Level of Salicylic Acid (mg/kg) of Irish potato leaves
KKR	3826.13a
TR	3766.56a
UR	3466.80b
KME	2991.97c
KKE	2793.47d
UE	2611.33e
TS	2605.20e
KMR	2579.07 e
TE	2082.43f
KKS	1976.80f
UW	1802.15g
US	1762.80g
KKW	1754.13g
KMS	1740.44g
TW	1718.29g
KMW	1516.95h
UO	1448.03hi
TO	1378.69i
KKO	1251.12jk
KMO	813.11k
Mean	2194.27
LSD	118.48
CV%	5.8%
R-squared	0.979738

[a]Means followed by the same letters are not significantly different at 5% significance level.

Where, KK = Kenya Karibu, KM = Kenya Mpya, U = Unica and T = Tigoni. R = attenuated *R. solanacearum*, E = *E. coli*, S = *S. cerevisiae*, W = sterilized distilled water and O = *R. solanacearum*.

4.4 Effect of *Escherichia coli*, *Saccharomyces cerevisiae* and Attenuated *Ralstonia solanacearum* on Tuber Yield of Irish Potato inoculated with *R. solanacearum*

The test of the fitted model indicated that it was adequate ($p < 0.05$) in explaining the linear relationship between different treatments (SAR inducing factors and Irish potato varieties) and the yield of Irish potato tubers (Appendix X). Tests on the effect of each factor and interaction between the factors showed that there was a significant effect ($p < 0.05$) of factors and their interaction on the yield of Irish potato tubers (Appendix XI). The blocking of the treatments was effective ($p < 0.05$) indicating that effect of arrangement of the treatments was effectively controlled. Analysis of the effect of SAR inducing factors on tuber yield revealed that attenuated *R. solanacearum* resulted to the highest yield of the tubers (0.5772 kg/plant) while *R. solanacearum* had the lowest yield of tubers (0.1507 kg/plant; Table 8). However, treatments of *E. coli* and attenuated *R. solanacearum* did not give significantly different yield of Irish potato tubers. The analysis of effect of Irish potato varieties on tuber yield revealed that the tuber yields ranged from 0.2240 kg/plant (in Tigoni) to 0.5915 kg/plant (in Unica; Table 8).

Table 8: Effect of induction factors and Irish potato varieties on tuber yield

SAR inducing factors	Tuber yield (kilogram/plant)	Variety	Tuber yield (kilogram/plant)
Attenuated *R solanacearum*	0.5772a	Unica	0.5915a
Escherichia coli	0.5442a	Kenya Karibu	0.4198b
Saccharomyces cerevisiae	0.4840b	Kenya Mpya	0.3698c
Sterilized distilled water	0.2779c	Tigoni	0.2240d
Ralstonia solanacearum	0.1507d		
LSD	0.037	LSD	0.0332

The analysis of the combined factors effect (interaction between SAR inducing factors and Irish potato varieties) on yield of Irish potato tubers showed that the mean ranged from 0.1505 kg/plant (in KKO treatment) to 0.7629 kg/plant (in UR treatment; Table 9). Generally attenuated *Ralstonia solanacearum* gave better yields as compared to the other SAR inducing factors. The overall mean for tuber yield was 0.410578 kg/plant. Plants that were not primed to induce SAR produced very low yields (0-0.4289 kg/plant). The study demonstrated that the infection of potato tubers by *Ralstonia solanacearum* altered tuber characteristics. The tubers harvested from inoculated Irish potatoes in the greenhouse experiment had the same morphological characteristics (colour and shape) as to those in the market (Appendix XII).

Table 9: Yield of tubers in treated Irish potato leaves infected with *R. solanacearum*

Treatment	Yield of Irish potato tubers (kilograms/plant)
UR	0.7629a
KKR	0.7042ab
UE	0.6978ab
US	0.6656cd
KKE	0.6011de
KME	0.5612ef
KMR	0.5437ef
KMS	0.5328ef
KKS	0.4885fg
UW	0.4289gh
UO	0.4022hi
TW	0.3367ij
TE	0.3167jk
TS	0.2492kl
KMW	0.2111m
TR	0.1583m
KKW	0.1544m
KKO	0.1505m
KMO	0
TO	0
Mean	0.410578
CV%	10.58211
LSD	0.0747
R-squared	0.978283

[a]Means followed by the same letters are not significantly different at 5% significance level.

Where, KK = Kenya Karibu, KM = Kenya Mpya, U = Unica and T = Tigoni. R = attenuated *R. solanacearum*, E = *E. coli*, S = *S. cerevisiae*, W = sterilized distilled water and O = *R. solanacearum*.

CHAPTER FIVE

DISCUSSION

5.1 Effect of Formaldehyde Concentration and Incubation Duration for Attenuation on the Number of Colonies of Attenuated *Ralstonia solanacearum.*

This study revealed that colony forming units of *R. solanacearum* significantly reduced with an increase in concentration of formaldehyde. All the formaldehyde concentrations utilized in this study had a positive impact in inactivating *Ralstonia solanacearum*. Although all the concentrations were effective in significantly reducing the number of colonies, the numbers reduced with increased incubation time after addition of formaldehyde. Concentrations of 0.4% and 0.6% were the most effective at two hours of incubation for complete inactivation of *R. solanacearum*. Treatment without formaldehyde (0% formaldehyde concentration) had the highest numbers of colony count due to lack of the inactivation agent with highest colony count being recorded after two hours of incubation for attenuation. Colony count in the absence of formaldehyde (0%) increased with an increase in incubation duration which was not the case in the 0.2%, 0.4% and 0.6% concentrations. This result is similar to a study by Chikere & Udochukwu (2014) where increase in incuation time increased the number of soil bacteria. Formaldehyde is known to react with nucleic acid to form crosslinks between protein and nucleic acids, a process which is mediated by an aldehyde group (Hoffman *et al*., 2015). The process leads to formation of methylene bridges, protein components and modifications related to Schiff bases and methyl groups thereby preserving the cell structures (Hoffman *et al*., 2015). Reactive dihydroxydimethyl peroxides and free radicals are formed in the presence of oxidizing molecules leading to oxidative stress and ultimately death of the cells. Unspecific binding of formaldehyde to different macromolecules results to inactivation of these molecules biological functions. This ends up damaging vital cell components like DNA, RNA and proteins through multistep formation of adducts and cross links leading to decline in the number of colony count of cells (Song *et al*., 2013; Kawanishi *et al*., 2014).

The results from this study is in agreement with those by Arshadi *et al*. (2020), which showed that concentrations of above 0.4% and 0.6% and incubation duration of one hour is ideal for killing pathogenic microbes. A study by Arshadi *et al*, (2020) on the most effective inactivation method between heat and formalin on *E. coli* revealed that both had variations in inactivating the microbe. However, formalin inactivation of *E. coli* showed better response compared to heat inactivated cells.

The duration of incubation was found to be directly proportional to the number of colonies, with number of colonies count decreasing with increase in incubation time and formaldehyde concentration. This finding was similar to a study by Chua *et al*. (2019) who examined the effectiveness of formalin in inactivating tissues infected by pathogenic bacteria. The study revealed that cells in formaldehyde stored for 14 days were completely fixed with one exception where *B. anthracis* spores were partially inactivated at 13 days of fixation but inactivated at 20 days. This current study demonstrated that short incubation durations (30 minutes and 1 hour) at 30℃ are not sufficient to completely inactivate *Ralstonia solanacearum*. A significant reduction in colony count to reach complete inactivation was found with a further increase of incubation time. Further modification like increasing temperature to above 30℃ could shorten the time required for complete inactivation even further. Increasing formaldehyde concentration could also accelerate inactivation and reduce incubation time (Möller *et al*., 2015). This current study also revealed that with increased incubation time, the effect of this chemical becomes more intense as the number of colonies reduce progressively.

Low concentration levels of formaldehyde inactivated the pathogenic microbe to some extent but also took a long incubation time in achieving this. In this study, an increase in formaldehyde concentration further inactivated *R. solanacearum*. Rutala *et al*, (2008) reported that different concentrations of formaldehyde destroy a wide range of microbes. The study showed that poliovirus subjected to a concentration of 8% takes 10 minutes to inactivate whereas other viruses inactivate with only 2% formalin. In bacteria, *M. tuberculosis* is inactivated with 4% formalin in 2 minutes while *Salmonella typhi* takes about 10 minutes to inactivate with 2.5% formalin. From all these findings, it is clear that almost all concentrations of formaldehyde inactivate microorganism and this concurs with the current study where very minimal amounts of formaldehyde were used in *R. solanacearum* inactivation.

5.2 Effect of *Escherichia coli*, *Saccharomyces cerevisiae* and Attenuated *Ralstonia solanacearum* on Disease Severity of Bacterial Wilt in Selected Irish Potato Varieties

Bacterial wilt symptoms in infected plants that had been primed with *Saccharomyces cerevisiae,* distilled water and *R. solanacearum* treatments started by wilting of the leaves at the tip of the branches. Wilting of young leaves is caused by loss of turgidity and drooping of plants due to colonization by pathogenic microbe that leads to blockage of water transport. Most of the infected plants were completely wilted by 9 days after infection with bacterial wilt. Infected vascular bundles became narrow with dark brown streaks visible on the potato stems which led to collapse of some, which is similar to an observation made by Manda *et al.* (2020). Sticky milky-white exudates were observed in *S. cerevisiae,* distilled water and *R. solanacearum* treatments. Cross-section cut of *E. coli* and attenuated *R. solanacearum* did not show any exudates.

There was very high disease severity in all the selected Irish potato varieties treated with *S. cerevisiae* treatment. The variation in infection rates was attributed to different varieties (Kenya Karibu, Kenya Mpya, Unica and Tigoni) and SAR inducing factors (*E. coli, S. cerevisiae, R. solanacearum,* water and attenuated *R. solanacearum*) used. The plants treated with *S. cerevisiae* showed very severe symptoms even at three days after inoculation, displaying partial chlorosis while others died as early as the sixth day after inoculation. Studies have shown that pathogenicity of fungal microbes on plants depend on filamentous process since they depend on the ability to undergo dimorphic transition process (Cullen & Sprague, 2012; Boyce & Andrianopoulos, 2015). Plants primed with *S. cerevisiae* to induce SAR were observed to have disease symptoms even before infection with the pathogen. This observation could be as a result of dimorphism that occurred in *Saccharomyces cerevisiae*. Dimorphism is essential for pathogenicity as it requires the fungus to sense and respond to the plant environment (Boyce & Andrianopoulos, 2015). The cAMP- dependent protein kinase A (PKA) and mitogen-activated protein kinase (MAPK) pathways govern dimorphic development in both pathogenic and non-pathogenic fungi such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (*Gerits et al*., 2008). These pathways have been found to control pseudohyphal growth that somehow contribute to the pathogenicity of fungus. It could be that the strain of *S. cerevisiae* utilized in this study was capable of forming pseudohyphae, which is associated with virulence in non-pathogenic fungi thus leading to higher disease severity in those treatments.

Plants with *Saccharomyces cerevisiae* showed symptoms of disease in almost all the varieties. A study by Gognies *et al*. (2001) on the potential virulence of *S. cerevisiae* on *Vitis vinifera* revealed that yeast with filamentous growth and endopolygalacturonase (endoPG) activity triggered necrosis of the host plantlets. Based on the study above, it can be summarized that filamentous differentiation is necessary for yeasts to penetrate plants tissues. This signifies that the strain of *S. cerevisiae* used in this study to trigger resistance was capable of filamentous growth and endoPG activity thereby being able to suppress immunity in Irish potato tissues causing infection. In this current study, Unica potato variety displayed partial resistance and was not overwhelmed by the two microbes as compared to the other varieties. Kenya Karibu variety was found to be moderately resistant while Tigoni was highly susceptible to both *S. cerevisiae* and *R. solanacearum*. The difference in disease severity among *S. cerevisiae* treatments could be attributed to the presence of effectors that could have triggered response in some genotypes (Unica and Kenya Karibu). High disease severity from this study is similar to a study carried out by (Oro *et al* 2014) on biocontrol of postharvest brown rot of sweet cherries by *S. cerevisiae* Disva 599, *M. pulcherrima* Disva 267 and *W. anomalus* Disva 2 strains which disqualified *S. cerevisiae* as a biological agent and characterized it as killer yeast.

The response of the four Irish potato varieties studied against *E. coli* treatments differed significantly. The disease severity of plants with *E. coli* treatments ranged from 0% - 60% indicating that the microbe triggered plant defense responses against bacterial wilt. Survival of *E. coli* is affected by plant defense response, which is triggered by the perception of bacterial PAMPs. It is probable that Irish potato plants detected multiple *E. coli* PAMPs and activated multiple defense pathways of the innate immune system. The result from this study suggested that certain factors associated with *E. coli* enhanced recognition of the enteric pathogen by plants. Conditions such as temperature and nutrient availability influence cell counts of bacteria and this could have had an impact on plant defense response. A study by Seo & Matthews (2012) to assess whether human pathogens regulate defense responses revealed that both *E. coli* and SL1344 induced an early expression of the PR1 gene. However, *E. coli* induces higher level of the gene than the later (Roy *et al*., 2013). A study has also demonstrated that this human pathogen (*E. coli*) induces stomatal closure in Arabidopsis leaves which reduces diseases severity (Melotto *et al*., 2006). Several populations of *E. coli* strains have been shown to lack cell surface structures hence inducing less PR gene than those with cell surface structures (Seo & Matthews, 2012), thus it is possible that some bacterial strains may induce stronger immunity than others depending on how they can recognize PAMPs and trigger defense responses.

Disease severity was low in attenuated *R. solanacearum* treated plants with Kenya Karibu variety having the lowest disease severity of 20%. Highest disease severity was realized in Tigoni variety (80%). Attenuated pathogens have been used to protect crops against various diseases (Nishiguchi & Kobayashi, 2011). This is a method that is based on cross interference of protection and has been proved to show resistance against severe strains of viruses and bacteria. A study by Karyn *et al*. (2007) on the virulence of plant pathogenic bacteria attenuated by degradation of fatty acid cell-to-cell signalling factors (DSF) revealed that overexpression of carAB in endophytes could be important for the control of diseases caused by pathogens that produce DSF. The study showed that inoculation with DSF-bacteria reduced disease incidence and severity in mustard and cabbage leaves.

5.3 Effect of *Escherichia coli*, *Saccharomyces cerevisiae* and Attenuated *Ralstonia solanacearum* on Salicylic Acid Levels in the Leaves of Induced Potted Potato Plants

The study demonstrated that *E. coli* and attenuated *R. solanacearum* could increase levels of SA in plants thus inducing SAR. The plants treated with attenuated *R. solanacearum* had the highest amount of SA followed by *E. coli, S. cerevisiae,* water and *R. solanacearum*. R-squared (0.98) obtained implied that 97.97% of the total variability observed in salicylic acid levels can be explained by change in SAR inducing factors and Irish potato varieties (Table 6). Consequently, the level of SA in leaves of induced potato plants can be attributed to the SAR inducing factors and well as the genotype of the studied potato varieties. This study showed that disease severity can be reduced by utilizing some of the pathogenic and non-pathogenic microbes to increase the levels of SA in plants. Upon inoculating the plants with *E. coli* and *R. solanacearum,* a change in cellular redox system occurs that induces nonexpressor of pathogenesis-related genes (NPR) 1-complex dissociation to monomers (Fu and Dong, 2013). These monomeric forms are translocated into the nucleus where they interact with transcription factors inducing transcriptional activation of the defense-related genes (Fu and Dong, 2013).

Escherichia coli and attenuated *R. solanacearum* treatments recorded high SA levels in all the varieties reducing the impact of bacterial wilt disease through expression of SA-induced defensive genes. At high SA concentration, cellular reduction potential is modified that leads to structure changes in NPR to monomers through the reduction of the intermolecular bridges by a change in redox (Dempsey and Klessig, 2017). This enables NPR1 to enter the nucleus binding to specific transcription factors inducing the expression of SA-induced defensive response genes. The activity of NPR1 is also regulated by proteasome-mediated degradation. There was high disease severity in the remaining treatments (*S. cerevisiae,* distilled water and *R. solanacearum*) due to low level of salicylic acid. At low SA intracellular concentration, NPR4 maintains low NPR1 levels hence the plants lack defense signals that leads to high disease severity in plants. Nonexpressor of pathogenesis-related genes 1 forms an oligomeric complex localized in the cytosol at low levels of SA. However, extremely high SA leads to NPR1 degradation when NPR3 binds to NPR1 (Dempsey and Klessig, 2017). After SA-triggered responses, plants are able to induce a long lasting defense response (SAR) that includes accumulation of PR proteins and biosynthesis of SA (Mishina and Zeier, 2007). *Escherichia coli* and *R. solanacearum* treated plants were able to provide resistance against bacterial wilt due to SA trigger.

Plants recognized PAMPs which triggered PTI (Yu *et al*., 2022). In this study, Irish potato plants were able to recognize *E. coli* and attenuated *R. solanacearum* flagellin thereby triggering PTI. Pathogen-associated molecular patterns in bacteria and fungi are bacterial flagellin and fungal chitin respectively (Zipfel & Robatzek, 2010; Ipcho *et al*., 2016). Flagellin is a structural protein of the flagellum containing as many as 20, 000 subunits that is responsible to bacterial motility (Imada, 2018). In situations where pathogens overcome the first layer of immune defense as in the case of *Saccharomyces cerevisiae* by evading recognition by PRRs or suppressing PTI signalling, the effector-triggered immunity (ETI) is triggered. This immunity recognizes pathogen effectors through polymorphic NB-LBR proteins resulting in HR to limit the pathogen spread (Jones & Dangl, 2006).

Plants primed with *E. coli* and attenuated *R. solanacearum* induced SAR leading to low disease severity in these treatments. The remaining SAR inducing factors (S. *cerevisiae*, water and *R. solanacearum* did not induce resistance against bacterial wilt in Irish potato plants. Primary signalling of SAR occurs through MeSA whose endogenous levels increase upon infection in a plant. Systemic acquired resistance requires expression of PR genes and accumulation of SA whose level increase locally and systemically in infected host tissues (Bektas & Eulgem, 2015; van Loon, 2016). Salicylic acid is mobile during induction of SAR and most of it usually accumulate on the upper non-infected leaves of induced plant. Biosynthesis of Salicylic acid from chorismate is via two reactions catalysed by isochorismate synthase and isochorismate pyruvate lyase. The ICS pathway is more important for sustained SA synthesis during SAR development (Kumar *et al*., 2015). Carboxylase methyltransferase (SAMT) enzyme converts SA into inactive MeSA in infected plant tissues which is later converted back to SA to induce SAR by the activity of SA binding protein 2.

5.4 Effect of *Escherichia coli, Saccharomyces cerevisiae* and Attenuated *Ralstonia solanacearum* on Tuber Yield of inoculated Irish Potato

Treatment of Irish potato with *E. coli*, *S. cerevisiae* and attenuated *R. solanacearum* resulted in varied yield of potato tubers of the studied varieties. R-squared was 0.98 implying that 97.83% of the total variability observed in tuber yields can be explained by change in SAR inducing factors and individual potato varieties (Table 8). Most plants treated with distilled water and *R. solanacearum* were severally infected by the pathogen and did not reach maturity. Yield of tubers was significantly high in plants that were treated with *E. coli* and attenuated *Ralstonia solanacearum* which could be majorly due to prior exogenous application of the mentioned treatments. This may have triggered systemic acquired resistance by increasing salicylic acid levels thereby conferring protection against the pathogen that was later inoculated into the plants. Systemic acquired resistance could also have been triggered by jasmonic acid and ethylene which are also major players in the regulation of signalling networks involved in induced defense responses against the microbes.

In this study, SAR was potently activated in the plants pre-treated with attenuated *R. solanacearum* and *E. coli* providing resistance against the bacterial wilt pathogen. Plants cope with virulent pathogens after triggering an array of defense reactions following a localized necrotizing infection, a process which is mediated by accumulation of SA in the plants. These two treatments acted as functional monologues of SA providing resistance against the intended pathogen and other unrelated pathogens that could have attacked the plants in the greenhouse such as late blight. There are several studies that have been carried out using different crops and the results reveal that high levels of SA in plants increases yields (Kaur *et al.,* 2022). A study by Alutbi *et al*. (2017), on the effect of SA on the growth and microtuberization of potato (*Solanum tuberosum* l.) cv. Arizona propagated in vitro revealed that it improves shoot number, leaf area, root length, chlorophyll content and micro tuber weight and number. Additionally, a study carried out by Tucuch-Haas *et al*. (2017), spraying maize plants with 1 µM and 0.1 µM SA resulted in 29 and 21 % increases in cob length, respectively. From that study, grain yield showed a highly significant and positive correlation with all the cob variables, suggesting that the increase of grain yield may be linked to the effect of SA on the cobs. Therefore, increase of yield of Irish potato tubers in this study could be attributed to the effect of various treatment that led to increase of SA in those plants.

High yields realized in attenuated *R. solanacearum* and *E. coli* treated plants was as a result of plant physiological processes and secondary metabolites that were stimulated by the increase of SA. Systemic acquired resistance is a SA dependent process where SA regulates plant growth and may also increase length, weight, perimeter and root area of a crop thereby increasing yield (Gozzo, 2003; Conrath, 2006). Salicylic acid also triggers a number of physiological processes in the plants; stomatal closure, reduction of photochemical capacity and increase photosynthetic activity. This compound induces proteins linked to PR that stimulates the activity of peroxidase, β-1, 3-glucanase and catalase and accumulation of some nutrients in the plants (Magnesium, Calcium and Potassium) (Fatima *et al*., 2014). Exogenous application of SA has been documented to protect plants against damage caused by certain diseases and increase yield and quality of crops such as tomato, pepper, wheat and cucumber (Martin-Mex *et al*., 2013; Fatima *et al*., 2014).

The genotypic variation may also have greatly contributed to difference observed in yield of potato tubers. Unica, gave the highest yield of potato tubers with attenuated *R. solanacearum* treatments while the Tigoni variety gave the lowest. Unica, gave the highest yield of potato tubers with attenuated *R. solanacearum* treatments while the Tigoni variety gave the lowest. Different varieties are expected to contain different genes combination that control trait variation such as disease resistance, yield and size of tubers. The variety, according to Zelalem *et al*. (2009), genetic makeup of a variety influences its stem density thereby increasing the number of tubers and/or size of tubers. Unica is a high yielding variety with large sized tubers as compared to Tigoni variety, which has very small tubers.

Size of the tubers, plant architecture, disease resistant levels, growth, development and yielding potential could be attributed to genetic variations among the varieties studied. Low yield in Tigoni variety could be attributed to the small size of planting material (tubers) used. Ebrahim *et al*. (2018) and Singh *et al*. (2022) study on effects of seed tuber size on growth and yield performance of potato revealed that medium to large sized tuber seeds produced higher tuber yield. Generally, Unica and Kenya Karibu varieties recorded better results over the other varieties with respect to SA levels and tuber yield. *Escherichia coli* and attenuated *R. solanacearum* offered the highest yield and SA as compared to *Saccharomyces cerevisiae*.

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary of the Findings

Bacterial diseases are major problems faced by Irish potato farmers in Kenya. Bacterial wilt disease has spread to all potato growing areas in Kenya attacking over 70% of potato farms resulting to yield losses of between 50% - 100%. *Ralstonia solanacearum*, the causative agent for bacterial wilt, has a wide host range including the weeds. The bacterium survives in the soil for a long period making it difficult to control even with the use of certified seeds and uncontaminated tubers. Several methods have been applied in the control *R. solanacearum*. These include biocontrol, chemical control, cultural practices, host resistance and phytosanitation have been used to manage *R. solanacearum*. However, these methods have not been effective. Therefore, there is need to come up with alternative methods of controlling bacterial wilt, especially non-chemical methods.

Inducing systemic acquired resistance could be an alternative method for the control of bacterial wilt. Systemic acquired resistance confers long-lasting protection against a broad spectrum of microorganisms. Thus, this study determined the induction of SAR against bacterial wilt in Irish potato through injection of a pathogen and non-pathogens. Induction of SAR by *Escherichia coli*, *Saccharomyces cerevisiae* and attenuated *Ralstonia solanacearum* can be an efficient, environment friendly and cost effective and can lead to increased and sustainable production of Irish potatoes. A study was also carried out to determine the effective formaldehyde concentrations that inactivate *R. solanacearum* and evaluate selected microbes that trigger resistance against bacterial in Irish potato.

A laboratory experiment was used to evaluate the effect of formaldehyde concentrations and incubation duration for attenuation on the number of colony counts of *R. solanacearum* in the laboratory experiment. To determine the induction of SAR against bacterial wilt in Irish potato, a controlled experiment in a greenhouse was carried out using a randomized complete block design with two factors; factor A (SAR inducing factors at 5 levels) and factor B (Irish potato varieties at four levels). The plants were inoculated with treatments of *E. coli*, *S. cerevisiae*, attenuated *R. solanacearum* and water then later on inoculated with the pathogen after a period of 7 days. Disease severity was then scored using disease score criteria scale of 0-6. salicylic acid was quantified using UV-spectrophotometry to determine the amount of SA produced by inoculated Irish potato leaves. The weight of potato tubers was taken 18 days after inoculation with *Ralstonia solanacearum*.

The level of attenuation was measured in terms of colony forming units. Colony forming units varied with different incubation time for attenuation of *R. solanacearum*. There was significant effect of both factors in attenuation of *Ralstonia solanacearum*. The highest colony count was recorded in 0% (179.25) followed by 0.2% (45.92 CFU/ml), 0.4% (5.0 CFU/ml) and lastly 0.6% (2.08 CFU/ml). Colony count was high at 120 minutes (66.5 CFU/ml) followed by 90 minutes (57.83 CFU/ml), 60 minutes (56.17 CFU/ml) and 30 minutes (51.75 CFU/ml). The result indicated that colony count varied in all the concentrations and incubation time. There was no significant difference between 0.4% and 0.6% FCs.

The results from disease severity indicated that there was a significant difference ($p < 0.05$) in SAR inducing factors (*E. coli*, *S. cerevisiae*, water, *R. solanacearum* and attenuated *R. solanacearum*) and varieties (Kenya Karibu, Kenya Mpya, Unica and Tigoni). *Escherichia coli* and attenuated *R. solanacearum* had the lowest percentage of disease severity thereby conferring resistance against bacterial wilt in Irish potato. Positively induced plants were healthy with dark green leaves from 3^{rd} to 12^{th} DAI. The highest rate of severity was recorded in treatments of *Saccharomyces cerevisiae*, water and *R. solanacearum* with as much as 40 % of disease symptoms on the first day after infestation. Kenya Karibu and Unica varieties had the lowest means from the 3^{rd} day to the 18^{th} DAI. These results lay groundwork for the development of biocontrol strategies of attenuated microbes and other plant non-pathogenic microbes in managing bacterial wilt disease in Irish potato.

The results from SA quantification indicated that the concentration of SA extract was high in attenuated *Ralstonia solanacearum* (3409.64 mg/kg of leaves) followed by *Escherichia coli* (2619.80 mg/kg), *Saccharomyces cerevisiae* (2021.31 mg/kg of leaves), distilled water (1697.88 mg/kg of leaves) and *R. solanacearum* (1222.74 mg/kg of leaves). Based on varieties, Kenya Karibu had the highest level of SA (2320.33 mg/kg of leaves) followed by Unica (2310.23 mg/kg of leaves), Tigoni (2218.22 mg/kg of leaves) and lastly Kenya Mpya (1928.31 mg/kg of leaves). Generally, there were significant effect of both treatments (SAR inducing factors and varieties) in the level of SA.

The effect of different treatments and varieties on yield was evaluated by measuring the yield of potato tubers in tonnes/ha. There was significant difference ($p < 0.05$) in all the treatments. Irish potato plants with *E. coli* and attenuated *Ralstonia solanacearum* had high yields with an average 0.5442 kg/plant and 0.5772 kg/plant respectively while those with *S. cerevisiae* had low yield (0.4840 kg/plant). Plants that were not primed to induce SAR produced very low yields (0.2142 kg/plant) due to the effect of the pathogen. Plants used in control produced very low yields due to the severe effect of the pathogen. The highest yield was recorded in Unica variety (0.5915 kg/plant) followed by Kenya Karibu (0.4198 kg/plant), Kenya Mpya (0.3698 kg/plant) and lastly Tigoni (0.2240 kg/plant).

6.2 Conclusion

The optimum concentration of formaldehyde and incubation time for attenuating *R. solanacearum* was within the range of 0.4 - 0.6% formaldehyde and at least two hours of incubation. The highest disease severity rate was recorded in *R. solanacearum* treatments while the lowest recorded in attenuated *R. solanacearum* treatments. All the treatments showed different rates of infection with distilled water and *R. solanacearum* treatments having high disease severity rates. Plants primed with attenuated *Ralstonia solanacearum* recorded the highest level of salicylic acid while *Ralstonia solanacearum* recorded the lowest SA level. All the Irish potato varieties that were screened in the greenhouse were susceptible to bacterial wilt. However Kenya Karibu and Unica varieties had the lowest disease severity with high levels of salicylic acid. The findings from this study suggested that overexpression of SA in potato induces SAR in plants reducing impact of diseases. Yield was high in Unica and Kenya Karibu varieties making them the most tolerant varieties. These findings lay groundwork for the development of strategies for generation of resistant varieties using these microbes.

6.3 Recommendation of the Study

This study has the following recommendations:

i. Attenuation of *Ralstonia solanacearum* requires a concentration ranging from 0.4 - 0.6% formaldehyde and incubation duration for attenuation of *R. solanacearum* of at least two hours.

ii. Induction of SAR by *Escherichia coli* and attenuated *Ralstonia solanacearum* reduce severity of bacterial wilt disease in Irish potato.

iii. Irish potato plants primed with *E. coli* and attenuated *R. solanacearum* increase SA levels thereby inducing systemic acquired resistance against diseases.

iv. Systemic acquired resistance primed plants with *Escherichia coli* and attenuated *Ralstonia solanacearum* improves yield by lowering disease severity in plants.

6.4 Suggested Further Study

i. There is need for identification of the specific proteins in attenuated *Ralstonia solanacearum* and *E. coli* that are responsible for induction of SAR against bacterial wilt in Irish potato.

ii. To find out why *Saccharomyces cerevisiae* though a non- pathogenic fungus, induced disease symptoms in plants.

iii. To carry out molecular characterization of *Saccharomyces cerevisiae* (baker's yeast) to find out the specific strain of Saccharomyces that causes disease symptoms in plants.

iv. Further studies should be done to come up with a method through which farmers can be able to use *Escherichia coli* and attenuated *Ralstonia solanacearum* for efficient management of bacterial wilt.

v. Additional research should be carried out to evaluate the mechanism of formaldehyde inactivation on bacterial pathogens.

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APPENDICES

Appendix I: Disease score criteria

Rating value	% of disease symptoms	Description	Disease reaction
1	0	Healthy plant; free from disease	Highly resistant
2	20	Plant starting to wilt	Resistant
3	40	Wilted plant	Moderately resistant
4	60	Partial chlorosis	Susceptible
5	80	Complete chlorosis	Susceptible
6	100	Dead	Highly susceptible

Disease score criteria modified version of (Trapero-Casas & Jimenez-Diaz, 1985)

Appendix II: National Commission for Science, Technology and Innovation Research Permit

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Appendix III: Test for the model adequacy for the effect of formaldehyde concentration and incubation duration on number of colony counts of *Ralstonia solanacearum* analysis

Source of variation	df	Sum of Squares	Mean Square	F. value	p-value
Model	17	269933.6042	15878.4473	646.16	<0.0001
Error	30	737.2083	24.5736		
Corrected total	47	270670.8125			

Appendix IV: Analysis of variance for the effects of formaldehyde concentration and incubation duration on number of colony counts of *Ralstonia solanacearum*

Source of variation	df	Sum of Squares	Mean Square	F. value	p-value
Replication	2	24.1250	12.0625	0.49	0.6169
Concentration	3	249398.7292	83132.9097	3383.02	<.0001
Duration	3	1376.2292	458.7431	18.67	<.0001
Concentration*Duration	9	19134.5208	2126.0579	86.52	<.0001

Appendix V: Kruskal-Wallis test for disease severity of bacterial wilt of selected Irish potato at different growth stages after infection

Days after infection	Chi-square value	df	p-value
3	93.5455	19	<0.0001
6	99.7282	19	<0.0001
9	106.5536	19	<0.0001
12	97.0129	19	<0.0001
15	94.3638	19	<0.0001
18	79.7284	19	<0.0001

Appendix VI: P-value from Kruskal-Wallis median test of severity of bacterial will in selected Irish potato varieties

Days after infection	Treatment	KMR	UR	KKR	KKE
3	KMO	0.0291	0.0246	0.017	0.0246
	UO	0.0463		0.0274	
	KKO			0.0458	
	TO	0.0349		0.0202	
	KMW	0.0299	0.0403		
	UW	0.0246	0.0441	0.014	0.0441
	KKW	0.0149	0.0379	0.0088	0.00379
	TW	0.0276	0.0428	0.0156	0.0428
6	KMO		0.036	0.02	0.0284
	UO			0.0274	0.0409
	KKO	0.0426	0.0358	0.016	
	TO		0.0492	0.02	
	KMW	0.031	0.0232	0.0136	0.0187
	UW	0.044	0.0426	0.014	0.0263
	KKW	0.0263	0.0201	0.0115	0.016
	TW			0.0177	0.0322
9	KMO		0.0468	0.02	0.0363
	UO			0.0274	
	KKO		0.0413	0.0173	0.0333
	TO			0.0215	
	KMW	0.0372	0.0296	0.0136	0.0234
	UW	0.0448	0.0352	0.014	
	KKW	0.0172	0.0156	0.0059	0.0116
	TW		0.0474	0.0173	0.0384
	KMS			0.0169	
12	KMO		0.043	0.0263	
	KKO		0.0362	0.0237	
	TO			0.0381	
	UW		0.0456	0.0264	
	KKW		0.0361	0.0201	
	TW			0.0284	
15	KMO		0.0416	0.0353	
	KKO	0.0339	0.0248	0.0211	
	TO			0.0458	
	KMW	0.0496	0.0351	0.026	
	UW	0.0496	0.0351	0.026	
	KKW	0.0496	0.0351	0.026	
	TW		0.0454	0.0296	

Where, KK = Kenya Karibu, KM = Kenya Mpya, U = Unica and T = Tigoni. R = attenuated *Ralstonia solanacearum*, S = *Saccharomyces cerevisiae*, E = *Escherichia coli*, W = sterilized distilled water and O = *Ralstonia solanacearum*.

Appendix VII: Medians of disease severity from three to eighteen days after infection of selected Irish potato varieties with *Ralstonia solanacearum*

Treatment	DS3 DAI	DS6 DAI	DS9 DAI	DS12 DAI	DS15 DAI	DS18 DAI
KKE	1 (0%)	1(0%)	1(0%)	2(20%)	2(20%)	3(40%)
KKO	4 (60%)	4(60%)	5(80%)	6(100%)	6(100%)	6(100%)
KKR	1(0%)	1(0%)	1(0%)	1(0%)	2(20%)	3(40%)
KKS	1(0%)	2(20%)	3(40%)	4(60%)	5(80%)	6(100%)
KKW	2(20%)	3(40%)	4(60%)	5(80%)	6(100%)	6(100%)
KME	1(0)	1(0%)	1(0%)	2(20%)	2(20%)	3(40%)
KMO	4(60%)	4(60%)	5(80%)	6(100%)	6(100%)	6(100%)
KMR	1(0%)	1(0%)	1(0%)	2(20%)	2(20%)	3(40%)
KMS	4(60%)	4(60%)	4(60%)	5(80%)	6(100%)	6(100%)
KMW	3(40%)	4(40%)	4(60%)	5(80%)	6(100%)	6(100%)
TE	1(0%)	2(20%)	2(20%)	3(40%)	5(80%)	6(100%)
TO	3(40%)	4(60%)	5(80%)	6(100%)	6(100%)	6(100%)
TR	1(0%)	1(0%)	1(0%)	2(20%)	3(40%)	6(100%)
TS	1(0%)	2(20%)	3(40%)	4(60%)	5(80%)	6(100%)
TW	2(20%)	3(40%)	4(60%)	5(80%)	6(100%)	6(100%)
UE	1(0%)	1(0%)	1(0%)	2(20%)	2(20%)	3(40%)
UO	4(60%)	4(60%)	5(80%)	6(100%)	6(100%)	6(100%)
UR	1(0%)	1(0%)	1(0%)	2(20%)	2(20%)	3(40%)
US	1(0%)	1(0%)	1(0%)	3(40%)	4(60%)	4(60%)
UW	2(20%)	4(60%)	4(60%)	5(80%)	6(100%)	6(100%)

Where, KK = Kenya Karibu, KM = Kenya Mpya, U = Unica and T = Tigoni. R = attenuated *Ralstonia solanacearum*, S = *Saccharomyces cerevisiae*, E = *Escherichia coli*, W = sterilized distilled water and O = *Ralstonia solanacearum*. DS = disease severity and DAI = days after infection. Figures in parenthesis indicates the percentage of disease severity according the disease score criteria

Appendix VIII: Test for the model adequacy for the effect of the systemic acquired resistance inducing factors and Irish potato varieties on the salicylic acid levels

Source of variation	df	Sum of Squares	Mean Square	F. value	p-value
Model	21	123703989.1	5892767.5	363.80	<0.0001
Error	158	2558377.3	16192.3		
Corrected total	179	126262366.4			

Appendix IX: Analysis of variance for the effects of systemic acquired resistance inducing factors and Irish potato varieties on the salicylic acid

Source of variation	df	Sum of Squares	Mean Square	F. value	p-value
Replication	2	2879.5	1439.8	0.09	0.9150
SAR inducing factors	4	103622228.3	25905557.1	1599.87	<0.0001
varieties	3	4529216.5	1509738.8	93.24	<0.0001
Inducing factors*varieties	12	15549664.8	1295805.4	80.03	<0.0001

Appendix X: Test for the model adequacy for effect of treatments on Irish potato tuber yield of selected Irish potato infected with bacterial wilt

Source of variation	df	Sum of Squares	Mean Square	F. value	p-value
Model	21	2.97619149	0.14172340	75.08	<.0001
Error	35	0.06606976	0.00188771		
Corrected total	56	3.04226124			

Appendix XI: Analysis of variance for the effects of systemic acquired resistance inducing factors and Irish potato varieties on potato tuber yield

Source of variation	df	Sum of Squares	Mean Square	F. value	p-value
Replication	2	0.00335933	0.00167966	0.89	0.4198
SAR inducing factors	4	1.41847283	0.35461821	187.86	<0.0001
Variety	3	0.94440787	0.31480262	166.76	<0.0001
Inducing factors*Variety	12	0.43082823	0.03590235	19.02	<0.0001

Appendix XII: Samples of different potato varieties harvested from the greenhouse experiment.

Where, A is Kenya Karibu variety with smooth red skin , B is Kenya Mpya variety that is cream white in colour, C is Tigoni variety which is white with a smooth skin and D is Unica variety having a skin that is red in colour.