BACTERIAL LEAF SPOT OF SWEET PEPPER CAUSED BY *XANTHOMONADS*: INCIDENCE, PATHOGEN CHARACTERIZATION, EPIDEMIOLOGY AND MANAGEMENT OPTIONS

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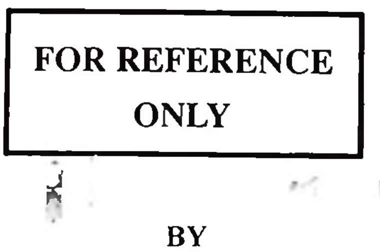
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BY

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A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHYLOSOPHY (PhD) IN AGRICULTURE OF SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

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ABSTRACT

A study was conducted in sweet pepper growing regions (Morogoro, Arusha, Tanga and Mbeya) in Tanzania to determine incidence and epidemiology of bacterial leaf spot disease (BLS), characterize bacterial spot-causing xanthomonad (BSX) strains and screen for resistance of the locally available sweet pepper varieties. One hundred sweet pepper fields were surveyed and diseased samples were collected for laboratory analysis. Bacterial isolates were identified based on physiological, biochemical, PCR and pathogenicity tests. Races were determined based on compatible or hypersensitive response on differential sweet pepper near-isogenic lines ECW-10R, ECW-20R and ECW-30R. Results indicated that, BLS disease was wide-spread in farmers' fields in the surveyed regions. Disease incidence ranged between 10 – 100 %, while the overall mean disease incidence was 69.3 %. Disease severity was statistically significant on village basis and the overall mean score was 4.6. High mean disease incidence (93.3 %) and disease severity score (6.5) were recorded in Lukozi and Kivulul villages, respectively. The lowest mean disease incidence (12 %) and severity (1.2) were recorded in Utengule village. Poor cultural practices and epiphytic survival of BSX on host and non-host plants were found to be the sources of inocula for successive crops. The RST2/RST3 primer sets detected 59 strains to genus level and the effector/avirulence gene primer sets detected 68 out of the 74 strains tested. The species-specific primer sets identified 63 out of 68 BSX to species level and were pathogenic on the susceptible cultivar Early Calwonder (ECW). The BSX were *X. euvesicatoria* (30), *X. perforans* (10) and *X. gardneri* (23). *Xanthomonas euvesicatoria* and *X. perforans* dominated in Tanga region whereas *Xanthomonas gardneri* dominated in Arusha region. Five strains were not pathogenic on cv. ECW. Seven sweet pepper races (P0 - P6) were identified. Race P3 (50 %) dominated

the strains of BSX in Morogoro, Arusha and Tanga regions. Race P6 (27.9 %) dominated in Tanga and Morogoro regions. Frequencies of races P0, P1, P2, P4 and P5 were considerably low. All the locally available sweet pepper varieties were susceptible to BLS disease. This is the first report to characterize BSX of sweet pepper in Tanzania.

DECLARATION

I, MAGDALENA NCHAGWA MAGERE WILLIAM, do hereby declare to the Senate of Sokoine University of Agriculture that, this thesis is my own original work, and has never been submitted, nor concurrently being submitted in any other institution.

23/11/2011

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The declaration above is confirmed by

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DEDICATION

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LIST OF ABREVIATIONS AND SYMBOLS

A_fSHC	African Seed Health Centre
ANOVA	Analysis of Variance
ARI	Agricultural Research Institute
ATPase	Adenosine triphosphate
avrBs	avirulence/effector gene in the pathogen corresponding to the resistance gene in the plants
AVRDC	Asian Vegetable Research and Development Centre
bp	base pair
BSX	Bacterial leaf spot- causing xanthomonads
cfu	colony forming unit
CRD	Complete randomized design
cv	Cultivar
CV	Coefficient of variation
^{o}C	Degree Celsius
DMRT	Duncan's Multiple Range Test
DNA	Deoxyribonucleic acid
dNTPase	Deoxynucleotide triphosphate enzyme
DRD	Director for Reseach and Development
DSHC	Danish Seed Health Centre
ECW	Early Calwonder
e.g	Example
EPPO	European and Mediterranean Plant Protection Organization
ERIC	Enterobacterial repetitive intergenic consensus

et al	et alei (and others)
FW	Forward
h	Hour
HCl	Hydrochloric acid
$HgHCl_2$	Mercury hydrochloride
hrp	Hypersensitive response and pathogenicity gene
HR	Hypersensitive reaction
IDM	Integrated Disease Management
i.e	That is
K	Potassium
kb	Kilobase
LSD	Least Significant Difference
KOH	Potassium hydroxide
MAbs	Monoclonal antibodies
MAFSC	Ministry of Agriculture, Food Security and Cooperatives
$MgCl_2$	Magnesium chloride
ml	Millilitre
min	Minutes
mm	Millimetre
m^2	Meter squared
N	Nitrogen
NA	Nutrient agar
NaOH	Sodium hydroxide
NCPPB	National Collection for Plant Pathogenic Bacteria
NYDA	Nutrient yeast dextrose agar

O/F	Oxidative fermentative metabolism of glucose
P	Phosphorous
P0 – P6	Sweet pepper races
PCR	Polymerase chain reaction
PI	Plant Introduction line
pv	Pathovar
REP	Repetitive extragenic palindromic
rep-PCR	Repetitive polymerase chain reaction
RFLP	Restriction fragment length polymorphism
R	Resistant
RNA	Rebonucleic acid
rpm	Rotations per minute
RV	Reverse
SAR	Systemic acquired resistance
SAS	Statistical Analysis System
SDW	Sterile distilled water
SDS-PAGE	Sodium dodecyl sulphate-polycrylamide gel electrophoresis
SE	Standard error
Sec	Second
SEM	Scanning electron microscope
SPSS	Statistical Package for Social Sciences
SUA	Sokoine University of Agriculture
TAE	Tris acetate ethylene diamine tetra acetic acid
Taq	Thermus aquaticus
T3SS/TTSS	Type III protein secretion system

T1 – T3	Tomato races 1 - 3
UK	United Kingdom
U.S.A	United States of America
UV	Ultraviolet
v	Volts
v/v/v	Volume per volume per volume
w/v	Weight per volume
XVT	*Xanthomonas vesicatoria* from tomato
YDC	Yeast dextrose carbonate agar
≤	Less than or equal to
μl	Microlitre
%	Percent
®	Trade name

CHAPTER ONE

1.0 INTRODUCTION

Bacterial leaf spot of sweet pepper (*Capsicum annuum* L.) is incited by a group of bacterial spot-causing xanthomonads (BSX) namely *Xanthomonas euvesicatoria, X. vesicatoria, X. perforans* and *X. gardneri* (Jones *et al.*, 2004). These pathogens were previously known as *X. campestris* pv. *vesicatoria* (Dye *et al.*, 1980) and more recently as *X. axonopodis* pv. *vesicatoria*. Separation from *X. vesicatoria* was based on amylolytic activity and other characteristics (Bouzar *et al.*, 1994; Jones *et al.*, 1995; Jones *et al.*, 1998; Obradovic *et al.*, 2004; Vauterin *et al.*, 1995). Bacterial spot-causing xanthomonads are seedborne. Infected seed, which is one of the major sources of the primary inoculum and a determinant of successful disease dissemination through seed distribution systems, has serious implications on sweet pepper production (Aysan and Sahin, 2003; Berke *et al.*, 2003; Obradovic *et al.*, 2004).

Bacterial leaf spot is one of the most economically important diseases of sweet pepper all over the world, especially in areas of high temperature and rainfall (AVRDC, 2004; Canteros *et al.*, 1995; Jones *et al.*, 1995; Jones *et al.*, 2000; Kousik and Ritchie, 1996; Obradovic *et al.*, 2004; Pernezny and Collins, 1997; Sahin and Miller, 1996; Vauterin *et al.*, 1995). The disease reduces plant growth, fruit yield and quality and it is characterized by necrotic lesions that occur on leaves, stems, petioles, flowers and fruits (Park *et al.*, 2007; El-Hendawy *et al.*, 2005; Jones *et al.*, 1998; Nesmith and Hartman, 2004). Severe infection has been reported to cause defoliation, resulting in reduced yield and pre-disposes fruits to sun scald (Aysan and Sahin, 2003; Berke *et al.*, 2003; Obradovic *et al.*, 2004; Pernezny and Collins, 1997). However, the main economic effect of this disease is

the reduction in fruit weight and quality. In Tanzania, disease incidence was reported to be in the range of 10 – 100 % during the year 2009. In Turkey, disease incidence has been reported to range from 50 – 95 % (Aysan and Sahin, 2003) and fruit losses due to bacterial leaf spot was reported to reach up to 100 % in Illinois (Babadoost, 1988: McGrath, 1997).

It is not clear when the disease was first identified in Tanzania. However, field surveys conducted in the country in 1997 and 1998 showed that the disease was widespread in tomato and sweet pepper fields in all the vegetable-growing regions in the Northern and Southern highlands (Black *et al.,* 2001). Other surveys conducted in Arusha, northern Tanzania in 1998 – 2000 (Kaaya *et al.*, 2003) confirmed the wide spread occurrence of bacterial leaf spot disease in the region. Furthermore, Shenge *et al.*. (2007) reported the existence of *X. axonopodis* pv. *vesicatoria* on tomato from the major vegetable growing regions of Tanzania. The economic impact of the disease on sweet pepper production in Tanzania has not been assessed.

For many years, the disease-causing organism was considered homogeneous (Jones *et al.*, 1998). However, in the 1990s, the bacterium was found to be composed of two genetically and phenotypically distinct groups (A and B), which Vauterin *et al.*, (1995) renamed as *X. axonopodis* pv. *vesicatoria* (group A) and *X. vesicatoria* (group B). Two other groups of (BSX) pathogenic on sweet pepper and tomato were successively described by Jones *et al.*, (2000) and assigned to groups C and D. Furthermore, Jones *et al.*, (2004) demonstrated that, groups A, C and D have less than 70 % DNA relatedness with each other, with the type strain *X. axonopodis* and *X. vesicatoria*. For these reasons, they renamed groups A, C and D as *X. euvesicatoria*, *X. perforans* and *X. gardneri*, respectively, whereas, group B remained as *X. vesicatoria*.

Genetic resistance in the host plant has been reported to offer the most economical and long–lasting solution to disease problems. However, the prevalence of races makes it difficult to manage this disease based only on host resistance. Furthermore, it does happen that, more virulent emerging strains of the pathogen compromise the genetic basis of such resistance (Gore and O'Garro, 1999; Kousik and Ritchie, 1998). Various investigations have shown that, there is wide degree of variation within BSX (Gore and O'Garro, 1999; Jones *et al.*, 2000; Jones *et al.*, 2004; Kousik and Ritchie, 1996; Obradovic *et al.*, 2004; O'Garro *et al.*, 1999; Shenge *et al.*, 2007; Stall *et al.*, 1994; Vauterin *et al.*, 1995). Several physiological races of BSX infecting pepper only (pepper strains), tomato only (tomato strains) and both sweet pepper and tomato (pepper-tomato strains) have been reported (O'Garro *et al.*, 1999).

Currently, there are eleven sweet pepper races that have been differentiated on the basis of resistance conferred by four single dominant genes Bs1, Bs2, Bs3 and Bs4 (; Hibberd *et al.*, 1987; Obradovic *et al.*, 2004; O'Garro *et al.*, 1999; Sahin, 2001; Sahin and Miller, 1998). These genes were identified in four different plant introduction (PI) lines: PI 163192 (*C. annuum*), PI 260435 (*C. chacoense*), PI 271322 (*C. annuum*) and PI 235047 (*C. pubescens*). Such diversity within this group of organisms causing bacterial spot disease of sweet pepper has complicated precise identification of the strains and made management strategies difficult.

Since phytopathogenic xanthomonads have been known to manifest a high degree of host specificity, knowledge of existing variation within the existing populations of the pathogen in any given environment is both exigent and expedient (Jones *et al.*, 1998). Considering the fact that, the occurrence and the economic importance of the bacterial leaf

spot disease on sweet pepper in Tanzania has not been established, therefore, this study aimed at investigating the status of the disease in the country. Since the pathogen infect both sweet pepper and tomato, it is important to have knowledge of existing variation within the pathogen, which is the first step in the development of host plant resistance. It is also important to determine if the sweet pepper seeds that farmers obtain through the various distribution outlets have a contributory effect in the spread of the pathogen. The study also assessed the level of resistance to BSX in locally available sweet pepper varieties in order to identify suitable varieties that may be recommended to farmers or for inclusion in breeding programme for disease resistance.

1.1 Objectives

1.1.1 Overall objective

The overall objective of the current study was to investigate the etiology, epidemiology, disease incidence of bacterial spot-causing xanthomonads and provide information for management of bacterial leaf spot disease of sweet pepper in Tanzania.

1.1.2 Specific objectives

i). To determine the incidence and epidemiology of bacterial spot on sweet pepper in Morogoro, Arusha, Tanga, and Mbeya regions

ii). To characterize *Xanthomonas* strains associated with bacterial spot disease of sweet pepper in Morogoro, Arusha, Tanga, and Mbeya regions

iii). To classify races of bacterial spot-causing *Xanthomonas* strains present in the study area

iv). To screen for resistance to bacterial leaf spot disease in locally available sweet pepper varieties.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Discovery, Distribution and Importance of Bacterial Leaf Spot

Bacterial leaf spot disease was first observed on tomato plants as early as 1914 in South Africa and later in 1921. The causal agent was originally identified as *Bacterium vesicatorium* (Jones *et al.*, 1998; Jones *et al.*, 2000). At approximately the same time, a similar disease on tomato and sweet pepper was described in the United States and referred to as bacterial leaf spot (Jones *et al.*, 1998). The disease was also described in Florida, Georgia, Indiana, Sudan and Korea (Gardner and Kendrick, 1923) and bacterial leaf spot is now reported to be worldwide-distributed (Jones *et al.*, 1998: Jones *et al.*, 2000; Vauterin *et al.*, 1995) (Figure 1). Significant losses on fruits, particularly in warm and humid environment have been reported to reach up to 100 % in Illinois (Babadoost, 1988; Bouzar *et al.*, 1994; McGrath, 1997; Vauterin *et al.*, 1995).

2.2 Symptomatology

All stages of sweet pepper growth are susceptible to bacterial spot disease and all portions of the plant may show symptoms. Infected plants in the seedbed usually have small, irregular, black or water-soaked spots along the edges of the first leaves. Older sweet pepper plants develop small, circular to irregular, pale green or water-soaked

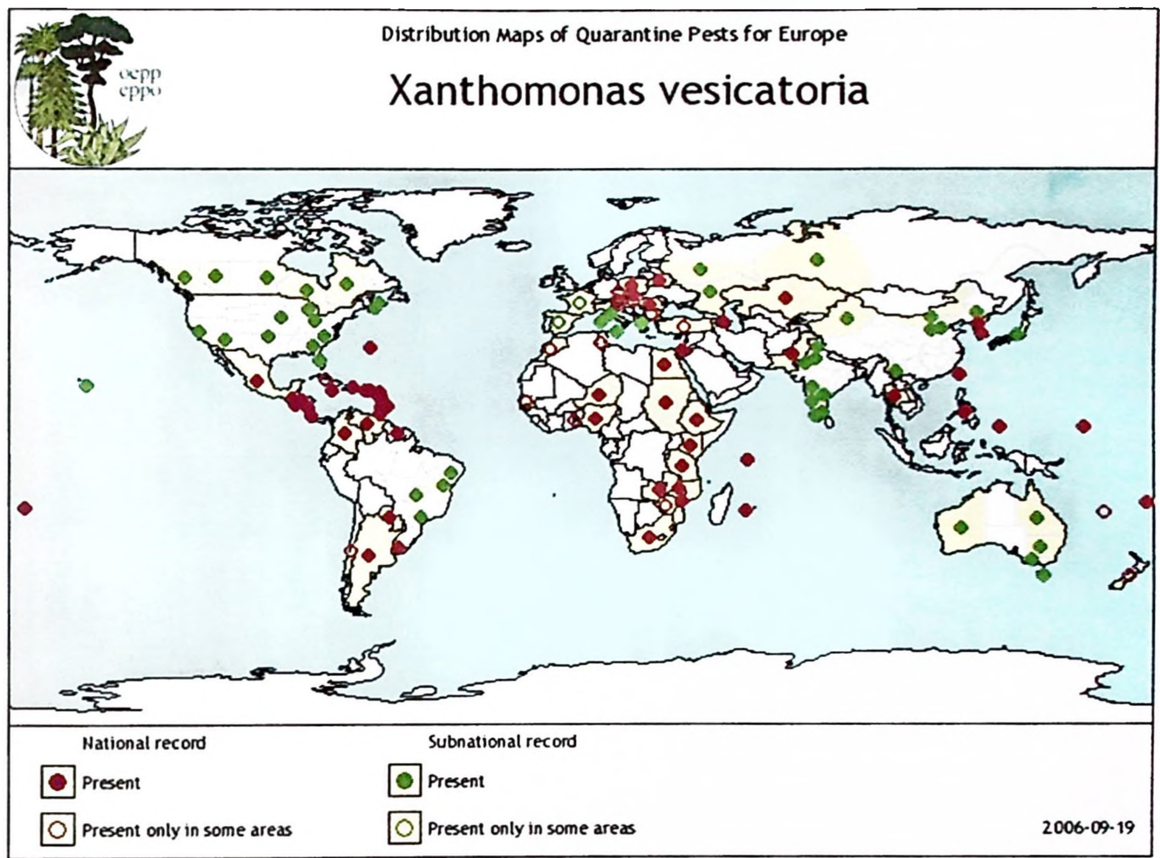

Figure 1: Distribution maps of bacterial leaf spot-causing xanthomonads in the world

Source: European and Mediterranean Plant Protection Organization (EPPO), (2006).

lesions that are sunken on the top surface and slightly raised on the lower surface. The lesions on leaves are usually smaller than 3 mm in diameter (sometimes larger in hot, humid conditions). When numerous lesions occur, they coalesce causing necrotic areas, and give the plants a blighted appearance. Affected leaves may turn yellow and drop off or become dry and remain on the plant. Some leaves may drop while still green and severe infections can result in defoliation of the plant. Young infected seedlings may lose all but their topmost leaves (AVRDC, 2004; Obradovic *et al.*, 2004). Stem and petiole spots are small, oval and raised. Flower infection results in severe blossom drop. On young fruit, spots are conspicuous, blister-like, roughly circular, small spots reaching a diameter of 2 –

3 mm. The spots on fruits are initially pale green, later turn brown with raised, coarse, wart-like surfaces (AVRDC, 2004; Babadoost, 1999) (Plates 1a and 1b).

Plate 1a: Bacterial leaf spot symptoms on sweet pepper leaves collected from farmers' fields

Plate 1b: Severely defoliated pepper plant with bacterial leaf spot symptoms on leaves, stem and fruit in one of the farmer's field in Luale village, Morogoro region

2.3 Etiology

2.3.1 Morphology of *Xanthomonas axonopodis* pv. *vesicatoria*

Xanthomonas axonopodis pv. *vesicatoria* is a Gram-negative, rod-shaped bacterium, 0.6 – 0.7 x 1.0 – 1.5 µm and occurring singly or in pairs. The cells are motile by means of one flagellum. The bacterium is obligately aerobic and produces yellow, smooth or viscid colonies on nutrient agar (Plate 2a) and a characteristic zone of lipolytic activity on Tween medium. Starch hydrolysis and pectolytic activity are variable (Plate 2b). The optimum temperature for growth is 25 – 30 °C (Aysan and Sahin, 2003; Bouzar *et al.*, 1994; Jones *et al.*, 1998; Vauterin *et al.*, 1995).

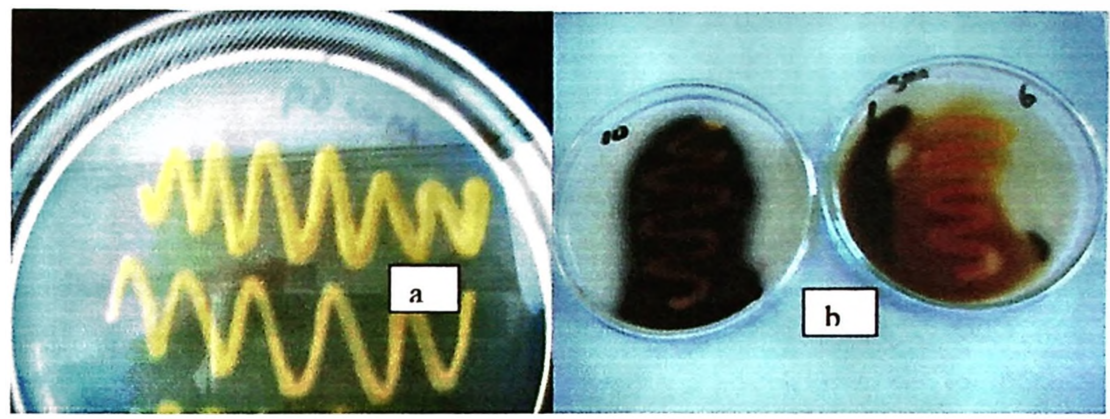

Plate 2 (a): Cultures of bacterial leaf spot-causing xanthomonads on nutrient agar medium. (b). Left: Negative starch hydrolysis test; right: Positive starch hydrolysis test

2.3.2 Races of *Xanthomonas* strains of sweet pepper

For over 70 years (since the organism was originally identified in 1921), *X. axonopodis* pv. *vesicatoria* was thought to be a relatively homogenous organism (Jones *et al.*, 1998). The variation within the pathogen was differentiated by physiological, biochemical and pathological characteristics. However, various investigations have shown that, there exists a remarkable degree of variation within the group of xanthomonads pathogenic on pepper and tomato (Jones *et al.*, 1998; Jones *et al.*, 2004; Kousik and Ritchie, 1996; Obradovic *et al.*, 2004). While strains of *X. axonopodis* pv. *vesicatoria* isolated by Doidge (1921), were feebly amylolytic, those isolated by Gardner and Kendrick (1921) strongly hydrolyzed starch. The investigation that was done by Vauterin *et al.* (1995) has shown that, within the group of xanthomonads pathogenic on pepper and tomato, four distinct phenotypic groups, A, B, C and D exist. Groups A (pepper) and B (tomato) strains are genetically and

phenotypically distinct, thus classified them as *X. axonopodis* pv. *vesicatoria* and *X. vesicatoria*, respectively.

The two groups can easily be distinguished reliably using rep-polymerase chain reaction (rep-PCR), genomic fingerprinting (Jones *et al.*, 2004; Louws *et al.*, 1995), and have limited genetic diversity within themselves. Furthermore, the two groups also differ phenotypically according to utilization of cis-aconitic acid, reaction to monoclonal antibodies (MAbs), hypersensitive reaction on host plant, amylolytic and pectolytic activity, and whole cell protein profiles in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Jones *et al.*, 2004; Bouzar *et al.*, 1994a; Bouzar *et al.*, 1994b). The third species (C group), *X. perforans* which causes symptoms on both pepper and tomato was originally described in 1957 by Sutic, and the pathogen is related to but distinct from the A group strains. Group D strains (*X. gardneri*) are phenotypically and genotypically distinct from the other three groups (Jones *et al.*, 2004) and cause lesions on both pepper and tomato.

Numerous strains of the bacterial spot pathogen have been identified on the basis of their pathogenic reaction on pepper and tomato genotypes (Bouzar *et al.*, 1994a; Bouzar *et al.*, 1994b; Minsavage *et al.*, 1990). According to Jones *et al.* (1998) and O'Garro *et al.* (1999), there were several emerging physiological races of *X. axonopodis* pv. *vesicatoria* infecting pepper only (designated pepper strains), tomato only (designated tomato strains) and a few are pathogenic on both pepper and tomato (pepper-tomato strains).

Currently, eleven races (designated P0 to P10) of *X. axonopodis* pv. *vesicatoria* virulent on pepper have been distinguished on the basis of the hypersensitive response (HR) using

'Early Calwonder' (ECW) plants (with no known genes of resistance) and the near-isogenic lines (ECW-10R, ECW-20R and ECW-30R). The near-isogenic lines contain one of the three single resistance genes (Bs1, Bs2 and Bs3, respectively) to bacterial leaf spot (Bouzar *et al.*, 1994b; Jones *et al.*, 1998; Kousik *et al.*, 1996; Minsavage *et al.*, 1990; Obradovic *et al.*, 2004; Sahin and Miller, 1995) and *Capsicum pubescens* a plant introduction line (PI) 235047, which carries a major resistance gene Bs4 (Aysan and Sahin, 2003; Obradovic *et al.*, 2004; Sahin and Miller, 1998; Wangsomboondee, U*npublished*).

Early Calwonder cultivar is susceptible to all pepper races, whereas the near-isogenic lines: ECW-10R is resistant to races 0, 2, and 5, ECW-20R is resistant to races 0, 1, 2, 3, 7 and 8, and ECW-30R is resistant to races 0, 1, 4, 7 and 9 (Aysan and Sahin, 2003; Kousik and Ritchie, 1998; Obradovic *et al.*, 2004; Sahin, 2001; Sahin and Miller, 1998). The PI 235047 is resistant to races 0, 1, 3, 4 and 6. Strains previously designated as race 6 but that defeat a major resistant gene (*Bs*4) in PI 235047 are designated as race 10 (Aysan and Sahin, 2003; Obradovic *et al.*, 2004; Sahin and Miller, 1998; Wangsomboondee, *unpublished data*). In addition, races P7 to P10 were discovered as a result of the recently identified resistance gene in PI 235047 (Sahin, 2001; Wangsomboondee, *unpublished data*).

Races 1 and 2 were first identified and reported in the United States in 1969 (Cook and Stall, 1969) and in Brazil in 1972 (Kimura *et al.*, 1972). Cook and Stall (1982) characterized pepper races in a worldwide collection and determined that; race 1 was widely distributed and continues to be the prevalent race in certain locations where as race 2 was restricted to Florida and Guadeloupe. Race P0 was originally detected in North

Carolina (Ritchie and Dittapongpitch, 1991) and has since been identified in Mexico (Bouzar *et al.*, 1996), Brazil, Oklahoma (Bouzar *et al.*, 1994) and the Barbados (Jones *et al.*, 1994). Race P3 is the prevalent race in Ohio and it has been found in other locations including Florida and the Caribbean (Sahin and Miller, 1996), Taiwan (Kousik and Ritchie, 1996) and Korea (Lee *et al.*, 1999).

Race P4 first identified in Australia (Hibberd *et al.*, 1989) has also been observed in South-eastern United States (Kousik and Ritchie, 1995), the Barbados and Florida (Jones *et al.*, 1994). Races P5 and P6 were identified in the United States (Kousik and Ritchie, 1995; Sahin and Miller, 1996; Sahin and Miller, 1998). Races P7 and P8 discovered within races P1 and P3 were first identified in Ohio, USA (Sahin, 1997) and were also reported in Turkey (Sahin, 2001). Races 9 and 10 were discovered within races P4 and P6 (Aysan and Sahin, 2003; Wangsomboondee, U*npublished*). However, a limited collection of isolates from Africa revealed only tomato races (Jones *et al.*, 1998).

The races known so far, and which have been characterized and classified are not stable due to the fact that: (a) avirulence genes in the pathogen interacting with resistance genes in the hosts result in existing pathogenic variation in xanthomonads pathogenic to pepper and tomato, (b) the avirulence genes in the pathogen are subject to mutation or loss, and (c) many of the avirulence genes are located on self-transmissible plasmids that can be gained naturally by conjugation between bacterial strains (Canteros *et al.*, 1995; Jones *et al.*, 1998; Kousik and Ritchie, 1996). Canteros *et al.* (1995) suggested that, as more resistance genes are identified in the host and additional strains are tested, more races of xanthomonads pathogenic on pepper and tomato will be identified.

2.3.3 Genotypic variation within bacterial spot-causing xanthomonads of pepper

Recent advances in molecular biology have made possible the genotypic and molecular characterization of several phytopathogenic bacteria. The application of these techniques has proved to be a fast and efficient way of detecting and identifying bacterial pathogens in infected plant materials.

The value of these techniques is based on the fact that, symptoms on the host plant are not always a reliable method of identifying disease, given that, symptoms may vary from host to host, and also due to environmental influences. Moreover, isolation and purification on traditional media may be difficult because the bacterial pathogen might not grow at all on these media (Lopes and Damann, 1994). Therefore, alternative approaches that are rapid, accurate and reliable must be found.

Some of the commonly used molecular techniques for determining genetic variation include the restriction fragment length polymorphism (RFLP) analysis, various types of the polymerase chain reaction (PCR) and DNA-DNA hybridization.

2.3.3.1 Restriction Fragment Length Polymorphism (RFLP) analysis

The technique involves the high resolution analysis of amplified genomic DNA using restriction enzymes that are specific for particular segments of the gene. By employing this technique in the differentiation of representative strains of *X. axonopodis* pv. *vesicatoria* with two different rare cutting enzymes, followed by phylogenic analysis, Stall *et al.* (1994) observed that strains formed two distinct clusters, which corresponded to the A and B phenotypic groups of *X. axonopodis* pv. *vesicatoria.*

In another study, representative strains of the A and B groups of *X. axonopodis* pv. *vesicatoria* were compared with C strains and *X. gardneri* (Jones *et al.*, 2004, Roberts *et al.*, U*npublished*). Based on phylogenetic analyses of strains, the C strains and *X. gardneri* formed clusters which were distinct from those in which the representative A and B strains fell. It was, thus, shown through the use of RFLP analysis that at least four major genetic groups exist within *X. axonopodis* pv. *vesicatoria*.

Employing the same technique, Leite *et al.* (1995) amplified genomic DNA from the *hrp* gene cluster of representative *X. axonopodis* pv. *vesicatoria* with the aim of assessing variation within the clusters of *X. axonopodis* pv. *vesicatoria* strains. The amplification was done using oligonucleotide primers specific for different regions of the *hrp* gene cluster. The PCR products were digested with restriction enzymes, followed by phylogenetic analyses. The resulting analyses placed the A and C strains in a cluster distant from the B group strains. This technique has been shown to be highly efficient, fast and reliable in the identification of *X. axonopodis* pv. *vesicatoria* strains.

2.3.3.2 The use of repetitive elements (polymerase chain reaction- PCR)

These techniques are based on the use of naturally occurring interspersed repetitive DNA elements in phytopathogenic bacteria as oligonucleotide primer binding sites for genomic DNA amplification and fingerprinting (Versalovic *et al.*, 1994). Genomic fingerprinting with interspersed repetitive sequence-based probes has been used to distinguish unrelated organisms on the basis that bacterial strains vary with respect to the distances between the repetitive sequences (Mitrev and Kovačević, 2006; Versalovic *et al.*, 1994).

Three families of repetitive sequences have been recognized that are unrelated at the DNA sequence level. These are the 35-40 bp (base pair) repetitive extragenic palindromic (REP) sequence, the 124-127 bp enterobacterial repetitive intergenic consensus (ERIC) sequence, and the 154 bp BOX element (Versalovic *et al.*, 1994). Nucleotide sequence determination of these repetitive regions has enabled the design of PCR primers specific to each region. Accordingly, PCR with these primers can give rise to amplification products that reflect the number and distribution of repetitive sequences. This approach to genomic fingerprinting (referred to as rep-PCR) represents a highly sensitive, efficient and reliable tool for the identification of bacterial pathogens to the pathovar and (in some cases) strain level (Louws *et al.*, 1994).

Louws *et al.* (1994, 1995) tested several strains of *X. campestris* pv. *vesicatoria* using rep-PCR, in order to determine genetic variation between them. The strains fell into four groups, with the majority falling into two groups that corresponded closely with the two major groups (A and B) within *X. campestris* pv. *vesicatoria* identified by Stall *et al.*, (1994). From a study of fingerprinting pattern generated by rep-PCR analysis, Louws *et al.*, (1995) noted that, group A was relatively homogenous, whereas group B formed six patterns, and was therefore, more heterogenous. Obradovic *et al.*, (2004) also used PCR to characterize *X. campestris* pv.*vesicatoria* strains from pepper and tomato in Serbia. They reported that the banding patterns generated following restriction enzymes *HaeIII* digestion of the PCR amplification product of the *hrp* region were identified to those of the groups A and C representative strains.

Although genomic fingerprinting is beginning to have an impact, most of the applications in phytobacteriology have to do with the design of primers that aid in pathogen detection

or diagnosis (Leite *et al*., 1995; Obradovic *et al*., 2004; Zacardelli *et al*., 2005). The detection protocols are especially ideal where the target bacteria cause diseases whose primary management strategy is exclusion or avoidance (Louws *et al*., 1995). By using specific primers combined with high sensitivity protocols, it is possible to support quarantine regimes that are able to implement zero tolerance of pathogens borne on/in seed, seed pieces or plant parts (Louws *et al*., 1995).

2.3.3.3 DNA-DNA Hybridization

This genomic technique involves the process of joining two complimentary strands of DNA or one each of DNA and RNA to form a double-stranded molecule. DNA-DNA hybridization techniques usually involve the use of DNA probes. Detection methods based on DNA probes are not as susceptible to interference by substances in plant specimens, and have more rapid turn-around times, when compared to PCR (Kuflu and Cuppels, 1997). Due to the fact that, probe detection limits are considerably higher than those of PCR (Kuflu and Cuppels, 1997), they are also less likely to give a false positive due to the presence of small amounts of DNA or non-viable cells (Deboer *et al*., 1996).

DNA- relatedness studies conducted over the years have contributed in increasing the understanding of the degree of variation within *X. campestris* pv. *vesicatoria* (Hildebrand *et al*., 1990; Ritchie and Dittapongpitch, 1991; Stall *et al*., 1994; Vauterin *et al*., 1995; Jones *et al*., 2000; Jones *et al*., 2004). Stall *et al*. (1994) compared 20 strains of *X. campestris vesicatoria* belonging to races T1 and T2 using DNA-DNA hybridization, and determined that, the strains formed two distinct genetic groups with less than 50 % DNA homology between groups and greater than 70 % within groups. The two groups of strains were phenotypically distinct. Thus, they were given phenotypic group designations in

which T1 and T2 strains were placed in groups A and B, respectively. These groups corresponded to the group designations used by Vaterin *et al.* (1993). Results of these and several other studies involving genomic techniques led many phytopathologists working with *X. campestris* pv. *vesicatoria* to suggest that, adjustments should be made in the classification of the pathogen to reflect the presence of homologies between the DNA characteristics of strains (Vauterin *et al.*, 1995; Bouzar *et al.*, 1996).

In another study that involved 52 geographically diverse bacterial spot-causing xanthomonad strains, Kuflu and Cuppels (1997), used a genomic subtraction technique with subtractor DNA from non-pathogenic epiphytic xanthomonads to enrich for sequences that could serve as diagnostic probes for the pathogens. In their study, a 1.75-kb *PstI-NotI* fragment (KK1750) that preferentially hybridized to *X. vesicatoria* DNA and *X. axonopodis* pv. *vesicatoria* DNA was identified. When they cloned the KK1750 fragment into pBluescriptII KS+, they found that, 46 of the 52 strains included in the study were hybridized. They also found that, six probe negative strains were genotypically and pathologically distinct from the other BSX strains studied. The results of all studies using this technique showed that, the technique is an effective method that is able to distinguish between pathogenic bacteria to the pathovar level (Kuflu and Cuppels, 1997).

2.3.4 Incidence of *Xanthomonas axonopodis* pv. *vesicatoria*

Xanthomonas axonopodis pv. *vesicatoria* has been reported in tomato and in pepper seeds (Bashan *et al.*, 1982; Gardner and Kendrick, 1921; Jones *et al.*, 1986; Sahin and Miller, 1998; Sijam *et al.*, 1991). The incidence was as high as 60 % on tomato, where as it was 5 % on pepper (Bashan *et al.*, 1982). Jones *et al.* (1986) recovered the pathogen from 53 commercial pepper seedlots and in one of 293 commercial tomato seedlots.

In a study in which fruit infection by *X. campestris* pv. *vesicatoria* was monitored by scanning electron microscope (SEM) and bacterial counts, possible sites for bacterial penetration were through dead flowers and proliferation of bacteria in the wart (small protuberance) area. Bacterial multiplication was observed in all parts of young, mature and ripened pepper fruits. Bacterial cells bound to the fruit surface by fibrillar material and multiplied in small aggregates, submerged in slime, which consisted mainly of sucrose units. At later stages of disease development, the slime covered the entire fruit surface in young fruits (Basham and Okan, 1986).

According to Bonas *et al.* (2000) and Thieme *et al.* (2005), bacterial leaf spot-causing *Xanthomonas* strains enter the plant tissue through stomata and wounds. Bacterial colonization of plant intercellular spaces is locally restricted and induces macroscopically visible disease symptoms, so-called water-soaked lesions that later become necrotic.

2.3.5 Host range

The principal hosts of bacterial spot-causing-xanthomonas strains are pepper (*Capsicum annum*, *C. frutescens*, and *C. pubescens*) and tomato (*Lycopersicon esculentum*). Various other members of the family Solanaceae, mainly weeds have been recorded as incidental hosts. They include: *L. pimpinellifolium*, *L. halimifolium*, *Solanum nigrum*, *S. rostratum*, *S. tuberosum*, *S. melongena*, *Datura stramonium*, *Physalis minima*, *Hyoscyamus niger*, *H. aureus*, *Nicandra physalodes*, *Lycium chinese* and *Argemone mexicana* (Dye *et al.*, 1964; Hayward, 1993; Jones *et al.*, 1998).

2.3.6 Survival

Bacterial leaf spot causing xanthomonads survive primarily in seeds, crop residues left on the soil after harvest, on volunteer pepper/tomato plants, rhizosphere of pepper/tomato and solanaceous weeds (Aysan and Sahin, 2003; Babadoost, 1988; Kousik and Ritchie, 1998; Mew and Natural, 1993; Pernezny and Collins, 1997). However, the bacterial spot organism has been reported to survive for periods of at least 10 years in the soil (Bashan *et al.*, 1982). Leben (1974) reported the bacterium as a resident organism which is often isolated from seedlings without any sign of infection on the cotyledons. In the glasshouse, seed-borne infection is the main source of inoculum. Less than 1 % seed-borne infection can lead to epiphytotics (Cox, 1966). In the field, dissemination occurs rapidly from diseased plants to healthy plants primarily by rain splash or by over-head irrigation (Blancard, 1997; Good and Sasser, 1980; Hibberd *et al.*, 1986; Obradovic *et al.*, 2004; Sahin and Miller, 1998). The pathogen has also been isolated from the soil (Blancard, 1997; Watterson, 1986). Isolation of the bacterium from seed indicates that, seed serves as a means of dissemination.

Bacterial leaf spot-causing xanthomonads have been reported on pepper and tomato seed (Bashan *et al.*, 1982b; Gardner and Kendrick, 1921, 1923; Jones *et al.*, 1986; Sahin and Miller, 1998). Information of the bacterium on weed hosts is not clear. Several authors reported that weed hosts were not hosts of the bacterial leaf spot pathogen (Jones *et al.*, 1986). Volunteer pepper plants are important sources of inoculum in pepper crops and crop residues may serve as an inoculum source for successive crops (Peterson, 1963). There is evidence that infected sweet pepper seed may be a source for introduction of new races of bacterial leaf spot-causing xanthomonads in areas where the pathogens do not occur (Aysan and Sahin, 2003; Pohronezny *et al.*, 1992). Race 2 of the pathogen infecting

pepper had been the prevalent race in Florida for over 20 years, where in 1988, approximately 90 % of the strains were race 2 and 11 % were race 3. From 1988 – 1990, 84 % of the pathogen strains were race 1 and less than 15 % were race 2.

2.4 Epidemiology

The source of inocula for primary infection is usually contaminated seed or transplants or carry-over inoculum in infested plant debris in the field (AVRDC, 2004; Blancard, 1997; Hibberd *et al.*, 1989; McGrath, 1997; Mew and Natural, 1993). Infection generally occurs through wounds, where the disease is favored by warm temperatures of about 25 °C or more, high humidity and heavy rains, and its spread is aided by driving rain and wind-blown debris and soil which cause mechanical injury to the leaves and fruits (Babadoost, 1988; Basim *et al.,* 2004; Blancard, 1997; Thieme *et al.*, 2005; Nesmith and Hartman, 2004) (Figure 2).

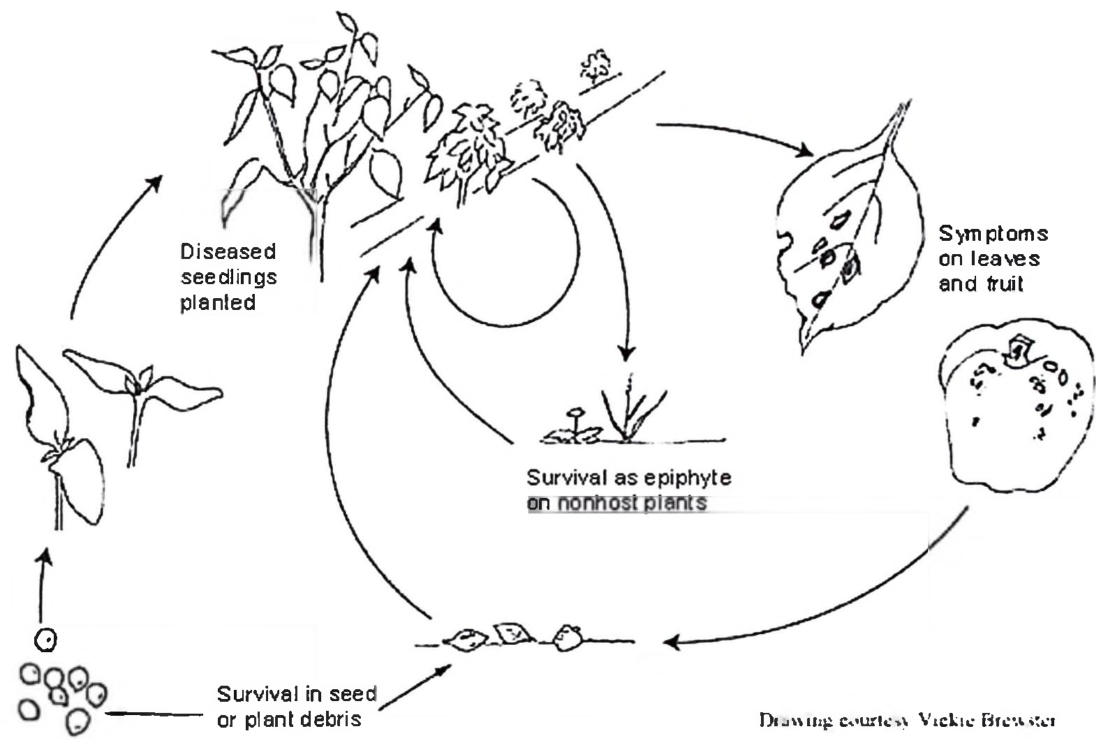

Figure 2: Disease cycle and epidemiology of bacterial leaf spot-causing xanthomonads, the causal agent of bacterial spot of sweet pepper. Drawing courtesy of Vickie Brewster
http://www.apsnet.org/education/lessonsPlantPath/BacterialSpot/images/cycle.jpg

2.5 Host-pathogen Interaction

The interaction of the gram-negative phytopathogenic bacterium *X. campestris* pv. *vesicatoria* with its host pepper and tomato is mediated by a type III protein secretion system (T3SS/TTSS) that translocates bacterial effector proteins into the plant cell. The T3S system which is highly conserved among plant and animal pathogenic bacteria is encoded by the chromosomal *hrp* (hypersentive response and pathogenicity) gene cluster (Bonas *et al.*, 1991; Büttener *et al.*, 2007; Leister *et al.*, 2005; Thieme *et al.*, 2005). The T3SS spans both bacterial membranes and is associated with an extracellular pilus-like appendage (He *et al.*, 2004; Koebnik, 2001). The secretion apparatus mediates the Sec-

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independent secretion of proteins into the extracellular milieu and the translocation of bacterial effector proteins into the host cell cytosol. Effector protein translocation depends on the presence of the T3SS translocon, which is probably inserted as a channel-like protein complex into the host plasma membrane (Büttener and Bonas, 2002b).

In natural infections, *X. axonopodis* pv. *vesicatoria* cells enter the plant through natural openings or wounds to reach the intercellular spaces of the tissue where they establish an intimate relationship with the plant cell. The pathogen remained localized in the intercellular spaces and does not invade the xylem (Bonas *et al.*, 2000; Büttener *et al.*, 2007; Thieme *et al.*, 2005). Pathogenicity of *X. axonopodis* pv. *vesicatoria* on susceptible pepper plants and the induction of HR on resistant plants requires a number of genes designated *hrp*, most of which are clustered in a 23-kb chromosomal region (Bonas *et al.*, 1991; Büttener *et al.*, 2007; Huguet and Bonas, 1997; Knoop *et al.*, 1991; Leister *et al.*, 2005). In susceptible plants, infection (compatible reaction) with a virulent strain of the pathogen leads to bacterial multiplication, giving rise to small water-soaked lesions, which later become necrotic. In the presence of a resistant gene (e.g. Bs3) in the plant, and a complementary avirulence gene (e.g. avrBs3) in the pathogen, a hypersensitive reaction is induced (incompatible interaction) (Bonas *et al.*, 2000; Knoop *et al.*, 1991; Thieme *et al.*, 2005). The HR is a local defense reaction accompanied by rapid and highly localized cell death reaction (necrosis) of the infected tissue, thus preventing multiplication of the bacteria in the infected region (Büttener *et al.*, 2007; Jones *et al.*, 2001; Leister *et al.*, 2005).

Xanthomonas axonopodis pv. *vesicatoria* has been recognized to employ two types of genes in its pathogenicity on susceptible pepper plants, and the induction of the

hypersensitive reaction on resistant hosts. These are *hrp* (hypersensitive reaction and pathogenicity) and *avr* (avirulence) genes (Bonas *et al.*, 1991; Büttener *et al.*, 2007; Leister *et al.*, 2005). The importance of the *hrp* genes has been demonstrated by the fact that, mutants are not only unable to establish a normal interaction with the plant, but they are also unable to grow in the plant tissue (Alfano and Collmer, 1997; Knoop *et al.*, 1991; Thieme *et al.*, 2005; Wengelnik *et al.*, 1999).

The large *hrp* gene cluster of *X. axonopodis* pv. *vesicatoria* contains 22 genes, nine of which encode proteins with similarity to components of type III protein secretion systems (Bonas *et al.*, 2000; Büttener *et al.*, 2007). Recognition of the pathogen by resistant host plants requires functional *hrp* genes, and is determined by a pair of corresponding genes, one for avirulence (*avr*) in the bacterium, and another for resistance (R) in the plant (Bonas *et al.*, 2000; Büttener *et al.*, 2007; Van den Ackerveken *et al.*, 1996). In line with this model, the *X. axonopodis* pv. *vesicatoria* avirulence gene *avrBs3* (a member of large family of highly homologous avr/pth (pathogenicity) genes in *X. axonopodis* pv. *vesicatoria*) specifically induces the HR in pepper genotypes with Bs3 gene (Bonas *et al.*, 1989; Knoop *et al.*, 1991). Proteins of the *avrBs3* family have been found to posses more than 10 nearly identical tandem repeats of 34 amino acids, each in the middle of the protein. The avrBs3 protein (122 kDa) contains 17.5 repeats, which have been shown to determine recognition specificity (Herbers *et al.*, 1992; Knoop *et al.*, 1991; Van den Ackerveken *et al.*, 1996).

Most *hrp* genes in *X. axonopodis* pv. *vesicatoria* are clustered in a 23-kb chromosomal region, and are organized in six operons (*hrpA*, *hrpB*, *hrpC*, *hrpD*, *hrpE* and *hrpF*) (Bonas *et al.*, 1991; Bonas *et al.*, 2000; Huguet and Bonas, 1997). Expression of *hrp* genes is

induced in the plant and in a particular minimal medium, XVM2 (Wengelnik *et al.*, 1996a). So far, two regulatory genes (*hrpX* and *hrpG*) which map outside the large *hrp* genes have been characterized (Huguet and Bonas, 1997; Wengelnik *et al.*, 1996). The bacterium *hrpG* protein shows similarity to the response regulatory proteins of the OmpR subclass of two component regulatory systems (Huguet and Bonas, 1997). The *hrpG*, which encodes a response regulator (OmpR-type) of two-component systems, is at the top of the cascade regulating the expression of *hrpA* and of *hrpX*, which in turn activates the other five known *hrp* loci, *hrpB* to *hrpF* (Bonas *et al.*, 2000; Huguet and Bonas, 1997; Wengelnik and Bonas, 1996). At least one more protein is predicted to be involved with this signal cascade, as the environmental sensor protein of the *hrpG* two-component system needed for sensing environmental stimuli has yet to be identified in *X. axonopodis* pv. *vesicatoria* (Wengelnik *et al.*, 1996b).

In addition to these genes, *X. campestris* pv. *campestris* (a close relative of *X. campestris* pv. *vesicatoria*) produces a number of enzymes needed for pathogenesis, including an endoglucanase protease, polygalacturonase lyase, amylase and lipase (Dow and Daniels, 1994). These proteins are not transported through the *X. campestris* pv. *campestris hrp* secretion system, but instead are secreted by a signal sequence-dependent mechanism encoded by a cluster of at least twelve genes (Dow and Daniels, 1994). A similar situation is also reported to be the case in *Erwinia* soft-rot bacteria for the secretion of pectolytic enzymes (Collmer and Bauer, 1994). Apart from the harpins and *Pseudomonas syringae* pv. *tomato hrpA*, *avr* proteins have also been suggested to represent a class of proteins that are secreted through the *hrp* system (Lindgren, 1997). Evidence for this view is the observation that when *avrBs2* were inactivated in *X. campestris* pv. *vesicatoria*, the resulting mutants were less virulent on susceptible pepper plants (Swords *et al.*, 1996).

The ability of *X. campestris* pv. *vesicatoria* to suppress papilla-formation in pepper through the secretion of protein factors has also been shown to be largely dependent upon functional *hrp* systems (Brown *et al.*, 1995).

2.6 Disease Management

Management of bacterial spot disease depends on a combination of practices including the adoption of cultural practices (the use of pathogen-free seed and transplants, crop rotation with non-host crops, field sanitation during rotation), use of resistant pepper genotypes, and chemical application to slow down the spread of disease (AVRDC, 2004; Hansen, 2000; Kuflu and Cupples, 1997; McGrath, 1997; Mew and Natural, 1993; Pernezny and Collins, 1997; Sahin and Miller, 1996; Sahin and Miller, 1998). However, it has been reported that, these practices are not always successful when environmental conditions are favorable for disease development (Sahin and Miller, 1998). Although the use of pesticides in agricultural practices is presently being discouraged (Stoll, 1998), pesticide application is still a useful option where bacterial leaf spot-causing xanthomonads have not developed resistance and when there is an urgent need to halt pathogen spread. However, copper compounds are not highly effective under environmental conditions optimal for disease development or when high inoculum levels are present (Sahin and Miller 1996; Sahin and Miller, 1998).

2.6.1 Cultural practices for managing bacterial leaf spot disease

The primary bacterial leaf spot disease management strategy is the use of cultural practices that can be integrated with sweet pepper crop management. Bacterial leaf spot disease can be managed by either removing the sources of inoculum or delaying and reducing the spread and development of the pathogen from the epiphytic (resting) stage to the infective

(pathogen) phases (Mew and Natural, 1993). The cultural methods of managing xanthomonads causing bacterial leaf spot include rotation with non-related crops and or new fields where possible, to avoid carryover of the inocula on volunteers and crop residues. Other cultural methods include the production of disease-free sweet pepper transplants raised in an area where sweet pepper and tomato production does not occur; seed treatment (with bactericides, hot water or other disinfectants) to reduce possible transmission of the bacterium, eliminating sweet pepper volunteers by cleaning fields periodically, and avoiding pepper cull piles near fields or greenhouses (CABI, 2005; Hansen, 2000; Kuflu and Cuppels, 1997; Sahin and Miller, 1996).

2.6.2 Resistant genotypes

Management of bacterial leaf spot by the development of disease resistant genotypes is the most efficient and environmentally friendly way to control the disease, as long as sufficient genetic variation for resistance is available (Hulbert *et al.*, 2001; Pernezny and Collins, 1997).

The main drawback of genetic resistance is that it takes a long time to develop the desired sweet pepper varieties. However, advances in molecular biology and biotechnology have enabled the molecular cloning of resistance genes, and the transfer of these genes between distantly related crop species. This development has considerably reduced the amount of time that would normally be spent in developing resistant genotypes by traditional breeding techniques. Another potential significance of interspecific gene transfer is that, plants are known to carry resistance genes which interact with avirulence genes from pathogens of the plant species (Cuppels *et al.*, 2006; Knoop *et al.*, 1991; Sahin and Miller, 1998; Wood *et al.*, 1994). This situation has been suggested to be the reason for the

existence of a significant portion of the hundreds of resistance gene sequences, which exist in plant genomes for which no function is known (Hulbert *et al.*, 2001). Many resistance genes have been demonstrated to function after they have been transferred transgenically to different but related species. Several resistance genes from the family Solanaceae have now been transferred successfully to another species of the same family (e.g. from tomato to tobacco), and have been demonstrated to be able to confer resistance to pathogens carrying the appropriate *avr* gene (Bendahmane *et al.*, 1999; Hammond-Kosack *et al.*, 1998; Jirage *et al.*, 1999; Tai *et al.*, 1999).

Another disadvantage of genetic resistance is that, its effects are often not durable as a result of genetic shifts in the pathogen population. In cases where resistance has remained stable and effective, the success of the resistance genes was attributed to the possible loss of the corresponding *avr* gene in the pathogen, rather than the effectiveness of the resistance genes. However, scientists are now beginning to target the resistance genes that are thought to interact with important or widespread *avr* genes. An example is the isolated Bs2 gene from pepper (Louws *et al.*, 2001; Tai *et al.*, 1999) whose cognate *avr* gene is very widespread in *Xanthomonas campestris* pathovars, and is also a virulence factor (Kearney *et al.*, 1990). Once these genes are successfully incorporated into commercially acceptable pepper genotypes, the durability of the resulting disease resistance would be reasonably guaranteed.

Generally, however, the dynamic nature of the pathogen populations demands that, their population studies are done regularly with a view to making an early identification of population shifts. Such a trend would then be followed up with breeding work to take care of the observed pathogen population shifts. However, none of the commercial cultivars are resistant to all known pepper races of bacterial leaf spot pathogen (Cuppels *et al.*, 2006).

2.6.3 Seed health and bacterial leaf spot disease management

The seeds of many agronomic crops are either infected or infested with xanthomonads. One of the major sources of the primary inocula for bacterial leaf spot disease is infected seed (Blancard, 1997; Kuflu and Cuppels, 1997; Mirik and Aysan, 2009; Sahin and Miller 1996; Watterson, 1986).

The role of seed as an important inoculum source of bacterial leaf spot-causing xanthomonads has been reported since 1920s. Gardner and Kendrick (1923) demonstrated the effectiveness of seed treatment with mercuric chloride in controlling what had been a serious bacterial spot problem in Indiana, USA. Peterson, (1963) discovered that, soil and tomato crop residues were also sources of inocula. This led to confusion as to the relative importance of seed-borne inoculum (Cox, 1982). However, the significance of seed-borne inoculum as a means of introducing and spreading the disease has been indicated by the random pattern of foci of infection noted in diseased fields that is consistent with seed-borne infection (Cox, 1966). There is also evidence that, infected pepper seed may be a source for introduction of new races of bacterial spot causing xanthomonads (Aysan and Sahin, 2003; Louws *et al.*, 2001; Pohronezny *et al.*, 1992; Sahin and Miller, 1996).

Infected seed has serious implications, because the seed-borne phase of the pathogen is not only a means of survival, but also a major determinant of successful pathogen dissemination. Therefore, seed health is an important aspect in the management of bacterial leaf spot disease. Suitable seed production procedures approaching to a zero tolerance are essential for the control of bacterial leaf spot disease (Gooder and Sesser, 1980). Isolation, crop rotation (2-3 years of rotation with non-hosts) and sanitation have been reported to be helpful in managing the disease. However, these cultural practices may

have a minimal impact when weather conditions for disease development are present (Pohronezny *et al.*, 1990). Various seed treatments that have been used to eradicate seed-borne infection include: 0.8 % acetic acid for 24 h, 5 % HCl for 5 – 10 h, 1.05 % sodium hypochlorite for 30 min and 0.05 % $HgHCl_2$ for 5 min. Hot water treatment at 56 °C for 30 minutes has also been recommended (EPPO, 1988).

In many countries, there is a strict enforcement of quarantine measures to restrict the entry of tomato/pepper germplasm that may be infected with bacterial leaf spot pathogens. Intra-national measures to exclude the pathogens from new areas include the strict enforcement of seed certification programmes.

2.6.4 Chemical application

Copper fungicides have been used in agriculture since early 1800's. Soluble copper ions are known to bind tightly to sulfhydryl groups, accounting for its biocidal properties. Free copper ions can penetrate through plant cuticles and cause severe phytotoxicity. Water-soluble copper salts ('fixed coppers') are the solution to this problem and has become the major chemical group for bacterial disease management. Some disadvantages of copper-based chemicals include phytotoxicity, reduced copper sensitivity among bacterial leaf spot-causing *Xanthomonas* strains (in some areas) and negative environmental impact. Copper ions are not degraded in the soil, and can accumulate to high levels at locations with a history of intensive copper application (Koller, 1998).

Chemical control originally relied on the application of streptomycin, an antibiotic, and copper compounds. However, streptomycin and copper compounds have been reported to lose their effectiveness due to the emergence of resistant bacterial leaf spot-causing

Xanthomonas strains (Martin *et al.*, 2004; Mirik *et al.*, 2007; Ritchie and Dittapongpithch, 1991; Sahin and Miller, 1996; Thayer and Stall, 1961, Ward and O'Garro, 1992). Marco and Stall (1983) reported the ineffectiveness of copper bactericides in some tomato production areas when used alone. However, it has been reported that, the addition of maneb or mancozeb fungicides to the copper bactericides enhanced efficacy (Canover and Gerhold, 1981; Marco and Stall, 1983). Thus, copper-mancozeb mixtures are now used for managing bacterial leaf spot of tomato and pepper. Bacterial leaf spot management using copper bactericides is not very effective due to poor spray coverage, incorrect timing of copper sprays, or both; the presence of bacterial strains tolerant to copper or favorable environmental conditions together with high disease pressure (Mirik *et al.*, 2007).

2.6.5 Integrated bacterial leaf spot management (IDM)

An integrated management program against bacterial leaf spot disease is a key factor for successful sweet pepper production. Two important approaches to reduce severity and incidence of bacterial leaf spot on pepper in the field have been reported (Louws *et al.*, 2001; Pernezny and Collins, 1997). These include reducing inoculum and minimizing plant susceptibility. Moreover, new environmentally friendly technologies can be utilized in IDM programmes as alternative management tools for bacterial spot. These technologies include a compound (Actigard) which induces systemic acquired resistance (SAR) (i.e. increasing natural defence mechanism of the existing commercial cultivars or minimizing susceptibility) and use of phages specific to the target bacterium (i.e. reducing inoculum on leaf and fruit surfaces) (Louws *et al.*, 2001).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Disease Surveys and Collection of Samples

A survey was conducted in farmers' fields to determine the current status of bacterial leaf spot disease in the major sweet pepper- producing regions (Tanga, Morogoro, Arusha and Mbeya) of Tanzania (Plate 3). One hundred fields (twenty-five fields per region) were surveyed in 2009 during the long rainy season (March – June). In each region, one village was selected based on the history of sweet pepper production. Thus, in Tanga region, the disease was surveyed at Lukozi village (ca. 1777 meters above sea level (masl) in Lushoto district. In Morogoro region, the survey was conducted at Luale village (ca. 1194 masl) in Mvomero district. In Arusha region, the survey was conducted at Kivulul village (ca. 1650 masl) in Arumeru district. In Mbeya region, the disease was surveyed at Utengule village (ca. 1320 masl) in Mbarali district. In each field, the incidence of bacterial leaf spot disease was assessed in five quadrants (of 2x2 m^2) by assessing an average of 10 plants along a diagonal within each quadrant. The number of plants that showed disease symptoms on leaves, stems or fruits was recorded. The size of the field was estimated and plant population was calculated. The incidence of bacterial leaf spot was determined by taking the number of infected plants in each field as a percentage of the total number of plants sampled (Shenge *et al.*, 2007).

Disease severity was assessed as the proportion of the area of the four mature leaves of the upper third of the foliage of each plant with bacterial leaf spot symptoms in each field (Ward and O'Garro, 1992). Disease severity scoring was done on stems, leaves and fruits

Plate 3: Map of Tanzania showing bacterial leaf spot disease surveyed regions

using the scale of 0 (no disease) – 9 (complete defoliation) according to Kousik and Ritchie (1996b) and Katawczik (*Unpublished*) where: 0 = no disease lesion, 1 = ≤ 10 % leaf area covered with lesions, but no defoliations, 2 = 11 – 20 % leaf area covered with lesions, but no defoliations, 3 = 21 – 30 % leaf area covered with lesions, one to two leaves defoliated, 4 = 31- 40 % leaf area covered with lesions, 3 - 4 leaves defoliated, 5 = 41 – 50 % leaf area covered with lesions, five to six leaves defoliated, 6 = 51 – 60 % leaf area covered with lesions, seven to eight leaves defoliated, 7 = 61 - 70 % leaf area covered with lesions, few leaves remaining on plant, 9 = 91 – 100 % plant covered with lesions,

plant dying or dead, complete defoliation. Scores 1 – 3 were classified as low severity, 4 – 6 as moderate severity and 7 – 9 as high severity. From each field, infected leaves, stems and fruits were collected, placed in paper bags and transported to the African Seed Health Centre laboratory, Sokoine University of Agriculture, for analysis. Epidemiological studies were done through evaluating the sanitary condition of each field. Other data on land use intensity, pesticides use and sources of sweet pepper seeds were provided by farmers through interviews. In addition, rainfall and temperature data for the year 2009 were obtained from the national meteorological head quarters in Dar es Salaam as follows: In Lushoto district (Tanga region) rainfall amount was 1067 mm, and the temperature ranged between 15.5 - 25 °C. In Arumeru district (Arusha region) rainfall amount was 1200 mm, and the temperature ranged between 24 - 28 °C. In Mvomero district (Morogoro region) rainfall amount was 1050 mm, and the temperature ranged between 23 - 27 °C. In Mbarali district (Mbeya region) rainfall amount was 1100 mm, and the temperature ranged between 10.2 - 22 °C.

3.2 Isolation of Bacterial Strains

Samples of sweet pepper leaves, stems and fruits were washed in tap water to remove soil and debris, and surface disinfected by dipping them in 1.25 % sodium hypochlorite for 20 seconds. Samples were then rinsed thoroughly in sterile distilled water (SDW) to remove the sodium hypochlorite (O'Garro *et al.*, 1999; Ward and O'Garro, 1992). Tissue segments of about 2 mm x 3 mm from bacterial spot symptomatic leaves, stems and or fruits were excised from advancing lesion margins, teased in a few drops of SDW, and allowed to stand for 10 -15 minutes in a lamina airflow bench. A loopful of the resulting suspension was streaked onto plates of nutrient yeast dextrose agar (NYDA) and incubated for 3 – 5 days at 25 – 28 °C to obtain presumptive colonies of bacterial spot-causing

Xanthomonas strains. Putative colonies of the pathogens were obtained by sub-culturing single colonies on YDC agar and stored at -80 °C on porcelain beads in Protect® tubes (Protect System, Bury, UK) (Bradbury, 1970) for further investigations.

3.3 Identification of Bacterial Strains

3.3.1 Identification and characterization of *Xanthomonas* strains

Presumptive colonies of bacterial spot causing *Xanthomonas* strains from pepper and seven reference strains of the pathogens (positive controls) (Table 1) provided by The Danish Seed Health Centre – Denmark and The World Vegetable Centre -Taiwan, were identified using physiological and biochemical characteristics, including colony morphology on NA, Gram-reaction based on potassium hydroxide solubility test (Lelliot and Stead, 1987), Kovac's oxidase reaction (Hildebrand and Schroth, 1968; Kovacs, 1959), gelatin liquefaction, nitrate reduction, potato soft rot, starch hydrolysis and oxidative/fermentative metabolism of glucose (Fahy and Persley, 1983). In addition, the bacteria were subjected to, Polymerase chain reaction (PCR) (Leite *et al.*, 1994; Versalovic *et al.*, 1994; Wichmann and Bergelson, 2003), pathogenicity test (Shakya and Chung, 1983; Kauffman *et al.*, 1973) and races determination (Minsavage *et al.*, 1990).

Table 1: List of bacterial reference strains used in the study

No.	Strain	Country	Host	Part	Race
1	*Xanthomonas vesicatoria* (XVT-12)	Taichung	Tomato	Leaf	T1 P3
2	*X. vesicatoria* (XVT-28)	Furan Co.	Tomato	Leaf	T1 P1
3	*X. vesicatoria* (XVT-48)	Nantou	Tomato	Leaf	T1 P2
4	*X. vesicatoria* NCPPB 422	Denmark			
5	*X. euvesicatoria* NCPPB 2968	Denmark			
6	*X. gardneri* NCPPB 881	Denmark			
7	*X. perforans* NCPPB 4321	Denmark			

NCPPB = National Collection for Plant Pathogenic Bacteria, XVT = *Xanthomonas vesicatoria* from tomato, T1P1/T2P2/T3P3 = Tomato Pepper races

3.3.1.1 Gram reaction

The purified bacterial isolates were tested for Gram reaction using three percent potassium hydroxide (KOH) solubility test (Lelliot and Stead, 1987). On a glass slide, a loopful of 24 - 48 hour-old bacteria cultures was mixed, for about 10 seconds, with a drop of 3 % KOH aqueous solution. A toothpick was used for picking bacteria from a colony and thoroughly mixing to create a bacterial suspension.

The toothpick was then raised a few centimetres from the suspension on the glass slide. Presence of strands of viscid material indicated that the bacteria tested were Gram-negative. Gram-positive bacteria did not produce such strands even on repeated strokes of the loop/toothpick.

3.3.1.2 Oxidase Test

Oxidase test was used to determine the presence of cytochrome-c (oxidase enzymes of the respiratory chain). A Whatman filter paper No. 1 was placed in a petri dish and 3-4 drops of freshly prepared 1 % aqueous solution of tetramethyl-p phenylenediamine dihydrochloride was added on the centre of the paper. Using a platinum loop wire, a loopful of 24 - 48 h-old bacterial cultures grown on NA were streaked onto the moist filter paper. Change in colour of the reagent to purple within 10 seconds of application of the culture was recorded as positive for oxidase test. No change in colour of the reagent was recorded as negative for oxidase test.

3.3.1.3 Gelatin hydrolysis

Proteolytic bacteria decompose gelatin with loss of gelling properties. Isolated bacteria were grown in a nutrient medium solidified by gelatin. The tubes containing gelatin were stab- inoculated by 24 hour-old bacterial cultures grown on NA medium. Un-inoculated control; negative and positive reference strains (Table 1) were also included. The inoculated test tubes were incubated at 20 °C for 7 – 14 days. Liquefication of the medium was recorded every 2-3 days. The inoculated tubes were cooled at 5 °C for 30 minutes, incubated in a tilted position at room temperature of 25 °C for 30 minutes before recording the results. Lack of cultures to solidify indicated that, the gelatin had been hydrolyzed and was recorded as a positive reaction. The negative reaction was indicated when the test culture liquefication as the control (Fahy and Parsley, 1983, Lelliot and Stead, 1987).

3.3.1.4 Starch hydrolysis

Starch hydrolysis was done following procedures described by Lelliot and Stead (1987) in order to assess an organism's ability to hydrolyze starch by enzymatic activity. Starch

medium agar plates were inoculated by streaking with a 24 hour-old pure cultures grown on NA medium. The reference strains (*Xanthomonas euvesicatoria*, *X. vesicatoria*, *X. perforans* and *X. gardneri*) were included as controls. The inoculated plates were incubated for 3 - 5 days. The inoculated plates were then flooded directly with Lugol's iodine and the results interpreted. Appearance of yellowish, clear zones around or under the bacterial growth indicated a positive reaction. Appearance of starch staining blue-black (indicating lack of starch hydrolysis) was recorded as a negative reaction.

3.3.1.5 Oxidative/Fermentative (O/F) metabolism of carbohydrates

The carbohydrate utilization tests following the procedures described by Fahy and Parsley, 1983) were used to determine whether a bacterium had a respiratory (oxidative) or fermentative metabolism (creating anaerobic conditions). A 24 hour-old culture grown on NA medium was stab-inoculated in duplicate test tubes containing O/F – semi- solid medium with 1 % glucose. Control tubes, un-inoculated and other tubes with reference strains were also included. The medium in one of the test tubes was covered with sterile mineral oil to a depth of two cm. The tubes were incubated at 28 °C and examined daily for 7 - 14 days.

Production of yellow discoloration (acid formation) only at the top, 1-2 cm, of the unsealed tube was recorded as oxidative metabolism for glucose. Yellow colour in both (sealed and unsealed) tubes indicated fermentation, and no colour change in both tubes indicated that neither oxidation nor fermentation took place.

3.3.1.6 Nitrate Reduction

Nitrate reduction test determines the ability of an organism to reduce nitrate to nitrite, which in turn can be further reduced to free nitrogen gas. The procedure described by Fahy and Parsley (1983) was used. A heavy loopful of 24 hour-old bacterial cultures grown on NA medium were inoculated in duplicate tubes containing molten nitrate–semi-solid medium and mixed by rotation between palms before the setting of agar. A non-inoculated, a positive and a negative reference strains were also included. The inoculated test tubes were incubated at 27 °C and observed daily for 7 days. Three drops of Nitrate reagent A (Starch iodide solution) together with three drops of Nitrate reagent B (Hydrochloric acid solution) were added to an incubated nitrate agar tubes and results were recorded. A change in colour of the reagent to blue-black within few seconds was considered as positive reaction (the organism reduced nitrate to nitrite). Lack of the blue-black colour was recorded as negative for nitrate reduction.

When negative results were observed, a speck of zinc dust was added to such test and further observations were done. Presence of the blue-black colour indicated that nitrate was not reduced (the results were negative) and development of the blue-black colour was considered positive (nitrate reduced beyond nitrite).

3.3.1.7 Potato soft rot (Pectolytic) test

The procedure as described by Lelliot and Stead, (1987) was used. This test was useful and determinative character in bacterial identification. Round potato tubers were washed and surface disinfected by dipping in 70 % alcohol followed by flaming briefly. The disinfected potato was sliced aseptically 7-8 mm thick. The slices were placed on moistened, sterile petri dishes. A groove was made in the slice and smeared with bacterial

growth from 24 hour-old agar cultures. Uninoculated control slices were also included. The petri dishes with inoculated potato slices were incubated in the darkness at 27 ^{0}C for 24 hours. Results were recorded by drawing an inoculating loop across the surface to determine whether the slices rotted beyond the inoculation point or not. Black rotted tissue was recorded as positive for potato soft rot and slight rot only at the inoculating point was considered as negative.

3.3.2 Identification and characterization of *Xanthomonas* strains using PCR

3.3.2.1 DNA Extraction from bacterial isolates and reference strains

Putative bacterial cultures isolated from diseased sweet pepper plant parts (leaves, fruits and/or stems) and the reference strains collected from The Danish Seed Health Centre - Denmark and The world Vegetable Centre -Taiwan were used for DNA extraction using the method described by Gomes *et al.* (2000).

The 48-h old bacterial cultures from NA were grown in 5 ml nutrient broth with 10 % glycerol v/v (to avoid Xanthan formation) for 72 h at 27 °C. The cultures were poured in 1.5 ml eppendorff tubes, centrifuged the cells at 13000xg for 5 minutes. The pellets obtained were suspended in 200 µl Tris 0.1 mol/l and added with 200 µl of lysis solution (NaOH 0.2 N and 1 % Sodium Dodecylsulphate (SDS), mixed and deproteinazed with 700 µl of phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v), homogenized and centrifuged at 13000xg for 10 minutes. DNA was precipitated by adding 700 µl of cold 95 % ethanol, mixed gently by inversion, spinned, washed again in 70 % ethanol, centrifuged at 13000xg for 10 minutes and spinned. The precipitated DNA was then dried at room temperature for 30 minutes, suspended in 100 µl of sterile distilled water and mixed well by shaking. The

extracted DNA of each isolate/strain was electrophoresed on 1 % agarose gel stained with ethidium bromide and photographed under ultra violet (UV) light.

3.3.2.2 Polymerase Chain Reaction (PCR) specificity

Amplification of the DNA extracted from the bacterial cultures was carried out using the highly conserved putative ATPase gene (hrpB6) primer pairs of an approximately 840-bp fragment, which is expected from most phytopathogenic xanthomonads (Gent *et al.*, 2005; Leite *et al.*, 1994; Mitrev and Kovačević, 2006). Four species-specific and four effector gene primer pairs were employed to characterize bacterial isolates and reference strains into different *Xanthomonas* strains (Wichmann and Bergelson, 2003) (Table 2). The PCRs were performed in 50 µl reaction mixture containing 39.0 µl of SDW, 2.5 µl of 10x buffer, 1.5 µl of $MgCl_2$, 0.5 µl of deoxynucleotide triphosphate (dNTPase), 1.0 µl of Primer F 1, 1.0 of Primer R 1, 0.5 µl of Taq-DNA polymerase and 4 µl crude bacterial lyase (DNA). The PCRs temperature profile for the general and four effector primer sets comprised of an initial denaturation time of 3 minutes at 95 °C, followed by 35 cycles of 30 sec at 95 °C, 30 sec at the specified annealing temperature and 72 °C for the specified extension time (Table 2), with a final 5-minutes extension at 72 °C and cooling at 4 °C. The PCRs temperature profile for the four species specific primers sets comprised of an initial denaturation time of 3 minutes at 95 °C, followed by 35 cycles of 20 sec at 95 °C, 15 sec at the specified annealing temperature and 72 °C for 15 sec extension time (Table 2), with a final 3 minutes extension at 72 °C and cooling at 4 °C. The PCR products were separated by 0.3 % (w/v) agarose gel (stained with ethidium bromide) electrophoresis in 0.5 x TAE buffer for 30 minutes at 50 V and visualized under ultra violet (UV) light.

Table 2: List of primer sets and Polymerase Chain Reaction conditions used to amplify ATPase gene and species-specific primers from genomic DNA of bacterial spot-causing *Xanthomonas* strains from sweet pepper

Primer name	Primer sequence (5'-3')	Annealing temp (°C)	Extension time	Base pair (bp)
RST2	(AGGCCCTGGAAGGTGCCCTGGA)	55	2 min	840
RST3	(ATCGCACTGCGTACCGCGCGCGA)			
avrBs 1 -642U	(TGAGCTCCTATGACGGACTTGTGCTCG)	58	2 min	1337
avrBs 1 -2069L	(TGCATGCGTGGCGGATACTTCTTCTCT)			
avrBs 2 -79U	(TGAGCTCGCAGGCATCGTTTCGCATC)	58	2 min	2156
avrBs 2 -2324L	(TGCATGCGAAGCCGTGATTGGAAGGT)			
avrBs 3 -187U	(TTCCCGGGCGACGGTAGGGGAATGCT)	53	4 min	500
avrBs 3 -4235L	(TTCCCGGGGCTTGGTGTACGGGTGTGG)			
avrBs 4 -50U	(TTCCCGGGCATCACGCACATAGTTCTGA)	53	4 min	3482
avrBs 4 -3963L	(TTCCCGGGTTGCCGCCACTGAACAAGC)			
X. euves - Fw	(CTGCTTACAATCCGGACCAT)	60	15 sec	153
X. euves - Rv	(GCCAGGGCATAATCAAGAGA)			
X. ves - Fw	(TATGGCCTGGACAAGAATGG)	66	15 sec	248
X. ves - Rv	(CGCTCTGCGGCATCTTTCAG)			
X. gardneri – Fw	(GATGAGGTGCAATCGGTTCT)	60	15 sec	189
X. gardneri – Rv	(TCTTTTCTTGCTGCTGCTGA)			
X. parforans – Fw	(CCTCTAAGCCTGGGGTTCTC)	59	15 sec	232
X. parforans –Rv	(GACGCTTGAGCGAGAGCTAC)			

X. euves = *Xanthomonas euvesica*toria, *X. ves* = *Xanthomonas vesicatoria*, FW = forward, RV = reverse

3.3.2.3 Pathogenicity test on host plant

Pathogenicity of xanthomonad isolates was confirmed by inoculating the susceptible sweet pepper cultivar Early Calwonder (ECW) (Mitrev and Kovačević, 2006; Vauterin *et al.*, 1995). Three seeds per pot (15 cm diameter) of cultivar Early Calwonder were sown in sterilized potting compost in the screenhouse, set at 25 – 28 °C and relative humidity of 80 – 90 %. In each pot, seedlings were reduced to two after establishment. Loopfuls of 24 – 48 hour-old bacterial cultures grown on NA medium were suspended in sterile distilled water at the concentration of 10^8 cfu/ml and vortexed at 600 rpm to form a uniform bacterial suspension. Fully expanded leaves of 4-week-old sweet pepper seedlings were infiltrated with each of the identified bacterial suspension by a syringe without a needle (Obradovic *et al.*, 2004; Ward and O'Garro, 1992). Each pot was labeled with isolate number and date inoculated. Reference bacterial strains (Table 1) and sterile distilled water were included as positive and negative controls, respectively. The plants were covered immediately with polyethylene bags to prevent drying, maintain high humidity and promote disease development; and were incubated individually for 24 h with 12-h-light regime and observed daily for disease symptom development for up to 14 days (Plate 4).

Plate 4: Pathogenicity test and race determination in the screenhouse at Sokoine University of Agriculture

3.3.3 Race determination

Capsicum annuum cultivar Early Calwonder (ECW) and three near-isogenic lines ECW-10R, ECW-20R and ECW-30R (Gore and O'Garro, 1999; Jones *et al.*, 1998; Mitrev and Kovačević, 2006; O'Garro *et al.*, 1999) obtained from World Vegetable Centre- Taiwan were used to differentiate *Xanthomonas* strains into races. The near-isogenic lines ECW-10R, ECW-20R and ECW-30R contain genes Bs1, Bs2 and Bs3, respectively, for resistance to *Xanthomonas* strains carrying the avirulence genes/effector proteins *avr*Bs1, *avr*Bs2, and *avr*Bs3, respectively; where as, cultivar ECW carries no known genes for resistance to bacterial spot (Bouzar *et al.*, 1994; Hibberd *et al.*, 1987; Mitrev and Kovačević, 2006). Five seeds per pot (15 cm diameter) of cultivar and near-isogenic lines

were sown in sterilized potting compost in the screenhouse set at 25 – 28 °C and relative humidity of 85 – 95 %, arranged in a completely randomized design (CRD) replicated three times. In each pot, seedlings were thinned to two after establishment. Bacterial cultures were suspended in sterile distilled water, adjusted to a concentration of 10^8 CFU/ml. Fully expanded leaves of 4-week-old plants were inoculated by infiltrating the intercellular spaces with bacterial suspension (10^8 CFU/ml) using a syringe without the needle and sprayed leaf surfaces with each of the identified bacterial suspension to runoff, using a hand-held sprayer 20 - 30 cm from the foliage (Obradovic *et al.*, 2004; Ward and O'Garro, 1992). Reference bacterial strains (Table 1) and sterile distilled water were used as positive and negative controls, respectively. After inoculation, plants were covered immediately with polyethylene bags to prevent drying and maintain humidity; and were incubated individually for 48 h under screen-house conditions. Races of BSX were identified based on the hypersensitive or compatible response tissue at the infiltration site within 24 – 48 h after inoculation (Mitrev and Kovačević, 2006; Obradovic *et al.*, 2004; Gore and O'Garro, 1999; O'Garro *et al.*, 1999; Ward and O'Garro, 1992).

3.4 Epidemiology of Sweet Pepper Bacterial Leaf Spot in Tanzania

3.4.1 Collection of sweet pepper seeds

Six seed samples of three different sweet pepper varieties produced by different seed companies from within and outside Tanzania were procured from agricultural input suppliers. Sweet pepper varieties included Yolo Wonder (from Pop Vriend Seeds and Bakker Brothers - Holland), California Wonder (from Kibo Seed Company Ltd. - Tanzania, Pop Vriend Seeds - Holland) and Yolo Wonder B (from Royal Sluis – Holland, and Mkulima Seeds – Tanzania).

3.4.2 Detection of *Xanthomonas* strains from sweet pepper seeds

From each sweet pepper seed samples purchased, 100 seeds were sown in 15 cm diameter plastic pots filled with sterile soil. The seeded pots were placed in the screen house and observed for germination. When plants were 2-week-old, the pots were covered separately with clear polyethylene bags for 36 h to provide high moisture conditions and maintain humidity. Seedlings were observed for typical bacterial spot symptoms on leaves seven days after emergence and after they were enclosed in the polyethylene bags up to four weeks.

3.4.3 Market surveys

Four markets, one from each of the four regions (Morogoro, Arusha, Tanga and Mbeya) were surveyed to determine the level of bacterial spot symptoms on sweet pepper fruits brought to the market. At each market, three fruits bearing typical bacterial spot symptoms were collected randomly from each of three sweet pepper lots sampled, placed in labeled paper bags and brought to the laboratory for isolation and characterization of the pathogen.

3.4 Screening Sweet Pepper Varieties for Resistance to Bacterial Spot Disease

The trial was conducted under screenhouse condition. Three sweet pepper varieties namely: California Wonder, Yolo Wonder and Yolo Wonder B, commonly planted by farmers were evaluated. Sweet pepper seeds were sown in plastic buckets of 15 cm diameter filled with sterile forest soil. The pots were arranged on a bench in completely randomized design (CRD) replicated three times. In each pot, 10 seeds were sown, and the numbers of seedlings per pot were reduced to two after seedling establishment (O'Garro *et al.,* 1999; Ward and O'Garro, 1992). The plants were watered, N (4 %), P (0.35 %) and K (4 %) fertilizers were applied as recommended. Inoculation was done when seedlings were

4-weeks-old by spraying the abaxial and adaxial leaf surfaces with the 10^8 cfu ml^{-1} bacterial suspension of each of the identified race to runoff, using a hand-held sprayer held 20-30 cm from the foliage (O'Garro *et al.*, 1999; Ward and O'Garro, 1992). Negative control sweet pepper plants were treated with SDW. The inoculated plants were thereafter, covered with a polyethlene sheet for 72 h and observed for bacterial spot disease symptom development for up to fruit maturity. Plants which showed typical symptoms of bacterial spot were scored for disease severity at 7, 14, 21, 28 days after inoculation and at fruit maturity (75 – 90 days after transplanting) using the scale as described by Kousik and Ritchie (1996b).

3.5 Data Analysis

Data obtained in all the experiments were analyzed using Statistical Package for Social Science (SPSS) and Statistical Analysis System (SAS). Means separations were done when necessary using Duncan's Multiple Range Test (DMRT) and Turkeys Studentized Range (HSD) Test.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Disease Survey

4.1.1 Incidence and severity of bacterial leaf spot disease of pepper

Survey results of bacterial leaf spot disease in farmers' fields are summarized in Table 3. The results indicate that bacterial leaf spot disease of sweet pepper is widespread in farmers' fields in the sweet pepper-producing regions studied (Morogoro, Arusha, Tanga and Mbeya). Disease incidence was very highly significant ($P \leq 0.001$) by village basis (Table 4) and ranged between 10 – 100 % (Figure 3). The overall mean disease incidence was 69.3 %. Kivulul, Lukozi and Luale villages in Arusha, Tanga and Morogoro regions, respectively, had the highest mean disease incidence. The lowest mean disease incidence (12.0 %) was recorded in Utengule village (Mbeya region). In Turkey, disease incidence was reported to range from 50 – 95 % (Aysan and Sahin, 2003).

The results also indicate that, disease severity was statistically significant ($P \leq 0.05$) between the villages surveyed (Table 5). The overall mean disease severity was 4.6. The highest mean disease severity score of 6.5 was found in Kivulul village in Arusha region. The lowest mean disease severity score of 1.2 was found in Utengule village in Mbeya region, where disease incidence was also low. Kivulul, Lukozi and Luale villages in Arusha, Tanga and Morogoro regions, respectively, had the highest bacterial leaf spot disease severity (Table 5 and Figure 4).

Table 3: Relationship between field history, sanitary condition and the occurrence of bacterial leaf spot disease of sweet pepper in the surveyed villages in Morogoro, Arusha, Tanga and Mbeya regions

Region	Village	No. of fields visited	Sanitary condition		Land use intensity (years)			Source of seeds		Farmers using pesticide	Farmers irrigating	Disease incidence		
			Clean	Weedy	Virgin	1-2	≥3	Bought	Extracted			10-30	31-50	>50
Morogoro	Luale	25	19	6	5	3	17	25	0	25	25	0	6	19
Arusha	Kivulul	15	11	4	0	0	15	15	0	15	15	0	2	13
Tanga	Lukozi	30	28	2	2	0	28	30	0	30	30	0	0	30
Mbeya	Utengule	05	3	2	0	5	0	5	0	5	5	5	0	0
	TOTAL	**75**	**61**	**14**	**7**	**8**	**60**	**75**	**0**	**75**	**75**	**5**	**8**	**62**

Table 4: Mean disease incidence of bacterial leaf spot of sweet pepper on village basis

Village	Mean
Lukozi	93.3a
Kivulul	90.6a
Luale	81.2a
Utengule	12.0b
Mean	**69.3**
CV	23.7
SE	19.7

Means with the same letter are not significantly different at $P \leq 0.05$ according to Duncan's Multiple Range Test (DMRT)

Table 5: Mean disease severity of bacterial leaf spot of sweet pepper on village basis.

Village	Mean score
Kivulul	6.5a
Lukozi	5.6a
Luale	5.2a
Utengule	1.2b
Mean	**4.6**
CV	39.1
SE	4.3

Means with the same letter are not significantly different at $P \leq 0.05$ according to Duncan's Multiple Range Test (DMRT)

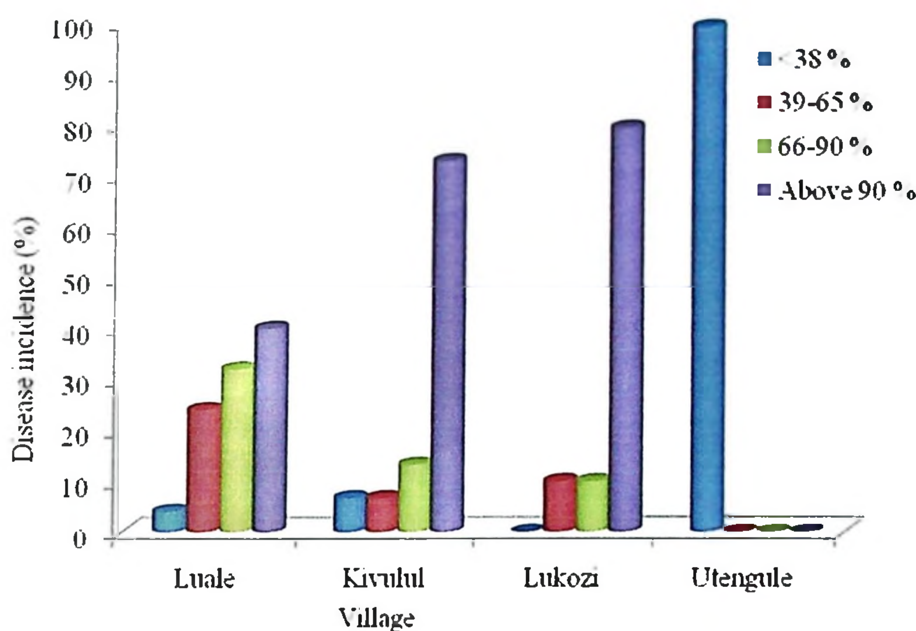


Figure 3: Incidence of bacterial leaf spot disease of sweet pepper in farmers' fields in the selected sweet pepper-producing areas in Tanzania during the 2008 – 2009 planting seasons

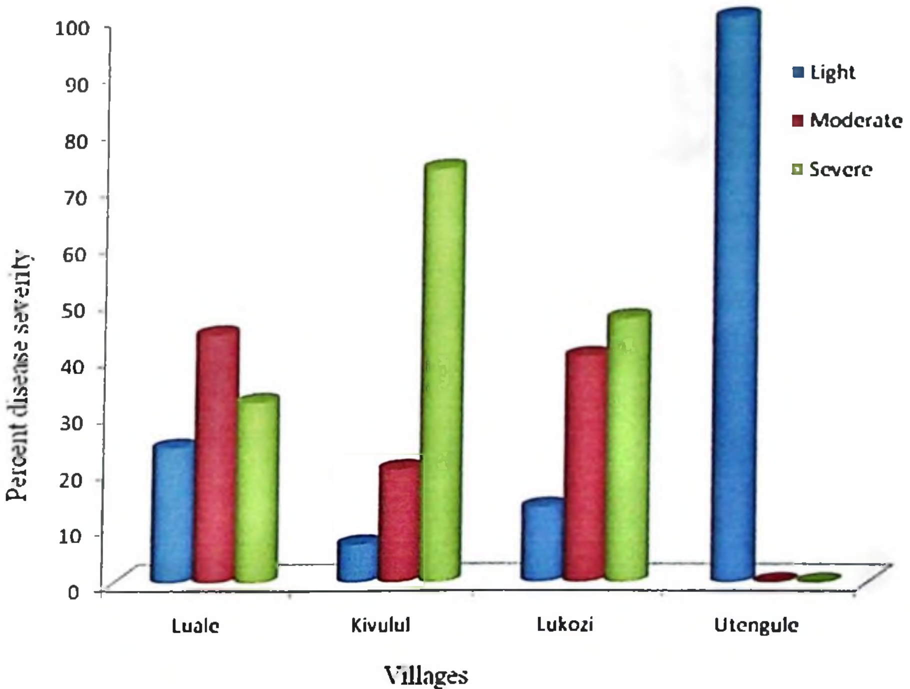


Figure 4: Severity of bacterial leaf spot disease of sweet pepper in farmers' fields in selected sweet pepper-producing areas in Tanzania during the 2008 - 2009 planting seasons

4.1.2 Effect of field sanitation on disease incidence and severity

The results of this study indicate that, field sanitation had statistically significant effect ($P \leq 0.05$) on disease incidence and severity. High disease incidence and severity were recorded in clean sweet pepper fields compared to weedy fields (Figures 5 and 6). The results of this study are contrary to results reported by Shenge *et al.* (2010). In their study they reported that, the incidence and severity of bacterial spot and bacterial speck of tomato were high in weedy fields. In this study, it was observed during the survey study they reported that, the incidence and severity of bacterial spot and bacterial speck of tomato were high in weedy fields. In this study, it was observed during the survey that, high disease incidence and severity of bacterial leaf spot in clean sweet pepper fields might be due to the continuous growing and rotation of solanaceae crops (such as sweet pepper, tomatoes, round potatoes and African eggplant) in such fields (Figure 7).

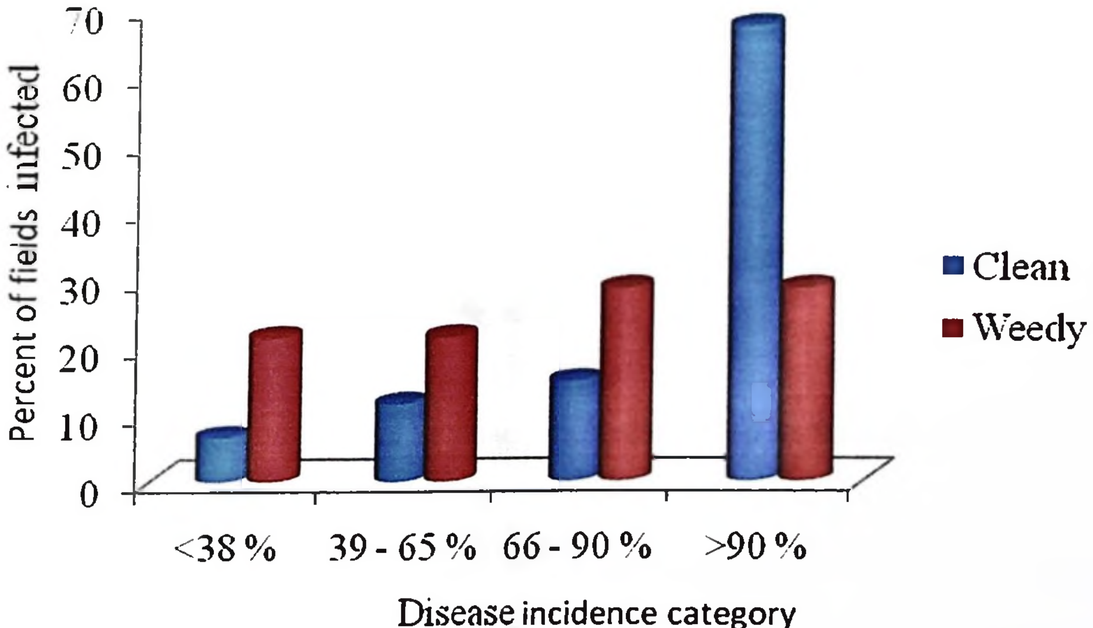


Figure 5: Effect of field sanitation on the incidence of bacterial leaf spot disease of sweet pepper in farmers' fields in the surveyed villages

The types of solanaceae plants (tomatoes, round potatoes African eggplant, *Bidens pilosa, Solanum nigrum*) surrounding the sweet pepper crop, crop debris left on the soil and volunteer sweet pepper/tomato plants (Plate 5) might also be the causes of high disease incidence and severity of bacterial leaf spot of sweet pepper. Other causes might be heavy shades of coffee and banana plants (especially in Arusha region), the use of susceptible varieties and the possibility of emerging new races.

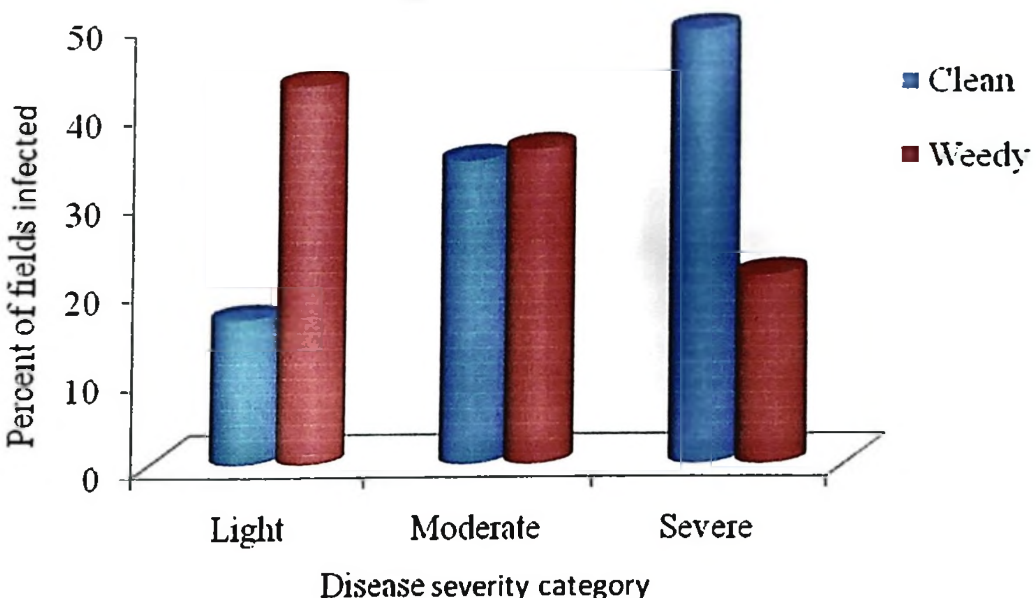


Figure 6: Effect of field sanitation on the severity of bacterial leaf spot disease in farmers' fields in the surveyed villages

4.1.3 Effect of previous cultivation history on incidence and severity of bacterial leaf spot disease

The results also demonstrate that, the effect of previous cultivation history was highly statistically significant ($P \leq 0.005$) and ($P \leq 0.019$) on disease incidence and severity, respectively (Appendices 4 and 5). Fields grown continuously with solanaceous crops and which were dominated by crops of the same family (such as sweet pepper, tomatoes,

round potatoes and African eggplant) had high disease incidence (above 90 %) and severity (Figures 7 and 8). Fallowed and virgin fields had low disease incidence and severity.

Plate 5: Recovered sweet pepper plants infected by bacterial leaf spot-causing xanthomonads in farmer's field in Luale village, Morogoro region

The results also indicate that, the majority of farmers (57.3 %) reported high disease incidence at sweet pepper flowering stage, when the rainfall was high, compared to when environmental conditions were dry. The results of this study imply that, there were many sources of inocula for bacterial leaf spot disease of sweet pepper. The ability of BSX to survive on volunteer sweet pepper/tomato plants, crop debris, soil and solanaceous weeds has been reported (Basim *et al.*, 2004; Blancard, 1997; Jones *et al.*, 1986; McGrath, 1997; Mew and Natural, 1993; Peterson, 1963).

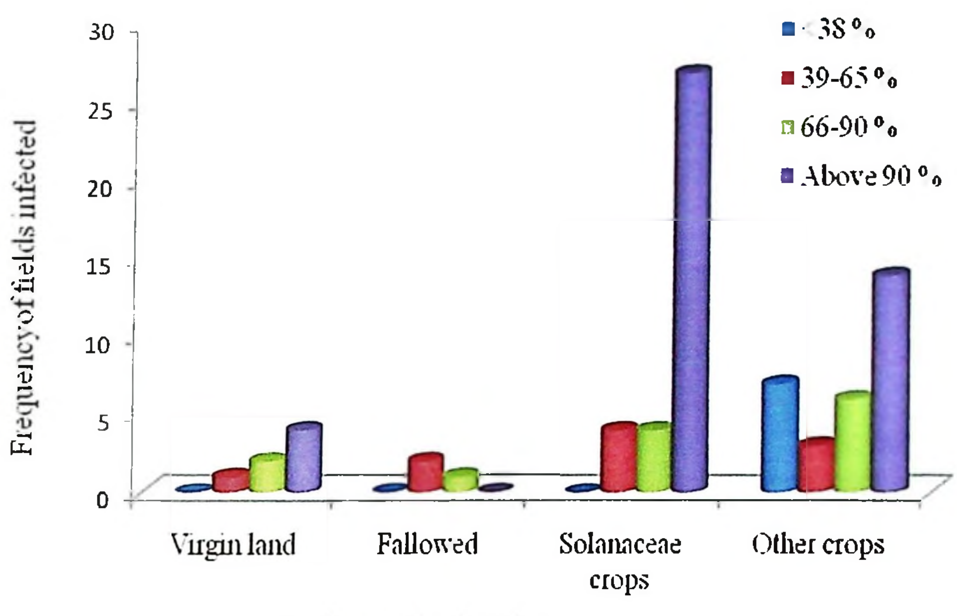


Figure 7: Effect of previous cultivation history on bacterial leaf spot disease incidence on sweet pepper in farmers' fields in Morogoro, Tanga, Arusha and Mbeya regions in Tanzania during the 2008 - 2009 planting seasons

Although sweet pepper and tomato were reported to be the sole hosts of bacterial leaf spot causing xanthomonads, it has also been reported to have the ability to epiphytically survive on protected plant organs (such as leaves, buds and flowers) of sweet pepper/tomato and non-host plants without causing visible symptoms (Jones *et al.*, 1986; Pernezny and Collins, 1997). The 'protected position' are reported to be more moist and receive less ultraviolet light, therefore, are better environments for bacterial survival and growth (Leben, 1981; Leben, 1988). Furthermore, Pernezny and Collins (1997) reported that, the susceptible sweet pepper cultivars support more epiphytic populations of BSX than did the resistant cultivars. Therefore, during the favourable environmental conditions (high temperatures, humidity and rainfall) the BSX multiply rapidly and spread, leading to high disease incidence during the sweet pepper flowering stage.

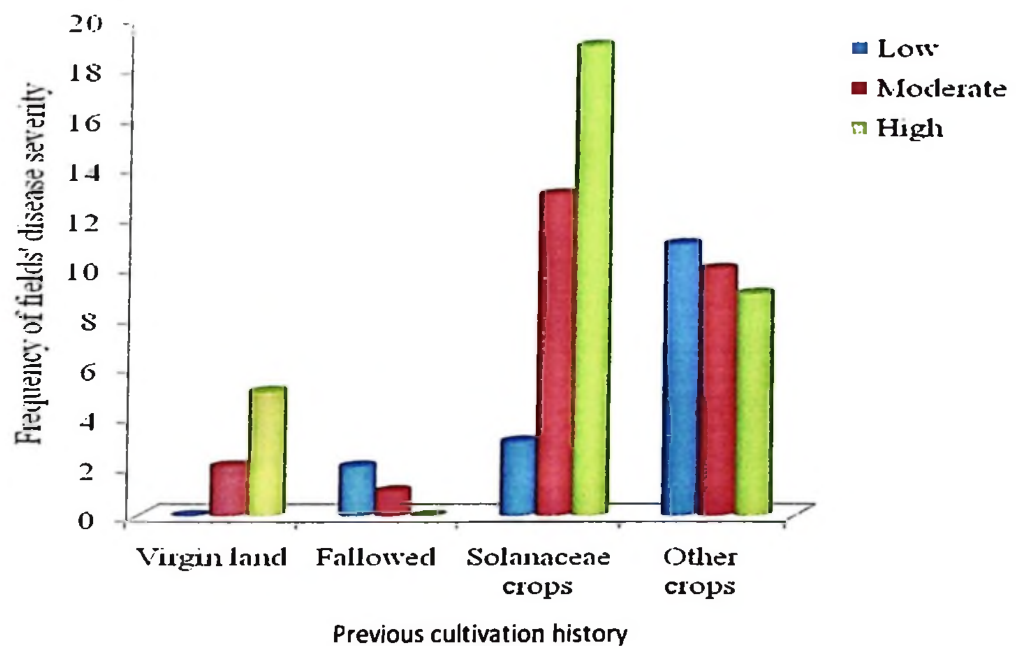


Figure 8: Effect of previous cultivation history on bacterial leaf spot disease severity on sweet pepper in farmers' fields in Morogoro, Tanga, Arusha and Mbeya regions in Tanzania during the 2008 - 2009 planting seasons

4.1.4 Sweet pepper varieties preferred by farmers

The sweet pepper varieties (Yolo Wonder, California Wonder and Yolo Wonder B) preferred by farmers had highly statistically significant difference ($P \leq 0.001$) between and within villages. Most of the farmers interviewed (54.7 %) preferred the sweet pepper variety Yolo Wonder (Figure 9) because of its early maturity, large fruits, high yield and resistant to bacterial leaf spot disease. In Arusha, all the sweet pepper farmers interviewed grew the variety Yolo Wonder (Figure 10) which was thought to be resistant to bacterial leaf spot disease. The variety California Wonder was found grown by some farmers in Luale and Lukozi villages in Morogoro and Tanga regions. Three other varieties grown but to a lesser extent were Yolo Wonder B, Capsicum Red and Yellow and Libert Bell (Figures 9 and 10) and were reported to be recent introductions.

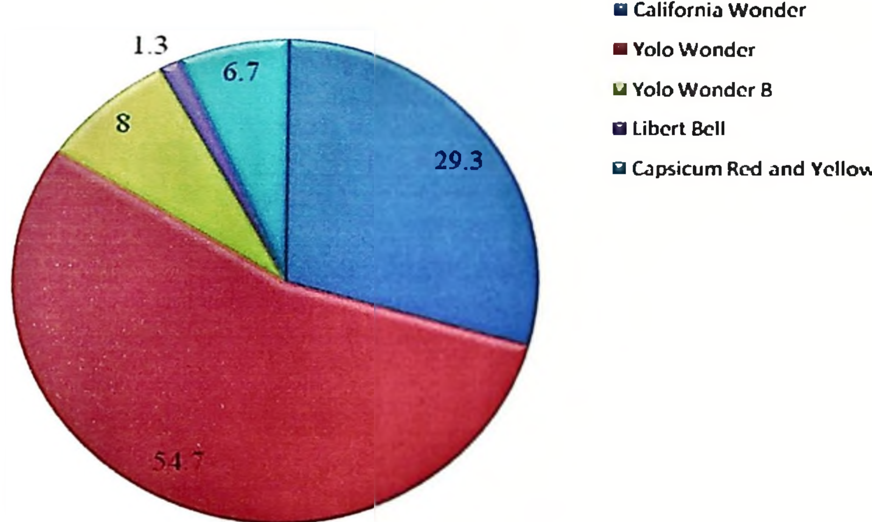


Figure 9: The preference of sweet pepper varieties (%) by farmers in Morogoro, Tanga, Arusha and Mbeya regions, Tanzania

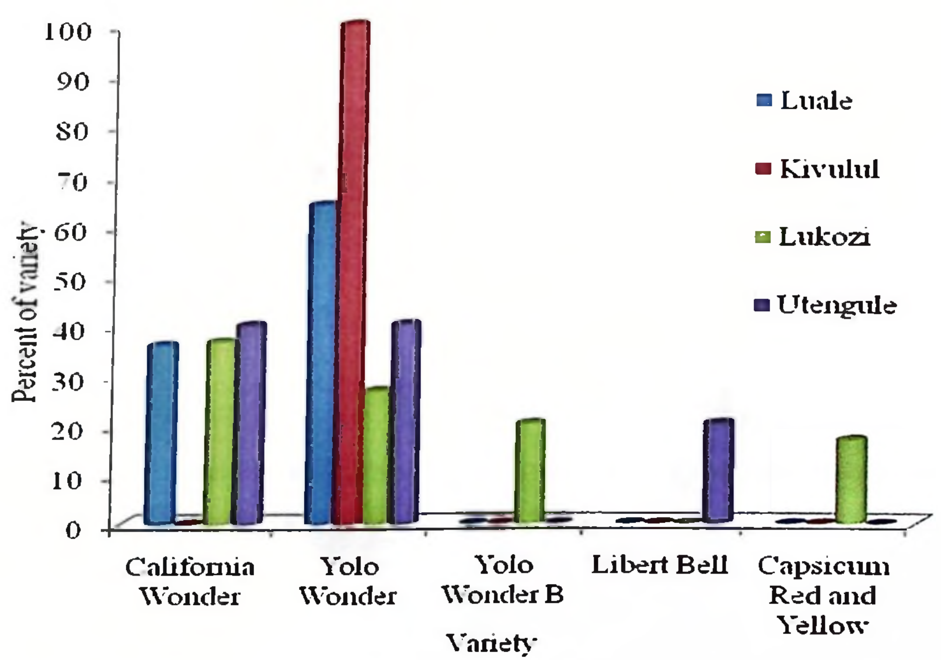


Figure 10: Percent of sweet pepper varieties preferred by farmers in Morogoro, Arusha, Tanga and Mbeya regions on a village basis

All the varieties grown were previously known to farmers to be resistant to bacterial leaf spot disease. However, the results of this study indicate that, all the varieties grown were susceptible to the bacterial leaf spot disease at varying injury levels (Figure 11). Unfortunately, the most preferred variety Yolo Wonder had high disease incidence and severity followed by California Wonder. Although the varieties Capsicum Red and Yellow and Yolo Wonder B were not extensively grown, they had also high disease incidence and severity. However, the variety Libert Bell had low disease incidence and severity (Figures 11 and 12).

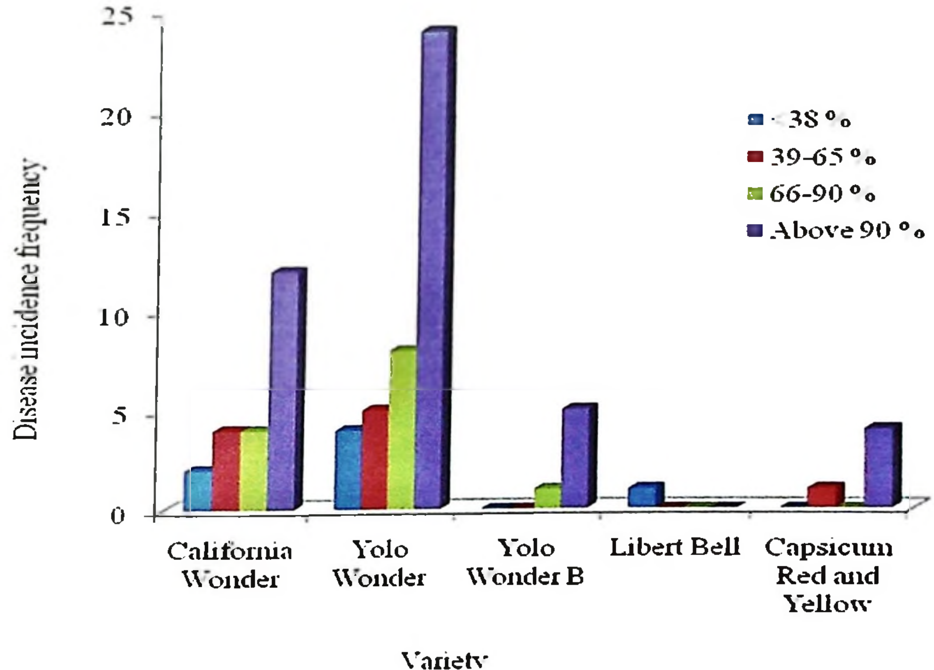
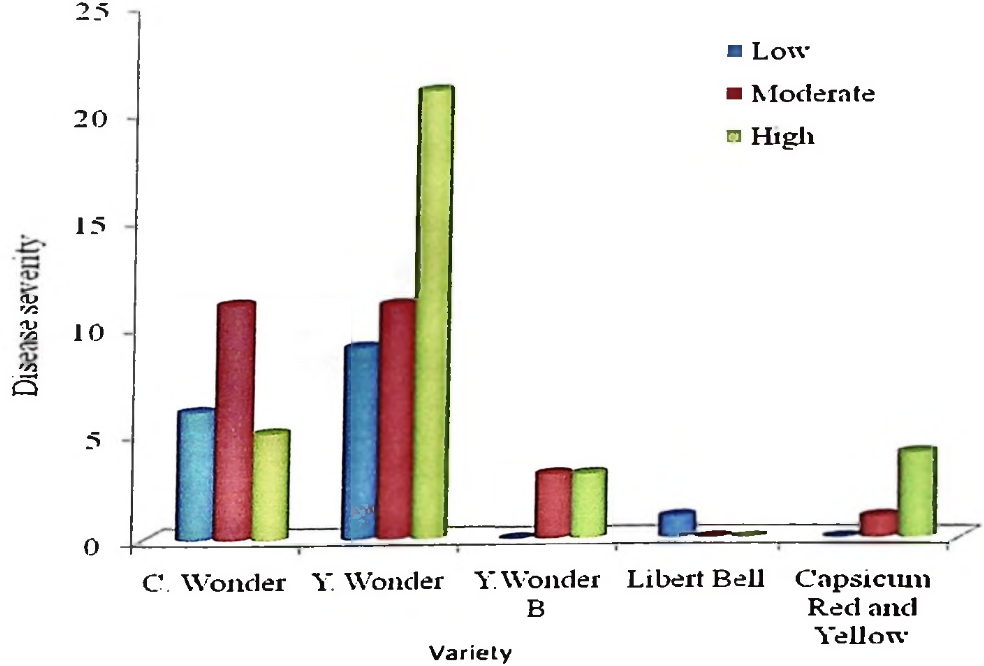


Figure 11: The reaction of locally available sweet pepper varieties to bacterial leaf spot disease in farmers' fields in Morogoro, Arusha, Tanga and Mbeya regions

The results also demonstrate that, all sweet pepper farmers interviewed (100 %), bought their sweet pepper seeds from the agricultural inputs shops, applied pesticides as a management strategy for the bacterial leaf spot disease and irrigated their crop (Table 3).

The majority of the farmers interviewed (80 %) used the same field for sweet pepper production every year and rotated with other solanaceous crops, especially tomatoes and round potatoes. Twenty percent of the farmers interviewed grew sweet pepper on the same field for two to three years, fallowed by rotation with non-related solanaceous crops (Table 3; Figures 7 and 8).



Key: C. Wonder = California Wonder, Y. Wonder = Yolo Wonder

Figure 12: Severity of bacterial leaf spot disease on different sweet pepper varieties in farmers' fields in Morogoro, Arusha, Tanga and Mbeya regions

Bacterial leaf spot disease of sweet pepper was widespread in the major sweet pepper-growing regions in the country. The disease prevailed where sweet pepper and tomato or other solanaceae crops were present in the same or adjacent fields. High disease incidence was observed in the farmers' fields during high rainfall season in Morogoro, Arusha and Tanga regions. In some fields, severe leaf infection caused defoliation and complete death of the plants (Plates 1b and 1c). However, a recovery of the plants occurred in some cases,

developing new foliage and fruits, although yield and quality were low (Plates 1c and 5). Black *et al.* (2001) reported disease incidence of 5 % in sweet pepper fields, whereas, in tomato fields the incidence of bacterial leaf spot was up to 90 % in the major sweet pepper/tomato-producing regions. In this study disease incidence was found to be up to 100 % in sweet pepper fields.

Previous studies (Hansen, 2000; Kuflu and Cupples, 1997; McGrath, 1997; Pernezny and Collins, 1997; Sahin and Miller, 1996; Shenge *et al.*, 2010) reported that, field sanitation is an effective option in the management of bacterial leaf spot disease of sweet pepper and tomato. However, other studies (Kousik and Ritchie, 1998: Martin *et al.*, 2004; Sahin and Miller, 1998) reported that, once the diseases are present in the field and disease pressure is high, field sanitation is not effective in managing bacterial diseases of vegetable crops. High incidence and severity of bacterial leaf spot disease caused by bacterial leaf spot-causing xanthomonads depend upon several factors such as contaminated seeds and transplants, continuous growing of sweet pepper and tomato on the same field, susceptible sweet pepper varieties, volunteer sweet pepper plants, infested crop debris and infected weeds (Canteros *et al.*, 1995; Hansen, 2000; Hibberd *et al.*, 1989; Jones *et al.*, 1986; Kousik and Ritchie, 1998; Leben, 1974; McGrath, 1997; Sahin and Miller, 1998), presence of bacterial strains tolerant to copper (Martin *et al.*, 2004), epiphytically survival of the pathogen on host and non-host plants (Kuflu and Cuppels, 1997; Louws *et al.*, 2001; Pernezny and Collins, 1997), high temperature, rainfall and humidity (Canteros *et al.*, 1995; Cuppels *et al.*, 2006; Hansen, 2000; Kousik and Ritchie, 1996; Louws *et al.*, 2001) and shades (McGrath, 1997). The results of this study demonstrate that, high disease incidence and severity were recorded in clean fields (Figures 5 and 6). Some fields in Luale, Kivulul and Lukozi villages were abandoned by the owners who reported to fail

managing the disease and therefore, decided to grow other crops (Plate 5). However, it was observed during the survey that, some farmers did not uproot the diseased sweet pepper plants, which later on recovered (Plate 5) and became sources of BSX inocula for the successive crops.

High disease incidence and severity of bacterial leaf spot in clean fields were also due to the use of susceptible varieties (Yolo Wonder and California Wonder), continuous growing of sweet pepper/tomato crops on the same fields for a long time (Table 3), ability of bacterial leaf spot-causing xanthomonads to survive on crop debris thus, causing contamination of transplants and volunteer sweet pepper/tomato plants which, served as sources of inocula for successive crops (Plate 5). Although sweet pepper and tomato were reported to be the sole hosts of bacterial leaf spot causing xanthomonads, it has also been reported to have the ability to epiphytically survive on protected plant organs (such as leaves, buds and flowers) of sweet pepper/tomato and non-host plants without causing visible symptoms (Jones *et al.*, 1986; Pernezny and Collins, 1997). The 'protected position' are reported to be more moist and receive less ultraviolet light, therefore, are better environments for bacterial survival and growth (Leben, 1981; Leben, 1988). Furthermore, Pernezny and Collins (1997) reported that, the susceptible sweet pepper cultivars support more epiphytic populations of BSX than did the resistant cultivars. Therefore, during the favourable environmental conditions (high temperatures, humidity and rainfall) the BSX multiply rapidly and spread, leading to high disease incidence during the sweet pepper flowering stage.

In this study, high disease incidence was recorded in Lukozi village (Tanga region) and high disease severity was recorded in Kivulul village (Arusha region) (Tables 4 and 5;

Figures 4 and 5). High disease severity in Arusha was due to sweet pepper grown under heavy shades of coffee and banana plants which increased humidity (moist) and plants received less ultraviolet light. Moist condition due to high humidity and less ultraviolet light, are better environments for bacterial survival (Leben, 1981; Leben, 1988). Therefore, when farmers worked in wet fields, they wounded plants, thus made easy penetration of bacteria through wounds.

In addition, the bacterial spot-causing xanthomonads have been reported to survive for periods of 10 years in seed (Bashan *et al*., 1982; Peterson, 1963) and in various solanaceae plants, mainly weeds which have been recorded as incidental hosts of bacterial spot-causing xanthomonads (Aysan and Sahin, 2003; CABI/EPPO, 1990; Jones *et al*., 1986; Kousik and Ritchie, 1998; Leben, 1974; Pernezny and Collins, 1997). In this study, round potatoes, sweet potatoes and other solanaceae weeds were found surrounding sweet pepper fields during the survey.

Furthermore, existence of new sweet pepper races make bacterial leaf spot disease management difficult (Bouzar *et al*., 1994; Cook and Stall, 1982; Kousik and Ritchie, 1995; Romero *et al*., 1996). In this study, seven sweet pepper races (P0 – P6) were identified. Therefore, integrated disease management strategies should be considered as an important option for managing bacterial leaf spot disease of sweet pepper.

It has been reported by several authors that, the current management strategies for bacterial leaf spot disease, including the use of "pathogen-free" seed and seedlings, sanitation, cultural practices, the use of bacterial spot-causing xanthomonads-resistant cultivars and chemical application (Cox, 1982; Jones *et al*., 1986; Kousik and Ritchie,

1996; Louws *et al.*, 2001; Sahin and Miller, 1998) are not always successful in managing the disease due to pathogenic variation within the pathogens (BSX). The use of host genetic resistant to BSX is economically and technically the most practical method for bacterial leaf spot management (Hulbert *et al.*, 2001; Kousik and Ritchie, 1996; Scott *et al.*, 1989). However, development of disease-resistant varieties requires the identification of the prevailing strains and incorporation of resistant genes into commercially acceptable pepper genotypes (Hibberd *et al.*, 1987; Obradovic *et al.*, 2004; Sahin and Miller, 1998). The results of this study demonstrate the need for breeding for resistant sweet pepper varieties in Tanzania by considering the existing pathogenic variation.

All farmers interviewed in this study indicated that, chemical application was their first choice for management of bacterial leaf spot disease outbreak in sweet pepper (Table 3). However, it was observed during the survey that, all farmers interviewed had little knowledge on the different chemicals used. Chemicals which were common to farmers interviewed included Dithane® (M-45 mancozeb), Karate® (cyhalothrin K), Ridomyl® (Metalyxl), Cupravit®/Blue copper® (Copper oxychloride), Thionex (Endosulfan: Hexachlorohexahydromethano-2,4,3- benzodioxathiepin-3-oxide), Rogor (Dimethoate), Thiodan (Endosulfan) and Ridomil Gold® Bravo® (Mefenoxam and Chlorothalonil). Four of the chemicals (Karate, Thiodan, Rogor and Thionex) mentioned were insecticides. During the interview, it was observed that, some sweet pepper farmers applied a combination of pesticides (cocktail of fungicides and insecticides) as a control measure against bacterial leaf spot disease. Similar observations on the indiscriminate application of pesticides have been reported for cabbage farmers in Arumeru district (Arusha region) in northern Tanzania; where insecticides were used to control black rot caused by *X. campestris*. pv. *campestris* (Massomo *et al.*, 2005). Tomato farmers in the major tomato-

growing regions have also been reported to use insecticides to control bacterial leaf spot caused by BSX (Shenge *et al.*, 2010).

The chemicals mentioned by farmers during this study were sold in the open market and agricultural input shops, where the majority of the pesticides end up in the hands of untrained farmers, leading to their indiscriminate application on sweet pepper and other crops (Plate 6). Since the indiscriminate applications of these pesticides have been reported for a long time, their long-term impact in the environment for some of the chemicals has not been assessed on sweet pepper except for copper compounds (Gore and O'Garro, 1991; Louws *et al.*, 2001; Martin and Hamilton, 2004; Ritchie and Dittapongpitch, 1991; Sahin and Miller, 1996; Sahin and Miller, 1998; Ward and O'Garro, 1992). However, Shenge et al. (2010) reported the increasing occurrence of copper sulphate resistance among population of BSX of tomato in Tanzania. Since sweet peppers and tomatoes are grown by the same farmers in the major sweet pepper/tomato growing regions in Tanzania where the sweet pepper/tomato farmers apply copper pesticides as a bacterial leaf spot disease management practice, it is possible that, copper pesticides may no longer be a viable option for the management of bacterial leaf spot disease of sweet pepper due to build-up of resistance.

Plate 6: Freshly applied pesticide on sweet pepper, Chinese cabbage and maize plants for disease management in a farmer's field at Kivulul, Arusha region, Tanzania

Bacterial leaf spot of sweet pepper is a seed-borne disease. The seed-borne nature of this pathogen is well documented (Bashan *et al.*, 1982; Cox, 1982; Jones *et al.*, 1995; Pohronezny *et al.*, 1992; Sahin and Miller, 1998). Contaminated seeds are not only a means of survival, but also a major source of inocula for bacterial leaf spot-causing xanthomonads (Bashan *et al.*, 1982a; Bashan and Okon, 1986; Bashan *et al.*, 1982b; Jones *et al.*, 1986; Mirik and Aysan, 2009; Peterson, 1963). Therefore, a key management strategy for the control of this disease should include the use of healthy planting material. The results of this study indicate that, all the tested sweet pepper seeds from the surveyed areas were free from BSX (Table 6).

Table 6: Sweet pepper varieties screened for seed-borne infection by bacterial spot-causing xanthomonads during the current study

Pepper variety	Origin	Collection site	Seed health status of BSX
California Wonder	Pop Vriend Seeds	Suba Seed supplier, Arusha	-
California Wonder	Kibo seed Company	Seed supplier, Lushoto	-
Yolo Wonder	Bakker Brothers	Suba Seed supplier, Arusha	-
Yolo Wonder	Pop Vriend Seeds	Suba Seed supplier, Arusha	-
Yolo Wonder B	Mkulima Seed	Seed supplier, Lushoto	-
Yolo Wonder B	Royal Sluis	Imuka Seed supplier, Morogoro	-

- = Bacterial leaf spot-causing xanthomonads not detected

All the sweet pepper seeds sold by agricultural input suppliers in the study areas were hermetically sealed in cans, and were found treated with thiram. However, previous studies by Kaaya *et al.* (2003) reported that 40.7 % of tomato seeds tested was contaminated with bacterial leaf spot-causing xanthomonads in Tanzania. Other studies (Miriki and Aysan, 2009) reported that, 21 (72.4 %) of the 29 sweet pepper seed samples in Turkey were contaminated by bacterial spot-causing xanthomonads. Sahin and Miller (1996) reported that the prevalence of P3 in Ohio was the result of distribution of contaminated seed and transplants. Therefore, screening of the sweet pepper seeds sold in Tanzania should be done on a regular basis to ensure that they are not infected with seed-borne bacterial pathogens.

Unlike in the developed parts of the world where vegetable production is mostly undertaken by large-scale farmers, production of vegetables in Tanzania is mostly

undertaken by small-scale, poor-resource farmers who face a lot of difficulties and constraints (such as diseases and inadequate knowledge) in production (Massomo *et al.*, 2005). In this study, bacterial leaf spot disease was identified by all sweet pepper farmers as the most important production constraint. In order to overcome the problem of bacterial leaf spot disease in sweet pepper production, farmers were reported to apply a mixture of pesticides in controlling the disease. However, the indiscriminate application of pesticides was an indication that, sweet pepper farmers did not attend formal school beyond the secondary level. Therefore, training farmers in disease management strategies is very important. The agricultural extension department should therefore, focus more on training farmers on pesticides handling, application and management of vegetable crop diseases in order to equip them with well-informed disease management decisions, including proper application of pesticides.

4.2 Identification of Bacterial Strains

Seventy four isolates from diseased sweet pepper plant parts were identified as xanthomonads based on morphological and biochemical characteristics. The strains were Gram negative and oxidase negative. They formed yellow, convex, mucoid colonies on YDC medium, metabolized glucose oxidatively, hydrolyzed gelatin, lypolytic positive, did not reduce nitrate and were pectolytic negative.

4.2.1 Starch hydrolysis

The results after characterization of the strains by PCR, using species specific primer pairs, and confirmation of their pathogenicity on the susceptible host plant (cv. Early Calwonder) revealed that, 43 of the 63 pathogenic bacterial leaf spot-causing xanthomonads isolated from sweet pepper were amylolytic positive (Amy^{+}) and non-

pectolytic (pec^-) similar to the two reference strains (*X. vesicatoria* NCPPB 422 and *X. perforans* NCPPB 4321). Twenty strains were amylolytic negative and non-pectolytic similar to the 5 reference strains (*X. euvesicatoria* NCPPB 2968, *X. gardneri* NCPPB 881, XVT 12, XVT 28 and XVT 48) (Table 7). These characteristics indicated that, there were two (amylolytic and non-amylolytic) groups of bacterial leaf spot-causing xanthomonads infecting pepper plants in Tanzania. All of the non-pathogenic strains were Amy^+ and pec^-.

Table 7: Grouping strains of bacterial leaf spot-causing xanthomonads of sweet pepper based on amylolytic activity

Species name	Number of strains	Amylolytic activity	
		+	-
X. euvesicatoria	30	21	9
X. perforans	10	5	5
X. gardneri	23	17	6
Total	**63**	**43**	**20**

+ = positive amylolytic activity; - = negative amylolytic activity

4.2.2 Polymerase chain reaction (PCR)

The selected general primer sets RST2 and RST3 generated a PCR product (Plate 7) from genomic DNA of 59 (79.7 %) out of 74 tested *Xanthomonas* strains isolated from sweet pepper. All of the reference strains tested generated similar PCR product. No PCR product was obtained from the 15 (20.3 %) isolates (Table 8). The PCR results confirm the presence of bacterial leaf spot-causing xanthomonads in Tanzania. However, the general primer sets used (formerly used to identify *X. axonopodis* pv. *vesicatoria*) were not so specific to identify the strains to species level. Therefore, further analysis using effector genes and species specific primer sets was employed.

Table 8: Polymarase Chain Reaction and pathogenicity tests of bacterial leaf spot-causing xanthomonads from sweet pepper collected from Morogoro, Tanga, Arusha and Mbeya regions, Tanzania

Isolate/Strain	Plant part	Primer name									Path. test
		RST2/3	avrBs1 F/R	avrBs2 F/R	avrBs3 F/R	avrBs4 F/R	X. e F/R	X. v F/R	X. p F/R	X. g F/R	ECW
MW01TA	Fruit	+	-	-	+	-	+	-	-	-	C
MW02TA	Fruit	+	-	-	+	-	+	-	-	-	C
MW03TA	Fruit	-	-	-	+	+	+	-	-	-	C
MW04TA	Fruit	+	-	+	+	-	+	-	-	-	C
MW05TA	Fruit	+	-	+	+	+	+	-	-	-	C
MW06TA	Fruit	+	-	+	+	+	+	-	-	-	C
MW07TA	Leaf	-	-	-	+	-	+	-	-	-	C
MW08TA	Leaf	+	-	-	+	+	+	-	-	-	C
MW09TA	Leaf	+	-	-	+	-	-	-	-	+	C
MW10TA	Leaf	+	-	+	-	-	-	-	+	-	C
MW11TA	Leaf	+	-	+	+	-	-	-	-	+	C
MW12TA	Leaf	+	-	+	+	+	+	-	-	-	C
MW13TA	Leaf	+	-	+	+	+	+	-	-	-	C
MW14TA	Leaf	+	-	+	+	+	+	-	-	-	C
MW15TA	Leaf	+	-	-	-	+	+	-	-	-	C
MW16TA	Fruit	+	-	-	+	-	+	-	-	-	C
MW17TA	Leaf	+	-	-	+	+	+	-	-	-	C

Key: C = compatible response (disease reaction); HR = hypersensitive response (resistance reaction); nr = no reaction; Tmt = tomato; ECW= Early calwonder; ECW-10R, ECW-20R and ECW-30R = ECW with incorporated resistance gene; NR = no reaction; NT = not tested; *X..e* = *Xanthomonas euvesicatoria*; *X. v* = *X. vesicatoria*; *X.p* = *X. perforans*; *X.g* = *X. gardneri*; Path = pathogenicity

Table 8: (Cont). Polymarase Chain Reaction and pathogenicity tests of bacterial leaf spot-causing xanthomonads from sweet pepper collected from Morogoro, Tanga, Arusha and Mbeya regions, Tanzania

Isolate/Strain	Plant part	Primer name									Path. test
		RST2/3	avrBs1 F/R	avrBs2 F/R	avrBs3 F/R	avrBs4 F/R	X. e F/R	X. v F/R	X. p F/R	X. g F/R	ECW
MW18TA	Leaf	+	-	+	+	-	+	-	-	-	C
MW19TA	Leaf	+	-	-	+	-	-	-	-	+	C
MW20TA	Leaf	+	-	-	+	+	-	-	+	-	C
MW21TA	Fruit	-	-	-	+	-	-	-	-	+	C
MW22TA	Fruit	+	-	+	+	-	-	-	-	+	C
MW23TA	Fruit	+	-	-	+	+	-	-	+	-	C
MW24TA	Fruit	+	-	-	+	+	-	-	-	+	C
MW25TA	Fruit	+	-	+	+	+	-	-	+	-	C
MW26TA	Leaf	+	-	+	+	+	-	-	+	-	C
MW27TA	Fruit	-	-	-	-	+	+	-	-	-	NR
MW28TA	Fruit	-	-	-	-	-	-	-	-	-	HR
MW29TA	Fruit	-	-	-	-	+	-	-	+	-	C
MW30TA	Fruit	+	+	+	+	-	-	-	+	-	C
MW31AR	Fruit	+	-	+	+	+	-	-	-	+	C
MW32AR	Fruit	+	-	+	+	-	-	-	-	+	C
MW 33MG	Leaf	-	-	-	-	-	NT	NT	NT	NT	NT
MW34MG	Leaf	+	+	-	+	-	-	-	+	-	C

Key: C = compatible response (disease reaction); HR = hypersensitive response (resistance reaction); nr = no reaction; Tmt = tomato; ECW= Early calwonder; ECW-10R, ECW-20R and ECW-30R = ECW with incorporated resistance gene; NR = no reaction; NT = not tested; *X..e* = *Xanthomonas euvesicatoria*; *X. v* = *X. vesicatoria*; *X.p* = *X. perforans*; *X.g* = *X. gardneri*; Path = pathogenicity

Table 8: (Cont). Polymarase Chain Reaction and pathogenicity tests of bacterial leaf spot-causing xanthomonads from sweet pepper collected from Morogoro, Tanga, Arusha and Mbeya regions, Tanzania

Isolate/Strain	Plant part	Primer name									Path. test
		RST2/3	avrBs1 F/R	avrBs2 F/R	avrBs3 F/R	avrBs4 F/R	X. e F/R	X. v F/R	X. p F/R	X. g F/R	ECW
MW35MG	Leaf	+	-	-	+	+	-	-	-	+	C
MW36MG	Fruit	+	-	-	+	+	-	-	-	+	C
MW37MG	Leaf	+	-	+	+	-	-	-	-	+	C
MW38MG	Leaf	+	+	+	+	-	-	-	-	+	C
MW39MG	Fruit	+	-	+	+	+	+	-	-	-	C
MW40MG	Leaf	+	-	+	+	+	+	-	-	-	C
MW41MG	Leaf	+	-	-	+	-	+	-	-	-	C
MW42MG	Leaf	+	-	-	+	-	+	-	-	-	C
MW43MG	Fruit	+	-	-	-	-	-	-	-	+	C
MW44MG	Fruit	+	-	-	-	+	-	-	+	-	C
MW45MG	Fruit	-	-	-	-	-	-	-	-	-	NR
MW46MG	Fruit	+	+	+	+	-	-	-		-	C
MW47MG	Leaf	+	+	+	+	-	-	-	-	+	C
MW48AR	Leaf	+	+	+	+	-	-	-	-	+	C
MW49AR	Leaf	+	+	-	+	+	-	-	-	+	C
MW50AR	Leaf	-	-	-	-	+	-	-	+	-	C
MW51AR	Leaf	+	-	+	-	+	+	-	-	-	C

Key: C = compatible response (disease reaction); HR = hypersensitive response (resistance reaction); nr = no reaction; Tmt = tomato; ECW= Early calwonder; ECW-10R, ECW-20R and ECW-30R = ECW with incorporated resistance gene; NR = no reaction; NT = not tested; *X..e* = *Xanthomonas euvesicatoria*; *X. v* = *X. vesicatoria*; *X.p* = *X. perforans*; *X.g* = *X. gardneri*; Path = pathogenicity

Table 8: (Cont). Polymarase Chain Reaction and pathogenicity tests of bacterial leaf spot-causing xanthomonads from sweet pepper collected from Morogoro, Tanga, Arusha and Mbeya regions, Tanzania

Isolate/Strain	Plant part	Primer name									Path. test
		RST2/3	avrBs1 F/R	avrBs2 F/R	avrBs3 F/R	avrBs4 F/R	X. e F/R	X. v F/R	X. p F/R	X. g F/R	ECW
MW52AR	Leaf	+	-	+	+	+	-	-	-	+	C
MW53AR	Leaf	+	+	-	+	+	+	-	-	-	C
MW54AR	Leaf	+	+	+	-	+	-	-	-	+	C
MW55AR	Stem	+	-	+	+	+	-	-	-	+	C
MW56AR	Leaf	-	+	-	-	-	-	-	-	-	HR
MW57AR	Leaf	-	-	-	+	+	-	-	-	-	NR
MW58AR	Leaf	+	-	-	+	-	-	-	-	+	C
MW59AR	Leaf	+	-	+	+	+	+	-	-	-	C
MW60AR	Leaf	+	-	+	+	+	-	-	-	+	C
MW61MG	Fruit	+	-	+	+	+	+	-	-	-	C
MW62MG	Fruit	+	-	+	+	+	+	-	-	-	C
MW63MG	Fruit	+	-	+	+	+	+	-	-	-	C
MW64MG	Fruit	+	-	+	+	+	-	-	-	+	C
MW65MG	Fruit	+	-	+	+	+	+	-	-	-	C
MW66MG	Fruit	+	-	+	-	-	+	-	-	-	C
MW67MG	Fruit	+	-	+	+	+	+	-	-	-	C
MW68MG	Fruit	+	-	-	-	-	+	-	-	-	C

Key: C = compatible response (disease reaction); HR = hypersensitive response (resistance reaction); nr = no reaction; Tmt = tomato; ECW= Early calwonder; ECW-10R, ECW-20R and ECW-30R = ECW with incorporated resistance gene; NR = no reaction; NT = not tested; *X..e* = *Xanthomonas euvesicatoria*; *X. v* = *X. vesicatoria*; *X.p* = *X. perforans*; *X. g* = *X. gardneri*; Path = pathogenicity

Table 8: (Cont). Polymarase Chain Reaction and pathogenicity tests of bacterial leaf spot-causing xanthomonads from sweet pepper collected from Morogoro, Tanga, Arusha and Mbeya regions, Tanzania

Isolate/Strain	Plant part	Primer name									Path. test
		RST2/3	avrBs1 F/R	avrBs2 F/R	avrBs3 F/R	avrBs4 F/R	X. e F/R	X. v F/R	X. p F/R	X. g F/R	ECW
MW69MG	Fruit	+	-	+	-	+	+	-	-	-	C
MW70MB	Leaf	-	-	-	-	-	NT	NT	NT	NT	NT
MW71MB	Leaf	-	-	-	-	-	NT	NT	NT	NT	NT
MW72MB	Leaf	-	-	-	-	-	NT	NT	NT	NT	NT
MW73MB	Leaf	-	-	-	-	-	NT	NT	NT	NT	NT
MW74MB	Leaf	-	-	-	-	-	NT	NT	NT	NT	NT
X v NCPPB 422		+	-	-	-	-	-	+	-	-	NR
X.e NCPPB 2968		+	-	+	-	+	+	-	-	-	C
X.g NCPPB 881		+	-	-	-	-	-	-	-	+	C
X.p NCPPB 4321		+	-	-	-	+	-	-	+	-	C
XVT12	Tmt	+	-	+	-	+	+	-	-	-	C
XVT28	Tmt	+	-	+	-	+	+	-	-	-	C
XVT48	Tmt	+	-	-	-	+	+	-	-	-	C

Key: C = compatible response (disease reaction); HR = hypersensitive response (resistance reaction); nr = no reaction; Tmt = tomato; ECW= Early calwonder; ECW-10R, ECW-20R and ECW-30R = ECW with incorporated resistance gene; NR = no reaction; NT = not tested; *X..e* = *Xanthomonas euvesicatoria*; *X. v* = *X. vesicatoria*; *X.p* = *X. perforans*; *X.g* = *X. gardneri*; Path = pathogenicity

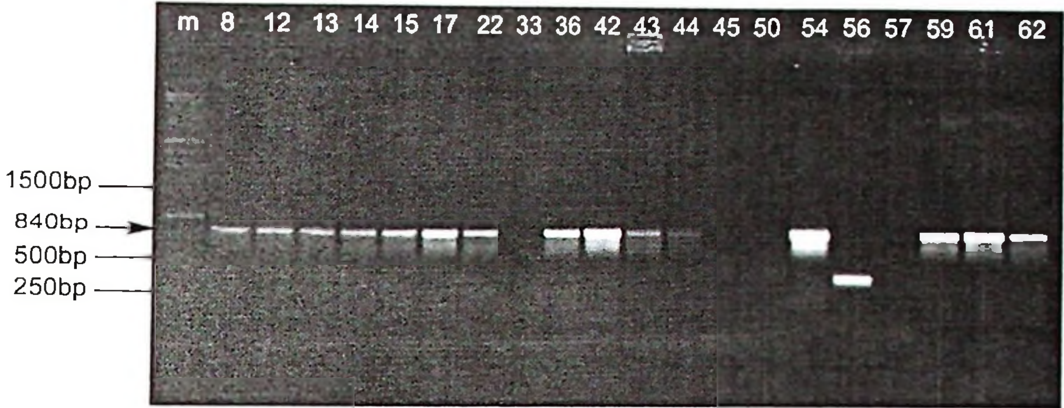


Plate 7: Gel electrophoresis analysis of polymerase chain reaction products amplified using primer set RST2/RST3 for bacterial leaf spot-causing xanthomonads (sweet pepper strains) collected from Morogoro, Tanga, Arusha and Mbeya regions in Tanzania

4.2.3 Characterization of bacterial leaf spot-causing xanthomonads using the effector proteins primer sets *avrBs*1, *avrBs*2, *avrBs*3 and *avrBs*4

The effector protein/avirulence genes primer pairs (*avrBs*1 F/R, *avrBs*2 F/R, *avrBs*3 F/R and *avrBs*4 F/R) generated PCR products from genomic DNA of 68 out of 74 strains tested [*avrBs*1 (10), *avrBs*2 (36), *avrBs*3 (53) and *avrBs*4 (39)] (Plates 8, 9, 10 and 11). The amplification fragment length of the primer pairs were 1337-bp, 2156-bp, 500-bp and 3482-bp, respectively (Table 8). When the effector proteins primer pairs were used, nine more strains which were not generated by the general primer set described by Leite *et al.* (1995), generated PCR products from genomic DNA. However, the effector gene primer sets were not specific enough to identify the strains. The results also indicate that, the xanthomonad strains characterized contained none or more than one of the effector genes indicating that, a pathogen genome can contain more than one effector genes that can be functional or non-functional for interaction with resistant (R) genes from the plant. Of the 74 strains characterized, 10 contained none, 17 contained 1, 19 contained 2 and 28 strains contained 3 effector genes. None of the strains contained 4 effector genes.

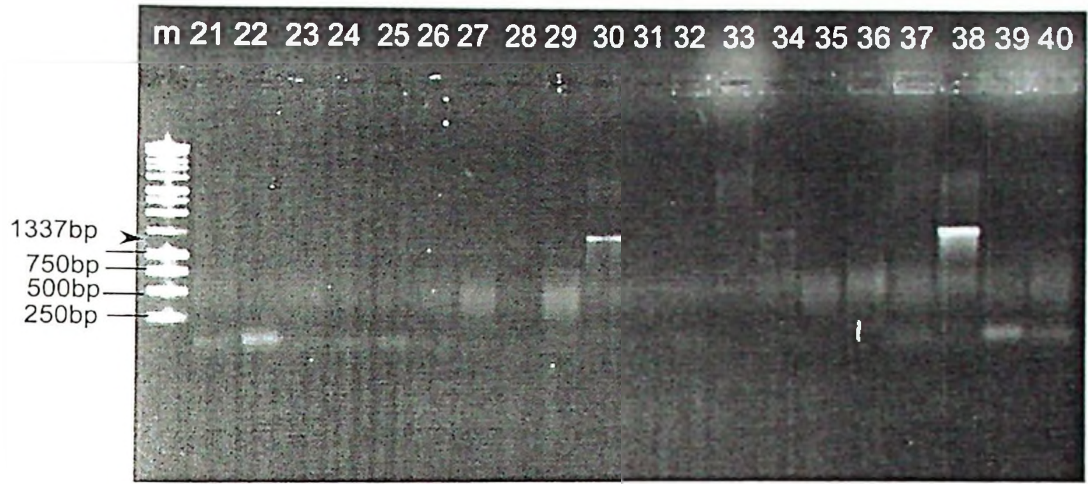


Plate 8: Gel electrophoresis analysis of polymerase chain reaction products amplified using primer set *avrBs*1 for bacterial leaf spot-causing xanthomonads (sweet pepper strains) collected from Morogoro, Tanga, Arusha and Mbeya regions in Tanzania

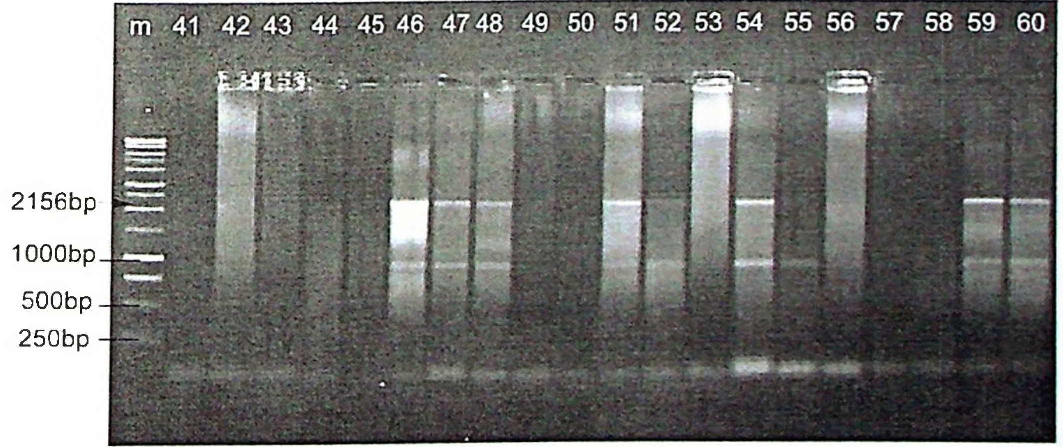


Plate 9: Gel electrophoresis analysis of polymerase chain reaction products amplified using primer set *avrBs*2 for bacterial leaf spot-causing xanthomonads (sweet pepper strains) collected from Morogoro, Tanga, Arusha and Mbeya regions in Tanzania

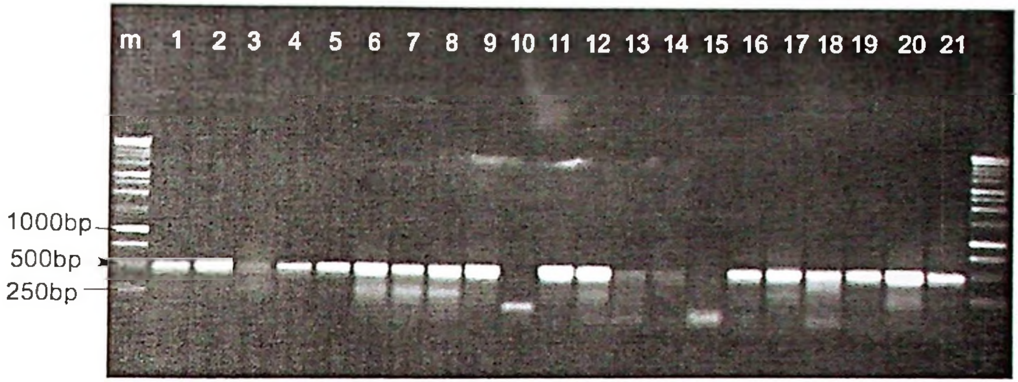


Plate 10: Gel electrophoresis analysis of polymerase chain reaction products amplified using a set of primer *avrBs3* for bacterial leaf spot-causing xanthomonads (sweet pepper strains) collected from Morogoro, Tanga, Arusha and Mbeya regions in Tanzania

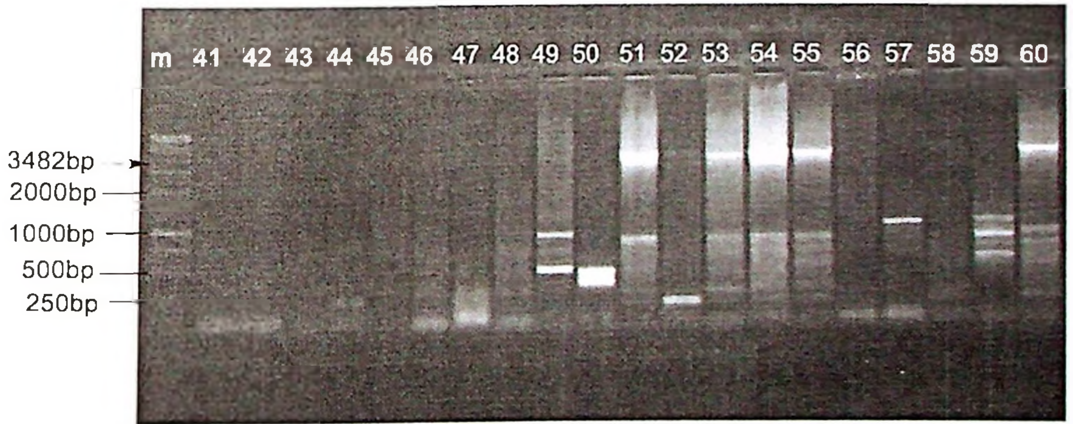


Plate 11: Gel electrophoresis analysis of polymerase chain reaction products amplified using *avrBs4* primer set for bacterial leaf spot-causing xanthomonads (sweet pepper strains) collected from Morogoro, Tanga, Arusha and Mbeya regions in Tanzania

All isolates from Mbeya region were not amplified by any of the primer sets used, indicating that the isolates were not pathogenic xanthomonads (Table 8). Of the reference strains characterized, *X. vesicatoria* and *X. gardneri* haboured none of the effector genes, *X. perforans* haboured 1, *X. euvesicatoria*, XVT12, XVT28 and XVT48 each haboured 2 effector genes (Table 8).

4.2.4 Characterization of bacterial leaf spot-causing xanthomonads using species specific primer sets

Of the 68 strains characterized using the effector gene primer sets, only 63 (92.6 %) strains generated PCR products from genomic DNA using three out of four species specific primer pairs (*X. euvesicatoria* F/R, *X. perforans* F/R, *X. gardneri* F/R and *X. vesicatoria* F/R) used (Plates 12, 13 and 14). The amplification fragment lengths were 153-bp, 232-bp and 189-bp, respectively. The species identified were *Xanthomonas euvesicatoria* (44.1 %), *X. perforans* (14.7 %) and X. *gardneri* (33.8 %) (Figure 12 and Plates 12, 13 and 14).

Furthermore, the reference strains XVT12, XVT28 and XVT48 from Taiwan, which were reported to be *X. vesicatoria* isolated from tomato, were characterized as *X. euvesicatoria* (Plate 15) and not as *X. vesicatoria* (Plate 16). No xanthomonad strains of sweet pepper were characterized as *X. vesicatoria*. These results indicate that, the strains identified fell into three groups: A (*X. euvesicatoria*), C (*X. perforans*) and D (*X. gardneri*), which were previously described by Jones *et al.* (2004). The species *X. euvesicatoria* dominated in Tanga (22.1 %) and Morogoro (17.6 %) regions, whereas, *X. perforans* dominated in Tanga region (10.3 %); and *X. gardneri* dominated in Arusha region (13.2 %) followed by Morogoro region (11.8 %) (Figures 13 and 15). The results also indicate that, the overall

153bp

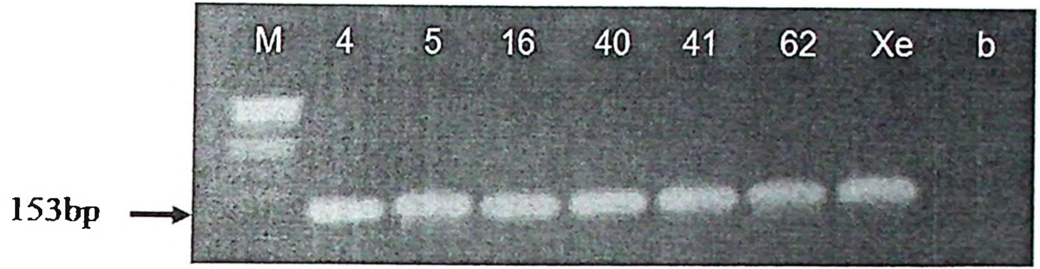


Plate 12: Gel electrophoresis analysis of polymerase chain reaction products amplified using primer set *Xanthomonas euvesicatoria* for bacterial leaf spot-causing xanthomonads (sweet pepper strains) collected from Morogoro, Tanga and Arusha regions, Tanzania

189bp

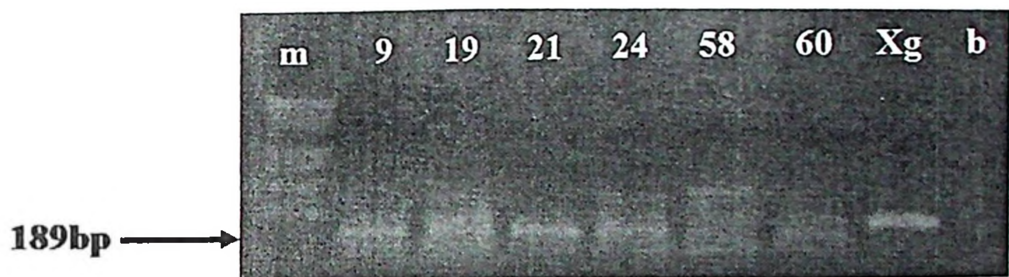


Plate 13: Gel electrophoresis analysis of polymerase chain reaction products amplified using primer set *Xanthomonas gardneri* for bacterial leaf spot-causing xanthomonads (sweet pepper strains) collected from Morogoro, Tanga and Arusha regions, Tanzania

232bp

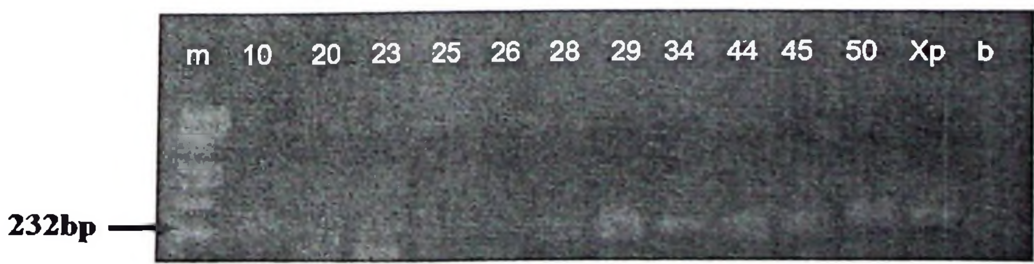


Plate 14: Gel electrophoresis analysis of polymerase chain reaction products amplified using *Xanthomonas perforans* primer set for bacterial leaf spot-causing xanthomonads (sweet pepper strains) collected from Morogoro, Tanga and Arusha regions, Tanzania

153bp

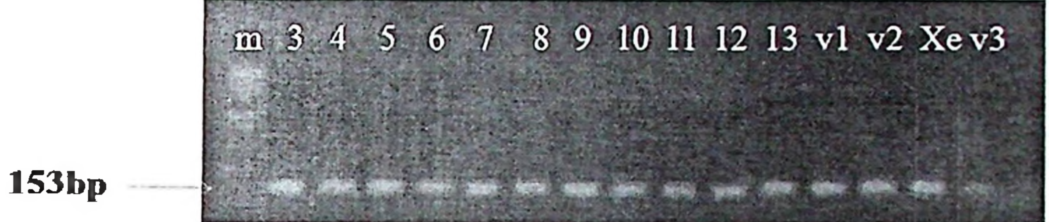


Plate 15: Gel electrophoresis analysis of polymerase chain reaction products amplified using *Xanthomonas euvesicatoria* (*Xe*) primer set for some pepper strains collected from Tanzania; and the reference strains from Taiwan

Key: v1 = XVT12, v2 = XVT28 and v3 = XVT48

248bp

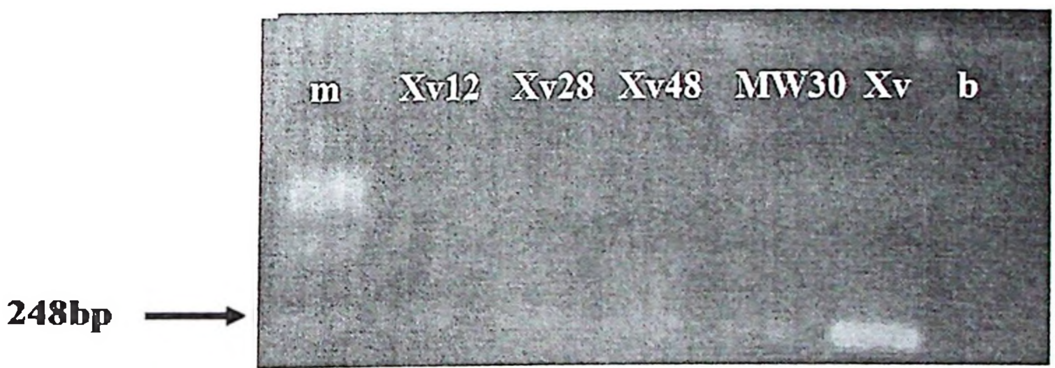
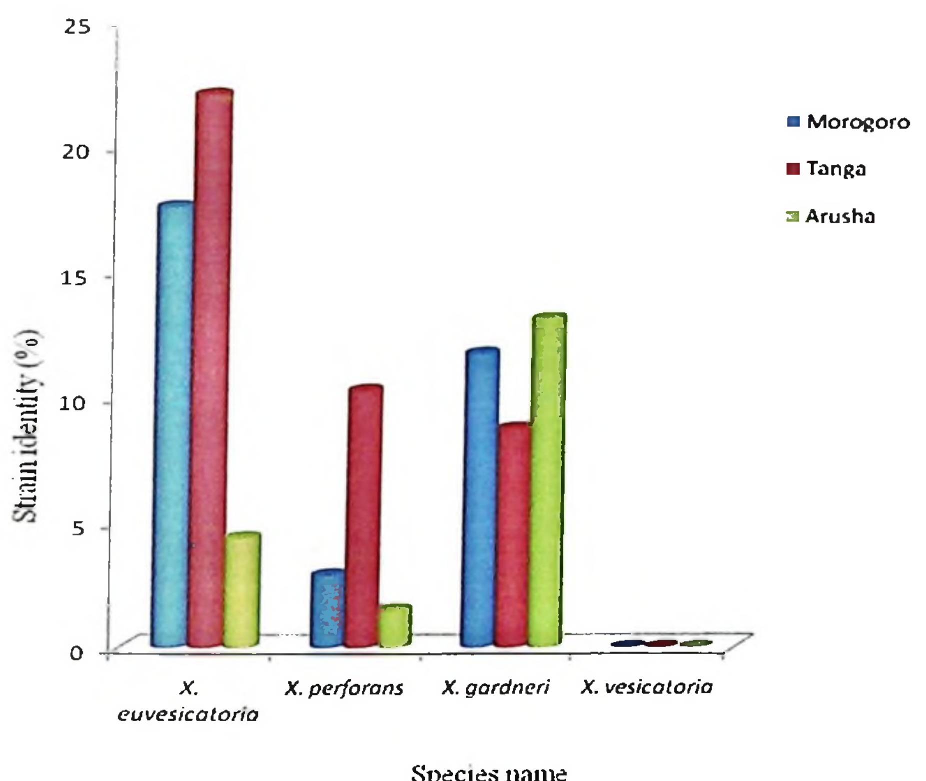


Plate 16: Gel electrophoresis analysis of polymerase chain reaction products amplified using *Xanthomonas vesicatoria* primer set for the reference strains collected from Taiwan

percentage of all species identified (41.2 %) were collected from Tanga region. More surveys are needed in other sweet pepper growing regions in the country in order to understand the population composition of bacterial leaf spot causing xanthomonads for proper management purposes.



Key: X = *Xanthomonas*

Figure 13: Characterization of bacterial leaf spot-causing *Xanthomonas* species isolated from sweet pepper collected from Morogoro, Tanga and Arusha regions in Tanzania

The amplification of genomic DNA using the rep-PCR has been a useful tool for the detection and identification of plant pathogenic xanthomonads (Jones *et al.*, 1998; Mitrev and Kovačević, 2006; Obradovic *et al.*, 2004). Although, a number of molecular methods have been developed for detection of bacterial leaf spot-causing xanthomonads, successful detection of the pathogen using PCR techniques depends on the specificity of the primers after restriction enzyme analysis of the amplicon produced (Cuppels *et al.*, 2006; De Boer *et al.*, 2007; Mitrev and Kovačević, 2006; Moretti *et al.*, 2009; Obradovic *et al.*, 2004). This study has revealed that, the primer set described by Leite *et al.* (1995) permitted the

detection of 79.7 % of bacterial leaf spot-causing xanthomonads to genus level and failed to detect other strains (Table 8). The effector/avirulence genes primer sets described by Wichmann and Bergelson (2003), used to detect effector genes residing in the pathogen genome, were able to detect 68 (91.9 %) out of 74 isolates tested (Table 8). However, the species-specific primers described by Mbega *et al.* (*Unpublished*) permitted successful identification of three species of bacterial leaf spot-causing xanthomonads of sweet pepper present in Tanzania. These species were *X. euvesicatoria*, *X. perforans* and *X. gardneri.*

Previous studies (Jones *et al.*, 2000; Jones *et al.*, 2005; Obradovic *et al.*, 2004; Vauterin *et al.*, 1995) reported that, bacterial leaf spot disease of sweet pepper and tomato is caused by four *Xanthomonas* species/groups namely: *X. euvesicatoria* (group-A) infecting pepper only, *X. vesicatoria* (group-B) infecting tomato only, *X. perforans* (group-C) infecting both pepper and tomato and *X. gardneri* (group-D) infecting both pepper and tomato. However, the results of this study indicate that, the three species: *X. euevesicatoria, X. perforans* and *X. gardneri* were associated with bacterial leaf spot of sweet pepper in Tanzania. Of the three species identified, *X. euvesicatoria* (44.1 %) was the predominant organism in the three sweet pepper-growing regions in the country, whereas, *X. gardneri* constituted 33.8 % and *X. perforans* was 14.7 %. The results also indicate that Tanga region had a high percentage of the strains (41.2 %) of all the bacterial leaf spot-causing xanthomonads identified, whereas, Morogoro had 32.3 % and Arusha had 19.1 % (Figure 13). However, the fourth species: *X. vesicatoria* (group-B) was not identified by PCR in this study, based on species specific primer sets used and pathogenicity tests on the susceptible sweet pepper cv. Early Calwonder.

Other studies (Büttner *et al.*, 2007; Katawczik (*Unpublished*); Kousik and Ritchie, 1996; Wichmann and Bergelson, 2004; Wichmann *et al.*, 2005; Wangsomboondee (*Unpublished*) reported that, the pathogens genome harbor a diverse array of the effector genes which include *avrBs*1, *avrBs*2, *avrBs*3 and *avrBs*4. These effector genes can either be functional or non-functional for interaction with (R) genes in the sweet pepper plant. In this study when the four effector genes (*avrBs*1, *avrBs*2, *avrBs*3 and *avrBs*4) primer sets were used for pathogen characterisation, similar results were found. Furthermore, it was found that, of the 74 strains characterized, 10 carried none of the effector genes, 17 carried 1, 19 carried 2 and 28 strains carried 3 effector genes. None of the strains carried 4 effector genes. All isolates from Mbeya region were not amplified by any of the primer sets used, indicating that the isolates obtained were not pathogenic xanthomonads (Table 8). In addition, the reference strains characterized, *X. vesicatoria* and *X. gardneri* haboured none of the effector genes, *X. perforans* haboured 1, *X. euvesicatoria*, XVT12, XVT28 and XVT48 each haboured 2 effector genes (Table 8).

In the study of re-classification of the xanthomonads associated with bacterial spot disease of sweet pepper and tomato, Jones *et al.* (2004) reported that *X. euvesicatoria* and *X. gardneri* were weakly amylolytic and pectolytic, whereas, *X. perforans* was strongly amylolytic and pectolytic. Furthermore, Quezado-Duval et al. (2004) reported that, strains of *X. gardneri* were unable to hydrolyse starch and pectate and were pathogenic to tomato but variable on sweet pepper. In this study, most of the *X. euvesicatoria* (21) and *X. gardneri* (17) were amylolytic positive and half of the *X. perforans* (5) were non-amylolytic (Table 7). However, all groups were pectolytic negative and pathogenic on susceptible sweet pepper plant cv. Early Calwonder. Although, the determination of tomato races was not done in this study, it is possible that some of the amylolytic sweet

pepper strains identified infect both sweet pepper and tomato plants. Aysan and Sahin (2003) reported similar results in their study of characterization of *X. axonopodis* pv *vesicatoria* of sweet pepper in the eastern Mediterranean region of Turkey, where the sweet pepper strains were Amy$^+$ and pathogenic on both sweet pepper and tomato plants.

In addition, in the study of the worldwide collection of *X. campestris* pv. *vesicatoria* strains from sweet pepper and tomato, Bouzar et al. (1994) found that the T2P3 (tomato/pepper) races were almost always Amy$^+$, whereas, the other tomato and sweet pepper races (T1P0, T1P1, T1P2, T1P3 and T2P1) were Amy$^-$. Furthermore, Sahin and Miller (1996) reported that *X. campestris* pv. *vesicatoria* sweet pepper strains T2P3 were Amy$^-$ and 66 % of the 65 race T1P_ (P0, P1, P2, P3 and P6) strains were Amy$^+$ and most of the two groups were pathogenic on both hosts. These results indicate that, the amylolytic activity vary among strains infecting both sweet pepper and tomato plants. Therefore, there is a need for further investigation on the differentiation of bacterial leaf spot-causing *Xanthomonas* races using tomato lines in order to confirm to which group they belong.

4.2.5 Pathogenicity test

Of the 68 strains tested for pathogenicity on the susceptible host plant (cv. Early Calwonder), 63 (92.6 %) strains induced disease symptoms (water-soaked lesions) within 24 to 48-h of incubation after inoculation (Plate 17). Similar symptoms were observed on the six reference strains (*X. euvesicatoria* NCPPB 2968, *X. gardneri* NCPPB 881, *X. perforans* NCPPB 4321, XVT12, XVT28 and XVT48) which indicated that, they were sweet pepper xanthomonad strains. Five strains (7.4 %) did not induce the bacterial leaf spot disease symptoms, similar to one of the reference strain (*X. vesicatoria* NCPPB 422)

and negative control plants inoculated with SDW. These results indicate that, three groups of bacterial leaf spot-causing xanthomonads that infect sweet pepper are present in the country. The reference strain *X. vesicatoria* (group-B) was not pathogenic on sweet pepper. This strain was previously reported to infect tomato only (Jones *et al.*, 2000; Jones *et al.*, 2005; Obradovic *et al.*, 2004; Vauterin *et al.*, 1995). Therefore, there is a need for further pathogenicity test on the susceptible tomato plant in order to determine the specificity of the crop they infect.

Plate 17: "Bacterial leaf spot symptoms (water-soaked lesions) on sweet pepper cultivar Early Calwonder observed 24 h after inoculation

4.3 Race Determination Using Differential Sweet Pepper Genotypes

Of the 68 bacterial leaf spot-causing xanthomonad strains tested for pathogenicity on sweet pepper plants, 63 (92.6 %) induced characteristic symptoms of bacterial leaf spot-causing xanthomonads on the near-isogenic sweet pepper lines 48 -72 h after inoculation.

Seven sweet pepper races (P0, P1, P2, P3, P4, P5 and P6) were detected based on compatible or hypersensitive response on differential sweet pepper plant cultivars ECW-10R, ECW-20R and ECW-30R (Plate 18). Furthermore, symptoms were observed for 14 days (Plate 19).

Race distribution has been shown in Figure 15. Race P3 (50 %) dominated the strains of bacterial leaf spot-causing xanthomonads in the three regions surveyed (Table 9 and Figures 14 and 15). The composition of race P3 in each of the Tanga and Morogoro regions was 19.1 %, whereas, in Arusha it was 11.8 %. Race P6 was detected in Tanga (17.6 %) and Morogoro (10.3 %). The frequencies of races P0, P1, P2, P4 and P5 were considerably low (Table 9 and Figure 14). Race P0 was detected in strains collected from Tanga (1.5 %) and Arusha (1.5%) regions. Race P1 was detected in Tanga (1.5 %), Arusha (1.5 %) and Morogoro (4.4 %). Race P2 (1.5 %) was only detected in Arusha region while races P4 (1.5 %) and P5 (1.5 %) were detected in Tanga region (Figures 13and 15). Of the seven races affecting sweet pepper, six races (P0, P1, P3, P4, P5 and P6) were detected in Tanga region; while, P3 and P6 were the predominant races (Figure 14). However, five strains which were amplified by PCR (Tanga (2), Morogoro (1) and Arusha (2)) were not pathogenic to the susceptible cv. Early Calwonder indicating that they were not sweet pepper bacterial leaf spot-causing xanthomonads.

Plate 18: Symptomatic leaves of Early Calwonder-10R defoliated 72 h after inoculation with bacterial leaf spot-causing xanthomonads isolated from sweet pepper

Plate 19: Bacterial leaf spot symptoms observed 14 days after inoculation on the sweet pepper near-isogenic line Early Calwonder -20R

Table 9: Race determination of bacterial leaf spot-causing xanthomonad strains isolated from sweet pepper samples collected from Morogoro, Arusha and Tanga regions, Tanzania

Race	Functional Avirulence gene (s)	No. of strains	Differential pepper lines and resistant gene (s)			
			ECW (none)	ECW-10R (Bs1)	ECW-20R (Bs2)	ECW-30R (Bs3)
P0	*avrBs*1, *avrBs*2, *avrBs*3	2	C	HR	HR	HR
P1	*avrBs*2, *avrBs*3	5	C	C	HR	HR
P2	*avrBs*1,*avrBs*2	1	C	HR	HR	C
P3	*avrBs*2	34	C	C	HR	C
P4	*avrBs*3	1	C	C	C	HR
P5	*avrBs*1	1	C	HR	C	C
P6	None	19	C	C	C	C
np		5	nr	nr	nr	nr

P = pepper race; np = non-pathogenic; C = compatible response (disease reaction); HR = hypersensitive response (resistance reaction); nr = no reaction; np = non pathogenic; ECW = Early Calwonder; ECW-10R, ECW-20R and ECW-30R = ECW with incorporated resistance genes Bs1, Bs2 and Bs3 respectively; *avr* = avirulence/effector gene in the pathogen corresponding to the resistance gene in the plants

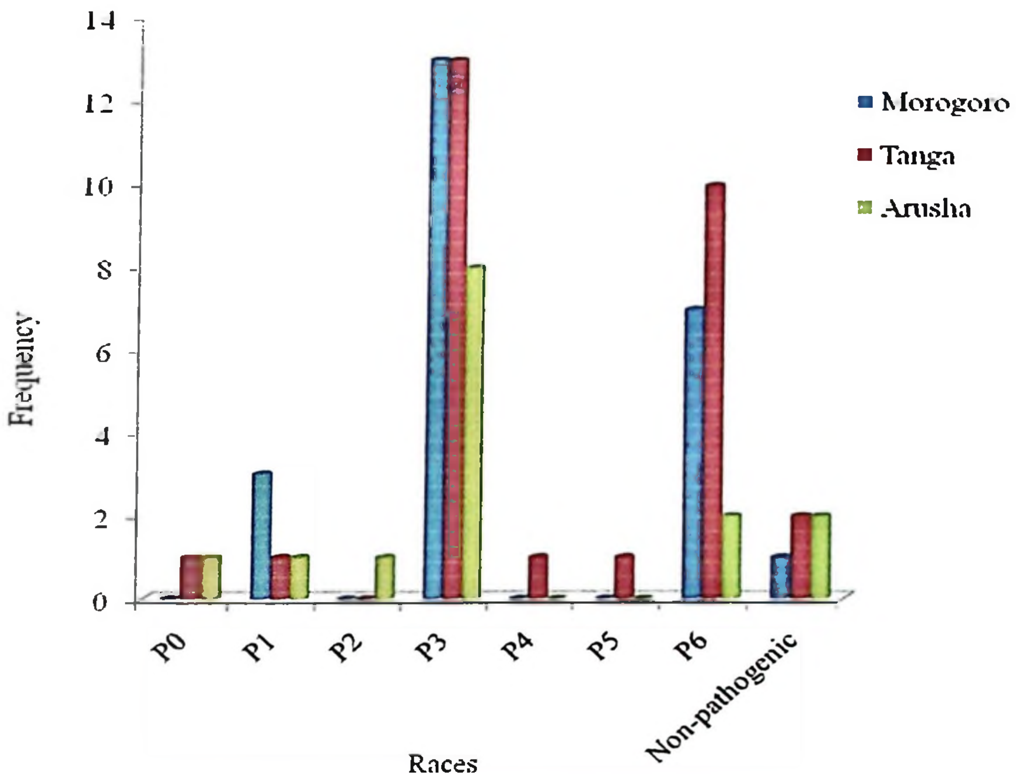


Figure 14: Frequencies of bacterial leaf spot-causing xanthomonad races detected from sweet pepper samples collected from Morogoro, Tanga and Arusha regions, Tanzania

P = Race affecting sweet pepper

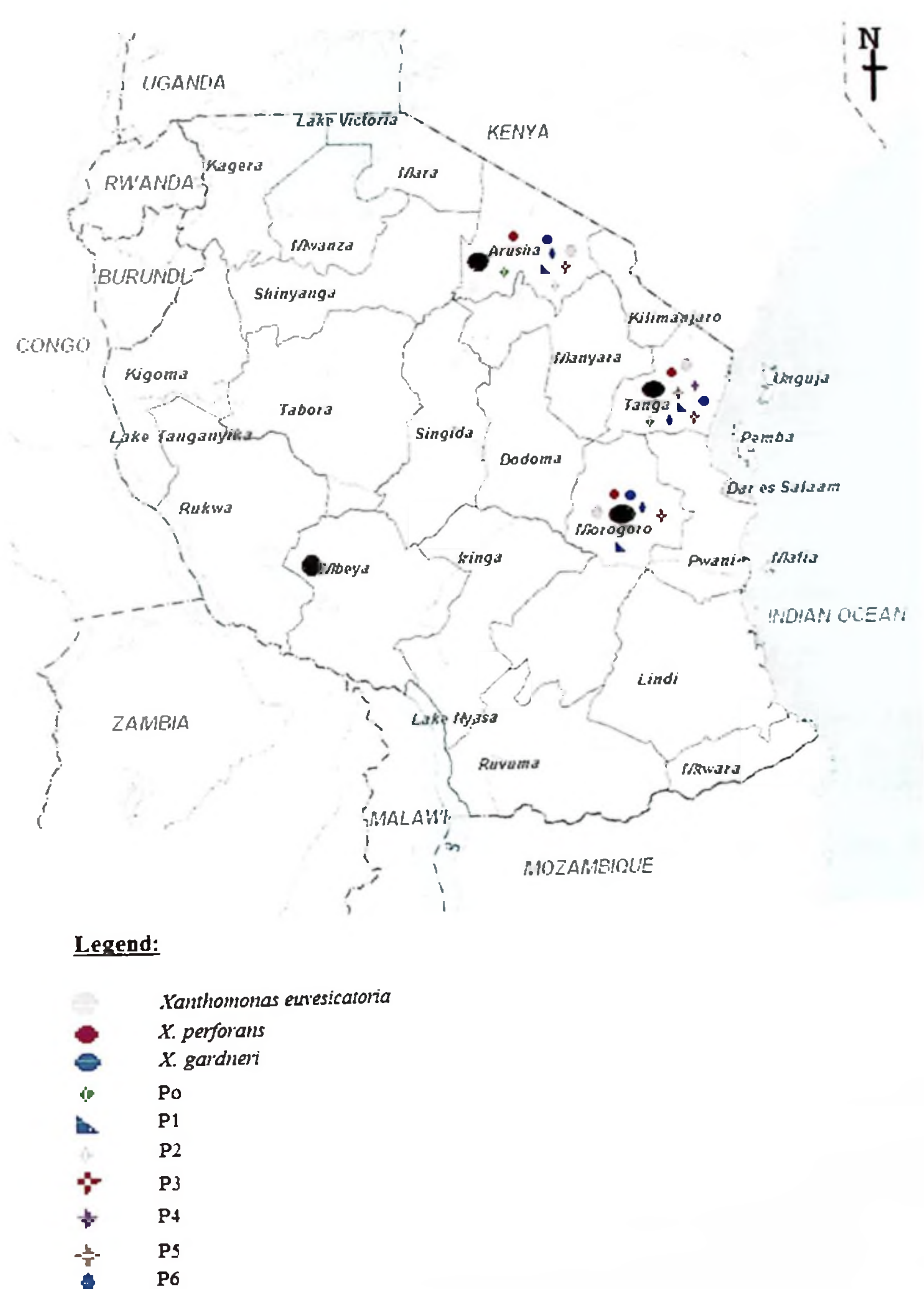


Figure 15: Map of Tanzania showing distribution of bacterial spot-causing xanthomonas strains of sweet pepper in the surveyed regions

Table 10: Grouping of sweet pepper races of bacterial leaf spot-causing xanthomonad strains isolated from sweet pepper samples collected from Morogoro, Tanga and Arusha regions, Tanzania

Species name	Races frequencies							
	P0	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**	**Total**
X. euvesicatoria	-	1	-	16	1	-	12	**30**
X. perforans	1	1	-	7	-	-	1	**10**
X. gardneri	1	3	1	11	-	1	6	**23**
Total	**2**	**5**	**1**	**34**	**1**	**1**	**19**	**63**

X = *Xanthomonas*

Studies on pathogenic variation of bacterial leaf spot-causing xanthomonads on sweet pepper near-isogenic lines identified seven races (P0, P1, P2, P3, P4, P5 and P6) in Tanzania. Similar races were reported in South-eastern United State (Kousik and Ritchie, 1995), North Carolina (Kousik and Ritchie, 1996; Kousik and Ritchie, 1998; Ritchie and Dittapongpitch, 1991), Barbados (Gore and O'Garro, 1999; O'Garro and Tudor, 1994; O'Garro *et al.*, 1999), Grenada (O'Garro *et al.*, 1999), Ohio (Sahin and Miller, 1995; Sahin and Miller, 1996; Sahin and Miller, 1998) and in the worldwide collection of *X. campestris* pv. *vesicatoria* strains (Bouzar *et al.*, 1994). The overall picture of the race determination study indicated the predominance of race 3 strains (50 %) of bacterial leaf spot-causing xanthomonads identified in the three regions followed by P6 strains (27.9 %) (Figure 14). Frequencies of races P0, P1, P2, P4 and P5 were considerably low (Tables 9 and 10 and Figure 14). The frequency of sweet pepper race 3 was high for *X. euvesicatoria* (16), *X. gardneri* (11) and *X. perforans* (7). The frequency of P6 for *X. euvesicatoria* was 12, *X. gardneri* 6 and *X. perforans* was 1 (Table 10). The results also indicate that, six

races (P0, P1, P3, P4, P5 and P6) affecting sweet pepper were detected in Tanga region and race 3 was predominant among the races detected, followed by race 6 (Figure 14).

The prevalence of bacterial leaf spot-causing xanthomonad races has not been studied; since this is the first report of race determination in the country. However, several authors (Gore and O'Garro, 1999; Katawczik (*Unpublished*); McGrath, 1997; Wangsomboondee (*Unpublished*)) reported that, race P3 emerged from races P1 and P2 by plasmid loss and became prevalent during one crop cycle. In addition, Sahin and Miller (1996) reported that race P3 may be better adapted for survival on sweet pepper and tomato than other bacterial spot-causing xanthomonad races and or may survive better as an epiphyte or pathogen on weed hosts. In this study, it was observed during the survey that, bacterial leaf spot disease prevailed where sweet pepper and tomato or other solanaceae crops (e.g. round potatoes and sweet potatoes) were grown in the same or adjacent fields. This was mostly observed in Tanga and Morogoro regions where race P3 dominated (Figure 14).

Previous studies (Büttner *et al.*, 2007; Katawczik (*Unpublished*); Kousik and Ritchie, 1996; Wichmann and Bergelson, 2004; Wichmann *et al.*, 2005; Wangsomboondee (*Unpublished*) and the results of this study revealed that bacterial leaf spot causing xanthomonad strains habour a diverse array of effector genes. However, the interaction between an effector and R genes is quite specific in race determination (Büttner *et al.*, 2007; Katawczik (*Unpublished*); Wichmann and Bergelson, 2004; Wichmann *et al.*, 2005; Wangsomboondee (*Unpublished*). Race P0 has the functional avirulence genes *avrBs*1, *avrBs*2 and *avrBs*3. The functional effector genes for race P1 are *avrBs*2 and *avrBs*3, whereas P2 has the functional effector genes *avrBs*1 and *avrBs*2. Races P3, P4 and P5 have the functional effector genes *avrBs2*, *avrBs*3 and *avrBs*1, respectively. Race P6 has

none of the functional effector genes (Table 9), but can harbour non-functional effector genes (Table 8). Several authors (Bouzar *et al.*, 1994; Katawczik (*Unpublished*); Kousik and Ritchie (1996); Minsavage *et al.*, 1995; Obradovic *et al.*, 2004 and Sahin and Miller, 1998) have reported similar results in race determination studies.

4.4 Screening the Locally Available Sweet Pepper Varieties for Resistance to Bacterial Leaf Spot Disease

The results from the screenhouse experiment indicate that, there were highly statistically significant differences ($P \leq 0.05$) in reaction to disease severity among the inoculated sweet pepper varieties California Wonder, Yolo Wonder and Yolo Wonder B. All the three locally available sweet pepper varieties (California Wonder, Yolo Wonder and Yolo Wonder B) were susceptible to bacterial leaf spot disease in varying levels (Table 11 and Figure 16).

Table 11: Mean disease severity ratings of bacterial leaf spot on locally available sweet pepper varieties

Variety	Days after inoculation				Mean
	7	14	21	28	
California Wonder	4.02a	4.83a	5.79a	6.40a	5.26
Yolo Wonder	2.81b	3.06b	3.71b	5.58c	3.79
Yolo Wonder B	2.17c	2.17c	3.42c	5.88b	3.41
Mean	**3.0**	**3.35**	**4.31**	**5.95**	**4.15**
$LSD_{0.05}$	0.09	0.09	0.03	0.04	
CV	6.2	5.5	4.2	3.4	

Means with the same letter are not significantly different at $P \leq 0.05$ according to Turkey's Studentized (HSD) Range Test.

The overall mean disease severity score for the three varieties was 4.15. The mean disease severity scores showed that, Yolo Wonder B was significantly less susceptible ($P \leq 0.05$) to the bacterial leaf spot disease compared to California Wonder and Yolo Wonder. However, 28 days after inoculation, the mean disease severity score for Yolo Wonder B was higher than that of Yolo Wonder (Table 11). The mean disease severity scores were 5.26, 3.79 and 3.41 for varieties California Wonder, Yolo Wonder and Yolo Wonder B, respectively (Table 11).

Among the three varieties screened in this study, California Wonder was more susceptible ($P \leq 0.05$) to the bacterial leaf spot disease, followed by Yolo Wonder (Table 11 and Figure 16). The mean disease severity scores for inoculated plants on 7, 14, 21 and 28 days after inoculation were 3.0, 3.35, 4.31 and 5.95, respectively (Table 11).

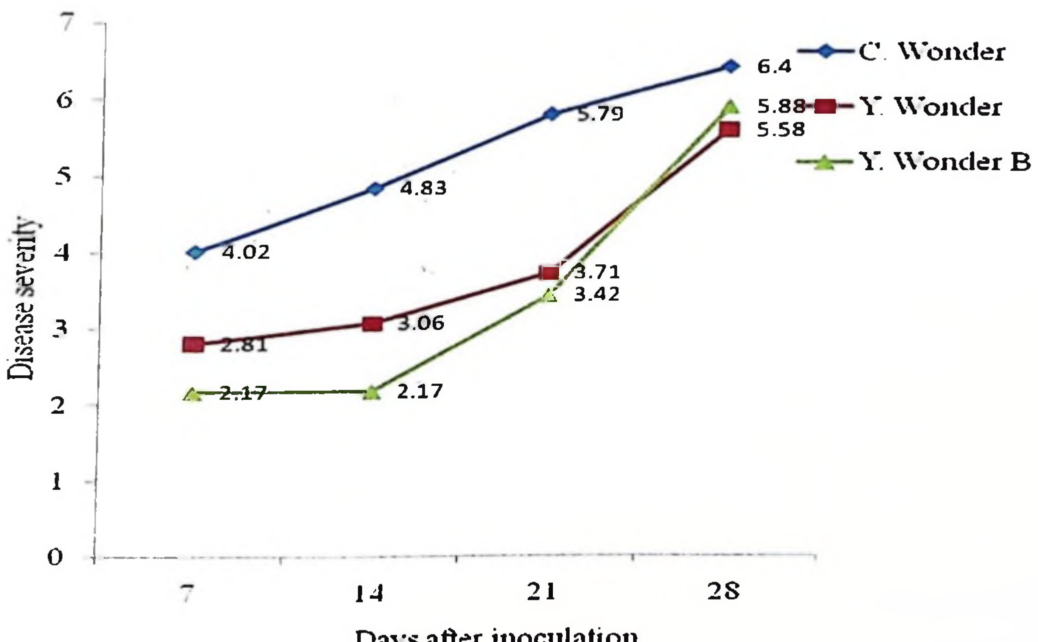


Figure 16: Mean disease severity of bacterial leaf spot on locally available sweet pepper varieties inoculated with bacterial leaf spot-causing xanthomonads

C. Wonder = California Wonder, Y. Wonder = Yolo Wonder

Disease symptoms on leaves were observed as early as 3 – 7 days after inoculation. On stems, the symptoms were observed 14 days after inoculation. The severity of bacterial leaf spot disease was high such that, most of the sweet pepper seedlings in the study suffered severe defoliation, stunted growth and complete death of the plants between 14 and 28 days after inoculation (Figure 16). However, a regeneration of the plants occurred in some cases, resulting in new foliage growth. The variety California Wonder had high disease severity scores from the seventh to 28 days after inoculation. Bacterial leaf spot disease symptoms were not observed on all plants inoculated with sterile distilled water (negative control).

Mean disease severity scores for Yolo Wonder B were low 7 and 14 days after inoculation. However, the severity of the disease increased between 14 and 21 and the plants were highly affected between 21 and 28 days after inoculation. The disease severity data of Yolo Wonder increased gradually between 7 to 21 days after inoculation and increased abruptly between 21 and 28 days after inoculation (Figure 16).

The results also indicate that there were some variations in the ability of bacterial leaf spot-causing xanthomonad races to cause disease on locally available sweet pepper varieties California Wonder, Yolo Wonder and Yolo Wonder B (Table 12). Races 2 (MW58AR), 3 (MW69MG), 5 (MW21TA) and 6 (MW40MG and MW05TA) detected in Tanzania caused significantly higher disease severity on California Wonder than race 1 (MW18TA). Races 0 (MW29TA), 3 (MW15TA) and 4 (MW17TA) caused low disease severity on California Wonder (Table 12). Race 5 of the reference strain XVT48 caused statistically significantly high disease severity ($P \leq 0.001$) on California Wonder compared to the reference strain race 6 (*X. euvesicatoria* and XVT28). Other races (P1, P3 and P6)

of the reference strains XVT12, *X. gardneri* and *X. perforans*, respectively, also caused low disease severity on California Wonder (Table 12).

Races 2 (MW58AR), 3 (MW69MG) and 5 (MW21TA) caused significantly high disease severity ($P \leq 0.001$) compared to race 3 (MW15TA) on the variety Yolo Wonder, while races 0 (MW29TA), 1 (MW18TA and XVT12), 3 (*X. gardneri*), 4 (MW17TA) and 6 (MW05TA, MW40MG, XVT28, *X. euvesicatoria* and *X. perforans*) caused low disease severity (Table 12). Furthermore, race 5 (MW21TA and XVT48) caused significantly high disease severity on Yolo Wonder B compared to race 1 (MW18TA). Other races 0, (MW29TA), 2 (MW58AR), 3 (MW15TA and MW69MG), 4 (MW17TA) and 6 (MW05TA and MW40MG) caused significantly low disease severity on Yolo Wonder B (Tables 12).

The results also indicate that, races 5 (MW21TA), 3 (MW69MG) and 2 (MW58AR) were more virulent on both California Wonder and Yolo Wonder varieties. However, race 5 (MW21TA) caused high disease severity on the three locally available sweet pepper varieties indicating that, race 5 detected in Tanzania was more virulent compared to other races. Races 0 (MW29TA), 3 (MW15TA) and 4 (MW17TA) caused low bacterial leaf spot disease severity on the three locally available varieties. These results indicate that, the locally available sweet pepper varieties were not resistant to bacterial leaf spot disease.

Table 12: Mean disease severity scores caused by races P0 – P6 of bacterial leaf spot-xanthomonads on locally available sweet pepper varieties

Race	Days after inoculation															
	7				14				21				28			
	CW	YW	YWB	Mean	CW	YW	YWB	Mean	CW	YW	YWB	Mean	CW	YW	YWB	Mean
P5 (MW21)	8.0	6.0	5.0	6.3^{a}	8.3	7.0	5.0	6.7^{a}	8.3	7.0	7.0	7.4^{a}	8.3	8.0	8.0	8.1^{a}
P2 (MW58)	8.0	8.0	3.0	6.3^{a}	8.0	8.0	3.0	6.3^{b}	8.0	7.6	5.0	6.8^{b}	8.0	7.6	7.0	7.5^{b}
P3 (MW69)	8.0	8.0	3.0	6.3^{a}	8.0	8.0	3.0	6.3^{b}	8.3	7.6	3.0	6.3^{c}	8.3	7.6	5.0	6.8^{d}
P5 (XVT48)	5.0	3.0	6.0	4.6^{b}	6.0	3.0	6.0	5.0^{c}	7.0	4.0	6.0	5.8^{d}	8.0	7.0	7.0	7.3^{bc}
P1 (MW18)	3.0	4.0	5.0	4.0^{c}	5.0	4.0	5.0	4.6^{d}	7.0	4.0	5.0	5.3^{e}	8.0	6.0	7.0	7.0^{cd}
P6 (MW40)	8.0	1.0	2.0	3.6^{d}	8.0	1.0	2.0	3.6^{f}	8.0	4.0	5.0	5.8^{d}	8.0	6.0	7.0	7.0^{cd}
P6 (MW05)	5.0	3.0	1.7	3.2^{e}	7.0	4.0	1.7	4.2^{e}	8.0	5.0	4.6	5.8^{d}	8.0	7.0	7.0	7.3^{bc}
P6 (XVT28)	5.0	1.0	1.0	2.3^{f}	5.0	1.0	1.0	2.3^{h}	6.0	3.0	1.0	3.3^{h}	6.0	5.0	6.0	5.6^{f}
P6 (X.euves)	4.0	1.0	2.0	2.3^{f}	5.0	1.0	2.0	2.6^{g}	6.0	2.0	4.0	4.0^{f}	7.0	7.0	6.0	6.6^{de}
SDW	0.0	0.0	0.0	0.0^{j}	0.0	0.0	0.0	0.0^{i}	0.0	0.0	0.0	0.0^{l}	0.0	0.0	0.0	0.0^{i}
P3 (MW15)	1.3	4.0	1.0	2.1^{fg}	3.0	4.0	1.0	2.6^{g}	5.0	6.0	5.0	5.3^{e}	6.0	7.0	7.0	6.6^{de}
P6 (X. perf)	4.0	1.0	1.0	2.0^{g}	4.0	1.0	1.0	2.0^{i}	4.0	1.0	1.0	2.0^{k}	4.0	1.0	5.0	3.3^{h}
P0 (MW29)	2.0	1.0	1.0	1.3^{h}	3.0	3.0	1.0	2.3^{h}	5.0	3.0	1.0	3.0^{i}	6.0	5.0	5.0	5.3^{fg}
P1 (XVT12)	1.0	2.0	1.0	1.3^{h}	1.0	2.0	1.0	1.3^{k}	3.0	2.0	1.0	2.0^{k}	5.0	5.0	5.0	5.0^{g}
P4 (MW17)	1.0	1.0	1.0	1.0^{i}	3.0	1.0	1.0	1.6^{j}	5.0	1.0	5.0	3.6^{g}	7.0	5.0	7.0	6.3^{e}
P3 (X. gard)	1.0	1.0	1.0	1.0^{i}	3.0	1.0	1.0	1.6^{j}	4.0	2.0	1.0	2.3^{j}	5.0	5.0	5.0	5.0^{g}
Mean				3.0				3.3				4.3				5.95

Means in the same column followed by the same letter (s) do not differ significantly at $P \leq 0.05$ according toTurkey's Studentized (HSD) Range Test. CW = California Wonder; YW = Yolo Wonder; YWB = Yolo Wonder B; X. euves = *Xanthomonas euvesicatoria*; X. perf = *Xanthomonas perforans*; X. gard = *Xanthomonas gardneri*; SDW = sterile distilled water

Of the six reference strains used as positive control, race 5 (XVT48) caused high disease severity on California Wonder and Yolo Wonder B, but low disease severity on the variety Yolo Wonder. In addition, race 6 (*X. euvesicatoria* and XVT28) caused high disease severity on California Wonder and low disease severity on Yolo Wonder and Yolo Wonder B. Other races, P1 (XVT12), P6 (*X. perforans*) and P3 (*X. gardneri*) caused low disease severity on the three sweet pepper varieties. All sweet pepper plants inoculated with sterilized distilled water (negative control) did not show bacterial leaf spot disease symptoms.

Generally, all the locally available sweet pepper varieties (California Wonder, Yolo Wonder and Yolo Wonder B) were susceptible to bacterial leaf spot disease. The level of disease severity varied among varieties studied (Tables 11, 12 and Figure 16). Variety Yolo Wonder B had significantly low bacterial leaf spot disease ($P \leq 0.05$) for most of the races inoculated compared to varieties California Wonder and Yolo Wonder. However, of the three sweet pepper varieties tested, California Wonder was highly susceptible compared to the other two varieties.

Kousik and Ritchie (1998) and McGrath (1997) reported that, several commercial sweet pepper varieties were bred carrying a combination of three R genes *Bs*1, *Bs*2 and *Bs*3, which provided a better protection against three sweet pepper races P1, P2 and P3. However, it was found that, the newly emerged sweet pepper races P4, P5 and P6 overcome the resistant genes and cause bacterial leaf spot disease (Kousik and Ritchie, 1998; Pernezny and Collins, 1997; Sahin and Miller, 1996; Sahin and Miller, 1998). In this study, the varieties California Wonder, Yolo Wonder and Yolo Wonder B which have been reported to carry a combination of 3 resistant genes against the sweet pepper races

P1, P2 and P3 (McGrath, 1997), were very susceptible to almost all the races used in this study. These results indicate that despite of these varieties reported to carry resistant genes against bacterial leaf spot, none of them were resistant to all the seven bacterial leaf spot causing-xanthomonad infecting sweet pepper used in the current study (Cuppels *et al.*, 2006, Kousik and Ritchie, 1996; Kousik and Ritchie, 1998; Pernezny and Collins, 1997; Pohronezny *et al.*, 1992; Sahin and Miller, 1996; Sahin and Miller, 1998).

The ability of these races to cause variable levels of disease on sweet pepper varieties was also observed in this study. Races 2 (MW58AR), 3 (MW69MG), 5 (MW21TA and the reference strain XVT48) and 6 (MW05TA and MW40MG) caused significantly high disease severity ($P \leq 0.001$) on California Wonder, while races 2 (MW58AR), 3 (MW69MG) and 5 (MW21TA) caused high disease severity on variety Yolo Wonder. Races 0 (MW35MG), 3 (MW15TA) and 4 (MW17TA) and the reference strains (*X. euvesicatoria,* XVT12, XVT28, *X. perforans* and *X. gardneri*) caused low disease severity (Tables 11 and 12). The variation in virulence of xanthomonad races has been reported by Sahin and Miller (1998). The authors reported that, half of the P1 and P3 strains tested varied in virulence on *Capsicum pubescens*. Such findings suggest existence of wide genetic diversity among the bacterial leaf spot-causing xanthomonads. It was observed in this study that, races 5 (carrying functional *avrBs*1) and 6 (carrying none functional gene) caused higher disease severity on both California Wonder and Yolo Wonder than the rest of the races. Several authors (Bouzar *et al.*, 1994; Cuppels *et al.*, 2006; Kousik and Ritchie, 1996; Sahin and Miller, 1998) reported similar observation in their studies that, the newly identified races of bacterial spot-causing xanthomonads (P4, P5 and P6) caused severe disease on sweet pepper cultivars lacking resistance gene Bs2. These results indicate that, the *Bs*2 resistance gene which has been reported to be durable and effective

against bacterial leaf spot disease on sweet pepper has been overcome by races 4, 5 and 6 (Kousik and Ritchie, 1996; Sahin and Miller, 1998).

It has been reported that, sweet pepper cultivars with resistance to the three races (P1, P2 and P3) of bacterial leaf spot-causing xanthomonads provide stable disease resistance (Kousik and Ritchie, 1998; Pernezny and Collins, 1997; Pohronezny *et al.*, 1992; Sahin and Miller, 1996). However, it has been observed in this study that, the locally available sweet pepper varieties (especially California Wonder and Yolo Wonder) that were reported to be resistant to races 1, 2 and 3 were susceptible to races 1 (MW18TA), 2 (MW58AR) and 3 (MW69MG) detected in Tanzania and races 1 and 3 of the reference strains XVT12 and *X. gardneri*, respectively. Such susceptibility has been suggested to emanate from mutation of the host-plant *Bs*2 resistant gene (Kearney and Staskawicz, 1990; Kousik and Ritchie, 1996; Sahin and Miller, 1998; Wangsombondee, *Unpublished*). It has been demonstrated that, the use of host-plant genetic resistance is economically and technically the most effective method of managing bacterial leaf spot disease of sweet pepper (Kousik and Ritchie, 1996a; Kousik and Ritchie, 1996b; Pernezny and Collins, 1997; Sahin and Miller, 1998). However, the prevalence of races makes the use of host-plant resistance to manage the disease difficult (Kousik and Ritchie, 1996; Kousik and Ritchie, 1998; Louws *et al.*, 2001). Furthermore, race shift in the pathogen population can overcome the effectiveness of resistance genes (major genes) when deployed in monoculture (Kousik and Ritchie, 1996; Kousik and Ritchie, 1998; Pohronezny *et al.*, 1992). In this study, seven races (P0 to P6) of bacterial leaf spot-causing xanthomonads were detected in the major sweet pepper growing regions of Tanzania. This suggests that, breeding for resistant varieties based on the prevailing races and geographical location is needed in order to address variation in pathogen races.

The presence of resistance in sweet pepper to bacterial leaf spot was described by Horsfall in 1939 (Kousik and Ritchie, 1998). The host-plant resistance gene *Bs*1 was identified in *Capsicum annuum* (Cook and Stall, 1963) while, *Bs*2 was identified in *Capsicum chacoense* (Cook and Guevara, 1984) and *Bs*3 was identified in *Capsicum annuum* (Kim and Hartmann, 1985). Resistance to bacterial leaf spot in commercial sweet pepper varieties has been derived from 1 or more of these 3 resistance genes (Kousik and Ritchie, 1998). This suggests that, breeders need to identify and incorporate resistant genes into widely acceptable pepper cultivars by screening a number of accessions (Cook and Guevara, 1984; Cook and Stall, 1963; Cook and Stall, 1982; Hibberd *et al.,* 1987; Kim and Hartmann, 1985; Kousik and Ritchie, 1998).

Additionally, development of resistant sweet pepper varieties should take into account consumer preferences. Findings of this study indicate that, the variety Yolo Wonder which is the most preferred and grown by 55 % of farmers in the surveyed sweet pepper-producing regions in the country (Figure 8), is susceptible to bacterial leaf spot disease. This variety was popular to sweet pepper farmers due to its preferred characteristics which include early maturity, large fruits, high yield and resistant to bacterial leaf spot disease. Unfortunately, in the current study the variety Yolo Wonder was also susceptible to bacterial leaf spot disease (Tables 11, 12 and Figure 16).

Prior to 1994, bacterial leaf spot resistance encoded by resistance gene *Bs*2 was expected to be durable and effective against bacterial leaf spot disease since compartible races of the bacteria were hitherto, undetected in sweet pepper (O'Garro, 1998). Only races P0, P1, P2 and P3 where known. Since 1994, it has been reported that, many of the bacteria leaf spot-causing xanthomonads of sweet pepper overcame the Bs2 gene through mutations in

the *avrBs*2 locus (Bouzar *et al.*, 1994; Kousik and Ritchie, 1995; O'Garro and Tudor, 1994; Sahin and Miller, 1995; Sahin and Miller, 1996). However, more studies are needed in order to properly understand the actual mechanism causing breakdown of resistance to bacterial leaf spot disease in sweet pepper.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Surveys were conducted in Morogoro, Arusha, Tanga and Mbeya regions to determine the current status of bacterial leaf spot disease of sweet pepper. The results of this study indicate that, the disease is widespread in farmers' fields in Morogoro, Arusha and Tanga regions. The incidence of the disease ranged from 10 – 100 %, whereas, disease severity ranged between 3 -7 (on a scale of 1 – 9).

Sixty three strains of bacterial leaf spot-causing xanthomonads were isolated, characterized using starch hydrolysis and PCR and confirmed to be pathogenic to the host. The identified xanthomonads included *X. euvesicatoria* (30) strains, *X. perforans* (10) strains and *X. gardneri* (23) strains. Characterization of bacterial leaf spot xanthomonads by PCR indicated that, the primer sets described by Leite *et al.* (1995) permitted the detection of 79.7 % of these pathogens to the genus level. The effector/avirulence gene primer sets were able to detect 68 (91.9 %) out of 74 strains tested. The species-specific primer sets *X. euvesicatoria*, *X. perforans* and *X. gardneri* described by Mbega *et al.* (*Unpublished*) permitted successful identification of bacterial leaf spot-causing xanthomonads of sweet pepper to species level. These results indicate that, successful detection of the pathogen using PCR techniques depends upon the specificity of the primers after restriction enzyme analysis of the amplicon produced. It was also found that, the amylolytic activity vary among *Xanthomonas* strains infecting sweet pepper, suggesting that, the strains might also infect tomatoes.

Race typing using differential sweet pepper genotypes ECW and near-isogenic lines ECW-10R, ECW-20R and ECW-30R was done. Seven races affecting sweet pepper (P0 to P6) were identified. Race P3 (50 %) dominated the strains of bacterial leaf spot-causing xanthomonads in Tanga, Morogoro and Arusha regions. The frequency of race P3 was high for *X. euvesicatoria* (16), *X. gardneri* (11) and *X. perforans* (7). The distribution of race P3 was 19.1 % in each Tanga and Morogoro regions, and 11.8 % in Arusha region. The distribution of race P6 was 17.6 % and 10.3 % in Tanga and Morogoro regions, respectively. Race P0 was detected in strains collected from Tanga (1.5 %) and Arusha (1.5%) regions. Race P1 was detected in Tanga (1.5 %), Arusha (1.5 %) and Morogoro (4.4 %). Race P2 (1.5 %) was only detected in Arusha region while races P4 (1.5 %) and P5 (1.5 %) were detected in Tanga region. This is the first report to characterize bacterial leaf spot-causing xanthomonads of sweet pepper in Tanzania.

The prevalence of races of bacterial spot-causing xanthomonads makes the use of host-plant resistance to manage the disease difficult. Therefore, breeding for resistance to bacterial leaf spot disease of sweet pepper requires knowledge of races of the pathogen likely to be present in an area of consideration. The results also indicate that, all the three locally available sweet pepper varieties grown in Tanzania and screened for resistance in the present study were susceptible to bacterial leaf spot disease.

Although the results of this study indicate that, sweet pepper seeds being sold in agricultural input shops were not infected with bacterial leaf spot-causing xanthomonads, other possible sources of inocula for bacterial leaf spot infection were continuous growing and rotation of solanaceae crops, types of solanaceae plants surrounding the sweet pepper crop, infected crop debris left on the soil and volunteer sweet pepper/tomato plants

observed during the survey, and epiphytically survival of bacterial spot-causing xanthomonads on protected organs (leaves, buds and flowers) of primary host and non-host plants .

5.2 Recommendations

i). There is a need for frequent surveys to monitor disease incidence as an important component for managing bacterial leaf spot disease of sweet pepper.

ii). The current study represents the first report of characterization of sweet pepper strains and races of bacterial leaf spot-causing xanthomonads in Tanzania. Therefore, there is a need for further investigation on the differentiation of BSX races using tomato differential lines in order to further confirm the current findings.

iii). The findings of this study indicate the occurrence of seven races of bacterial leaf spot-causing xanthomonads in Tanzania. Therefore, more surveys are needed in other sweet pepper growing regions in the country in order to understand the population composition of bacterial spot-causing xanthomonads.There is a need for sweet pepper breeding programmes to consider the existing pathogenic variation.

iv). There is also a need to conduct yield loss studies to determine the economic impact of the disease on sweet pepper production in the country based on the race structure identified.

v). Although the sweet pepper seeds sold in agricultural input shops were not contaminated by bacterial leaf spot-causing xanthomonads, it is recommended to have

regular monitoring and screening of sweet pepper seeds sold in the country in order to ensure that, they continue to meet the sanitary standards required. In addition, more studies on screening for sweet pepper seed infection by bacterial leaf spot-causing xanthomonads should be done using samples collected country-wide.

vi). The results of this study reported the prevalence of different races of bacterial leaf spot-causing xanthomonads in Tanzania. Furthermore, the locally available sweet pepper varieties were not resistant to these races. Therefore, there is a need to develop an integrated management program for bacterial leaf spot disease that include the use of pathogen-free seeds and transplants, proper cultural practices, sanitation, use of resistant varieties and judicious use of chemicals.

vii). Three locally available sweet pepper varieties grown in Tanzania were susceptible to bacterial leaf spot disease. Breeding programmes to incorporate resistance genes against bacterial leaf spot disease in Yolo Wonder, California Wonder and Yolo Wonder B varieties, as one of the most sustainable strategies for managing the disease are urgently needed.

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APPENDICES

Appendix 1: Plant Disease Diagnostic Form

Name of interviewer_______________________________ Date________________

Personal details

Farmer's name: ____________________ Date: ______________________________

Village: _____________ District: ______________________Region:_______________

Altitude: ____________________ Temperature: _____________ Rainfall:

Plant/crop

Plant: ____________________ Variety: ______________________________ Size of the field___________

Planting date/approximate age:_____ Plant population: _____________Numbers of ha affected:__

Disease Symptoms	Affected parts	Distribution	Planting	Weather
Wilting	Whole plant	Entire field	Field/farm	Clear
Yellowing	New growth	Single plant	Nursery	Cloudy
Dieback	Stems	Scattered plants	Landscape	Rainy
Rootrot	Leaves/needles	Group of plants	Garden	Windy
Stemrot	Flowers	Edge of field	Flowers	Heavy dews
Leaf spots	Fruit	High areas	Other	Drought
Blight	Twigs/branches	Low areas		Adequate moisture
Canker	Crown/collar	Wet areas	**Drainage**	Excess moisture
Streak	Buds	Dry areas	Good	**Degree of injury**
Mosaic	Other	Sunny areas	Fair	Light
Galls	**Soil type**	Shaded areas	Poor	Moderate
Leaf/needle drop	Sandy	Next to drive		severe
Stunting	Loam	Other		
Fruit spots/decay	Potting mix			
Distortion	Mix			
Other	Clay			
	Mulch			

Tick the applicable one (s)

Describe the problem:____

____

____

____

History

Chemicals used in the planting or vicinity:______________________________

Application time: ____

Fertilizer application____________________________________

Previous planting and cultivation history: ______________________________________
____
When did the problem first appear:____
Do you irrigate: _______

Observe field sanitation _______________ and types of plant species surrounding the field____

Appendix 2: One–way ANOVA results comparing incidence of bacterial leaf spot of sweet pepper in villages covered by the study.

Village	n	Mean	Sum of squares		df	Mean square	F	Sig.
Luale	25	81.2	Between groups	29362.6	3	9787.5	26.8	0.001
Kivulul	15	90.7	Within groups	25904.0	71	364.8		
Lukozi	30	93.3						
Utengule	5	12.0						
Total	75	83.3		55266.7	74			

Mean difference is significant at the 0.05 level

Appendix 3: One–way ANOVA comparing severity of bacterial leaf spot disease of sweet pepper in villages covered by the study

Village	n	Mean	Sum of squares		df	Mean square	F	Sig.
Luale	25	5.2	Between groups	94.0	3	31.3	7.4	0.001
Kivulul	15	6.5	Within groups	299.4	71	4.2		
Lukozi	30	5.6						
Utengule	5	1.6						
All	75	5.3		393.5.7	74			

Mean difference is significant at the 0.05 level

Appendix 4: Chi-square tests on effect of previous cultivation history on incidence of bacterial leaf spot of sweet pepper

	Value	df	Asymptomatic Sig. (2-sided)
Pearson chi-square	23.4	9	0.005
Likelihood Ratio	24.3	9	0.004
N of valid cases	75		

Appendix 5: Chi-square tests on effect of previous cultivation history on severity of bacterial leaf spot of sweet pepper

	Value	df	Asymptomatic Sig. (2-sided)
Pearson chi-square	15.2	6	0.019
Likelihood Ratio	17.1	6	0.009
N of valid cases	75		

Appendix 6: Mean disease severity scores caused by races of bacterial leaf spot-xanthomonads on sweet pepper variety California Wonder

Race	Days after inoculation				Mean
	7	14	21	28	
P5 (MW21)	8.0	8.3	8.3	8.3	8.2
P2 (MW58)	8.0	8.0	8.0	8.0	8.0
P3 (MW69)	8.0	8.0	8.3	8.3	8.1
P5 (XVT48)	5.0	6.0	7.0	8.0	6.5
P1 (MW18)	3.0	5.0	7.0	8.0	5.8
P6 (MW40)	8.0	8.0	8.0	8.0	8.0
P6 (MW05)	5.0	7.0	8.0	8.0	7.0
P6 (XVT28)	5.0	5.0	6.0	6.0	5.5
P6 (X.euvesic)	4.0	5.0	6.0	7.0	5.5
SDW	0.0	0.0	0.0	0.0	0.0
P3 (MW15)	1.3	3.0	5.0	6.0	3.8
P6 (X. perf)	4.0	4.0	4.0	4.0	4.0
P0 (MW29)	2.0	3.0	5.0	6.0	4.0
P1 (XVT12)	1.0	1.0	3.0	5.0	2.5
P4 (MW17)	1.0	3.0	5.0	7.0	4.0
P3 (X. gard)	1.0	3.0	4.0	5.0	3.3
Mean	**4.02**	**4.83**	**5.79**	**6.40**	**5.26**

Appendix 7: Mean disease severity scores caused by races of bacterial leaf spot-xanthomonads on sweet pepper variety Yolo Wonder

Race	Days after inoculation				Mean
	7	14	21	28	
P5 (MW21)	6.0	7.0	7.0	8.0	7.0
P2 (MW58)	8.0	8.0	7.6	7.6	7.7
P3 (MW69)	8.0	8.0	7.6	7.6	7.8
P5 (XVT48)	3.0	3.0	4.0	7.0	4.3
P1 (MW18)	4.0	4.0	4.0	6.0	4.5
P6 (MW40)	1.0	1.0	4.0	6.0	3.0
P6 (MW05)	3.0	4.0	5.0	7.0	4.5
P6 (XVT28)	1.0	1.0	3.0	5.0	2.5
P6 (X.euvesic)	1.0	1.0	2.0	7.0	2.8
SDW	0.0	0.0	0.0	0.0	0.0
P3 (MW15)	4.0	4.0	6.0	7.0	5.3
P6 (X. perf)	1.0	1.0	1.0	1.0	1.0
P0 (MW29)	1.0	3.0	3.0	5.0	3.0
P1 (XVT12)	2.0	2.0	2.0	5.0	2.8
P4 (MW17)	1.0	1.0	1.0	5.0	2.0
P3 (X. gard)	1.0	1.0	2.0	5.0	2.5
Mean	**2.81**	**3.06**	**3.71**	**5.58**	**3.79**

Appendix 8: Mean disease severity scores caused by races of bacterial leaf spot-xanthomonads on sweet pepper variety Yolo Wonder B

Race	Days after inoculation				Mean
	7	14	21	28	
P5 (MW21)	5.0	5.0	7.0	8.0	6.3
P2 (MW58)	3.0	3.0	5.0	7.0	4.5
P3 (MW69)	3.0	3.0	3.0	5.0	3.5
P5 (XVT48)	6.0	6.0	6.0	7.0	6.3
P1 (MW18)	5.0	5.0	5.0	7.0	5.5
P6 (MW40)	2.0	2.0	5.0	7.0	4.0
P6 (MW05)	1.7	1.7	4.6	7.0	3.7
P6 (XVT28)	1.0	1.0	1.0	6.0	2.4
P6 (X.euvesic)	2.0	2.0	4.0	6.0	3.5
SDW	0.0	0.0	0.0	0.0	0.0
P3 (MW15)	1.0	1.0	5.0	7.0	3.5
P6 (X. perf)	1.0	1.0	1.0	5.0	2.0
P0 (MW29)	1.0	1.0	1.0	5.0	2.0
P1 (XVT12)	1.0	1.0	1.0	5.0	2.0
P4 (MW17)	1.0	1.0	5.0	7.0	3.5
P3 (X. gard)	1.0	1.0	1.0	5.0	2.0
Mean	**2.17**	**2.17**	**3.42**	**5.88**	**4.15**

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