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Characterization and management of bacterial wiltcausing pathogen(s) of tomato in Tanzania

Aloyce, Agatha

NM-AIST

CHARACTERIZATION AND MANAGEMENT OF BACTERIAL WILT-CAUSING PATHOGEN(S) OF TOMATO IN TANZANIA

Agatha Aloyce

A Thesis Submitted in Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Life Sciences of the Nelson Mandela African Institution of Science and Technology

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ABSTRACT

Bacterial wilt disease (BWD), caused by *Ralstonia solanacearum* (Smith) is one of the most destructive tomato diseases globally. A research was carried out from 2017 to 2019 to assess the incidence and severity of tomato BWD, characterize the pathogen and evaluate effects of selected pesticidal plants against BWD. Results indicated that 55.17% of the surveyed fields in different districts in Tanzania were significantly ($p \leq 0.05$) infected by BWD with overall disease incidences and severities ranging from 5.8 to 44.6% and 10.70 to 59.30% respectively. Majority (70%) of farmers used synthetic chemicals, 13% used botanical, 10% did crop rotation and 7% did not use any BWD management measure. A total of 29 Ralstonia isolates were isolated from infected tomato plants out of which 19 (52%) were pathogenic on tomato variety Tanya. Carbohydrate oxidation test showed that most (90%) predominating isolates in the main agro-ecological zones belong to biovar 3 while the rest (10%) belong to biovar 2 and prevail in the southern zone of Tanzania. This is the first report of prevalence of biovar 2 of Ralstonia in Tanzania and it alerts global plant health regulators to prevent new introduction and/or spread of Ralstonia. Phylogenetic analysis based sequence data generated from the 16S-23S internal transcribed spacer (ITS) of isolates revealed that most (80%) were phylotype I and III and the rest (20%) were phylotype II of *R. pseudosolanacearum* and *R. solanacearum* geno-species respectively. Such results implied that BWD in the country is caused by diverse Ralstonia populations. In screening the effect of 20 plant extracts, the findings revealed that *R. solanacearum* can be managed using extracts from one of the tested plants (Patent Application ID Number G190916-2061) which was as effective as the positive control (ampiclox) in inhibiting growth of Ralstonia in *in-vitro* conditions. It produced inhibition zone of 19.25 mm almost similar to 20.58 mm by the ampiclox compared with 0.00 mm of the negative control. Similarly, the application of the extracts of the most effective plant managed BWD severity on tomato seedlings grown in screen-house 100% compared with the untreated seedlings which wilted. Therefore the extract from the most effective plant will be recommended after patenting for developing effective and sustainable management strategies of BWD in Tanzania based on the pathogen characteristics as generated during this study.

DECLARATION

I **Agatha Aloyce**, do hereby declare to the Senate of the Nelson Mandela African Institution of Science and Technology that this thesis is my own work and that it has neither been submitted nor being concurrently submitted for degree award in any other institution.

_____ _____

Name and signature of candidate **Date**

The above declaration is confirmed

_____ _____

Name and signature of supervisor 1 **Date**

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Name and signature of supervisor 2 **Date**

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CERTIFICATION

The undersigned certify that they have read the thesis entitled "Characterization and management of bacterial wilt-causing pathogen(s) of tomato in Tanzania" and found it to be acceptable for examination in fulfilment of the Award of Doctor of Philosophy in Life Sciences majoring in Sustainable Agriculture of the Nelson Mandela African Institution of Science and Technology.

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Name and signature of supervisor 1 **Date**

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Name and signature of supervisor 2 **Date**

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DEDICATION

I dedicate my thesis to the Almighty God.

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LIST OF ABBREVIATIONS AND SYMBOLS

ANOVA	Analysis of Variance
ASM	Acibenzolar-S-Methyl
AVRDC	Asian Vegetable Research and Development Centre
BLAST	Basic Local Alignment Searching Tool
bp	base pair
BRELA	Business Registration and Licensing Agency
BWD	Bacterial Wilt Disease
β	Beta
cfu	colony forming unit
CRD	Completely Randomized Block Design
cv	cultivar
dNTP	Dinuclo-triphosphate
DNA	Deoxyribonucleic Acid
DW	Distilled Water
FAO	Food and Agriculture Organization of the United Nations
EDTA	Ethylenediaminetetraacetic Acid
EPPO	European and Mediterranean Plant Protection Organization
EPS	Exopolysaccharide
F	Statistical F- value
Fig.	Figure
HR	Hypersensitive Reaction
IITA	International Institute of Tropical Agriculture
µl	microlitre
µM	micromolar
L	Litre
LSD	Least Significant Difference
MEGA	Molecular Evolutionary Genetics Analysis
min	minute(s)
MS	Mean Score
N	Total number
NCBI	National Centre for Biotechnology Information
NM-AIST	Nelson Mandela African Institution of Science and Technology

ns	non-significant
P	P value (Statistical significance level)
PC	Phenotypic Change
PCR	Polymerase Chain Reaction
RAPD	Random Amplification of Polymorphic DNA
rpm	revolution per minute
RSSC	*Ralstonia solanacearum* Species Complex
s	second(s)
spp.	species
SUA	Sokoine University of Agriculture
TARI	Tanzania Agricultural Research Institute
TBE	Tris Borate -EDTA
TTC	Triphenyl Tetrazolium Chloride
T2SS	Type Two Secretion System
T3SS	Type Three Secretion System
USDA	United States Department of Agriculture
VBNC	Viable but Non Culturable
v/v	volume by volume
w/v	weight by volume
WVC	World Vegetable Centre
∑	Sum of

CHAPTER ONE

INTRODUCTION

1.1 Background of the problem

Tomato (*Lycopersicon esculentum* Mill.) is one of the most important vegetable crops worldwide. It is one of the world's most eaten vegetables as a source of minerals, vitamins, essential amino and organic acids, lycopene, β-carotene and dietary fibres (AVRDC, 2015). The β-carotene and lycopene contained in tomato have anti-cancer and antioxidant characteristics and are regarded as healthy food (Wang *et al*., 2013). Economically, tomato is among the cash crops and source of employment especially for youth in peri-urban areas of developing countries (AVRDC, 2015). Moreover, tomato has become one of the most important agenda in the international horticultural forums due to its nutritive and economic importance (Ayandiji & Adeniyi, 2011). Due to those benefits, cultivation of tomato in terms of acreage, production and consumption has been increasing globally (FAO, 2016).

In Tanzania, tomato ranks the first most important vegetable crop produced for consumption and income generation (Maerere *et al*., 2006; Allen *et al*., 2016). However, contrary to the global production of tomato which is substantially increasing, tomato production in Tanzania is decreasing with the production of 255 000 t/year (FAO, 2016). The average yield of tomato in Tanzania is 17.5 t/ha compared with the global average yield of 33.6 t/ha (FAO, 2016). Factors for low production and yield of tomato include low soil fertility, drought, poor quality inputs, unreliable markets and pests (GoK, 2010; Anang *et al*., 2013) and of these, disease pests caused by pathogens have been cited as among the most limiting (Maerere *et al*., 2006; Sang *et al*., 2016).

Bacterial wilt disease (BWD) caused by complex species of *Ralstonia solanacearum* (RSSC) has been categorized as one of the most significant plant diseases in the world. The BWD is considered a serious plant disease because of the biology of RSSC which composes several genomic-species. For example, based on host range, pathological, biochemical and molecular descriptors, the RSSC can be classified into races (Buddenhagen, 1962; Pegg & Moffet, 1971; He *et al*., 1983), virulent/avirulent and pathogenic/non-pathogenic (Kelman, 1954; Janse & Ruissen, 1988), biovars (Hayward, 1964; Hayward, 1991; Hayward & Hartman, 1994) or phylotypes (Remenant *et al*., 2010; Safni *et al*., 2014; Prior *et al.,* 2016).

The RSSC usually invades plant roots from the soil through injuries or natural openings (Alvarez *et al*., 2010). It invades the root cortex and parenchyma vascular and ultimately reaches the xylem vessels and extends into the stem and buds which subsequently cause the infected plants to wilt and die (Agrios, 2005). Losses due to RSSC are known to be huge but cannot be estimated correctly due to differences in host plant species infected by RSSC, geographical location, pathogen strains, farming environments and soil types. Research shows that about 1.5-2 million ha equivalently estimated to an annual loss of United States Dollars ($) 950 million is caused by BWD in 80 countries (Elphinstone, 2009). This study aimed at characterizing bacterial wilt-causing pathogen(s) and developing sustainable management strategies for tomato growers in Tanzania. The study was important and urgently needed since there was little or no information on Ralstonia species composition and on bactericides that can be used for managing the BWD in Tanzania.

1.2 Statement of the problem

The BWD is a serious problem of tomato and it causes significant economic losses in Tanzania. Tomato yield losses caused by BWD range from 10-100% contingent on cultivar, soils, climate, cropping practices and strain of RSSC (Elphinstone, 2005; Katafiire *et al*., 2005, Radhi *et al*., 2016). It is among the serious tomato production challenges in different areas including the East African region (Aloyce *et al*., 2019). In Uganda for instance, BWD has been associated with a loss of about 88% of tomato (Katafiire *et al*., 2005) but research has not been informed on the current situation. In Tanzania, a total tomato yield loss was observed in one of the screen-houses in Arumeru district for three consecutive growing seasons of 2015/16/17 (Aloyce *et al*., 2019), but actual data about the incidence and severity of BWD of tomato was not quantified.

Famers' level of understanding of BWD management is key for a successful management of the disease in both open field and screen-house environments, however, there exists infrequent information about this knowledge in Tanzania. Unskilled farm workers can be the source of introduction and spread of RSSC in the environments as they participate in various farming practices (Alvarez *et al*., 2010; Shamayeeta & Sujata, 2016; Prior *et al*., 2016; Gaofei *et al*., 2017; Aloyce *et al*., 2017) and under suitable environmental conditions such as temperature and humidity, the RSSC cells multiply and spread quickly (Kinyua *et al*., 2014; Mwaniki *et al*., 2017). Contaminated soil, seedlings, irrigation water, plant debris and weeds

are also associated with the spread of RSSC (Alvarez *et al*., 2008; Heuvelink *et al*., 2008; VonZabeltitz, 2011; Singh *et al*., 2014; Radhi *et al*., 2016).

Several management options of BWD have been proposed varying from the use of physical, biological and chemical approaches (Yuliar & Koki, 2015). Nevertheless, use of such methods has not successfully addressed the BWD in Tanzania (Aloyce *et al*., 2019). The biology of the causative pathogen has been associated with difficulties that exist in managing the BWD (Wang *et al*., 2013; Aloyce *et al*., 2017). For instance, the unusual genetic diversity of the RSSC, its ability of inhabiting the hidden plant parts such as the xylem vessels and surviving in a diverse environment with extremely wide host range (Alvarez *et al*., 2008; Nguyen & Ranamukhaarachchi, 2010). The characteristics of RSSC in the Coastal agro-ecological zone of the country has been reported by Baitani (2017) but no study has been steered to determine species, biovars and phylotypes of RSSC affecting tomato production in the main agro-ecological zones of Tanzania. There is no commercial pesticide apart from chemical fumigants reported for the management of BWD. The use of tolerant/resistant varieties has been the main management strategy but the stability of resistance is highly affected by density, strains of pathogen, temperature and moisture of soil and the presence of root-knot nematode (Wang *et al*., 2013). Management approaches such as soil manipulations, crop rotation and field cleanliness are often not practically feasible due to resource scarcity by small-scale tomato farmers (Alvarez *et al*., 2010; Yuliar & Koki, 2015).

1.3 Rationale of the study

Development of effective and sustainable management strategies should be designed based on the characteristics of the targeted pathogen. Accurate diagnosis of the pathogen to detect its presence in the environment is believed to be the strongest foundation towards its effective management (Kinyua *et al*., 2014). Understanding composition and characteristics of the prevailing Ralstonia species, biovar(s) and phylotypes will help to tailor management measures towards the particular traits of strains prevailing in a specific geographical location, thus designing a pathogen-targeted management strategy.

Extracts from plant species have been reported as a source of agricultural bio-pesticides through the antimicrobial properties of the phytochemical compounds they possess (Yuan *et al*., 2012; Paret *et al*., 2012). Effective plant extracts can be used as manures and/or dried powders (Naz *et al.*, 2015) and/or as liquid extracts (Yesmin *et al*, 2008; Balestra *et al.*, 2009). Plant bio-pesticides are relatively safe, biodegradable, non-phytotoxic thus eco-

friendly, majority are systemic in action, affordable and viable economically and unlikely cause harm or pathogen resistance to pesticides (Tripathi & Dubay, 2004; El-Ariqi *et al*., 2005; Abo-elyousr & Asran, 2009).

Despite the fact that some farmers use crude extracts from certain plant species in the country (Mkenda *et al*., 2015), there is no study that has investigated their effect against Ralstonia in Tanzania. Consequently, the current study was conducted to generate information on status of tomato BWD, characteristics of the causative isolates and explore the anti-bacterial effect of selected plant extracts against the most predominating isolate of Ralstonia in Tanzania to draw implications for plant health regulators and inspectors, pathologists, breeders, extension workers and farmers to manage BWD. Knowledge of farmers about BWD management and management methods used against BWD in Tanzania were also captured to wrench implications for researchers, extension officers and farmers. The information generated is thus valuable in developing effective and sustainable management approaches for BWD to improve tomato production in Tanzania.

1.4 Objectives

1.4.1 General objective

To characterize and manage tomato bacterial wilt disease (BWD) in order to improve tomato production in Tanzania.

1.4.2 Specific objectives

(i) To assess BWD incidence and severity on tomato growing in the main agro-ecological zones of Tanzania.

(ii) To assess knowledge of famers growing tomato on BWD management in the main agro-ecological zones of Tanzania.

(iii) To detect the BWD-causing pathogen(s) from tomato plants collected from the main agro-ecological zones of Tanzania using pathological and molecular approaches.

(iv) To identify Ralstonia biovar(s) associated with BWD from infected tomato samples.

(v) To establish the phylogenetic relationships of the BWD-causing pathogen(s) isolated from infected tomato plants collected from main agro-ecological zones.

(vi) To test the effect of selected pesticidal plant extracts on managing the BWD-causing pathogen(s) under *in-vitro* and *in-vivo* environments

1.5 Research questions

Each specific objective of the study was guided by respective research questions (Table 1).

Table 1: Research questions

S/N	Objective	Questions
i.	To assess BWD incidence and severity on tomato growing in the main agro-ecological zones of Tanzania	• Is BWD present in all the main tomato agro-ecological zones of Tanzania? • How severe is BWD in Tanzania? • At what extent is BWD affecting tomato production in Tanzania? • How much do farmers understand about BWD? • What are the common methods used by farmers to manage BWD?
ii.	To assess knowledge of tomato-growing farmers on BWD management in the main agro-ecological zones of Tanzania	• What is the farmers' level of understanding regarding key principles of BWD management? • Can farmers distinguish BWD from wilting caused by other factors? • What management options are used by farmers against BWD in the study area? • Are the management options used effectively?
iii.	To detect the BWD-causing pathogen(s) from tomato plants collected from the main agro-ecological zones of Tanzania using pathological and molecular approaches	• Do the pathogens of BWD in Tanzania vary in virulence and pathogenesis? • What % is virulent and avirulent? • What does the PCR analysis results of the pathogenic isolates tell?
iv.	To identify Ralstonia biovar(s) associated with BWD from infected tomato samples	• What biovar(s) of RSSC exist in Tanzania? How many biovar (s) of RSSC prevail in different agro-ecological zones?
v.	To establish the phylogenetic relationships of the BWD-causing pathogen(s) isolated from infected tomato plants collected from main agro-ecological zones	• What are similarities or differences among isolates of RSSC in Tanzania? • What and how many groups, sub-groups of RSSC prevail in Tanzania? • How do they relate with the other strains in NCBI • What management implications can be gained from the phylogenetic study?
vi.	To test the effect of selected pesticidal plant extracts on managing the BWD-causing pathogens) under *in-vitro* and *in-vivo* environments	• What plant species can be used to combat BWD of tomato in Tanzania? • How effective are such plant species?

1.6 Significance of the study

The present study was significant because it aimed in determining the pathogen characteristics and the management of BWD of tomato in Tanzania. Tomato is the first most important vegetable crop for improving the nutrition and income of the small-holder farmers in the country, thus the study was of particular interest to the tomato farmers. In addition, the strains of RSSC have a quarantine status in different countries worldwide. Therefore, results of this study will inform the plant health regulators in different countries to implement necessary standard phytosanitary measures in order to prevent the pathogen from being introduced and/or spread to the uninfected areas.

Specifically the study has:

(i) Generated information on the status of tomato BWD and this will assist in planning and implementing management measures to reduce yield losses in Tanzania.

(ii) Documented the diversity of the BWD-causing pathogens as a key step which can guide formulation of a strategy towards the development of effective and sustainable management measures of the disease in Tanzania.

(iii) Determined the effect of potential pesticidal plant extracts against Ralstonia for potential bio-bactericide formulation in Tanzania.

(iv) Earmarked one pesticidal plant extract that can be developed as an antibacterial product for farmers' use to manage BWD of tomato in Tanzania and the application for patent has been made and decision is on progress.

(v) Made production of this thesis possible for Award of a PhD degree.

1.7 Delineanation of the study

The present study was conceptualized and can be described as in Fig. 1.

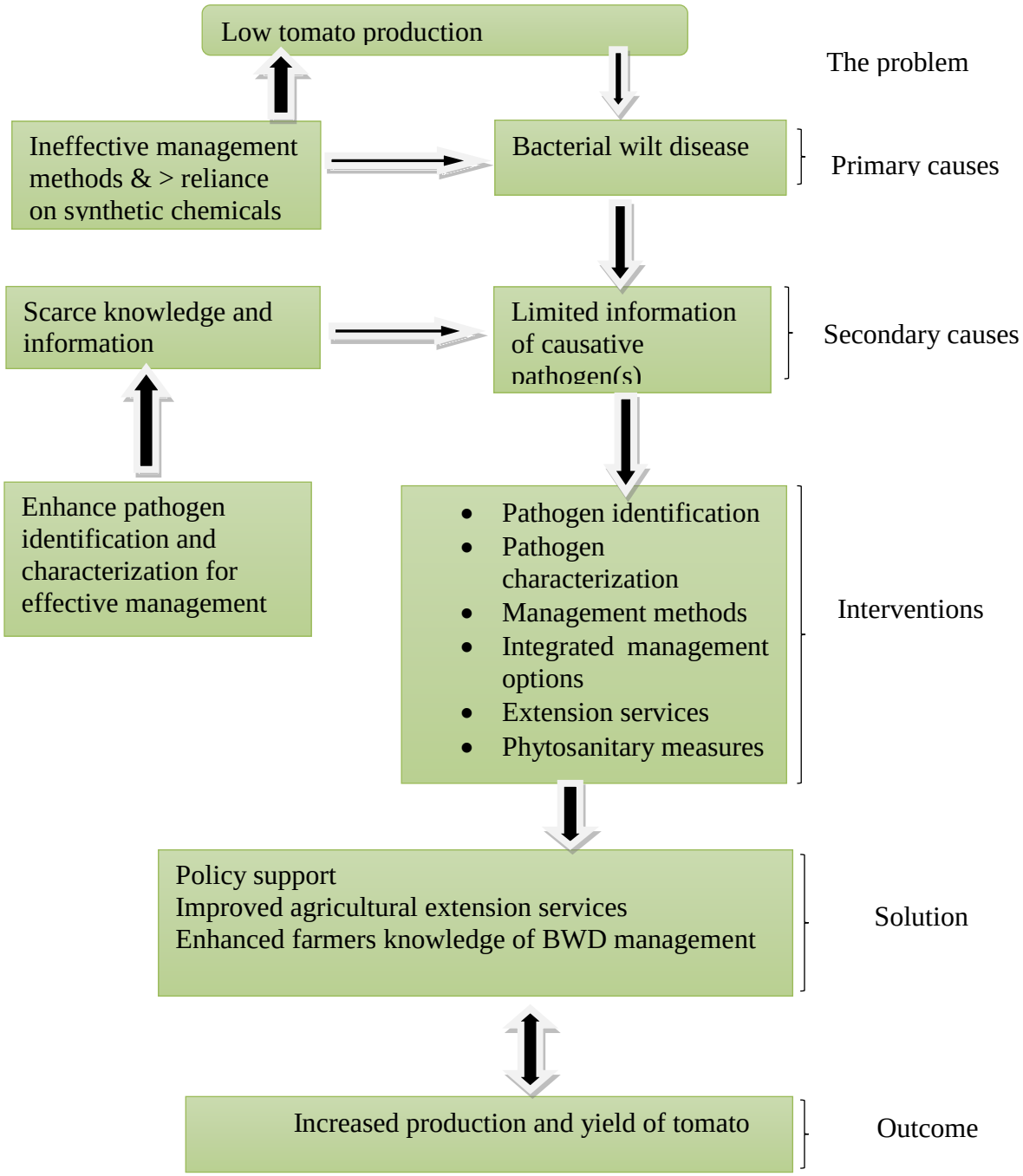

Figure 1: Description of the study

CHAPTER TWO

LITERATURE REVIEW

2.1 Importance of tomato

Tomato (*Lycopersicon esculentum* Mill.) is a subsequent world's most significant vegetable crop next to potato (FAO, 2005). It is one of the most disbursed vegetables as a source of vitamins, minerals and fibres worldwide (Gaofei *et al*., 2017). Tomato contains lycopene and β-carotene that have anti-cancer and antioxidant properties and hence considered as a healthy food and provides much higher income and more jobs per hectare than staple crops especially under small-holder production system (AVRDC, 2015).

2.2 Tomato production constraints

Tomato production is challenged by several factors such as low soil fertility, drought, poor quality inputs, unreliable markets and pests (GoK, 2010; Anang *et al*., 2013). Of these, disease pests have been cited as among the most limiting (Maerere *et al*., 2006; Sang *et al*., 2016) and one of the most challenging diseases of tomato production is BWD.

2.3 Bacterial wilt disease symptoms and its importance in tomato production

The BWD symptoms includes wilting of the youngest leaves which appear during hot (>25^{o}C) daytime temperatures and infected crops can momentarily recover at night when temperatures are cooler (Agrios, 2008). After three to four days, sudden and permanent wilt of roots and lower portion of the stem occurs making the vascular system brown (Yuliar & Koki, 2015). The infected roots can rot because of secondary pathogens such as nematodes, bacteria and fungi. Diseased stems that are expurgated and placed in a container of water show whitish milky streaming (Kinyua *et al.*, 2014). When conditions are less favourable (temperature < 25^{o} C and humidity < 80%) for disease development, infected plants may only indicate signs of stunting and develop adventitious roots on the main stems (Agrios, 2008). The BWD is cited to be one of the most challenging to manage among other factors affecting tomato production from 55.17% to 88% of tomato fields in Sub Saharan Africa (James *et al*., 2010; Kinyua *et al*., 2014; Sang *et al*., 2016; Aloyce *et al*., 2019).

2.4 Bacterial wilt disease status globally

It is usually recognized that BWD is caused by *R. solanacearum*, a species complex consisting of several genomic species (Safni *et al*., 2014; Prior *et al*., 2016). It is a soil-borne bacterium known to have a large group of strains varying in their geographical origin, host range and pathogenic behavior worldwide (Fegan & Prior, 2005). It has a quarantine status in different countries globally and reported as the second most important plant pathogenic bacteria after *Pseudomonas syringae pathovars* because of its economic importance (Mansfied *et al*., 2012), lethality, persistence, wide host and geographic distribution (Denny, 2006; Mansfied *et al.,* 2012).

2.4.1 Diversity of *Ralstonia solanacearum* species complex

There exists a wide genetic diversity of RSSC worldwide and several authors have described the pathogen using different criteria. For instance, Buddenhagen and Kelman (1964) grouped strains of RSSC into five races based on geographical location while Hayward (1964) and Remenant *et al.* (2010) described the pathogen biovars based on their ability to utilize and/or oxidize hexose alcohols and disaccharides. The RSSC has extremely wide host range infecting more than 200 species from over 50 plant families (Prior *et al*., 2016). Recently, RSSC was re-classified into several species and four phylotypes based on DNA analysis (Fegan & Prior, 2005; Safni *et al*., 2014; Prior *et al*., 2016). The races, biovars and phylotypes of RSSC are distributed differently and affect various host plants in the world (Elphinstone, 2009; Yuliar & Koki, 2015).

2.4.2 Virulence and host infection by *Ralstonia solanacearum* species complex

When available in the environment, RSSC can survive within or without the host. Through flagella, it travels to the roots of plant, confers to epidermis, infects the cortex, inhabits the xylem systems and causes plant wilting and death (Alvarez *et al*., 2008). The possession of flagella facilitates its access to the injuries by swimming, chemotaxis and aerotaxis (Yao & Allen, 2007; Alvarez *et al.,* 2010). It has genes characterized in gram-negative bacteria for all six protein secretion pathways (Alvarez *et al*., 2010) and exports extensive of pathogenicity effectors repertoires through type two (T2SS) and three (T3SS) secretion systems (Poueymiro & Genin, 2009). It produces hydrolytic enzymes which degrade the plant cell wall (Hikichi *et al*., 2007) and hence colonization of its host (Alvarez *et al*., 2010). The pathogen has *hrp*

genes which govern the process of both disease development and the hypersensitive reaction (HR) (Boucher *et al*., 2001).

The virulent strains of RSSC produce the exopolysaccharide (EPS) (Poussier *et al*., 2003) that mainly contain acid and high molecular mass of extracellular polysaccharide as their virulence factor (Alvarez *et al.*, 2010). The EPS mainly block xylem vessels and hence interfere with the normal fluid movement of the plant or break the vessels due to hydrostatic excessive pressure (Hikichi *et al*., 2007) thereby causing plant wilting and dying.

2.4.3 Survival of *Ralstonia solanacearum* species complex in the environment

After killing the host, RSSC is released to the environment, where it can survive in soil, water and/or reservoir plants, awaiting another contact with the new plant roots (Alvarez *et al*., 2010). In soil environment, RSSC survives for up to one year even after treatment with herbicides (Van Elsas *et al*., 2005). It can be noticed for up to two years after cultivation and can withstand a four-year period of intercropping (Alvarez *et al*., 2010). Major factors for the survival of RSSC in the soil include suitable soil temperatures and moisture (Kelman, 1953; Hayward, 1991). Survival of the pathogen is enhanced in favourable environment for its development such as in deeper soil layers due to lower temperature fluctuation, lower protozoan or weeding or competition or else the pathogen change and form of various survival mechanisms such as biofilms, viable but non-culturable (VBNC), phenotypic Conversion (PC) type and starvation-survival response to overcome the unfavourable environment (Alvarez *et al*., 2010).

2.5 Identification methods of *Ralstonia solanacearum* species complex

Proper identification of the pathogen is believed to be the strongest foundation for developing disease management strategies (Miller *et al*., 2009; Kinyua *et al*., 2014). Techniques for the detection of RSSC include symptomatology, bacterial streaming, semi-selective medium and immunodiagnostic assay by species-specific antibodies and polymerase chain reaction (PCR) (Elphinstone, 2005; Terblanche & Villiers, 2013; Kinyua *et al*., 2014; Yuliar & Koki, 2015). Worldwide, growth medium particularly triphenyl tetrazolium chloride (TTC) is a commonly used method for the identification and detection of RSSC (Kelman, 1954) due to relative affordability and simplicity of use. In addition, tomato bioassay and pathogenicity tests are recommended by European Plant Protection Organization (EPPO) for detection of Ralstonia (Elphinstone *et al*., 1996). Also, currently the molecular techniques such as PCR are

increasingly being used in commercial laboratories where accurate and timely diagnosis are critical. With the development of specific PCR procedures, viable cells can be detected (Opina *et al*., 1997; Ito *et al*., 1998). Integration of the three approaches (growth medium, bio-assaying and molecular) so that the merits of each method could be utilized is recommended. However, the use of such techniques is challenged by several factors such as costs, lack of technical know-how and low investment in science and technology especially in developing countries (Miller *et al*., 2009).

2.5.1 Identification challenges of bacterial wilt disease of tomato-based on symptoms

It is often challenging to differentiate symptoms of BWD from those caused by other disease-causing factors (Agrios, 2005; Mbega, 2011). Plant wilting can be a result of vascular bundles failing to function, high salinity, saturated soil or infection by bacteria, fungi and/or nematodes (Agrios, 2008). Secondary infections by other pathogens may interfere with those of RSSC (Alvarez *et al*., 2010). There are cases where some BWD infected plants do not show up symptoms (Mbega, 2011; Jonathan *et al*., 2014) and consequently, increasing spread of disease in the farming system. Hence, studies should be conducted to complement symptoms with other plant disease diagnosis methods.

2.5.2 Identification challenges based on the bacterial streaming technique

Bacterial streaming is an initial step to detect RSSC in a plant tissue showing wilting symptoms under the condition of adequate soil moisture. A cut plant tissue exhibits bacterial slime after suspending vascular vessels in clean water (Kinyua *et al*., 2014). The technique is simple and convenient to be performed in the field or laboratory (Singh *et al*., 2014; Shamayeeta & Sujata, 2016). However, it gives a generalized indication for the infection caused by bacteria but cannot be informative on the bacterial species or strain (Alvarez *et al*., 2010). In addition, visibility of bacterial streaming by naked eye depends on the bacterial population in the xylem which should not be low (Kinyua *et al*., 2014). Research is needed to advance this technique in such a way that bacterial species can be detected as they are present even at low population for example ways can be investigated on how visibility of bacterial oozing may be improved by incorporating the colouring agents.

2.5.3 Identification challenges based on species-specific antibodies

This is a commercially developed diagnosis kit for the detection of RSSC in plant tissue and culture in the field or laboratory. Test kit which usually contains Immunostrips, sample extraction bags and user guide requires to be stored at lower temperatures of 2-8^{o}C and

should be tightly stored in the desiccated container at all times. Prior to use, Immonostrips and extraction buffer need to be warmed at temperatures of 18-30^{o}C to make test components ready for use (USDA, 2015). In performing the test, a plant tissue of 0.15 g is taken from a wilting plant and put into an extraction buffer of 3 ml in a sample extraction bag. Presence or absence of RSSC can then be detected from the strips as a positive or negative result. The test is sensitive to the bacterial population from 10^5 cfu/ml. The whole process usually takes about 5-30 minutes depending on pathogen titer in the sample.

The species-specific antibodies technique of RSSC could be one of the quicker and cheaper methods of detecting BWD, however its application faces certain challenges in developing countries. First, Immunostrips are not readily available at the community level and hence expensive, this has limited their application and adoption as a majority of farmers cannot afford (Miller *et al*., 2009). Second, Immunostrips is incapable of detecting bacterial population which is below 10^5cfu/ml and can only detect *R. solanacearum* species complex to the species level. Since *R. solanacearum* has a quarantine status, presence of bacterium even at low population has to be detected for prevention and management measures (EPPO, 2014). Third, the recommended storage temperature range of 2-8^{o}C may not be achievable in tropical and subtropical countries where the average temperature is high. Therefore, Immunostrips technology requires harmonization for the farming community in Sub-Saharan Africa to use effectively and efficiently.

2.5.4 Identification challenges based on carbon source and semi-selective medium

The carbon source utilization method uses disaccharides and hexose alcohols for the determination of biovars of RSSC (USDA, 2015). Disaccharides used are maltose, cellobiose and lactose while hexose alcohols are sorbitol, dulcitol and Mannitol (Kinyua *et al*., 2014). Biovars determination is imperative in the development of management strategies (Jonathan *et al*., 2014). The procedure is mainly performed by experts in specialized laboratories (Miller *et al*., 2009; Kinyua *et al*., 2014).

The semi-selective medium method constitutes isolation of RSSC from plant tissues on a specific diagnostic media. A major challenge of this technique is that it takes time (at least 3-6 days) to carry out and obtain diagnosis results. This may look to be a long period to implement the required management measure as by then the plant will have wilted resulting in huge yield reduction (Janse, 2012).

2.5.5 Identification challenges based on polymerase chain reaction (PCR)

With PCR technique plant, soil or water samples suspected to contain RSSC is subjected to DNA testing for identification purposes (Kinyua *et al*., 2014) and various methods can be used (Denny & Hayward, 2001; Inoue & Nakaho, 2014). The technique is however considered as one of the most complicated and costly pathogen detection (Grover *et al*., 2011) as it depends on bacterium pure culture isolation, DNA extraction and testing which all resources are demanding. This limit technological application as well as adoption to benefit from its use in developing countries. Use of isothermal amplification which is more affordable and appropriate than the PCR-based methods could be exploited in Sub-Saharan Africa. In addition developing biosensors could be a way forward for timely implementation of management measures in Sub-Saharan Africa where techniques such as Immunodiagnostic assays still faces some challenges.

2.6 Management challenges of tomato bacterial wilt disease

Different management strategies are used against BWD of tomato such as the use of chemical, biological, cultural and physical practices (Table 2). Being a complex plant pathogen, Ralstonia can infect crops as soil, water and/or seed-borne pathogen (Alvarez *et al*., 2010; Nguyen & Ranamukhaarachchi, 2010; Wang *et al*., 2013). The pathogen is able to form genetically different strains and survive in an extremely diverse environment traveling along waterways (Alvarez *et al*., 2008).

Table 2: Bacterial wilt disease management approaches and mechanisms

Method	Examples	Mechanisms used
Chemical	**Algicide** e.g. 3-3-indolyl butanoic acid, **Fumigants** e.g. metamsodium, 1,3-dichloropropene, Chloropicrin, Validoxylamine, Validamycin, Acibenzolar-S-methyl(ASM), Thymol, Silicon, chitosan and Sodium chloride and **Bactericides** e.g. Triazolothiadiazine, streptomycin sulfate, bleaching powdersor weak acidic electrolyzed water and phosphoric acid solution.	Induce systemic resistant, increase the amount of soil microorganisms and soil enzyme activity or increase seedling vigor and tolerance to *R. solanacearum* and act as sterilizers, antibacterial and bacteriostatic.
Biological	**Bacteria:** Avirulent species of *R. solanacearum*, *Sterptomyce*s spp., *Acinetobacte*r sp., *Burkholderia nodosa*, *B. sacchari*, *B. tericola*, *B. pyrrocinia*, *Bacillus thruringiensis*, *B. cereus*, *B. amyloliquefaciens*, *Chryseobacterium* daecheongense, C. indologenes, Chryseomonasluteola, Clostridium sp., Delfia acidovorans, Anterobacter spp., Flavo *bacterium johnsoniae*, *Myroides odoratimimus*, *Paeni bacillusmarcerans*, *P. polymxa*, *Pseudomonas* spp., *Brassica cearum*, *R. picketii*, *Serrati* asp., *Sphingom onaspaucimobilis*, *Staphylococcus auricularis*, *Stenotrophomonas*, *maltophilia*, *Streptomyces rochei*, S. *virginiae* and *Xenorhabdusne matophila.*	Competition for nutrients and space, antibiosis, parasitism, induced systemic resistance and root colonization.
	Fungi: *Glomusversiforme*, *Pythiumoligandrum*, Shiitake mycelia, *Gigaspora margarita, Glomusmosseae, and Scutellospora* sp. *and Parmotrematinctorum*.	Increase in the contents of soluble phenols and cell-wall bound phenols in the root tissue, cell wall proteins which induce resistance and antibiotic ingredient.
	Plant products: Chili (*Capsicum annum*), Chinese gall (*Rhuschinensis*), clove (*Szygyumaromaticum*), cole (*Brassica* sp.), eggplant (*Solanum melongena*), eucalyptus (*Eucalyptusglobules*), geranium (*Geranium carolinianum*), guava (*Psidium guajava* and *P .quineense*), hinoki (*Chamaecyparis obtusa*), Japanesecedar (*Cryptomeria japonica*), lemongrass (*Cimbopogon citratus*), marigold (*Tagetes patula*), neem (*Azadirachta indica*), palmarosa (*Cimbopogon martint*), pigeon pea (*Cajanuscajan*), sunhemp (*Crotalaria juncea*), tamarillo (*Cyphomandrabetacea*), thyme (*Thymus* spp.), woodwax tree (*Toxicodendronxylvestre*), and worm killer (*Aristolochiabracteata*).	Antimicrobial activities, and the indirect suppression of the pathogen through improved physical, chemical, and biological soil properties.
	Animal wastes: pig slurry, poultry and farmyard manure	Shifts in bacterial community profiles and higher microbial activity and numbers of cultural bacteria and fungi.
	Simple organic compounds: amino acids, sugars and organic acids e.g. lysine, riboflavin, aminobutyric acid and methyl gallate.	Shifts in the soil microbial community and induction of resistance and bactericidal effects.
Cultural	Resistant cultivar, crop rotation, multi-cropping, soil amendment and grafting.	Limited pathogen movement in xylem, reduced disease inoculum, induced uptake and distribution of nutrients.
Physical	Solarization, hot water treatment, and biological soil disinfection.	Killing *R. solanacearum* with high or low temperatures.

2.6.1 Management challenges due to the diversity *R. solanacearum* species complex

Despite the availability of several BWD management methods, this disease has not been successfully managed in Sub-Saharan Africa countries (Aloyce *et al*., 2019). Breeding resistant cultivars for example has been popularly promoted as one of the best strategies (Yang *et al*., 2016) but the success of breeding resistant cultivars is hampered by the RSSC genetic diversity (Fegan & Prior, 2005; Denny, 2006; Wang *et al*., 2013; Yuliar & Koki, 2015) [Table 3].

Classification of RSSC based on race or host often overlap due to a wide range of strains, environments and host range, therefore, isolate biovars have been used to determine pathogen phylotype (Fegan & Prior, 2005; Kinyua *et al*., 2014). Phylotyping which is based on DNA sequence analysis divides strains of RSSC in 4 phylotypes according to their geographical origin namely phylotype I, II, III and IV correspond to strains from Asia, Americas, Africa, and Indonesia respectively (Agrios, 2008). Recent research suggests grouping RSSC into 3 species: *R. solanacearum* (phylotype II), *R. pseudosolanacearum* (phylotype I and III), and *R. syzgii* (phylotype IV) [Denny *et al*., 1994]. It is thus evident that the environment in which RSSC is found can determine the predominating isolate, biovar or its virulence. The ability of *R. solanacearum* to change genetically and form new strains over time challenges management approach(es) but the information on the prevailing or emergence of new strains of RSSC is limited in Sub-Saharan Africa thus calls for research in order to improve BWD management.

Table 3: Race, biovars, distribution and host plants of *R. solanacearum* species complex

Race	Biovar	Distribution	Examples of common host plants	
			Common name	**Scientific name**
1	1,3,4	Asia, Australia, and America	Tomato	*Solanum lycopersicum*
			Groundnut	*Archishy pogaea*
			Peper	*Capsicum* spp.
			Coleus	*Plectranthus scutellarioides*
			Banana	*Musa* spp.
			Tobaco	*Nicotiana tabacum*
			Roses	*Rosa spp.*
			Egg plant	*Solanum melongena*
			Potato	*Solanum tuberosum*
			Sunflower	*Helianthus annuus*
			Anthrium	*Anthurium* spp.
			Dahlia	*Dahlia* spp.
			Heliconia	*Heliconia*,spp.
			Hibiscus	*Hibiscus* spp.
			Lilium	*Lilium* spp.
			Pothos	*Pothos* spp.
			Strelitzia	*Strelitzia* spp.
			Verbena	*Verbena* spp.
			Zinnia	*Zinnia* spp.
			Marigold	*Tagetes* spp.
			Eucalyptus	*Eucalyptus* spp.
			Neem	*Azadirachta indica*
			Cowpea	*Vigna unguiculata*
			Cucurbits	*Curcurbita* spp.
			Hyacinth beans	*Lablab purpureus*
			Jute mallow	*Corchorusolitorius*
			Moringa	*Moringa oleifera*
			Mulberry	*Morus spp.*
			Nutmeg	*Myristica fragrans*
			Patchouli	*Pogostemon cablin*
			Sesame	*Sesamum indicum*
			Strawberry	*Fragaria ananassa*
			Water spinach	*Ipomoea aquatic*
2	1	Caribbean, Brazil and Philippines	Taro	*Colocasia esculenta*
			Pumpkin	*Cucurbita máxima*
			Goosegrass	*Eleusine indica*
			Cocoa	*Gliricidia sepium*
			Banana	*Musa* spp.
			Guava	*Psidium guajava*
			Heliconia	*Heliconia* spp.
3	2	Worldwide except US and Canada	Tomato	*Solanum lycopersicum*
			Pepper	*Capsicum* spp.
			Garden cosmos	*Cosmos bipinnatus*
			Tree tomato	*Cyphomandra betacea*
			Jimsonweed	*Datura stramonium*
			Bittersweet nightshade	*Portulaca gleracea*
			Egg plant	*Solanum dulcamara*
			Black nightshade	*Solanum melongena*
			Potato	*Solanum tuberosum*
			Stinging nettle.	*Urtica dioica*
4	3,4	Asia	Ginger	*Zingiber officinales*
			Mioga	*Zingibe rmioga*
			Patumma	*Curcuma alismatifolia*
5	5	Asia	Mulberry	*Morus spp.*

Denny *et al*. (1994); Elphinstone (2009); EPPO (2014)

2.6.2 Management challenges due to the persistence of broad host range

The RSSC infects different host plants that are common in tomato farming systems and the host plants overlap as well (Fegan & Prior, 2005; Singh *et al*., 2014). The management of pathogen which is host to several and commonly cultivated plant species is challenging in the farming system. The use of crop rotation for example is challenged by the long period that RSSC can strive in the soil (Alvarez *et al*., 2010). Active crop rotation to manage RSSC in infected land requires abandoning of land to grow host plants for 2-5 years (Yao & Allen, 2007). This is in practice infeasible for the most of small-holder farmers in Sub-Saharan Africa due to land scarcity concerns. Crop rotation can be more challenging to growers who have ventured in protected vegetable cropping where tomato is grown in screen-house structures with intense investment (Nguyen & Ranamukhaarachchi, 2010). Once the screen-house soil is infected by RSSC, eradication is challenging and a grower suffers from crop losses which range from 10-100% depending on farming systems and pathogen strains (Yuliar & Koki, 2015). The mechanism of RSSC to concur wide range of host plant species is not well known, therefore study should be conducted to improve understanding for the improved disease management.

2.6.3 Management challenges due to endophytic nature of *R. solanacearum* species complex

The RSSC enters plants via wounds, root tips or cracks at the sites of lateral root emergence (Denny, 2006; Kinyua *et al*., 2014; Genin, 2010). Unlike many phytopathogenic bacteria, RSSC potentially requires only one entry site to establish a systemic infection that results in BWD (Hiroki *et al*., 2016). The bacterium subsequently colonizes the root cortex, invades the xylem vessels and reaches the stem and leaves through the vascular system (Alvarez *et al*., 2010). It can then rapidly multiply in the xylem causing rapid irreversible plant wilting and death (Kinyua *et al*., 2014; Williams *et al*., 2014). Within xylem for example, high densities of the pathogen increase expression of pathogenicity genes such as the hrp genes which control the induction of disease and hypersensitive reaction (Lin *et al*., 2010) and the endophytic nature of inhabiting within the hidden plant tissues challenges management options. Chemical control for instance, apart from being potentially harmful to the environment, has been reported to be inefficient (Van Elsas *et al*., 2005). This can be explained by the fact that the bacterium is sheltered in xylem vessels. Ways should be explored by targeting management strategies which can be applied via the xylem system.

2.6.4 Management challenges due to pathogen ability to survive without a host

Association of RSSC with either reservoir plants or plant debris has been frequently suggested to promote its survival in soil and water (Yao & Allen, 2007). The RSSC has the ability to persist in toxic environments. The cells of RSSC are capable of changing to various survival forms in unfavourable environments and the most frequently reported are as discussed hereunder.

(i) Viable but non-culturable (VBNC) form

The RSSC in the soil can change and became VBNC within a month after exposure to low temperature of 4°C with cold-exposed cells gradually dropping wilting ability (Hong *et al*., 2005; Alvarez *et al*., 2010). The VBNC state has also been reported to occur in infected plant where the proportion of cells becoming VBNC increase after the plant's extensive necrosis (Grey & Steck, 2001).

(ii) The starvation-survival response

This is a physiological survival state in energy-deficient condition, in which bacterial cells starve to maintain a non-growing but culturable condition (Heim *et al*., 2002; Alvarez *et al*., 2010). Starved cells of RSSC remain pathogenic in the water microcosms for > 4 years (Yao & Allen, 2007).

(iii) Phenotypic conversion (PC) type

This is a form that describes a morphological change of the RSSC colonies from fluidal to afluidal form (Brumbley & Denny, 1990). The phenotypic conversion type which occurs in most strains of RSSC can be easily observed by prolonged culture on agar plates and when the bacterium is grown in a non-aerated liquid medium with glucose and organic source of nitrogen (Popoola *et al*., 2005). The phenotypic conversion type variants have a selective advantage over the non PC-type. For example PC-types have higher motility for aerotaxis in oxidative stress environment (Alvarez *et al*., 2010).

(iv) Biofilms forms

Some cells of RSSC form biofilms on host xylem vessel walls to protect them from host defenses. Biofilms also filter nutrients from the flow of xylem fluid (Inoue & Nakaho, 2014). Different strains of RSSC form biofilms on polyvinyl chloride (PVC) wells at the liquid-air interface and on the surface of tomato seedlings (Kang *et al*., 2002). Aerotaxis deficient

mutants overproduce biofilms on abiotic surfaces which lead cells to avoid toxic oxygen levels at the liquid-air interface by forming protective thicker biofilms to facilitate survival (Alvarez *et al*., 2010; Inoue & Nakaho, 2014).

The ability of RSSC to form different survival strategies to live and cope with unsuitable environmental conditions may raise new concerns about the epidemiology of BWD in tomato farming systems. Although these infecting populations are not as high as those from wilted plants, the continuous formation of these forms would contribute to the RSSC persistence in the environment. Knowledge on the ability of RSSC to transform differently with environmental conditions may have certain vital implication towards its management. For instance, when an environmental conditions e.g. soil temperature is made unsuitable RSSC become avirulent (Alvarez *et al*., 2010), meaning that cannot cause infection to plants. Further research is required to investigate the potential of this knowledge to manage BWD.

2.6.5 Management challenges due to pathogen ability to travel along waterways

The RSSC can enter the surrounding soil, water or plants and spread to uninfected environment through the moving water (Coupat *et al*., 2008). Plants which grow along waterways are mostly reported to facilitate RSSC movement in waterways. The typical examples include bittersweet nightshade, black nightshade and stinging nettle (Hayward, 1991). Roots and stems of bittersweet nightshade for example can shelter cells of RSSC and continuously release them into the water system (Elphinstone, 2009). The use of contaminated water for field irrigation has been associated to most outbreaks of BWD (Hong *et al*., 2005; Caruso *et al*., 2005; Yao & Allen, 2007). Irrigation water could be treated prior to crop irrigation, but there are still some challenges associated with this approach such as exposure of the community to the health risks of chemicals, costly and contamination of water system. Use of management methods which are environmentally friendly like the use of plant extract could be the best approach to combat BWD in the farming system (Mbega *et al.,* 2012).

The present literature has highlighted the importance of tomato for enhancing nutrition and income and challenges associated with the identification and management of BWD in Sub-Saharan Africa. The literature has exposed the reality that RSSC is indeed challenging and thus urgent need to find ways for simple and quick identification options and sustainable disease management methods including the use of pesticidal plants.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Assessment of bacterial wilt disease incidence and severity on tomato growing in the main agro-ecological zones of Tanzania

3.1.1 Survey and sampling

A field survey was conducted in the main agro-ecological zones of Tanzania to assess the incidence and severity of BWD of tomato in September 2017 to February 2018. Ten districts namely Arumeru, Babati, Manyoni, Nyamagana, Kilolo, Temeke, Chake Chake, Mbeya Urban, Kibondo, and Kongwa covering the major agro-ecological zones of Tanzania were visited (Fig. 2) and selection of the study areas based on the tomato production history by targeting the tomato production areas. A multistage random sampling procedure was used which involved selection of the wards within the District, then the villages and the fields. Four wards were selected at random in a district and five fields were identified from each village. Fields were surveyed when plants were at flowering stage and within the field; five (four at each corner and one at the centre of the field) plots of 50 m^2 were measured as quadrant and assessed by critically observing symptoms of BWD. The bacterial streaming test was carried out in order to distinguish BWD from other wilting (Agrios, 2005; Kinyua *et al.*, 2014).

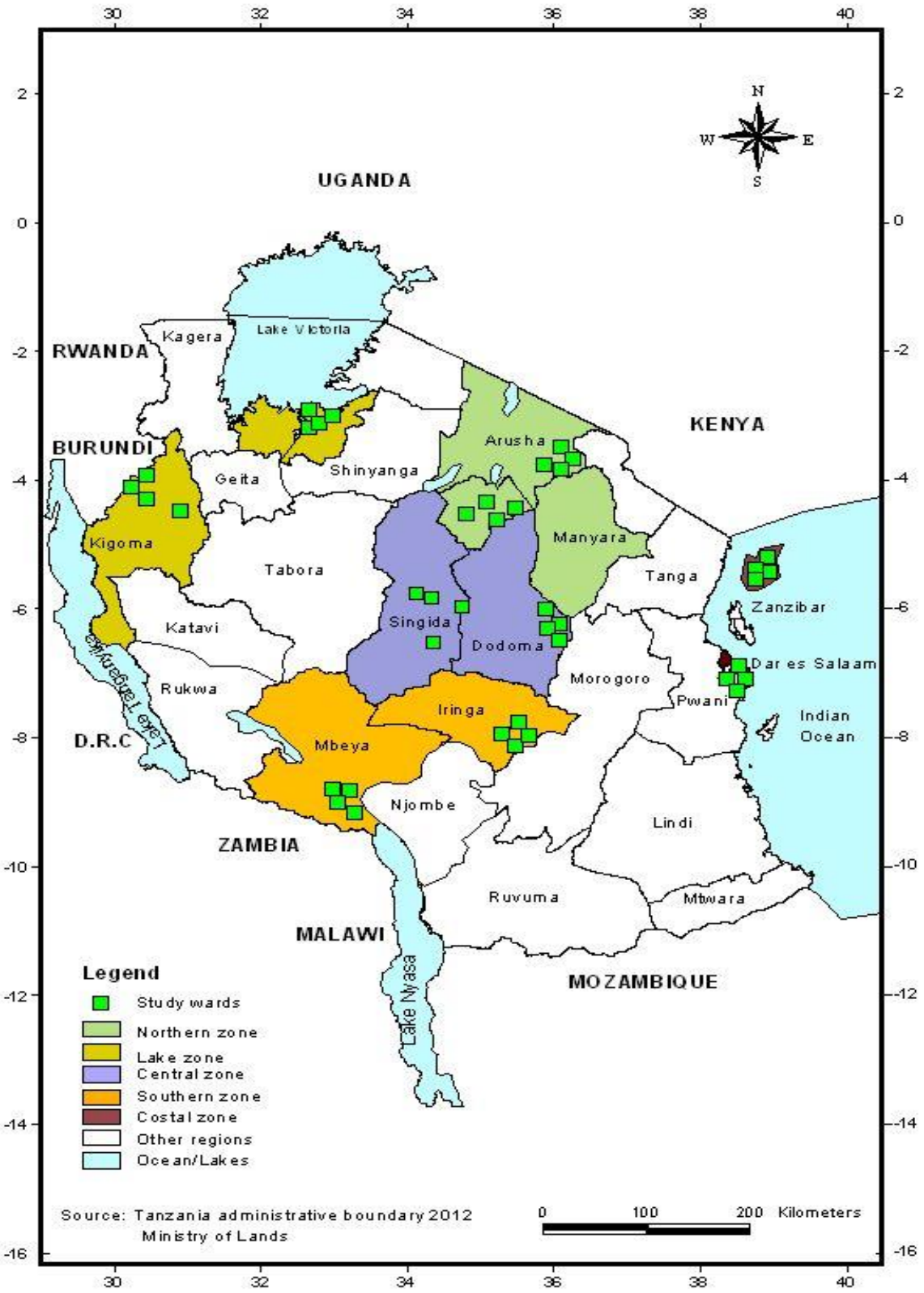

Figure 2: Main agro-ecological zones visited during bacterial wilt disease survey in Tanzania

3.1.2 Assessment of bacterial wilt disease incidence and severity

Data on wilt incidence were recorded by counting a number of plants with BWD symptoms in a plot and percentage was calculated by the following formula:

$$\text{Wilt incidence (\%)} = \frac{\text{Mean of wilted plants in a field}}{\sum \text{plants assesed in a field}} \text{ x } 100 \quad \text{..... (1)}$$

Disease severity was recorded based on a one to five scale (Hyakumachi *et al.*, 2013; Sang *et al.*, 2016). Briefly, one = No symptom; two = Top young leaves wilted; three = Two leaves wilted; four = Four or more leaves wilted and five = Plant died.

Average disease severity per district was calculated using a formula

$$\text{Disease severity (\%)} = \frac{5A+4B+3C+2D+E}{5N} \text{ x } 100 \quad \text{..... (2)}$$

Where: A = number of plants on scale 5; B = number of plants on scale 4; C = number of plants on scale 3; D = number of plants on scale 2; E = number of plants on scale 1; N = total number of plants evaluated. Twenty samples of tomato stem with characteristic of bacterial wilt symptoms were sampled by cutting the stem at 5 cm above the soil and a total of 200 out of 4,800 plant samples assessed for BWD from each of the ten districts were collected and sent in a cool box to the NM-AIST laboratory where samples were kept in a refrigerator at 4°C before the isolation and characterization of RSSC. Temperature and relative humidity in the study areas were measured and recorded during the research.

Data of disease incidence and severity were pooled together by calculating the average of disease incidence and severity of each ward within district, this resulted into 40 samples which were subjected to the analysis of variance (ANOVA). Mean separation was carried based on different locations by using the Fisher Least Significant Difference (LSD) procedure ($p = 0.05$). The CoStat software program facilitated analyses (Gomez *et al.*, 1984; Ver, CoStat, 2005).

3.2 Assessment of knowledge of tomato-growing farmers on BWD management

Discussion to capture farmers' knowledge on principles of BWD management was carried out with 220 farmers by using a semi-structured questionnaire with both closed and open-ended questions (Appendix 1). To cross-check information collected from farmers and ensure that responses reflect farmers' knowledge and experience, 20 development agents (10 district extension and 10 research officers) were selected as key informants and interviewed by using the same semi-structured questionnaire. These development agents work closely with farmers

and are familiar with principles of tomato BWD management. The consent of respondents was inquired before each discussion session (Appendix 2). The mean score of farmers' responses was computed as:

$$\text{Mean score (MS)} = \frac{\text{Responses scored}}{\text{The highest score}} \quad \text{..... (3)}$$

$$\text{MS} = \frac{4+3+2+1}{4} = 2.50$$

Using the interval score of 0.05, the upper and lower limits were determined as 2.50 + 0.05 and 2.55-0.05 = 2.45 respectively (Gomez *et al*., 1984). On the basis of this, MS < 2.45) were ranked 'low', those 2.45 ≥ MS ≤ 2.54 was ranked 'medium' while the MS ≥ 2.55) were considered 'high' (Bangor *et al*., 2009).

3.3 Detection of the BWD-causing pathogen(s) from tomato plants

3.3.1 Isolation of bacteria from infected plants

The collected infected tomato samples were surface cleaned to remove soils in running tap water and then immersed in 70% ethanol for 3 minutes to remove any saprophytic bacteria from stem surface. The surface-sterilized samples were macerated in distilled sterile water and bacterial suspension was obtained. Using a sterile loop, samples of suspension were plated onto triphenyl tetrazolium chloride (TTC) medium and incubated for 48 h at 28°C in the incubator (Kelman, 1954; Kinyua *et al*., 2014). Pure colony isolation on new plates of TTC medium yielded 40 RSSC looking bacterial isolates and then three approaches (Table 4) were used for detection of the pathogen.

Table 4: Approaches used for the detection of *Ralstonia solacearum* species complex of tomato in Tanzania

Approach	Method	Component	Descriptor	Pathogenesis		Reference
				Virulence	Avirulence	
Pathological	Cultural	TTC medium	Colony appearance	Pink centre with white margins	Red colony with off white margins	Kelman (1954)
	Pathogenicity test	Seedlings bioassay	Wilt symptoms	Pathogenic	Non Pathogenic	Janse and Ruissen (1988)
Molecular	PCR	Species specific primers	Base pair size	≈ 281 bp	≠ ≈ 281 bp	Kinyua *et al*.,2014)
Biochemical	Carbohydrate oxidation	Carbon sources	Medium colour change	Biovars		Hayward, 1964; He *et al*., 1983

PCR = Polymerase chain reaction and TTC = Triphenyl tetrazolium chloride.

3.3.2 Approaches used for the identification of *R. solacearum* species complex of tomato in Tanzania

(i) Evaluation of the virulence of isolates on growth medium

The triphenyl tetrazolium chloride (TTC) medium was prepared as detailed in Table 5. Thereafter, the bacterial suspension was streaked into the TTC medium agar plates and incubated at 28°C for 48 h (Kinyua *et al*., 2014). Single growing colonies were selected and sub-cultured on a fresh medium to acquire clean cultures and identification of the colonies were made based on colony appearance (Kelman, 1954). The RSSC colonies appear reddish in colour different from other bacterial species on TTC medium also TTC medium specifically allows growth of RSSC but not of other bacteria. Virulent isolates were selected for a subsequent experiment.

Table 5: Components and procedures used to prepare triphenyl tetrazolium chloride medium

S/n	Components	Amount	Procedure
i.	Casein	1g	a) Components i-iii were dissolved in DW and
ii.	Bacteriological agar	15g	b) Autoclaving at 121ºC for 15 minutes
iii.	MR-VP Medium	17g	c) Filtered TTC were added to about 45-50ºC cool medium
iv.	Distilled water (DW)	1000ml	d) Medium was poured into sterile petri dishes
v.	TTC	5ml of 1% solution/1L medium (50mg/l)	

(ii) Pathogenicity test

This trial was designed in a completely randomized design (CRD) in three replications. Tanya tomato seed variety, a popularly grown, preferred but susceptible to BWD were used (Shenge *et al*., 2007). Seeds were planted in a one litre pot filled with forest soil and sand at a ratio of three to one respectively. The pots were placed in screen-house with the surrounding temperature of about 25-28°C. Watering was conducted after every other day but seedlings were not irrigated a day before inoculation. The three weeks old seedlings were inoculated with the inoculum of the virulent and predominating isolates of RSSC infecting tomato in Tanzania. Inoculation of seedlings was carried with the suspension of isolates of approximately 10^9 cfu/ml by using a sterile needle through puncturing the stem (Janse & Ruissen, 1988). Seedlings not inoculated with RSSC were included as a negative control.

Development of wilting symptoms of BWD was observed (Agrios, 2005) and severity was recorded weekly for 8 weeks on a scale of zero to five (He *et al*., 1983; Horita & Tsuchiya, 2001). Briefly 0 = no wilting symptoms; 1 = one wilted leaf; 2 = two wilted leaves; 3 = three wilted leaves; 4 = four or more wilted leaves and 5 = all leaves wilted or plant died. Bacterial wilt disease severity was computed using the formula in section 3.1.2 and was correlated with the BWD severity recorded in the field, all data analysis were facilitated by CoStat software (Ver CoStat, 2005). Re-isolation of the bacteria was carried out on TTC medium and incubated at 28°C for 48 h. Presence of RSSC typical colony was examined and recorded.

(iii) Polymerase chain reaction (PCR)

The 10 bacterial isolates that produced both typical wilting symptoms of BWD in tomato bioassay and characteristic of RSSC colonies on TTC medium were subsequently subjected to PCR experiment. The deoxyribonucleic acid was extracted from the bacterial cells by using a bacterial DNA extraction kit as per the manufacturer's instructions (info@zymoresearch.com).

The non-template DNA response mixtures were included as a negative control. The primer pairs used were AU759F (5′-GTC GCC GTC AAC TCA CTT TCC-3′) and AU760R (5′-GTC GCC GTC AGC AAT GCG TCG-3′) (Opina *et al*., 1997; Lin *et al*., 2009). Primers and master mix were synthesized by the inqaba Biotec East Africa Ltd. (IBE002). The PCR was conducted in the final 25 µl reaction mixture with components and mixing procedures shown in Table 6 according to the manufacturer's instruction and number of reactions performed.

Table 6: Polymerase chain reaction components for Ralstonia isolates detection

Component	**1 µL Reaction**	**12+1 µL Reaction**	**Procedure**
OneTaq® Quick-Load 2X Master Mix with standard Buffer	12.50	162.50	1. The primers and One Taq® Quick-Load 2X Master Mix were thaw with standard Buffer
10 µM Forward Primer	0.50	6.50	2. Primers were vortexed for 3-5 seconds
10 µM Forward Primer	0.50	6.50	3. All tubes were pulse spined and hold on ice
Template DNA	0.50		
Nuclease free-Water	11.00	143.00	4. One Taq® Quick-Load 2X Master was mixed by gently pipetting it up and down, then pulse spined and hold on ice
			5. Components 2-4 were combined in appropriate tube
Total volume	**25**		

A DNA thermal cycler C 1000 Touch®, (BIO-RAD) was used with PCR amplification conditions set as denaturation and anneling at 94°C and 53°C for 3 and 1min respectively, and extension at 72°C for 1.5 min, lagged by 30 cycles of 94°C, 60°C and then 72°C for 15 s each and a final extension of 72°C for 5 min (Grover *et al*., 2011). Subsequent PCR products were analysed in a 1.5% agarose gels stained with 0.5 µg/ml of vision blue light DNA dye solution in TBE (Tris-borate-EDTA (Ethylenediaminetetraacetic acid) buffer at 100 V and a 100bp DNA ladder was used. Results were visualized under ultraviolet light by measuring and recording the length of base pair (bp) of each isolate.

3.4 Identification of Ralstonia biovar(s) associated with BWD from infected tomato samples

3.4.1 Preparation of isolates and inoculum

The 10 most virulent, pathogenic and confirmed to be Ralstonia isolates by PCR were subjected to biovar(s) determination (Hayward, 1964). A sole colony was speckled on TTC medium and incubated at 28°C for 2 days (Kelman, 1954) to get fresh RSSC colonies. A loop-full of two days old colony of each isolate was taken and mixed into 1ml sterile water in 2.0 ml centrifuge tube (Eppendorf) to make suspension containing about 10^9 cfu/ml (Kinyua *et al*., 2014). Biovars of RSSC were determined by the carbohydrate oxidation test using the as detailed in Table 7 (Hayward, 1964; He *et al*., 1983).

Table 7: Description of carbohydrate oxidation test to determine biovars of Ralstonia

Approach	Method	Component	Descriptor	Biovar					
				1	2		3	4	5
					2A	2T			
Carbohydrate oxidation test	Disaccharides	Dextrose	Colour change	Y	Y	Y	Y	Y	Y
		Salicin	Colour change	G	G	G	G	G	G
		Lactose	Colour change	G	Y	Y	Y	G	Y
		Maltose	Colour change	G	Y	Y	Y	G	Y
		Cellobiose	Colour change	G	Y	Y	Y	G	Y
		Trehalose	Colour change	Y	G	G	Y	Y	Y
		D-Ribose	Colour change	Y	G	Y	Y	Y	Y
		D-Trehalose	Colour change	Y	G	Y	Y	Y	Y
		L-Tryptophan	Colour change	Y	G	Y	Y	Y	Y
	Sugar alcohols	Mannitol	Colour change	G	G	G	Y	Y	Y
		Sorbitol	Colour change	G	G	G	Y	Y	G
		Dulcitol	Colour change	G	G	G	Y	Y	G

Y = Yellow and G = Greenish

3.4.2 Preparation of mineral medium and carbohydrates solution

The solution of mineral medium and carbohydrates were prepared as indicated in Table 8. D-Trehalose, L-Tryptophan and D-Ribose were incorporated in the test to distinguish between biovar 2 sub-phenotypes 2A and 2T. Negative controls were set up without any carbohydrate, where salicin and distilled water (DW) were used. Dextrose, the most commonly utilized carbohydrate by all biovars was included as a positive control.

Table 8: Preparation of disaccharides and sugar alcohols solution

Medium	Component	Amount	Procedure
Mineral medium	i. Ammonium dihydrogen phosphate ($NH_4H_2PO_4$)	1.0 g	1. Components i-vi were dissolved in vii
	ii. Potassium chloride (KCl)	0.2 g	2. Medium was boiled with constant stirring
	iii. Magnesium sulphate (MgSO4.7H2O)	0.2 g	3. Medium pH was raised to 7.0 by addind component viii drop wise
	iv. Peptone	1.0 g	4. Medium was divided (90ml) into container
	v. Bromothymol blue	0.03 g	
	vi. Agar	3.0 g	5. Autoclaved at 121°C, 15 psi for 20min
	vii. DW	1.0 L	
	viii. Sodium hydroxide (NaoH)	1.0 N	
Disaccharides solution	Cellobiose	1.0 g	1. Each disaccharide or sugar alcohol was dissolved in 10 ml DW separately
	Lactose	1.0 g	
	Maltose	1.0 g	
	Salicin	1.0 g	2. Filter-sterilize with 0.22µm filters
	Dextrose	1.0 g	
	D-Trehalose	1.0 g	
	L- trptophan	1.0 g	
	D- ribose	1.0 g	
Sugar alcohols solution	Dulcitol	1.0 g	
	Sorbitol	1.0 g	
	Mannitol	1.0 g	

DW = Distilled water

3.4.3 Allocation of disaccharides and sugar/alcohol solution into mineral medium

After autoclaving, the media was cooled to 65°C. Thereafter 10 ml of each carbon source were added in a mixture of 90 ml of the mineral medium to make sugar/alcohol-amended medium. The 300 µl of sugar/alcohol-amended medium were dispensed in a 2.0 ml centrifuge tube.

3.4.4 Inoculation of sugar/alcohol-amended medium and data collection

The sugar/alcohol-amended medium was inoculated with 10 µl of suspension a bacterial isolate and incubated at 28°C (Kinyua *et al*., 2014). Observations on changing pH of the amended medium as indicated by the colour change were recorded for seven days (Schaad, 1988).

3.5 Establishment of the phylogenetic relationships of the BWD-causing pathogen(s) isolated from infected tomato plants collected from main agro-ecological zones

3.5.1 Preparation of bacterial DNA

The 19 isolates of RSSC that were recognised as most pathogenic were used for the phylogenetic study (Table 9). The XYZ, an out-group isolated from the infected potato stems were included for comparison. All isolates were stored at 20°C and revived on TTC medium (Kelman, 1954). After growing bacterial isolates on TTC medium, a single colony of each isolate was used for DNA extraction using the bacterial DNA extraction kit as per the manufacturer's instructions (info@zymoresearch.com). The DNA was used as a PCR template for PCR and a non DNA template reaction mixture were included as the negative control.

Table 9: Description of isolates used during the phylogenetic study of *Ralstonia solanacearum* species complex in Tanzania

Agro-ecological zone	District	Code of Isolate	Agro-ecological zone	District	Code of isolate
Northern	Arumeru	NAA1	Central	Kongwa	CDK1
Northern	Arumeru	NAA4	Central	Kongwa	CDK4
Northern	Babati	NMB1	Lake	Nyamagana	LMN1
Northern	Babati	NMB2	Lake	Nyamagana	LMN3
Southern	Kilolo	SIK1	Lake	Kibondo	LKK1
Southern	Kilolo	SIK3	Lake	Kibondo	LKK4
Southern	Kilolo	SIK4	Coastal	Chake Chake	CZC1
Southern	Mbeya	SMM2	Coastal	Chake Chake	CZC3
Central	Manyoni	CSM2	Coastal	Temeke	CDT4
Central	Manyoni	CSM4	Northern	Arumeru	XYZ

3.5.2 Repetitive sequence –based polymerase chain reaction (Rep-PCR)

The phylogenetic relationship of RSSC infecting tomato in Tanzania was determined by using the repetitive sequence-based polymerase chain reaction (rep-PCR) method. The random amplification of polymorphic DNA (RAPD) marker type known as the entero-bacterial repetitive intergenic consensus (ERIC) was selected due to its robust, reproducible and highly discriminatory fingerprint characteristic (Louws *et al*., 1994; Sander *et al*., 1998; Horita *et al*., 1999; Zhao *et al*., 2000; Horita & Tsuchiya, 2001; Peters *et al*., 2004; Shutt *et al*., 2005; Poliakoff *et al*., 2005; Horita *et al*., 2005). The forward (5'-AAGTAAGTGGGG GTGGGG-3') and reverse (5'-ATGTAAGCTCCTGGG GGATTCAC-3 ') pairs of primers (Lamessa *et al*., 2010), synthesized by the inqaba Biotec East Africa Ltd. (IBE002), Nairobi,

Kenya were used. The PCR amplification was carried out in a final 25 µl volume comprising PCR response buffer (Table 10).

Table 10: Reaction mixture for the rep-PCR experiment for phylogenic analysis

Component	1 µL Reaction	20 ±1 µL Reaction	Procedure
OneTaq® Quick-Load 2X Master Mix with standard Buffer	12.50	262.50	1. The primers and One Taq® Quick-Load 2X Master Mix were thaw with standard Buffer
10 µM Forward Primer	0.50	10.50	2. Primers were vortexed for 3-5 seconds
10 µM Forward Primer	0.50	10.50	3. All tubes were pulse spined and hold on ice
Template DNA	0.50		
Nuclease free-Water	11.00	231.00	4. One Taq® Quick-Load 2X Master was mixed by gently pipetting it up and down, then pulse spined and hold on ice
			5. Components 2 and 4 were combined in appropriate tube
Total volume	**25**		

Rep-PCR amplification was performed with automated C 1000 Touch® Thermal Cycler BIO-RAD) programmed as an initial denaturation of 95° C for 2 min, followed by 30 cycles of 94° C designed for 30 s, 50° C for one min and 65° C for four min with a final extension of 65° C for five min, followed by a holding time of 4° C until specimens were collected (Versalovic *et al*., 1991).

3.5.3 Repetitive Sequence-based polymerase chain reaction product analysis

The Rep-PCR products were visualized in a 1.5% agarose gels stained with 0.5 µg / ml of blue vision DNA dye solution in a 1% TBE buffer using a 100bp DNA ladder at 100 V. The results were viewed under ultraviolet light and base pair length of each isolate was measured and recorded. Genetic similarity between isolates was assessed using the fingerprint profile of Rep-sequence- based PCR in which a position number was allocated to each group with distinct electrophoretic mobility and scored as either one (presence of band) or zero (lack of band).

The similarity coefficients were predicted by the Dice technique for all possible pairs of isolates based on the finger-print groups (Dice, 1945). The dendogram was produced by the unweighted pair group method with arithmetic means (UPGMA) clustering from the similarity coefficient information (Sneath & Sokal, 1973; Pyiyambada *et al*., 2017) and was facilitated by the Molecular Evolutionary Genetics Analysis (MEGA.7.0.14). Finally, the aligned sequences were contrasted with the standard strains in the National Centre for

Biotechnology Information (NCBI) database of taxonomic browser to determine resemblance using the Basic Local Alignment Search Tool (www.ncbi.nih.gov/BLAST).

3.6 Evaluation of the effect of selected plant extracts on managing the BWD-causing pathogen(s) in *in-vitro* and screen-house environments

3.6.1 Assessment of the antibacterial activity of selected plant species water extracts

(i) Preparation of water extracts of plant species

Twenty pesticidal plant species known for their antibacterial properties were collected in Arusha Region and the plant part constituting the most used part was selected for extraction purposes (Table 22). Each plant part was washed with distilled water, cut in pieces, dry shade and ground into fine powder with a grinder (Mahlo *et al*., 2010). Then 10 g of the ground powder of each plant part was soaked in 10 ml distilled water (DW) at a ratio of one to one (water: plant material). The final volume was made up to 10 ml with the plant material and water at the ratio of one to one and rotated at low speed for 15 min in a blender till the material formed a fine texture (Frey & Meyers, 2010). The resulting crude liquid extract was then pressed through a sterilized muslin cloth, filtered through Whatman paper filter No. 1 and through sterilized Seitz filter. The sterilized filtrate was collected in sterilized glass tubes and was sealed, labelled and kept into the refrigerator at 4°C until when used for further analysis.

(ii) Evaluation of the antibacterial effect water extracts of plant species

The inhibitory effect of 20 plant species extracts on the growth of the most virulent and pathogenic isolate and the most widely distributed biovar i.e. biovar 3 of RSSC affecting tomato production in Tanzania was evaluated using an agar disc diffusion technique (Balestra *et al*., 2009). The suspension (1.5×10^8 cfu/ml) of Ralstonia was spread (lawn plating) uniformly on the Kelman's TTC medium (Kelman, 1954). The inoculated petri-plates were partitioned and marked to accommodate each extract accordingly.

Discs of Whatman filter paper measuring 8 mm diameter was loaded with 20 µL of each plant extract and air-dried and was respectively positioned on the surface of the inoculated TTC agar medium in petri-dish plate. Negative and positive control paper discs were loaded with 20 µL of DW and Ampiclox (ampicillin and cloxacillin) respectively. Each plate received 8 paper discs i.e. 6 discs of different plant extracts, 1 disc of antibiotic and 1 disc of DW (Frey & Meyers, 2010). The Petri-plates were sealed with parafilm and incubated at

room temperature (25-28°C) for 24 h. The diameter of the zones of inhibition produced around the disc was measured by using a tape measure. The zone of inhibition measurements were subjected to the analysis of variance (ANOVA) and mean separation was carried out by using the LSD test facilitated by CoStat data analysis software program.

3.6.2 Identification of the most effective part, form and extraction solvent of the most effective plant species

(i) Preparation of water and alcohol extracts of the most effective plant species

The plant extract with significantly the highest inhibitory effect was considered the most effective and was further investigated. Its leaves, roots and fruits were collected from the bush around the Arusha Region. Similar sampling location and plants were maintained to minimize variation. Samples for extract preparation from the dried plant parts were collected 14 days prior to the collection of samples for extracts from the undried plant parts in order to allow a shade-drying period.

The water extract from these plant materials was prepared as outlined earlier in section 3.6.1. The ethanolic and methanolic extracts were prepared by mixing 10 g of dried leaves/fruit/root with 10 ml of 70% ethanol or methanol. The mixture was macerated in a pestle and mortar, blended into fine texture, transferred to a beaker with a final volume made up to 10 ml with the plant material and ethanol/methanol in the ratio of one to one (Frey & Meyers, 2010). They were left to dissolve for 24 h and then pressed through a sterile muslin cloth to get a crude liquid extract. The crude extract was filtered through Whatman filter paper No. 1 and then through sterile Seitz filter. The filtrate was collected in sterile glass tubes and was sealed, labelled and stored into the refrigerator at 4°C for future use.

(ii) Evaluation of the effect of parts and forms of water and alcohol plant extracts of the most effective plant species against *Ralstonia solanacearum* species

The suspension (1.5×10^8 cfu/ml) of the most predominating isolate of RSSC in Tanzania was spread uniformly on the Kelman's TTC medium (Kelman, 1954; Balestra *et al*., 2009). The autoclaved discs of Whatman filter paper No.1 measuring 8 mm diameter was loaded with 20 µL of each extract of specific part or form or extraction solvent of the most effective plant species (Table 11) and air-dried for 2 min. Thereafter, each disc was placed on the surface of the inoculated TTC medium in the petri-dish plate. Negative and positive control paper discs with 20 µL of DW and Ampiclox respectively were included (Table 11). The petri-dish plates

were sealed with parafilm and incubated at 25-28°C designed for 24 h. The diameter of the inhibition growth region was measured and data were subjected to ANOVA and mean separation was conducted by LSD test, all facilitated by CoStat data analysis software program.

Table 11: Treatments used to determine the most effective part and extraction solvent for the most effective plant species

Treatment Description	Dilution	Treatment Description	Dilution
Dried (w/v)		**Fresh (v/v)**	
Leaves: DW	1:1	Leaves: DW	1:1
Leaves: Ethanol	1:1	Leaves: Ethanol	1:1
Leaves: Methanol	1:1	Leaves: Methanol	1:1
Leaves: DW	1:1	Leaves: DW	1:1
Fruits: Ethanol	1:1	Fruits: Ethanol	1:1
Fruits: Methanol	1:1	Fruits: Methanol	1:1
Roots: DW	1:1	Roots: DW	1:1
Roots: Ethanol	1:1	Roots: Ethanol	1:1
Roots: Methanol	1:1	Roots: Methanol	1:1
Ampiclox: DW	1:1	DW	1 ml

DW = Distilled water

3.6.3 Determination of the most effective concentration of most effective plant extract

(i) Preparation of plant extract concentrations

The fresh sample of the most effective plant part was collected and washed in distilled water. Five levels of concentrations were prepared by diluting 1 ml of plant extract into 6 ml, 4 ml, 2 ml, 1 ml and 0 ml of the most effective extraction solvent to form 6.25%; 12.5%, 25%, 50% and 100% levels respectively.

(ii) Evaluation of the extract concentrations against the growth of Ralstonia isolate

Each concentration of the plant extract was verified for its inhibitory effect against the growth of the most virulent and pathogenic isolate by using paper disc diffusion method (Eloff, 1998). The suspension (1.5×10^8 cfu/ml) of the most virulent and pathogenic isolate of RSSC was spread uniformly on the Kelman's TTC medium (Kelman, 1954; Balestra *et al*., 2009). Paper disc was loaded with 20 µL of each concentration and air-dried for 2 min. Negative and positive control paper discs were loaded with 20 µL of DW and Ampiclox respectively. The plates were gestated for 24 h at 25-28°C and the diameter of zone of inhibition were measured across the discs by using the tape measure. The experiment was repeated three times in a CRD (Fig. 3). Data of inhibition zone were subjected to the

ANOVA and means were separated by LSD test facilitated by CoStat software program of data analysis.

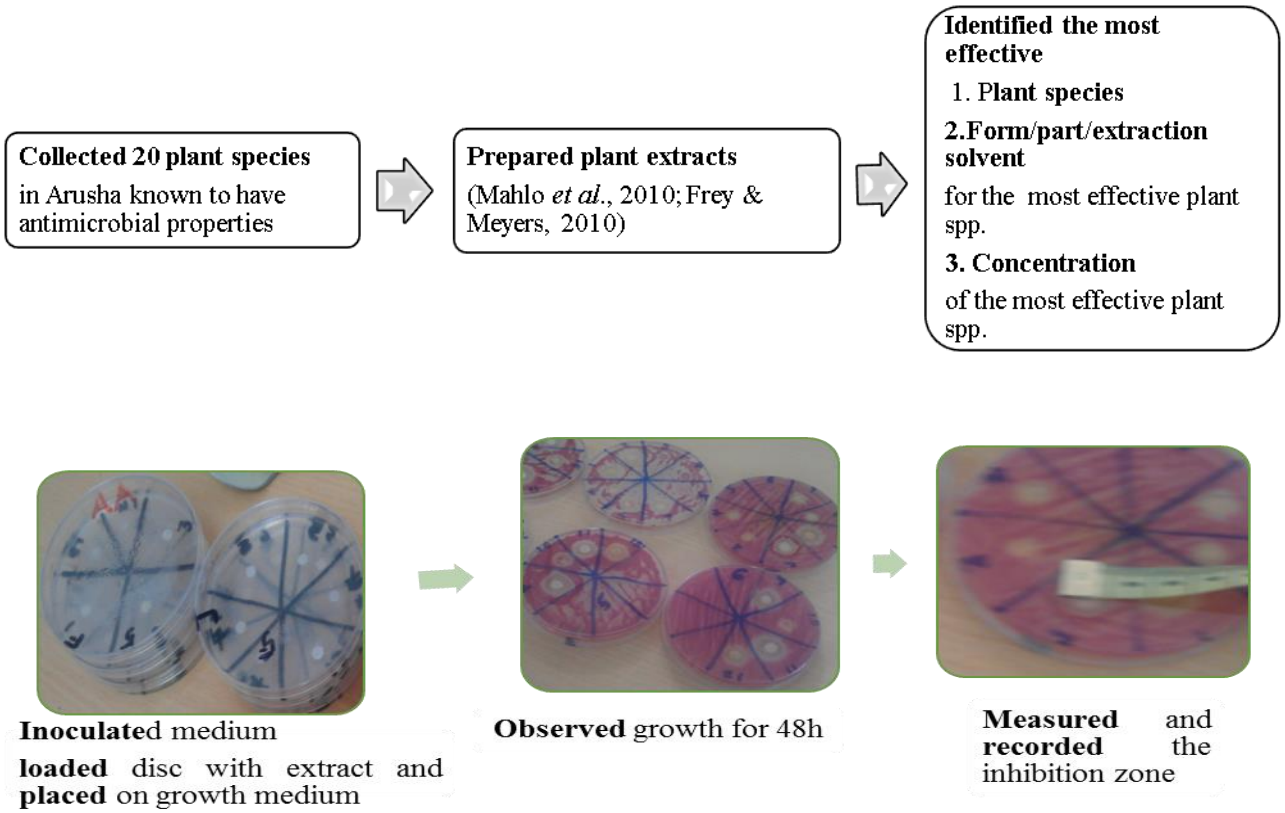

Figure 3: Determination of effect of plant species against Ralstonia *in-vitro*

3.6.4 Assessment of the efficacy of the most effective extract in managing bacterial wilt disease in screen-house environment

(i) Raising of tomato seedlings

A screen-house trial was established in a completely randomized design in three replications (Fig. 4). Seeds of Tanya tomato variety, a commonly cultivated and preferred but susceptible to bacterial wilt disease variety were used (Shenge *et al*., 2007). The seeds were planted in a 1 L pots filled with forest soil and sand at a ratio of 3:1 respectively. The pots were placed in screen-house at the temperature of 25-28°C and relative humidity of 75-80%. Watering of seedlings was done after every one day but before inoculation, seedlings were not irrigated for 24 h.

(i) Seedlings inoculation with Ralstonia and treating with plant extract

The two weeks old seedlings were inoculated with bacterial suspension (≈10^9 cfu/ml) of the predominating Ralstonia isolate coded as NAA1 (Table 17) infecting tomato in Tanzania by puncturing in a stem with a sterile needle (Janse & Ruissen, 1988). A week after inoculation, seedlings were treated once with 6.25%, 12.5%, 25%, 50% and 100% concentrations of the most effective plant extract as prepared and described in Section 3.6.3. Through soil drenching, 5 ml of each concentration of the extract was applied around the seedling stem in the pot. The untreated seedlings with plant extract were used as negative control but there was no positive control for this experiment because of lack of effective product against BWD. An antibiotic, Ampiclox was avoided in this experiment due to the concerns of the environment to antibiotic exposure especially under the *in-vivo* environment. The seedlings were held at 25-28°C in screen-house for 8 weeks after inoculation to monitor for the occurrence of BWD symptoms.

1. Raised seedlings

2. Inoculated seedlings with NAA1 isolate

3. Treated seedlings with C15 extract

4. Collected data
-Disease severity weekly
-Plant height (11 weeks)
-Root length (11 weeks)
-Xylem vessels arrangement (11 weeks)

Figure 4: **Determination of effect of plant extract C15 against bacterial wilt disease *in-vivo***

(i) Evaluation of bacterial wilt disease severity and plant growth

Wilting symptoms was assessed and disease severity was recorded weekly on a scale of 0-5 (Hyakumachi *et al*., 2013), where; 0 = no symptoms; 1= one wilted leaf; 2 = two wilted leaves; 3 = three wilted leaves; 4 = four or more wilted leaves and 5 = whole or all leaves wilted and plant died. The mean of bacterial wilt disease severity was computed using the following formula:

$$\text{Disease severity } (\%) = \frac{5A+4B+3C+2D+E}{5N} \times 100$$; (Hyakumachi *et al.*, 2013), where A = average amount of plants on scale 5; B = number of plants on scale 4; C = number of plants on scale 3; D = number of plants on scale 2; E = number of plants on scale 1; N = total plants evaluated. Also, the plant height and root length were measured at the termination of the experiment to determine the general plant growth.

Data of disease severity and plant height and root length were analysed by the analysis of variance (ANOVA) and means were compared by LSD at $p = 0.05$, all facilitated by the CoStat software program. Furthermore, a cross-sectional part of the stem was made and examined under the electron microscope (40x) to investigate the xylem vessels of the healthy (treated with 100% plant extract concentration) and untreated and infected tomato stem with Ralstonia. This is because RSSC cells inhabit and block the xylem vessels of the plant (Agrios, 2005).

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Bacterial wilt disease incidence and severity on tomato growing in the main agro-ecological zones of Tanzania

Out of 4800 plants assessed in all districts, 2648 (55.17%) were infected by the BWD at different levels of disease incidence and severity. The disease incidence and severity significantly differed among districts ($p \leq 0.05$) (Table 12). Incidence (44.6%) and severity (59.3%) were significantly higher in Arumeru as compared with other districts that ranged from 5.8-20.8% and 10.7-46% respectively (Table 12). Mean values of temperature and relative humidity in the BWD most affected districts were ≥ 28ºC and ≥ 85% as compared with ≤ 20.5°C and ≤75%°C in the BWD less affected districts, respectively and rainfall data for during the study period were as indicated (Table 12).

Table 12: The means of bacterial wilt disease incidence and severity, temperature, relative humidity and rainfall in different districts in Tanzania during the field study period

District	Incidence (%)	Severity (%)	Temperature (ºC)	Relative humidity (%)	Rainfall (ml)
Arumeru	44.60a	59.30a	31.00a	89.00a	10.00c
Babati	38.70a	44.00a	29.00a	87.00a	0.00e
Manyoni	22.60b	36.00b	27.00a	85.00a	10.00c
Nyamagana	14.48c	25.90c	28.00a	81.00b	10.00c
Mvomero	27.80b	33.80b	26.00b	80.00b	5.00d
Kilolo	10.60b	20.80c	22.00b	79.00b	22.00a
Temeke	19.10c	14.65c	29.00a	83.00b	15.00b
Chake Chake	22.40b	30.10b	28.00a	83.00b	10.00c
Mbeya urban	8.80d	9.70d	21.00c	80.00b	20.00a
Kibondo	11.70c	14.30c	27.00a	81.00b	0.00e
Tandahimba	6.30d	4.10d	20.00c	70.00c	0.00e
Kongwa	23.10b	31.20b	28.00a	80.00b	8.00c
Mean	**20.85**	**26.99**	**26.33**	**81.50**	**10.83**
F-test	*	*	*	*	*
CV (%)	**19.60**	**20.07**	**11.17**	**13.42**	**9.62**

* = Means are significant at $p \leq 0.05$ and mean of incidence and severity with the same letter within the column are not significantly different based on LSD test ($p = 0.05$)

Similarly, the incidence and severity of BWD were significantly ($p \leq 0.05$) higher in the screen- houses than in the open field tomato production environments and was higher in Arumeru as compared with other districts both in open field and screen-house environments (Table 13).

Table 13: Bacterial wilt disease incidence and severity in open-field and screen-house in different Districts in Tanzania

District	Environment	Incidence (%)	Severity (%)	District	Environment	Incidence (%)	Severity (%)
Arumeru	Screen-house	58.71a	54.33a	Temeke	Screen-house	21.47a	19.49a
	Open field	23.89b	24.97b		Open field	7.63b	15.16a
	Mean	**41.30**	**39.65**		**Mean**	**14.55**	**17.33**
	F-test	*	*		**F- test**	**	**ns**
Babati	Screen-house	24.59a	52.81a	Chake Chake	Screen house	18.40a	36.80a
	Open field	4.11b	21.19b		Open field	12.00b	24.30a
	Mean	**14.35**	**37.00**		**Mean**	**15.20**	**30.55**
	F-test	**	**		**F- test**	*	**ns**
Manyoni	Screen house	24.80a	52.00a	Mbeya	Screen house	9.67a	11.70a
	Open field	13.80b	34.00b		Open field	7.04a	8.43a
	Mean	**19.30**	**43.00**		**Mean**	**8.36**	**10.07**
	F- test	*	*		**F -test**	**ns**	**ns**
Nyamagana	Screen-house	26.34a	42.85a	Kibondo	Screen-house	16.52a	25.62a
	Open field	12.96b	24.49b		Open field	12.78a	19.65b
	Mean	**19.65**	**33.67**		**Mean**	**14.65**	**22.64**
	F- test	*	**		**F-test**	**ns**	*
Mvomero	Screen-house	26.30a	34.31a	Tandahimba	Screen-house	8.48a	15.70a
	Open field	13.33b	22.83b		Open field	2.42b	7.62b
	Mean	**19.82**	**28.57**		**Mean**	**5.45**	**11.66**
	F - test	*	*		**F- test**	*	***
Kilolo	Screen-house	17.44a	38.50a	Kongwa	Screen-house	25.81a	28.53a
	Open field	15.73a	36.42b		Open field	9.50b	13.31b
	Mean	**16.59**	**37.46**		**Mean**	**17.66**	**20.92**
	F test	**ns**	**ns**		**F test**	**	**

***; **; * = Means are significant at $p \leq 0.001$, 0.01 and 0.05 respectively and mean of incidence and severity with the same letter(s) within the column are not significantly (ns) different based on LSD test ($p = 0.05$)

Mean Temperature and RH values in the visited screen-houses were > 29 °C and >85% respectively except in Mbeya, Tandahimba and Chake Chake. In linking the BWD incidence and severity in screen-houses with temperature and RH, the results indicated that incidence and severity increased with temperature and relative humidity (Fig. 5).

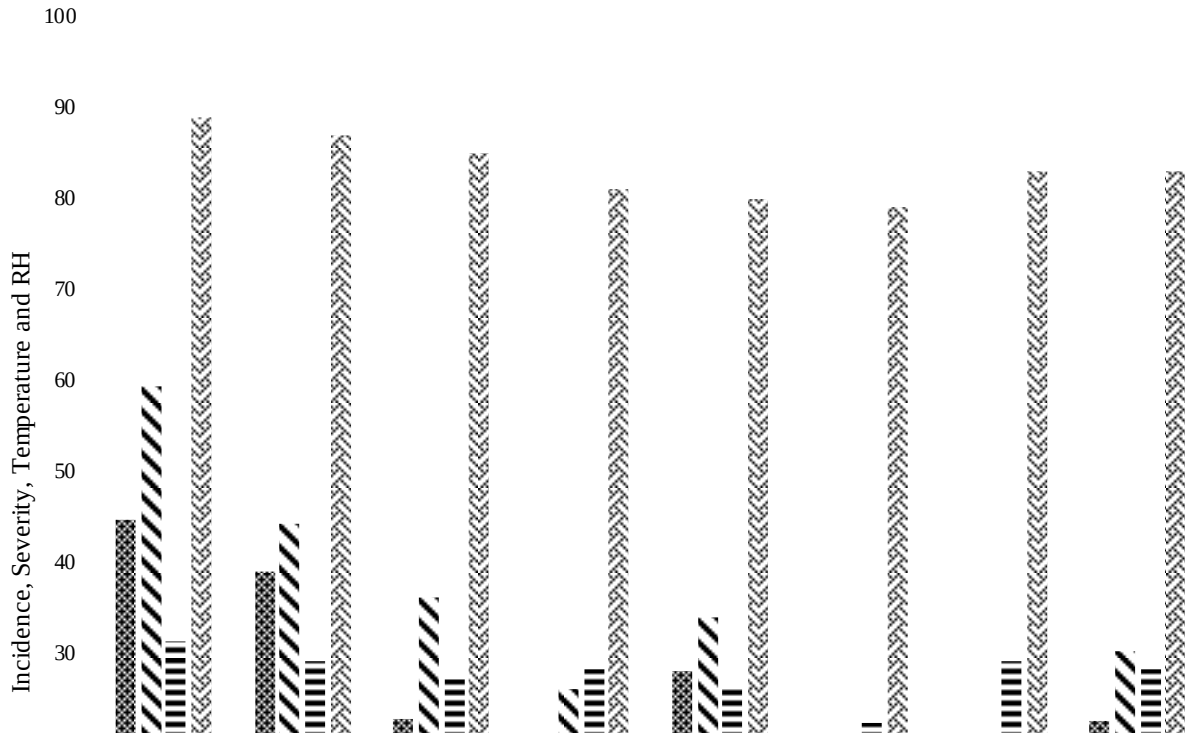

Figure 5: Relationship of BWD incidence, severity, temperature and relative humidity in screen-houses in Tanzania

4.1.2 Knowledge of tomato-growing farmers on bacterial wilt disease management in the main agro-ecological zones of Tanzania

The mean score of farmers' level of knowledge for different principles practiced varied from 0.0-3.12 (Table 14). Respondents indicated that practices such as testing of soil and irrigation water to determine their suitability for tomato production were not considered necessary and thus not done. Most respondents recognized BWD problem and believed to be caused by the cold weather and have named it as ice disease "*barafu*". They can describe the disease by symptoms but they could not identify its source in screen-houses or predict its outbreak but believed it is not a new tomato disease in their environment.

Table 14: Mean score for principles of BWD management in screen-house in Tanzania

Stage	Principle	Mean score (Max = 4)
Before growing cycle	Test soil for its status – soil microbes and fertility	0.00
	Identify the source of irrigation water and test its suitability for tomato irrigation	0.00
	Clear the screen-house of plant debris and weeds	2.11
	Wash and disinfect benches, potting tables, storage shelves, tools and equipment	0.44
During the growing cycle	Avoid recontamination with pathogen	0.33
	Purchase certified seeds from reliable sources	0.03
	Use cell grown seedlings grown by using sterilized growth media	2.27
	Use resistant cultivars whenever possible	1.41
	Close the green-house door all the time	0.96
	Have a foot bath at the door with disinfectant	2.43
	Disinfect farm tools and equipment e.g. hoe, pruning knives and scissors before use or when moving from one plant to another	1.80
	Avoid unnecessary visits/visitors inside the green-house	0.23
	Maintain a disease prevention program for tomato plants	0.19
	Inspect plants for diseases - look for localized symptoms such as root lesions, cutting end rot, leaf spots, and shoot blights	0.46
	Monitor seedlings for damping off	0.31
	Inspect incoming seedlings	3.12
	Properly identify the diseases	2.93
	Select a well-drained soil	1.27
	Apply water for optimum growth plant growth	2.08
	Space plants for good air movement and sunlight	1.79
	Irrigate early enough in the day to allow foliage to remain dry overnight	1.06
	Do not reuse growing media	0.01
	Use clean gloves	0.34
	Use un-contaminated (disinfected) drip irrigation system	0.06
	Use separate screen-houses for vegetable plants and ornamental plants	0.01
	Maintain required relative humidity and temperature	0.53
End of cycle	Discard unused seedlings, fruits and plant debris	0.05

Furthermore, the results of mean score of respondents' knowledge were ranked as low in all districts (Table 15).

Table 15: Farmers' level of knowledge and rank on BWD management in screen-houses in Tanzania (N = 220)

District	High(4)	Medium(3)	Low(2)	No(1)	Mean score	Level	Rank
Arumeru	2(0.40)	6(0.90)	9(0.90)	3(0.15)	2.35	Low	1
Babati	0(0.00)	1(0.15)	10(1.00)	9(0.45)	1.60	Low	6
Nyamagana	1(0.20)	6(0.90)	9(0.90)	4(0.20)	2.20	Low	2
Manyoni	0(0.00)	2(0.30)	8(0.80)	9(0.45)	1.55	Low	7
Mvomero	0(0.00)	0(0.00)	3(0.30)	17(0.85)	1.15	Low	8
Kilolo	3(0.60)	6(0.90)	6(0.60)	5(0.25)	2.35	Low	1
Temeke	0(0.00)	5(0.75)	10(1.00)	5(0.25)	2.00	Low	4
ChakeChake	0(0.00)	3(0.45)	11(1.10)	6(0.30)	1.85	Low	5
Kibondo	0(0.00)	0(0.00)	7(0.18)	13(0.65)	0.83	Low	9
Tandahimba	0(0.00)	0(0.00)	3(0.3)	17(0.85)	1.15	Low	8
Kongwa	0(0.00)	3(0.45)	11(1.10)	6(0.30)	1.85	Low	5
Mbeya Urban	1(0.20)	4(0.60)	11(1.1)	4(0.20)	2.10	Low	3

Results revealed that a number of management methods are used by farmers such as chemicals (70%), botanicals (13%) and crop rotation (10%) (Fig. 6). Imidacloprid, metalaxyl, profenofos and thiamethoxam were regularly reported chemicals used by farmers which are applied on tomato on a weekly basis in the morning from transplanting to harvesting. Most of respondents (90%) (Fig. 6) reported that chemicals are ineffective against BWD and declared their willingness to adopt other management methods if available. Moreover, some farmers (7%) do not use any method to manage BWD (Fig. 6). The crude extract from Neem (*Azadrachta indica*) leaves and seeds were the commonly used pesticidal plant and were applied at least twice to thrice weekly. Farmers reported being uncertain on the performance of botanical extracts on BWD management.

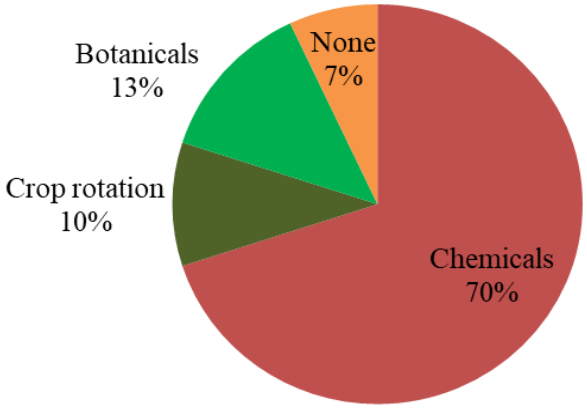

Figure 6: Number of farmers using different management methods for bacterial wilt disease in Tanzania

Statistically, the number of farmers using various methods to manage BWD was significant ($p \leq 0.05$) (Table 16).

Table 16: Mean comparison of number of farmers using different management methods against bacterial wilt disease in Tanzania

Method	Mean
Chemical	12.83a
Do not use any practice	3.67b
Botanicals	2.42b
Implement crop rotation	1.83b
Mean	**5.19**
F- test	***
LSD	**4.73**
CV (%)	**14.04**

*** = significant means at $p \leq 0.001$ and mean with the same letter within the column are not significantly different based on LSD ($p = 0.05$)

4.1.3 Bacterial wilt disease-causing pathogens from tomato plants originating from the main agro-ecological zones of Tanzania

(i) Virulence and pathogenesis of isolates

Results showed that out of 40 isolates of RSSC evaluated for virulence from different zones, 29 produced typical colonies of virulent isolates on TTC medium (Kelman, 1954) (Table 15). Irregular and fluidal colony appearance with a pink centre and white margins colour were consistently observed (Fig. 7A) while the rest were avirulent by producing off white non-fluidal colonies (Fig. 7B).

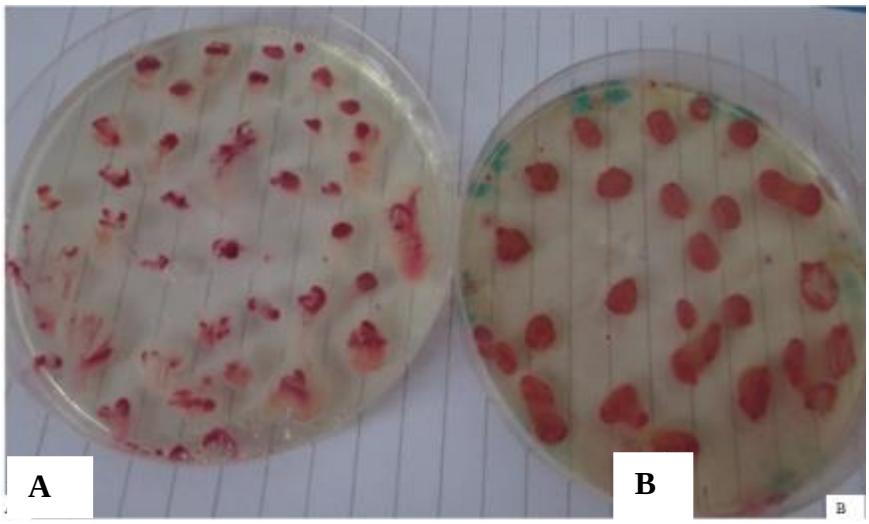

Figure 7: Colony appearance of *R. solanacearum* species complex on triphenyl trazolium chloride growth medium: Virulent (A) and avirulent (B)

In the pathogenicity test, 19 isolates showed typical wilting symptoms of BWD and the remaining 11 were non-pathogenic and could be regarded as saprophytic despite the comparable appearance of the RSSC colony (Table 17) [Agrios, 2008]. Afterwards, in re-isolating the isolates on TTC medium, they produced virulent colonies of RSSC.

Table 17: Virulence and pathogenesis reaction of isolates of *Ralstonia solanacearum* species complex

Zone	Isolate code	Virulence	Pathogenesis	Koch's rule
Northern	NAA1	+	+	+
Northern	NAA2	+	-	-
Northern	NAA3	-	-	-
Northern	NAA4	+	+	+
Northern	NMB1	+	+	-
Northern	NMB2	+	+	-
Northern	NMB3	+	-	-
Northern	NMB4	-	-	-
Southern	SIK1	+	+	+
Southern	SIK2	+	-	-
Southern	SIK3	+	+	-
Southern	SIK4	+	+	+
Southern	SMM1	-	-	-
Southern	SMM2	+	+	-
Southern	SMM3	+	-	-
Southern	SMM4	-	-	-
Central	CSM1	+	-	-
Central	CSM2	+	+	-
Central	CSM3	-	-	-
Central	CSM4	+	+	+
Central	CDK1	+	+	-
Central	CDK2	+	-	-
Central	CDK3	-	-	-
Central	CDK4	+	+	-
Lake	LMN1	+	+	+
Lake	LMN2	-	-	-
Lake	LMN3	+	+	-
Lake	LMN4	-	-	-
Lake	LKK1	+	+	+
Lake	LKK2	+	-	-
Lake	LKK3	-	-	-
Lake	LKK4	+	+	-
Coastal	CZC1	+	+	+
Coastal	CZC2	+	-	-
Coastal	CZC3	+	+	+
Coastal	CZC4	+	-	-
Coastal	CDT1	+	-	-
Coastal	CDT2	-	-	-
Coastal	CDT3	-	-	-
Coastal	CDT4	+	+	+
∑ Virulent/pathogenic isolates (+)		**29**	**19**	**10**

+ = virulence/pathogenic and - = avirulence /non-pathogenic

Furthermore, wilting severity in tomato seedlings significantly differed among isolates. *Ralstonia solanacearum* species complex isolates from the Southern zone were relatively more pathogenic than those from other agro-ecological zones (Table 18).

Table 18: Differences in wilting severity of the pathogenic isolates of Ralstonia in Tanzania

Agro-ecological zone	Code of isolate	Mean of disease severity
Northern	NAA1	33.00a
Northern	NAA4	20.00b
Northern	NMB1	33.30a
Northern	NMB2	20.00b
Southern	SIK1	40.00a
Southern	SIK3	33.30a
Southern	SIK4	43.70a
Southern	SMM2	26.70b
Central	CSM2	16.70c
Central	CSM4	16.70c
Central	CDK1	26.70b
Central	CDK4	23.30b
Lake	LMN1	20.00b
Lake	LMN3	16.70c
Lake	LKK1	13.30c
Lake	LKK4	16.70c
Coastal	CZC1	13.30c
Coastal	CZC3	23.30b
Coastal	CDT4	20.00b
Mean		**2.84**
F-test		*****
LSD		**1.47**

* = Significant at $p \leq 0.05$ and means of with the same letter within the column are not significantly different based on LSD test ($p = 0.05$)

There was a strong positive association (r = 0.84) between the severity of BWD in the field and pathogenesis of the causing isolates as illustrated by a scatter graph (Fig. 8).

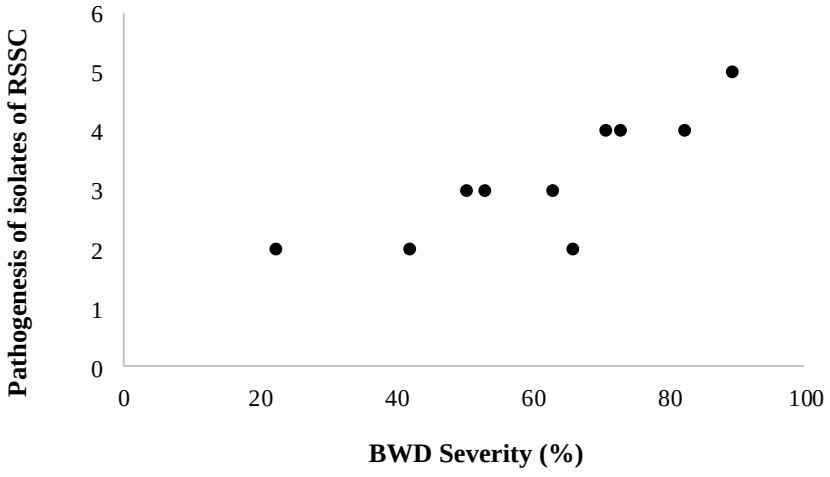

Figure 8: Relating bacterial wilt disease severity in the field and pathogenesis of Ralstonia

(ii) Polymerase chain reaction (PCR) of isolates

The PCR profile analysis indicated that, 10 isolates of RSSC that supported Koch's postulates i.e. produced typical colony characteristics of RSSC and wilting symptoms of BWD (Table 17) produced a single band of approximately 281bp fragment (Fig. 9).

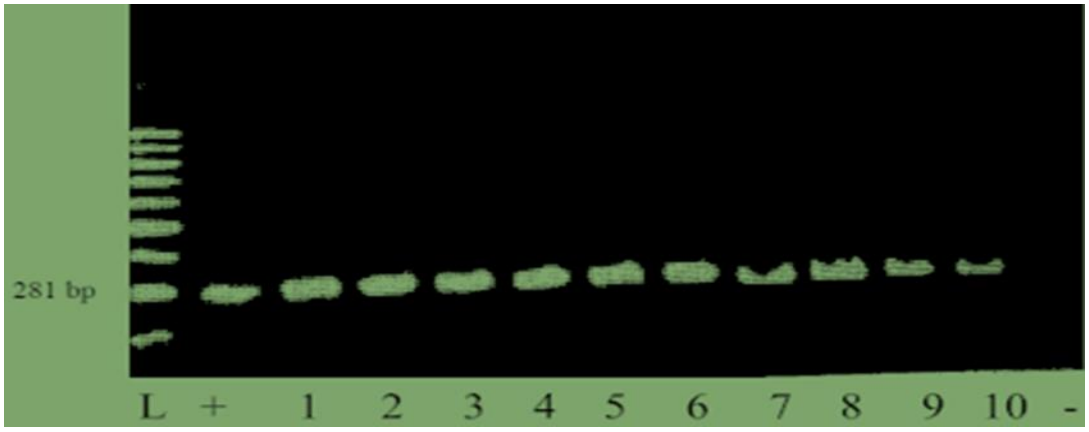

Figure 9: Polymerase chain reaction (PCR) profile produced by *Ralstonia solanacearum* species complex isolates collected from the infected tomato in Tanzania

L = 100 bp DNA ladder, 1-10 = test isolates (Table 19) + = Positive control - = Negative control

4.1.4 Biovars of tomato bacterial wilt disease-causing pathogen(s) identified from the collected tomato samples

All the investigated isolates were able to oxidize the four basic carbon sources i.e. Dextrose, sucrose, mannitol and lactose within 3 days of inoculation and this implied that they were all RSSC (Table 19).

Table 19: Utilization reaction of basic carbon sources by isolates of Ralstonia in Tanzania

S/n	Isolate	Sucrose	Lactose	Mannitol	Dextrose	Salicin	DW	Inference
1	NAA1	+	+	+	+	-	-	RSSC
2	NAA4	+	+	+	+	-	-	RSSC
3	SIK1	+	+	+	+	-	-	RSSC
4	SIK4	+	+	+	+	-	-	RSSC
5	CSM4	+	+	+	+	-	-	RSSC
6	LMN1	+	+	+	+	-	-	RSSC
7	LKK1	+	+	+	+	-	-	RSSC
8	CZC1	+	+	+	+	-	-	RSSC
9	CZC3	+	+	+	+	-	-	RSSC
10	CDT4	+	+	+	+	-	-	RSSC

+ Positive reaction, - Negative reaction and RSSC = *Ralstonia solanacearum* species complex

Similarly, the colour of the media changed from greenish to yellow in tubes with Dextrose (positive) while the medium in tubes with Salicin or distilled water (negative) were unchanged (Fig.10) revealing the utilization of carbon sources in dextrose and vice versa, hence confirming the presence of RSSC.

Figure 10: Utilization of the basic carbon sources as indicated by a colour change

Yellow = Lactose, Sucrose and Dextrose and greenish = Salicin and Distilled water

Nine isolates oxidized both disaccharides namely sucrose, lactose and maltose and sugar alcohols namely mannitol, sorbitol and dulcitol while one isolate (SIK4) was not able to utilize sugar alcohols (Table 20). The isolate coded as XYZ (isolated from potato stems from the Northern agro-ecological zone) behaved similarly with SIK4 by not utilizing the sugar alcohols. On the other hand, the colour of the medium in the positive control tubes with dextrose changed to yellow while media colour in the negative control tubes with salicin and DW remain unchanged (Table 20 and Fig. 11).

Table 20: Differentiation of isolates of Ralstonia into biovar(s) in Tanzania

Isolate	Maltose	Lactose	Cellobiose	Mannitol	Dulcitol	Sorbitol	Ribose	Trehalose	Tryptophan	Dextrose	Salicin	DW	Biovar
NAA1	+	+	+	+	+	+	+	+	+	+	-	-	3
NAA4	+	+	+	+	+	+	+	+	+	+	-	-	3
SIK1	+	+	+	+	+	+	+	+	+	+	-	-	3
SIK4	+	+	+	-	-	-	+	+	+	+	-	-	2(2T)
CSM4	+	+	+	+	+	+	+	+	+	+	-	-	3
LMN1	+	+	+	+	+	+	+	+	+	+	-	-	3
LKK1	+	+	+	+	+	+	+	+	+	+	-	-	3
CZC1	+	+	+	+	+	+	+	+	+	+	-	-	3
CZC3	+	+	+	+	+	+	+	+	+	+	-	-	3
CDT4	+	+	+	+	+	+	+	+	+	+	-	-	3
XYZ	+	+	+	-	-	-	+	+	+	+	-	-	2(2T)

+ = Positive reaction, - = Negative reaction and 2T = Sub-group of biovar 2

Figure 11: Biochemical reaction of Ralstonia isolates from Tanzania

XYZ = Out group isolated from the infected round potato stems

4.1.5 Phylogenetic relationships of the bacterial wilt disease-causing pathogens isolated from infected tomato plants

(i) Fingerprint profile of the RSSC isolates

The ERIC primer pair generated genomic PCR profiles for all 20 isolates tested. The polymorphic bands that were observed at different base pair were used to group isolates and at about 150 bp monomorphic single band was noted (Fig. 12).

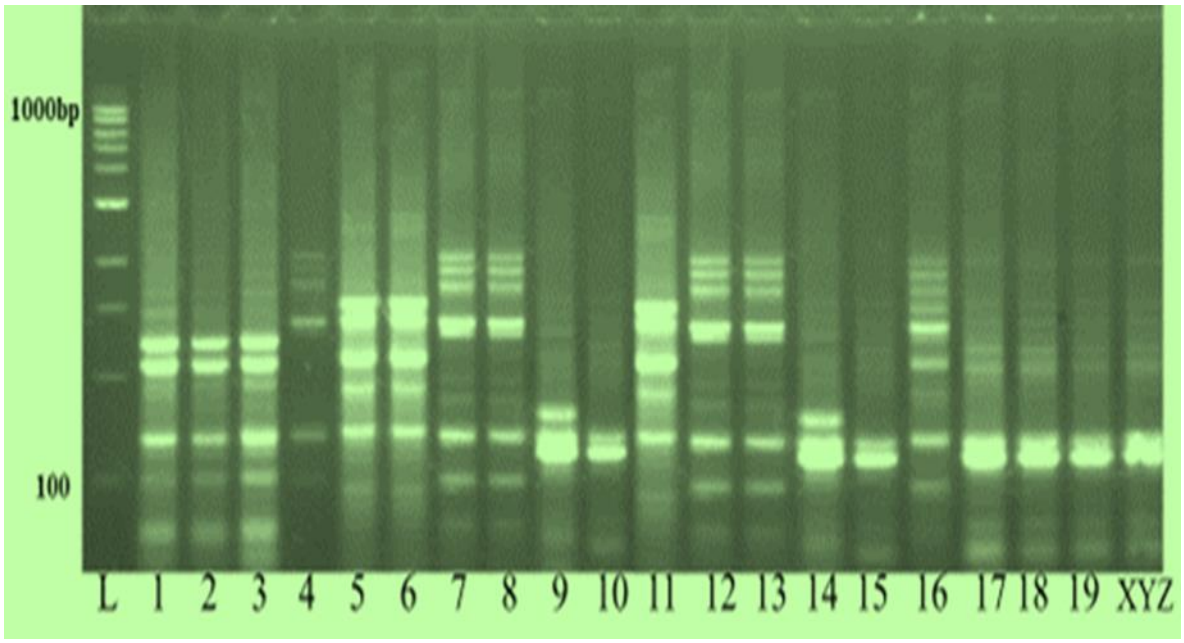

Figure 12: Band patterns of Ralstonia isolates collected in Tanzania

L = DNA ladder (100 bp), 1-19 = Isolates of RSSC and XYZ =out group potato isolate

The 20 isolates of RSSC investigated were classified in 3 main groups at 55% similarity level (Fig. 13). At similarity point of 90%, group 1 isolates were categorized into 5 sub-groups (1a, 1b, 1c, 1d and 1e) with the majority (38%) in subgroup 1a followed by 1b and 1e (23%) and 1c and 1d (1%). Group 2 isolates consisted of 5 isolates and group 3 isolates separated into 2 subgroups (3a and 3b) (Fig. 13).

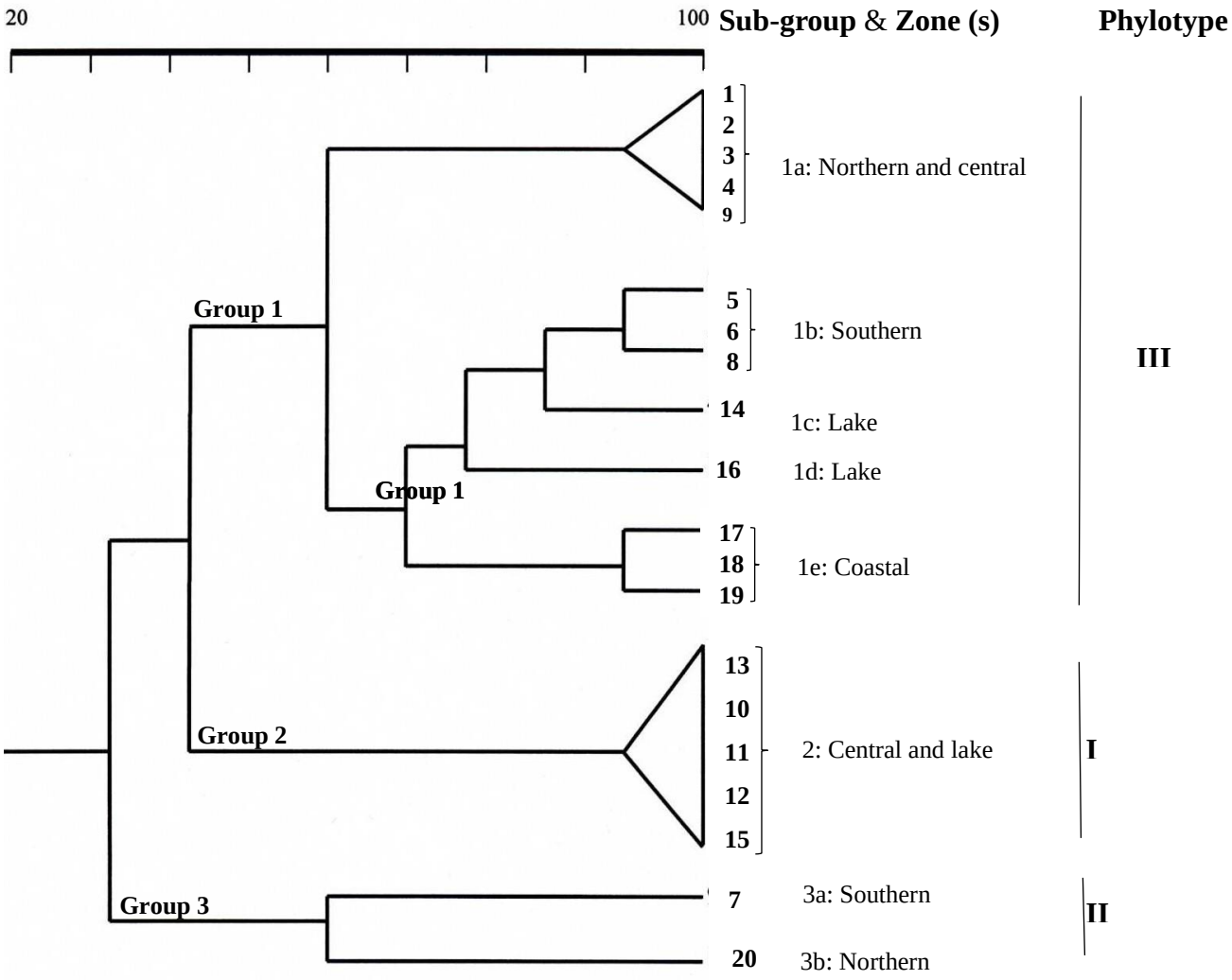

Figure 13: Phylogenetic relationship of *Ralstonia solanacearum* species complex in Tanzania

(ii) Comparison of isolates with standard strains in the National Centre for Biotechnology Information (NCBI) database

Identity of RSSC isolates collected in Tanzania was compared with standard strains in the NCBI database through BLAST (Fig. 14). Maximum (100%) query cover was obtained with a degree of identity from 98.67 to 100% (Table 21).

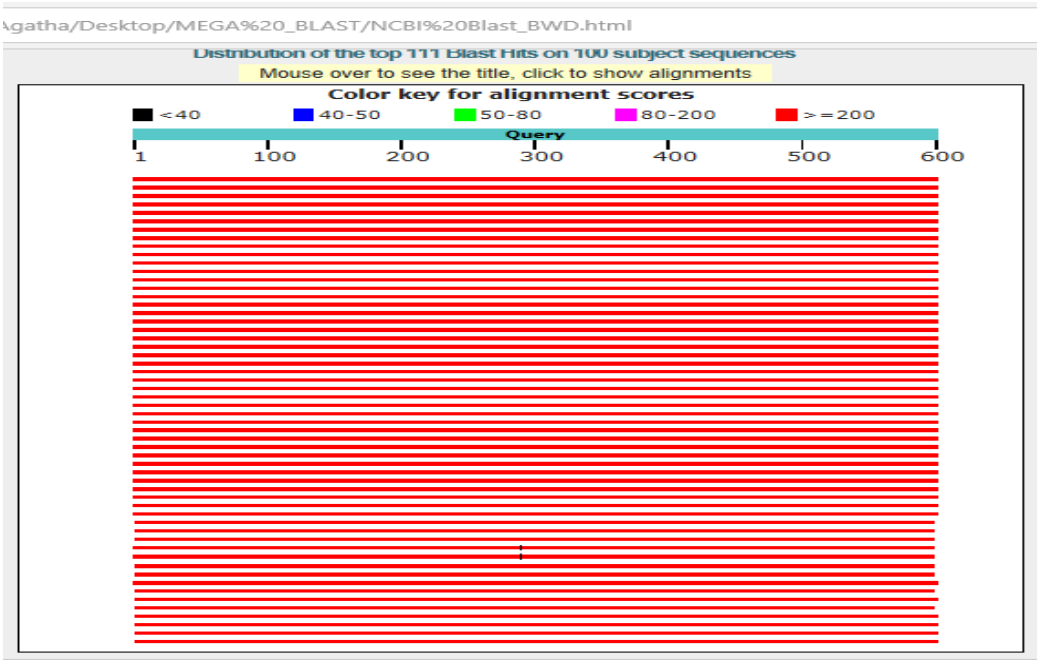

Figure 14: Graphical summary of identity of *Ralstonia solanacearum* species complex isolates in Tanzania with standard strains in the national centre for biotechnology information (NCBI) database (www.ncbi.nih.gov/BLAST)

Table 21: Similarity index of the Tanzanian isolates of Ralstonia with standard strains in the national centre for biotechnology information (NCBI)

Species	Strain	Max score	Total score	Query cover (%)	E value	Identity (%)	Accession
R. solanacearum	FJAT 1458	1110	2220	100	0.0	100	CP016554.1
R. solanacearum	SL2330	1103	2205	100	0.0	99.83	CP022794.1
R. solanacearum	SL3755	1103	2205	100	0.0	99.83	CP022782.1
R. solanacearum	T25	1103	2205	100	0.0	99.83	CP023014.1
R. solanacearum	T110	1103	2205	100	0.0	99.93	CP023012.1
R. solanacearum	SL2729	1064	2127	100	0.0	98.67	CP022792.1
R. solanacearum	SL3300	1064	2127	100	0.0	98.67	CP022786.1
R. solanacearum	SL3730	1064	2127	100	0.0	98.67	CP022784.1
R. solanacearum	SL3822	1064	2127	100	0.0	98.67	CP022780.1
R. solanacearum	SL3882	1064	2127	100	0.0	98.67	CP022778.1
R. solanacearum	T42	1064	2127	100	0.0	98.67	CP022772.1
R. solanacearum	T60	1064	2127	100	0.0	98.67	CP022768.1
R. solanacearum	T78	1064	2127	100	0.0	98.67	CP022765.1

www.ncbi.nih.gov/BLAST

4.1.6 Effect of selected pesticidal plants on managing the bacterial wilt disease-causing pathogen under *in-vitro* and screen-house environments

(i) Antibacterial effect of selected pesticidal plant species to reduce the *in-vitro* growth of *Ralstonia solanacearum* species complex

The antibacterial effect of extracts of some pesticidal plant species to inhibit the growth of RSSC was as shown in Fig. 15.

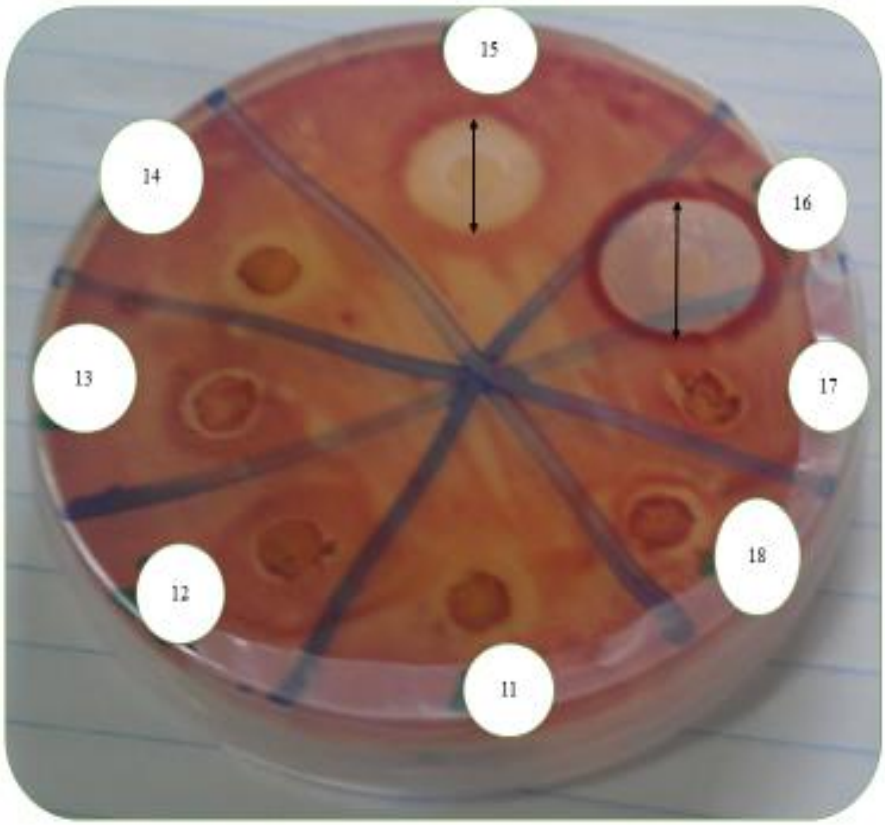

Figure 15: Comparative inhibition zone produced by selected common botanical extracts (11-18) against growth of the most predominating isolate of *Ralstonia solanacearum* species complex in Tanzania

13 = *Ocimum* spp., *15 = C15*= Patent application ID Number G190916-2061, 16 = Ampiclox antibiotic and 17 = *Allium sativum*

The growth inhibitory effect of RSSC significantly ($p \leq 0.05$) differed with plant species (Table 22). The plant extract *C15* was the most highly inhibiting botanical plant species by producing an inhibition zone of 19.25 mm and was as effective as Ampiclox (20.58 mm) followed by *Allium sativum* (17.20 mm). *Moringa oleifera* (6.59 mm), *Jatropha curcas* (7 mm) and *Bidens pilosa* (7.41 mm) were the least effective plant species.

Table 22: Effect of different plant extracts on *in-vitro* growth of *Ralstonia solanacearum* species complex

Common Name	Scientific Name	Part used	Inhibition zone (mm)
Bitter leaf	*Vernonia* spp.	Leaf	8.05d
Ocimum	*Ocimum* spp.	Leaf	11.50c
Coffee	*Coffea* spp.	Bean	11.01c
Alovera	*Aloe vera*	Leaf	8.83d
Garlic	*Allium sativum*	Bulb	17.20b
Lemon grass	*Cymbopogon citratus*	Leaf	8.57d
Plant C15	Patent application ID Number G190916-2061		19.25a
Black jack	*Bidens pilosa*	Leaf	7.41de
Neem	*Azadrachta indica*	Leaf	9.00d
Onion	*Allium cepa*	Bulb	12.19c
Ginger	*Zingiber officinale*	Rhizome	12.47c
Clove	*Syzygium aromaticum*	Bark	12.42c
Black pepper	*Piper nigrum*	Seed	6.10e
Artemisia	*Artemisia* spp.	Leaf	9.53d
Moringa	*Moringa oleifera*	Leaf	6.59e
Pawpaw	*Carica papaya*	Seed	12.33c
Hot pepper	*Capsicum annuum*	Seed	9.72d
Jatropha	*Jatropha curcas*	Leaf	7.00de
Tumeric	*Curcuma longa*	Bark	8.65d
Guava	*Psidium guajava*	Leaf	10.34d
Ampiclox (positive control)			20.58a
DW (negative control)			0.00
Mean			**10.89**
F-test			****
LSD			**3.84**

** = Significant at $p \leq 0.01$ and means inhibition zone with different letter(s) are significantly different based on LSD test ($p = 0.05$), DW = Distilled water

(ii) Effect of parts and forms of extracts of plant *C15* in growth inhibition of Ralstonia

Different extracts of plant *C15* reduced the growth of RSSC as measured by the zone of inhibition which differed significantly ($p \leq 0.05$) with extracts (Table 23). With respect to fresh form of *C15*, the methanolic extract of fruits was highly (24.03 mm) inhibitory to the growth of RSSC and was followed by ethanolic (22.07 mm) fruit extract. Also, significant differences of growth inhibition were recorded among the methanolic and ethanolic or distilled water extracts of leaf, fruit and root while no significant differences were recorded between the water and ethanolic extracts of dried leaf or root (Table 23).

Regarding the dried forms, the methanolic extract of fruit outperformed those of root and leaf methanolic extracts by recording the zones of inhibition of 22 mm, 17 mm and 14 mm, respectively (Table 23). In the case of extraction solvents used to dissolve both dried and fresh parts of plant *C15*, the fruit, leaf and root dissolved in sterile distilled water performed poorly in inhibiting the growth of RSSC as compared with those extracted in either methanol or ethanol (Table 23).

Table 23: Effect of forms and parts of plant extract *C15* dissolved in different solvents in reducing the growth of *Ralstonia solanacearum* species complex *in-vitro*

Form	Part: solvent	Inhibition (mm)	Part: solvent	inhibition (mm)	Part: solvent	Inhibition (mm)
Dried	Leaf: DW	7.02c	Fruit: DW	16.42b	Root: DW	11.02c
	Leaf: Ethanol	9.07c	Fruit: Ethanol	17.07b	Root: Ethanol	12.07c
	Leaf: Methanol	14.00b	Fruit: Methanol	22.00a	Root: Methanol	17.00b
	DW	0.00d	DW	0.00c	DW	0.00d
	Ampiclox	20.00a	Ampiclox	20.90a	Ampiclox	20.00a
	Mean	**12.52**		**19.09**		**15.02**
	F-Test	**		*		**
	LSD	**1.79**		**3.79**		**2.31**
Fresh	Leaf: DW	12.14c	Fruit: DW	19.42c	Root: DW	13.02c
	Leaf: Ethanol	13.00c	Fruit: Ethanol	22.07b	Root: Ethanol	16.07b
	Leaf: Methanol	18.33b	Fruit: Methanol	24.03a	Root: Methanol	21.00a
	DW	0.00d	DW	0.00d	DW	0.00d
	Ampiclox	20.33a	Ampiclox	20.67c	Ampiclox	20.00a
	Mean	**15.95**		**21.55**		**17.52**
	F-Test	**		**		*
	LSD	**1.39**		**1.82**		**1.27**

*, ** = Significant at $p \leq 0.05$ and 0.01 respectively, mean with the similar letter in a column are not meaningfully different based on least significant difference (LSD) test ($p = 0.05$) and DW = Distilled water

Of the three parts (leaf, root and fruit), the methanolic fruit extract was highly inhibitory and was followed by root and leaf methanolic extracts (Table 24).

Table 24: Effect of forms and parts of plant *C15* methanolic extracts on reducing Ralstonia growth

Form	Treatment	Zone of inhibition (mm)
Dried	Leaf: Methanol	14b
	Fruit: Methanol	22a
	Root: Methanol	17b
Mean		**18**
F – test		*****
LSD		**2.39**
Fresh	Leaf: Methanol	17b
	Fruit: Methanol	24a
	Root: Methanol	20b
Mean		**20**
F – test		*****
LSD		**4.01**

* = Significant at $p \leq 0.05$ and means with a similar letter in a column are not significantly different based on LSD test ($p = 0.05$)

(iii) Effect of concentrations of plant *C15* on inhibiting growth of *Ralstonia solanacearum* species complex

The mean zone of inhibition produced by various concentrations of methanolic extracts of plant *C15* differed significantly ($p \leq 0.05$) (Table 25). The undiluted (100% extract) highly inhibited the growth of RSSC by recording inhibition zone of 26.42 mm. It was followed by the 50% extract concentration that measured 24.06 mm while the least zone of inhibition of 12.94 mm was recorded in the 6.25% extract concentration (Table 25).

Table 25: Effect of fruit extract concentrations of plant *C15* in inhibiting the growth of *Ralstonia solanacearum* species complex

Dilution Juice: Methanol (v/v)	Concentration (%)	Mean of the zone of inhibition (mm)
1:6	6.25	12.94cd
1:4	12	15.02c
1:2	25	19.67b
1:1	50	24.06a
1:0	100	26.42a
DW 1ml		0.00e
Ampiclox 1:1DW(w/v)		20.42b
Mean		**12.83**
F-Test		*****
LSD		**2.18**

* = Significant ($p \leq 0.05$), means with different letter(s) in a column are significantly different based on least significant different (LSD) test ($p = 0.05$) and DW = Distilled water

(iv) Effect of plant *C15* on bacterial wilt disease management in screen-house

A similar trend of results was recorded for bacterial wilt disease severity on tomato seedlings treated with 100% of methanolic extract of plant *C15* and those untreated (Fig.16A-B). Severe wilting symptoms were observed in the untreated tomato seedlings while no wilting was reported on seedlings treated with the 100% of extract concentration of plant *C15*. A comparative assessment of the xylem vessels of stems of tomato seedlings untreated and treated with extracts of plant *C15* showed that, xylem vessels of treated plants were intact and well-arranged while those of untreated and wilted stems were malformed (Fig.16C-D). Among the treated seedlings with extract concentrations of plant *C15*, wilting symptoms varied with concentrations and severe wilting and plant death was reported with 0% i.e. untreated (Fig. 16E).

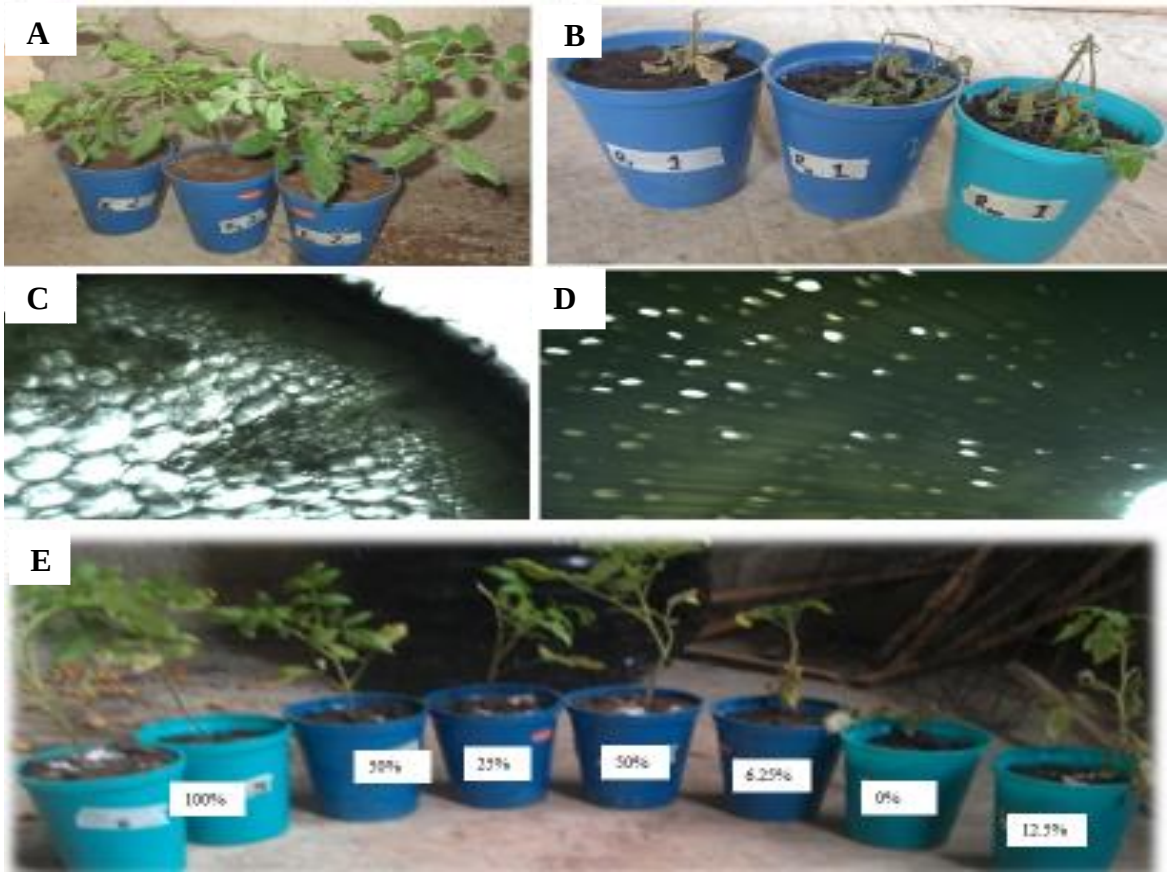

Figure 16: Effect of plant extract *C15* on bacterial wilt disease severity management

A = treated seedlings with 100% of plant *C15* extract, B = Untreated seedlings, C = Xylem vessels of treated seedlings with 100% of plant *C15* extract, D = Xylem vessels of untreated seedlings and E = Seedlings supplied with various concentrations of plant *C15* extracts to manage tomato bacterial wilt disease

Statistically, the 100% and 50% methanolic extract concentrations of *C15* significantly ($p \leq 0.05$) managed bacterial wilt disease of tomato at seedlings stage. Efficacy of bacterial wilt disease management was non-significant between 100% and 50% extract concentrations of plant *C15*. The 25% concentration reduced disease severity as compared with 12.5% and 6.25% which performed poorly and were statistically insignificant in managing bacterial wilt disease of tomato seedlings (Table 26).

Correspondingly, there was significant ($p \leq 0.05$) increase in root length and plant height of seedlings treated with 100% or 50% as compared with 25% and 12.5% of extract concentrations of plant *C15* (Table 26). While there was stunted growth of seedling supplied with 6.25% of the extract and untreated seedlings severely suffered from wilting (Table 26).

Table 26: Effect of fruit extracts of plant *C15* on bacterial wilt disease management and plant growth

Treatment (v/v)	Concentrations (%)	Severity (%)	Root length (cm)	Plant height (cm)
1:0	100	13d	24a	33a
1:1	50	15d	22a	31a
1:2	25	20c	21ab	27b
1:4	12.5	36b	20ab	26b
1:6	6.25	41b	18b	23c
Untreated		54a	7d	9d
F – test		**	*	*
LSD		**2.71**	**1.43**	**2.93**

*, ** = Significant at $p \leq 0.05$, *0.01* correspondingly, means in the same column with different letter(s) are significantly different according to LSD test at $p = 0.05$ and DW = distilled water

4.2 Discussion

The findings of this study have shown that BWD is a serious problem of tomato production affecting about 55% of farmers' fields surveyed in Tanzania. The ability of farmers to talk about BWD could indicate how economically important this disease is to them. The RSSC cells can be transmitted through soil, surface water, infected plant materials, insects, field-workers and cultural practices such as weeding and pruning or mono-cropping (Alvarez *et al*., 2010; Remenant *et al*., 2010; Yang *et al*., 2012). Under suitable environmental conditions such as temperature and relative humidity of > 29°C and 85% respectively, BWD development is accelerated through multiplication of the pathogenic cells (Alvarez *et al*., 2008; Jonathan *et al*., 2014). Higher incidence and severity of BWD disease in Arumeru as compared with other districts could be attributed to multiple factors such as soil and strain type, suitable temperature and relative humidity (Alvarez *et al*., 2010; Mrema *et al*., 2017).

Similarly, higher incidence and severity of BWD reported in screen-house than open field environments could be ascribed by increased temperature and relative humidity in the screen-house than in open field environments. Temperature range of 25-30°C enhances development of lateral roots in tomato and thus significantly increasing the entry points of *R. solanacearum* which increases disease incidence and severity (Agrios, 2005). This is unfortunate and a major challenge to farmers in developing countries such as Tanzania where in most cases farmers ventured in screen-house tomato production without adequate knowledge of BWD management (Mwaniki *et al*., 2017).

Respondents were affirmative that they ventured into the screen-house tomato production by imitating from neighbours without technical guidance. Allen *et al*. (2016) reported that adequate knowledge and technical guidance are key for successful management of BWD in screen-houses. Limitation in knowledge on key principles of BWD management can contribute to the increased disease incidence and severity (Qian *et al*., 2011), soils and water for example constitute a major transmission source of RSSC (Alvarez *et al*., 2008; Kinyua *et al*., 2014; Yuliar & Koki, 2015), but farmers in the study area never consider testing soils and irrigation water suitability for the tomato production i.e. soil and water to be free from RSSC.

Due to inadequate knowledge, farmers in the study area were unaware of the effect of temperature and relative humidity on BWD development in the screen-house instead they associate BWD with a cold-weather. By maintaining unfavourable environmental conditions for BWD such as temperature (< 25°C), relative humidity (< 80%) and soil (pathogen free) in screen-house, the BWD incidence and severity can be decreased (Alvarez *et al*., 2010; Mbega *et al*., 2012). This could be achieved by developing appropriate screen-house structures to allow proper air circulation, irrigation and drainage systems.

Moreover, farmers indicated inadequate understanding about the importance of using quality seeds and seedlings, immediately uprooting infected plants and discarding debris, disinfecting contaminated tools, identifying sources of RSSC, using foot bath and important of disease outbreak prediction and prevention program as good practices recommended by the extension and plant health regulation officers. Weeding is important as some weeds such as *Solanum dulcamara*, *Ipomoea* sp. and *Portulaca oleraceae* are hosts of RSSC that remain asymptomatic (Pradhang *et al*., 2005) which aid in long term survival of the pathogen in the environment. Destroying plant debris at the end of a cropping season can starve RSSC from nutrition and habitation hence protection of the subsequent crop against the pathogens. All

these are important and hence farmers should be guided and research is needed to understand how farmers could be empowered with needed skills for improved BWD management.

Although the majority (70%) of farmers used chemical pesticides, they reported the chemicals to be ineffective. One of the explanations for this could be limitation in farmers' knowledge and use of inappropriate chemicals or inappropriate dosses, resistance by the pathogens and planting susceptible tomato cultivars (Ngowi *et al*., 2007; Aloyce, 2013). Apart from being ineffective, use of chemicals as pesticides have been claimed to be environmentally unsafe (Yuliar & Koki, 2015). Some of the alternative methods for BWD management include use of botanicals, resistant/tolerant varieties, microbes and proper sanitation measures. Farmers reported to use some plant extract to manage BWD however, they were unsure of their effectiveness. None of the farmers reported using resistant varieties or biological control agents. Information on performance of specific methods used by farmers to manage BWD was still limited in Tanzania. Thus this study lays a foundation of the pathogen current status for further steps towards disease management.

The study findings of RSSC characterization revealed that the causing pathogens of tomato BWD are diverse in Tanzania and could be among the causes of variations of disease incidence and severity in the study area. The pathogen population differ with geographical locations in terms of virulence, pathogenesis, biovar and phylotypic traits of isolates. The virulent and avirulent and pathogenic and non-pathogenic (Kelman, 1954; Agrios, 2005; Champoiseau & Momol, 2008; Rahman *et al*., 2010) groups were encountered in different surveyed main agro-ecological zones. The non-pathogenic isolates can be classified as saprophytic despite of comparable colony appearance with RSSC on TTC medium. Avirulent strains of RSSC have a potential antagonist effect on virulent strains (Yuliar & Koki, 2015) and hence can be explored as microbial-based bactericide for BWD management. In this study, a number of avirulent isolates have been found. Such findings open a window for further research towards exploring these isolates for development of microbial based bactericides.

Biovars differentiation based on the oxidation of carbon sources as described by Hayward (1964) and He *et al.* (1983) reported that biovar 1 oxidizes hexose alcohols only, biovar 2 oxidizes only disaccharides, biovar 3 oxidizes both disaccharides and sugar alcohols and biovar 4 oxidizes alcohols only (Fig. 17). The RSSC utilize different carbon sources for their maintenance and growth (Dhital *et al*., 2001). This study found that 90% of isolates from the

infected tomato stems belong to biovar 3 while 10% belong to biovar 2 of RSSC. One isolate SIK4 behaved similar with the potato isolate XYZ and they belong to biovar 2 (2T) of RSSC. Biovar 3 isolates were reported from the infected samples collected from all the surveyed agro-zones while biovar 2 was found in infected tomato samples collected from the Southern agro-ecological zone in Tanzania.

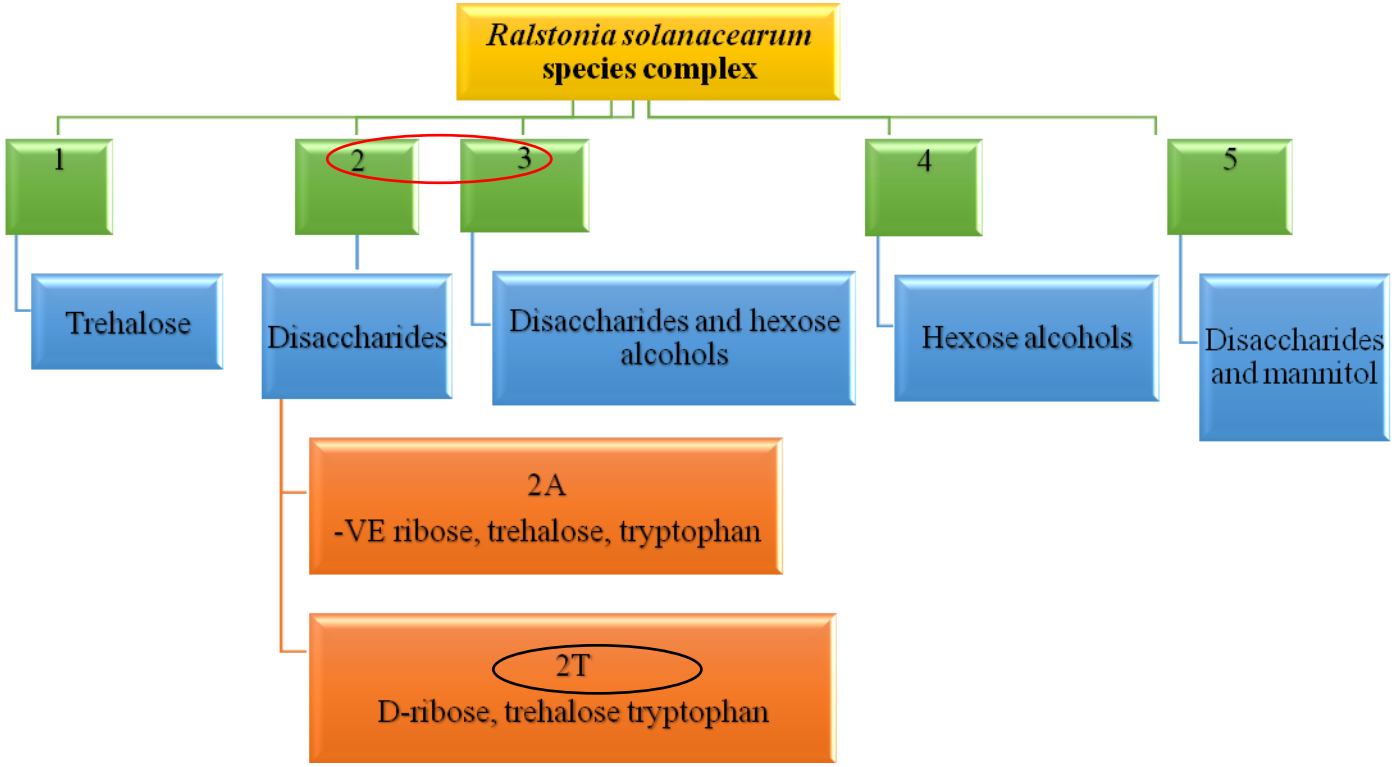

Figure 17: Biovars of Ralstonia, types of carbon sources they utilize and biovars associated with tomato bacterial wilt in Tanzania

To the best of the acquaintance as of this study, this is the first report of the prevalence of biovar 2 of RSSC in Tanzania, and hence it is an alarm to plant health regulators to design and implement stronger standard phytosanitary procedures to avert the new and/or spread of the disease to other geographical locations. The RSSC biovar 2 infects both tomato and potatoes and thus considered of relatively more economic importance than biovar 3 which infect tomato only. The prevalence and survival of biovar 2 in the Southern agro-ecological zone of Tanzania could be associated with the continuous cultivation of potato (Alvarez *et al*., 2010) and thus farmers should be guided on plant protection practices such as crop rotation to discourage survival of RSSC persistence in the environment.

In the present study, various identification methods of RSSC were used and from the findings it can be construed that, the TTC medium as a detection and identification method was less accurate despites of being simple and less costly. This is because certain saprophytic bacteria with comparable colony characteristics as RSSC can curtail the accuracy of TTC medium test. Conversely, the pathogenicity test enhanced the identification results and is recommended by the European and Mediterranean Plant Protection Organization (EPPO) as justified by the findings of this study that all the isolates which produced characteristic wilting symptoms in pathogenicity test were confirmed as RSSC with the most consistent modern technique of PCR. In addition, results for some carbon sources such as dextrose were obtained within 1 h and this could be providing potential information in developing a quick diagnosis kit of RSSC especially for the developing countries such as Tanzania.

Regarding the phylogenetic relationship, results indicated a considerable genetic variation among the isolates according to the agro-ecological zones. For instance, isolates belonging to phylotype III were found from all the agro-ecological zones except the Central agro-ecological zone. Phylotype I isolates was found in Central and Lake zones while phylotype II was found to prevail in the Southern and Northern zones. Based on the present study and literature, two major species and three phylotypes of RSSC namely Pylotypes I and III of *R. pseudosolanacearum* and Phylotype II of *R. solanacearum* prevail in Tanzania (Fegan & Prior, 2005; Safni *et al*., 2014; Prior *et al*., 2016) indicating that there exist a huge genetic diversity of RSSC infecting tomato in Tanzania.

In linking the phylotyping and biochemical results of this study, biovar 2 isolates of RSSC have been described from different geographical locations globally as the most homogenous group and form one group by rep-PCR analysis (Smith *et al.*, 1995; Poussier *et al.*, 1999).

However, in the present study, isolates of biovar 2 were sub-grouped into 3a and 3b at about 62% level of similarity. These isolates formed two sub-groups contrary to Smith *et al.* (1995) and Poussier *et al.* (1999). This discrepancy with the earlier results may be caused by the presence of genetic transformation of isolates implying that they have undergone certain genetic modification(s) and thus a need for epidemiological surveillance to update the knowledge of pathogen characteristics for effective and sustainable disease management with time and location. The fact that most of the researches were conducted primarily on isolates collected from conserved phytopathogenic bacterial collections may also decrease the real population reflection.

Unlike biovar 2, biovar 3 isolates has been indicated to be heterogenous and isolates may be clustered into two groups (Poussier *et al*., 1999) or in five groups (Horita & Tsuchiya, 2001). The cluster analysis assembled biovar 3 strains in six subgroups (1a-1e, and 2) at approximately 95% level of similarity indicating their phylogenetic relationship.

Similarly, results indicated that group 1 and 2 could be clustered together while group three formed another cluster at about 30% level of similarity which agreed with previous research (Cook *et al.*, 1989; Taghavi *et al*., 1996). Cook *et al.* (1989) and Taghavi *et al.* (1996) identified two major genetically distinctive divisions of RSSC strains namely group one comprising all isolates of biovars 3-5, and suggested to be Asian in origin (Asiaticum) and group two included all isolates of biovars 1-2, and proposed to American in origin (Americanum).

Therefore, biovar 2 and 3 isolates reported in this study could be classified in the Americanum and Asiaticum divisions respectively. Relating the Ralstonia isolates collected from the infected tomato sample in Tanzania with the standard strains in NCBI database revealed that the isolates of RSSC found in Tanzania are closely aligned to those available in the NCBI database. Therefore, it is a collaboration avenue for scientists across the world in designing effective management strategies against BWD. The variation of isolates of RSSC reported in the present study has major implications in developing effective and sustainable management strategies for BWD and thus should be considered as valuable. For instance, specific management strategies for disease should be developed based specific characteristics of the pathogen prevailing in a given location as pathogen isolates differed significantly with locations and therefore management strategies may as well be different.

To manage tomato BWD, farmers use several approaches including the use of synthetic chemical pesticides but the majority reported that chemicals are ineffective. Pathogen characteristics such as genetic complexity/genetic transformation and/or resistant to pesticides could have contributed to the failure of synthetic chemicals to manage BWD. Some of the alternative strategies of BWD management used by farmers were such as crop rotation and botanicals. Despite the fact that farmers are using some pesticidal plants to manage BWD, they could not tell about their effectiveness justifying the need for research.

In the present study, out of the twenty extracts of plant species screen against the predominating isolate of RSSC in Tanzania, plant extract *C15* (Patent Application ID Number G190916-2061) was identified as the most effective extract of plant species. The plant extract *C15* strongly reduced the growth of Ralstonia as indicated by the inhibition zone measurement and further by the suppression of bacterial wilt disease severity in the screen-house experiment. It is anticipated that El-Ariqi *et al*. (2005) and Abo-Elyousr and Asran (2009) reported on several plant species with varying levels of antibacterial properties against the growth of RSSC. Finding of the present research revealed that plant *C15* extract outperformed some common botanical plant species such as *Allium sativum* and *Ocimum* spp. that were previously reported to have strong antibacterial activity against RSSC.

These common plant species were included in the comparative research due to differences in strains (Hayward, 1991; Yuliar & Koki, 2015), as prevalence of different strains in specific geographical location may lead to different results, hence necessitating for multi-locational studies. The differences in antibacterial activity of different plant species could be one of the reasons for the strong antibacterial activity of plant extract *C15*.

In this study methanolic and ethanolic extracts of plant *C15* provided higher inhibitory activity compared to extract of water. Inhibition zone of Ralstonia growths increased by approximately 51% in the 100% as compared with the 6.25% methanolic extract concentration of plant *C15*. Methanol and ethanol are used in the extraction process to facilitate extraction of a wide range of phytochemical compounds both polar and non-polar (Ju & Howard, 2003, Sultana *et al*., 2009).

However, smallholder farmers may hesitate to use commercial solvents in extraction process as water is their preference because of economic and environmental viability (Mkenda *et al*., 2015). Therefore, there is need to explore mechanism(s) on how to improve the water

extraction process of phytochemical compounds in plant materials for the interest of smallholder farmers to improve technology adoption (Karani *et al*., 2017; Harouna *et al*., 2019) and develop quantity of raw materials required to manage BWD per unit area. Such activities are pending until a patent and intellectual property rights clearances are completed.

In the screen-house experiment, no wilting was reported on tomato seedlings treated with plant extract *C15* while the untreated seedlings wilted and died. The management of BWD severity recorded in seedlings treated with 100% and 50% concentration of plant extract *C15* is attributed to more antibacterial activity as compared with those treated with < 50% of concentrations. The findings of the current study are consistent with those of Bang *et al*. (2003) who indicated using undiluted extract concentration is recommended for maximum disease management. Application of 50% and 100% extract concentrations of plant *C15* increased plant height and root length which could be attributed to better water and nutrient uptake and enhanced photosynthesis due to reduced bacterial infection. Further studies of the extract are needed to establish mechanism of action of plant extract *C15* and whether other than inhibiting growth of the pathogen the plant extract *C15* could have plant growth promoting role.

Contrarily, there was a decrease in plant height and length of the root in plants treated with 6.25 to 25% concentrations of plant *C15* and was attributed to poor water and nutrient uptake because of the root deformation caused bacterial cells (Kinyua *et al*., 2014). The infected plants exhibit wilting symptoms and ultimately die because of poor nutrient and water uptake due to malformation and blockage in the xylem vessels by bacterial cell. The RSSC as many other soil-borne pathogens are known to affect water and nutrient uptake due to the bacterial cells blocking the xylem systems (Vasse *et al*., 1995; Prior *et al*., 2013). Well-functioning xylem vessels would lead to better water uptake, hence a vigorous plant growth. From the economic and environmental perspectives, plant *C15* can be a viable strategy for both farmers and the innovator by the developing an eco-friendly bio-bactericide product which is cost-effective but yet realizing innovator(s)' royalty towards BWD management.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Findings from this study have quantified that bacterial wilt disease is indeed a challenge of tomato production in Tanzania, the disease is spread in the surveyed main agro-ecological zones and management methods used by farmers are ineffective.

Population of RSSC causing tomato BWD is diverse in Tanzania. Two species, three phylotypes and two biovars of tomato BWD-causing pathogens were detected prevailing in different main agro-ecological zones of the country. The detected isolates were phylotypes I and III of biovar 3 of *Ralstonia pseudosolanacearum* and phylotype II of biovar 2 of *Ralstonia solanacearum*. The biovar 2 and phylotype II found in this study are reported for the first time in Tanzania. Such findings are an alert to tomato growers and plant protection experts to propose appropriate measures to policy makers that can minimize spread or new introduction of new pathogen of tomato in Tanzania.

Farmers' knowledge regarding BWD was found inadequate and this could be one of the variables that challenge disease management in the surveyed main agro-ecological zones. There is a need to train farmers on BWD and how to manage it in Tanzania.

The plant extract *C15* was earmarked to potentially manage BWD after indicating significantly high effectiveness against the predominating isolate of RSSC in Tanzania. The patent development and bactericide formulation from plant extract *C15* work are on-going.

5.2 Recommendations

The study findings have revealed that BWD is spread in all the main agro-ecological zones at significantly varying disease incidence and severity with considerable diversity of the causing pathogen(s) call for tomato stakeholders in Tanzania to combine efforts on how they can minimize the BWD levels in tomato fields by developing effective management options.

Due to the first report of biovar 2 and phylotype II of RSSC in Tanzania, there is a need for plant health regulators globally to implement strong standard phytosanitary measures to avert disease introduction or spread to uninfected areas.

The study used one primer pair for the phylogenetic analysis there is thus a need to validate the results with other primer systems by using the modern sequencing methods. It is also proposed to extend the characterization of the identified phylotypes of RSSC in Tanzania into sequevars based on nucleotide variations found in the partial sequencing of endoglucanase (*egl*) gene to enhance knowledge on RSSC in Tanzania.

Plant extract *C15* earmarked is of immediate interest to the author of this thesis to develop a bactericide-based product that is cognizant in terms of cost-effectiveness for use by smallholder farmers to manage BWD and yet income realization for the innovator. A patent application has been made and once approved, the products to be developed from plant *C15* is highly recommended for managing BWD in Tanzania.

Field experiments to validate results of the present study are recommended in future and similar study is recommended to understand the effect of plant extract C15 in managing biovar 2 of RSSC since this study focused on RSSC biovar 3 because of its wide distribution in the tomato agro-ecological zones of Tanzania.

Further research is recommended for the avirulent strains of RSSC identified to be characterized for developing a microbial-based product against the virulent isolates of BWD-causing pathogens in Tanzania.

Future research is recommended on how the knowledge of farmers on BWD and its management can be enhanced so that they venture in tomato farming without threat of BWD.

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APPENDICES

Appendix 1: Study questionnaire

Agro-ecological zone: ……. District…… Ward ……..

Open field/Screen - house …… Date……....

NO	SECTION A: FAMERS' INFORMATION	CODE
1	Name of the farmer:	ID. No.
2	Age of the farmer:……..(years)	
3	Total number of people in the household (including yourself)	
4	Gender of the farmers: 1. Female 2. Male	
SECTION B: SOCIO-ECONOMIC AND DEMOGRAPHIC CHARACTERISTICS		
5	Type of the household? 1. Female-headed 2. Male headed	
6	Number of years gone to school……..	
7	Occupation of the head of the household? a) Farmer, b) Self-employed, c) Paid employed, d) Casual labour and e) Others (Mention)	
8	Main sources of vegetables for the household a) Own farm b) Buying from market	
9	What is the most important vegetable crop for you? Why?	
10	Do you grow a tomato? Yes/No.	
11	Main occupation a) Subsistence farming b) Pastoralist c) Employment d) Business 5. other (specify)	
12	Who owns the land? a) My Self b) Government c) Landlord d) Village authority e) others (mention)	
SECTION C: BACTERIAL WILT DISEASE AND MANAGEMENT METHODS USED BY FARMERS		
13	Where do you commonly grow your tomato a) Open field b) Screen-house	
14	Did you attended any training on tomato production?	
15	Are you aware of the principles of tomato production in the screen – house structures? Where did you learn from?	
16	What is the source of planting materials (seeds or seedlings)?	
17	Do you sterilize soils for raising your seedlings?(Yes/No)	
18	What is the source of irrigation water for your tomato?	
19	Do you consider knowing the suitability of soil and water for tomato production? (Yes/Know). If Yes, what do you do to know if soil or water is suitable for tomato production?	
20	Do you conduct practices such as pruning and stacking for your tomato? Yes/No.	
21	Do you routinely sterilize the pruning tools? Yes/No. Why?	
22	What irrigation method do you use?	
23	What consideration do you take when irrigating your tomato as plant disease management is concerned?	
24	What do you do with the tomato debris after harvest?	
25	Do you know bacterial wilt disease? (Explain what you know about tomato bacterial wilt).	
26	How is BWD pathogens spread in the environment?	
27	In the last season, where you or your neighbour challenged by BWD?	
28	What did you do to manage BWD?	
29	What management methods did you use?	
30	Which management method do you commonly use? a) Chemicals, b) Crop rotation, c) Botanicals (Mention name, how much and how long) d) Resistant/tolerant varieties, e) Antagonists and f)None	
31	For each method (Mention name(s) of product, how much, when applied and for how long). What are the challenges in using such approaches?	
32	How can the spread of BWD minimize in the environment?	

Appendix 2: Consent statement

I read the above information or it was read to me. I had the chance to talk to the researcher about this research, and I had her answer my questions in a language that I comprehend.

I agree to participate in this research.

_____ _____

Farmer's name and signature / thumb print **Date**

_____ _____

Researcher's name and signature **Date**