

**OCCURRENCE AND DISTRIBUTION OF POTATO BACTERIAL WILT  
DISEASE AND VARIABILITY OF ITS CAUSAL AGENT IN SOUTHERN  
HIGHLANDS OF TANZANIA**

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**A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE  
REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN CROP  
SCIENCE OF SOKOINE UNIVERSITY OF AGRICULTURE.  
MOROGORO, TANZANIA.**

## ABSTRACT

A survey was carried out in three potato growing Districts of the Southern Highland Zone of Tanzania to determine incidence and severity of bacterial wilt of potato caused by *Ralstonia solanacearum* and characterizing the strains of the causing pathogen. Ten villages from each district and five fields from each village were surveyed. The fields and plants were randomly selected and bacterial wilt symptoms identified. Wilt incidence was recorded based on number of plants showing symptoms and expressed as percentage of the total number of plants observed. Disease severity was recorded by severity score as described by Horita and Tsuchiya (2001). Asymptomatic and symptomatic tubers samples were collected. Asymptomatic for the detection of latent infection whereas symptomatic for characterization of *R. solanacearum* respectively. The results showed the highest incidence in Mbeya Rural (27.7%) followed by Rungwe (26.7%) and lastly Njombe (19.4%). The highest and lowest bacterial wilt severities recorded were 4.0 and 2.3 both in Mbeya rural fields. Asymptomatic tubers were (18.8%) latently infected after being tested by NCM-ELISA. Molecular characterization confirmed the isolates to be *R. solanacearum* by species specific and grouped to phylotype 2 by phylotype specific PCR respectively. Upon isolation in TZC medium twenty of thirty isolates were virulent whereas ten were avirulent. Pathogenicity test showed the isolates to be highly virulent on potato and tomato and slightly virulent on eggplant. No symptoms in tobacco and pepper was produced and therefore grouped as race 3. The isolates were grouped to biovar 2 as they oxidized the tested carbohydrates but not the sugar alcohols. The disease was observed to occur in all three surveyed districts. Use of certified and disease free seeds, area for seed production, development of resistant varieties and training on symptoms and bacterial wilt control were recommended.

**DECLARATION**

I, Mwankemwa Zera, do hereby declare to the senate of Sokoine University of Agriculture that this dissertation is my original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

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## **ACKNOWLEDGEMENTS**

My special thanks is to the ALMIGHTY GOD for granting me life, good health and strength to move on. I also acknowledge my late parents Prof S. A. Mwankemwa and Mrs. B. Mwankemwa, for their well upbringing and support.

I thank Tanzania Official Seed Certification Institute (TOSCI) and Seed Potato Development Project Tanzania (SPDP- TZ) for providing financial support, without them the study wouldn't be possible.

My sincere gratitude is to my supervisors Dr. Mamiro, D and Dr. Kudra, A for their guidance, consultation and constructive advice whenever needed. I also acknowledge Dr. Kakuhenzire, R of SPDP (TZ) for discovering master's opportunity and his tireless efforts to guide and advice me through the whole period of the study. I really appreciate for his massive contributions.

I also thank my fellow colleagues from Sokoine University of Agriculture especially Mr. Mushobozi Baitani for his guidance and opinions towards the success of the study.

I also extend my sincere appreciation to the District Agricultural Irrigation and Cooperatives officers (DAICO) of Njombe, Mbeya Rural and Rungwe Districts for allowing me to conduct the survey in their districts. And lastly, to farmers for their time, fields and tuber samples.

## **DEDICATION**

This work is dedicated to my son Nassir.

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## LIST OF ABBREVIATIONS

BWD	Bacterial wilt disease
CABI	Centre for Agriculture and Biosciences Internation
CbhA	Cells with m-carboxynnamic acid bis hydroxamide
C.F.U	Colony forming units
CIP	International Centre for Potato
CPG	( Casamino acid Peptone and Glucose) media
CWDE	Cellwall degrading enzymes
DAS ELISA	Double Antibody Sandwich ELISA
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycydine triphosphate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribose nucleic acid
dTTP	Deoxythyodine triphosphate
Egl	Endoglucanase gene
ELISA	Enzyme Linked Immunosorbent Assay
EPPO	European and Mediterranean Plant Protection Organization
EPS	Extracellular polysaccharides
G+ve	Gram positive
G-ve	Gram negative
H <sub>2</sub> O <sub>2</sub>	Hydrogen Peroxide
HR	Hypersensitive reaction
NCM-ELISA	Enzyme Linked Immunosorbent Assay on Nitrocellulose membrane
O.D	Optical density

PCR	Polymerase Chain Reaction
<i>Rs</i>	<i>Ralstonia solanacearum</i>
SAR	Systemic acquired resistance
SPDP-TZ	Seed Potato Development Project Tanzania
SUA	Sokoine University of Agriculture
T <sub>2</sub> SS	Type three secretion system
T <sub>3</sub> SS	Type three secretion system
TE	Tris EDTA
TOSCI	Tanzania Official Seed Certification Institute
URT	United Republic of Tanzania

## CHAPTER ONE

### 1.0 INTRODUCTION

Potato (*Solanum tuberosum* L.) is the main root and tuber crop and the third most important food crop in the world after rice and wheat. It is grown in over 125 countries and is consumed by over a billion people (CIP, 2008). Tanzania produces about 504,000 tons annually most of which comes from the Southern Highlands (URT, 2007). Potatoes in Tanzania are essentially a food crop with growing domestic urban demand. Ninety percent of the national crop is grown in the Southern Highlands and predominantly around Mbeya and Njombe. Most of the crop produced in Tanzania is consumed as food at household level and through food service in outlets such as restaurants, street and food vendors. The crop can serve both as food as well as income to the rural population (Kadigi *et al.*, 2012).

Studies on potato have shown that production and consumption of the crop is increasingly becoming popular (Anderson, 1996; Namwata *et al.*, 2010). Round potato is grown mainly by small scale farmers as a cash and food crop and therefore plays an important role in food security and creating employment.

Despite its importance the crop has not achieved its full potential because of a number of production constraints; including low soil fertility, inadequate supply of certified seeds, use of low yielding varieties and infection by diseases. The common diseases include late blight, bacterial wilt and viral infections (Kaguongo *et al.*, 2008).

Bacterial wilt disease caused by *Ralstonia solanacearum* (Smith, 1896) is the second most important potato disease in tropical and sub-tropical regions of the world after late blight (Champoiseau *et al.*, 2010). The disease is also known to affects over 200 plant species from more than 50 families (Hayward, 1993). It is widely distributed in tropical, sub-tropical and warm temperate climates of the world. In addition to potatoes, the disease also affects over 200 plant species from more than 50 families (Hayward, 1991).

Bacterial wilt occurs in about 45 countries in the southern hemisphere. In Africa, it is found in Angola, Burkina Faso, Burundi, Cameroon, Congo, Ethiopia, Gabon, Gambia, Kenya, Madascar, Malawi, Mauritius, Mozambique, Nigeria, Rwanda, Senegal, Seychelles, Sierra-Leone, Somalia, South- Africa, Swaziland, Tanzania, Tunisia, Uganda, Zaire, Zambia and Zimbabwe (EPPO, 2004).

## **1.1 Problem Statement and Justification**

### **1.1.1 Problem statement**

Bacterial wilt caused by *Ralstonia solanacearum* is a serious disease affecting potato. It is the second important constraint to potato production after Late blight disease in tropical and sub-tropical countries of the world. Yield losses due to this disease are estimated to be as high as 75 % in seed potato and 50 % in ware house potato. Such losses have been reported to be serious due to ability of the seed tuber to harbour latent infection. This type of infection is considered the main source of disease outbreaks, ensuring carryover of the disease into subsequent growing seasons and new regions (Ajanga, 1993).

Bacterial wilt disease of potato is difficult to control or to eradicate because of the soil borne nature of its causal organism. Stanburry *et al.* (2001) reported various ways to be



considered in managing the disease; which include approaches of minimizing the occurrence and those of minimizing the spread. Management approaches which involve minimizing the disease occurrence include adopting rotation with pastures, cereals and non-solanaceae crops for the period exceeding five years. Crop rotation adoption is impractical in many areas particularly Africa where there is land pressure (Ajanga, 1993).

Other way of minimizing disease occurrence includes the use of certified seeds from reliable sources which in most cases is expensive and not affordable by the majority of farmers. Minimizing the pathogen spread can be done through quarantine or other legislative measures, avoiding deep ploughing as the organism survive in deep, cool layers of soil, regular crop inspection for disease symptoms and removing and destroying diseased plants.

### **1.1.2 Justification**

There is no enough information on the occurrence, incidence and distribution of potato bacterial wilt in Tanzania. However, the occurrence of this disease in neighboring countries of Kenya and Uganda together with poor quarantine measures implies high probability of disease occurrence. Ability of the pathogen to stay long in soils together with its wide host range and farmers practice of saving their own seed potato for planting the following season also increase the probability of the disease occurrence in Tanzania in particular in the Southern Highland zone where major potato production is taking place.

A study was therefore needed to fill the current knowledge gap on occurrence, incidence and distribution of the disease and variability of the disease causing organism in the

major potato producing areas of the Southern Highlands zone of Tanzania so that locations for producing clean seed potato may be identified.

Using pathogen free tubers can prevent the loss of both potato quality and quantity of about 30-100 % with a long term advantage of preventing soil contamination (Smith *et al.*, 2003).

## **1.2 Objectives**

### **1.2.1 Overall objective**

To study the occurrence and distribution pattern of potato bacterial wilt in the Southern Highlands of Tanzania so that control measures can be developed for increasing potato production and improving its quality through reducing bacterial wilt infection.

### **1.2.2 Specific objectives**

- i) To determine the incidence and severity of potato bacterial wilt disease in potato cultivars grown in the Southern Highlands of Tanzania.
- ii) To characterize strains of *Ralstonia solanacearum* occurring in the Southern Highlands of Tanzania.

## CHAPTER TWO

### 2.0 LITERATURE REVIEW

#### 2.1 Potato bacterial wilt disease

Bacterial wilt disease is one of the most destructive diseases of potato, it is also known as brown rot, sore eye or jammy eye (Osborn, 1995; De Boer, 2011). It is caused by a soil-borne bacterium known as *Ralstonia solanacearum* which was formerly known as *Pseudomonas solanacearum* (Tahat and Sijam, 2010). The disease is generally favoured by temperatures between 25 to 37°C and usually does not cause problems in areas where the mean soil temperature is below 15°C. Under condition of optimum temperature, infection is favoured by soil wetness. However, once infection has occurred severity of symptoms is increased with hot and dry conditions which facilitate wilting (Osborn, 1995).

#### 2.2 *Ralstonia solanacearum*

The history of *R. solanacearum* goes back to 1896 where it was first named *Bacillus solanacearum* as it seemed to have peritrichous flagella (Smith, 1896). Later on, it was named *Pseudomonas solanacearum* as it was found to possess a single polar flagellum (Smith, 1914). For nearly 80 years *R. solanacearum* was a member of Pseudomonads rRNA homology group I that included many other non-fluorescent Pseudomonads such as *P. mallei*, *P. caryophylli*, *P. cepacia*, *P. pickettii* and *P. gladioli* (Palleroni and Doudoroff, 1971). In 1992, this group was proposed to be placed in the new genus *Burkholderia* based on 16S rRNA sequences, DNA-DNA homologies, fatty acid analyses and other phenotypic characteristics (Yabuuchi *et al.*, 1992). Phylogenetic and polyphasic phenotypic analyses allowed accommodating the bacterium in a new

established genus *Ralstonia*. The latest, revision has settled on the name *R. solanacearum* which is described as a non-spore forming, gram negative, nitrate-reducing, ammonia-forming, aerobic, and rod-shaped (0.5-1.5  $\mu\text{m}$ ) with one polar flagellum (Yabuuchi *et al.*, 1995).

*Ralstonia. solanacearum* is considered a species complex-a heterogeneous group of related but genetically distinct strains (Meng, 2013). *Ralstonia. solanacearum* was ranked second in a list of the top 10 most scientifically/economically important plant pathogenic bacteria in Molecular Plant Pathology Journals (Mansfield *et al.*, 2012).

### **2.3 Economic Importance**

Globally the bacterial wilt disease is estimated to affect about 1.7 million hectares of potatoes in approximately 80 countries with global damage estimates of over USD 950 million per annum (CABI 2015, Champoiseau, 2009). The disease is formidable constraint on potato and accounts for significant losses in Sub-Saharan Africa (Olanya *et al.*, 2012). It can cause losses up to 75 % in potato crops (Stanbury *et al.*, 2001). In countries like Kenya, the disease has been reported to affect over 70 % of potato farms causing yield losses of about 50-100 % (Muthoni *et al.*, 2012). Bacterial wilt disease also affects over 200 plant species from more than 50 families (EPPO, 2004). The disease can cause damage at two stages, the first being killing the standing plants by causing wilt and by causing rot of infected tubers in storage, and another indirect loss is spread of the disease through planting of healthy looking tubers harvested from infested fields (Sagar and Bakade, 2007).

## 2.4 Host Range

*Ralstonia solanacearum* attacks almost 200 plant species in 50 different plant families. It constitutes one of the largest known host range for any plant pathogenic bacterium (Moorman *et al.*, 2003). Plants that can host the bacterium include tobacco, potato, tomato, eggplant, pepper, banana, peanut and beans, thorn apple and nightshade are two common weed hosts that are attacked by the disease (Hayward, 2000).

## 2.5 Races

*Ralstonia solanacearum* is divided into races based on host range. Race 1 is known to affect wide range of specie including potato, tomato, eggplant, tobacco, chillies and several weed species families (French, 1994; Denny, 2006). Race 1 is more frequent in warm areas and lower elevations of the tropics. It has a high optimum temperature requirement of 35-37°C as races 2, 4, and 5 (Martin and French, 1985, EPPO, 2004).

Race 2 of the *R. solanacearum* is indigenous to Central and South America, and attacks members of the Musaceae family such as plantain, triploid bananas, and heliconia (French, 1994). It causes moko disease on bananas and heliconia in Central and South America, and bugtok disease on plantains in the Philippines (Martin and French, 1985; EPPO, 2004). Race 3 occurs at higher altitudes (in the tropics) and higher latitudes than race 1 (EPPO, 2004). It mainly attacks potato, tomato (especially when planted after infected potato), geranium, occasionally *Pelargonium zonale*, eggplants, Capsicum, and some solanaceous weeds like *Solanum nigrum* and *S. dulcamara* (Martin and French, 1985; Janse, 1991; French, 1994). Race 3 also infects a number of non-solanaceous weed asymptotically (Wenneker *et al.*, 1999; Pradhanang *et al.*, 2000). This race has a long association with potatoes and has an optimum temperature range of 27 - 28°C (French,

1994). Race 4 has been reported to affect ginger in Asia and Hawaii, while race 5 affects mulberry in China (EPPO, 2004).

## **2.6 Biovars**

The *R. solanacearum* species is further subdivided into biovars based on utilization of the disaccharide cellobiose, lactose and maltose and oxidation of the hexose alcohols dulcitol, mannitol and sorbitol (Oslon, 2005). Martin and French, (1985) added that five biotypes I to V can be distinguished depending on biochemical tests. Biotype II coincides with race 3, biotype V with race 4 and Biotype I, III and IV are in race I.

## **2.7 Disease Cycle**

The source of inoculum can be infected potato (seed tubers), harvest leftovers, irrigation water and infected plants or infested soils or both. Infection is through the root system. The pathogen enters through wounds occurring during cultivation or natural growth of secondary roots, the root-knot and other nematodes also promote penetration of the bacterium by injuring the roots. Once the bacterium penetrates the roots it multiplies and moves through the plant via the xylem vessels of stems and petioles (Sagar and Bakade, 2007).

The pathogen can survive in soils mostly in plant debris and in the rooting system and rhizosphere of many hosts weeds and other host crops such as potato volunteers (Priou *et al.*, 1999). Infected seed potato tubers are the most common source of inoculum especially latent infection. Seed tubers which are grown in high elevations above 2500 m may not show any symptoms. The bacterium is also transmitted through infected soils and is native to many tropical soils (Martin and French, 1985).

## 2.8 Pathogenicity and Virulence Factors of the Pathogen

Many factors have been found to contribute to the virulence of the *R. solanacearum*. The commonest one include a heterogeneous polymer of N-acetylated Extracellular Polysaccharide I (EPS I), which causes direct wilting by physically blocking the vascular system and there by alters water movement in the vascular bundles (Denny and Baek, 1991). It also protects the pathogen from plant anti-microbial defenses by cloaking bacterial surface feature that could be recognized by hosts (Razou *et al.*, 1998).

The Type III Secretion System (T<sub>3</sub>SS) is another factor which has a central role in contributing to the pathogenicity and virulence of the pathogen. It is encoded by the *hrp* gene cluster which spans a 23- kb region on the mega plasmid (Van Gijsegem *et al.*, 1995). As in other major groups of Gram negative bacteria, *R. solanacearum* *hrp* genes are key determinants for disease development on compatible host and for induction of the defensive hypersensitive response (HR) on resistant plants (Meng, 2013).

The pathogen also possesses flagella driven swimming motility and type IV pilli- driven twitching motility that are important to its ecological fitness and virulence (Tans-Kersten *et al.*, 2004). Swimming motility contributes to virulence in the early stage of host colonization and invasion (Tans-Kersten *et al.*, 2001).

Another factor contributing to its virulence is the ability of the pathogen to secrete Cell Wall Degrading Enzymes (CWDE's). CWDEs secreted include three polygalacturonases PehA, PehB and PehC (Huang and Allen; 1997), an endoglucanase (Egl), a pectinmethylesterase (Pme) and cellobiohydrolase (CbhA). Gene disruption analysis

revealed that Egl, Peh A, Peh B, Peh C and CbhA, each contribute to the pathogen ability to cause wilt (Zhang *et al.*, 2005).

## **2.9 Symptoms of the Disease**

### **2.9.1 Above ground symptoms**

Above ground symptoms are wilting, stunting, and yellowing of the foliage (De Boer, 2011). Wilting caused by *R. solanacearum* resembles that which is caused by lack of water, other pathogens such as *Fusarium* or *Verticillium* spp., as well as insect or mechanical damage (IOSAT, 2005). In early stages of the disease development, there is a sudden wilting of only part of the stems of a plant, or even one side only of a leaf or stem. If disease development is rapid, entire plants wilt quickly (Murithi, 2003). A cross section through a young diseased potato stem reveals brown discoloration of the vascular system. Upon slight pressure, a milky slime may exude. In a longitudinal section, the vascular system may show dark, narrow stripes beneath the epidermis.

### **2.9.2 Below ground symptoms**

The infected tuber releases the bacteria on its eyes. Cross section cutting of the diseased tuber shows a browning and a death of the vascular ring and the immediate surrounding tissues. On the cut surface, a creamy fluid usually appears on the vascular ring (IOSAT, 2005). Symptoms in the tuber are very specific; brownish-grey areas are seen on the outside, especially near the point of attachment of the stolon (Martin and French, 1985). Cut tubers may show pockets of white to brown ooze or browning of the vascular tissue which, if left standing, may exude dirty white globules of bacteria. As the disease progresses bubbly globules of bacteria may exude through the eyes; soil will often adhere to the exuded bacteria, hence the name 'sore eyes' or 'jammy eyes' (De Boer,



2011). External symptoms may or may not be visible, depending on the state of development of the disease (CABI, 2015).

## **2.10 Signs of the Pathogen**

The disease signs in the tuber include slimy, sticky ooze tan- white to brownish beads where the vascular tissue is cut, when an infected stem is cut across and the cut ends held together for a few seconds, a thin thread of ooze can be seen as the cut ends are slowly separated. If one of the cut ends is suspended in a clear container of water, bacterial ooze will form a thread in water (Moorman, 2003).

## **2.11 Diagnosis and Identification of the potato Bacterial wilt disease**

Symptoms identification is the first step of early diagnosis of potato bacterial wilt, The screening tests can also facilitate detection and identification of bacterium in potentially infected plants or contaminated soil and water, they include streaming, planting on semi-selective medium, immuno-diagnostic assays, pathogenicity assessment, biochemical growth tests and several nucleic acid based methods such as DNA probe hybridization especially polymerase chain reaction with specific probes and primers (Champoiseau, 2008).

### **2.11.1 Simple disease diagnostic techniques**

A simple Bacterial wilt disease diagnostic technique can be done by observing rapid wilting of plant leaves and stem during the warmest day time (EPPO, 2004). At the initial stage the leaves can recover at night or when it is cold (Lemay *et al.*, 2003). In later stages of the disease development the leaves show necrotic areas and the vascular

bundles look brownish (Osborn, 1995) which later on extends to the whole plant and never recovers.

In the field wilting caused by bacterial wilt can be distinguished from other forms of wilting by cutting a fresh living infected plant transversally like stem and leaf stalk and deep it into transparent container containing clean water, if the infection is bacterial wilt a milky substance ooze out from the specimen (Gildemacher *et al.*, 2007). This method is popularly called vascular flow test, it is fast and convenient in the field or anywhere when fast results are needed. Its drawback however, is that it only manages to detect an infection of a certain degree. The infection degree that could not be detected by the vascular flow test can be detected by high sensitive method such as Enzyme Linked Immunosorbent Assay -ELISA and Polymerase Chain Reaction - PCR (Skoglund, 1993). ELISA is a technique used to measure the concentration of an analyte usually antibodies or antigens in solution (Priou, 2001). PCR is a technology in molecular biology which is used to amplify a single copy or few copies of a piece of DNA across several orders of magnitude, generating millions of copies of a particular DNA sequence.

### **2.11.2 Serological techniques**

Latent infections of *R. solanacearum* have assisted the trans-global transmission of bacterial wilt. In the efforts to combat latent infection problem very sensitive methods of *R. solanacearum* detection have been developed. Some of these methods are ELISA and PCR as previously described to apply. The choice of the method however, depend on the competency of researchers to use the method, the cost to be incurred, the demand of the type of facilities for the successful operation of the test, the amount of samples to be tested and the degree of sensitivity needed (French *et al.*, 1995).

### 2.11.3 Enzyme-Linked Immunosorbent Assay (ELISA)

This method is becoming common in most pathogen detection experiments due to its sensitivity, easiness, quickness and reliability to produce quantifiable results from a variety of samples (French *et al.*, 1995). The method can be used to detect very low concentration of *Ralstonia* as far as  $10^8$  c.f.u /ml if tuber extract is enriched (Priou *et al.*, 2006). There are two common types of ELISA test namely Nitro-Cellulose Membrane (NCM-ELISA) and Double Antibody Sandwich (DAS-ELISA). NCM-ELISA is an immuno enzymatic assay that uses nitrocellulose membrane as a support for the membrane instead of a microtitre plate as support for the samples and reagents. DAS-ELISA uses antibodies which are bound to the surface of a microtitre plate to capture the antigen of interest. A specific antibody- enzyme conjugate is then used to detect the trapped antigen.

The two methods have four main steps respectively: coating the micro titration plates with soluble antigens from bacterial cells, addition of the sample extract to the plates; if the pathogen is in the extract it will stick to the antigen, the addition of the second conjugated antibody to enzyme which reacts to the bound antigen-antibody complex. The unbound and excess materials are removed through washing with well prepared buffer after each step. The fourth step then involves the addition of enzyme substrate of which produces color as an indication of the presence of reaction in third step (French *et al.*, 1995; Priou *et al.*, 2006). The intensity of the color tells the concentration of *R. solanacearum* found in the extract.

### 2.11.4 Polymerase chain reaction

This method is a highly sensitive and less labour demanding to come up with pathogen-specific DNA (French *et al.*, 1995). PCR has been developed for the detection of *R.*

*solanacearum* race 3 biovar 2 (Lemay *et al.*, 2003). The results from this method can be obtained within 2-5 hours (French *et al.*, 1995; Lemay *et al.*, 2003).

There are three steps involved in PCR. DNA is first extracted from the cell, it is then unwound and its strands separated by high temperature, as the temperature is lowered, a short single stranded DNA sequences called primers are free to bind to the DNA strands at region of homology, this allows polymerase enzyme (Taq) to make a new copy of the molecule ( Alberts *et al.*, 1994). The cycle of denaturation- annealing elongation is repeated 30-40 times yielding millions of identical copies of the segment. The method uses designed primers which through heating cycles are able to detect even one copy of *Ralstonia* DNA in the sample (French *et al.*, 1995; Lemay *et al.*, 2003; Pastrik and Maiss, 2000). In researches the method is used to develop interactions between the pathogen and its hosts. It is more sensitive than ELISA but its drawbacks rely on technical complexity and expensiveness.

## **2.12 Survival**

The pathogen survives through infected seed tubers and in plant debris in soil. Symptomless plants may also harbour the bacterium and transmit it to progeny tubers as latent infection. This could lead to severe disease outbreaks when the tubers are grown at disease free sites. High soil moisture, temperature, oxygen stress and soil type affect the survival of the pathogen. The pathogen population decline gradually in soil devoid of host plants and their debris (Sagar and Bakade, 2007).

## **2.13 Disease Management and Control**

Despite decades of efforts by many national and international organizations to control bacterial wilt disease, It has continued to be a considerable problem throughout the

world. The variability of both the pathogen and the agro-ecosystems has undoubtedly hampered progress in controlling the disease (Horita *et al.*, 2005). The bacterium is very difficult to control because it is a soil borne pathogen, ability to stay long in soils, has wide host range and wide biological variation (Martin and French, 1985). No single control method has been found to be 100 % effective, although in locations where the pathogen is established, some level of bacterial wilt disease control has been possible through the use of a combination of diverse methods (EPPO, 2004; Champoiseau *et al.*, 2010).

To control and eradicate the disease, main components are to use healthy seeds and planting in clean soils. Many other ways can also be used such as the use of less susceptible varieties, rotation with non- host plants, cultural practices such as nematode control, sanitation and chemical control (Priou *et al.*, 1999). An integrated strategy of these methods is recommended in reducing bacterial wilt incidences (Martin and French, 1985; Champoiseau, 2010).

### **2.13.1 Phytosanitation**

This is one of the strategies widely used for controlling bacterial wilt disease in the field (Champoiseau *et al.*, 2010). The method is mostly practiced in areas where the disease is endemic or in areas where the disease is present but not yet established (French 1994; Champoiseau *et al.*, 2010). Phytosanitation practices include the use of planting disease-free tuber seeds, rouging affected plants, weeding and quarantine measures (Kinyua *et al.*, 2001; EPPO, 2004; Champoiseau *et al.*, 2010). Quarantine measures may prevent introduction of the pathogen into disease-free areas (Champoiseau *et al.*, 2009).

### **2.13.2 Cultural practices**

The use of cultural methods is also very effective in managing bacterial wilt disease. They include crop rotation, intercropping, delayed planting and soil amendments (Kinyua *et al.*, 2001; EPPO, 2004; Champoiseau *et al.*, 2010). Various cultural practices, whether deliberate or not, are effective in reducing the occurrence of bacterial wilt disease. Rotation with a non- host crop forces pathogens to starve. Starvation of the pathogen is a key mechanism of crop rotation. Rotation with maize, okra, cowpea, French marigolds are examples of cropping system that were reported to reduce the incidence of bacterial wilt in infested potato, groundnuts, eggplant and tomato fields in Nepal, Taiwan, China, India and other Asian countries (Saddler, 2005). Solarization assays have demonstrated some efficacy against *R. solanacearum* biovar 2, but not against other biovars (French, 1994).

### **2.13.3 Chemical control**

The use of chemicals and other forms of treatments, in particular treatment of soils have been investigated in addition to cultural methods. A number of soil fumigants like chloropicrin and methyl bromide have been tested for bacterial wilt control to in many crops; however fumigation is not economically feasible over large areas (Muthoni, 2012). There are increasing concerns about application hazards of chemicals and environmental problems, including destruction of the protective ozone layer by methyl bromide and groundwater contamination with toxic compounds. On the other hand, products called "plant activators" that induce SAR (Systemic Acquired Resistance) in plants have been identified, and have shown to induce host resistance in tomato to bacterial wilt disease (Qui *et al.*, 1997).

The most commonly used chemical treatment in many countries has been fumigation of contaminated soil/portions of the farm with methyl bromide (Champoiseau *et al.*, 2010). This is a very expensive and tedious exercise and cannot be used in large areas. In addition, methyl bromide has been banned in most countries in the world. The other product commonly used at field level is sodium hypochlorite; it is appropriate for spot treatment of the holes left behind after rouging of the wilting plants, and for general field sanitation. However, it was also found expensive and tedious and therefore impractical (Kaguongo *et al.*, 2008).

#### **2.13.4 Integrated disease management**

Bacterial wilt incidence can be reduced only if various control components are combined. These involve planting healthy seeds in clean soils, planting tolerant varieties, rotation with non-susceptible crops and application of various sanitation and cultivation practices as well as nematode control. An integrated disease management approach can lead to significant reduction or even eradication of the disease (Priou *et al.*, 1999).

#### **2.13.5 Use of Resistant varieties**

The growth of cultivars that are resistant to bacterial wilt is considered to be the most economical, environmentally friendly, and effective method of disease control. Breeding for resistance to bacterial wilt has been concentrated on crops of wide economic importance such as the tomato, potato, tobacco, eggplant, pepper, and peanut, and has commonly been influenced by factors such as the availability of resistance sources, their diversity, genetic linkage between resistance, and other agronomic traits, differentiation and variability in pathogenic strains, the mechanism of plant-pathogen interactions, and breeding or selection methodology (Boshou, 2005; Elphistone, 2005). For example,

the *Arabidopsis* NPR1 (non-expresser of *PR* genes) gene was introduced into a tomato cultivar, and enhanced resistance to bacterial wilt and reduced the incidence of wilt by approximately 70% 28 d after the inoculation (Lin *et al.*, 2004). Potato genotype BP9, which is a somatic hybrid between *Solanum tuberosum* and *S. phureja*, successfully reduced bacterial wilt by 90–100% (Fock *et al.*, 2000). Somatic hybrids between *S. melongena* cv. Dourga and two groups of *S. aethiopicum* were produced by the electrical fusion of mesophyll protoplasts and were found to be tolerant to *R. solanacearum*. Prior *et al.* (1996) showed that resistant plants were heavily invaded by *R. solanacearum* without displaying wilt symptoms. Nakaho *et al.* (2004) revealed that bacterial multiplication in the stems of resistant tomato plants was suppressed due to limited pathogen movement from the protoxylem or primary xylem to other xylem tissues (Nakaho *et al.*, 2004). A proteomic approach was used to elucidate molecular interactions in the cell walls of resistant and sensitive plants inoculated with *R. solanacearum* (Dahal *et al.*, 2010). Resistance to bacterial wilt in many crops has generally been negatively correlated with yield and quality. Thus, the release of resistant cultivars may be poor because of other agronomic traits and are not widely accepted by farmers or consumers. The breeding of a good resistant cultivar is expected in the future through stronger efforts in the genetic enhancement of bacterial wilt resistance through biotechnology approaches in order to improve crop yield.



## **CHAPTER THREE**

### **3.0 MATERIAL AND METHODS**

#### **3.1.1 Incidence and severity of potato bacterial wilt disease in cultivars grown in Southern Highlands of Tanzania**

##### **3.1.1.1 Location and sampling**

The survey was conducted in potato growing season in Mbeya rural, Njombe and Rungwe districts of Southern highlands of Tanzania. Sampling was done at random in 5 farms per village. Ten (10) villages were surveyed in each district, the distance between one field and another was 3-5 km. Most of the potato fields surveyed were at flowering.

In each field 10 rows of about 100 plants were selected randomly, bacterial wilt symptoms was identified by visual observation of typical bacterial wilt disease symptoms such as wilting, vascular discoloration, bacterial streaming in glass of water and browning of the vascular bundles of the tuber.

Bacterial wilt incidence was recorded based on number of plants showing symptoms and were expressed as % of the total number of plants observed.

Disease severity was done by recording on severity score as described by Horita and Tsuchiya (2001) as 1= no symptoms, 2= top young leaves wilted, 3= two leaves wilted, 4= 4 or more leaves wilted and 5= plant died.

During the survey field altitudes and their corresponding geographical position were recorded. Two (2) samples were collected in each farm, one comprised of 25 healthy

looking tubers and the other of 4 diseased tubers, packed in paper bags and labeled in each farm for further tests.

### **3.2 Characterizing races of *Ralstonia solanacearum* occurring in the Southern Highlands of Tanzania**

#### **3.2.1 Isolation of the bacteria and cultures**

Infected potato tubers collected from different locations were washed with running tap water to remove soils and any dirty materials and then they were then dipped in 70% alcohol for 5 minutes for surface sterilization and were dried by tissue paper. The tubers were cut into half by a sterile knife; vascular ring was removed from the tuber by scoop and was then placed in test tubes containing 5 ml of sterile water. Bacteria were allowed to flow from the vascular bundles for 5 to 10 minutes.

One loopful of bacterial suspension was streaked into 2, 3, 5 Triphenyl Tetrazolium Chloride (TZC) agar medium and incubated at 28°C for 48 h. Single colony of *R. solanacearum* showing virulent, fluidal, irregular and creamy white with pinkish at the centre was picked from the TZC Petri-plates and streaked to Casamino acid, Peptone and Glucose (CPG) medium and were incubated for 48 h. Virulent cultures were maintained in distilled water in screw taped tubes at room temperature after 48 h of incubation.

#### **3.2.2 Pathogenicity test**

Virulent strains were inoculated on 5 host plants namely, potato, tomato, eggplant, pepper and tobacco. Host plants were planted in pots containing soil, sand and compost mixture (1:1:1) treated with formalin, and kept in screen house until they attained a height of 15-20 cm. Five plants of each host were injected with 100 µl of bacterial

suspension by inserting a sterile micropipette tip at the axil of fully expanded leaf from the top. The micropipette tips were left in a position until all inoculum was absorbed. Inoculated plants were observed daily for pathogenicity and severity. Disease severity was assessed by a scale of 1- 5 described by He *et al.* (1983) where 1= no symptom, 2= two leaves wilted, 3 = three leaves wilted, 4= four or more leaves wilted and 5= plant died.

### **3.2.3 Biochemical characterization of the pathogen**

#### **3.2.3.1 Gram staining**

A loop full of bacterium was picked from maintained virulent cultures and was spread on a glass slide and fixed by heating on a very slow flame. Aqueous crystal violet solution of 0.5 % was then spread over the smear for 30 seconds, and then washed with running tap water for a minute. Iodine (95%) was then flooded for a minute followed by rinsing with tap water. Then the slides were decolorized with 95% ethanol until colorless runoff. The slides were then counter stained with safranin for 10 seconds and washed with water. The slides were dried under the laminar flow cabinet and placed under the light microscope at 10X, 40X and 100X for observation using oil (Schaad, 1980).

#### **3.2.3.2 Potassium hydroxide test**

Bacteria were picked from petri-plates by wire loop and placed on glass slide containing a drop of 3% KOH solution, stirred for 10 seconds and observed for the formation of slime threads (Suslow *et al.*, 1982)

### **3.2.3.3 Catalase oxidase test**

A loop full of bacterial culture obtained from young agar cultures of 18-24 h were mixed with a 3% hydrogen peroxide ( $H_2O_2$ ) on a glass slide to observe production of gas bubbles with a naked eye and under a dissecting magnification of 25X (Schaad, 1988).

### **3.2.3.4 Kovacs oxidase test**

Oxidase reagent (1% tetra-methyl-p-phenyl diamine dihydrochloride) solution of 100ml was prepared and kept in rubber stopper dark bottle. A drop of reagent was added to a piece of filter paper placed within a glass Petri dish. Small quantity of inoculum was rubbed on the filter paper containing oxidase reagent solution. Bacteria were then observed for the development of purple color in 10-60 seconds.

### **3.2.3.5 Oxidation of glucose**

Basal medium constituents were dissolved as described by Hayward (1964). The pH was adjusted to 7.0 to 7.1 and the medium was prepared and then for identification of acid production from sugars by aerobic, Gram negative bacteria. The contents of the medium were Difco Bacto agar (0.3 g) which was mixed with 100 ml quantities of the basal medium. Glucose (1 g) was added into each Pyrex screw capped tubes containing 10 ml sterile distilled water. The solutions were heated to 100°C for 30 minutes for sterility. Semi solid basal medium in bottles were melted in microwave oven and cooled to 60-70°C. 10 ml of 1% glucose was mixed in the basal media by rotation and 5 ml were dispensed into each sterile screw capped tubes. The medium was allowed to solidify at room temperature.

Inoculum from bacterial cultures was picked by sterile wire loop and was stab inoculated onto the tubes. Half of the tubes were sealed with 3 ml molten sterile

Difco Bacto agar cooled to 45°C. Sealed and unsealed tubes were then incubated at 28°C and examined after 1, 2 and 7 days for gradual pH change at the surface of the open tube.

### **3.2.4 Biovar Determination**

Biovar test was done as follows; mineral medium was prepared in a litre of water as follows;  $\text{NH}_4\text{H}_2\text{PO}_4$  1.0 g, KCl 0.2 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.2 g, Difco Bacto peptone 1.0 g, Agar 3.0 g, Bromothymol blue 80.00 mg. The pH was adjusted to 7.0-7.1 (an olivaceous green colour) by drop- wise addition of 40 % Sodium Hydroxide solution. The medium was heated to melt the agar, autoclaved at 121°C for 20 minutes and cooled to 60°C.

Ten millilitres (10 ml) of each 10 % lactose, maltose, cellobiose, mannitol, Sorbitol and dulcitol were added in sterilized screw capped tubes and they were heated to 100 °C for 30 minutes to sterilize the solutions. Bottles of semi-solid basal medium were melted in water bath and cooled to 70°C. Ten millilitres (10 ml) of carbohydrate solutions were added into 90 ml of media. About 200 µl of the melted medium was dispensed into the wells of microtitre plate. The media was then allowed to solidify under room temperature. Inoculum of each isolate was prepared by adding 2 loopful of bacteria from 48 h old TZC cultures to distilled water to make suspension containing about  $10^8$  c.f.u/ml, then 20 µl of bacterial suspension was added to the wells of the microtiter plate. Control was kept by adding 10 ml of distilled water instead of sugar solution. The plates were incubated at 28°C and examined 3-5 days for pH color change from olivaceous green to yellow (Schaad, 1988).

### **3.2.5 Serological Detection of the Isolates**

Samples of healthy tubers from the fields consisting of 25 tubers from each field were tested using procedures outlined in CIP, NCM-ELISA (Enzyme Linked Immunosorbent

Assay on Nitrocellulose Membrane) protocol for detection of *R. solanacearum* in potato (Priou, 2001). The test consisted of sample preparation, loading a very small amount of plant extract (20 µl) on a nitrocellulose membrane (Dot blotting), blocking the area of the membrane that was free of samples, binding the samples with *R. solanacearum* (*Rs*) with specific rabbit antibodies, binding the *Rs*-antibodies complex with enzyme-labeled goat anti-rabbit antibodies and revealing the bound enzymes by adding the substrate leading to colouration reaction.

### **3.2.6 Molecular Characterization of the Isolates**

Positive samples of tubers that were infected by *R. solanacearum* that were detected by NCM-ELISA were subjected to molecular characterization to species specific PCR to confirm if they were really *R. solanacearum*. The procedures used for molecular characterization are as indicated below;

#### **3.2.6.1 DNA Extraction**

Bacterial DNA was extracted as follows; two tubers per sample were washed under running water and air dried for an hour. The skin was removed at the heel end of the tuber with a clean disinfected vegetable knife so that the vascular tissues become visible. A small conical core (3-5 mm diameter) of vascular tissue at the heel end was carefully cut out. A sample of a heel end was crushed with the help of pestle and mortar and collected in a sterile Falcon tube containing 5 ml of double distilled water. The content was allowed to stand for 30 min. The supernatant was then centrifuged for 2 min in 1.2 ml microfuge tube. The pellets were resuspended in a 500 µl tube by vortexing. Then 30 µl of Sodium Dodecyl Sulphate (SDS) and 3 µl of 20 mg/ml Proteinase K was added. The mixture was vortexed and incubated at 35°C for one hour. One hundred microlitre

(100 µl) of 5M CTAB/NaCl solution was added and the mixture was incubated at 65°C for 10 min. Phenol, chloroform and isoamyl was added in the ratio (25:24:1) and the tubes were centrifuged for 5 min. The supernatants were then transferred to new microfuge tubes and 0.6 volume of Isopropanol was added. Centrifugation was again done for 5 min at 16000 g. The supernatant was then discarded and the DNA pellets were resuspended in 100 µl of 1X T.E. buffer (Grover *et al.*, 2012).

### **3.2.6.2 Species specific PCR**

Species specific PCR was done by using a Random Amplified Polymorphic DNA (RAPD) Primer set AU 759/760. Master mix (readymade) from Thermo Scientific containing 0.4 Mm of dATP, dCTP, dGTP, dTTP, 4 Mm  $MgCl_2$  and 5 U/µl Taq Polymerase was used. Primer concentration in the master mix was 0.1 Mm. One µl of 10 ngµl<sup>-1</sup> DNA The reaction volume was 25 µl.

The PCR conditions were set as one cycle of 94°C for 3 min., 53°C for 1 min. and 72°C for 1.30 min., followed by 30 cycles of 94°C for 15 s, 72°C for 15 s, one cycle of 72°C for 5 min and held at 4°C. Gel electrophoresis was done for 40 minutes in which 1.2% Agarose gel in TAE buffer stained with gel red was prepared and the samples were run against 1 kb DNA ladder for 45 min.

### **3.2.6.3 Phylotype determination**

Phylo-typing was determined by Multiplex PCR (pmx PCR). The reaction involved four forward primers, one reverse primer and a species specific primer. The four forward primers involved were Nmult21:1F, Nmult21:2F, Nmult23: AF, and Nmult22:1nF, one reverse primer used was Nmult 22: RR and species specific primer pairs AU 759/760. The reaction mixture contained 6 p moles of the forward primers, 12 p moles of the

reverse primer, 4 p moles of species specific primer, 12.5  $\mu$ l of Thermo-scientific Taq Green Mastermix and 1  $\mu$ l of 20 ng $\mu$ l<sup>-1</sup> DNA template making a total 25  $\mu$ l volume of reaction mixture. The PCR conditions were set as one cycle of 96°C for 5 min., 59°C for 30 s and 72°C for 30 s. The final extension was done at 72°C for 10 min and samples were held at 4°C. For 45 minutes Agarose gel electrophoresis was done in which 1% of Agarose gel was prepared in TAE buffer stained with gel red and the samples were run against a 1 kb plus DNA ladder.



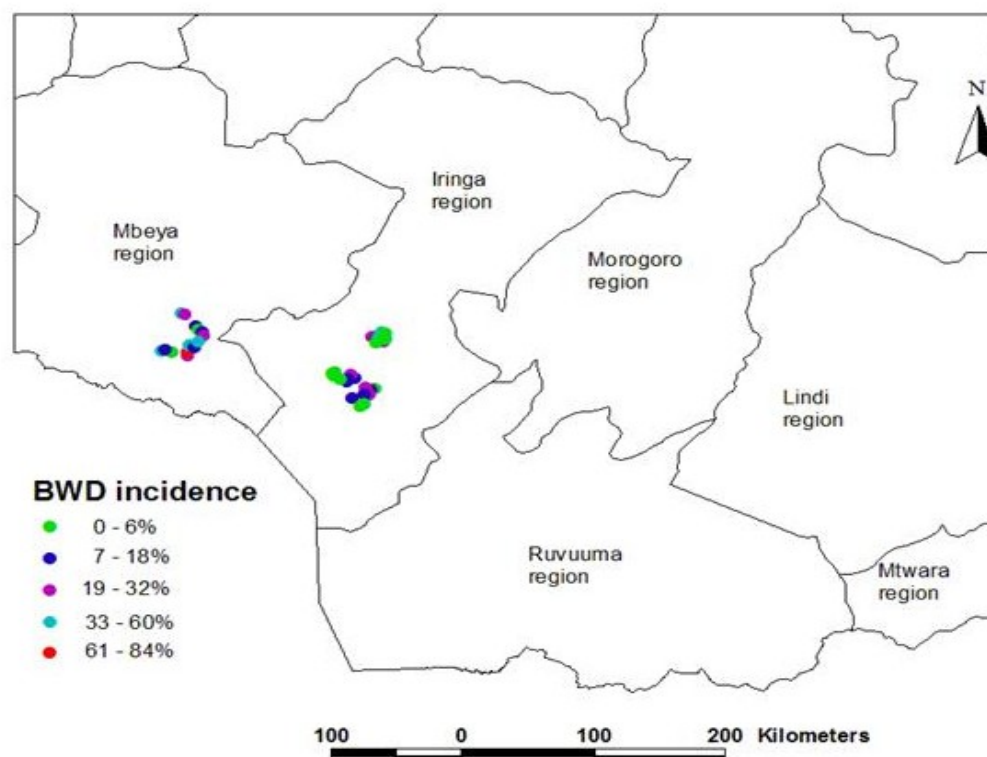
## CHAPTER FOUR

### 4.0 RESULTS AND DISCUSSION

#### 4.1.1 Bacterial Wilt Disease Incidence and Severity

Bacterial wilt disease of potato was found in all districts surveyed but not in all fields (Table 1). Common bacterial wilt symptoms observed in the fields were wilting of one side of a leaf and/ or stem, general whole plant wilting and brown discoloration of the vascular system in young stems (Fig. 2a and b). The infested tubers released the bacteria on their eyes, and showed a browning and a death of the vascular ring and the immediate surrounding tissues when cut (Fig. 2c–f). Wilting was the most obvious symptom in the field. Such conditions are a result of restricted water movement due to the formation of slime that surrounds the bacterial masses in the xylem vessels (Martin and French, 1985).

The survey results showed that the highest mean (27.7%) bacterial wilt incidence was observed in Mbeya Rural followed by Rungwe (26.6%) and Njombe district (19.4%) (Table 1). In terms of villages, bacterial wilt incidence ranged from (0- 84 %). The highest (84 %) disease incidence was observed in Lukata B village in Rungwe where all 5 fields assessed had typical symptoms of bacterial wilt infection. The incidences of bacterial wilt disease in all other fields were consistently  $\leq 60$  % (apparent infection). The bacterial wilt incidences in different districts were: 0-84 % (n =50, Rungwe), 0-60 % (n = 50; Njombe), and 0 – 60 % (n = 50; Mbeya rural (Table 1). Bacterial wilt disease occurred in all districts .Statistical analysis revealed that the means of incidence of bacterial wilt disease in the districts surveyed were not statistically significant different (Table 1).



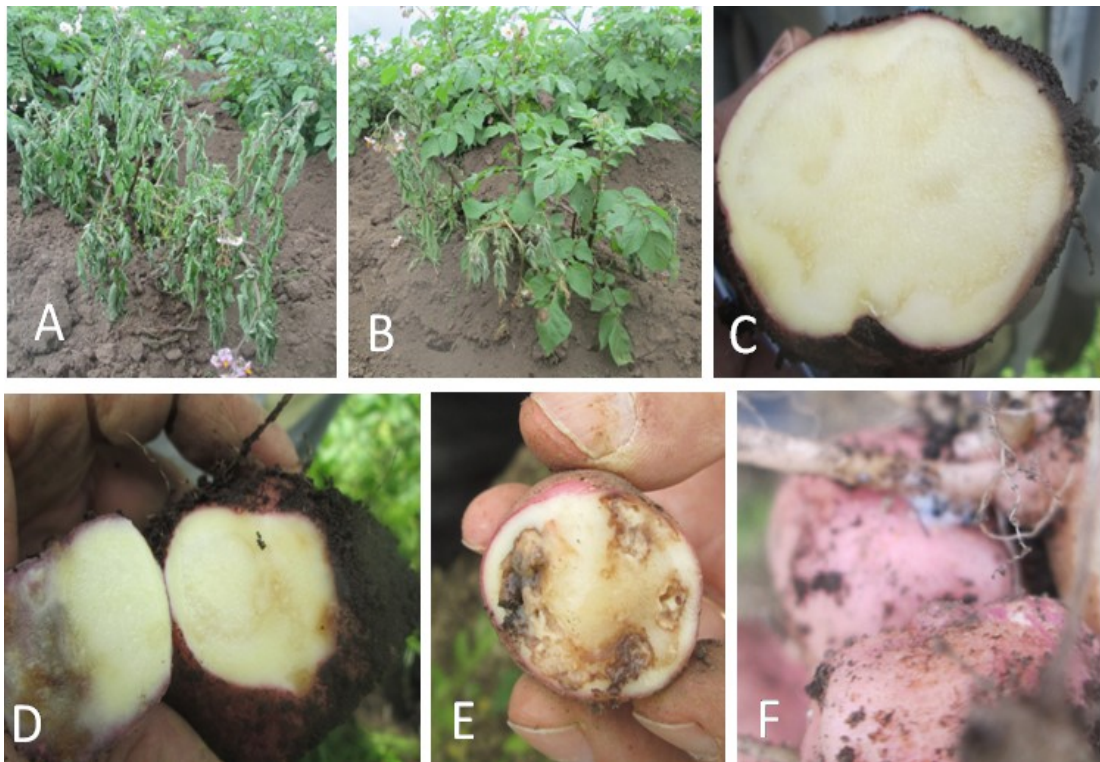
**Figure 1:** A map showing some regions of Tanzania indicating incidence and distribution of bacterial wilt disease in Mbeya and Njombe (Njombe was a part of Iringa).

**Table 1: Disease incidence and severity in Southern Highlands Zone Tanzania**

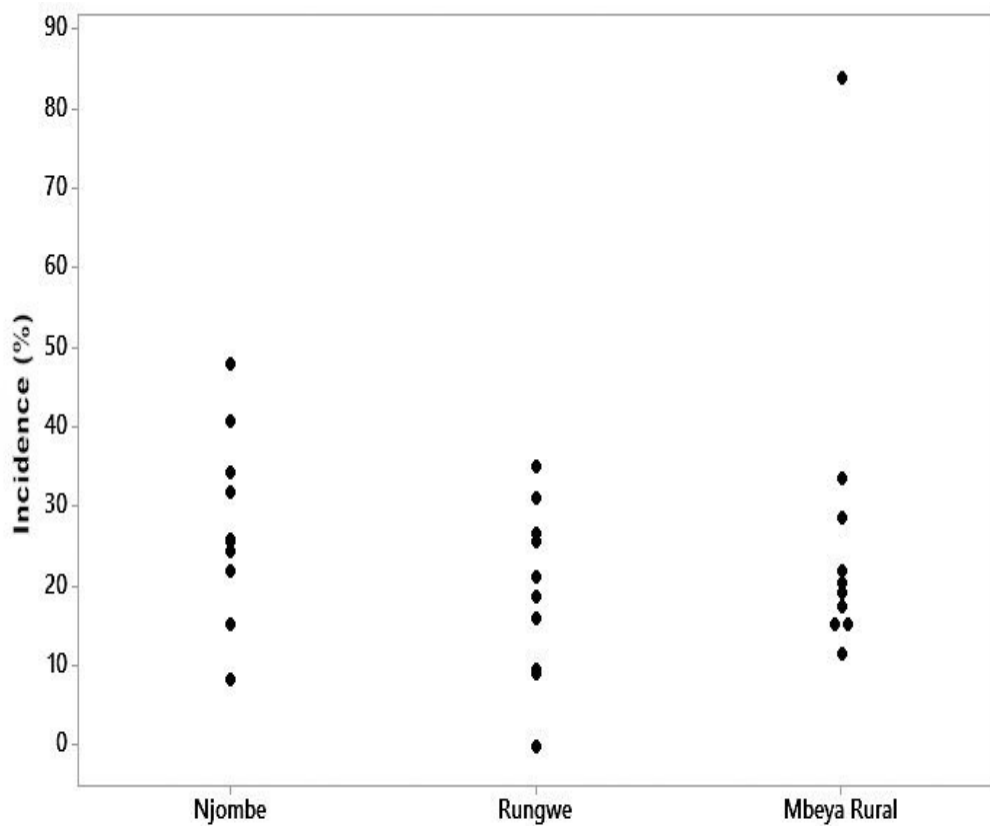
<b>District</b>	<b>Village</b>	<b>Disease incidence (%) per village</b>	<b>Disease severity per village</b>
<b>Rungwe</b>	Ndaga	15.2	3.92
	Malangali	15.2	3.52
	Ishinga	11.6	2.74
	Isebelo	28.8	3.14
	Ntokela	19.2	3.44
	Swaya	20.4	2.46
	Mbeye one	33.6	3.52
	Lukata A	17.6	2.58
	Lukata b	84.0	3.70
	Ushirika	22.0	3.22
<b>Mean</b>		26.7	3.22
<b>Njombe</b>	Welela	9.6	2.14
	Mtwango	26.8	3.08
	Kichiwa	21.2	3.10
	Lunguya	16.0	2.78
	Ilunda	25.6	2.5
	Ulembwe	35.2	3.34
	Usalule	31.2	3.26
	Igeri	0.00	0.00
	Magoda	18.8	3.02
	Luponde	9.2	2.8
<b>Mean</b>		19.4	2.60
<b>Mbeya Rural</b>	Itizi	8.4	2.7
	Sanje	40.8	3.66
	Jojo	48	2.82
	Santilya	15.2	2.34
	Idimi	34.4	2.82
	Haporoto	32	3.3
	Ifiga	22	3.1
	Hatwelo	25.6	3.3
	Simambwe	26	2.9
	Usoha	24.4	4.0
<b>Mean</b>		27.7	3.10

The mean severity scores for bacterial wilt disease symptom based on the scale by Horita and Tsuchiya (2001) ranged from 2 to 4.8. The lowest mean severity score was 2.0 in all the districts. The results show that bacterial wilt is indeed present in the surveyed areas however with a district variation in disease incidence and severity. Differences of wilt

incidence and severity is due to great diversity of host plants affected by the pathogen, phenotype, genotype, its wide geographical distribution and the range of environmental conditions conducive to bacterial wilt (Chalterfee *et al.*, 1997). Severity of the disease depends upon soil temperature, moisture, soil type, host susceptibility and virulence of strains. (Momol, 2003).



**Figure 2:** Bacterial wilt and brown rot symptoms caused by *R. solanacearum* on potato plants in the Southern Highlands Zone of Tanzania: A: Wilting of the whole plant; B: Wilting of a few stems of the potato; C: Oozing of the vascular tissues and brown rot; D and E. Further rotting of tubers infected with *R. solanacearum* and F: Oozing on the eyes of potato tubers



**Figure 3:** Disease incidence (%) of potato bacterial wilt in Southern highlands of Tanzania. One dot is representing more than one score.

## 4.2 Morphological characteristics of the isolates

### 4.2.1 Bacterial colony colour

When bacterial suspensions from potato tuber that had vivid symptoms of the disease were streaked to TZC medium large and elevated fluidal, colonies which were creamy with pinkish centre were observed indicating that they were virulent cultures. There were also colonies which were entirely white or with a pale red centre, smaller and non-fluidal which indicated that they were avirulent. These findings are in line with French *et al.* (1995) whom described virulent colonies as large, elevated, fluidal that are either white or with pale red centre and avirulent mutant colonies that are butyrous and deep red centers. The findings were also similar to Kelman (1954) who described virulent

colonies as being pink or light red colour or characteristic red centre and whitish margin.

Avirulent colonies were described as smaller, off white and non fluidal.



**Figure 4:** Virulent colony of *R. solanacearum* on TZC medium

#### 4.2.2 Pathogenicity test

Using 18 bacterial isolates tested, N1, N4, MBY R2, MBY R4, R3, R4 and R10 were highly virulent on potato and tomato and they were slight virulent on eggplant after 3 weeks of inoculation. Strains N7, MBY R5, MBY R6 and R5 were highly virulent on potato and moderate to tomato and eggplant (Table 2). Other strains N9, MBY R7, MBY R8, MBY R9, MBY R10, R2 and R7 were highly virulent on tomato and moderate to low on both potato and eggplant. None of the strains expressed wilting symptoms on tobacco and pepper. The strains were therefore grouped as Race 3. This race usually causes damage at lower temperature and attacks plants at higher altitudes (Oslon, 2005). It is more cold tolerant than race 1 and 2 in tropical highlands and temperate areas of East Africa (Oslon, 2005). *Ralstonia solanacearum* possesses hrp encoding the type III secretions system (T<sub>3</sub>SS) and pathogenicity depends on interactions between the host plant and the type III effectors. According to Hichiki *et al.* (2007), once the pathogen is introduced, it invades intercellular spaces of roots through openings such as wounds. After invasion they accumulate around the stele before breaking into and filling the

xylem vessels. Upon invasion of the xylem vessels, the bacteria grow and travel rapidly to the upper parts of the plants resulting in extensive wilting because of reduced sap flow caused by the presence of a large number of bacteria cells and Exopolysaccharides (EPS) slime produced by the bacteria in some xylem vessels.

**Table 2: Pathogenicity test of host plants inoculated with potato strains from the Southern Highland Tanzania.**

Origin	Isolate name	Potato		Tomato		Eggplant		Pepper		Tobacco	
		Score	Virulence rate	Score	Virulence rate	Score	Virulence rate	Score	Virulence rate	Score	Virulence rate
Njombe	N1	4.0	H	4.5	H	2.0	L	0.0	None	0.0	None
	N4	2.0	L	4.2	H	2.2	L	0.0	None	0.0	None
	N7	5.0	H	3.0	M	3.0	M	0.0	None	0.0	None
	N9	3.0	M	5.0	H	3.0	M	0.0	None	0.0	None
Mbeya Rural	MBY R2	5.0	H	3.8	H	2.5	L	0.0	None	0.0	None
	MBY R 4	4.2	H	4.1	H	1.5	L	0.0	None	0.0	None
	MBY R5	5.0	H	3.0	M	3.0	M	0.0	None	0.0	None
	MBY R6	4.1	H	2.7	M	3.5	M	0.0	None	0.0	None
	MBY R7	3.0	M	4.5	H	3.0	M	0.0	None	0.0	None
	MBY R8	3.0	M	4.8	H	3.0	M	0.0	None	0.0	None
	MBY R9	3.0	M	5.0	H	3.0	M	0.0	None	0.0	None
	MBY R10	3.0	M	4.0	H	3.0	M	0.0	None	0.0	None
Rungwe	R2	3.0	M	5.0	H	3.0	M	0.0	None	0.0	None
	R3	4.0	H	4.5	H	1.3	L	0.0	None	0.0	None
	R4	4.8	H	4.5	H	1.1	L	0.0	None	0.0	None
	R5	4.0	H	3.0	M	3.0	M	0.0	None	0.0	None
	R7	4.4	H	4.0	M	3.5	M	0.0	None	0.0	None
	R10	5.0	H	4.3	H	3.0	M	0.0	None	0.0	None

MBY R = Mbeya Rural, N= Njombe, R = Rungwe. Rating scales were as follows: H= high (4.1-5.0), M= moderate (2.6-4.0), L=low (1.1-2.5) and 0=none (He *et al.*, 1983).



*Ralstonia solanacearum* strains differ considerably in host range as well as aggressiveness to different hosts. Race 1 strains have a wide host range including numerous ornamentals and are present in most regions of the world. Race 2 is pathogenic to *Musa* spp and *Heliconia* spp. and occurs in tropical areas of the Central and South America, Hawaii and Philippines (Kelman, 1954; Bradbury, 1986; Elphistone, 2005). Race 3 strains are pathogenic mainly to potato and eventually infect tomato or other Solanaceous host. Race 4 affects ginger (Pegg and Moffet, 1971) and race 5 is pathogenic to mulberry (Rodrigouz *et al.*, 2012). Race 3 is an extremely destructive potato pathogen and it has been reported to cause Bacterial wilt disease in Highland tropics of Africa, Latin America and Asia. (Champoiseau, 2009). In Europe it disrupted seed potato production and caused serious quarantine related losses (Champoiseau, 2009). Measure to control this race is necessary in areas where its presence was not known like in the Southern Highlands of Tanzania so as to minimize the losses.

**Table 3: Definition of races and biovars of *Ralstonia solanacearum* by host range**

<b>Race</b>	<b>Natural host</b>	<b>Biovars</b>
1	Many Solanaceae, some diploid bananas numerous other crops weeds and many families	1,3 or 4
2	Triploid bananas numerous other crops and weeds in many families	1 or 3
3	Potato, tomato and rarely a few other host	2
4	Ginger	5
5	Mulberry	5

**Source:** EPPO, 2004

### **4.3 Biochemical Characterization of the Strains**

#### **4.3.1 Colour on TZC media**

All eighteen (18) virulent potato strains from Southern highland zone of Tanzania produced fluidal and irregular creamy colonies with pinkish centre on TZC agar media. These results are similar to those of Dhital *et al.*, (2000) in which potato strains from Nepal were also fluidal and irregular with pinkish or light red centre on TZC Media.

#### **4.3.2 Gram staining**

All 18 isolates gave negative response when tested for Gram staining (Table 4). Bacteria retained reddish pink colony colour when counter stained with safranin. This showed that they were Gram negative. In gram reaction as described by Schaad (1988) bacteria retaining reddish pink colony colour are Gram negative (G-ve) while Gram positive (G+ve) stain the blue violet colour.

#### **4.3.3 KOH test**

The isolates tested were negative on KOH loop test as they formed slime threads when the bacterial cultures (48 h) were mixed with 3 % KOH solution (Table 4).

Gram negative bacteria have relatively fragile cell walls which are bounded by an outer membrane. The outer membrane is disrupted by exposing it to 3 % KOH solution which results in releasing slime threads which is actually the viscous DNA. On the other hand, the Gram positive bacteria possess thick and more rigid cell wall which resists the disruptive effect of KOH. This test is useful supplement to the gram staining for the initial classification of anaerobic bacteria. Suslow *et al.*, (1982) reported that the KOH technique is easier and faster to distinguish Gram negative and positive bacteria than the traditional Gram stain in which dyes are employed.

#### 4.3.4 Catalase oxidase test

The bacterial isolates tested produced gas bubbles when mixed with a drop of H<sub>2</sub>O<sub>2</sub> on a glass slide, indicating that they might be *R. solanacearum* (Table 4). Production of gas bubbles is a tendency of all Gram negative bacteria, and it gives a clue for presence of aerobic and facultative anaerobic bacteria (Schaad, 1988). Catalase is a hemi-enzyme capable of decomposing hydrogen peroxide to water and oxygen gas (Klement *et al.*, 1964).

#### 4.3.5 Kovac's Oxidase Test

The tested isolates varied on the development of purple colour after being rubbed with Kovacs oxidase reagent (Table 4). Thirteen (13) isolates produced the purple colour within 10 seconds whereas 3 isolates showed the purple colour in 60 seconds. Those which developed colour in 10 seconds were categorized as positive for this reaction whereas those which developed colour in 60 seconds were categorized as delayed positive isolates. Two isolates were negative on this test as they did not develop purple colour.

In Kovac's oxidase test bacterial isolates which give purple colour when mass of bacterial growth was rubbed on filter paper impregnated with oxidase reagent were categorized as positive whereas negative ones do not produce purple colour (Table 4). This test is useful for differentiating aerobic and anaerobic bacteria (Kovacs, 1956) and is particularly important for differentiating Gram –negative bacteria. *R. solanacearum* gives a positive reaction.

#### 4.3.6 Oxidation of glucose

Tested bacterial isolates were positive for oxidation of glucose. In positive isolates there was colour change from green to yellow at the surface of the open tubes (Table 4). This indicated that there was gradual change of pH. There was no colour change in sealed tubes and therefore no pH change and thus glucose was not oxidized in sealed tubes.

Bacteria utilize glucose and other carbohydrates using certain metabolic pathways. Some are oxidative (respiratory) while others involve a fermentation reaction. Most bacterial plant pathogen of genus *Pseudomonas* and *Ralstonia* are oxidative as compared to genus *Erwinia* which are fermentative. Oxidative organisms can only metabolize glucose or other carbohydrates under aerobic condition, that is, oxygen is the ultimate hydrogen acceptor. Other organisms ferment glucose and the hydrogen acceptor is substance e.g. Sulphur. The oxidation fermentation process is used to differentiate species especially Gram-negative rods (Agrios, 2005).

#### 4.4 Biovar Determined

Results of isolate tests with dextrose and hexose alcohols showed that they all belonged to Biovar 2. The dextrose (Carbohydrates) were utilized as there was colour change from olivaceous green to yellow. The colour change was observed in microtitre plates containing carbohydrates maltose, cellobiose and lactose. This indicated that there was utilization of carbohydrates. In the inoculated tubes containing hexose alcohols such as Dulcitol, Mannitol and Sorbitol no colour change was observed and it implied that they were not oxidized. These results agree with French *et al.* (1995) whom described Biovar 2 group as one which utilizes disaccharides but not oxidizing alcohols.

**Table 4: Biochemical tests of different isolates obtained from potato samples used in this study**

Origin	Isolate name	Colour on TZC media	Gram reaction	KOH	Catalase oxidase	Kovacs oxidase	Oxidation of glucose	Inference
Njombe	N1	+	G-ve	Slime threads	Bubbles	Purple	Colour change	<i>R. solanacearum</i>
	N4	+	G-ve	Slime threads	Bubbles	Purple	Colour change	<i>R. solanacearum</i>
	N7	+	G-ve	Slime threads	Bubbles	Purple	Colour change	<i>R. solanacearum</i>
	N9	+	G-ve	Slime threads	Bubbles	Purple	Colour change	<i>R. solanacearum</i>
Mbeya Rural	MBY R2	+	G-ve	Slime threads	Bubbles	Purple	Colour change	<i>R. solanacearum</i>
	MBY R4	+	G-ve	Slime threads	Bubbles	Purple	Colour change	<i>R. solanacearum</i>
	MBY R5	+	G-ve	Slime threads	Bubbles	Purple	Colour change	<i>R. solanacearum</i>
	MBY R6	+	G-ve	Slime threads	Bubbles	Not purple	Colour change	<i>R. solanacearum</i>
	MBYR7	+	G-ve	Slime threads	Bubbles	Purple	Colour change	<i>R. solanacearum</i>
	MBY R8	+	G-ve	Slime threads	Bubbles	Purple	Colour change	<i>R. solanacearum</i>
	MBY R9	+	G-ve	Slime threads	Bubbles	Not Purple	Colour change	<i>R. solanacearum</i>
	MBYR10	+	G-ve	Slime threads	Bubbles	Purple	Colour change	<i>R. solanacearum</i>
	R2	+	G-ve	Slime threads	Bubbles	Purple	Colour change	<i>R. solanacearum</i>
	R3	+	G-ve	Slime threads	Bubbles	Purple	Colour change	<i>R. solanacearum</i>
Rungwe	R4	+	G-ve	Slime threads	Bubbles	Purple	Colour change	<i>R. solanacearum</i>
	R5	+	G-ve	Slime threads	Bubbles	Purple	Colour change	<i>R. solanacearum</i>
	R7	+	G-ve	Slime threads	Bubbles	Purple	Colour change	<i>R. solanacearum</i>
	R10	+	G-ve	Slime threads	Bubbles	Purple	Colour change	<i>R. solanacearum</i>

**+ Positive reaction, - Negative reaction**

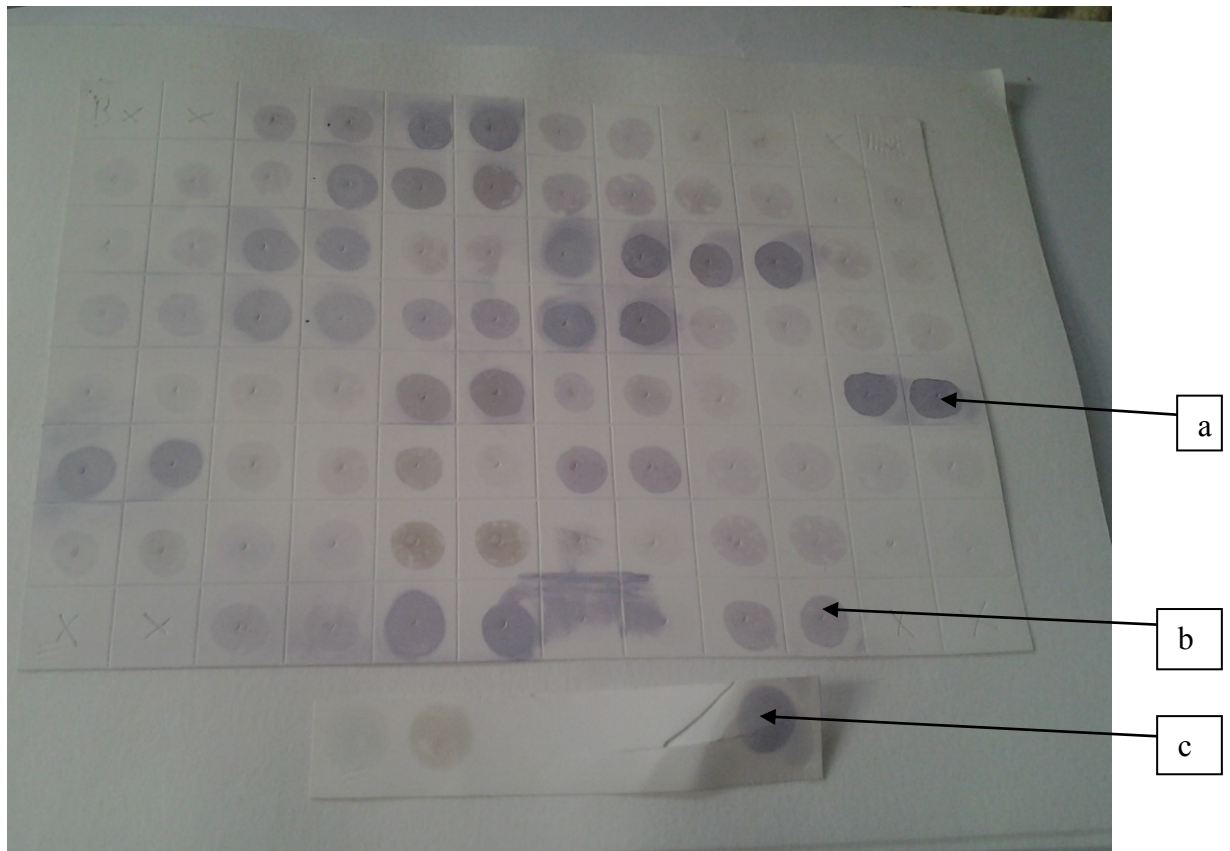
#### **4.5 Serological Detection of the Isolates**

Most of the sampled healthy looking tubers were found not to be healthy after being tested serologically by NCM-ELISA (Table 5). Using 50 samples collected from each district 22 %, 36 % and 46 % of them from Njombe, Mbeya rural and Rungwe respectively were found to be latently infected. Different range of bacterial concentrations was observed in the positive samples. The concentration ranged from  $10^7$

to  $10^8$  cfu/ ml based on colour intensity of the positive and negative control strips of the NCM-ELISA kit. Results also indicated that out of 150 samples tested, 52 (34.6 %) were latently infected implying that the seed with symptomless infection will look absolutely healthy. But once planted, the plant will develop bacterial wilt and die. Even worse, it will also infect the soil with the bacteria where it is planted. Bacteria will also spread from the initial symptomless tuber to neighbouring plants and infect them. Whether these plants will actually show symptoms depends on temperatures and soil humidity. They can also become symptomless carriers again, and spread the disease to yet another crop in a different field.

**Table 5: Number of potato tuber samples infected by *Ralstonia solanacearum* as revealed by NCM-ELISA test.**

<b>District</b>	<b>No. of Sample taken</b>	<b>No. of samples with latent infection</b>
Rungwe	50	23
Njombe	50	11
Mbeya rural	50	18
<b>Total</b>	<b>150</b>	<b>52</b>

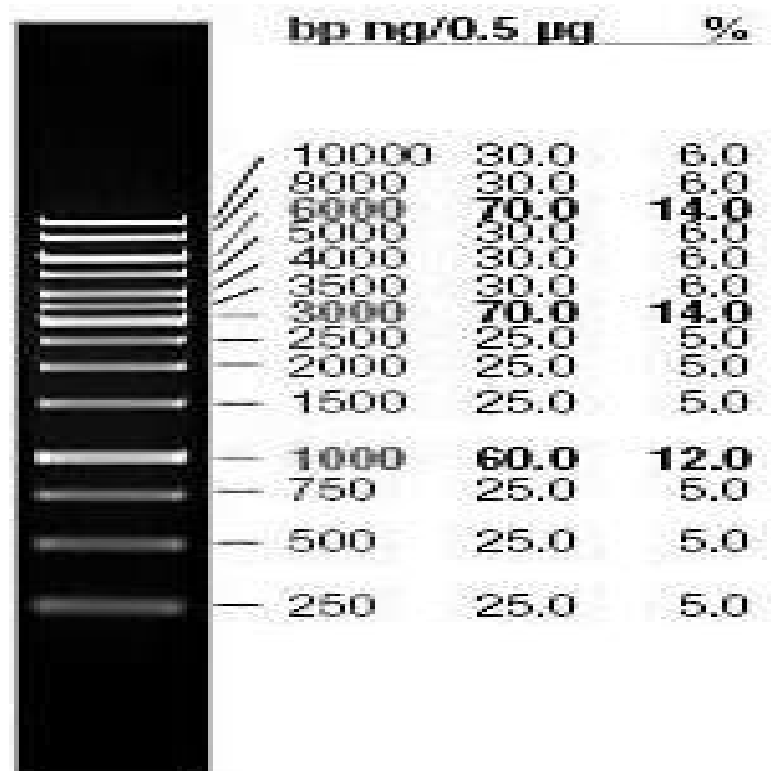


**Figure 5:** NCM-ELISA membrane of tested potato samples with different concentrations, a) deep purple ( $10^8$  bact /ml) b) light purple ( $10^6$  bact/ml), c) the control strip

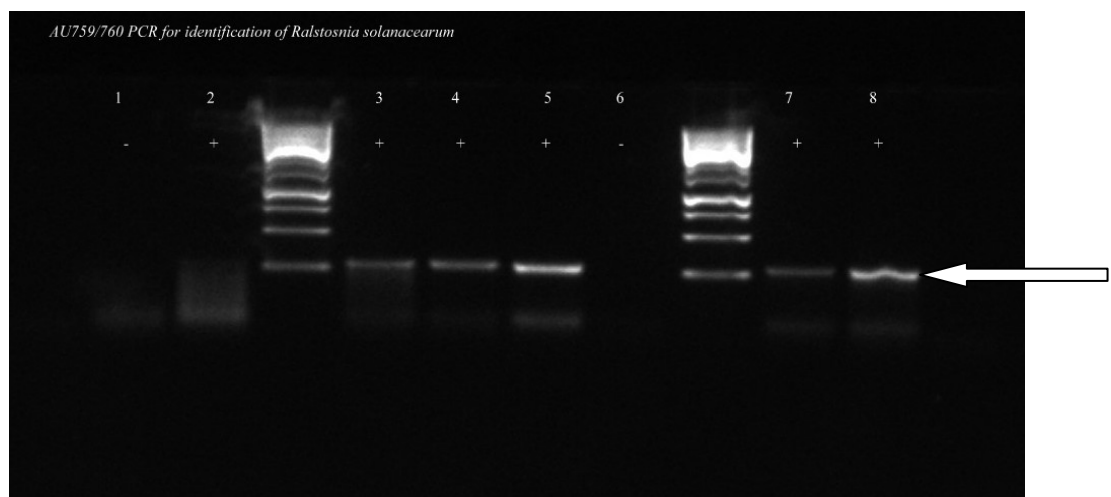
#### 4.6 Molecular Characterization of the Isolates

##### 4.6.1 Species-specific PCR

Out of Fifteen (15) strains tested from each district; 7, 4 and 3 strains from Rungwe, Njombe and Mbeya Rural respectively were confirmed to be *R. solanacearum* using species specific PCR. The PCR produced a product of about 280 bp band was observed (Figures 6 and 7). The isolates that produced band size were selected for further characterization.



**Figure 6:** Ladder map (1 kb) as a reference used in the experiment to determine the size of the PCR product.



**Figure 7:** An agarose gel image showing 280 bp PCR product using primer pair AU759/760 confirming bacteria in question as *R. solanacearum*. An arrow indicates 280 bp produced.



#### 4.6.2 Phylotype determination

Phylotype specific multiplex PCR revealed that *R. solanacearum* strains from Southern Highlands of Tanzania belonged to Phylotype III as 280 and 91 bp amplicon was observed in all the strains when Phylotype specific multiplex (Pmx- PCR) products of these strains were subjected to electrophoresis on 1.2% Agarose gel (Figure 8).

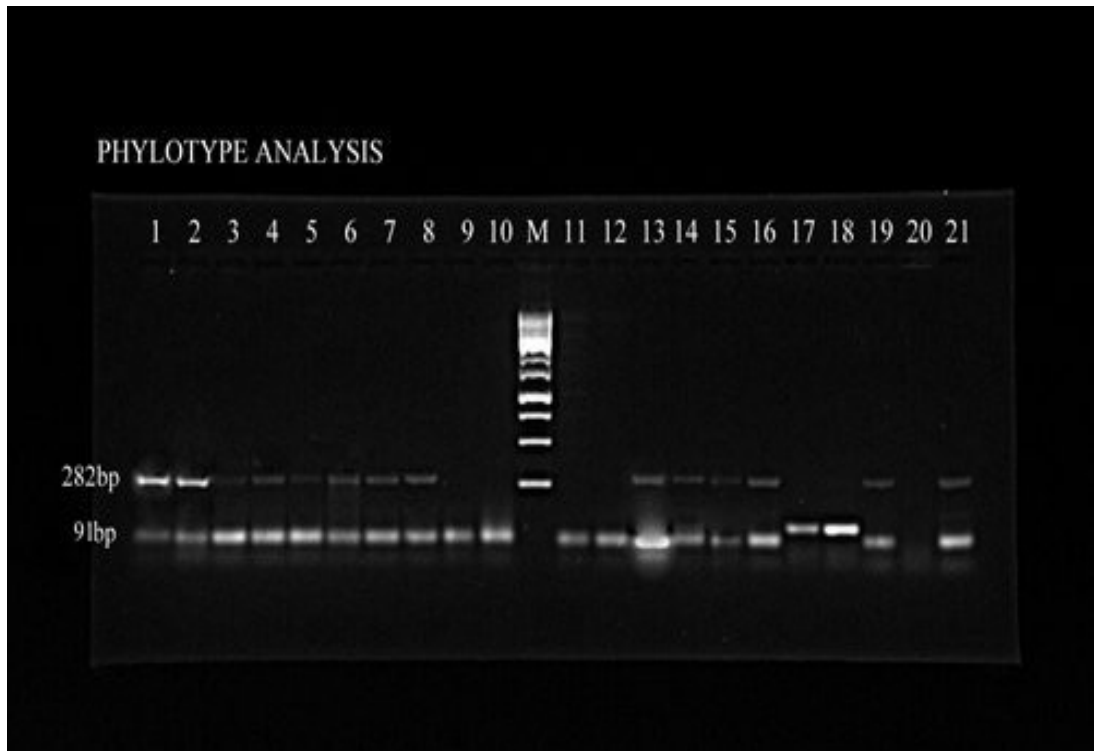
In the concept of integrated disease management, the use of resistant cultivars is of prime importance. A variety may be resistant to one phylotype but susceptible to the other and therefore it is very important to know the diversity of local strains of the pathogen (Sagar *et al.*, 2014). Phylo-typing is needed for successful disease management and control strategies.

The results obtained correspond to those of Fegan and Prior (2005) whom described a phylotypic classification system consisting of four phylotypes, in which Phylotype I are those from Asia and are characterized by production of 280 and 144 bp amplicon, Phylotype II strains are from America and they produce 280 and 372 bp amplicons. Phylotype III are mainly from Africa and nearby islands such as Reunion and Madagascar which produce 280 and 91 bp and Phylotype IV strains which are from Indonesia, Japan and Australia which produce 280 and 213 bp amplicon. Phylotyping helps in the concept of integrated disease management where the use of resistant cultivar is of prime importance. A variety may be resistant to one phylotype but susceptible to another therefore it is very important to know the diversity of local strain of the pathogen (Sagar *et al.*, 2014).

**Table 6: Phylotype categorization of *Ralstonia solanacearum* strains from potato grown in the Southern Highland zone of Tanzania.**

Primer name	Primer sequence	Expected band size	Phylotype category
Nmult:21:1F	5'-CGTTGATGAGGCGCGCAATTT-3'	144	Phylotype I (Asia)
Nmult:21:2F	5'-AAGTTATGGACGGTGGAAGTC-3'	372	Phylotype II (America)
Nmult:22InF	5'-ATTGCCAAGACGAGAGAAGTC-3	213	Phylotype IV (Tropical)
Nmult:23:AF	5'ATTACGACAGCAATCGAAAGATT3'	91	Phylotype III (African)
Nmult:22:RR	5'-TCGCTTGACCCTATAACGAGAGTA-3		Amorce reverse unique
759R	5-GTCGCCGTCAACTCACTTTCC3'		
760F	GTCGCCGTCAGCAATGCGGAATCG3	280	Universal <i>R. solanacearum</i> specific primers

Source: Sagar *et al.*, 2014



**Figure 8:** Phylotype specific multiplex PCR of 20 isolates of *R. solanacearum* from the Southern highlands zone of Tanzania. Samples 17 and 18 were above 91 bp therefore, not *R. solanacearum*, lane M is 1kb ladder, and sample 21 was a positive control.

## **CHAPTER FIVE**

### **5.0 CONCLUSION AND RECOMMENDATIONS**

#### **5.1 CONCLUSION**

Potato bacterial wilt disease was observed to occur in all three surveyed districts where major potato growing is taking place in the Southern Highland zone of Tanzania. Race 3, biovar II and phylotype III were prevalent in the study area.

#### **5.2 RECOMMENDATIONS**

Southern Highland zone of Tanzania know the disease but they find no single effective control. So long as the disease is a seed borne, necessary measure such as use of disease free seed tubers should be encouraged. In this study, potato seeds from Igeri in Njombe region are recommended for use by farmers in the southern of Tanzania as it was the location where no disease incidence was observed during the survey and which its samples were negative for serological test.

Use of certified seed potatoes at prices that small holder farmers can afford is recommended. To ensure quality seed potato availability there is a need of legalizing quality declared seed potato as in other crops.

Developing resistant varieties to bacterial wilt of potato is also recommended so as to minimize its spread.

Training on symptoms and bacterial wilt control is recommended to farmers. It was observed that most potato farmers in the Southern Highlands zone of Tanzania know the disease but they find no single effective control method.

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