# CHARACTERIZATION OF *Ralstonia solanacearum* INFECTING TOMATO (*Solanum lycopersicum* L.) ALONG THE COAST OF TANZANIA AND REACTION TO LINES BRED FOR RESISTANCE

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## EXTENDED ABSTRACT

*Ralstonia solanacearum* is a bacterium recognized as a major wilt causing phytopathogen with an unusual broad host range. This study aimed at determining the distribution of the bacteria along the coast of Tanzania and environmental factors influencing incidence and disease severity. It also aimed to define the population structure of the strains in terms of its biology and phylogenetic make up in order to effectively screen for variety tolerance. The study revealed that contaminated irrigation water and temperature are the key environmental factors determining incidence and severity. Bacterial wilt was found in Tanga, Pwani, Unguja ukuu and Morogoro where temperature varied within the range of 25°C to 32°C. *R. solanacearum* was detected in river water by DAS-ELISA. At a temperature of 18-20°C, no infected plants were found in Mtwara and Lindi. Due to high diversity in its genotypic and phenotypic traits, *Ralstonia solanacearum* is considered a species complex. Strains from different locations vary in virulence, hence its control using resistant varieties is usually strain specific. The study also included characterization of *R. solanacearum* isolates in order to determine strains present in the selected study areas. The strains were tested for their ability to utilize hexose sugars and alcohols and found to be biovar 3. Multiplex PCR showed that strains found along the Tanzanian coast are phylotype I which are predominantly Asian. A representative strain from each region was chosen and inoculated on tomato lines from AVRDC bred for resistance to *R. solanacearum*. Lines AVTO 1010, 9221, 0301, and 1226 had varying levels of resistance against different strains but were significantly resistant to the pathogen compared to the susceptible variety Cal J. These lines can be disseminated appropriately for farmers' seed testing and used to improve yield. The collection of strains can be used for selection purposes when screening for available local sources of resistance and mapping genes strongly linked to strain virulence.

## DECLARATION

I, Mushobozi Davis Baitani, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own 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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## COPYRIGHT

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## LIST OF ABBREVIATIONSAND SYMBOLS

| | |
|---|---|
| AVRDC | Asian Vegetable Research and Development Centre |
| BDB | Blood Disease Bacterium |
| c.f.u | Colony forming units |
| CPG | Casamino Peptone and Glucose |
| CWDE | Cell Wall Degrading Enzymes |
| DAS-ELISA | Double Antibody Sandwich Enzyme Linked Immunosorbent Assay |
| DNA | Deoxyribose Nucleic Acid |
| EDTA | Ethylene Di-Amine Tetra Acetic Acid |
| EPPO | European Plant Protection Organization |
| EPS | Exopolysaccharide |
| FAO | Food and Agriculture Organization |
| Hrp | Hypersensitive response and pathogenicity |
| IITA | International Institute of Tropical Agriculture |
| ITS | Internal Transcribed Spacer |
| KOH | Potassium Hydroxide |
| LSD | Least Significance Difference |
| O.D | Optical density |
| PBST | Phosphate Buffer Saline in Tween 20 |
| PCR | Polymerase Chain Reaction |
| PehA | Pentaethylenehexamine |
| PgIA | Phosphoglucose isomerase |

| | |
|---|---|
| Pme | Pectin methyl esterase |
| QTL | Quantitative Trait Loci |
| R3bv2 | Race 3 Biovar 2 |
| RFLP | Restriction Fragment Length Polymorphism |
| RIL | Recombinant Inbred Line |
| rRNA | Ribosomal Ribose Nucleic Acid |
| SDS | Sodium Dodecyl Sulphate |
| SMSA | Semi- selective Medium South Africa |
| T.E | Tris-EDTA |
| T3SS | Type three secretion system |
| TAE | Tris-Acetate Ethylene diamine tetra acetic acid |
| TE | Tris Ethylene diamine tetra acetic acid |
| TMB | Tetramethylbenzidine |
| TZC | Tetrazolium Chloride |
| UV | Ultraviolet |
| β | Beta |

# CHAPTER ONE

## 1.0 GENERAL INTRODUCTION

### 1.1 Taxonomic background and pathogenic potential of *Ralstonia solanacearum*

*Ralstonia solanacearum* is a phytopathogenic bacterium that causes wilting of many crops and ornamentals of economic importance (Belena *et al*., 2010). It can cause wilting in over 300 plant species belonging to over 30 botanical species. The major hosts of *Ralstonia solanacearum* belong to the family Solanaceae (nightshades) which include crops like tomato (*Solanum lycopersicum* L.), potato (*Solanum tuberosum* L.), eggplant (*Solanum melongena* L.), chilli (*Capsicum* spp.) and tobacco (*Nicotiana tabacum*) (Khavkar *et al*., 2011). Other major hosts include banana and groundnut.

*Ralstonia solanacearum* was initially known as *Bacillus solanacearum* (Smith, 1896) due to its rod shape and its predilection to members of the family Solanaceae. It was later named *Pseudomonas solanacearum* due to cultural and biochemical similarities to the members of the genus *Pseudomonas* (Smith, 1896). Its inability to fluorescence like the rest of the Pseudomonads led to its transferring to a new genus *Burkholderia* (Yabuuchi *et al*., 1992). The bacteria came to be known as *Ralstonia solanacearum* in 1995 after sequence analysis of the 16S rRNA, RNA-DNA hybridization, cellular lipid and fatty acid analysis revealed significant similarities between the *Burkholderia* and members of the genus *Ralstonia* (Yabuuchi *et al*., 1995).

*Ralstonia solanacearum* is a gram negative, rod shaped, anaerobic and motile bacterium. It was grouped into the beta subclass of Proteobacteria and falls within rRNA homology group II (non-fluorescent) of the taxon Pseudomonas (Ralston *et al*.,

1973). Within the genus *Ralstonia*, it is closely related to *Pseudomonas syzygii*, *R. pickettii* and Banana Blood Disease Bacterium (BDB).

*Ralstonia solanacearum* was classified into races and biovars whereby the race depends on host range and the latter is based on utilization and oxidation of hexose sugars (French *et al*., 1995). The common susceptible plant hosts used to define the race of *R. solanacearum* are tomato, potato, mulberry, banana and ginger (Floyd, 2008). The hexose sugars used in classifying *R. solanacearum* into biovars are cellobiose, maltose, lactose and the hexose alcohols dulcitol, mannitol and sorbitol (French *et al*., 1995). There are five races and six biovars of *R. solanacearum*. With respect to the predominant geographical origin, *R. solanacearum* was divided into four phylotypes. The pathogen is spread worldwide due to its ability to adapt to tropical, sub-tropical and temperate region climates (Lebeau *et al*., 2011). *Ralstonia solanacearum* is found across the globe in many countries including Tanzania and its neighboring countries Uganda and Kenya (EPPO, 2004).

*Ralstonia solanacearum* was described as a species complex due to its diversity and the variability of the strains aggressiveness in different hosts (Peeters *et al*., 2013). This essentially makes controlling *R. solanacearum* difficult. The ideal control method is to use resistant varieties tested against well characterized local *R. solanacearum* strains. The strains that can cause disease below 20℃ are considered a threat to agriculture in temperate areas and some countries have placed quarantine measures naming it as a select agent (EPPO, 2004).

## 1. 2 Problem statement

Bacterial wilt in tomatoes causes high yield loss. The disease is caused by a wide range of strains of *Ralstonia solanacearum*. Collectively, they infect all tomato varieties grown by farmers in Tanzania. Specific varietal resistance to *R. solanacearum* strains is unclear. *Ralstonia solanacearum* is highly flexible and capable of rapid adaptation to environmental changes and counteracting plant resistance (Wicker *et al.*, 2007). The locally preferred varieties are susceptible to *the pathogen* and there are no efficient chemical control strategies. Cultural practices such as fallowing and crop rotation are ineffective since the bacteria is able to endure harsh conditions and survive for a long time in soil (Tahat and Sijam, 2010; Ishihara *et al.*, 2012). Grafting tomatoes on resistant rootstocks had success on a commercial scale in Japan, Bangladesh, Philippines (Champoiseau *et al.*, 2009) and in experiments in the south eastern United States of America (Rivard and Louws, 2012). However, when subjected to a different environment, different strains of *R. solanacearum* have shown ability to break the resistance of the rootstocks (Nakaho *et al*., 2004).

## 1. 3 Justification

Tomato is the most important vegetable crop in Tanzania. The largest growers of tomato in Tanzania are small holder farmers (FAO, 2011). The threat *Ralstonia solanacearum* poses has adverse impacts on the livelihood of small holder farmers. Characterizing the strains will provide vital biological information that can be used in screening for resistance. *Ralstonia solanacearum* is a quarantine organism in countries like the United States of America, the European Union, Ghana and Nigeria. This denies the country a potential international trade market. The problem can be solved by introducing new resistant varieties whose response to different strains is known.

## 1.4 Objectives

### 1. 4.1 Overall objective

To reduce yield loss caused by *Ralstonia solanacearum* in tomato farms along the coast of Tanzania.

### 1. 4.2 Specific objectives

i. To document the distribution of *R. solanacearum* along the coast.

ii. To identify strains of *R. solanacearum* affecting tomato plants in the targeted growing areas

iii. To determine the pathogenicity and virulence of the strains of *R. solanacearum* against different tomato lines bred for resistance

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# CHAPTER TWO

## 2. 0 Distribution and biochemical characterization of *Ralstonia solanacearum* in tomato (*Solanum lycopersicum* L.) farms along the coast of Tanzania

### 2.1 Abstract

*Ralstonia solanacearum* causes wilting of tomatoes in farms in the Tanzanian coastal regions. Management of the bacteria must begin with a survey to determine areas infected. During the survey, tomato bacterial wilt disease was found in Tanga, Morogoro, Pwani and Unguja Ukuu (Zanzibar). The distribution of the bacteria was influenced by epidemiological factors favoring its transmission. Natural water sources, soil type, and temperature are the main factors influencing the presence of the bacteria in different areas. All fields visited that had ferralsols and used river water as the source of irrigation were infected. No tomato plants infected with *Ralstonia solanacearum* were found in Mtwara and Lindi regions. The atmospheric temperature was 19℃ and 20°C in Mtwara and Lindi respectively. This was attributed to the fact that the temperature there was below the optimum temperature for infection by the bacteria. Biovar classification revealed presence of *R. solanacearum* belonging to biovar 3 which are strains incapable of causing wilting in regions with temperature below 25°C.

**Keywords:** *Ralstonia solanacearum*, Distribution, soil type, water source, biovar, tomato

## 2.2 Introduction

*Ralstonia solanacearum* is a plant pathogenic bacterium which causes wilting in tomato in lowland tropical or subtropical areas (Champoiseau *et al.*, 2009). It is a major production constraint of tomato in East Africa, including Kenya and Tanzania. It is soil borne and can cause up to 100% loss of yield (Champoiseau *et al.*, 2009). *Ralstonia solanacearum* invades plant roots through wounds or emergence points of lateral roots (Yao and Allen, 2007). Root exudates released from wounds initiate the bacteria's' chemotactic mediated swimming toward the roots (Belena *et al*., 2010). It invades the vascular vessels and grows systemically in the plant (Schell, 2000). Plants infected by *R. solanacearum* appear shrunk with severe leaf epinasty while still freshly green. When the plant stem is vertically split, vascular discoloration can be observed, while lateral sections are dipped in water, the bacteria can be seen streaming.

The biochemical and anatomical attributes of the bacteria aid its ability to cause damage upon invasion. Pathogenic *R. solanacearum* produce catalase enzyme for protection against the bactericidal hydrogen peroxide (Schmid, 2015). The production of an exopolysaccharide (levan) is also a major biochemical property which it poses as a significant virulence factor (Agrios, 2005). The levan aids wilting due to its high viscosity which in turn occludes the xylem vessels and blocks water uptake (Champoisseau, 2009). The ability to utilize different sugars in root exudates and in plant ensures constant supply of energy needed for cell division and proliferation of the bacteria. Structural features such as flagella aid *R. solanacearum* in chemotactic mediated swimming along a chemical gradient of root exudates. In this manner, the

bacterium swims to the point of infection (Belena *et al.*, 2010). The outer membrane of its cell wall allows more resistance to toxins that could damage the pathogen.

The host pathogen interaction is greatly influenced by the environment (Gilbert and Parker, 2010). *Ralstonia solanacearum* strains have varying abilities to survive in different climatic zones (Chandrashekara *et al*., 2012). Most plant pathogens of a given species are active within a limited range of temperature. However, some strains are virulent across a wider range due to adaptive and perhaps evolutionary pressures. Introduction of such strains to new crop zones could be economically devastating (Bocsanczy, 2014). Some strains such as R3bv2 cause wilting in areas with temperate and tropical climates while some are restricted to areas with tropical climate *R. solanacearum* is transmitted through soil, surface water, infected plant materials and insects (Remenant *et al*., 2010).

After the plant collapses due to wilting, *R. solanacearum* is released to a saprophytic life in the soil until it encounters a new host to infect. It survives in soil by feeding on dead plant debris and shows an unusual ability of surviving in a nutrient depleted environment (Meng, 2013). It was shown that strains of *R. solanacearum* can be altered based on soil type, its texture, pH and moisture content (Yadessa *et al.*, 2010). During heavy rainfall on infested soil, water runoff contaminates rivers which are the sources of irrigation water (Adebayo *et al*., 2009).

The survival and physiology of the bacteria in water sources is highly affected by epidemiological factors such as temperature, light, canal sediments, sea water salts

and competing microorganisms (van Elsas *et al*., 2001). Temperature and light in water sources vary according to the seasons. The bacteria in irrigation schemes are more exposed to the sun during the dry season. A survey was conducted in order to determine areas infected by *R. solanacearum* and the epidemiology linked to its distribution. Biochemical characterization was carried out to confirm the identity of the pathogen, its virulent nature and determine the biovar of the strains present in the study areas.

## 2.3 Materials and methods

### 2.3.1 Determination of the distribution of *Ralstonia solanacearum*

A survey was conducted in the regions of Tanga, Mtwara, Lindi, Pwani on main land Tanzania and Unguja Ukuu (Zanzibar). These regions have a hot humid climate which boosts the bacteria's pathogenic potential. Samples were also collected from Morogoro region for biochemical comparison to assess the existence of a biological gradient away from the coast. A total of thirteen districts were surveyed. These included three districts from each region except Morogoro and Unguja North where only two districts were surveyed in each. The districts surveyed were Ilala, Kinondoni and Temeke in Dar es Salaam; Bagamoyo, Kibaha and Mkuranga in Pwani; Korogwe, Tanga and Handeni in Tanga; Morogoro and Mvomero in Morogoro and Kaskazini A and Kaskazini B in Unguja North. In Lindi, the districts surveyed were Lindi rural, Kilwa and Liwale while Mtwara rural, Masasi and Nanyumbu districts were surveyed in Mtwara region. In each district, three villages were surveyed. Sampling was done at random in three farms per village. The distance between farms was fifty meters. The Garmin Etrex GPS device was used to record locations and altitudes of the farms.

Locations where the disease was found were recorded. These were later plotted on the map of Tanzania using Googles' online software "My Maps". The survey was done during the wet and dry seasons. Upon observation of symptoms typical to bacterial wilt such as wilting, vascular discoloration, leaf epinasty and softening, a piece of stem (5 cm) was cut from the crown, immersed in clear water to observed the presence of bacterial streaming. The bacteria were allowed to stream in extraction buffer in a mesh bag for immunostrip test using kits from Agdia INC, 52642 Co. Rd, Elkhart, IN 46514, Indiana. This was used for preliminary confirmation of bacterial species. Each kit contained 25 immunostrips embedded with monoclonal antibodies specific to *Ralstonia solanacearum*. Samples that test positive due to interaction of the surface antigen of the bacteria with embedded antibodies on the strip show a confirmatory test line and a control line to confirm validity of the test (Figure 2.1). The plants which tested positive were then placed in a paper bag and labeled appropriately with the date of collection, location and GPS coordinates. In locations where farmers refrain from growing tomatoes during the wet season in order to avoid diseases, soil samples were collected instead.

The collected samples (soil and plant) were taken to the IITA laboratory in Mikocheni Dar es Salaam for isolation and characterization of *Ralstonia solanacearum*. The air temperature was recorded using a digital thermometer during sampling.

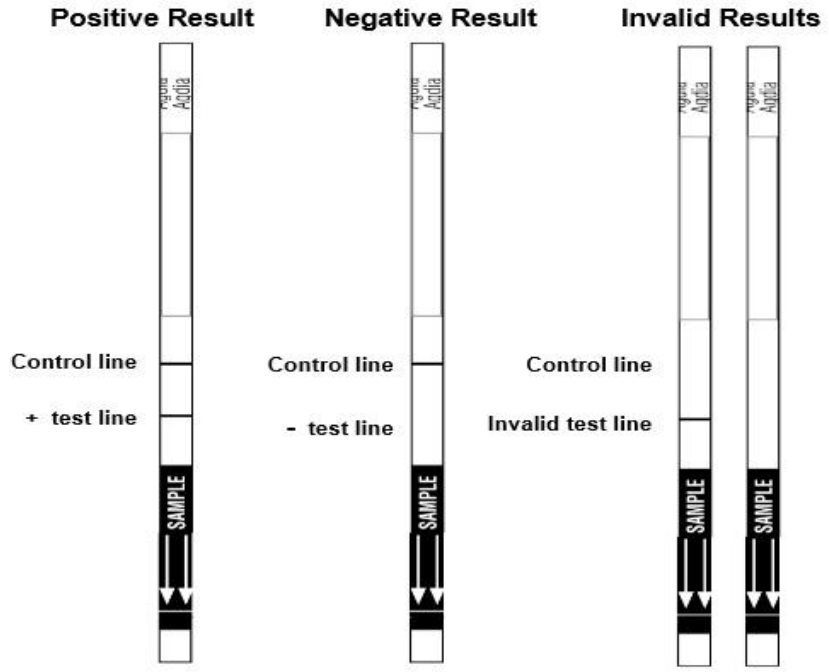


**Figure2. 1: Instructions of interpretation of results of a *Ralstonia solanacearum* species specific immunostrip test**

### 2.3.2 Serological detection *of Ralstonia solanacearum* in river water by Double Antibody Sandwich Enzyme Linked Immunosorbent Assay (DAS-ELISA)

This was performed according to Priou *et al.*, (2010) with few modifications. The protocol, which was meant for detection of *Ralstonia solanacearum* in stems of asymptomatic potato plants, was modified to suit detection of the pathogen in river water. River water was collected in a 50 ml screw cap tube. The sample aliquot of 25 ml was mixed with 25 ml of CPG broth (casamino acids 1 g, peptone 10 g, glucose 5 g, distilled water 1000 ml) instead of SMSA broth and incubated for 48 h in 28℃. This would enrich the bacteria to a detectable population density in case of their presence. From this sample, 500 µl was transferred to a microfuge tube containing 50

µl of the general extraction buffer and mixed thoroughly. Using a spectrophotometer at the absorbance of 600 nm the cells were adjusted to a concentration of $10^5$ c.f.u/ml.

An aliquot of 100 µl of this sample was added to a microliter well coated with anti-*Ralstonia solanacearum* antibodies. The plate was incubated for one hour at room temperature in a humid chamber. After incubation, the wells were dumped with a quick flipping motion without mixing the contents. The wells were then washed with 1 X PBST wash buffer eight times and tapped firmly on a folded paper towel to remove any remaining drops. A hundred microliters of peroxidase enzyme conjugate per well was added and the plate was again incubated for one hour in a humid box. The wells were dumped again after incubation and washed eight times with PBST buffer. The plate was moved to a dark humid chamber and 100 µl of TMB substrate was added to each well. The plate was incubated for fifteen minutes. Thereafter, the colorless aliquots in the plate were observed for change to the color blue.

### 2.3.3 Isolation of *Ralstonia solanacearum* from diseased plants and soils

Isolation of the bacteria from the plant was carried out according to Kelman (1954). A 5 cm stem piece was cut using a sterile knife and washed with tap water. It was later rinsed with sterile water and sprayed with 70% alcohol in a laminar air flow chamber. After 3 minutes. the alcohol was rinsed off with sterile water and the stem piece was dried. Upon immersion in sterile water, bacterial streaming from the stem piece was observed. This suspension was streaked on a TZC agar (Casamino acids 1 g, Peptone 10 g, Glucose 2.5 g, Tetrazolium chloride 5 ml, Agar 18 g, distilled water 995 ml) and incubated in 28°C for two days. The plates were observed for growth of irregular shaped and mucoid colonies with red/pink centers.

From the soil samples, isolation was done according to Elphinstone and Gutarra (1995). A serial dilution was performed and the suspensions plated on a SMSA (Semi selective South Africa) medium (1% Crystal violet 5 ppm, 1% Polymyxin B Sulphate 100 ppm, 1% Tetrazolium 50 ppm, 1% Bacitracin 25 ppm, 0.1% Penicillin 0.5 ppm, 1% Chloramphenicol 5 ppm). This media included antibiotics in addition to the TZC media to inhibit growth of fungi and unwanted bacteria.

### 2.3.4 Biochemical characterization of the bacteria isolated from plants and soil

#### 2.3.4.1 Gram reaction

Gram staining was achieved by the step wise protocol described by Schaad (1980). A smear of a pure colony from TZC plates was prepared on a grease free glass slide by mixing it with 6 µl of water and air drying for 2 min. In order to stain the bacterial cell wall, the slide was flooded with crystal violet and left for 30 seconds. It was then rinsed gently by running tap water. The smear was then covered by lugol iodine for 30 seconds to increase affinity of the crystal violet crosslinking to the peptidoglycan wall. The slide was then rinsed with tap water to wash off any loose crystal violet. Soon after that it was flooded with the decolorizing agent ethanol for fifteen seconds and rinsed. It was then flooded with the pink counterstain safranin. The slide was viewed under 10X magnification on a compound microscope (BA310E Elite Compound Microscope).

#### 2.3.4.2 KOH loop test

The KOH test is a confirmation test for the gram stain. Three pure colonies were picked using wire loop and mixed rigorously with few drops of KOH solution (3%) on a glass slide in order to lyse gram negative bacteria. The loop was frequently raised 1 cm off the surface so as to see if the mixture had become viscous and formed a sticky

slime with the ability to "string out" (Suslow *et al.*, 1982). Ruptured gram negative bacteria release DNA into the mixture and make it viscous. The mixture stays non-viscous if the bacteria is gram positive.

**2.3.4.3 Levan production**

Bacterial colonies were streaked on 523 media (Agar 8 g, Casein hydrolysate 8 g, Magnesium Sulphate anhydrous 0.035 g, Potassium phosphate monobasic 2 g, sucrose 10 g, yeast extract 4 g, distilled water 1000 ml). The levan is an exopolysaccharide (EPS) which on media is fluidy and spreads. In *Ralstonia solanacearum* the EPS is a pathogenicity factor (Agrios, 2005). The plates were incubated at 28°C for 2 days and observed for Levan production (Schaad, 1980).

**2.3.4.4 Catalase activity**

Three pure colonies of bacteria were picked from TZC plates and mixed with two drops of hydrogen peroxide solution on a glass slide. The slide was observed for formation of bubbles indicating release of oxygen by enzymatic reaction of catalase.

**2.3.4.5 Oxidase activity**

Pure bacterial suspension of concentration of $5x10^8$ c.f.u/ml was prepared. This was spotted on an autoclaved filter paper and soaked with few drops of Kovacs reagent (Kovacs, 1956). After 30 seconds, the filter paper was observed for formation of a purple ring.

**2.3.4.6 Pigment production**

Pure colonies were streaked on Kings B Medium (Peptone 20 g, Di-potassium hydrogen phosphate 1.5 g, Magnesium sulphate. Heptahydrate 1.5 g, Agar 20 g,

distilled water 1000 ml incubated at 30°C for two days and observed for brown pigmentation. Fluorescence was observed under UV lamp at a wave length of 356 nm (King *et al*., 1954).

#### 2.3.4.7 Determination of Biovar

Ninety six well microliter plates were purchased from BioRad Laboratories, 3, Boulevard Raymond Poincare, 92430, Marnes la Coquette, France. Two hundred microliters of the basal medium ($NH_4H_2PO_4$ 1 g, KCl 0.2 g, $MgSO_4.7H_2O$ 0.2 g, Peptone 1.0 g, Bromothymol blue 0.03 g, Agar 3 g, $dH_2O$ 1000 ml, pH 7) with ready added disaccharides (maltose, cellobiose and sucrose) and hexoses (dulcitol, mannitol and sorbitol) was distributed in the wells. The concentration of bacteria stored in sterile distilled water was set to $10^8$ c.f.u/ml. With the help of a micropipette, 200 µl of bacterial suspension was added to the wells and the plate was incubated at 28°C for five days.

## 2.4 Results

### 2.4.1 Determination of the distribution of *Ralstonia solanacearum*

The surveyed areas showing distribution of *Ralstonia solanacearum* are presented in Fig. 2.2. Bacterial wilt was found in some farms in Tanga, Morogoro, Pwani and Zanzibar (Tables 2.1 – 2.3). Disease incidence was highest in Tanga at a range of 57-100% (Fig. 2.3). An exemplary symptomatic wilting tomato plant is shown in Fig. 2.4 and an immunostrip positive results samples in Fig. 2.5. There were no plants infected with *Ralstonia solanacearum* in Mtwara and Lindi. Mtwara and Lindi had the lowest temperatures (19 and 20°C respectively) unlike the others which had more than 25°C and (Fig. 2.6).

**Table2. 1: Bacterial wilt (*Ralstonia solanacearum*) status in Tanga**

| S/N | District | Village | Farm location (GPS) | | ST | *Rs* |
|---|---|---|---|---|---|---|
| | | | Longitude | Latitude | | |
| 1 | Korogwe | Bungu | E38.24326° | S5.04618° | Plant | + |
| 2 | Korogwe | Bungu | E38.24326° | S5.04618° | Plant | + |
| 3 | Korogwe | Bungu | E38.24326° | S5.04618° | Plant | + |
| 4 | Korogwe | Bungu | E38.24326° | S5.04618° | Plant | + |
| 5 | Korogwe | Bungu | E38.24326° | S5.04618° | Plant | + |
| 6 | Korogwe | Bungu | E38.24326° | S5.04618° | Plant | - |
| 7 | Korogwe | Bungu | E38.24326° | S5.04618° | Soil | + |
| 8 | Korogwe | Bungu | E38.24326° | S5.04618° | Soil | + |
| 9 | Korogwe | Bungu | E38.24326° | S5.04618° | Soil | - |
| 10 | Korogwe | Bungu | E38.24326° | S5.04618° | Plant | + |
| 11 | Korogwe | Bungu | E38.24326° | S5.04618° | Soil | + |
| 12 | Korogwe | Bungu | E38.24326° | S5.04618° | Soil | + |
| 13 | Korogwe | Bungu | E38.24326° | S5.04618° | Soil | - |
| 14 | Korogwe | Bungu | E38.24326° | S5.04618° | Plant | + |
| 15 | Korogwe | Bungu | E38.24326° | S5.04618° | Plant | + |
| 16 | Korogwe | Bungu | E38.24326° | S5.04618° | Plant | + |
| 17 | Korogwe | Bungu | E38.24326° | S5.04618° | Plant | + |
| 18 | Korogwe | Bungu | E38.24326° | S5.04618° | Plant | - |
| 19 | Korogwe | Bungu | E38.24326° | S5.04618° | Plant | - |
| 20 | Korogwe | Bungu | E38.24326° | S5.04618° | Plant | - |
| 21 | Korogwe | Bungu | E38.24326° | S5.04618° | Plant | + |
| 22 | Korogwe | Bungu | E38.24326° | S5.04618° | Soil | + |
| 23 | Korogwe | Bungu | E38.24326° | S5.04618° | Soil | + |
| 24 | Korogwe | Bungu | E38.38929° | S5.03651° | Soil | + |
| 25 | Korogwe | Bungu | E38.38929° | S5.03651° | Plant | + |
| 26 | Korogwe | Bungu | E38.38929° | S5.03651° | Plant | - |
| 27 | Korogwe | Bungu | E38.38929° | S5.03651° | Plant | - |
| 28 | Korogwe | Bungu | E38.38929° | S5.03651° | Plant | - |
| 29 | Korogwe | Bungu | E38.38929° | S5.03651° | Plant | - |
| 30 | Korogwe | Bungu | E38.38929° | S5.03651° | Plant | - |
| 31 | Korogwe | Bungu | E38.38929° | S5.03651° | Plant | - |
| 32 | Korogwe | Bungu | E38.38929° | S5.03651° | Plant | - |
| 33 | Korogwe | Bungu | E38.38929° | S5.03651° | Plant | - |
| 34 | Handeni | Pogwe | E38.02946° | S5.46643° | Plant | - |
| 35 | Handeni | Pogwe | E38.02946° | S5.46643° | Plant | + |
| 36 | Handeni | Pogwe | E38.02946° | S5.46643° | Plant | + |
| 37 | Handeni | Pogwe | E38.02946° | S5.46643° | Soil | + |
| 38 | Handeni | Msaje | E38.03467° | S5.46722° | Plant | + |
| 39 | Handeni | Msaje | E38.03467° | S5.46722° | Plant | + |
| 40 | Handeni | Msaje | E38.03467° | S5.46722° | Plant | - |

**Table2. 2: Bacterial wilt (*Ralstonia solanacearum*) status in Pwani**

| | | | Farm location (GPS) | | | |
|---|---|---|---|---|---|---|
| **S/N** | **District** | **Village** | **Longitude** | **Latitude** | **ST** | ***Rs* status** |
| 1 | Kibaha | Mpiji | E39.19114° | S7.08645° | Plant | - |
| 2 | Bagamoyo | Mtoni | E38.88523° | S6.46413° | Plant | - |
| 3 | Bagamoyo | Mtoni | E38.88523° | S6.46410° | Plant | - |
| 4 | Bagamoyo | Chemi-chemi | E38.88562° | S6.45297° | Plant | + |
| 5 | Bagamoyo | Mtoni | E38.82672° | S6.48877° | Plant | - |
| 6 | Bagamoyo | Mtoni | E38.82596° | S6.48836° | Plant | + |
| 7 | Mkuranga | Kisse | E39.11092° | S7.15657° | Plant | + |
| 8 | Mkuranga | Kisse | E39.11093° | S7.15657° | Plant | + |
| 9 | Mkuranga | Kisse | E39.11094° | S7.15657° | Plant | - |
| 10 | Mkuranga | Kisse | E39.11095° | S7.15660° | Plant | - |
| 11 | Mkuranga | Hoyoyo | E39.19304° | S7.8199° | Plant | - |
| 12 | Mkuranga | Hoyoyo | E39.19305° | S7.8244° | Plant | - |
| 13 | Mkuranga | Hoyoyo | E39.19306° | S7.8201° | Plant | + |

**Table2. 3: Bacterial wilt (*Ralstonia solanacearum*) status in Unguja Ukuu, Lindi and Mtwara**

| | | | Farm location (GPS) | | | |
|---|---|---|---|---|---|---|
| **S/ N** | **District** | **Village** | **Longitude** | **Latitude** | **ST** | ***Rs* status** |
| 1 | Kibaha | Mpiji | E39.19114° | S7.08645° | Plant | - |
| 2 | Bagamoyo | Mtoni | E38.88523° | S6.46413° | Plant | - |
| 3 | Bagamoyo | Mtoni | E38.88523° | S6.46410° | Plant | - |
| 4 | Bagamoyo | Chemi-chemi | E38.88562° | S6.45297° | Plant | + |
| 5 | Bagamoyo | Mtoni | E38.82672° | S6.48877° | Plant | - |
| 6 | Bagamoyo | Mtoni | E38.82596° | S6.48836° | Plant | + |
| 7 | Mkuranga | Kisse | E39.11092° | S7.15657° | Plant | + |
| 8 | Mkuranga | Kisse | E39.11093° | S7.15657° | Plant | + |
| 9 | Mkuranga | Kisse | E39.11094° | S7.15657° | Plant | - |
| 10 | Mkuranga | Kisse | E39.11095° | S7.15660° | Plant | - |
| 11 | Mkuranga | Hoyoyo | E39.19304° | S7.8199° | Plant | - |
| 12 | Mkuranga | Hoyoyo | E39.19305° | S7.8244° | Plant | - |
| 13 | Mkuranga | Hoyoyo | E39.19306° | S7.8201° | Plant | + |

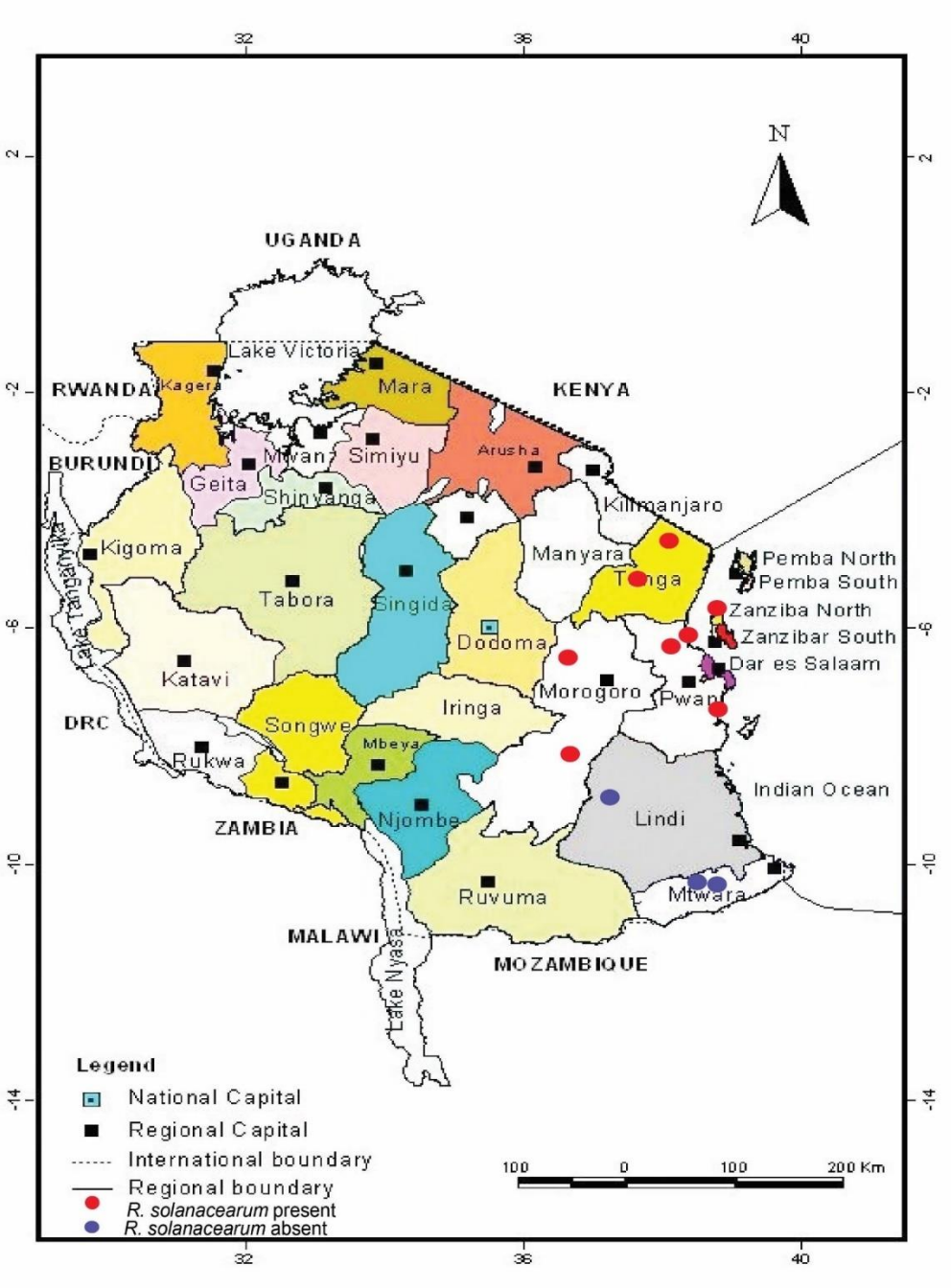


**Figure2. 2: Distribution of *Ralstonia solanacearum* in surveyed tomato farms along the coast and Morogoro**

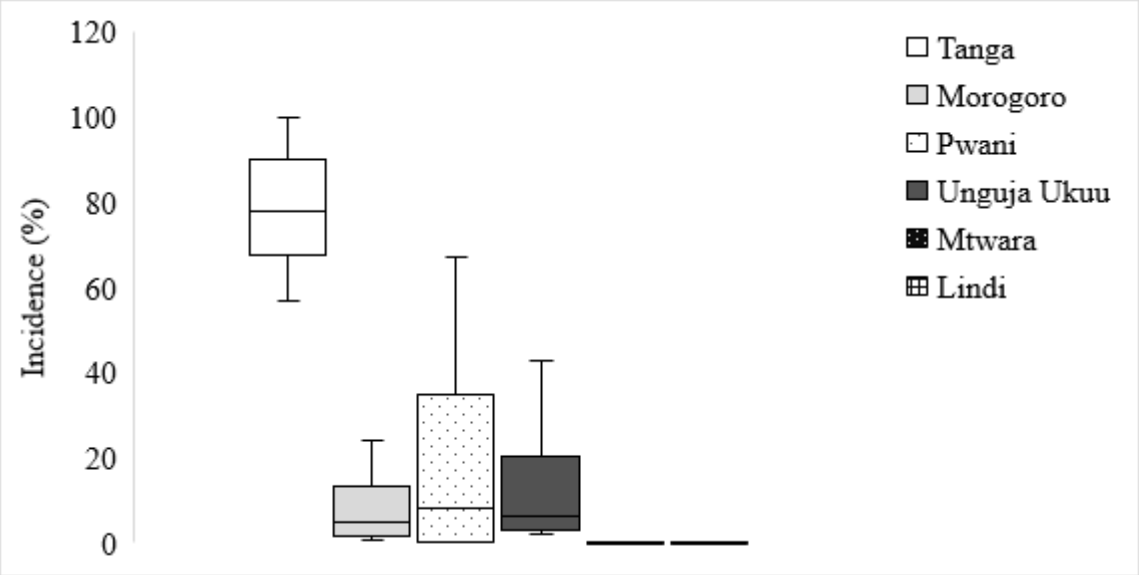


**Figure2. 3: Incidence of bacterial wilt in tomato farms found infected with *Ralstonia solanacearum*.**

**Figure2. 4: A symptomatic wilted tomato plant which was found in Tanga**


**Figure2. 5: An immunostrip test showing positive for *Ralstonia solanacearum* in the field.**

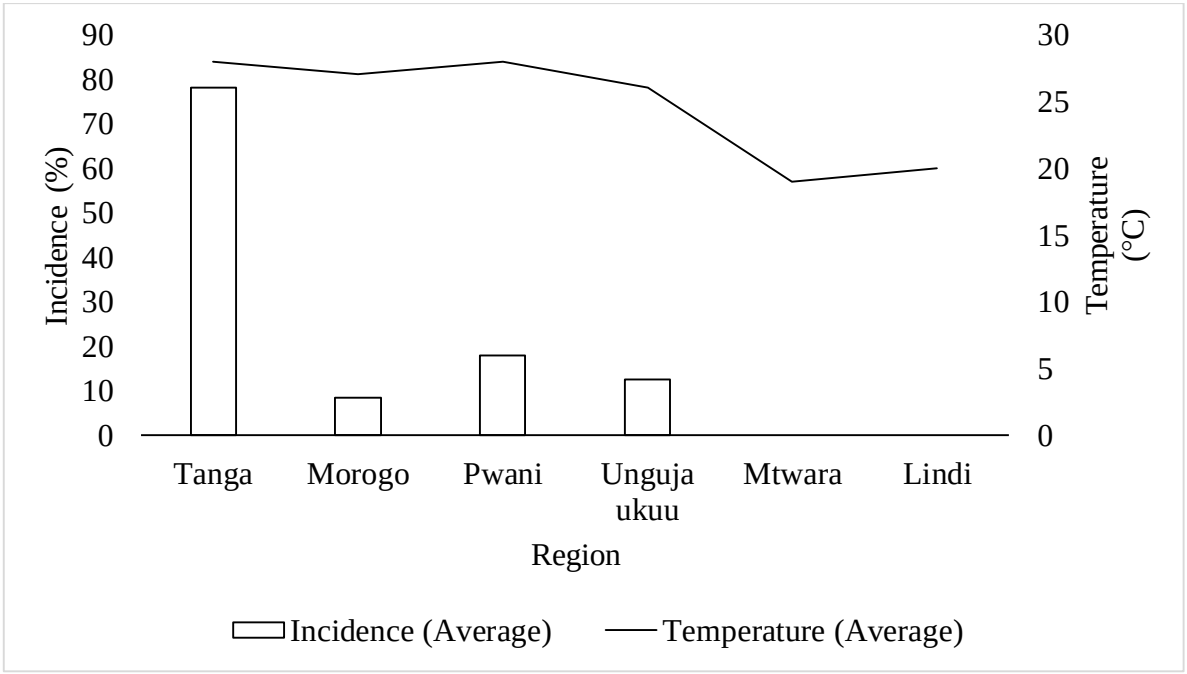


**Figure2. 6: Regional average incidences and trend of mean temperature in *Ralstonia solanacearum* sampling sites.**

### 2.4.2 Serological detection of *Ralstonia solanacearum* in river water by Double Antibody Sandwich Enzyme Linked Immunosorbent Assay (DAS-ELISA)

DAS ELISA plates specific for *Ralstonia solanacearum* detected *Ralstonia solanacearum* from samples collected from rivers Rufiji in Pwani and minor rivers in Wami, Kwa bululu and Vuje in Tanga.

### 2.4.3 Isolation of *Ralstonia solanacearum* from diseased plants and soils

After 48 hours of incubation in 28℃, 21 isolates (Table 2.2) had irregular shaped mucoid colonies with pink centers on the TZC agar plates (Fig. 2.5). The remaining had no pink centers indicating their avirulent status.

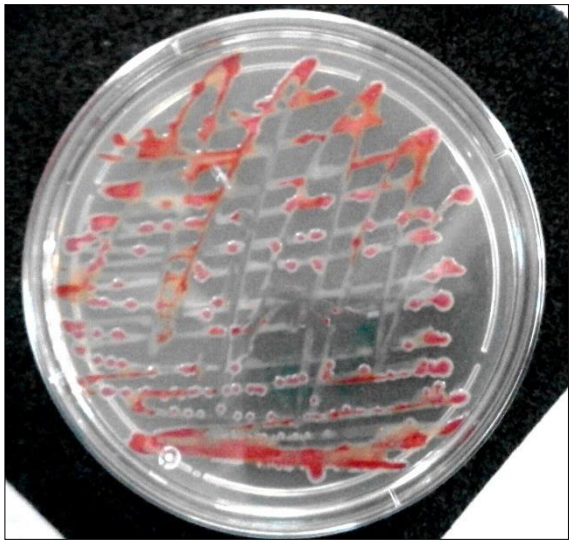

**Figure2. 7: *Ralstonia solanacearum* virulent colonies on TZC media**

### 2.4.4 Biochemical characterization of *R. solanacearum*

When viewed under microscope, all bacteria samples were rod shaped and stained pink. Thus, the isolates were gram negative. The bacteria samples formed slimy threads when mixed with 3% KOH solution. This confirmed the results of the Grams' test.

Variable responses were observed when isolates were further tested for Levan production. Twenty-one isolates produced Levan, which is a main trait for pathogenic *Ralstonia solanacearum* isolates. When the bacteria isolates were mixed with a drop of $H_2O_2$ on a glass slide they all produced gas bubbles indicating release of oxygen. This showed presence of the catalase enzyme. Using Kovacs solution, 21 isolates out of 66 formed a purple ring on the filter paper indicating oxidase activity, produced brown pigmentation on Kings B medium and were non–fluorescent. This indicated their virulent nature hence responsible for the wilting found in the field. They were further classified using the biovar test.

The biovar test was positive for the selected twenty-one isolates for both utilization and oxidation of all the disaccharides and hexose alcohols respectively. This is biovar3 (Table2.4).

**Table 2.1: Biochemical tests results of *Ralstonia solanacearum* isolates collected**

| S. No | Isolate | Region | District | Village | Gram | KOH | Levan | Catalase | Kovac | Pigment | Biovar |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | RsTNG1 | Tanga | Korogwe | Bungu | - | + | + | + | + | + | 3 |
| 2 | RsTNG2 | Tanga | Korogwe | Bungu | - | + | + | + | + | + | 3 |
| 3 | RsTNG3 | Tanga | Korogwe | Bungu | - | + | + | + | + | + | 3 |
| 4 | RsTNG4 | Tanga | Korogwe | Bungu | - | + | + | + | + | + | 3 |
| 5 | RsTNG5 | Tanga | Korogwe | Bungu | - | + | + | + | + | + | 3 |
| 6 | RsTNG6 | Tanga | Korogwe | Bungu | - | + | + | + | + | + | 3 |
| 7 | RsTNG7 | Tanga | Handeni | Pogwe | - | + | + | + | + | + | 3 |
| 8 | RsTNG8 | Tanga | Handeni | Pogwe | - | + | + | + | + | + | 3 |
| 9 | RsTNG9 | Tanga | Handeni | Msaje | - | + | + | + | + | + | 3 |
| 10 | RsMR1 | Morogoro | Morogoro | Misufini | - | + | + | + | + | + | 3 |
| 11 | RsMR2 | Morogoro | Morogoro | Misufini | - | + | + | + | + | + | 3 |
| 12 | RsMR3 | Morogoro | Morogoro | Misufini | - | + | + | + | + | + | 3 |
| 13 | RsMR4 | Morogoro | Morogoro | Misufini | - | + | + | + | + | + | 3 |
| 14 | RsBG1 | Morogoro | Morogoro | Misufini | - | + | + | + | + | + | 3 |
| 15 | RsBG2 | Pwani | Bagamoyo | Chemichemi | - | + | + | + | + | + | 3 |
| 16 | RsBG3 | Pwani | Bagamoyo | Chemichemi | - | + | + | + | + | + | 3 |
| 17 | RsBG4 | Pwani | Bagamoyo | Mtoni | - | + | + | + | + | + | 3 |
| 18 | RsBG5 | Pwani | Bagamoyo | Mtoni | - | + | + | + | + | + | 3 |
| 19 | Rs BG6 | Pwani | Bagamoyo | Mtoni | - | + | + | + | + | + | 3 |
| 20 | RSZN1 | Unguja Ukuu | Kaskazini A | Donge | - | + | + | + | + | + | 3 |
| 21 | RSZNZ4 | Unguja Ukuu | Kaskazini A | Donge | - | + | + | + | + | + | 3 |

## 2.5 Discussion

The establishment of disease is the result of successful interaction between a susceptible host and a virulent pathogen under a conducive environment (Jeger, 2009). Observation of symptoms should go hand in hand in establishing virulence of suspect pathogen upon isolation. Variation within a pathogen population can determine compatibility in a host-pathogen interaction. Biovar 3 strains were found in tomato fields along the coastal regions of Tanzania. This is in conjunction with the results of Burney (1995) on strains of *R. solanacearum* affecting tomato and pepper. Begum (2005) found that both biovar 2 and 3 can infect tomato but biovar 3 are the most aggressive ones while biovar 2 strains mainly infect potatoes.

The main environmental factors affecting soil borne pathogens like *Ralstonia solanacearum* are temperature and soil moisture (Hayward, 1991). Most strains of *R. solanacearum* are pathogenic at 25-30℃ (Ghini *et al*., 2007), a temperature range corresponding to that of Tanga, Morogoro, Zanzibar and Pwani in the month of October (TMA, 2013). Some strains of *R. solanacearum* are known to cause disease in tomato at lower temperatures but most strains are non-pathogenic below 20℃. This is supported by proteomic studies which showed the effect of temperature on the expression of hrp B and hrp G genes, key determinants of pathogenicity in *Ralstonia solanacearum* (Bocsanczy*et al*., 2014). The failure to isolate the bacteria from soil in Mtwara and Lindi also doesn't conclusively advocate the absence of the pathogen. *Ralstonia solanacearum* enters into a viable but non culturable (VBNC) state when subjected to unfavorable conditions, including low temperatures. The VBNC state was attributed to its ability to survive for a long time and resuscitate in soil (Kong *et al.*, 2014). Also, the rate of pathogen movement in the stem depends on soil moisture and temperature (Kelman, 1953).

During tomato infection by *R. solanacearum*, wounds sustained by the plant from formation of lateral roots are used as entry points for the pathogen. The pathogen attaches at two precise root sites that is the root elongation zones and axils of emerging or developed lateral roots. Tomato lateral roots development is highest at the temperature range of 25-30°C (Shoaib *et al*., 2012) therefore significantly increasing pathogen entry points.

The distribution and incidence of bacterial wilt was in favor of locations with ferralsols, which are soils of the humid tropics. These are deeply weathered yellow or red soils with great depth, low pH, good permeability and stable microstructure. They have poor chemical fertility with a residual concentration of resistant primary minerals such as quartz along with sesquioxides and kaolinites (FAO, 2014). These conditions favor the pathogens' multiplication, especially low pH (Ramesh and Bandyopadhyay, 1993). The regions with ferralsols namely Tanga, Pwani and Morogoro were found to have bacterial wilt as opposed to Mtwara and Lindi which have cambisols and vertisols. Nevertheless, the main reason for absence of bacterial wilt incidence in Mtwara and Lindi remains to be the low temperature in spite of the soil type being less favorable to the pathogen as well. Smallholder tomato farmers in Bungu (Tanga) and Bagamoyo (Pwani) have resorted to crop rotation much to their disappointment. The detection of *R. solanacearum* in river water by DAS ELISA means that farmers have been using *R. solanacearum* infected water for irrigation. *Ralstonia solanacearum* can survive for over four years in a nutrient depleted environmental water microcosm and maintain its pathogenic status (Belena *et al*., 2008) which makes the farmers' one-year rotation program ineffective.

It is thus recommended that farmers make use of safe seedlings lacking latent infection and resistant to *Ralstonia solanacearum*.

Cooling soil temperature by grass mulching can help reduce infection. Weeding is important as some weeds such as *Solanum dulcamara*, *Ipomoea* sp. and *Portulacaoleraceae* are good hosts of *R. solanacearum* and remain asymptomatic (Pradhang *et al.,* 2001). They aid in long term survival of the pathogen in the soil. Use of wells as source of irrigation water instead of rivers would significantly reduce infection. The burning of plant debris would deny the bacteria of a source of food as it enters the saprophytic stage of its life cycle.

Further studies are needed to develop an epidemiological model of tomato bacterial wilt disease. Studies on soil chemical composition and how soil amendment can be done to hamper invasion would be useful. Yadessa *et al*. (2010) proposed amending top soil with coco peat, farmyard manure and compost to control *Ralstonia solanacearum*. Grafting of susceptible varieties onto resistant rootstocks has shown great promise in controlling the pathogen. *Solanum sisymbrifolium*, *S. integrifolium* and *S. torvum* as rootstocks have proved to be resistant to *Ralstonia solanacearum* in Bangladesh. Seedlings successfully grafted onto them gave rise to healthy plants (Rashid and Zaman, 2005). Grafting tomato on *Solanum sisymbrifolium* also increases marketable yield even under disease free conditions (Miller *et al.,* 2005).

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# CHAPTER THREE

## 3.0 Molecular characterization of *Ralstonia solanacearum* strains infecting tomato along the coast of Tanzania: Their pathogenicity and virulence against lines bred for resistance

### 3.1 Abstract

Bacterial wilt disease caused by *Ralstonia solanacearum,* is a serious threat to tomato production in Tanzania. It's highly diverse nature in terms of virulence makes it difficult to manage. The use of resistant breeding lines is an attractive management option, but due to the genetic and phenotypic variability of the bacterium, assessment against local strains is necessary in order to ensure suitability of the resistance. A molecular study of 21 isolates of *Ralstonia solanacearum* was conducted with the aim of determining the phylotype they belong to. They were later inoculated on cultivars bred for resistance whilst using Cal J as a susceptible check. The breeding lines were also evaluated in the field. Molecular confirmation of 21 isolates to be *Ralstonia solanacearum* was performed by a species-specific PCR using primer pair AU759/760. A Phylotype specific multiplex PCR (pmx-PCR) was carried out using Nmult series primers. Four tomato breeding lines from the World Vegetable Center (AVRDC) were evaluated against five of these strains of *R. solanacearum*. Upon amplification with species specific primer AU759/760, isolates produced a 282 bp band after electrophoresis. All the isolates produced a 144bp and 282bp which is consistent with strains belonging to phylotype I. Such strains mainly originate from Asia and have a wide host range. They varied in virulence on the susceptible check as well as the breeding lines, causing varying levels of damage. Disease progression

derived from severity scores showed variations in virulence across the breeding lines were of no specific pattern. AVTO 0301 performed well both in the screen house and field where no wilting symptoms were observed. AVTO 0922 had a 59% less disease progression than the susceptible check. All breeding lines were significantly less affected by the *R. solanacearum* strains from coastal Tanzania than the susceptible check.

### 3.2 Introduction

*Ralstonia solanacearum* is responsible for the devastating bacterial wilt disease in many solanaceae crops and has also been described as a species complex due to its high genotypic and phenotypic variability. DNA-DNA homology analysis showed that *R. solanacearum* isolates have a relatedness of just fewer than 70% of the threshold level expected within a species (Roberts *et al*., 1990). In bacteria, polymorphism can be caused by mutations. It is redistributed by recombination (Peeters *et al*., 2013) and horizontal gene transfer (Coupat-Goutland *et al*., 2011; Fall *et al.*, 2007; Guidot *et al*., 2009). Horizontal gene transfer enhances aggressiveness of *R. solanacearum* on tomato (Coupat-Goutland *et al*., 2011) while multi locus sequence analysis concluded that recombination played a major role in its evolution (Wicker *et al*., 2012). This makes it difficult to control the pathogen. Failure to control the pathogen by traditional methods, such as crop rotation and synthetic pesticides has increased focus towards using resistant breeding lines or resistant rootstocks for grafting onto *Solanum sisymbrifolium* (Miller *et al*., 2005). When breeding for tolerance or resistance to *Ralstonia solanacearum,* one must take note that these two phenomena are strain specific hence it's important to be able to distinguish between different strains (Wang *et al*., 2000). Initially, *R. solanacearum* strains were

distinguished based on host range (race) and their preference in utilization of sugars (biovar). The race system collapsed due to the pathogens' ability to adapt and cause infection on new hosts hence making it taxonomically useless (Meng, 2013). The biovars do not correspond to phylogenetically coherent groups, with the exception of biovar 2A, which corresponds to R3bv2. A subgroup of biovar 2, known as biovar2T is present in strains from Asia and Africa as well as strains in the original "biovar 2" group from South America. This means that the traditional biovar test is not completely reliable for diagnostic purposes (Champoiseau *et al.*, 2009).

Fegan and Prior (2005) introduced a flexible meaningful classification system for *Ralstonia solanacearum*. The system was based on the analysis of the sequences of the endoglucanase (*egI*), hypersensitive response and pathogenesis B (*hrp*B) and the internal transcribed spacer (ITS) genes. These are conserved genomic regions universal for all *R. solanacearum*. In spite of its flexibility, the classification offers stability. The analysis would group *Ralstonia solanacearum* strains into phylogenetically related groups known as phylotypes regardless of their races or biovars.

The ability to distinguish epidemiological and ecological grouping by phylotype analysis helps predict biological properties of unknown strains. This feature is important during onset of planning a disease management program.

The phylotypes correlate with the geographical origin of the population and therefore describe the epidemiological position of a strain and predict its' characteristics. Phylotype I is predominantly found in Asia, phylotype II in America, phylotype III in

Africa and phylotype IV in Japan, Indonesia and Australia. Based on the ITS region, a rapid multiplex polymerase chain reaction (PCR) has been developed to determine the phylotype of a population (Fegan and Prior, 2005). It is important to confirm isolates as those belonging to *Ralstonia solanacearum* by a species-specific PCR before phylotyping.

This is done by using species specific primers AU759/760 to amplify conserved genomic regions of the bacteria (Opina *et al*., 1997). While bacterial wilt is a major threat to tomato production, no information is available on the phylotype infecting tomato in Tanzania. To identify suitable resistant or tolerant materials for the target region, they must be tested with strains whose phylotype is known, hence the purpose of this study. Strains which infect tomatoes are mostly reported as biovar 3, although not exclusively (Cook *et al*., 1994). Wang *et al*. (2000) were able to map a locus on chromosome 12 on the genome of the tomato variety cv. Hawaii 7996 that specifically confers resistance to race 1 biovar 3 strains. Other quantitative trait loci (QTL) have major or minor and broad spectrum effects (Lebeau *et al*., 2011). In order to cause infection, the *R. solanacearum* must be able to utilize the host resources to ensure successful multiplication inside the plant tissue amidst the plant defense mechanisms (Peeters *et al.,* 2013). Thus, *R. solanacearum* has to deploy energy and be metabolically proficient (Plener *et al.*, 2012). Different *R. solanacearum* strains have different levels of aggressiveness and subsequently different breeding lines differ in their levels of resistance (Meng *et al.,* 2013). A cultivar may be resistant against *R.*

*solanacearum* in one geographical location but susceptible elsewhere (Mimura *et al*., 2009). Hence, when screening for resistance, it is important to take into consideration both the virulence of individual strains as well as level of resistance of the breeding lines as two separate factors contributing to the nature of the host-pathogen interaction. In the present study, tomato breeding lines bred for resistance to *R. solanacearum* were inoculated with *R. solanacearum* strains recovered from coastal Tanzania and their reactions were observed.

### 3.3 Materials and methods

#### 3.3.1 Study locations

Phylotyping and assessment of virulence were conducted at the East African hub of the International Institute of Tropical Agriculture (IITA) in Mikocheni, Dar es Salaam, Tanzania from March to mid-June, 2014. The temperature in the screen-house was 29°C. Re-isolation and molecular detection was carried out in the pathology and molecular labs respectfully at the hub. Field trial was conducted at Bungu (E38°.24326´, S5°.04618´) in Tanga region. The area is a hotspot for the disease from mid-June to September 2014 so that plants get naturally inoculated. The temperature in Bungu fluctuated from 28°C to 30°C during the experiment.

#### 3.3.2 DNA extraction

*Ralstonia solanacearum* DNA purification was carried out according to Grover *et al*., (2012). *Ralstonia solanacearum* was sub-cultured from the TZC agar plates by streaking onto a Petri dish with CPG Agar with the help of a wire loop. After 48 h

white mucoid, fluidal shiny colonies typical of *R. solanacearum* were observed on the plates. Pure colonies were transferred to 500 µl sterile distilled water in a 1.2 ml microfuge tubes to get *R. solanacearum* cells suspensions. The suspensions were boiled for 5 min then vortexed for 2 min, before adding 500 µl of phenol: chloroform:iso-amylalcohol (25:24:1) and then vortexing the tubes again for 2 min. Tubes were centrifuged at 16000 g for 5 min and 200 µl of the supernatant was transferred to a new microfuge tube. To the supernatant 20 µl of ammonium acetate and 400 µl of chilled ethanol were then added. The tubes were centrifuged again at 16000 g for 5 min and the supernatant was discarded. DNA pellets were suspended in 100 µl of 1×TE buffer (1MTrisHCl, 0.1 M EDTA) and the concentration was measured using Nanodrop.

### 3.3.3 *Ralstonia solanacearum* identification by PCR

A species-specific PCR was done according to Opina *et al.* (1997) in order to confirm the isolates as those of *R. solanacearum* by using the AU 759/760 primer pair. Thermo Scientific green master mix was used in this experiment. The master mix was obtained from Inqaba Biotech, East Africa in Nairobi, Kenya. A volume of 0.25 µl of 10 mM AU 759/760 primer pair was added to aliquots of 12.5 µl of the master mix. Upon addition of 1 µl of 20 ng $\mu l^{-1}$ DNA sample, 11.25 µl of PCR water was added to bring the volume of the reaction mixture to 25 µl. This gave the primers a final concentration of 0.1 mM. The PCR conditions were one cycle of 94°C for 3 min., 53°C for 1 min. and 72°C for 1 min. 30 s followed by 30 cycles of 94°C for 15 s, 60°C for 15 s, 72°C for 15 s, and one cycle of 72°C for 5 min. The reaction was held at 4°C.

Agarose gel electrophoresis was carried out by preparing 1.2% Agarose gel in TAE buffer stained with gel red and running the samples along with a 1kb ladder for 40 min. at 120 V. The gel was later viewed under UV light.

### 3.3.4 Determination of phylotype

Phylotyping was conducted by a multiplex PCR (Pmx PCR) profile where the PCR reaction mixture included four forward primers; Nmult21:1F, Nmult21:2F, Nmult23: AF, Nmult22: InF and one reverse primer Nmult22: RR (Table 3.1). Species specific primer pairs AU759/760 were also included in the reaction mixture (Table 3.1). The total volume of the reaction mixture was 25 µl which contained 12.5 µl of Thermoscientific Taq Green Master mix, 6 pmoles of primers Nmult21:1F, Nmult21:2F, Nmult23: AF, Nmult22: InF, 12 pmoles of Nmult22: RR, 4 pmoles of AU759/760 and 1 µl of 20 ngµl$^{-1}$ DNA template. The PCR conditions were one cycle of 96℃ for 5 min., 59℃ for 30 s and 72°C for 30 s followed by 35 cycles of 94℃ for 15 s, 59℃ for 30 s, and 72℃ for 30 s.

The final extension was done at 72℃ for 10 min. and samples were held at 4℃. Electrophoresis of PCR products was carried out in a 1% Agarose gel prepared in a 1×TAE buffer stained with gel red. The samples were run at a voltage of 80V against a 1kb plus DNA ladder for 45 minutes. The gel was later viewed under 350λ of UV light in a gel documentation system.

**Table 3. 1: List of primers used in Phylotype analysis of *Ralstonia solanacearum* strains**

| Primer Name | Primer sequence | Expected band size (bp) | Remark |
|---|---|---|---|
| Nmult: 21:1F | 5´-CGTTGATGAGGCGCGCAATT-3´ | 144 | Phylotype I |
| Nmult: 21:2F | 5´-AAGTTATGGACGGTGGAAGTC-3´ | 372 | Phylotype II |
| Nmult:22: lnF | 5´-ATTGCCAAGACGAGAGAAGTA-3´ | 213 | Phylotype IV |
| Nmult:23:AF | 5´-ATTACGAGAGCAATCGAAAGATT-3´ | 91 | Phylotype III |
| Nmult:22: RR | 5´-TCGCTTGACCCTATAACGAGTA-3´ | | |
| 759R | 5´-GTCGCCGTCAACTCACTTTCC-3´ | | |
| 760F | 5´-GTCGCCGTCAGCAATGCGGAATCG-3´ | 280 | Universal |

### 3.3.5 Assessment of strain virulence and host resistance

Repeated experiments were conducted to evaluate the virulence of *R. solanacearum* strains and resistance of the breeding lines. The trial units were laid out in a randomized complete block design (RCBD) arranged in a split plot manner with five *R. solanacearum* strains (Table 3.2) as the main factor and five tomato varieties and breeding lines plus a susceptible check as the sub-factor (Table 3.3). The trial had four replications.

Inocula from each of four (4) strains were prepared according to Pradhang *et al.* (2000) by streaking *Ralstonia solanacearum* on sterile CPG media (casamino acids 0.5 g, peptone 10 g, glucose 5 g, distilled water 1000 ml, bactoagar 18 g) in 90 mm diameter Petri dishes. The petri dishes were incubated for 48 hours at 28℃. *Ralstonia*

*solanacearum* was then suspended in 500 ml of sterile water. The suspension was set to $5x10^8$ c.f.u/ml by measuring the absorbance at 600 nm and setting the optical density to 0.3 OD using a spectrophotometer. Inoculation was carried out by soil drenching. Pots filled with 80 g of potting mixture with three weeks old tomato seedlings were soaked with 80 ml of *R. solanacearum* inoculum.

In the screen-house, the disease rating was performed according to the scale of Winstead and Kelman (1952). A score of 0 = no symptoms, 1 = one leaf partially wilted, 2 = two or three leaves have wilted, 3 = all except two - three youngest leaves wilted, 4 = all leaves wilted, 5 = fully wilted dead plant. The most aggressive strain was used for screening tomato varieties and lines bred for resistance.

**Table 3. 2: List of strains used for screening of tolerant varieties**

| Strain | Region | Host | Biovar | Phylotype |
|---|---|---|---|---|
| RsMSFS1 | Morogoro | Tomato | 3 | I |
| RsMSFS2 | Morogoro | Tomato | 3 | I |
| RsBGYS3 | Pwani | Tomato | 3 | I |
| RsZNZS4 | Unguja | Tomato | 3 | I |
| RsBNGS5 | Tanga | Tomato | 3 | I |

**Table 3. 3: List of tomato breeding lines and control variety used in the experiment**

| Tomato ID | Source of resistance |
|---|---|
| AVTO 1010 | Hawaii 7996 |
| AVTO 0922 | Hawaii 7996 |
| Cal J (control) | N/A |
| AVTO 1226 | Hawaii 7996 |
| AVTO 0301 | Hawaii 7996 |

*N/A =Not Applicable

### 3.3.6 Re-Isolation and confirmation of *Ralstonia solanacearum*

Five diseased tomato plants of the susceptible check, each representing plants inoculated with one of the *R. solanacearum* strains were uprooted and taken to the laboratory. Stem pieces of 2 cm above the crown were cut and washed with tap water followed by 70% ethanol. After five minutes, they were rinsed with sterile distilled water in a sterile beaker and left to dry in a biosafety level two laminar air flow chamber under aseptic conditions. The stem pieces were dipped in 500 µl sterile water in a microfuge and *R. solanacearum* was allowed to stream. These solutions were streaked onto a TZC media and incubated for 48 h at 28°C.

Five diseased tomato plants from the tolerant breeding lines, each representing plants inoculated with one of the *R. solanacearum* strains were uprooted and taken to the laboratory. Stem pieces of 2 cm above the crown were cut and washed with tap water followed by 70% ethanol. The stem pieces were rinsed with sterile distilled water after 5 minutes, as the minimal streaming required enrichment of the *R. solanacearum*. After the *R. solanacearum* was allowed to stream the microfuge tube was spun at 16000 g for two minutes in a centrifugation machine. The water was discarded and 800 µl of autoclaved CPG broth was added in the tubes and later incubated at 28 °C for 48 h. After 48 h, the solution was streaked on tetrazolium chloride (TZC) agar.

Confirmation of *R. solanacearum* was carried out by using PCR. *Ralstonia solanacearum* DNA purification was carried out according to Grover *et al*. (2012) as detailed in section 3.3.2.

The isolates were confirmed to be *R. solanacearum* by doing a species specific PCR using a species specific primers set AU 759/760 (Opina *et al*., 1997) as described in section 3.3.3.

### 3.3.7 Field trial

The trial was laid out in a RCBD with three replications. A replication had five blocks separated by furrows whereby four blocks each represented a resistant breeding line from the four lines tested and one represented the susceptible check (Table 3.3). There were twenty plants in each block. Plant to plant spacing was 0.3 m and that of row to row was 0.6 m. The furrows were 0.3 m wide. The total trial area was 23.31 $m^2$. The number of infected plants and total number of plants were counted based on visual observation of symptoms.

### 3.3.8 Data analysis

Data collected from the screen-house trials were analyzed by calculating the area under the disease progression curve (AUDPC). This was developed based on the severity scores recorded. The AUDPC was calculated using "R for statistics" package "AGRICOLAE" (Mendiburu, 2014) where low AUDPC indicates higher resistance. The significance of the variation between the means of AUDPC were measured by ANOVA using the software R package "Rcmdr". Means were separated using Fishers' Lowest Significant Difference (LSD) test using the R package.

Field data was analyzed by computing the percentage incidences in Microsoft excel and the results were subjected to one way ANOVA using the R software package "Rcmdr". Mean separation was performed using Fishers' LSD test.

## 3.4 Results

### 3.4.1 DNA extraction and identification of *Ralstonia solanacearum* by PCR

DNA was successfully isolated. The final DNA concentration was 20 ngμl$^{-1}$. A 280 bp band was observed enabling the selection of *Ralstonia solanacearum* amongst the samples for analysis. Twenty one (21) samples were selected from these results for further characterization.

### 3.4.2 Determination of phylotype

A PCR product of 144kb was observed along with the 280 kb product for species identification (Fig. 3.1). This indicates that *Ralstonia solanacearum* strains from coastal Tanzania belong to phylotype I.

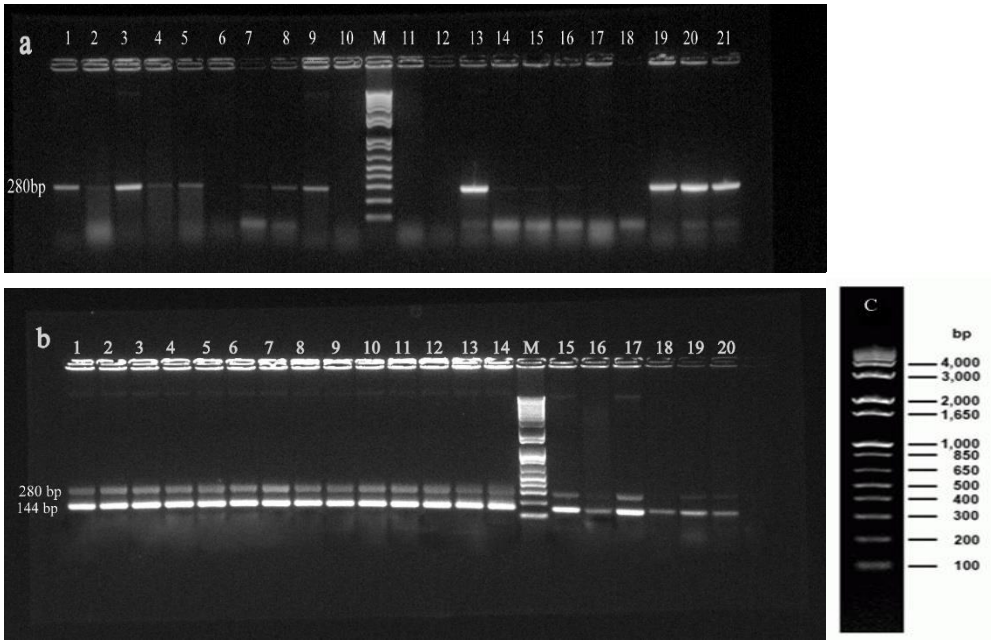


**Figure3. 1: (a) Gel image showing 280 bp PCR product of primer pair AU 759/760 (b) Gel image showing multiplex PCR products of 280 bp and I44 bp indicating species and phylotype (c) 1 kb plus DNA ladder map used in the experiment to determine size of PCR products.**

### 3.4.3 Assessment of strain virulence and host resistance

Table 3.4 shows the results of pathogenicity test. The most aggressive strain was $RsBNGS_5$. The least aggressive strain was $RsZNZS_4$. All plants of the susceptible check wilted. They had disease scores of above 3. Figures 3.2 a-e show disease progression curves of the breeding lines and the susceptible check Cal-J when challenged with $RsBNGS_5$. The disease progression between the breeding lines and the susceptible check varied significantly at the level ($p<0.01$). The F- value was 58 at a degree of freedom of 4. The LSD value computed was 3.2 disease units of AUDPC. In respect to this value, disease progression on resistant breeding lines upon inoculation with strains $RsMSF_{S1}$, $RsMSF_{S2,}$ $RsBNG_{S5}$ and $RsBGY_{S3}$ was significantly lower than the susceptible check (Table 3.5). The AUDPC of the susceptible check was 24.5. The most resistant breeding line was AVTO 1010 which had an AUDPC of 6 (Table 3.6).

**Table 3. 4: Reaction of tomato varieties and lines challenged with *R. solanacearum* strains in the screen house**

| Tomato | Tomato wilting disease score (Scale 0-5) *R. solanacearum* strains | | | | |
|---|---|---|---|---|---|
| | **$RsMSFS_1$** | **$RsMSFS_2$** | **$RsBGYS_3$** | **$RsZNZS_4$** | **$RsBNGS_5$** |
| **AVTO1010 (C1)** | 1.7 | 3 | 2.5 | 1.7 | 2.6 |
| **AVTO0922 (C2)** | 2.2 | 2.3 | 2.2 | 2 | 2.4 |
| **Cal J (C3)** | 5 | 4.7 | 3.2 | 4.6 | 5 |
| **AVTO1226 (C4)** | 2 | 2.1 | 2.4 | 2.7 | 3.8 |
| **AVTO0301 (C5)** | 2.4 | 2.2 | 2.6 | 2.1 | 2.3 |

**Table 3. 5: ANOVA table of AUDPC evaluation in the screen house**

| | Degrees of freedom | F - value | P -value |
|---|---|---|---|
| Breeding line | 4 | 58.14 | 2.20E-16 |
| Population | 4 | 5.7389 | 0.0004384 |
| B. line* Population | 16 | 9.45 | 2.85E-12 |
| Residuals | 75 | | |

**Table 3. 6: Pairwise comparison of means of disease progression on tomato breeding lines and Cal J challenged with RsBNGS5 in the screen house**

| | AUDPC | LSD |
|---|---|---|
| AVTO 1010 | 6 | a |
| AVTO 0301 | 9.5 | a |
| AVTO 0922 | 12.9 | a |
| AVTO1226 | 19 | b |
| Cal J | 24.5 | c |

[a] Values in the same column with different letters are significantly different at 95% level of confidence.

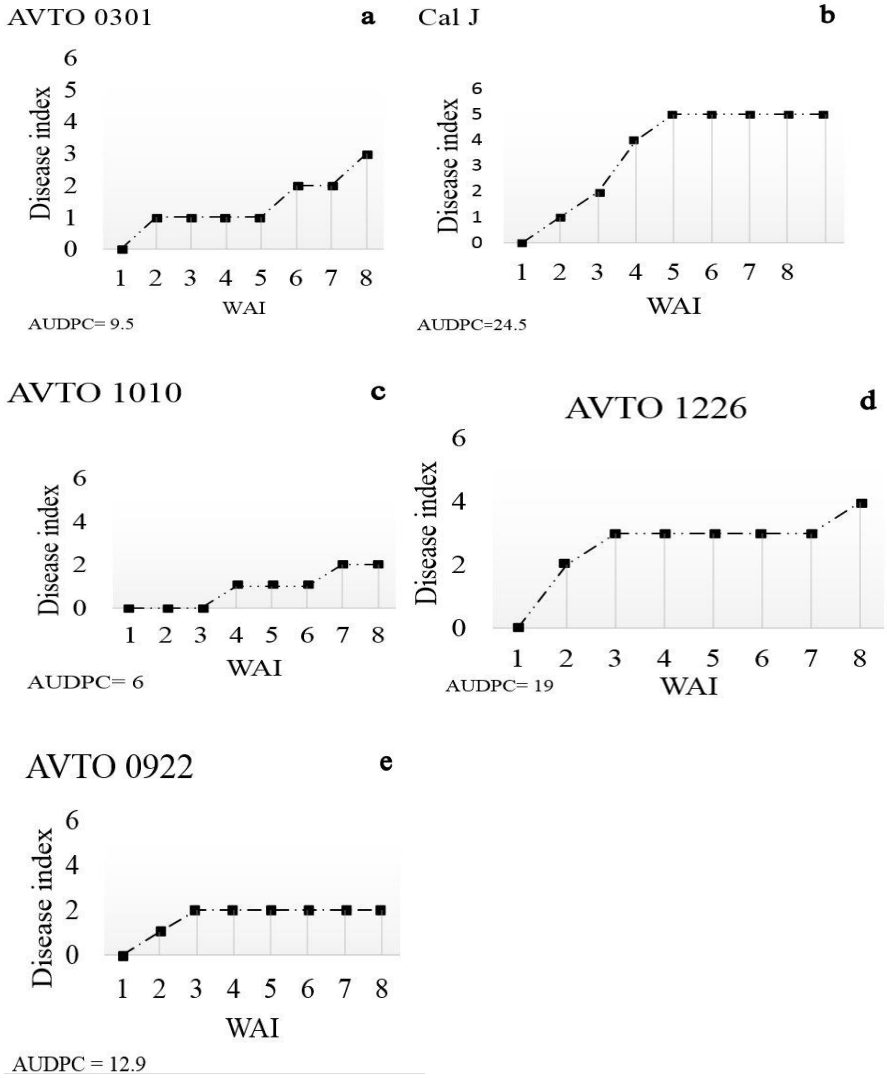


**Figure3. 2: a-e Disease progression curves used to estimate the resistance of tomato breeding lines and the susceptible check Cal J to *Ralstonia solanacearum* strain RsBNGS$_5$**

### 3.4.4 Re-isolation and confirmation of *Ralstonia solanacearum*

Agarose gel electrophoresis of PCR products revealed a 280 bp band. This is the expected base pair size for detection of *R. solanacearum* (Figure 3.3) hence confirming it as the source of wilting during the experiment.

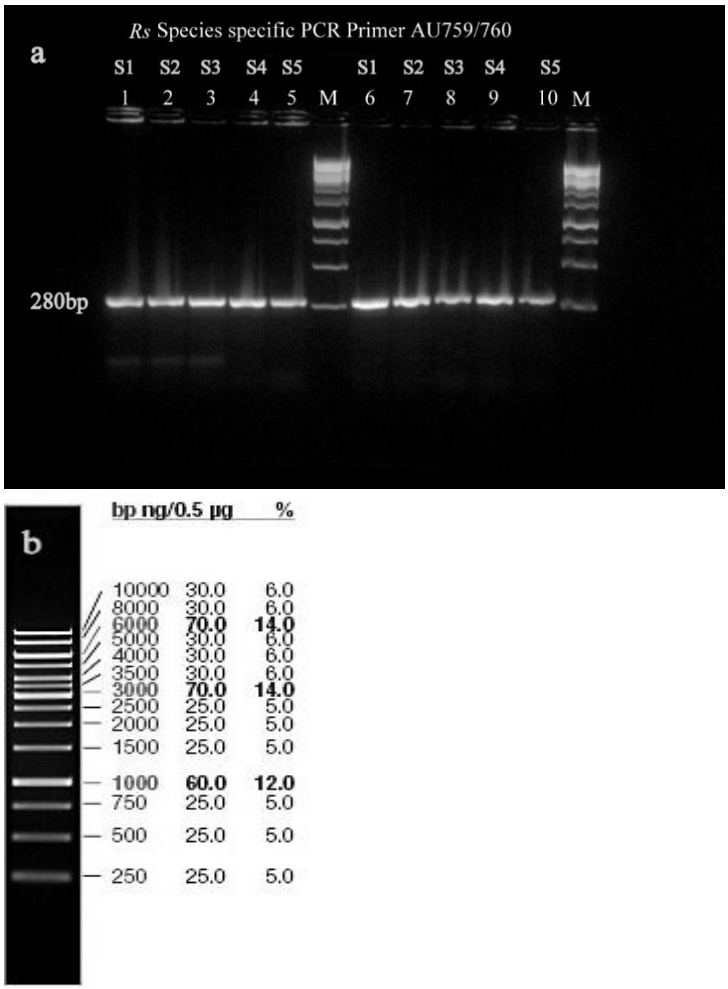


**Figure3. 3: (a) Gel image showing 280 bp band after amplification of *R. solanacearum* DNA with primer AU 759/760 (b) DNA ladder map used to estimate band size of the PCR product.**

### 3.4.5 Assessment of breeding lines in the field

All plants of the susceptible check variety Cal J wilted within three weeks after transplanting. The test breeding lines were found to be resistant to the pathogen (Fig. 3.4). After performing ANOVA, the variation in incidence did not differ across the test breeding lines ($p$>0.05). The F value was 114783.8 at 4 degrees of freedom. The LSD was found to be 32%. Based on this, the difference in incidence between the resistant breeding lines and the susceptible check was highly significant ($p$>0.001).

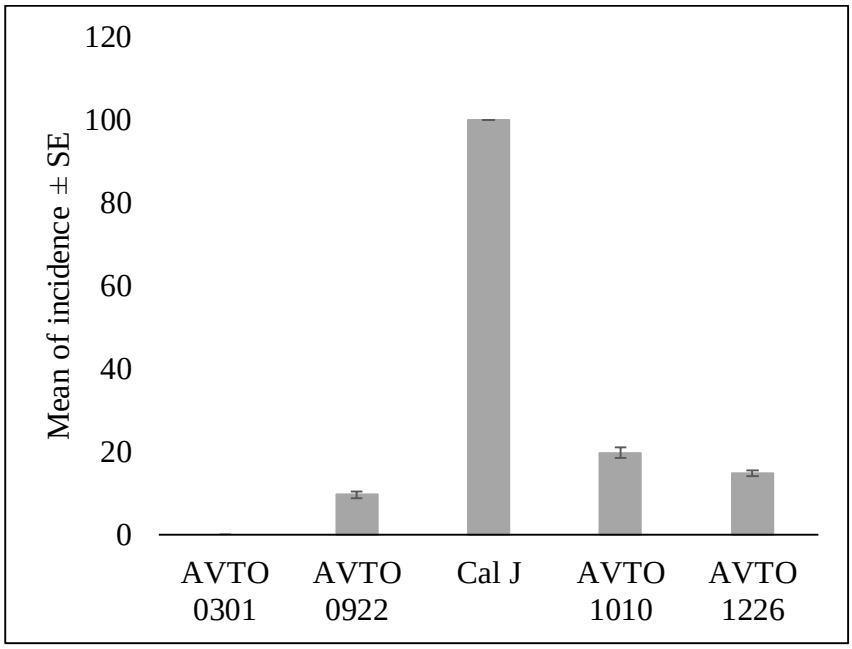


**Figure3. 4: Bacterial wilt disease incidence on tomato variety and lines**

### 3.5 Discussion

Key characteristics of Phylotype I strains of *R. solanacearum* include a wide host range and are mostly found in tropical climates (Cellier and Prior, 2010). Fegan and Prior (2005) found that African strains are mostly clustered in Phylotype III. However, there are reports of presence of Phylotype I strains in the continent, notably in Cameroon and Ghana as reported by Mahbou Somo Toukam *et al.* (2009) and Subedi *et al.* (2014) respectively. This is due to introduction of the strains via latently infected planting materials thus highlighting the importance of improving phytosanitary measures.

Phylotype I strain are originally classified as biovar 3, 4 and 5 strains from race 1 (Cook and Sequeira, 1994). Race 1 strains have a wide host range including chili, potato, tobacco and other non-solanaceae hosts such as beans, groundnuts, eggplant,

sunflower and cowpeas (Belena *et al.*, 2010). This means the threat is not only to tomato farmers and leaves little room for control by crop rotation to be effective. This information is useful for breeders who would make attempts to identify quantitative trait loci (QTL) for resistance to *R. solanacearum* Phylotype I strains. Quantitative trait loci for resistance to *Ralstonia solanacearum* Phylotype II were identified using a population of recombinant inbred lines (RILs) from across of Hawaii 7996 (partially resistant) and local variety of *L. pimpinellifolium* in France (Carmeille *et al*., 2006). In similar fashion, the same can be emulated using RILs from Hawaii 7996 and a local susceptible variety like Cal J or Tengeru 97. Screening and use of a local source of resistance would lead to attaining plants with stable resistance and better adaptability (Laine *et al*., 2011). The local variety Tengeru 97 is partially resistant to *Ralstonia solanacearum* in Tanzania but the information is yet to be scientifically verified and published.

*Ralstonia solanacearum* rapidly proliferates in stems of susceptible tomato hosts (Coutinho, 2005). The concentration of *R. solanacearum* in vascular vessels of susceptible hosts is high in comparison to its concentration in the vessels of resistant plants. In the screen-house trial as opposed to observations made in the susceptible variety Cal J, *R. solanacearum* streaming from resistant breeding lines was minimal. Hence, *R. solanacearum* had to be multiplied by CPG broth for further analysis. This indicates the ability of the resistant breeding lines to suppress proliferation of the pathogen within its vascular system (Grimault and Prior, 1994). The minor variations amongst these resistant breeding lines help to select breeding lines for dissemination

to where they will be most successful. In spite of having the same background of resistance (Hawaii 7996), the variation of host response towards different strains is explained by the polygenic nature of plants resistance to *R. solanacearum* (Thoquet *et al.*, 1996). Genin and Denny (2012) concluded that transferring all quantitative trait loci (QTL) to desirable breeding lines has proven difficult. The variation of QTL's acquired by the breeding lines during their development leads to their variability in response towards different strains.

With a focus on the susceptible check in the current study, the virulence diversity of the strains was established. The variation in strain virulence is attributed to the exopolysaccharide (EPS1) and plant cell wall degrading enzymes (CWDEs). The EPS1 is a major virulence factor. It contributes to the occlusion of the xylem vessels. Strains missing the EPS1 gene are rarely pathogenic (Agrios, 2005). The more the expression of the EPS1 the more virulent the strain. Enzymes β-1, 4- endoglucanase (EgI), endo-poly-galacturonase (PehA or PgIA), exoglucanase (ChbA) and pectin methyl esterase (Pme) have been identified as important CWDEs involved in invasion of host plants. Separately, none of these exoenzymes are essential for infection but their contribution relative to each other varies with strains (Gonzalez *et al*., 2007). In *R. solanacearum*, polymorphism is caused by mutations and is redistributed by recombination (Peeters *et al.,* 2013) and horizontal gene transfer (Coupat-Goutland *et al*., 2011; Fall *et al*., 2007; Guidot *et al*., 2009).

Field trial in Tanga showed that all the resistant breeding lines are able to resist *R. solanacearum.* Moreover, their variation in the ability to do so is insignificant. Future

prospects in controlling *R. solanacearum* include use of biocontrol agents. Biocontrol agents such as antagonistic bacteria or avirulent mutant strains of *R. solanacearum* have potential to compete with the virulent strains for nutrients and space (Saddler, 2005) but this is yet to prove effectiveness in field conditions. A non-pathogenic soil born Oomycete *Pythium oligandrum* is known for colonizing crop root ecosystems and producing antimicrobial compounds. This showed promise for effectively controlling *R. solanacearum* in the future (Akira *et al.,* 2009).

Ideally, the best approach is to use clean planting materials and uncontaminated irrigation water on *R. solanacearum* free soil. Otherwise, the most practical approach available is to use resistant varieties (Wang and Lin, 2005). To breed resistant cultivars, one must effectively maintain the desired agronomic traits of the farmer preferred variety. The cultivar should also be able to cope with the diverse strains which vary in virulence from different agro ecological zones (Chakrabarti, 2011). It is therefore important to undertake trial in multiple locations. It is also important that the cultivars are highly resistant to avoid further *R. solanacearum* dissemination by latently infected tolerant cultivars (Huet, 2014). Another complication is that resistance to bacterial wilt is polygenic and this risks the transfer of a number of undesirable genes (Wang *et al*., 2000; Ben *et al*., 2013). Nevertheless, this remains the best solution to bacterial wilt disease.

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# CHAPTER FOUR

## 4.0 GENERAL CONCLUSIONS AND RECOMMENDATIONS

### 4.1 Conclusions

i. The variation of incidence between sites was significant. This is due to the differences in epidemiological factors and farmers practice. However, the incidence of the disease is highest in Tanga region.

ii. The disease is absent in Lindi and Mtwara due to the low temperature in these regions (< 25℃).

iii. River water used for irrigation in observed diseased farms is infected with *Ralstonia solanacearum*.

iv. The study showed that tomato farms in the coastal zone have phylotype I biovar 3 strains of *Ralstonia solanacearum* except for Lindi and Mtwara where disease is absent. These are historically classified as race I strains which are known to have a wide host range.

v. The tomato lines AVTO 1010, AVTO0922, AVTO1226 and AVTO0301 are resistant to the *R. solanacearum* strains isolated in the study areas, though at different degrees, but of no significant variation.

### 4.2 Recommendations

i. To reduce loss due to bacterial wilt, it is imperative that an integrative approach is employed. Farmers should practice regular weeding to get rid of alternate hosts for the pathogen.

ii. Farmer associations should consider raising income and digging deep irrigation wells and use this source as an “inoculum free” source of water.

iii. Burning of dead plants will help reduce population levels. *Ralstonia solanacearum* feeds on dead plants during the saprophytic phase of its life cycle.

iv. There is a need to conduct more field trials in other sites (Morogoro, Pwani and Zanzibar) using the resistant lines from AVRDC before their multiplication and dissemination to farmers. The use of resistant lines will improve yield.

v. Authorities should improve phytosanitary measures to avoid entrance of latently infected planting materials