Phenotypic and biochemical characterisation of the causal agent of bacterial leaf streak of maize

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DECLARATION

I declare that this dissertation submitted for the degree of *Master of Science in Environmental Sciences* at the North-West University, Potchefstroom Campus, has not been submitted by me for a degree at this or another university, that it is my own work in design and execution, and that all material contained herein has been duly acknowledged.

_____ _____

Jesse Jay Nienaber Date

In loving memory of my late mother, Laura Nienaber, and grandmother Winnie Arlow. My constant sources of strength, support and inspiration.

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SUMMARY

Maize is the staple food for a majority of people in Southern Africa, but plant diseases are responsible for at least 10% of crop production losses. Bacterial leaf streak (BLS) of maize was first reported in South Africa in 1949 and has not been reported elsewhere. Very little is known about the pathogen involved and therefore it is deemed necessary to compile a characteristic profile for the pathogen to prevent the possibility of major crop losses as a result of this disease.

This study aimed to use biochemical and phenotypic methods to determine the specific characteristics of the causal agent of BLS. Diseased plant material showing symptoms of BLS were collected during the maize production seasons of 2012 and 2013 within South Africa's maize production regions namely the North West, Free State, Gauteng and Northern Cape provinces. To prevent contamination, maize leaves were surface sterilised thoroughly before bacterial isolation commenced. Sections of the infected maize leaves were placed on GYC agar plates on which yellow, mucoid bacterial colonies after incubation for 24 to 48 hrs. The isolated bacteria were purified and the molecular identification of the bacteria was conducted in a related study. Although literature indicates that *Xanthomonas campestris* pv. *zeae* is the causal agent of BLS, pure cultures obtained from maize leaves showing characteristic symptoms of BLS were identified as species of *Xanthomonas*, *Pantoea*, and *Enterobacter*. To elucidate the pathogenicity of the isolated strains, pathogenicity tests based on Koch's postulates were performed. Results from the pathogenicity tests confirmed that only the isolate *Xanthomonas* species was capable of inducing the characteristic BLS symptoms when healthy maize plants were inoculated with the suspected pathogens. It is important to inoculate the maize seedlings at the correct age (four-leaf stage) and the spray method is recommended. Re-isolation was repeated from the same plant material used during the initial isolation process but the isolation method was amended. The optimised isolation method involved the use of a dilution range and spread plate method. Colonies from this isolation technique grew as bright yellow colonies that were identified as *Xanthomonas* spp. This outcome indicates the importance of surface sterilisation,

pulverisation and subsequent dilution of plant materials for isolation of bacterial pathogens from diseases plants.

These isolates were used to create protein profiles with SDS-PAGE electrophoresis and carbon utilisation patterns with the Biolog® GN2 system. Protein profiling banding patterns was assessed based on presence/absence criteria. Highly similar protein profiles were observed among the *X. campestris* pv. *zeae* isolates but groupings of different protein profiles were determined when minor differences in the protein profiles was taken into account. *Xanthomonas campestris* pv. *zeae* was successfully distinguished from the *X. axonopodis* pv. *vasculorum* reference strain through unique SDS banding patterns. Banding patterns obtained from cultures grown in a liquid medium (tryptic soy broth) were of a higher quality than the banding patterns obtained from bacteria harvested from solid media (CYG agar plates).

Carbon source utilisation data was used to evaluate the average well colour development obtained from each isolate. Statistically significant differences were found among some of the isolates, with some isolates being metabolically more active than other isolates. Substrate utilisation patterns produced by the isolates corresponded to previously published studies on various *Xanthomonas* species. The cell count of the samples used during carbon utilisation patterns must be standardised in order to obtain reliable results.

During this study, the application of Koch's postulates and two inoculation techniques confirmed that *Xanthomonas campestris* pv. *zeae* is the pathogen responsible for bacterial leaf streak of maize. Members of the *Pantoea* and *Enterobacter* genera were found on the leaf surface of maize plants infected with BLS but inoculations of healthy maize plants with these bacteria did not result in bacterial leaf streak symptoms on the maize plants. These bacteria were not pathogenic and were considered endophytes. The identified pathogen was characterised through protein and metabolic profiling. The protein profiles of the pathogen obtained through analysis of the major bands of the SDS-PAGE gels were highly similar and distinguishable from the *Xanthomonas* reference culture. Groupings within the *X. campestris* pv. *zeae* group was found when major and minor

bands were considered, this may however be altered when the intensities of the bands are used during analysis. Carbon utilisation patterns were assessed using Biolog® GN2 plates. A metabolic fingerprint was created for the pathogen of BLS, it was possible to distinguish between *X. campestris* pv. *zeae* and other *Xanthomonas* strains based on the fingerprint. This fingerprint could be used to identify the pathogen.

Keywords: maize, bacterial leaf streak, *Xanthomonas, X. campestris* pv. *zeae,* pathogenicity tests, SDS-PAGE, protein profiling, Biolog GN2, metabolic fingerprinting.

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LIST OF SYMBOLS AND ABBREVIATIONS

% - percentage

°C – Degrees Celsius

µm – micrometer

AWCD – Average well colour development

BLS – Bacterial leaf streak

EPS - Extracellular polysaccharides

GYCA - glucose–yeast extract–calcium carbonate agar

hr – hour

hrp - hypersensitive response and pathogenicity

HSD - Honest Significant Difference

ITS - internal transcribed spacer regions

min – minutes

mm – millimetre

PCA – Principal Component Analysis

PCR – polymerase chain reaction

PM – Phenotype microarrays

pv. – pathovar

SDS-PAGE - sodium dodecyl sulfate-polyacrylamide gel electrophoresis

sec – seconds

spp. – species

TIVSS - type IV secretion system

v/v – volume per volume

w/v – weight per volume

xg – times gravity

α – Alpha

β – Beta

1.1 Introduction

The impact of bacterial leaf streak (BLS) of maize on crop production is still unknown. The first report of the disease occurred in South Africa in 1949 (Dyer, 1949). BLS has not been reported in any other country than South Africa (Qhobela *et al.*, 1990). Stewart's wilt, a serious maize disease that is very similar to BLS has been reported in many other countries. *Pantoea stewartii* subsp. *stewartii* is the causal agent of Stewart's wilt (Orio *et al.*, 2012) which is very difficult to distinguish from BLS visually. Bacterial leaf streak has caused the withdrawal of a commercial variety of maize due to its susceptibility (Coutinho & Wallis, 1990). To prevent the disease from becoming a threat to the South African maize production industry and to ensure food security for the country, there needs to be more research done on the causal pathogen.

Xanthomonas campestris pathovar *zeae* is the causal agent of BLS of maize. Infected maize plants have yellow-brown lesions on the leaves. The lesions are 2 to 3mm broad and have wavy, irregular margins. On one occasion, the disease caused the wilting of an entire maize plant (Coutinho & Wallis, 1990).

Before the impact of the disease on the maize industry can be determined, the pathogen needs to be characterised. A thorough understanding of the physiology and biochemistry of the pathogen is required to establish the mode of transmission of the disease and to determine which factors render a plant susceptible to infection. Knowledge of a potentially threatening plant disease enables the scientific community to work hand-in-hand with the agricultural sector to minimize the risks of the plant disease.

Very little research has been done on the disease and the causal agent of the disease, therefore information on this disease is very limited. This may be because the disease was not seen as a major problem over the past six decades. There are thus less than five peer reviewed references that are available in literature

databases. Information from recent reports of the disease symptoms of BLS indicates that this disease could have a significant effect with calamitous consequences on the South African maize production industry. This necessitates studies such as this one.

The hypothesis for this study is to confirm through Koch's postulates that *Xanthomonas campestris* pv. *Zeae* is the causative agent for BLS symptoms observed.

The aim of the present study was to characterise isolates of *Xanthomonas campestris* pv. *zeae* based on selected phenotypic and biochemical properties.

The objectives of the study were:

(i) to isolate and identify the causal agent of BLS.

(ii) to confirm the pathogenicity of *X. campestris* pv. *zeae* by applying Koch's postulates,

(iii) to detect differences and similarities among the isolates by comparing the protein profiles of the *X.campestris* pv. *zeae* isolates, and

(iv) to create a metabolic profile of the pathogen using the Biolog® GN2 system

1.2 Maize in Africa

South Africa is suitable for growing a large assortment of crops. The primary crops cultivated in South Africa include maize, wheat, soybeans, sorghum and sugarcane. Oats, groundnut, sunflowers, tobacco and dry beans are some of the minor crops cultivated in the country (Gbetibouo & Hassan, 2004). In terms of production, maize is the third most important crop in Africa as only cassava and sugar cane are produced in larger quantities. Maize, cassava, groundnut and other plant species such as sugar cane constituted two-thirds of the gross value of the agricultural output of Africa in 2004 (Gabre-Madhin & Haggblade, 2004). The 2010 and 2011 figures indicate that this trend has not changed (FAOSTAT, 2013). In figure 1 the total production in tons is provided for cassava, sugar cane, maize, clover and yams, respectively. Maize production has increased slightly from 2010 to 2011, but it is still just more than $6x10^6$ tons.

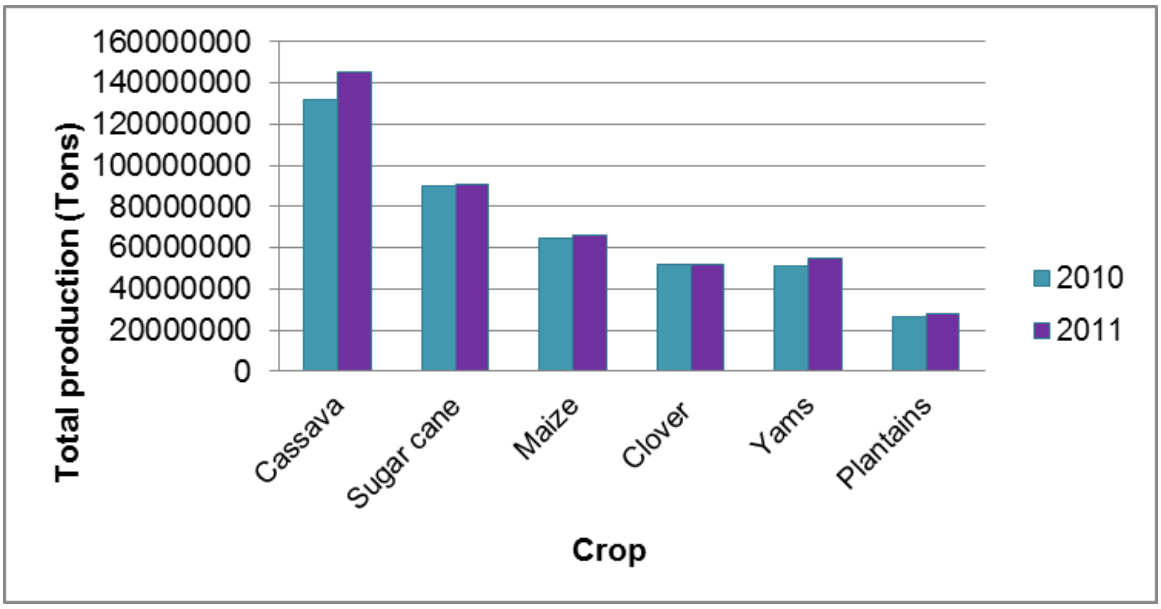

Figure 1: The top five crops in Africa based on production quantity (compiled from FAOSTAT, 2013).

Subsistence farmers in Africa typically practice intergrated crop-livestock systems. These farmers plant a variety of crops including maize (*Zea mays* L.), sorghum (*Sorghum bicolour (*L. Moench), wheat (*Triticum* spp.), teff (*Eragrostistef*), and barley

(*Hordeum* spp.) (Ali *et al.*, 2009). The most important of these crops is maize, because it is used for both food and feed (Jones & Thorton, 2003; Tittonell *et al.*, 2008).

Over the past few decades the demand for maize by urban and rural consumers in food deficit areas in Africa stimulated the production of maize as both food and cash crop (De Groote *et al.*, 2013). Western, Eastern and Southern African countries are the main maize producers in Africa (FAOSTAT, 2013). Maize accounts for at least 50% of the calories provided by starchy staples in eight of the countries in these regions; roots and tubers provide 20% of the remaining energy, while animal products offer another 7%. Specifically, in Malawi and Zambia maize accounts for over 80% of the food staple calories (FAO, 1996; Byerlee & Heisy, 1996; World Bank, 2007).

1.3 Maize production in South Africa

South Africa is the largest maize producer in Southern Africa (Van Tienhoven *et al.*, 2006). Maize is the leading grain commodity grown and consumed in South Africa (Qhobela *et al.*, 1990; FAOSTAT, 2013) and is also the primary staple food of the country (Byerlee & Heisey, 1996). In South Africa, maize constitutes approximately 70% of grain production and 60 percent of the country's cropping area is covered by maize (Akpalu *et al.*, 2008). Maize production contributed 11.3% to the total gross agricultural production value of South Africa for 2011 (DAFF, 2012). The country's agricultural export figures for the year 2011 indicated that in terms of value, maize is one of the most important export products. The export of citrus fruit from South Africa accounted for R7 067 million and was ranked as the most important export crop in terms of value. Maize was ranked as the second most important export crop with the export value reaching R6 038 million (DAFF, 2012). While in 2008 it was estimated that South Africa produced at least 50% of the total maize output of Southern Africa (Akpalu *et al.*, 2008), figure 2 (based on FAOSTAT, 2013) shows that it is closer to 90-98%. From figure 2 it is evident that production across this region remained proportionally constant over the past decade.

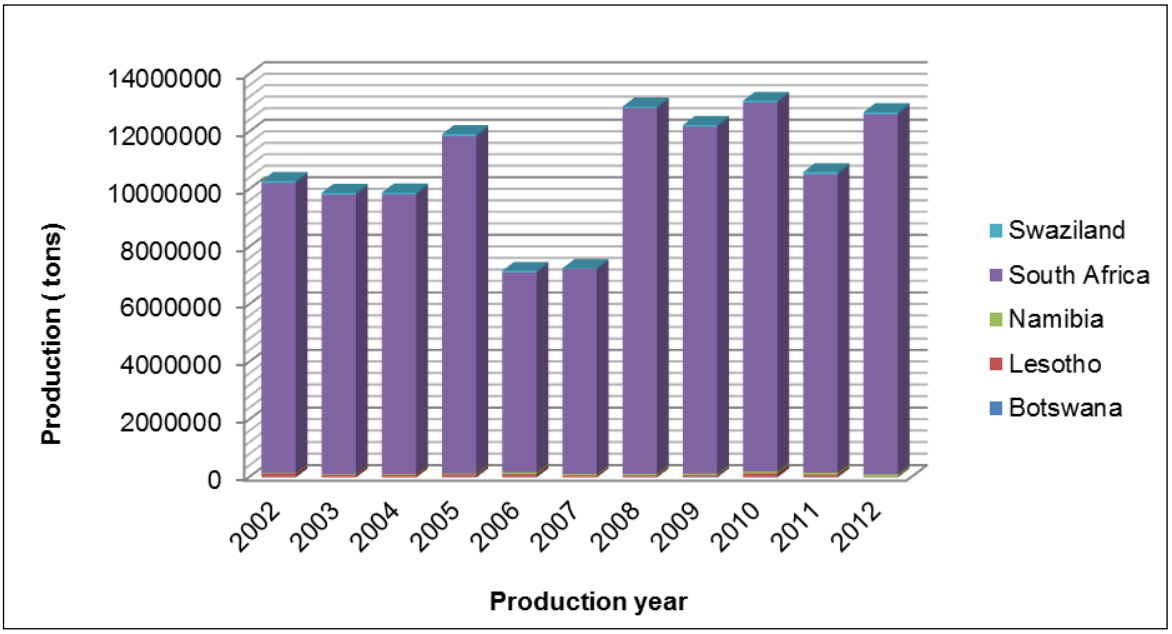

Figure 2: Maize production in Southern Africa including only South Africa and selected neighbouring countries (FAOSTAT, 2013).

Botswana produces the least maize, followed closely by Lesotho and Swaziland (figure 2). Over the past decade Namibia and South Africa consistently produced most of the maize for this region. However, South Africa produced significantly more maize than Namibia. The surplus maize produced by South Africa could be exported to neighbouring countries. Figure 3 shows that a total of 12% of South Africa's maize production is exported while 52% is used as food supply for the country's population.

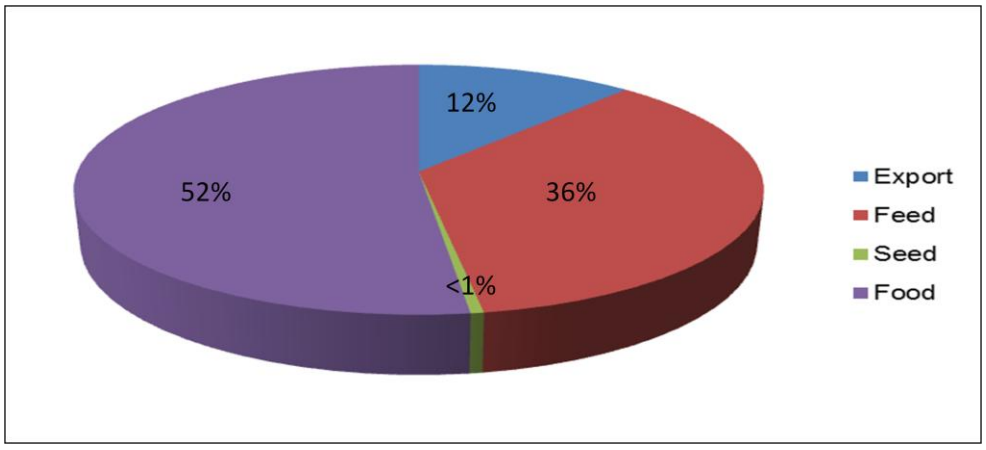

Figure 3: The average utilisation of maize in South Africa for the 2003-2009 periods (FAOSTAT, 2013).

Less than 1% of the maize produced is used for seeds for the next maize growing season and 36% is utilised as feed for livestock (Figure 3). Maize in South Africa is mostly produced through dry land agriculture and production under irrigation is limited to less than 10% (ARC-GCI, 2008). Within South Africa, 4 primary grain production regions have been identified which includes 36 magisterial districts. Vaalharts and eight other areas are situated in the North West province while eight other regions are placed within both the North West and Free State provinces. Mpumalanga contains five grain producing areas while Gauteng contains only two. Limpopo and Kwazulu-Natal each contains one grain producing area. The winter rainfall areas (Western and Eastern Cape) contain nine grain producing areas while Griqualand West contains one area for grain production (Du Toit, 1997; NDA, 2005). North West province and Free State are the major maize producing areas and the combined production is more than 60% of the total maize output of South Africa. The Highveld region of South Africa includes part of North West, Free State, Mpumalanga and the whole Gauteng province. This area is of critical importance as almost 90% of the commercially cultivated maize is grown here (Walker & Schulze, 2008).

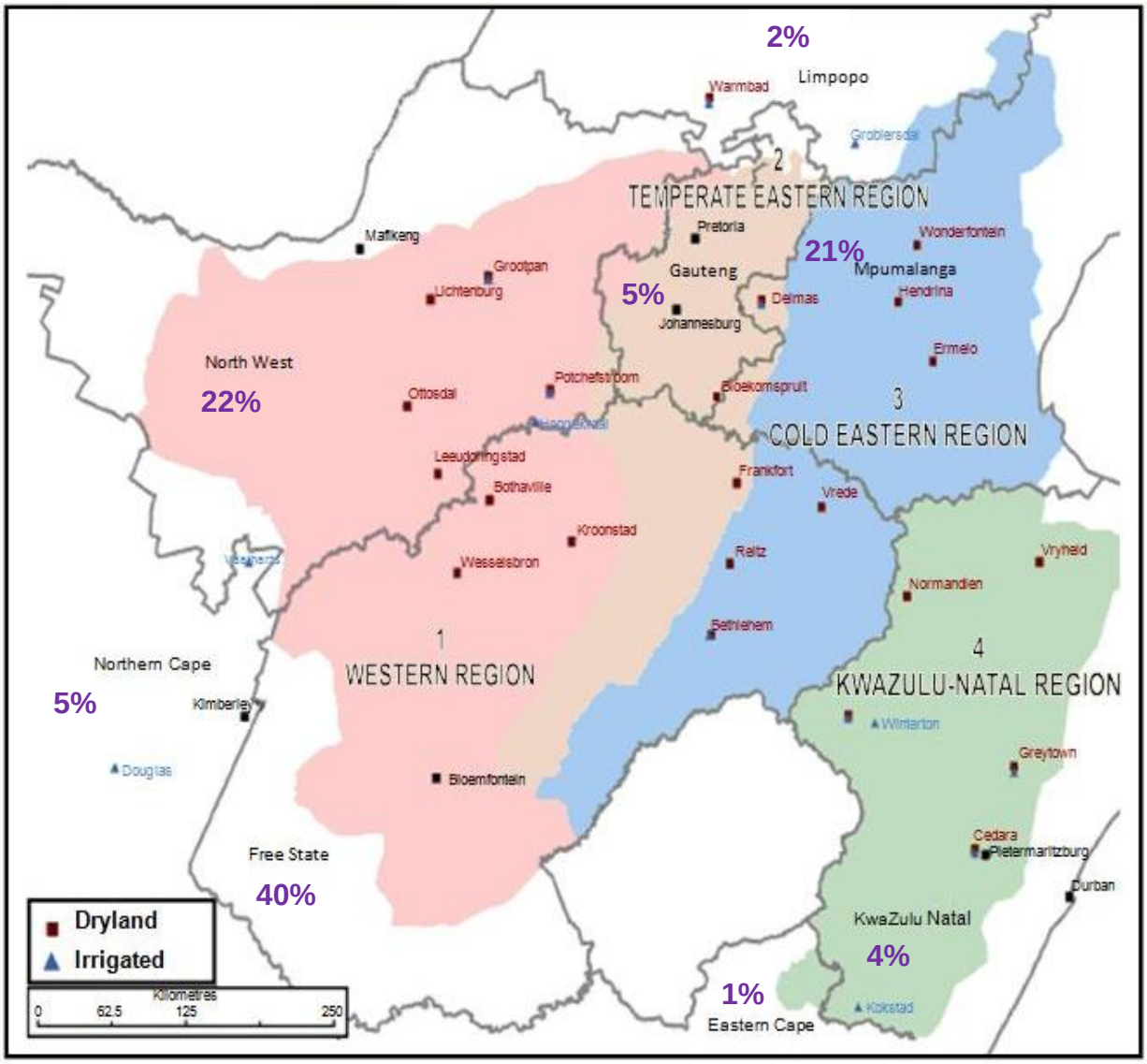

Figure 4: Maize production regions of South Africa (adapted from JAWF, 1999; DAFF, 2012; ARC-GCI, 2013). 1. Pink= Western Region, 2. Beige= Temperate Eastern Region, 3. Blue= Cold Eastern Region, 4. Green= Kwazulu-Natal Region.

Figure 4 illustrates the maize producing areas in the country together with the percentage of maize produced by each of the eight maize producing provinces. As Western Cape does not produce maize it is omitted from this figure. Areas where maize is produced under irrigation are also shown.

1.4 The climate of South Africa

South Africa has a warm and dry climate and is situated in a subtropical high pressure zone. Dry descending winds dominate the area. Rainfall patterns in South Africa have an uneven distribution, with an average annual rainfall of less than 500

mm, which is around 60% of the world average (Preston-Whyte & Tyson, 1993; Durand, 2006; DoA, 2007; Ziervogel *et al.*, 2010). Rainfall patterns are exceptionally variable and sometimes insufficient (Cook *et al.*, 2004).

The main maize producing provinces (North West and Free State) falls within three different climate zones (figure 4) based on spatial variance in temperature and rainfall. These zones are (1) the dry and warm western region, (2) the temperate eastern region and (3) the wet and cool eastern region (ARC-GCI, 2008). A majority of the interior and western part of the country is arid or semi-arid. Western regions of the country endures dry, desert like weather patterns. In contrast, eastern regions are prone to experience subtropical conditions and are humid (DoA, 2007). Rainfall decreases during summer in an east to west direction (Preston-Whyte & Tyson, 1993; Durand, 2006). Three rainfall regions have been identified in South Africa, namely summer rainfall regions, winter rainfall regions and regions with rainfall irrespective of the season (Schulze & Maharaj, 2007). The majority of dryland crop production takes place in the semi-arid zones of the country. A large portion of the country receives summer rainfall which is poorly distributed and droughts are common within these areas (Bennie & Hensley, 2000).

The majority of the precipitation in the Highveld region is received between October and March as this region is situated in a summer rainfall area (Walker & Schulze, 2008). The month in which maximum precipitation is received by different parts of the Highveld varies. During December, the eastern Highveld receives its maximum rainfall and is thus designated as an early summer rainfall area. The central Highveld is a mid-summer rainfall area, receiving the majority of the rain during January while the western Highveld is a late-summer rainfall area, only receiving the maximum rain during February (Schulze, 1997).

Agriculture in South Africa is highly dependent on environmental temperature and rainfall patterns (Behnin, 2008). It is estimated that approximately 5% of white maize and 11% of yellow maize is cultivated under irrigation while the remainder of the

maize is produced through dryland cropping (Durand, 2006). Dryland crop production covers 11 million hectare (ha) of the country's surface and utilises 12% of the potentially available rainfall (Bennie & Hensley, 2000). The production of maize as a staple grain requires rain as a critical input in rain-fed agricultural systems (Nicholson *et al*., 2000). Water availability is of critical importance to maize in any phonological stage and a water deficit could affect maize growth and development (Cakir, 2004).

Particular sensitivity to climate variability has been shown by the average maize yields in the western half of the Highveld. The maize yield in this area is highly dependent on farming practices and rainfall received during the growing season (Du Toit *et al.*, 2000).

1.5 Factors affecting maize growth, development and yield

Maize production is influenced by genotype, environment, crop management and maize prices (Du Toit, 1997; Damata *et al.*, 2010; Thitisaksakul *et al.*, 2012). Although grain yield and quality is significantly affected by the amount of sunshine a plant receives, temperature and rainfall patterns, there are many other factors affecting maize cultivation (Lu *et al.*, 2013). The main environmental conditions influencing maize agriculture are: (i) daily maximum and minimum temperatures, soil type and fertility, (ii) soil moisture levels, the ambient humidity surrounding the plant and wind movement, (iii) the day length along with light intensity, (iv) air quality and pollution, (v) competing plants and (vi) pathogen-insect complexes (Brown *et al.*, 1985).

Crop growth and development are highly dependent on temperature (Lu *et al.*, 2013). Maize is cultivated best in areas with warm weather. An average daily temperature of 19°C or more is required, while the mean of summer months should not be less than 23°C (Du Toit, 1997). A temperature range of 27 to 32°C is vital for optimal grain development in maize (Commuri & Jones, 1999). Environmental

temperatures that exceed 32°C combined with water stress resulting from higher evaporation rates may severely affect maize cultivation (Du Toit, 1997; Du Plessis, 2003). In tropical and temperate zones where maize is cultivated, an average temperature of 32°C is common during the reproductive development stage. Grain yield is reduced when high temperatures (exceeding 30°C) are experienced during grain filling. The high temperatures cause abortion of some kernels while decreasing grain weight (Engelen-Eigles *et al.*, 2000; Commuri & Jones, 2001; Barnabas *et al.*, 2008; Lobell *et al.*, 2014). South Africa has a warm climate with most regions experiencing an annual temperature above 17°C. Monthly temperature variations appear to occur gradually throughout the country with little to no sudden changes occurring frequently. Monthly averages of daily temperatures in the summer months range from 26-30°C for both eastern and western regions. The minimum temperatures during summer months range between 12 and 16°C (Schulze, 1997; Tadross *et al.*, 2011; Luhunga & Mutayoba, 2013).

Water is a limiting resource required during crop growth (Mukhala, 1998). Despite being adaptable to adverse conditions, maize production is negatively affected by low rainfall and droughts (Akpalu *et al.*, 2008). It is estimated that maize in South Africa requires between 450mm and 600mm of water per growing season, depending on the local environment (Du Plessis, 2003) while the annual rainfall in South Africa is less than 500 mm (Ziervogel *et al.*, 2010). Drought stress in maize causes the abortion of ovules and a reduction in invertase activities resulting in changes in hexose sugars and hormone balances (Zinselmeier *et al.*, 2000; Zinselmeier *et al.*, 2005; Chourney *et al.*, 2010). It is possible to produce maize in areas that receive at least 350 mm of rain during the growing season and in medium to high soil potential (NDA, 2005). The mean annual rainfall of the main maize producing areas range between 400-600 mm which is mostly received during the summer months when maize is cultivated (Bennie & Hensley, 2000).

Soil and climatic conditions experienced during a growing season is directly related to the harvest of a crop (Du Toit, 1997). To obtain high yields it is imperative for the

soil, nutrient and climatic conditions to be favourable during every stage of the development of the maize plant (Sun *et al.*, 2007).

Plants endure a variety of environmental challenges. Shade, high light levels, droughts, floods, freezing, low temperatures, high temperatures, high salinity, infections, predation, inorganic nutrient imbalances and natural or artificial toxic compounds could all be stressful to plants if the conditions are maintained (Bohnert & Sheveleva, 1998; Quinet *et al.*, 2010). The accumulation of ions and an increased production of metabolites are general stress responses in all kingdoms (Bohnert & Sheveleva, 1998). These metabolites include a range of sugars, sugar alcohols, carbohydrates, amino acids, amines and sulfomium compounds (Bohnert & Jensen, 1996; Quinet *et al.*, 2010). Stress perception and signalling could be directly translated into the re-programming of the plant system. Biochemical reactions, altered metabolic patterns and adjusted physiological states of the plants are triggered by prolonged environmental stresses (Bohnert & Sheveleva, 1998; Quinet *et al.*, 2010).

Proline, mannitol, sorbitol, glutamine, aspargine and putrescine are all included in the Biolog® GN2 test panel and play potential roles during plant stress. These substrates are produced more rapidly and accumulate within the plant tissue during stress conditions. Accumulation of proline upon dehydration due to water deficit or upon decreasing osmotic potential has been recorded in bacteria (Measures, 1975), algae (Measures, 1975) and higher plants such as maize (Mohammadkhani & Heidari, 2008; Anjum *et al.*, 2011). Putrescine, spermine and spermidine are all important in the physiological processes and development of all living organisms (Quinet *et al.*, 2010). The concentrations of diamines (putrescine) and polyamines (spermine and spermidine) in plants have been shown to increase during salinity stress (Sairam & Tyagi, 2004). In reaction to water stress, the stomata of the plant are closed and transpiration rates are lowered. During this, growth is inhibited and the water potential of the plant tissue and photosynthesis are decreased. Abscisic acid, proline, mannitol and sorbitol accumulate within the plant while radical scavenging compounds such as ascorbate, glutathione and α-tocopherol are formed

(Yordanov *et al.*, 2003). Early in the dehydration process altered cell carbon metabolism occurs (Lawlor, 2002).

1.6 Plant diseases and effects of water imbalance

Environmental conditions can influence host plant growth and susceptibility; pathogen reproduction, dispersal, survival and activity; as well as host-pathogen interaction (Ghini *et al.*, 2008). The classic disease triangle establishes the conditions for disease development, i.e. the interaction of a susceptible host, a virulent pathogen and a favourable environment. This relationship is evident in the definition of plant disease itself. Plant disease involves dynamic processes in which a host and a pathogen intimately relate to the environment and are mutually influenced, resulting in morphological and physiological changes (Gaumann, 1950). Changes within plants as a result of exasperated heat or water stress affect the susceptibility of the plant to pathogens (Garrett *et al.*, 2006). It is possible for plant pests and diseases to reduce the attainable yield of crops with up to 82% for cotton and up to 50% for other major crops such as maize (Oerke, 2006). It is estimated that plant diseases are responsible for at least 10-16% of global food production losses (Strange & Scott, 2005; Oerke 2006). The losses due to pests and diseases coupled with post-harvest spoilage and quality deterioration creates serious damage to production especially, in regions that have limited resources (Chakraborty & Newton, 2011).

1.7 Disease of maize crops

In spite of major technological advances and progress over the last few years, disease remains a limiting factor during maize production (Stuckey *et al.*, 1993; Gregory *et al.*, 2009). All crops are vulnerable to a range of plant disease, in the field as well as post-harvest. Diseases affecting maize can be classified into six groups of which the first four (i-iv) could be caused by either bacteria or fungi (Stuckey *et al.*, 1993): (i) seed and seedling diseases, (ii) leaf diseases, (iii) stalk rots, (iv) ear rots, (v) viral diseases and (vi) nematode diseases. Diseases of seeds, seedlings and leaves could be caused by both fungi (Mitchell *et al.*, 2002) and bacteria (Maude,

1996; Kloeppe *et al.*, 1999) while ear rots and stalk rots are mostly caused by fungi (Tesso *et al.*, 2012).

These pathogens create biotic constraints that could severely threaten food security. Viruses, bacteria, fungi, nematodes and parasitic plants are the major pathogen groups that can affect crops such as maize (Strange & Scott, 2005). Different sub-groups are distinguishable within the group of bacterial pathogens. Some of these pathogens are polyphagous which enables the pathogen to have a widespread distribution due to the non-specificity of its host range, while others have limited distribution ranges due to host-specificity (Krawczyk *et al.*, 2010). Epiphytic or endophytic bacterial species sometimes have the capability to be opportunistic pathogens (Stock *et al*., 2001; De Baere *et al*., 2004; Rezzonico *et al*., 2009).

The incidence and severity of bacterial pathogens of maize differs throughout the world. Bacterial pathogens that occur worldwide include pathogens such as *Pseudomonas avenae* subsp. *avenae* (Manns) (Claflin, 2000; Giester & Rees, 2004), *Erwinia carotovora* subsp. c*arotovora*, and *Erwinia chrysanthemi* pv. z*eae* (Sabet) (Claflin, 2000; Giester & Rees, 2004). Although these organisms are associated with many plant species they are also the causal agents of bacterial leaf blight, bacterial stalk rot and bacterial top rot of maize, respectively.

Diseases that occur on maize internationally include Stewart's wilt, Goss's wilt and bacterial leaf streak. *Pantoea stewartii* is the causal organism of Stewart's wilt (Lamka *et al*., 1991; Mergaert *et al*., 1993) which has been reported in USA, Brazil, Italy, Peru, Poland, Russia, Romania, Thailand, Austria, Canada, China, Costa Rica, Guyana, Greece, Malaysia, Mexico, Puerto Rico, Vietnam and former Yugoslavia (Claflin, 2000; Giester & Rees, 2004; CABI EPPO, 2008). Stewart's wilt caused between 40 -100% yield loss on susceptible maize lines (Suparyono & Pataky, 1989), but since the development of resistant maize lines losses have been limited (Pataky *et al.*, 1990).

Clavibacter michiganensis subsp. *nebraskensis* (Vidaver & Mandel, 1974), the agent of Goss's bacterial wilt and blight (Smidt & Vidaver, 1986), has a limited distribution range. Goss's wilt and blight was only reported from ten states in the USA and two provinces in Canada namely Manitoba and Ontario (Ruhl *et al.*, 2009; Korus, 2011). The maximum yield loss attributed to Goss's wilt and blight was reported as 43.5% during inoculation studies (Korus, 2011). Goss's wilt is to a lesser degree a seed borne disease which could be seed transmitted (Ruhl *et al.*, 2009). Similarly, bacterial leaf streak of maize, which is caused by *Xanthomonas campestris* pv. *zeae* (Elliott) Dye (Coutinho & Wallis, 1990; Claflin, 2000; Giester & Rees, 2004) has only been reported in South Africa to date. Although it is still unknown whether or not bacterial leaf streak is seed borne, it has been determined that some *Xanthomonas campestris* pathovars are seed borne such as *X. campestris* pv. *campestris* (Vincente *et al.*, 2012) and *X. campestris* pv. *carotae* (Meng *et al.*, 2004).

Pseudomonas avenae subsp. *avenae* (Manns) (syn. *Acidovorax avenae* subsp. *avenae*) causes bacterial leaf streak on many maize lines internationally (Schaad & Kado, 1975; Claflin, 2000: Schaad *et al.*, 2008). Bacterial leaf streak of maize caused by *P. avenae* was first reported in Thailand (Prathuangwong *et al.,* 2004). Major economic losses reaching 30% have been reported on a susceptible sweet corn cultivar, Insee2 (Techati, 2008). Under field conditions the pathogen does not always produce distinct symptoms making the diagnosis of the disease difficult. A bio-PCR assay was used in recent studies to detect *P.* avenae in commercial maize seeds and the transmission of the disease from seed to seedlings was confirmed. Inoculation studies on field maize infected with the disease was determined as 30% disease severity (Krittidetch *et al.*, 2013). The symptoms of bacterial leaf streak of maize caused by *P. avenae* are similar to the symptoms of Stewart's wilt. These symptoms are long, elliptical water soaked lesions with haloes, parallel to the leaf veins (Prathuangwong *et al.,* 2004). *Xanthomonas campestris* pathovar *zeae* is also responsible for a disease known as bacterial leaf streak of maize (Coutinho & Wallis, 1990).

1.8 Xanthomonas

1.8.1 General characteristics of *Xanthomonas*

Xanthomonas is a genus of bacterial plant pathogens that belong to the gamma-subdivision of the Proteobacteria (Coutinho & Wallis, 1990). A diverse range of species are4 found within the *Xanthomonas* genus and more than 27 plant associated species have been identified. *Xanthomonas* members are of significant economic importance as this group infects at least 124 monocotyledonous and 268 dicotyledonous plant species. They cause a variety of symptoms including necrosis, cankers, spots, and blight in a variety of plant parts including leaves, stems, and fruits (Leyns *et al.*, 1984). These pathogens are known to cause several diseases on a range of economically important crops and ornamentals including tomatoes (Dye & Lelliott, 1974; Ignatov *et al.*, 2007), bananas (Rangaswami & Ranga-Rajan, 1965; Jogo *et al.*, 2013), lettuce (Dye & Lelliott, 1974; Barak *et al.*, 2001), rice (Egli *et al.*, 1975; Bradbury, 1984; Yu *et al.*, 2011), watermelon (Dye & Lelliott, 1974; Dutta *et al.* 2013), cabbage (Dye & Lelliott, 1974; Bila *et al.*, 2013), radish (Dye & Lelliott, 1974; Ojha *et al.*, 2012), grape (Bradbury, 1984; Girase *et al.*, 2012), roses (Huang *et al.*, 2013) and maize (Dye & Lelliott, 1974; Egli *et al.*, 1975; Hashimi & Birch, 2010)

Usually the host specificity of *Xanthomonas* species or pathovars is limited to one genus or closely related genera of plants (Leyns *et al.*, 1984; Ryan *et al.*, 2011). Pathovars of *Xanthomonas* also display tissue specificity by infecting either the vascular system or the mesophyll tissue of the specific host. The pathovars are therefore classified as vascular or mesophyllic pathogens (Ryan *et al.*, 2011).

Colonies of *Xanthomonas* on solid media are usually observed as round, yellow mucoid colonies with diameters ranging between 2 to 5 mm. These colonies tend to have raised centres and continuous, smooth margins (Qhobela *et al.*, 1990). The yellow colour of the colonies is due to a yellow pigment - referred to as xanthomodin - that is produced by members of this genus. This unique "*Xanthomonas*- cartenoid" has not been found in any yellow non-xanthomonad (Starr & Stephens, 1964). Cells of the members of *Xanthomonas* appear as short, straight rods when viewed under a microscope. The size of the cells ranges between 0.4 - 1.0 ×1.2 - 3.0 µm. Species

within the genus are Gram-negative and have monotricous flagella, which contributes to their motility. *Xanthomonas* members do not form endospores. Members of this genus are able to depolymerise natural polysaccharides and proteins. Xanthomonads use compounds with low molecular weights and are described as chemoorganotrophic, facultative aerobic organisms. These bacterial cells make use of aerobic respiration and are not able to catabolise glucose, which classifies the bacteria as non-lactose fermenters. Xanthomonads do not utilise asparagine as sole carbon source (Qhobela *et al.*, 1990; Gracelin *et al.*, 2012). Although much research has been conducted on the genus *Xanthomonas*, very little is known about *Xanthomonas campestris* pathovar *zeae*, the causal agent of bacterial leaf streak of maize.

The genus of *Xanthomonas* has a remarkable diversity range and is uncommon in its phenotypic uniformity (Vauterin *et al.*, 2000). The identification of these pathovars rely mainly on the knowledge of its host since the pathovars are indistinguishable solely based on its phenotypic properties (Massomo *et al.*, 2003; Brenner *et al.*, 2005). Conversely, using pathogenicity tests to differentiate between pathovars of *X. campestris* have proven difficult. This is because these pathovars occasionally develop lesions that seem identical (Alvarez *et al.*, 2004; Massomo *et al.*, 2003). The naming of these pathovars is still facing major obstacles since taxonomic studies are controversial and have yet to be completed (Vauterin *et al.*, 1995; Zhao *et al.*, 2000; Massomo *et al.*, 2003; Young, 2008, Young *et al.*, 2008).

In the past, a *Xanthomonas* variant that causes different symptoms or exploited a different range of hosts were classified as a completely separate species based on the “new host-new species” concept. A very complex genus containing over 100 species ensued as a result of this naming concept (Starr, 1981). Later on, due to the phenotypic similarity and the lack of information on the phytopathogenic specialisation of these organisms (Burkholder & Starr, 1948; Dye, 1962; Van den Mooter & Swings, 1990), all the members of the genus were merged to form a single species, *Xanthomonas campestris* (Dye & Lelliot, 1974). It was proposed to reclassify the former nomenspecies into pathovars (Young *et al.*, 1978; Vauterin *et*

al., 2000). This was accepted as a temporary solution until it was possible to classify these organisms on more generally accepted principles (Vauterin *et al.*, 2000). Not only is it considered unacceptable in modern taxonomy to define a pathogen on a single feature but the reclassification into pathovars faces three other major problems: (i) the host range of many pathovars have not been investigated by performing cross-inoculations (Vauterin *et al.*, 2000); (ii) significant genomic heterogeneity has been confirmed among a number of pathovars (Hildebrand *et al.*, 1990; Vauterin *et al.*, 1992; Rademaker *et al.*, 2000) and (iii) it is not possible to classify endophytic xanthomonads isolated from plants into a pathosystem (Vauterin *et al.*, 2000). It is the task of The International Society of Plant Pathology Committee on the Taxonomy of Plant Pathogenic Bacteria (ISPP-CTPPB) to create and interpret the rules for the naming of plant pathogenic bacteria. To catalogue and verify the names of the phytopathogens (Bull *et al.*, 2012), the committee uses the International Code of Nomenclature of Bacteria (Lapage *et al.*, 1992) and the International Standards for Naming Pathovars of Plant Pathogenic Bacteria (Dye *et al.*, 1980; Young *et al*., 1991). The list of valid names include a variety of *Xanthomonas* members (Bull *et al.*, 2010), however, *Xanthomonas campestris* pv. *zeae* has not yet been included in any of these lists.

Xanthomonas campestris pathovar *zeae* caused the withdrawal of a commercial variety of maize due to the susceptibility of the variety to the disease termed bacterial leaf streak (Coutinho & Wallis, 1990). The first report of this disease occurred in South Africa during 1949 (Dyer, 1949). This disease has not been reported in any other country (Qhobela *et al.*, 1990). Occurrence of this disease has been found to be primarily in the warmer and drier regions of the country (Qhobela *et al.*, 1990). Symptoms that appear on naturally infected plants consist of yellow-brown lesions on the leaves of the plants as represented in figure 5. These lesions could range from 2 to 3 mm in width. The margins of the lesions are wavy and irregular and the lesions tend to run parallel to the veins of the leaves (Qhobela *et al.*, 1990; Coutinho & Wallis, 1990). In some cases the lesions could be present along the entire length of the leaf. Necrotic regions that resemble drought symptoms form when multiple lesions coalesce. The expression of symptoms is enhanced when daily temperatures exceed 32˚C. Irrigation during high environmental temperatures

tends to increase disease incidence (Nowell, unpublished, cited by Qhobela *et al.*, 1990). These conditions that promote the development of symptoms can result in up to 40% death of leaf tissue.

Figure 5: Maize leaf showing typical BLS symptoms, photographed during fieldwork.

Occasionally, yellow droplets of bacterial exudates form on the lesions and have been identified as xanthan. The formation of xanthan increases during humid conditions (Coutinho & Wallis, 1990). Members of the *Xanthomonas* genus produce this complex exopolysaccharide (Katzen *et al.*, 1998). Xanthan consists of pentasaccharide subunits that form a cellulose backbone with trisaccharide side chains that are composed of mannose (β1,4) glucoronic-acid (β1,2) mannose. These side chains are attached to alternate glucose residues in the backbone by α1,3 – linkages (Becker *et al.*, 1998; Katzen *et al.*, 1998).

1.8.2 Mode of infection utilised by *Xanthomonas*

The *Xanthomonas* genome is a single circular chromosome and encodes more than 4 000 proteins, including the proteins responsible for cellular functions such as energy production. Within the genome, gene clusters encode the type II secretion system (*xps* and *rpf*) which regulates the secretion of pathogenicity factors (Pieretti *et al.*, 2012). Pathogenicity factors are essential for disease establishment and include substances such as bacterial toxins and extracellular enzymes (Buttner & Bonas, 2010). The synthesis of xanthan by *Xanthomonas* spp. is induced by the hypersensitive response and pathogenicity (*hrp*) genes that encode both the type III

secretion system and the *gum* genes. For *Xanthomonas* to be able to induce disease, other encoded genes are also present. These genes are responsible for the recognition of the host by the pathogen and adhesion to the plant surface. The invasion and subsequent colonisation of the host tissue is regulated by these genes. The type III secretion system enables the pathogen to obtain nutrients and the pathogen's response to plant defence mechanisms. Additional plasmids that are associated with virulence could also be possessed by *Xanthomonas* spp. These plasmids can encode type III effector proteins, the secretion of extracellular enzymes and type IV secretion systems (Comas *et al.*, 2006; Lima *et al.*, 2008; Buttner & Bonas, 2010).

There are currently six recognised protein secretory systems for Gram-negative bacteria and in particular *Xanthomonas*. The classification of these systems is based on their structure, function and the recognition of secretion substrates (Preston *et al.*, 2005; Gerlach & Hensel, 2007). The first secretory system is the type I or ATP-binding cassette (ABC) system which includes secreted toxins, proteases, lipases and other degrading enzymes. Secretions of these substances take place through the periplasmic membrane fusion protein (Gerlach & Hensel, 2007). The type II secretory system, also known as the general secretory pathway, secretes toxins, extracellular enzymes and cell wall degrading enzymes such as cellulases, cellobiosidases, polygalacturonases, xylanases and proteases. These enzymes are passed through the periplasmic pseudopilus (Jha *et al.*, 2005; Johnson *et al.*, 2006). Substrates from the type II secretion systems may induce plant defence responses such as callose deposition in the cell wall (Jha *et al.*, 2005).

The type IV secretion system (TIVSS) delivers extracellular components such as DNA and proteins through a protein channel in the outer membrane. This system is a two-partner secretion system and makes use of autotransporters (Henderson *et al.*, 2004; Gerlach & Hensel, 2007; Cascales, 2008). Proteins and DNA in eukaryotic cells are also transported by the multicomponent secretion type VI system (Filloux *et al.*, 2008).

The most important secretory system for phytobacteria is the type III secretory system (Galan & Collmer, 1999; Ghosh, 2004). The bacterial type III secretion system (T3SS) is used by bacteria to directly insert effector proteins into host cells to manipulate the host cell function (Coburn *et al.*, 2007). Extracellular components and effector proteins of this system are secreted through a multimeric transmembrane channel (Ghosh, 2004).

The T3SS is encoded for by the hypersensitive response and pathogenicity (*hrp)* genes which are organized in roughly 20 genes with numerous operons (Cornelis & Van Gijsegem, 2000; Arnold *et al.*, 2003). The *hrp* genes of phytobacteria are differentiated into two groups based on the similarities of the *hrp* operon and the regulation of gene expression. The *hrp* genes of *Erwinia, Pantoea* and *Pseudomonas* species falls within group I, while *Xanthomonas* and *Ralstonia* members are found in group II (Alfano & Collmer, 1997). The type III system occurs in most Gram-negative pathogenic bacteria including the genera *Erwinia*, *Pantoea*, *Ralstonia*, *Pseudomonas* and *Xanthomonas* (Alfano & Collmer, 1997). These genes are used to alter the host cell to allow the pathogen to invade, colonise and multiply within the host. Therefore, these genes are essential for disease development and the induction of a hypersensitive response in resistant plants and non-hosts (Hueck, 1998; Coburn *et al.*, 2007).

Host factors and environmental conditions such as temperature and pH, dictate the level of *hrp* gene expressions during bacterial infection (Arlat *et al.*, 1992; Rahme *et al.,* 1992; Schulte & Bonas 1992; Wei *et al.*, 1992; Xiao *et al.*, 1992; Tang *et al.*, 2006). High pH, osmolarity and complex carbon and nitrogen nutrient sources have an inhibiting effect on the expression of the *hrp* genes. *Hrp* gene expression is induced by acidity, low osmotic pressure and simple sugars as nutrient sources. Optimal expression of the type III-SS genes takes place at 20 to 30°C (Van Dijk *et al.*, 1999). The environmental conditions inducing *hrp* gene expression most probably simulate the conditions during bacterial infection (Arlat *et al.*, 1992; Rahme *et al*., 1992; Schulte & Bonas 1992; Wei *et al.*, 1992; Xiao *et al.*, 1992; Tang *et al.*, 2006)

Plant openings form a natural entry/exit point for many plant pathogens. It has been found that *X. campestris* pv. *campestris* (Pammel) Dowson, the causal agent of black rot, actively enters leaves through the hydathodes (Hugouvieux *et al.*, 1998). Similarly, several foliar pathogens such as *P. syringae* and *Xanthomonas* pathovars could move from the inside of the leaf to the leaf surface, through the stomata, and occasionally through lesions and wounds (Roos & Hattingh, 1987; Beattie & Lindow, 1999).

A number of plant pathogens, including xanthomonads, could possibly be transmitted to a new host. For the transmission to a new host to take place, it is important for the pathogen to survive in the absence of the usual host (Soudi *et al.*, 2011). There are many ways for the pathogens to survive during the season when the host is not available. The bacteria could survive in seeds, plant debris and perennial hosts (Schaad & Dianese, 1981; Kocks *et al.*, 1998). Some pathogens also survive by living epiphytically or saprophytically in soil or on insects (Brenner *et al.*, 2005). Although the most important sources of inoculum for black rot are reported to be infected plant residues, seeds and weeds it is possible for infection to occur through infested soil (López *et al.*, 1999). The pathogens easily spread to surrounding plants through water splashing such as rain (Soudi *et al.*, 2011). The dispersal of bacteria from guttation droplets and the resulting spread of disease through field crops increase during wet, windy conditions (Kocks *et al.*, 1998). Warm and humid climates are ideal for the spread of xanthomonads (Duveiller *et al.*, 1997; Jones *et al.*, 2000).

It was postulated that *Xanthomonas campestris* pv. *zeae* enters the leaf through stomata and hydathodes and therefore the risk of infection is higher in the middle of the day when these openings are completely open (Kloppers & Tweer, 2009). An innate part of a plant's response to pathogens is to close the stomatal openings. It was found that living *Xanthomonas campestris* pv. *campestris* bacteria and the ethyl acetate extracts form the culture supernatant of these bacteria are able to interfere with the stomatal closure immune response in order to enter the leaf of the host through these openings (Gudesblat *et al.* 2009). The primary source of the inoculum

is believed to be remaining post-harvest crop debris. As with bacterial spot of pumpkin pathogen, *Xanthomonas cucurbitae* (Babadoost & Zitter, 2009), it is possible for the pathogen to survive in the debris for several months. During dry periods the pathogen remains dormant within plant debris but active development occurs when the environmental conditions become favourable (Kloppers & Tweer, 2009; Ryan *et al.*, 2011). Bacteria present in irrigation water or on leaf surfaces could serve as a secondary source of inoculum. Primary infection usually occurs during seedling development. During the growth cycle, younger leaves are vulnerable to secondary infection. After infection, the characteristic lesions develop on the leaves and during moist conditions bacterial exudates may form on the leaf surface (Kloppers & Tweer, 2009; Ryan *et al.*, 2011). These exudates desiccate under dry conditions and could serve as long distance inocula when it comes into contact with irrigation water (Kloppers & Tweer, 2009). The most effective dissemination of the pathogen within fields is through wind, rain and irrigation. Alternatively, the pathogen may be spread through aphids and other sucking insects and direct plant-to-plant contact (Kloppers & Tweer, 2009; Ryan *et al.*, 2011). It has not yet been proven whether or not bacterial leaf streak of maize could be seed-borne (Kloppers & Tweer, 2009).

1.8.3 The role of xanthan in pathogenicity

Although the role of xanthan in the pathogenomics of *X. campestris* pv. *zeae* has not been studied, it has been proposed that *X. campestris* requires both extracellular enzymes and xanthan for pathogenicity (Dow & Daniels, 2000). The ability to produce exopolysaccharide has been shown to play an important role of survival of *X. campestris* in soil environments (Soudi *et al.*, 2011). It is suggested that xanthan also plays a role in pathogenicity, survival in a plant during stress conditions and survival of the pathogen in plant exudates (Chun *et al.*, 1997).

Extracellular polysaccharides (EPS), such as xanthan, possibly aid cells to adhere to the leaf surface and could prevent bacterial cell desiccation by promoting the binding of water (Costerton *et al.*, 1995; Donlan, 2002; Annous *et al.*, 2009; Vu *et al*., 2009). EPS production could also alter the immediate surroundings of the bacterial cells to

create an environment that promotes bacterial growth and survival, thereby creating a matrix similar to a biofilm found in aquatic environments. This matrix could provide the same advantages to the bacterial community as a biofilm (Beattie & Lindow, 1999). Many microorganisms favour living in biofilms to take advantage of the multitude of possible benefits that biofilms offer (Costerton *et al*., 1995; Donlan, 2002; Annous *et al*., 2009; Vu *et al*., 2009). Biofilms are responsible for the concentration of otherwise diluted nutrients and safeguards the community against predators (Costerton *et al.*, 1995). Organisms living as part of a biofilm have shown greater antibiotic resistance and were better protected against other inhibitory compounds such as lytic enzymes (Costerton *et al.*, 1995; Donlan, 2002; Annous *et al*., 2009; Vu *et al*., 2009). Bacteria residing in biofilms are also protected from environmental stress factors including (but not limited to) extreme pH, high or low oxygen levels, osmotic shock, heat, freezing and UV radiation. Nutrients, metabolites and genetic material are more rapidly exchanged between cells within biofilms due to the adherent nature of the cells (Costerton, *et al.*, 1995; Donlan, 2002; Annous *et al.*, 2009; Vu *et al.*, 2009). The production of xanthan protects the bacteria from environmental stresses and also aids in adhesion to the leaf surface. It is critical for phytopathogenic bacteria to adhere to the host tissue to infect the host successfully (Cao & Saier, 2001). The role of bacterial adhesion in virulence was demonstrated for plant pathogens (Ojanen-Reuhs *et al.*, 1993; Rosenblueth & Martinez-Romero, 2006).

Although it has been proven that xanthan is not necessary for pathogenesis in citrus, it does aid in epiphytic survival of the pathogen (Dunger *et al.*, 2007). Studies on the role of xanthan during the infection of crucifers have revealed that xanthan plays a role in pathogenesis by preventing callose formation within the plant which makes the plant susceptible to *Xanthomonas* strains (Yun *et al.*, 2006). Callose is usually localised in the pollen grains and tubes, dead elements of the phloem, plasmodesmatas, and tracheids of plants. Mechanical wounding, physiological stress and phytopathogen infection induce callose synthesis (Stone & Clarke, 1992). Callose is essential during a variety of processes in plant development and as a stress response (Chen & Kim, 2009). Xanthan suppresses callose deposition and induces susceptibility of plants to *Xanthomonas* spp. Xanthan is essential for

virulence and necrosis in plants (Yun *et al.*, 2006). Wilting of leaves is caused by xylem vessels that are blocked by xanthan and could rupture as a result of the high osmotic pressure generated. Water flow is restricted and cell membrane leakage is increased by xanthan production, which could also result in the wilting of leaves. The water saturation of intercellular spaces is encouraged by the EPS, which in turn promotes bacterial colonization (Denny, 1995; Kiraly *et al.*, 1997). Xanthan production thus enhances the pathogenicity of xanthomonads by protecting the bacteria, preventing the activation of various plant defence reactions and facilitating dissemination of the pathogen (Braun, 1990; Saile *et al.*, 1997; Dow *et al.*, 2003).

1.9 Methods used to study plant pathogens

1.9.1 Physiological and biochemical methods

It is vital for all aspects of plant pathology to accurately detect and identify plant-pathogenic bacteria, especially to control the disease and the spread of the inoculum (López *et al.*, 1999). Taxonomic classification, identification and characterisation of bacteria are achievable through a broad range of laboratory techniques (Sintchenko *et al.*, 2007). Detection and identification of plant pathogenic xanthomonads mostly relied on predetermined biochemical, serological and pathological tests after the target organism has been isolated in a pure culture. Thus modern methods still require the isolation of the bacterium in a pure culture and are culture dependent (Leite *et al*., 1994; Sauer & Kliem, 2010). The isolation process is vulnerable to contamination by other bacteria that may be associated with plant tissue and act as fast-growing contaminants. In some cases when isolating xanthomonads, it is necessary to increase the sensitivity of isolation by adding non-selective or selective supplements. In recent years, the identification of phytopathogenic bacteria was accomplished with techniques based on metabolic and protein profiling and on fatty acid analysis (Leite *et al*., 1994).

Conventional methods to identify phytopathogenic bacteria are time consuming and may be costly. More rapid, reliable and inexpensive techniques are being developed (Jones *et al.*, 1993). Earlier, bacteria were identified by a range of phenotypic and biochemical tests that were done individually which was laborious and time-

consuming. Global phenotyping with the use of “Phenotype MicroArrays” (PM) were developed. Nearly 2000 assays were developed to productively characterise and identify bacteria (Bochner *et al.*, 2001; Bochner, 2003). Useful information on the biological properties of bacteria is generated through phenotypic testing. Physiological studies of bacterial cells enumerate various subsystems that function within cells making it a valuable approach in characterisation studies. The development of PM enabled the same set of tests to be used across a range of microbial species of which the results could be compared (Bochner, 2009).

There has been a major increase in carbon substrate utilisation patterns in both environmental and ecological microbiology due to the reproducibility and reliability of the technique. For years, the abundant range of biodegradable substrates has enabled the characterisation and identification of pure bacterial cultures based on the ability of the organism to catabolise certain substrates (Konopka *et al.*, 1998). Carbon utilisation assays have also been used to study microbial community function (Di Giovanni *et al.*, 1999; Lyons & Dobbs, 2012). A range of bacterial species has been identified with carbon utilisation patterns, including some species of *Mycobacterium* (Conville & Witebsky, 2001), *Erwinia* (Jiménez-Hidalgo *et al.*, 2004), *Pseudomonas* (Grayston *et al.*, 1998), *Pantoea* (Goszczynska *et al.*, 2007), *Xanthomonas campestris* (Nunez *et al.*, 2002; Roumagnac *et al.*, 2004), *Xanthomonas campestris* pv. *vesicatoria* (Bouzar *et al.*, 1994) and *Xanthomonas campestris* pv. *begoniae* (Zhou & Ji, 2013).

1.9.2 Genotypic methods

Genomic fingerprinting is useful for the identification of bacteria. The use of 16S rDNA is most commonly used for phylogenetic and taxonomic studies (Maggi & Breitschwerdt, 2005). The 16S rDNA gene is found in almost all bacteria. This gene is highly conserved while maintaining relatively slow evolution rates. A wide range of sequences has been deposited for this gene, which establishes a reliable database for the comparison of sequences. Universal primers are available and it is fairly rapid and easy to sequence this gene. Discrimination between taxa based on the conserved and variable regions is possible (Weisburg *et al.*, 1991; Cilia *et al.*, 1996;

Relman, 1999; Patel, 2001; Petti *et al.*, 2005; Mignard & Flandrois, 2006; McInerney *et al.*, 2008). Substantial interspecies differences and minor intra-species differences create the basis for identification using the 16S rDNA gene (Clayton *et al.*, 1995; Woo *et al.*, 2008). This technique is completely reliant on the deposit of absolutely unambiguous nucleotide sequences into databases and the assigning of the exact taxon, species or gene to each sequence (Kolbert & Persing, 1999; Janda & Abbott, 2007).

Although 16S rDNA sequences possibly allow genus identification in more than 90% of cases and 65-80% identification up to species level, it is possible for almost 14% of analysed sequences to remain unidentified; at the species, subspecies or strain levels (Mollet *et al.*, 1997; Drancourt *et al.*, 2000; Sontakke *et al.*, 2009).

The use of the 16S rDNA gene for the identification of bacteria and phylogenetic studies has a few shortcomings. These include (i) the gene is very small in comparison to the entire genome; (ii) the differentiation of closely related strains of bacteria is difficult due to the lack of informative characters; (iii) the slow rate of evolution complicates the resolution of evolutionary trees (Rogall *et al*., 1990; Bennasar *et al*., 1996); (iv) sequence alignment may be complicated by possible insertions or deletions within the gene; and (v) the relationship between organisms may be misrepresented due to secondary structures causing sequence convergence and saturation (Hillis & Dixon, 1991; Dixon &Hillis, 1993; Kjer, 1995). Comparative 16S rDNA analysis is strongly affected by the quality of the data set. Although a database is available for various microorganisms, the database contains a percentage of errors as a result of reading mistakes, PCR artefacts and the presence of chimera (Stackebrandt, 2011).

As with the 16S rDNA gene, the 23S rDNA gene has a universal distribution, is reasonably conserved and contains variable regions useful in identification and phylogenetic studies. Similar challenges associated with 16S rDNA gene also apply to the 23S rDNA gene. Longer sequences with unique insertions or deletions are

obtained from the 23S rDNA gene. As there are higher sequence variations, this gene is better equipped to differentiate between closely related species (Leffers *et al*., 1987; Ludwig & Schleifer, 1994; Hunt *et al.*, 2006). More information could be obtained when both the 23S rDNA and 16S rDNA genes are used in phylogenetic studies (Nochi *et al.*, 2009). Alternative genes are sometimes necessary, because differentiation at the species, subspecies or strain level may not always be achieved with the use of the 16S rDNA or 23S rDNA genes (Sontakke *et al.*, 2009).

Alternative molecular markers such as *gyrB* protein encoding genes are able to detect phylogenetic relationships without bias. The *gyrase B* gene is relatively conserved and provides a higher phylogenetic resolution, even at pathovar level due to more nucleotide variations within this gene (Fukushima *et al.*, 2002; Yin *et al.*, 2008; Nochi *et al.*, 2009). The loss of phylogenetic resolution is limited with the use of the *gyrB* gene because it is able to detect intragenomic heterogeneity or the lack of sequence heterogeneity (Case *et al.*, 2007; Mondal *et al.*, 2013). However, the *gyrB* gene was not able to distinguish between *X*. *euvesicatoria*, *X. perforans* and *X. alfalfae* (Parkinson *et al.*, 2009).

Homologous recombination gives more sequence variability to the internal transcribed spacer (ITS) regions (Leblond-Bourget *et al.*, 1996; Klappenbach *et al.*, 2000; Sadeghifard *et al.*, 2006). Multiple rRNA operons are included in this region for which multiple primers have been designed (Anton *et al.*, 1998; Gurtler & Stanisich, 1996; Acinas *et al.*, 2004; Liguori *et al.*, 2011). This region plays a role in ribosomal RNA maturation and transcriptional anti-termination (Apirion & Miczak, 1993; Rocap *et al.*, 2002). Closely related species are distinguished by direct gene sequencing or length polymorphisms (Grimont & Grimont, 1986; Rajendhran & Gunasekaran, 2011). Sequence heterogeniety of this region is also possible due to the type and number of rRNA present (Anton *et al.*, 1998; Garcia-Martinez *et al.*, 1999; Boyer *et al.*, 2001).

PCR protocols for rapid and specific identification of *X. campestris,* using hypersensivity response and pathogenicity (*hrp*) genes from *X. campestris* as the molecular targets, are available (Leite *et al.*, 1994; Berg *et al*., 2005; Lewis *et al.*, 2010; Soudi *et al.*, 2011). Other molecular markers for the genus *Xanthomonas*, in general or specific species, are available based on xanthan encoding genes (Mbega *et al.*, 2011) as well as xanthomonadin encoding genes (Poplawsky, 1993). Identification based on the *hrp* gene or Hrp/type III secretion system has been done on many plant-pathogenic bacteria including *R. solanacearum* (Poussier *et al.*, 2000), *X. campestris* pv. *vesicatoria* (Obradovic *et al.*, 2004), *X. fragariae, X. campestris* pv. *secalis, X. campestris* pv. *translucens, X. albilineans* (Leite *et al.*, 1994) and *X. campestris* pv. *campestris* (Zaccardelli *et al.*, 2007).

As very little information is available on the studied organisms, a polyphasic approach was selected for the current study. This approach included biochemical and phenotypic methods to serve as a foundation for future studies.

1.10 The principles of the methods used during the current study

1.10.1 Pathogenicity testing and Koch's postulate

Research on infectious diseases, such as anthrax and tuberculosis, gave rise to Koch's postulates. Koch's postulates were intended as a standard practice to prove the cause of an infectious disease. These postulates are summarised as follows: (i) the pathogen occurs in every case of the disease incidence taking into account the circumstances for the pathological changes and course of the disease; (ii) the pathogen is not present in any other disease as a non-pathogenic organism and (iii) after complete isolation from a diseased host, the pathogen is repeatedly grown in pure culture and when it is placed in an infected host, only after symptom development occurs and the same pathogen is re-isolated from the new host, the subject microorganism could be deemed as the causal pathogen (Fredericks & Relman, 1996).

It has often proved difficult to synthetically replicate a disease in attempts to establish the pathogenicity of a suspected pathogen. It is not only challenging to provide a favourable environment for disease development, but also to develop an inoculation technique which is easily executable while delivering good results (Bhat *et al.*, 2010). Inoculation techniques are usually based on the mode of penetration of the pathogen. It is also important to acquire knowledge about the host range of a pathogen to determine the crops that are vulnerable to infection. Pathogenicity studies also aid in understanding the pathogen's mode of survival during times when the main host is not present (Bhat *et al.*, 2010).

Couthino & Wallis (1990) successfully isolated *X. campestris* pv. *zeae* causing bacterial leaf streak of maize and Koch's postulates was used to establish pathogenicity. The disease was previously not described and the name *X. campestris* pv. *zeae* was proposed for the pathogen re-isolated from the diseased maize plants (Couthino & Wallis, 1990).

1.10.2 Protein profiling and SDS-PAGE

In molecular biology, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a very valuable tool (Wilson & Walker, 2000). The separation, visualisation and comparison of proteins were made possible by the discontinuous buffer system (Laemmli, 1970). It is possible to quantify the amount of a particular protein in a sample and to obtain molecular mass information through the use of SDS-PAGE separation (Wilson & Walker, 2000). The resolutions of proteins are influenced by (i) the ratio of acrylamide to bis-acrylamide cross-linker; (ii) the ratio of acrylamide to cross-linker used in the stacking and separation gel; (iii) the pH of the system and (iv) the method used during sample preparation. Proteins ranging from 200 kilo-Daltons (kDa) to about 2 kDa in molecular mass, can be resolved when a system that contains glycine in its buffers is used.

Polyacrylamide separates proteins on the basis of their size or shape, which relates to the molecular mass of the protein. A lattice of cross-linked, linear polyacrylamide

strands is formed by the co-polymerization of monomeric acrylamide and the *bis*-acrylamide cross-linker. The concentration of acrylamide and the acrylamid/*bis*-acrylamide ratio determines the pore size of the separating gel (Wilson & Walker, 2000).

Samples intended for use in the SDS-PAGE system are treated with a buffer containing an anionic detergent sodium dodecyl sulfate (SDS) and β-mercaptoethanol as a reducing agent. The SDS dissociates most multi-chain proteins by binding with the proteins. Most protein chains coated with SDS exhibit the same charge-to-mass ratio. The reducing agent is responsible for the reduction of disulphide bridges holding the tertiary structure of proteins together (Wilson & Walker, 2000). SDS-PAGE proved to be an important tool for species differentiation studies (Thomson-Carter & Pennington, 1989).

The SDS-PAGE technique has been widely applied to verify strain authenticity, rapid identification as well as classification, sample purity determinations, monitoring protein compositions, ecological and epidemiological studies, blotting applications and finally, the establishment of protein sizes (Plikaytis *et al.*, 1986; Schägger & Von Jagon, 1987; Pot *et al.*, 1994; Coenye *et al.*, 2001). Cellular proteins provide valuable information concerning bacterial pathogenesis (Bhaduri & Demchick, 1983). Highly standardized SDS-PAGE of whole-cell proteins allows fast screening of large numbers of strains for comparative purposes. Data in the literature support the overall correlation patterns and DNA : DNA hybridization studies (Vauterin *et al.*, 1990; Pot *et al.*, 1994). SDS-PAGE can only be used on a relatively large number of cells, derived from a pure culture (Pot *et al.*, 1994). The use of SDS-PAGE as a tool in the analysis of whole-cell proteins to confirm taxonomic status and distinguishing between strains and/or subspecies has been proved (Gómez-Zavaglia *et al.*, 1999). Protein profiling of whole-cell proteins have been used to characterise 51 isolates from respiratory secretions of cystic fibrosis patients. Some of these isolates included members of the *Xanthomonas* genus (Coenye *et al.*, 2001). SDS-PAGE profiles were used to group *Xanthomonas campestris* pathovars (Vauterin *et al.*, 1991), *X. campestris* pv. *citri* strains (Vauterin *et al.,* 1991) and to assess the

intrapathovar variability of *Xanthomonas axonopodis* pv. *commiphorae* (Samanta & Mandal, 2014).

1.10.3 Biolog® GN 2 Carbon substrate utilisation

The Biolog® technique is one of the methods that rely on measurements of the utilisation of different carbon substrates by microorganisms (Yu & Yu, 2000; Morgan *et al.*, 2009). A range of Biolog® plates is available to suit different applications. Biolog® assays are based on a redox system. Different carbon sources are found within a 96-well plastic microtiter plate. One well is reserved as a control and does not contain any substrate, while the other 95 wells each contain a different carbon source (Stefanowicz, 2006; Khatri *et al.,* 2013). A few different groups of chemical compounds are distinguishable among these substrates, including carbohydrates, amino acids, carboxylic acids, amines, amides and polymers. A colourless tetrazolium dye is also included in each of the wells. The organism to be studied is cultured and suspended in saline. The saline solution is inoculated into the plate wells and then incubated at a constant temperature. During incubation, the sample microorganisms oxidise the carbon source in the wells and simultaneously reduce the previously colourless tetrazolium dye to a violet formazan. Spectrophotometers are used to measure the colour development in terms of absorbance (optical density). Different groups of microorganisms utilise the carbon sources at different rates and consequently high variability in the rate and intensity of colour development is observable. A characteristic microbial fingerprint is obtained for the specific group or species of bacteria by assesing the colour pattern on the Biolog® plates (Stefanowicz, 2006; Khatri *et al.,* 2013).

Advantages of the Biolog® system include that the characteristic metabolic patterns are generated rapidly, reasonably economically and effectively (Konopka *et al.*, 1998). The Biolog® assay is much less laborious than other available techniques (Soudi *et al.*, 2011). Several phytopathogenic bacteria have been analysed with the Biolog® system (Khoodoo *et al.*, 2004; Nowbuth *et al.*, 2005; Samanta *et al.*, 2013). A metabolic profile for *Pantoea stewartii,* has been created (Wilson & Dillard, 1999). A total of 26% of the available substrates were utilised by all strains of *P. stewartii*

tested (Wilson & Dillard, 1999). Metabolic fingerprints for various *Xanthomonas* strains causing onion blight (Nowbuth et al., 2005) and bacterial blight of Anthurium (Khoodoo *et al.*, 2004) were generated. Recently, the phenotypic characterisation of a novel *X. axonopodis* pathovar was done. This pathogen causes gumming of Guggal (*Commiphorawightii*) (Samanta *et al.*, 2013). Results from these studies is summarised in the table.

Table 1 contains all 95 substrates found in the Biolog GN2 plates. Table 1 indicates the substrates that are utilised by *P. stewartii* (*P. s*), *Xanthomonas* (X) and *X. axonopodis* (*X. a*) as well as substrates that are seldom utilised by these organisms.

Table 1: Substrate utilisation by *P. stewartii* (*P. s), Xanthomonas* strains (*X),* and *X. axonopodis (X.a).*

Substrate	***P.s***	***X***	***X. a***	**Substrate**	***P.s***	***X***	***X. a***
α-Cyclodextrin		○	○	Itaconic Acid	○		○
Dextrin				α-Keto Butyric Acid	○		●
Glycogen			○	α-Keto Glutaric Acid	○		●
Tween 40			●	α-Keto Valeric Acid		○	○
Tween 80	○		●	D,L-Lactic Acid			
N-acetyl-D-galactosamine		○	○	Malonic Acid	○		○
N-acetyl-D-glucosamine	●	●	○	Propionic Acid			
Adonitol	○	○	○	Quinic Acid	○		○
L-Arabinose	●		○	D-Saccharic Acid	○	●	○
D-Arabitol	○		○	Sebacic Acid	○	○	○
D-Cellubiose	○	●	●	Succinic Acid	●	●	●
i-Erythritol		○	○	Bromo Succinic Acid	●		●
D-fructose	●	●	●	Succinamic Acid			●
L-Fucose			●	Glucuronamide			○
D-Galactose	●		●	L-Alaninamide			●
Gentiobiose	○	●	●	D-Alanine			●
α-D-Glucose	●	●		L-Alanine	●		●
m-Inositol	●		○	L-Alanyl-glycine	●	●	●
α-D-Lactose			○	L-Asparagine	●	●	○
Lactulose			○	L-Aspartic Acid		●	○

Maltose			○	L-Glutamic Acid	●	●	●
D-Mannitol				Glycyl-L-Aspartic Acid	●		
D-Mannose	●	●		Glycyl-L-Glutamic Acid	●		●
D-Mellibiose	●		●	L-Histidine	○		○
β-Methyl-Dglucoside			○	Hydroxy-L-Proline	○		○
D-Psicose	●		●	L-Leucine	○		○
D-Raffinose	●			L-Ornithine	○		○
L-Rhamnose	○		○	L-Phenylalanine	○	○	○
D-Sorbitol			○	L-Proline		●	●
Sucrose	●		●	L-Pyroglutamic Acid	○		○
D-Trehalose	●	●	●	D-Serine			○
Turanose	○	○	●	L-Serine	●	●	●
Xylitol	○	○	○	L-Threonine	○		○
Methyl Pyruvate		●	●	D,L-Carnitine	○		○
Mono-Methyl-Succinate	●		●	γ-Amino Butyric Acid	○		○
Acetic Acid			○	Urocanic Acid	○		○
Cis-Aconitic Acid		●		Inosine			○
Citric Acid		●		Uridine			○
Formic Acid			○	Thymidine			○
D-Galactonic Acid	○		○	Phenyethylamine	○		○
D-Galacturonic Acid	○	●	○	Putrescine	○		○
D-Gluconic Acid	●	●	○	2-Aminoethanol	○		○
D-Glucosaminic Acid	○		○	2,3-Butanediol	○	○	○
D-Glucuronic Acid		●	○	Glycerol	●	●	○
α-Hydroxy Butyric Acid			○	D,L-α-Glycerol Phosphate	○		○
β-Hydroxy Butyric Acid	○		○	Glucose-1-Phosphate	●		○
γ-Hydroxy Butyric Acid	○	○	○	Glucose-6-Phosphate	●		○
ρ-Hydroxy Phenylacetic Acid	○	○	○				

*For table 1: ● = substrate is almost always used; ○ =substrate not used; no symbol = variable results

Information obtained from systems such as Biolog® GN2, could be used to develop biological control strategies based on the knowledge of pathogenic nutritional requirements (Nowbuth *et al.*, 2005). Pathogen population sizes and the successive disease incidence and severity have been shown to be considerably reduced as a result of the utilisation of nutrients by non-pathogenic bacteria. The pathogens are reduced through pre-emptive competitive exclusion (Ji & Wilson, 2002). As the characterisation of a plant pathogen aids in understanding the plant-host interactions as well as the factors contributing to disease development, ample information on emerging plant pathogens obtained from molecular, phenotypic and biochemical studies could prove valuable in the management of possibly devastating crop diseases.

2.1 Study area and sampling

The sampled locations included commercial maize production farms from the North West, Free State, Gauteng and Northern Cape provinces of South Africa. These provinces are the primary maize producing areas of the country. Figure 6 is a map of the sampled area where each of the sample points was recorded using a global positioning system (Garmin, USA).

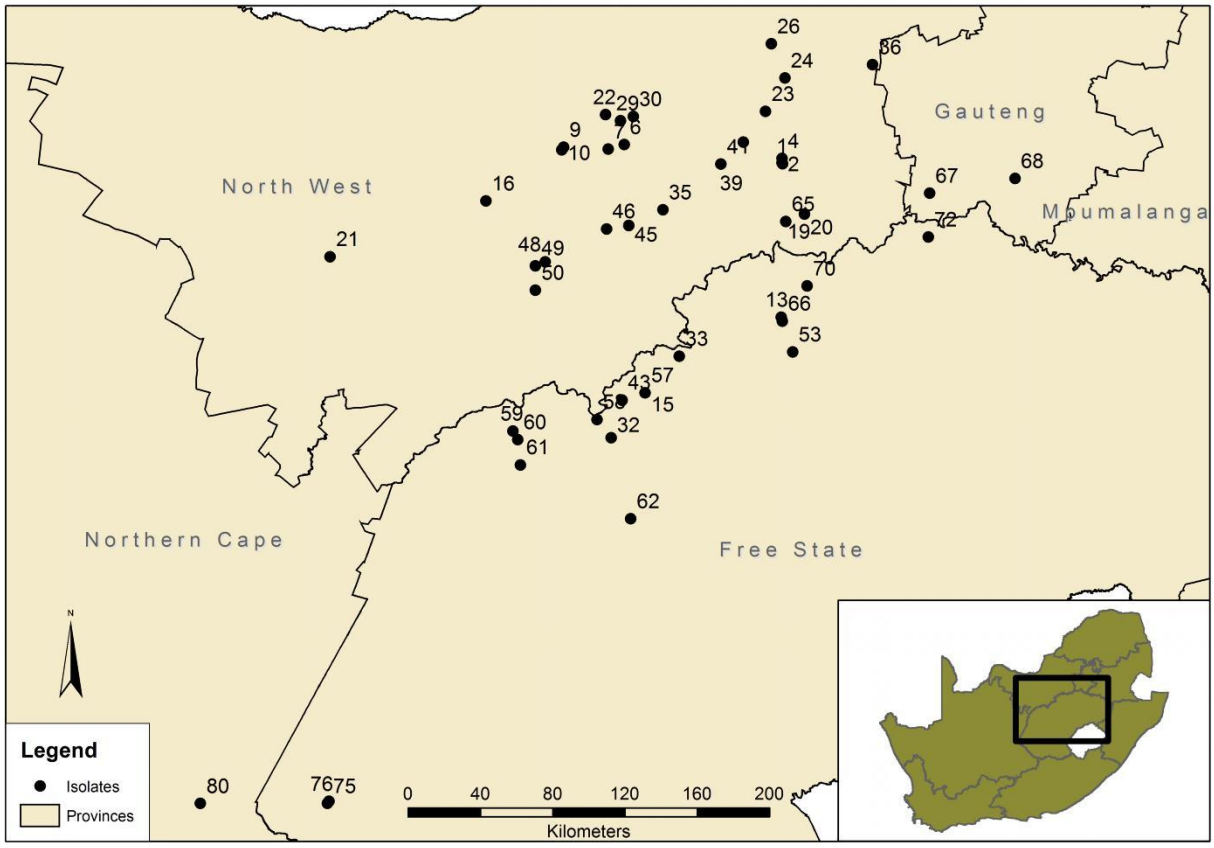

Figure 6: A map of the various localities from which maize leaf samples were obtained.

Leaf samples from 47 farms in total were used. Of these, 26 samples were collected in the North West, 18 in the Free-State, 2 in Gauteng and 1 in the Northern Cape. Samples were only collected from farms having maize plants with BLS symptoms. Maize leaves with yellow-brown lesions were indicative of BLS symptoms. The sampling process included visual observations in maize fields where leaves showing symptoms were aseptically collected. These were placed in a dark container and

transported to the laboratory within 12 hrs. Immediately upon arrival at the laboratory, these leaf samples were prepared for isolation of the pathogen.

2.2 Bacterial isolation

2.2.1 Primary isolation

The initial process of pathogen isolation as performed and prescribed by the ARC-Grain Crops Institute is briefly summarised below. Diseased maize leaves were surface sterilised by soaking in sodium hypochlorite (3 min) and 95% ethanol (5 min). The leaves were then rinsed in distilled water to remove any residue of the disinfectant. After rinsing, the leaves were cut into pieces of approximately 3 x 5 cm and placed in petri dishes containing GYCA (glucose–yeast extract–calcium carbonate agar) (Biolab, South Africa). The plates were incubated at 28°C for 72 hrs in an incubation chamber. After incubation, mucoid yellow colonies were identified, selected and subcultured with the streak plate method. In this case the media was also GYC agar. At least three sub-culturing steps were required before the isolate could be considered purified. Standard Gram-stain method was used to determine the Gram reaction, purity and cell-morphology.

2.2.2 Secondary isolation

A second isolation procedure that was followed included the following: the infected leaves were surface sterilised as described in Section 2.2.1 above. The rest of the isolation procedure was different in the following ways. A number of distinct lesions were removed from the diseased leaf and pulverised with a sterile mortar and pestle. Sterile distilled water (10 ml) was added to the pulverised leaves. This solution was collected in a sterile 10 ml bottle. And this was serially diluted three times using a dilution factor of 10. Dilutents were prepared and spread plated onto GYCA containing petri dishes (Biolab, South Africa). The succeeding steps remained the same as described in section 2.2.1.

2.3 Gram stain

Gram staining was performed as described by the Burke method (Arbeit *et al.*, 1995; Bagheri *et al.*, 2013). Briefly: Bacterial smears were prepared from 24-hr cultures. Smears were heat fixed. Crystal violet (Merck, Germany) was used to cover smears (1 min). Tap water was used to rinse smears. Smears were covered with Gram-iodine (Merck, Germany) (1 min) and destained with acetone alcohol (Merck, Germany) until the runoff was clear. Safranin (Merck, Germany) was used as a counter stain (1 min) and rinsed with tap water. Smears were examined using an Eclipse E200 optical microscope (Nikon, Japan).

2.4 Identification of isolates

All 47 isolates were identified as members of the genus *Xanthomonas* during a related study using 16S rDNA PCR amplification and subsequent sequencing of the amplicons. This was carried out in a parallel study (Niemann, 2014)

2.5 Storage of the isolates

Bacterial cultures were stored in 40% (v/v) glycerol at – 80ºC for long term preservation (Mhedbi-Hajri, 2013). Working cultures were continuously sub-cultured and maintained on GYCA plates, incubated at 28ºC for 2-4 days where after it was stored at 4ºC.

2.6 Koch's postulate analysis

2.6.1 Planting of seedlings

A single maize line was grown from sterilised seed under sterile laboratory conditions. Seedlings were planted in 300 mm pots containing sterile soil. Natural light/dark cycles were used during the growing period. All plants were held in a greenhouse at a temperature range of 17- 35°C and average daily relative humidity of 45.5%.

2.6.2 Inoculation of the seedlings

In order to confirm which of the isolated bacteria were the causal agent of BLS, an experiment was performed to demonstrate Koch's postulates. Two different

inoculation techniques were employed during these experiments. Six randomly chosen representative *Xanthomonas* samples, three *Pantoea* and three *Enterobacter* isolates obtained from maize leaves collected during the survey were used for both methods. *X. axonopodis* pv. *vasculorum* (ATCC 35938) (here after referred to as XA) is closely related to *X. campestris* pv. *zeae*, hence this strain was used as a reference strain throughout this study.

The first technique made use of bacteria from 72 hr-old cultures from GYCA plates. These cultures were suspended in sterile distilled water to prepare the inoculum containing approximately $5x10^7$ cells/ml (Coutinho & Wallis, 1990). Inoculation was performed at the four leaf stage (25 days after planting) by using a needle to puncture the stem into which 1ml of the inoculum was introduced using a syringe (Vidaver, 1977). Six replicates were used for each isolate.

For the second technique, seedlings in the three-to-four leaf stage were wounded by needle puncture, after which 10 ml of an aqueous suspension of $5 x10^8$ cells/ml was sprayed onto the wounded leaf surface. Inoculated plants were maintained at average temperature of 28°C during the day and 23°C during the night in a greenhouse (modified from Wallin *et al.*, 1979).

A total of 18 control plants were used during the study. Six of the plants were injected with 1 ml sterile saline solution into the stem of the plants. Another six plants were wounded by puncture with a sterile needle and the remaining six plants were left without any injury. These last six plants that were uninjured were used to help control the effect of the environmental conditions on the plants.

2.6.2 Scoring of disease severity

Disease severity was rated visually on a weekly basis for 7 weeks after inoculation. An interval scale was used to assess the severity on the leaves of the maize plants (modified from Horsfall & Barratt, 1945; Xie *et al.,* 2012). The visual scale is based on the percentage of the leaf area showing disease symptoms (0 = no visible symptoms, 1 =1-10%, 2 = 11-20%, 3 =21-30%, 4 = 31-40%, 5 = 41-50%, 6 = 51-60%, 7 = 61-70%, 8 = 71-80%, 9 = 81-90%, 10 = 91-100%).

2.7 Biochemical fingerprinting and carbon utilisation

2.7.1 Fingerprinting with the Biolog® GN2 system

The Biolog® GN2 system (Biolog, USA) was used to create metabolic fingerprints based on the carbon utilisation patterns of each of the isolates. The plates were specifically designed to analyse Gram-negative bacterial cultures. Bacteria from 24 hr-old trypticase soy agar (TSA) plate cultures were suspended in sterile saline (0.85% (w/v) NaCl) (Saarchem, South Africa) and compared to the Biolog® GN2 turbidity standard (Biolog, USA). The microplates were inoculated with 125µl of the homogenous bacterial suspension per well. The plates were incubated at 28°C for 24hr (Zhao *et al.*, 2000; Nowbuth *et al.*, 2005). The absorbance of each well was read at 590nm using a PowerWave X (BIO-TEK, USA) microplate scanning spectrophotometer. Absorbance was determined at three different intervals during incubation (4hr, 16hr and 24hr). All isolates were tested in triplicates.

2.7.2 Statistical analysis of Biolog data

Parametric and nonparametric statistical analyses were performed on all the carbon utilisation data by using Statistica 11.0 (StatSoft, Inc., Tulsa, Oklahoma, USA, 2012). A one-way ANOVA breakdown was performed on parametric data (distributed normally). Tukey's Honest Significant Difference (HSD) test was used to determine statistically significant differences between the various carbon sources ($p<0.05$). Multivariate ordination analysis specifically Principal Component Analysis (PCA) using CANOCO (Canoco for Windows Version 4.5, Biometris- Plant Research International, Wageningen, The Netherlands) were conducted on the data to identify the correlations between the various carbon sources and the different isolates. The standardised patterns were obtained by subtracting the absorbance of the control (water) well from the substrate wells (Garland & Mills, 1991). An average well colour development (AWCD) value of 0.35 absorbance units (AU) was used as reference point for the multivariate analysis (Péchy-Tarr *et al.*, 2005). Principal Component Analysis was performed on the Biolog® data to investigate the comparison of the various isolates by means of the carbon utilisation patterns of the microbial samples.

2.8 SDS-PAGE of whole-cell proteins

2.8.1 Protein extractions

Preparation and electrophoresis of whole-cell protein extracts were performed by the SDS-PAGE method of Laemmli (1970) as modified by Bhaduri & Demchick (1983). Cultures were grown in 10 ml trypticase soy broth (Merck, Germany) for 24 h at 28°C. Thereafter, 2 ml of the overnight cultures were pipetted into sterile 2 ml microfuge tubes and centrifuged at 8030 *xg* for 5 minutes using an M-240R centrifuge (Boeco, Germany). The supernatant was discarded and the steps were repeated four times with the remaining culture. The resulting pellet was washed and centrifuged at 8030 *xg* for 5 minutes using an M-240R centrifuge (Boeco, Germany). Phosphate-buffered saline (0.1 mM K_2HPO_4, 0.1 mM KH_2PO_4, 0.85% (w/v) NaCl; pH 7.4) was used for washing. As the bacterial cells did not adhere to the vessels, the phosphate-buffered saline (PBS) did not contain Mg^{2+} or Ca^{2+}. The supernatant was discarded and the pellet was suspended in 1 ml ice-cold acetone (Merck, South Africa) and allowed to stand on ice for 5 minutes (Mahlatsi, 2012). The cells were collected by centrifugation (8030 *xg* for 5 min) and the supernatant was discarded. Microfuge tubes were left on bench tops at room temperature to allow remaining acetone to evaporate. The pellet was re-suspended in extraction buffer (0.125 mMTris, pH 6.8, 4% (w/v) sodium dodecyl sulphate, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol) with 2 µl protease inhibitor cocktail1 (Melford, UK) to extract proteins from the pellet. The suspension was incubated at 100ºC (10 min). Small quantities of glass beads were added to each sample. The samples were vortexed for 2 minutes and centrifuged (8030 *xg*, 90 sec) and the supernatant was transferred to a sterile microfuge tube.

2.8.2 Gel based separation of proteins by SDS-PAGE

Protein samples (18 µg/µl) were mixed with a loading buffer (0.125 mMTris, pH 6.8, 4% (w/v) sodium dodecyl sulphate, 20% (v/v) glycerol, 10 % (v/v) β-mercaptoethanol, 0.002% (w/v) bromophenol blue). Samples were incubated at 100ºC (10 min) and afterwards immediately transferred to ice and loaded into gels consisting of a 12% (w/v) acrylamide resolving gel and a 6% (w/v) acrylamide stacking gel. The pH of the resolving gel was 8.8, while the pH of the stacking gel

was 6.8. Precision Plus Protein Western C standards (Bio-rad, USA) ranging from 10 to 250 kDA was used, of which 5 µl was loaded onto each gel. The electrophoresis buffer was a Tris-Glycine buffer (0.25 M Tris base, 1.92M glycine, 1% (w/v) SDS). Electrophoresis was done for 2 hrs at 110V using a Mini-PROTEAN 3 cell and PowerPac Universal (Bio-Rad, USA). The gel was stained with 0.13% (w/v) Coomassie Brilliant blue R-250 (Saarchem, SA) in 50% (v/v) methanol, 10% (v/v) glacial acetic acid and 40% ulta-pure water (ddH_2O). Destaining of the gel was done overnight in 50% (v/v) methanol, 10% (v/v) glacial acetic acid and 50% ultra-pure water (ddH_2O). The gel images were captured using a Chemidoc MP imaging system (Bio-Rad, USA) and Image Lab 4.0.1 (2011) software (Bio-Rad, USA).

2.8.3 Analysis of SDS-PAGE gels

Images of the SDS-PAGE gels were analysed using Phoretix 1D software-trial version (Nonlinear Dynamics, Durham, USA). Lanes and bands were selected manually. Inter- and intragel distortion was reduced through band alignment. Band similarities were quantified as a Dice similarity coefficient (Dice, 1945). The lanes were clustered based on banding patterns using an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) to construct a Dice coefficient distance tree (Kaakoush *et al.*, 2011).

3.1 Introduction

In this study the characteristics of *Xanthomonas* isolates associated with BLS of maize were investigated. Various tests were conducted on all the isolates obtained. The isolates were all Gram negative and were subjected to 16S rRNA gene sequencing analysis (Niemann, 2014). These included various species from the genera *Xanthomonas, Pantoea and Enterobacter*. The *Xanthomonas* isolates were in the minority and the others in the majority when the ARC isolation technique (Section 2.2.1) was used. The second isolation procedure used as described in Section 2.2.2 generated mostly *Xanthomonas* spp. To determine which of the isolates were responsible for the characteristic symptoms of BLS observed in maize fields, representatives of these isolates were subjected to Koch's postulate analysis.

3.2 Testing Koch's postulates

In this part of the study *Xanthomonas, Pantoea* and *Enterobacter* members obtained during field work were used for these tests. Six of the isolates obtained during fieldwork that were positively identified as *Xanthomonas* members, three *Pantoea* and three *Enterobacter* members were used to inoculate maize seedlings. *Xanthomonas axonopodis* pv. *vasculorum* (ATCC 35938) was used as a reference strain. Two different methods were employed as described in Section 2.6.1. which included the stab and the spray methods. Weekly observations on disease development were made and results were recorded and presented in table 2, table 3 and figure 7.

Table 2: Disease severity ratings observed over seven weeks on plants inoculated by the spray method

Week	X1	X2	X3	X4	X5	X6	P1	P2	P3	E1	E2	E3	C1	C2	C3
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0
3	1	2	1	1	1	1	0	0	0	0	0	0	0	0	0
4	2	2	1	1	2	1	0	0	0	0	0	0	0	0	0
5	2	2	2	1	2	2	0	0	0	0	0	0	0	0	0
6	2	3	2	2	3	2	0	0	0	0	0	0	0	0	0
7	2	3	2	2	3	3	0	0	0	0	0	0	0	0	0

*X= *Xanthomonas* samples, P= *Pantoea* samples, E= *Enterobacter* samples, C= Control. (Six replicates of each were used. For simplicity only 3 replicates are presented in the table.)

Table 3: Ratings of disease severity observed on plants inoculated by the stab method.

Week	X1	X2	X3	X4	X5	X6	P1	P2	P3	E1	E2	E3	C1	C2	C3
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0
5	1	2	1	1	1	1	0	0	0	0	0	0	0	0	0
6	1	2	1	2	2	1	0	0	0	0	0	0	0	0	0
7	1	2	1	2	2	1	0	0	0	0	0	0	0	0	0

***X= *Xanthomonas* samples, P= *Pantoea* samples, E= *Enterobacter* samples, C= Control.** (Six replicates of each were used. For simplicity only 3 replicates are presented in the table.)

Samples representing three genera were selected randomly. Ratings were made as a percentage of the leaves covered in symptoms and were then translated into an interval scale from 0 to 10 (modified from Horsfall & Barratt, 1945; Xie *et al.*, 2012).

None of plants inoculated with the *Pantoea*, *Enterobacter* or the *Xanthomonas axonopodis* reference strain developed any symptoms during the 7 week period and as such results were omitted from the figure.

All of the plants inoculated with the other *Xanthomonas* members developed the characteristic symptoms of BLS. The first symptoms started to develop after week 1 for the plants that were inoculated by the stab-and-spray method. For the stab-and-inject method the symptoms only developed after week 3.

The line graph in figure 7 compares the rate of symptom development between the two different inoculation methods. From figure 7, it is evident that plants that were subjected to the stab-and-spray method (as described in the first method in section 2.6.1) started to develop symptoms earlier and more severely than plants subjected to the stab-and-inject method (second method in section 2.6.1). Although the plants inoculated with the spray method, exhibited symptoms earlier, the rate of symptom development for the two methods (after initial symptoms developed) were similar as reflected by the slope (gradient) of the lines. The disease severity of the plants inoculated with the stab method also reached a plateau between week 6 and 7, as

no increase in symptoms severity was observed after week 6. In contrast to the stab method, the disease severity of the stab-and spray method did not reach a plateau and increased between week 6 and week 7.

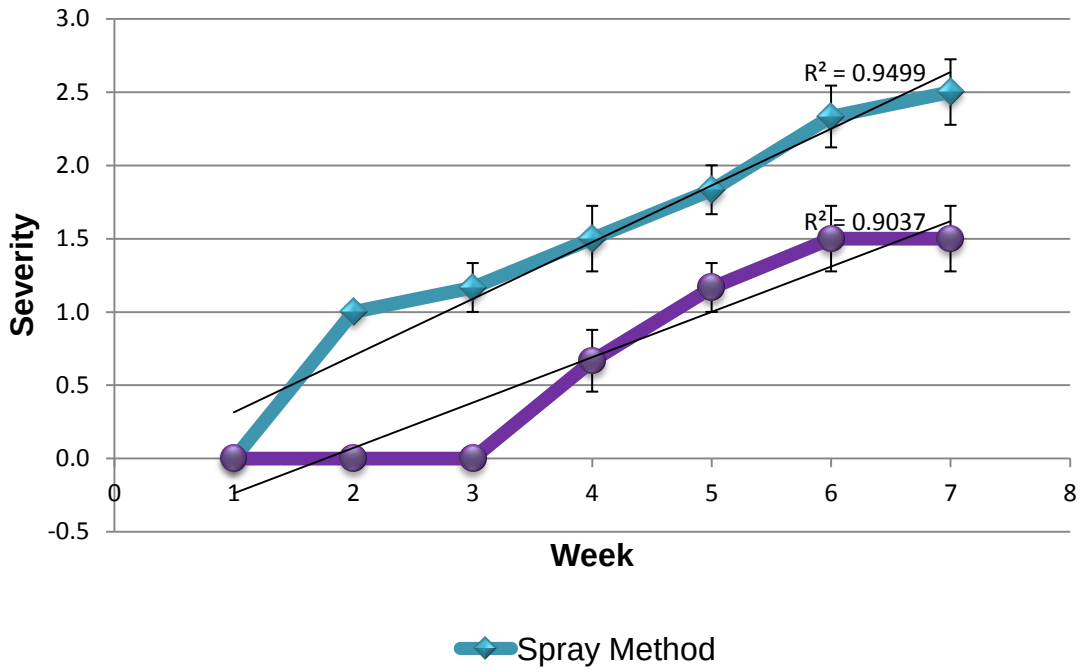

Figure 7: Line graph representing disease development of maize plants over a period of 7 weeks. Severity plant disease was assessed on an interval scale. Data from table 2 and 3 was used during the construction of the line graph. Data was transformed by creating homogenous groups where after the average for each week were used.

In this study, the stab-and-spray method was effective because symptoms were visible 1 week after inoculation and disease severity increased almost every week until the last visual assessment during the seventh week after inoculation. The spray method is more analogous to the natural mode of infection used by many *Xanthomonas* members as these pathogens may normally enter the plants through leaf openings such as stomata and hydathodes (Kloppers & Tweer, 2009). *Xanthomonas campestris* pv. *zeae* has been observed to cause 40% death of leaf tissue in some cases (Nowell, unpublished, cited by Qhobela *et al.*, 1990). In this study, leaf damaged reached a maximum of about 30%.

Figure 8: Photographs showing symptom development as a result of spray inoculation with *Xanthomonas campestris* pv. *zeae* during pathogenicity testing . Where (a) was taken after the initial onset of symptoms (2 weeks post-inoculation), (b) 4 weeks after inoculation, (c) 7 weeks post-inoculation and (d) control plant inoculated with saline 7 weeks after inoculation.

Couthino & Wallis (1990) used *Xanthomonas campestris* pv. nov isolates to inoculate maize lines in an attempt to establish the pathogenicity of the isolates. After 5 days water-soaked lesions appeared on the leaf surface and only after 14 days did typical yellow-brown lesions of BLS appear on the leaves. *Xanthomonas campestris* pv. *holicola* was included as a control in that study but produced minimal symptoms on the maize plants. The pathogenicity of *X. campestris* pv. nov was confirmed by Coutinho & Wallis (1990) and this new pathovar was proposed to be *X. campestris* pv. *zeae* due to host specificity of this pathovar to maize.

As none of the plants inoculated with *Pantoea*, *Enterobacter* and *Xanthomonas axonopodis* developed any symptoms of BLS these microorganisms did not conform to the requirements of Koch's postulates and were then recognised as endophytes. Microorganisms that are present after surface sterilisation of a plant (Hallman *et al.*, 1997) and which exist in the internal portion of the host plant without causing harmful reactions or diseases, while forming a lifelong association with the host plant, are recognised as endophytic bacteria (Bacon *et al.*, 2001).

The *Pantoea* and *Enterobacter* isolated during this study complied with the definition of endophytic bacteria. Endophytic bacteria are not limited to monocotyledonous plants and have also been isolated from dicotyledonous plants such as oak trees (Brooks *et al*., 1994) and crop plants, such as maize (Fischer *et al*., 1992; Gutierrez-Zamora & Martinez-Romero, 2001). Endophytes can reside within cells (Jacobs *et al.*, 1985), in the intercellular space (Patriquin *et al.*, 1978), or in the vascular system (Bell, 1995) of a plant. Some endophytes produce plant growth-promoting substances and fix nitrogen from the atmosphere (Sturz et al. 2000).The roots, leaves, stems, and fruits of different plants have been used to isolate endophytic bacteria over a number of years (Hallman *et al.*, 1997; Reinhold-Hurek & Hurek 1998; Lodewyckx *et al.*, 2002). Although several endophytic bacteria have been isolated from the seeds of commercially important crops and woody plants (Van Dijk & Nelson, 1998; Bacilio-Jimenez *et al.*, 2001: Seghers *et al.*, 2004) plant seeds as a source of endophytic bacteria is still under dispute (Hallman *et al.*, 1997; Cankar *et al.*, 2005). Genera isolated from seeds include *Bacillus*, *Corynebacterium*, *Enterobacter*, *Acinetobacter*, *Pseudomonas*, and *Rahnella* (Van Dijk & Nelson, 1998; Bacilio-Jimenez *et al.*, 2001; Seghers *et al.*, 2004).

Asis and Adachi (2003) isolated endophytic *Pantoea agglomerans* and *Enterobacter asburiae* from plants. *Enterobacter* has previously also been isolated from maize and has been acknowledged as endophytic (McInroy & Kloepper, 1995; Fischer *et al.*, 1992). Rijavec *et al.* (2007), who studied maize endophytes and found that maize kernels were accompanied by many previously recognised endophytes. Most of those endophytes have never been associated with seed tissue of maize. Among these endophytic genera newly associated with seed tissue, were members of *Pantoea*. The application of Koch's postulates confirmed that the *Xanthomonas campestris* pv. *zeae* isolates were obtained during fieldwork. The suspected pathogen was successfully isolated from diseased plant material, grown in pure culture, placed in a healthy host, caused the expected disease symptoms and the same pathogen was again isolated from the now diseased host. The molecular identification of the isolates was completed in a related study (Niemann, 2014). *Xanthomonas campestris* pv. *zeae* was previously confirmed as the pathogen responsible for BLS.

3.3 Protein profiling

In this study protein extractions were performed on 47 *Xanthomonas* isolates obtained during fieldwork and one reference culture *Xanthomonas campestris* pv. *axonopodis*. Protein extractions were conducted by the following two methods. Initially, bacterial plate cultures containing sufficient growth were scraped and bacteria were harvested. To remove any residue from the growth media, the samples were washed three times with ultrapure water and three times with PBS by repeatedly centrifuging and resuspending the culture at 8030 *xg* in 1ml PBS or ultrapure water. The concurrent steps of the extraction process remained the same as described in section 2.8.1. The concentration of the protein samples was adjusted to 18 µg/µl as the best bands were obtained using these concentrations. These samples were loaded onto a SDS-PAGE gel as described in section 2.8.2 and were subjected to electrophoresis. After staining of the gels, very light bands with a large amount of smearing were observed. The analysis of the gel was complicated by the faint bands and smearing. The protein extraction method was subsequently altered in an attempt to obtain clearer bands for analysis. The altered method as described in section 2.8.1 made used of overnight broth cultures. In figure 9 a Coomasie-Blue SDS-PAGE gel is shown. Proteins extracted with the altered method were analysed in this gel. The gel contains 17 *X. campestris* pv. *zeae* isolates and a molecular weight marker was loaded in the first lane on the left.

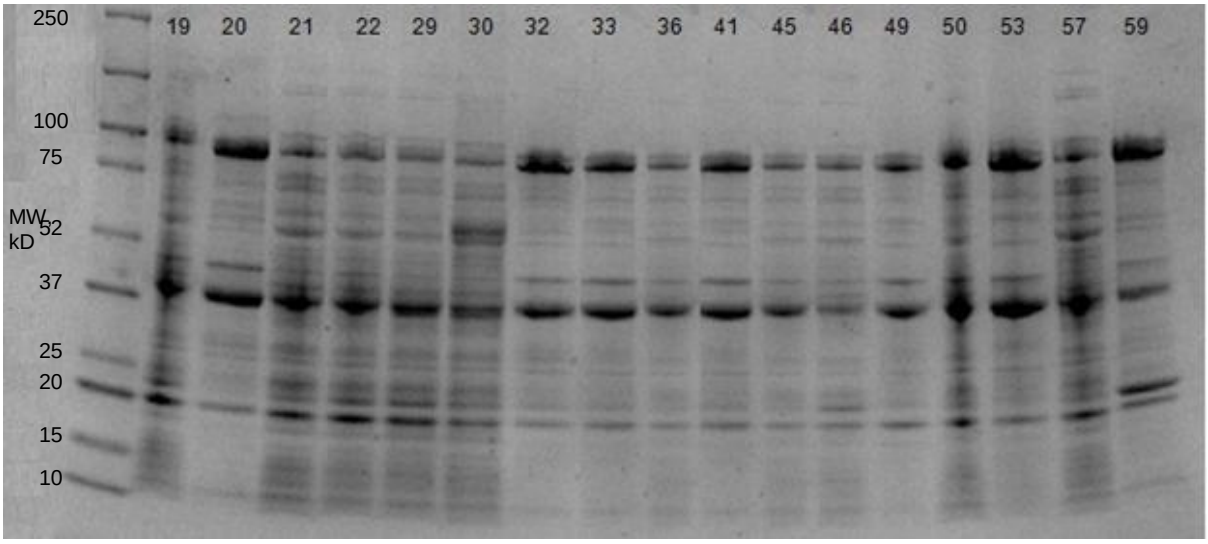

Figure 9: A representative SDS-PAGE gel image showing the protein profiles of selected isolates. A molecular weight marker ranging from 10 to 250 kilodalton (kD) was used.

The overall protein profile of the isolates appeared to be highly similar. On closer inspection, differences in some of the banding patterns were observed. The sizes of the bands ranged from 10 to 150 kD. From figure 9 it appears that three distinct groups were found. Isolates 19, 21, 22, 29, 53 and 57 group together while isolates 30 and 59 grouped together. Isolates 20, 32, 33, 36, 41, 45, 46 and 49 formed a separate group. Major bands on the gel are easily visible, while minor bands become visible when the contrast of the gel image is increased and the image is enlarged. The banding patterns observed in the photographs of the SDS-PAGE gels were analysed to construct a dendrogram based on the isolates' protein profiles.

Three clades were observed within the Dice coefficient distance tree. The dendrogram can be described as simplicifolious as in figure 10, *Xanthomonas axonopodis* (XA) formed a single leafed clade when only the major bands were selected during analysis of the banding patterns. When considering the presence of protein bands, the banding pattern of the reference culture was 77% similar to the isolates obtained during the study.

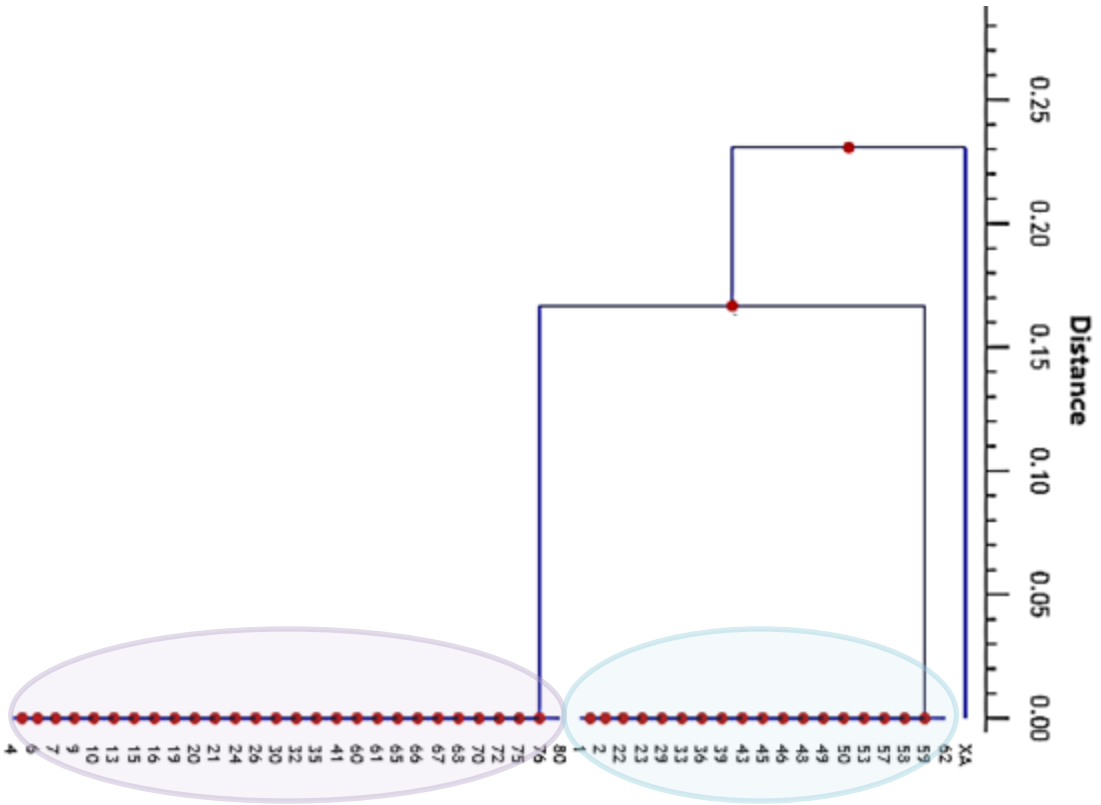

Figure 10: Dendrogram obtained from SDS-PAGE profiles using the UPGMA algorithm and Dice coefficient of similarity. Only major bands were selected.

During this banding pattern analysis slight differences were observed between the isolated *Xanthomonas* cultures. Although the cophenetic correlation is indicated as 100, the similarity matrix of the dendrogram showed that the banding patterns of the two clusters of isolates (represented on the dendrogram in the purple and blue ovals respectively) were 83% similar. All the test isolates fall within these 2 clusters. The clustering of isolates observed in figure 10 is supported by the patterns observed in figure 9. The reference strain was the least similar to the other two clusters with a co-efficient of 77%.

In an effort to distinguish the isolates from one another, the same gel images were analysed taking all major and minor visible bands into account. Nine distinct clusters were observed among the isolates and reference culture as in figure 11. Different colours on the dendrogram represent each cluster observed. The lowest similarity coefficient to distinguish between clusters was 33%.

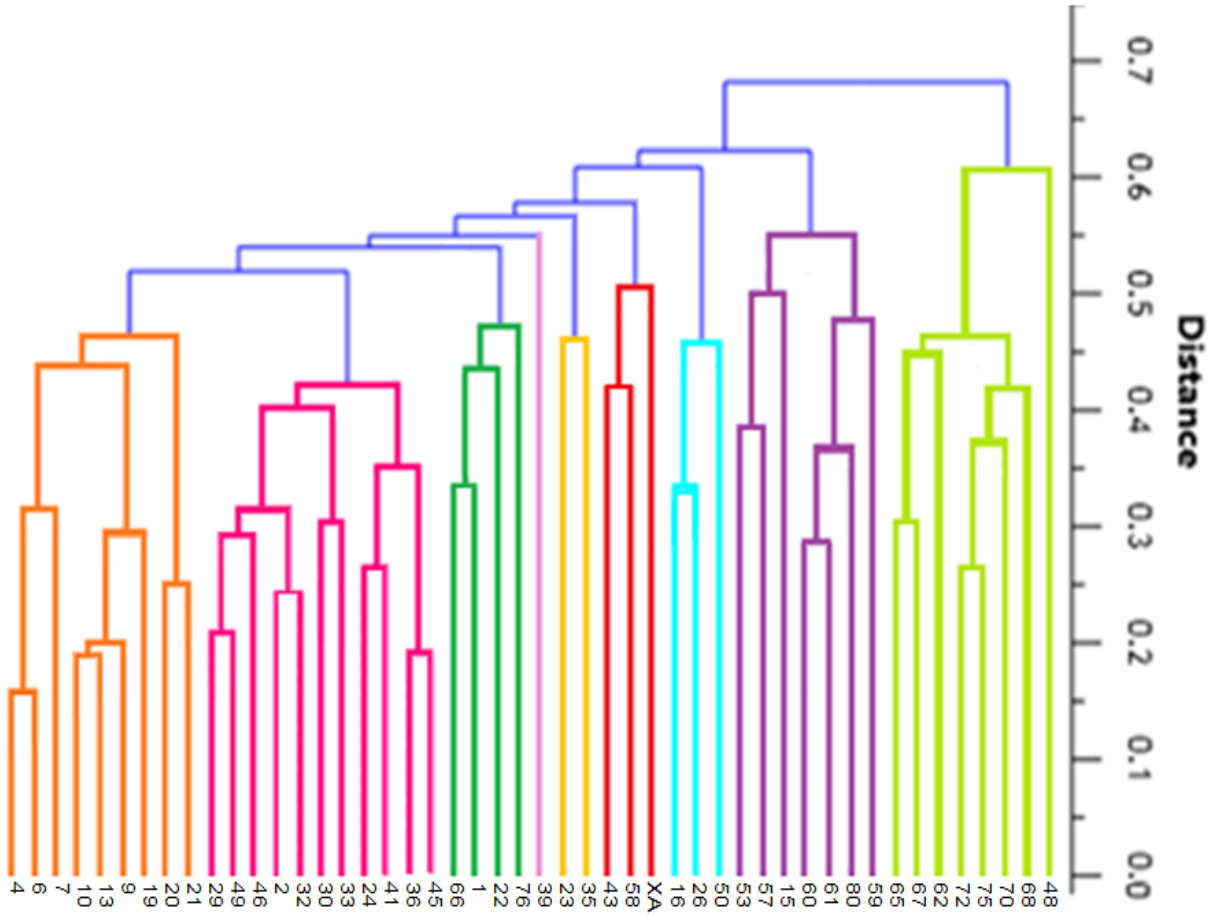

Figure 11: Dice coefficient UPGMA distance tree obtained when major and minor bands were considered during gel image analysis. (Cluster A= Orange, Cluster B= Pink, Cluster C= Dark green, Cluster D= Light Pink, Cluster E=Yellow, Cluster F= Red, Cluster G= Turquiose, Cluster H= Purple, Cluster I=light Green)

In figure 11 the reference culture (XA) had a strong correlation with isolates 58 and 43. Isolates 48, 68, 70, 75, 72, 62, 67, and 65 (cluster I) had a similarity correlation coefficient of only 33% with the remaining clusters and are thus observed as a separate cluster. In figure 11, isolates 19, 20 and 21 assemble together within cluster A; the protein profiles of these isolates are highly related even when faint bands are taken into account. All three of these isolates group together in figure 10 as well. The protein profiles of these three isolates remain highly similar irrespective of which bands (major or minor) are selected. Although isolate 22 is one of only four isolates found within the cluster C, when major bands are selected this isolate is closely related to isolates 33, 57, 45 and 53 as these isolates are also clustered into one cluster in figure 10.

Vauterin *et al.* (1991) used SDS-Page of whole cell proteins to group *Xanthomonas* pathovars. During their study, numerical analysis was performed on the banding patterns of 307 *Xanthomonas* strains which included a total of 27 *X. campestris* pathovars. In some cases, the groupings found in the study were supported by the available pathovar information; however, this was not true for all cases. In some cases the banding patterns of pathovars constituted a homogenous group but one culture (in some cases the reference culture) gave atypical results. Although many *X. campestris* pathovars were included in their study, *X. campestris* pv. *zeae* was not included. However, at least six of their *X. campestris* pathovars were found to display two different electrophoretic types. This indicates that these pathovars were heterogeneous.

Both the current study and the study conducted by Vauterin *et al.* (1991) found fairly high similarity values between different *Xanthomonas* species. It seems that xanthomonads display highly similar SDS-PAGE protein profiles. Only two species, *X. albilineans* and *X. maltophili,* produced highly distinct profiles.

In the current study, when only the major bands were taken into account, the *X. campestris* pv. *zeae* were heterogeneous and displayed two electrophoretic types. There is a high correlation among the *X. campestris* pv. *zeae* isolates but differences in protein profiles do occur. The *X. campestris* pv. *zeae* isolates were found to be related to the reference culture with a high similarity, but it was possible to distinguish between *X. campestris* pv. *zeae* and *X. axonopodis* pv. *vasculorum.*

3.4 Metabolic fingerprinting

In this study the characterisation of the *Xanthomonas* isolates based on metabolic activity and the subsequent reduction of carbon sources was carried out by employing Biolog® GN2 plates.

3.4.1 Average well colour development

The AWCD of all the utilised carbon substrates available in the plates was estimated as an average optical density value across all wells, for each isolate after 16 hrs of

incubation (Bernard *et al.*, 2012). The reference point of 0.35 AU was reached by the majority of the isolates after 16 hrs of incubation.

The AWCD reflects the metabolic activity of each isolate. From figure 12 and table 4 it is evident that isolate 29 utilised greater quantities of the available substrates in the GN2 plates while showing higher metabolic activity during the incubation time as indicated by the AWCD. Isolate 29 achieved an AWCD of 1.850 AU. Isolates 1, 2, 7, 19, 35, 39, 62, 66, 68, 70 and 80 all had an AWCD that exceeded 1.000 but was less than 1.850 AU while isolates 20, 30, 33, 48, 59, and the reference strain *Xanthomonas axonopodis* were the least metabolically active with AWCD values less than 0.200. This indicated that isolates 1, 2, 7, 19, 35, 39, 62, 66, 68, 70 and 80 were metabolically more active than the rest of the isolates excluding isolate 29. More than 58% of the isolates had AWCD ranging between 0.200 and 1.000, these isolates were 4, 6, 9, 10, 13, 15, 16, 21, 22, 23, 24, 26, 32, 36, 41, 44, 49, 50, 53, 57, 58, 60, 61, 65, 67, 72, 75 and 76. Although it was attempted to inoculate each well with the same amount of bacterial cells, differences do occur. These dissimilarities in cell density could result in variations in the AWCD. Isolates such as 20, 30, 33, 48, 59, and *Xanthomonas axonopodis* pv. *vasculorum* that displayed a very low AWCD could have been affected by a number of factors. Statistically significant differences (table 4) have been found (represented as alphabetical letters) and resulted in 21 subgroups within the tested isolates. The largest sub-group is ^{efghi} (12.5%) containing 6 isolates that are similar to each other and statistically distinguishable from the rest of the isolates. Seven of the subgroups within the table each contain 4.72% of the isolates. These seven subgroups all contain two isolates such as isolates 29 and 53 in subgroup ^{efghi} that are similar to each other but which differ significantly from all the other isolates. On the basis of these subgroupings it can be observed that the majority of the isolates are similar but differences are seen and allow the samples to be distinguished from one another. Although some similarities do occur, based on the observed AWCD, a total of 10 isolates were significantly different from all the other isolates including each other based on AWCD. These isolates are each separated into their own group namely; 1, 7, 10, 41, 46, 50, 57, 62, 72, 76. Two of the remaining subgroups each contain 3 isolates (^{abc} and ^{ijkl}) while three other

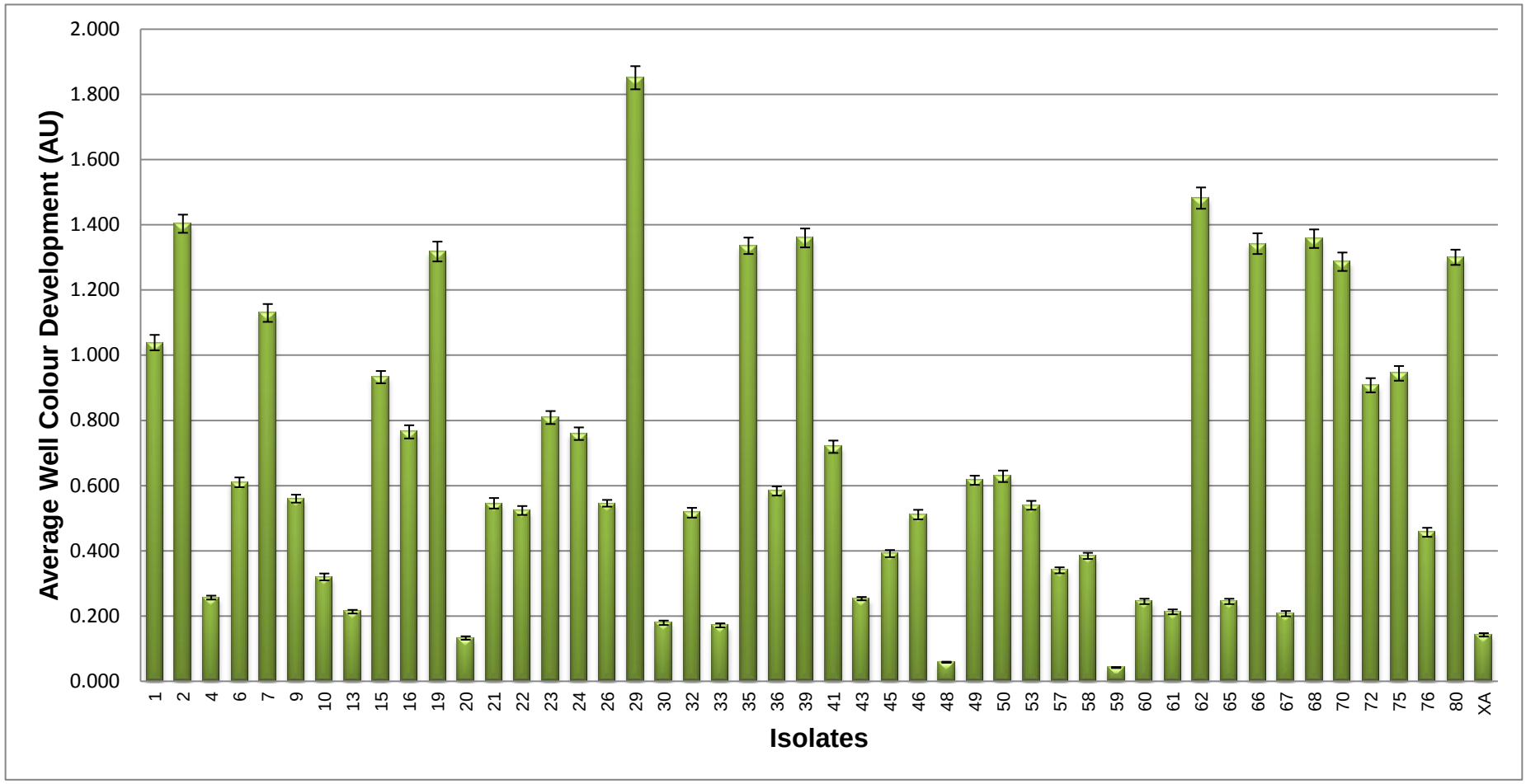

Figure 12: Average well colour development calculated on carbon source utilisation in the Biolog® GN2 plates for each sample after 16 hrs of incubation. Standard errors are indicated by the vertical bars.

subgroups each contain 4 isolates ([abcde, nop, op]). Groupings within the pathovar are observed. These groupings are not reliant on the type of substrates utilised but on the average metabolic capability of the bacteria.

Table 4: Average well colour development calculated on carbon substrate utilisation in the Biolog® GN2 plates for each isolate after 16 hrs of incubation.

Isolate	AWCD, SEM, Ssig	Isolate	AWCD, SEM, Ssig
1	1.038 ± 0.024 [lmn]	**70**	1.286 ± 0.028 [nop]
2	1.403 ± 0.028 [op]	**20**	0.132 ± 0.005 [ab]
39	1.359 ± 0.029 [op]	**XA**	0.0142 ± 0.005 [ab]
66	1.342 ± 0.032 [op]	**21**	0.546 ± 0.016 [efghi]
68	1.357 ± 0.028 [op]	**22**	0.523 ± 0.014 [efghi]
4	0.256 ± 0.006 [abcde]	**26**	0.546 ± 0.101 [efghi]
60	0.245 ± 0.008 [abcde]	**29**	1.850 ± 0.035 [efghi]
65	0.245 ± 0.008 [abcde]	**32**	0.517 ± 0.015 [efghi]
43	0.253 ± 0.005 [abcde]	**53**	0.539 ± 0.014 [efghi]
6	0.610 ± 0.015 [fghij]	**30**	0.179 ± 0.007 [abc]
49	0.616 ± 0.014 [fghij]	**33**	0.171 ± 0.006 [abc]
7	1.129 ± 0.027 [mno]	**67**	0.207 ± 0.008 [abc]
9	0.560 ± 0.012 [fghi]	**36**	0.583 ± 0.014 [ghi]
10	0.320 ± 0.010 [abcdef]	**41**	0.719 ± 0.019 [hijk]
13	0.213 ± 0.006 [abcd]	**45**	0.391 ± 0.011 [bcdefg]
61	0.212 ± 0.008 [abcd]	**58**	0.384 ± 0.009 [bcdefg]
15	0.932 ± 0.019 [klm]	**46**	0.511 ± 0.015 [defghi]
75	0.944 ± 0.023 [klm]	**48**	0.058 ± 0.002 [a]
16	0.765 ± 0.020 [ijkl]	**59**	0.042 ± 0.002 [a]
23	0.808 ± 0.020 [ijkl]	**57**	0.340 ± 0.009 [abcdefg]
24	0.759 ± 0.019 [ijkl]	**50**	0.628 ± 0.018 [ghij]
19	1.317 ± 0.030 [nop]	**62**	1.481 ± 0.033 [p]
35	1.335 ± 0.025 [nop]	**72**	0.907 ± 0.022 [jklm]
80	1.300 ± 0.023 [nop]	**76**	0.457 ± 0.014 [cdefgh]

*All values ± SEM (standard error of mean) (n=288). Statistically significant differences (Ssig) are indicated by alphabetic letters ($p < 0.05$) based on Tukey's HSD test. A different sequence of letters indicates statistically significant differences among isolates ($p<0.05$). The same letter sequence indicates no significant differences.

As the substrates within the wells are not always found in the natural environment of the bacteria and the carbon sources are present in very high concentrations within the Biolog® plates (Campbell *et al.*, 1997) the AWCD could thus be affected. Not all bacteria are capable of reducing the tetrazolium dye used as the indicator of substrate utilisation (Winding, 1994). The tri-phenyl-tetrazolium chloride (TTC) indicator dye is toxic to certain bacteria (Winding, 1994; Yao *et al.*, 2000) and the pH of the medium is buffered at 6.5 (Yao *et al.,* 2000).

Tosiah (2013) used Biolog® FF plates designed for fungi to study 10 isolates of *Exserohilum monoceras*. These 10 isolates were of varying virulence. When the AWCD were compared it was found that 3 isolates were significantly different from the remaining 7 isolates. There were no statistically significant differences among the aforementioned 3 isolates. The isolate deemed the most virulent, did not show significant difference when compared to the other isolates with lower virulence. Variations among *E. monoceras* isolates were illustrated by the differences in the substrate utilisation patterns generated as a result of their ability to integrate the carbon source. Verniere *et al.* (1992) also found differences among *Xanthomonas campestris* pv. *citri* isolates when employing the Biolog® system to create metabolic fingerprints for these isolates. These differences were, however, not dependant on location of the collected isolates or the year in which the isolates were collected. Tosiah (2013) concluded that the virulence was not influenced by the number of carbon sources assimilated or the ability to assimilate carbon sources. Differences observed in the present study could thus also not be ascribed to geographical or virulence characteristics.

3.4.2 Principal component analysis of Biolog® data

Principal component analysis (PCA) is used when a large number of variables are present in a data set. This data analysis technique receives observed variables as inputs and produces composite variables known as principal components. By yielding composite variables, the dimensionality of a large data set is reduced without a major loss of information. Principal component analysis can be used to

determine which of the available variables play the larger role in the total sample variance (Joliffe, 2002).

Figure 13 is a PCA biplot of all the isolates and the *X. axonopodis* (XA) reference culture (represented by the points) and the substrate groups as vectors (represented by the arrows). The vectors are linearly independent. The colours of the samples are related to the colour of the clusters on the dendrogram in figure 11.

The majority of the isolates cluster around the centre of the axis in figure 13 especially in the negative quadrants of the PCA biplot, while most of the vectors are directed towards the positive quadrant. The relevant position of the isolates and the vectors show a negative correlation between the isolates and most of the substrate groups namely, alcohols and polyols (*Alcohol*), nucleosomes (*Nucleo*), phosphorylated compounds (*Phospho*), carboxylic acids (*carbox_a*), organic acids (*OA*), amino acids (*Amino*), esters (*Esters*) and ketones (*Keto*). In figure 13, the majority of the isolates correlated strongly with the substrates found in the carbohydrate and amine groups of the carbon sources. This can be seen through the grouping of the specific isolates around the vector annotated as *"Carboh*" and "*Amines*". The relationship between the isolates is illustrated by the proximity of the isolates to one another. When isolates display similar assimilation of carbon sources at similar rates, the isolates lie close to one another as isolates 4, 41, 46, 21, 76 and 50 do in figure 13.

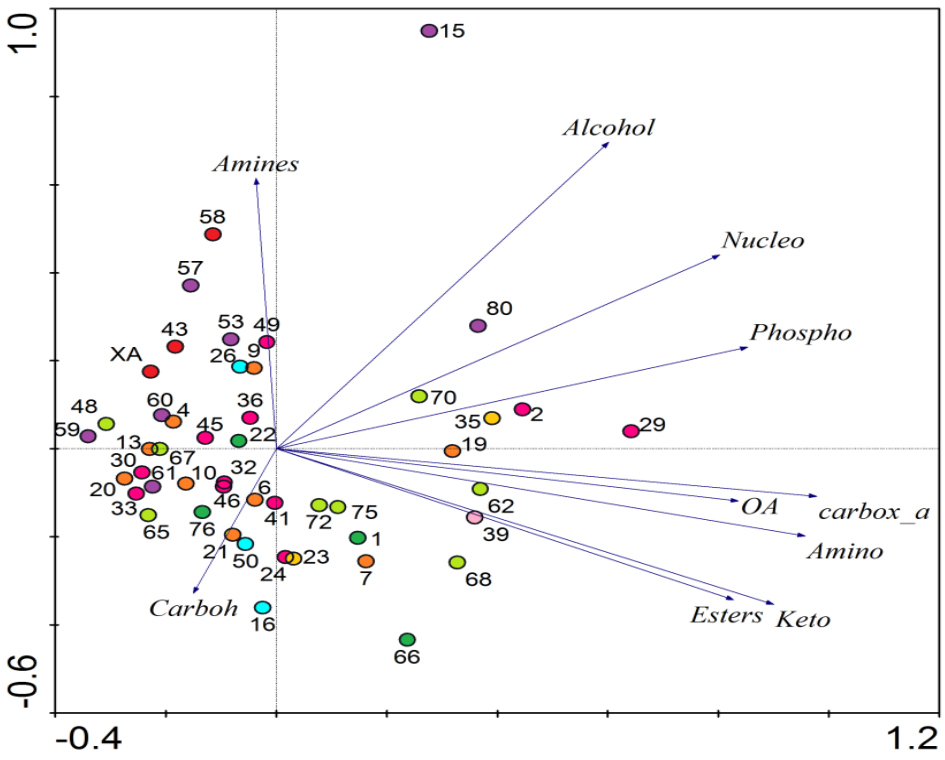

Figure 13: Principal component analysis (PCA) ordination diagram of all 48 isolates based on the average utilisation of all carbon sources divided into classes (Eigen values: PCA1 - 0.582, PCA2 - 0.153).

Isolate 29 is located in the positive quadrant of the PCA. As previously seen with the AWCD - isolate 29 correlates well with most of the substrate groups. Isolate 29 is positioned towards the end of all but two vectors (amines and carbohydrates). Furthermore, in the PCA isolate 15 is well removed from the rest of the groupings.

PCA ordination diagrams of each substrate group were generated. Substrates were arbitrarily sorted into corresponding groups based on their chemical classifications. Three of these ordination diagrams for selected substrate groups are shown. Figure 14 shows the variance between the samples on the basis of the assimilation of all the substrates classified as carbohydrates. The utilisation of alcohols and polyols are depicted in figure 15 while the utilization of carboxylic acid is shown in figure 16. Within all 3 of these diagrams, many of the samples are located in close proximity to one another, as is expected when members of the same pathovar are analysed with

the Biolog® system. Again as in figure 13, isolate number 15 is different from the rest of the isolates.

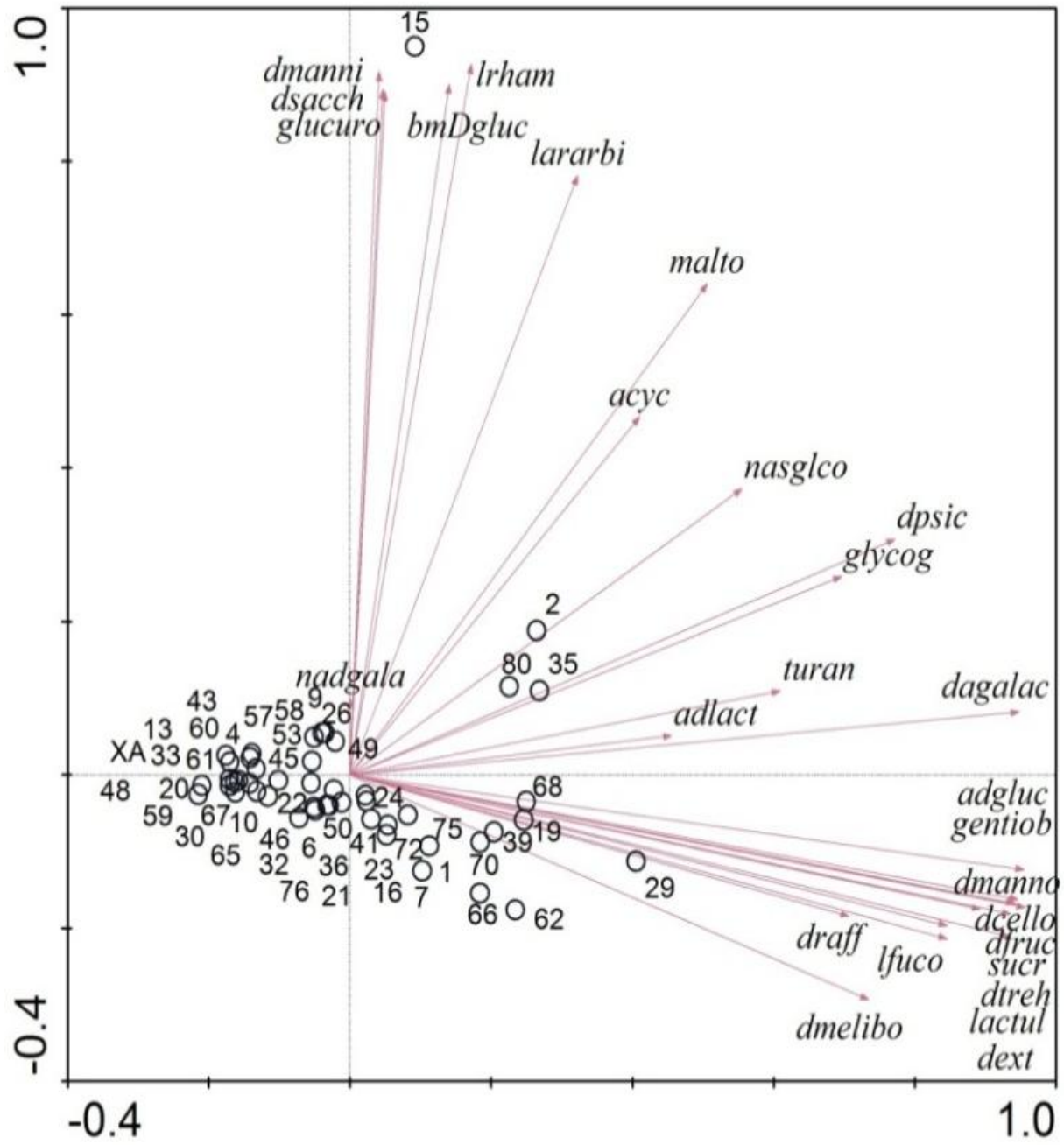

Figure 14: PCA ordination diagram based on the carbohydrate utilisation patterns of all the isolates.

From figure 14 it can be seen in the biplot that most of the isolates grouped together except for one. This isolate was the only isolate to utilise α-D-Lactose, D-Mannitol, ß-Methyl-D-Glucoside, L-Rhamnose, ß-Hydroxy Butyric Acid, Itaconic Acid, D-

Saccharic Acid, Glucuronamide and L-Arabitol while the majority of the isolates displayed stronger correlations with D-melibose, α-D-lactose and D-raffinose.

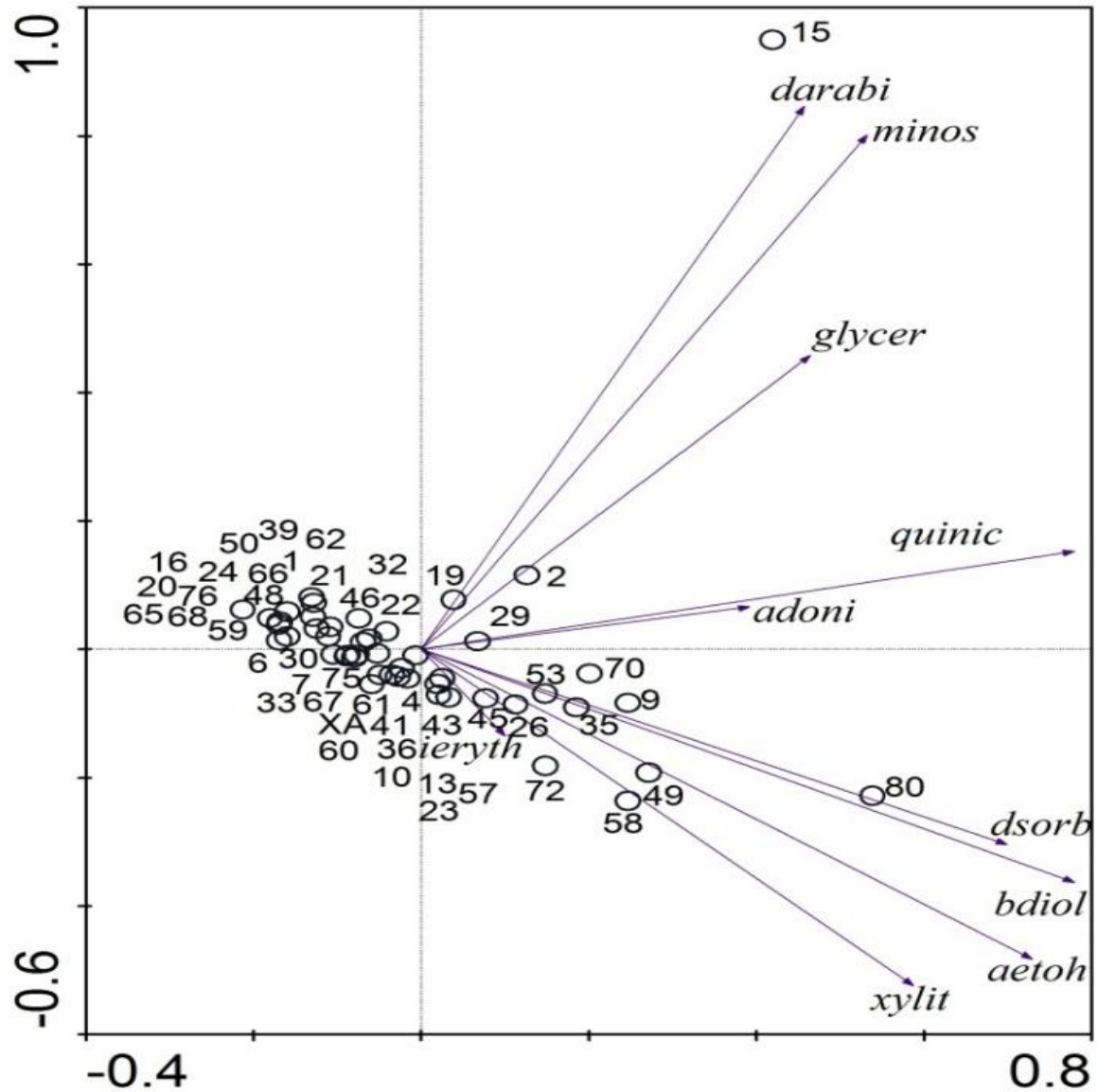

Figure 15: PCA ordination diagram of the utilisation of the alcohols and polyols substrate group by all the isolates.

From figure 15 it is evident that the majority of the isolates cluster together around the centre of the axis while isolate 15 and isolate 80 are located away from this cluster. These two isolates correlated stronger with D-Arabitol and D-sorbitol respectively. The reference strain *Xanthomonas axonopodis* correlates closely to the rest of the isolates as it is in close proximity to the rest the isolates on the biplot. The

eigen values for axis 1 and axis 2 of this ordination diagram is 0.346 and 0.235 respectively. Axis 1 thus showed higher variance than axis 2 as the eigen value for axis 1 was higher than the eigen value for axis 2.

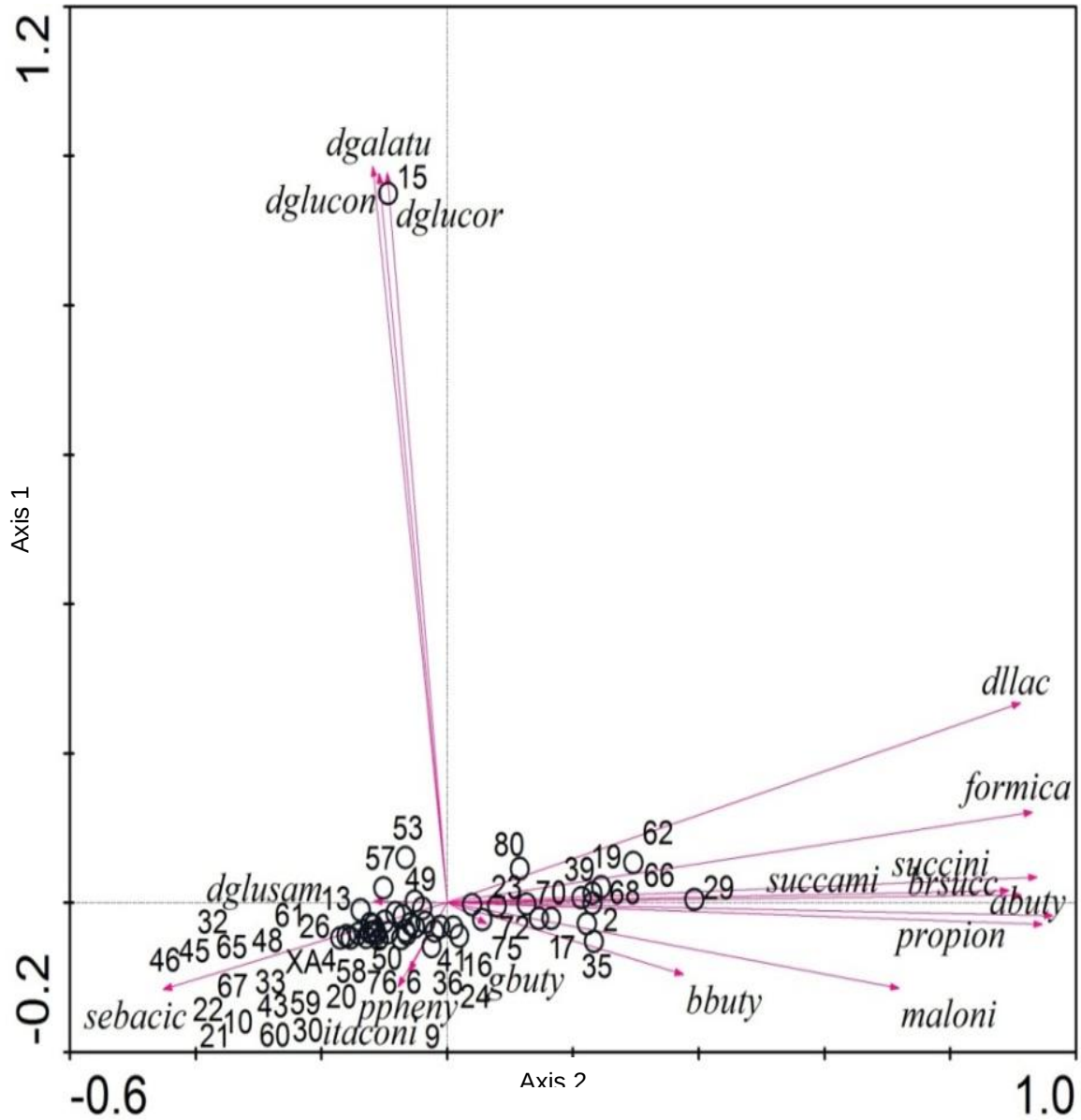

Figure 16: PCA ordination diagram of all the isolates in relation to carboxylic acid utilisation.

From figure 16 it can be observed that as with the other PCA ordination diagrams in figures 13, 14 and 15 the majority of the isolates (including the reference strain *Xanthomonas axonopodis*) grouped together in relation to each other except for isolate 15. Isolate 15 displayed a stronger association with D-Galacturonic Acid, D-

Gluconic Acid and D-Glucuronic Acid than the other isolates. The majority of the isolates displayed a stronger association with sebacic acid, itaconic acid and D,L-Lactic acid since most of the isolates are located in close proximity to the vectors representing these specific substrates.

Table 5: Eigen values for figures 14, 15 & 16

PCA	PCA1	PCA2
Fig. 14: Carbohydrates	0.480	0.227
Fig. 15: Alcohols and polyols	0.346	0.235
Fig.16: Carboxylic acids	0.409	0.178

Table 5 represents the Eigen values of the PCA bi-plots in figures 14, 15 and 16. The Eigen value of a bi-plot indicates the amount of variance found within the data. As the lowest Eigen values were calculated on the bi-plot in figure 15, the substrates classified as alcohols and polyols showed the least variance on the axis 1 (PCA1) while showing the highest variance on the axis 2 (PCA2). When tested with the Biolog system, the reactions of the sample organisms were more similar to one another based on the utilisation of alcohols and polyols although important differences were also found. As such, the tested organisms were more easily identified based on the utilisation of alcohols and polyols. Since greater variance of PCA1 as determined by the higher eigen values were found among the utilisation of patterns of carboxylic acid and carbohydrate substrate groups, these substrate groups could serve as a basis to distinguish possible strains within the pathovar from one another.

As highlighted through the PCA ordination diagrams based on the carbon utilisation of the different substrate groups, it is possible to determine which carbon sources are favoured by *Xanthomonas campestris* pv. *zeae* isolates when these carbon sources are available. From the results the Biolog® GN2 plates patterns were observed which could potentially aid in the identification of *Xanthomonas campestris* pv. *zeae*. As expected when isolates from the same pathovar were tested using the

Biolog® GN2 system, most of the isolates displayed strong preference or indifference to a carbon source. However, it is possible for some carbon sources to display variable results among the isolates. These results are further summarised in table 6 which indicate the list of various carbon sources that were tested.

Table 6: Summary of carbon source utilisation by *X. campestris* pv. *zeae*.

Substrate	**U/N**	**Substrate**	**U/N**
α-Cyclodextrin	○	α-Keto Glutaric Acid	●
Dextrin	●	D,L-Lactic Acid	●
Glycogen	○	Malonic Acid	●
Tween 40	●	Propionic Acid	●
Tween 80	●	Quinic Acid	○
N-acetyl-D-galactosamine	○	Sebacic Acid	○
N-acetyl-D-glucosamine	●	Succinic Acid	●
Adonitol	○	Bromo Succinic Acid	●
D-Cellubiose	●	Succinamic Acid	●
i-Erythritol	○	L-Alaninamide	●
D-fructose	●	D-Alanine	●
L-Fucose	●	L-Alanine	●
D-Galactose	●	L-Alanyl-glycine	●
Gentiobiose	●	L-Aspartic Acid	●
α-D-Glucose	●	L-Glutamic Acid	●
Lactulose	●	Glycyl-L-Aspartic Acid	●
Maltose	●	Glycyl-L-Glutamic Acid	●
D-Mannose	●	Hydroxy-L-Proline	●
D-Mellibiose	●	L-Ornithine	○
D-Sorbitol	○	L-Phenylalanine	○
Sucrose	●	L-Proline	●
D-Trehalose	●	L-Pyroglutamic Acid	○
Turanose	○	D-Serine	○
Xylitol	○	L-Serine	●
Methyl Pyruvate	●	L-Threonine	●
Mono-Methyl-Succinate	●	D,L-Carnitine	○
Acetic Acid	●	γ-Amino Butyric Acid	○
Cis-Aconitic Acid	●	Thymidine	○
Citric Acid	●	Phenyethylamine	○
D-Galactonic Acid	○	Putrescine	○
D-Glucosaminic Acid	○	2-Aminoethanol	○
α-Hydroxy Butyric Acid	●	2,3-Butanediol	○
γ-Hydroxy Butyric Acid	○	Glycerol	●
ρ-Hydroxy Phenylacetic Acid	○	D,L-α-Glycerol Phosphate	●
α-Keto Butyric Acid	●		

● = substrate is almost always used; ○ =substrate not used Carbon sources displaying variable results are omitted from the table.

The typical pattern observed by the utilisation of these substrates by the isolates from the present study is illustrated in figure 17. The Biolog® system is based on a redox reaction. When the sample bacteria oxidises the carbon source within the microplate wells, the tetrazolium dye is reduced and forms a purple colour. The absorbance of the purple colour increases as more of the carbon source is utilised. A distinctive metabolic fingerprint is obtained for the pathogen. The results presented here suggest that for rapid detection of the BLS pathogen, a pure culture can be inoculated into a Biolog® GN2 plate and after 16-24 hrs the isolates could be identified as *Xanthomonas campestris* pv. *zeae* if the same characteristic pattern is obtained as that seen in figure 17.

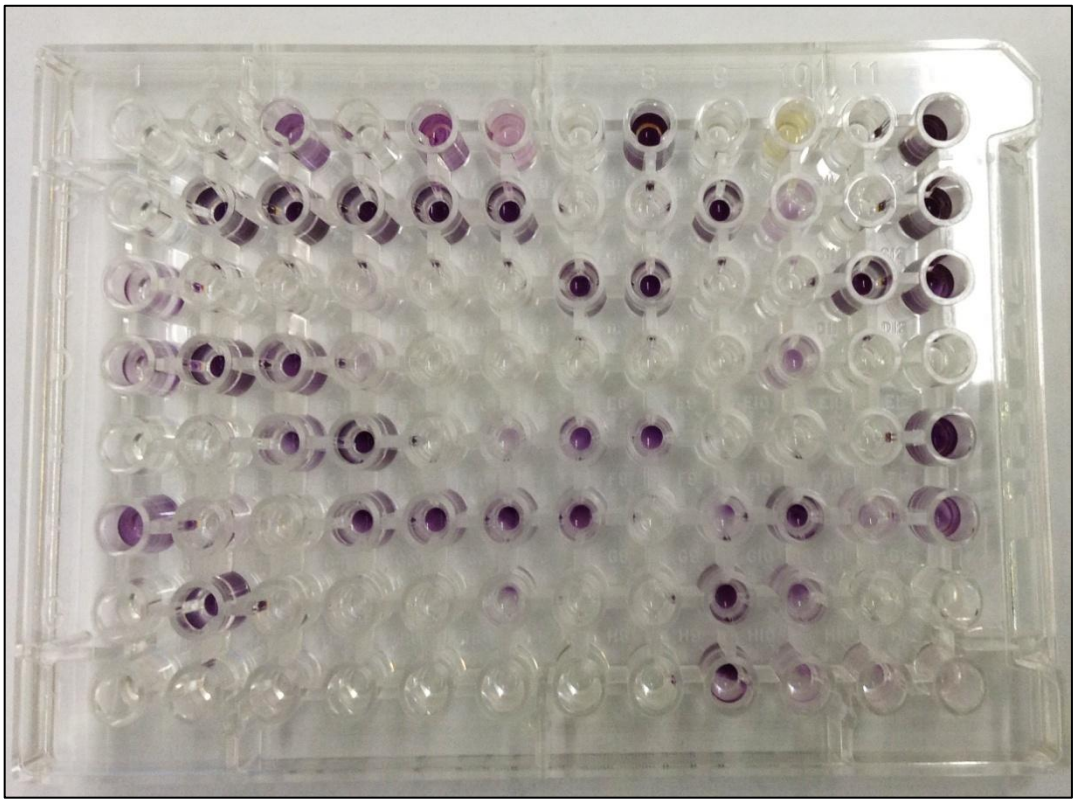

Figure 17: Photograph of Biolog® GN2 plate inoculated with *Xanthomonas campestris* pv. *zeae* after 24 hrs of inoculation.

In figure 18 the metabolic profile of *Xanthomonas* members isolated from onion blight is shown. Nowbuth *et al.* (2005) isolated these bacteria in Mauritius to study the phenotypic diversity of *Xanthomonas* in onion blight. To elucidate the differences between the metabolic profiles obtained during this study (figure 17) and the metabolic profiles for the *Xanthomonas* obtained from onion blight (figure 18) (Nowbuth *et al.*, 2005), the substrates utilised by *Xanthomonas campestris* pv. *zeae* is highlighted with a yellow margin in figure 18.

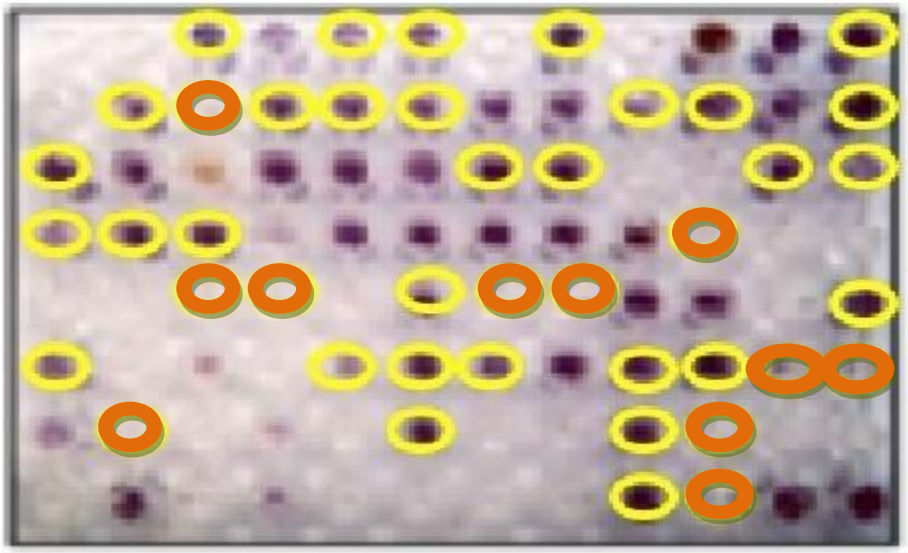

Figure 18: Metabolic profiles of Xanthomonas isolates associated with onion blight in Mauritius (Nowbuth *et al.*, 2005).

The substrates within the wells that are purple were utilised by the isolates from onion blight. Eleven of the wells utilised by *Xanthomonas campestris* pv. *zeae* were colourless when the onion pathogen was analysed and is indicated by orange in figure 18. The wells containing the purple colour appeared to constitute a unique pattern which allowed discrimination between the *Xanthomonas* isolates obtained during this study and the isolates obtained by Nowbuth *et al.* (2005).

Despite the fact that all the substrates included in the Biolog® plates are not abundant in the natural surroundings of the plants (Campbell *et al.*, 1997), many of these carbon sources occur in nature and are produced by plants (Bohnert & Sheveleva, 1998). Common osmolytes occurring in higher plants are sugars, organic

acids, polyols, amino acids and amides (Ashraf & Harris, 2004). *Xanthomonas campestris* pv. *zeae* utilises a range of substrates that is normally available within plants. During stress conditions the production of some of these substrates increases and the substrates accumulate within the plant. Plant metabolism changes in warm weather, as a result of water stress, salinity stress and desiccation (Bohnert & Sheveleva, 1998; Lawlor, 2002; Quinet *et al.*, 2010). As a natural stress response, plants produce greater amounts of normal metabolites (Bohnert & Sheveleva, 1998) such as sugars, polyols, carbohydrates, amino acids and amines (Bohnert & Jensen, 1996; Quinet *et al.*, 2010).

N-Acetyl-D-glucosamine that is utilised by *X. campestris* pv. *zeae* is an important building block of chitin in the cell walls of fungi and the exoskeleton of insects. Although plants do not contain chitin, it is the second most common natural polymer found in nature (Mehtre, 2010). Recently, chitin found in crab-shells has been employed to aid plants in coping with salinity stress (Brotman *et al.*, 2012). Since *X. campestris* pv. *zeae* is able to utilise N-Acetyl-D-glucosamine as a sole carbon source, the addition of chitin to plants could encourage colonisation by the pathogen. Kielak *et al.* (2013) found that although the total diversity of bacteria in soil treated with chitin amendments were decreased, the addition of chitin resulted in the increase in certain soil-borne plant pathogens.

During plant stress, changes in the plant tissue and plant metabolism occur. When plants experience stress, increases of certain polyols, amines, amino acids and amides takes place within their tissue. The formation of substrates such as ascorbate, glutathione and α-tocopherol are triggered by water stress (Yordanov *et al.*, 2003). Several plant species have been reported to accumulate polyols such as mannitol in response to drought and salinity stress. Salinity stress has shown to increase amine concentrations in plants, specifically putrescine, and the polyamines spermine and spermidine (Mansour, 2000). Salinity stress has also been reported to result in the accumulation of amino acids (Ashraf, 1994; Mansour, 2000). Glutamine and asparagine (amides) have also been reported to accumulate in plants subject to salt stress (Dubey, 1997). Alanine, arginine, glycine, serine, leucine, valine, proline,

are the most important amino acids that accumulate in plants due to salinity stress. As a response to herbivorous insects, higher plants form α-ketobutyrate and ammonia during the dehydration of threonine to form isoleucine. Trehalose occurs as an osmolyte and has been shown to increase in plants during abiotic stress conditions (Crowe *et al.*, 1984; Hounsa *et al.*, 1998; Jang *et al.*, 2003).

The purpose of these substrates is to presumably increase the ability of plants to tolerate the environmental stress. Plants, such as maize, experience various types of stress imposed on it by insects, animals and environmental factors. The stress response of a plant is dependent upon the type of stress that it experiences. Naturally occurring metabolites such as putrescine, spermine and spermidine are important for the physiological processes and development of all living organisms (Quinet *et al.*, 2010). L-amino acids are important for nitrogen (N) metabolism of terrestrial plants (Hill *et al.,* 2011). D-amino acids, especially d-alanine and D-alanyl glycine occur in the leaves of many higher plants. Threonine is also readily available within the plants (Gonzales-Vigil *et al.*, 2011).

Mayek-Perez *et al.* (2004) suggested that the increased production and storage of carbohydrates and other metabolites in plant host tissues as a result of stress may benefit plant pathogens since these metabolites could serve as a carbon source and encourage colonisation of the host. In this study, *X. campestris* pv. *zeae* was shown to be able to utilise proline as a sole carbon source. The accumulation of proline and other stress related metabolites within maize plants could encourage disease development and influence plant-host relationships. Gleason *et al.* (2011) stated that stress could predispose a plant to disease caused by plant pathogenic bacteria therefore environmental factors such as drought, salinity, osmotic pressure and other types of stresses could be correlated to an increase in plant disease.

Agriculture in South Africa is vulnerable to climatic change because of (i) the semi-arid nature of the country with increased farming on marginal lands, (ii) the frequency

of droughts, and (iii) the scarcity of water, which is exacerbated by a high spatial variability of rainfall (Behnin, 2008).

As climatic conditions are major contributing factors to plant stress it is important to consider the effect of climate change on plant stress and disease. South Africa became about 2% warmer and 6% drier over the 1997-2006 period (Blignaut *et al.*, 2009). The direct effect of a 1% decrease in rain within South Africa could lead to a 1.1% decline in maize production. This does not take into account the yield loss due to pests and disease. The disease triangle identifies the role of the physical environment in plant disease since no pathogen can induce disease on a host if weather conditions are not favourable. All stages of the host and pathogen's life cycles are influenced by weather. Disease severity can fluctuate as a result of climatic variation (Coakley, 1979: Scherm &Yang, 1995). Climate change, changes in precipitation events, temperature and atmospheric conditions, will significantly influence the interaction between technological and socio-economic changes that influences plant diseases (Chakraborty & Newton, 2011).

4.1 Conclusions

The aim of this study was to characterise isolates of *Xanthomonas campestris* pv. *zeae* isolated from maize plants infected with bacterial leaf streak of maize, based on selected phenotypic and biochemical properties. The following was achieved during this study:

(i) to isolate and identify the causal agent of BLS.

(ii) to confirm the pathogenicity of *X. campestris* pv. *zeae* by applying Koch's postulates,

(iii) to detect differences or similarities among the isolates by comparing the protein profiles of the *X.campestris* pv. *zeae* isolates, and

(iv) to create a metabolic profile for the pathogen using the Biolog GN2 system.

(i) isolation and identify the potential causal agent of BLS

Several Gram negative bacteria were isolated from diseased maize leaves showing bacterial leaf streak symptoms using two slightly different isolation procedures. The bacteria were identified using 16S rRNA as *X. campestris* pv. *zeae, Pantoea* spp. and *Enterobacter* spp., in a separate unrelated study.

(ii) Koch's postulates analysis

Representatives of the three genera as well as the negative control, *X. axonopodis* pv. *vasculorum,* were successfully inoculated into healthy maize plants, again using two different techniques. No symptoms developed on plants inoculated with *Pantoea* spp.*, Enterobacter* spp. or *X. axonopodis* pv. *vasculorum*. *Pantoea* and *Enterobacter* were recognised as potential bacterial endophytes of maize plants and are not the pathogens responsible for bacterial leaf streak of maize. Within one week of inoculation, plants that were injured and sprayed with an inoculum containing *X. campestris* pv. *zeae,* started to develop yellow-brown lesions on the leaf surface. These first lesions appeared in close proximity to the sites of injury. Three weeks after inoculation, the first plants that were inoculated with *X. campestris* pv. *zeae* through needle puncture in the stem, started to show symptoms of bacterial leaf

streak. Symptom development and severity increased over the seven week period. Symptoms caused by the stab method reached a plateau after week six.

It was observed that the symptoms induced by the stab-and-spray method developed earlier and had a higher average disease severity than the symptoms induced by the stab-and-inject method. For these reasons, the stab-and-spray method was recognised as more effective than the stab-and-inject method. *Xanthomonas campestris* pv. *zeae* complied to the requirements set in Koch's postulates to be recognised as the causal agent of bacterial leaf streak of maize.

(iii) Protein profiling

Proteins was successfully extracted from *X. campestris* pv. *zeae* and the X. *axonopodis* pv. *vasculorum* reference strain using two relatively similar methods. One method used plate cultures for direct protein extraction. The protein profiles generated by this technique resulted in smears on the gel. A similar method that used fresh (overnight) broth culture provided similar profiles as the first one but the gel images did not develop any smears. Gel images were captured and analysed with computer software. Bands were manually selected for profiling. Protein profiling was based on the presence or absence of the bands obtained after polyacrylamide gel separation of the isolated bacterial proteins. When only major bands that were easily observed, were used to create a dendrogram, two clusters within the *X. campestris* pv. *zeae* isolates were observed. The two *X. campestris* pv. *zeae* clusters were 87% similar to each other. The reference culture was a single-leafed cluster but had a similarity of 77% to the rest of the isolates.

When the contrast of the gel images was adjusted and the images were enlarged, many feint bands became visible. When these bands were selected in conjunction with the major bands nine distinguishable clusters were observed for all the isolates tested. The grouping of the isolates on average corresponded well between the two analysis methods. Isolates that grouped together in the dendrogram based on the analysis of the major bands, mostly grouped together when dendrograms were drawn based on minor bands. However, *X. axonopodis* pv. *vasculorum* was easier to distinguish from the *X. campestris* pathovars when only the major bands were used during analysis.

It was found that although the protein profiles of *X. campestris* pv. *zeae* was highly similar, differences were detectable. It was also possible to distinguish between different groups within the pathovar which could possibly indicate intra-pathovar differences or different phenotypic groups within the pathovar.

(iii) Metabolic profiling

The ability of *X. campestris* pv. *zeae* to oxidise an assortment of carbon sources was assessed by using Biolog® GN2 plates. When the isolates utilised a carbon source, a purple colour developed within the relevant well. The optical density of each well was measured at 4, 16 and 24 hrs after inoculation at a wavelength of 590 nm. In order to correct the absorption values for an increase in optical density due to a population increase within the wells, the absorption of the control well was subtracted from the optical density of the other 95 wells. Readings taken at 16 hrs of incubation were used for statistical analyses because most of the isolates had reached the reference point of 0.35 by this time.

The average well colour development was determined for each isolate. Groupings were found among the isolates when statistically significant differences were assessed. Isolate 29 had a very high AWCD while isolates 45 and 59 had very low AWCD. Isolates with high AWCD values were metabolically more active than isolates with low AWCD values.

PCA biplots were used to reduce the number of variables contained in the carbon utilisation data set without compromising the integrity of the data set. Substrates were divided into chemical classes to simplify the biplot. When all the chemical classes and all the samples were included on the biplot, atypical results were observed. It was expected that all the isolates would group together in close proximity to one another. However, isolate 15 was separated entirely from all the other isolates. As seen with the AWCD isolate 29 was strongly associated with a number of chemical classes while the rest of the isolates showed a weaker relationship. Three substrate groups correlated well with a majority of the isolates namely, alcohols and polyols, carbohydrates and carboxylic acids. As expected with

isolates from the same pathovar, the isolates were found to group closely together. Substrates from these chemical classes were utilised at the same rate by most of the isolates and the reference culture. Biolog® plates that were assessed visually displayed a characteristic pattern of substrate utilisation. It is potentially possible to distinguish *X. campestris* pathovars on the basis of the metabolic fingerprint created with the Biolog® plates. A metabolic fingerprint for *X. campestris* pv. *zeae* was produced and should be evaluated for use in other studies to identify the pathogen. As concluded from the results of the protein profiling, it was found that although it was possible to distinguish *X. campestris* pv. *zeae* from other *X. campestris* pathovars, slight differences in the rate of carbon utilisation was found within the *X. campestris* pv. *zeae* isolates which could indicatefurther intra-pathovar differences or races within the pathovar.

4.2 Recommendations

Identifying and analysing plant disease and the causal organisms involve several steps. The isolation of the assumed pathogen from diseased material takes place after which the culture must be purified, microscopically examined and analysed at the molecular and phenotypical levels (Goszczynska *et al.*, 2007; Alvarez, 2004). Usually a polyphasic approach is followed, especially when definite species identification is required, description of new species takes place, population genetics is analysed and phylogenetic relationships among closely related species are considered (Kämpfer *et al.*, 2003; Ah-You *et al.*, 2009). Results of this study provided information regarding the identification and characteristics of *X. campestris* pv. *zeae* isolates from maize.

From this study the following recommendations are made:

- It is of critical importance to use a suitable technique for the isolation of foliar pathogens. It is recommended that plant material is surface sterilised, pulverised and a dilution series is prepared.
- The spray inoculation method is recommended to be used during pathogenicity tests and/or screening of maize germplasm when foliar pathogens are investigated. This

method is easier to apply and earlier symptom development is obtained. This method may also be closer to the pathogens natural method of infection.

- The age of the maize plants used for pathogenicity testing is of critical importance, it is recommended that the seedlings are inoculated no later than the four-leaf stage.
- To obtain clear banding patterns suitable for proteomic analysis, it is recommended that liquid cultures are used for protein extractions.
- When the presence/absence analysis of protein profiles of a species or pathovar that could contain sub-groupings, is conducted it is recommended that only major bands are selected. This creates more concise groupings and enables the differentiation of closely related pathovars while recognising inter-pathovar similarities.
- It is recommended that more distant and closely related species are used during protein profiling and that the gels are analysed based on the intensity of the bands to obtain a weighted distance tree.
- In order to obtain reliable results from metabolic profiling assays, it is of utmost importance to standardise the cell count in each of the inoculation fluids for each sample.
- Additional studies to determine the possibility of an expanded host-range of this pathogen through the use of cross-inoculations is recommended.

It can be concluded that experiments conducted in this study were successful and useful results were obtained. Findings from this study create the foundation for future studies on the bacterial leaf streak of maize and its pathogen.

REFERENCES

ACINAS, S.G., KLEPAC-CERAJ, V., HUNT, D.E., PHARINO, C., CERAJ, I., DISTEL, D.L. & POLZ, M.F. 2004. Fine-scale phylogenetic architecture of a complex bacterial community. *Nature*, 430:551-554.

AH-YOU, N. GAGNEVIN, L., GRIMONT, P.A.D., BRISSE, S., NESME, X., CHIROLEU, F., BUI THI NGOC, L., JOUEN, E. & LEFEUVRE, P. 2009. Polyphasic characterization of xanthomonads pathogenic to members of the *Anacardiaceae* and their relatedness to species of *Xanthomonas. International Journal of Evolutionary Microbiology*, 59:306-318.

AKPALU, W., HASSAN, R.M. & RINGLER, C. 2008. Climate variability and maize yield in South Africa: results from GME and MELE methods. (IFPRI Discussion Paper No. 843). http://www.ifpri.org/publication/climate-variability-and-maize-yield-south-africa. Date of access: 27 Jun. 2012.

ALFANO, J.R. & COLLMER, A. 1997. The type III (*Hrp*) secretion pathway of plant pathogenic bacteria: trafficking harpins, *Avr* proteins, and death. *Journal of Bacteriology*, 179(18): 5655–5662.

ALI, M.A., ABBAS, A., NIAZ, S., ZULKIFFAL, M. & ALI, S. 2009. Morpho-physiological criteria for drought tolerance in sorghum (*Sorghum bicolor*) at seedling and post-anthesis stages. *International Journal of Agriculture and Biology,* 11:674–680.

ALVAREZ, M.A. 2004. Integrated approaches for detection of plant pathogenic bacteria and diagnosis of bacterial diseases. *Annual Review of Phytopathology*, 42:339-66.

ANJUM, S.A., WANG, L.C., FAROOQ, M., HUSSAIN, M., XUE, L.L. & ZOU, C.M. 2011. Brassinolide application improves the drought tolerance in maize through modulation of enzymatic antioxidants and leaf gas exchange. *Journal of Agronomy and Crop Science,* 197(3):177-185.

ANNOUS, B.A., FRATAMICO, P.M. & SMITH, J.L. 2009. Quorum sensing in biofilms: why bacteria behave the way they do. *Journal of Food Science*, 74:24–37.

ANTON, A.I., MARTINEZ, A.J. & RODRIGUEZ-VALERA, F. 1998. Sequence diversity in the 16S–23S intergenic spacer region (ISR) of the rRNA operons in representatives of *Escherichia coli* ECOR collection. *Journal of Molecular Evolution,* 47:62–72.

APIRION, D. & MICZAK, A. 1993. RNA processing in prokaryotic cells. *Bioessays*, *15*(2):113-120.

ARBEIT, R.D. 1995. Laboratory procedures for the epidemiologic analysis of microorganisms. (*In* Murray, P. R., Baron, E.J., Pfaller, M.A., Tenover, F.C., & Yolken, R.H. *eds*. Manual of clinical microbiology. 6th ed. Washington: American Society for Microbiology. p. 190-208.)

ARC-GCI (Agricultural Research Council – Grain Crops Institute). 2008. Maize information guide, Pretoria: Department of Agriculture. p. 11-17.

ARLAT, M., GOUGH, C.L., ZISCHEK, C., BARBERIS, P.A., TRIGALET, A. & BOUCHER, C.A. 1992. Transcriptional organization and expression of the large *hrp* gene cluster of *Pseudomonas solanacearum*. *Molecular Plant-microbe Interactions*, 5:187-193.

ARNOLD, D.L., PITMAN, A. & JACKSON, R.W. 2003. Pathogenicity and other genomic islands in plant pathogenic bacteria. *Molecular Plant Pathology*, 4(5):407-420.

ASHRAF, M. & HARRIS, P.J.C. 2004. Potential biochemical indicators of salinity tolerance in plants. *Plant Science,* 166:3-16.

ASHRAF, M. 1994. Organic substances responsible for salt tolerance in *Erucasativa*. *Journal of Plant Biology*, 36:255-259.

ASIS, C.A & ADACHI, K. 2003. Isolation of endophyticdiazotroph *Pantoea agglomerans* and non diazotroph *Enterobacteras buriae* from sweetpotato stem in Japan. *Letters in Applied Microbiology* 38:19-23.

BABADOOST, M. & ZITTER, T.A. 2009. Fruit rots of pumpkin: a serious threat to the pumpkin industry. *Plant Disease*, 93:772-782.

BACILIO-JIMENEZ, M., AGUILAR-FLORES, S., DEL VALLE, M.V., PÉREZ, A., ZEPEDA, A. & ZENTENO, E. 2001. Endophytic bacteria in rice seeds inhibit early colonization of roots by *Azospirillum brasilense*. *Soil Biology and Biochemistry*, *33*(2):167-172.

BACON, C.W., YATES, I.E., HINTON, D.M. & MEREDITH, F. 2001. Biological control of *Fusarium moniliforme* in maize. *Environmental Health Perspectives*,109:325-332.

BAGHERI, M., DIDARI, M., AMOOZEGAR, M.A., SCHUMANN, P., SANCHEZ-PORRO, C., MEHRSHAD, M. & VENTOSA, A. 2012. *Bacillus iranensis* sp. nov., a moderate halophile from a hypersaline lake. *International Journal of Systematic and Evolutionary Microbiology*, *62*:811-816.

BARAK, J.D., KOIKE, S.T. & GILBERTSON, R.L. 2001. Role of crop debris and weeds in the epidemiology of bacterial leaf spot of lettuce in California. *Plant Disease*, *85*(2):169-178.

BARNABAS, B., JAGERM, K. & FEHER, A. 2008. The effect of drought and heat stress on reproductive processes in cereals. *Plant, Cell and Environment*, 31:11-38.

BEATTIE, G.A. & LINDOW, S. E. 1999. Bacterial colonization of leaves: a spectrum of strategies. *Phytopathology*, 89:353-359.

BECKER, A., KATZEN, F., PUHLER, A. & IELPI, L. 1998. Xanthan gum biosynthesis and application: a biochemical/genetic perspective. *Applied Microbiology and Biotechnology*, 50(2):145-152.

BEHNIN, J.K.A. 2008. South African crop farming and climate change: an economic assessment of impacts*. Global Environmental Change*, 18:666-678.

BELL, C. R., DICKIE, G.A., HARVEY, W.L.G & CHAN, J.W.Y.F. 1995. Endophytic bacteria in grapevine.*Canadian Journal of Microbiology*, 41:46-53.

BENNASAR, A., ROSSELLO-MORA, R., LALUCAT, J. & MOORE, E.R. 1996. 16S rRNA gene sequence analysis relative to genomovars of *Pseudomonas stutzeri* and

proposal of *Pseudomonas balearica* sp. nov. *International Journal of Systematic bacteriology*, 46(1):200-205.

BENNIE, A.T.P. & HENSLEY, M. 2000. Maximizing precipitation utilization in dryland agriculture in South Africa — a review. *Journal of Hydrology 241:124-139.*

BERG, T., TESORIERO, L. & HAILSTONES, D.L. 2005. PCR-based detection of *Xanthomonas campestris* pathovars in *Brassica* seed. *Plant Pathology*, 54(3): 416-427.

BERNARD, E., LARKIN, R.P., TAVANTZIS, S., ERICH, M.S., ALYOKHIN, A., SEWELL, G., LANNAN, A. & GROSS, S.D. 2012. Compost, rapeseed rotation, and biocontrol agents significantly impact soil microbial communities in organic and conventional potato production systems. *Applied Soil Ecology*, 52:29-41.

BHADURI, S. & DEMCHICK, P.H. 1983. Simple and rapid method for disruption of bacteria for protein studies. *Applied and Environmental Microbiology*, 46(4):941-943.

BHAT, N.A., SYEED, N., BHAT, K.A. & MIR, S.A. 2010. Pathogenicity and host range of *Xanthomonas campestris* pv. *campestris* – incitant of black rot of crucifers. *Journal of Phytology*, 2(10):01-05

BILA, J., MORTENSEN, C.N., ANDRESEN, M., VICENTE, J.G. & WULFF, E.G. 2013. *Xanthomonas campestris* pv. *campestris* race 1 is the main causal agent of blackrot of *Brassicas* in Southern Mozambique. *African Journal of Biotechnology*, 12(6):602-610.

BLIGNAUT, J., UECKERMANN, L. & ARONSON, J. 2009. Agriculture production's sensitivity to changes in climate in South Africa. *South African Journal of Science*, 105:61- 68.

BOCHNER, B.R. 2003. New technologies to assess genotype phenotype relationships. *Nature Reviews Genetics,* 4:309-314.

BOCHNER, B.R. 2009. Global phenotypic characterization of bacteria. *FEMS Microbiology Reviews,* 33:191-205.

BOCHNER, B.R., GADZINSKI, P. & PANOMITROS, E. 2001. Phenotype microarrays for high-throughput phenotypic testing and assay of gene function. *Genome Research,* 11:1246-1255.

BOHNERT, H.J. & JENSEN, R.G. 1996. Strategies for engineering water stress tolerance in plants. *Trends in Biotechnology*, 14:89-97.

BOHNERT, H.J. & SHEVELEVA, E. 1998. Plant stress adaptations- making metabolism move. *Current Opinion in Plant Biology*, 1:267-274.

BOUZAR, H., JONES, J.B., MINSAVAGE, G.V., STALL, R.E. & SCOTT, J.W. 1994. Proteins unique to phenotypically distinct groups of *Xanthomonas campestris* pv. *vesicatoria* revealed by silver staining. *Phytopathology*, *84*(1):39-43.

BOYER, S. L., FLECHTNER, V.R. & JOHANSEN, J.R. 2001. Is the 16S–23S rRNA internal transcribed spacer region a good tool for use in molecular systematics and population genetics? A case study in cyanobacteria. *Molecular Biology and Evolution*, *18*(6):1057-1069.

BRADBURY, J.F. 1984. *Xanthomonas* Dowson 1939. (*In* Krieg, N.R. & Holt, J.G. *eds*. Bergey's manual of systematic bacteriology. vol. 1. Baltimore: The Williams & Wilkins Co. p. 199-210).

BRAUN, E.J. 1990. Colonization of resistant and susceptible maize plants by *Erwinia stewartii* strains differing in exopolysaccharide production. *Physiological and Molecular Plant Pathology*, 36(5):363-379.

BRENNER, D.J., KRIEG, N.R. & STALEY, J.T. 2005. Bergey's manual of systematic bacteriology. 2nd ed. New York: Springer.

BROOKS, D.S., GONZALEZ, C.F., APPEL, D.N. & FILER, T.H. 1994. Evaluation of endophytic bacteria as potential biological-control agents for oak wilt. *Biological Control*, *4*(4):373-381.

BROTMAN, Y., LANDAU, U., PNINI, S., LISEC, J., BALAZADEH, S., MUELLER-ROEBER, B. & VITERBO, A. 2012. The LysM receptor-like kinase LysM RLK1 is

required to activate defense and abiotic-stress responses induced by overexpression of fungal chitinases in Arabidopsis plants. *Molecular Plant*, *5*(5):1113-1124.

BROWN, V.K. 1985. Insect herbivores and succession. *Oikos,* 44:17-22.

BULL, C.T., DE BOER, S.H., DENNY, T.P., FIRRAO, G., FISCHER-LE SAUX, M., SADDLER, G.S. & TAKIKAWA, Y. 2012. List of new names of plant pathogenic bacteria (2008-2010). *Journal of Plant Pathology*, *94*(1):21-27.

BULL, C.T., DE BOER, S.H., DENNY, T.P., FIRRAO, G., FISCHER-LE SAUX, M., SADDLER, G.S. & TAKIKAWA, Y. 2010. Comprehensive list of names of plant pathogenic bacteria, 1980-2007. *Journal of Plant Pathology*, *92*(3):551-592.

BURKHOLDER, W.H. & STARR, M.P. 1948. The generic and specific characters of phytopathogenic species of *Pseudomonas* and *Xanthomonas*. *Phytopathology*, 38:494.

BUTTNER, D. & BONAS, U. 2010. Regulation and secretion of *Xanthomonas* virulence factors. *FEMS Microbiology Reviews*, 34(2):107-133.

BYERLEE, D. & HEISEY, P.W. 1996. Past and potential impacts of maize research in sub-Saharan Africa: a critical assessment. *Food Policy*, 21(3):255–277.

CABI EPPO. 2008. Distribution maps of plant diseases. 1st ed. Wallingford: CABI.

CAKIR, R. 2004. Effect of water stress at different development stages on vegetative and reproductive growth of corn. *Field Crops Research,* 89(1):1-16.

CAMPBELL, C.D., GRAYSTON, S.J. & HIRST, D.J. 1997. Use of rhizosphere carbon sources in sole carbon source tests to discriminate soil microbial communities. *Journal of Microbiological Methods*, 30:33-41.

CANKAR, K., KRAIGHER, H., RAVNIKAR, M. & RUPNIK, M. 2005. Bacterial endophytes from seeds of Norway spruce (*Picea abies L*. Karst). *FEMS Microbiology Letters*, *244*(2): 341-345.

CAO, T.B. & SAIER, M.H. 2001. Conjugal type IV macromolecular transfer systems of Gram-negative bacteria: organismal distribution, structural constraints and evolutionary conclusions. *Microbiology*, 147(12):3201-3214.

CASCALES, E. 2008.The type VI secretion toolkit. *EMBO Reports*, 9(8):735-741.

CASE, R.J., BOUCHER, Y., DAHLLOF, I., HOLMSTROM, C., DOOLITTLE, W.F. & KJELLEBERG, S. 2007. Use of 16S rRNA and *rpoB* genes as molecular markers for microbial ecology studies. *Applied and Environmental Microbiology*, 73(1): 278-288.

CHAKRABORTY, S. & NEWTON, A.C. 2011. Climate change, plant diseases and food security: an overview. *Plant Pathol*ogy, 60(1):2-14.

CHEN, X.Y. & KIM, J.Y. 2009. Callose synthesis in higher plants. *Plant Signaling & Behavior*, *4*(6):489-492.

CHOURNEY, P.S., LI, Q.B. & KUMAR, D. 2010. Sugar-hormone cross-talk in seed development: two redundant pathways of IAA biosynthesis are regulated differentially in the invertase-deficient minature1 (mn1) seed mutant in maize. *Molecular Plant, 3:1026-1036.*

CHUN, W., CUI, J. & POPLAWSKY, A. Purification, characterization and biological role of a pheromone produced by *Xanthomonas campestris* pv. *campestris. Physiological and Molecular Plant Pathology, 51*:1-14.

CILIA, V., LAFAY, B. & CHRISTEN, R. 1996. Sequence heterogeneities among 16S ribosomal RNA sequences and their effect on phylogenetic analyses at the species level. *Molecular Biology and Evolution*, 13(3):451-461.

CLAFLIN, L.E. 2000. Diseases caused by Prokaryotes. (*In* White, D.G., *ed.* Compendium of Corn Diseases. Minnesota: APS. Press.p. 3-9).

CLAYTON, R. A., SUTTON, G., HINKLE, P.S., BULT, C. & FIELDS, C. 1995. Intraspecific variation in small-subunit rRNA sequences in GenBank: why single sequences may not adequately represent prokaryotic taxa. *International Journal of Systemic Bacteriology. 45:595-599.*

COAKLEY, S.M. 1979. Climate variability in the Pacific Northwest and its effect on stripe rust disease of winter wheat. *Climatic Change*, 2(1):33-51.

COBURN, B., SEKIROV, I. & FINLAY, B.B. 2007. Type III secretion systems and disease. *Clinical Microbiology Reviews*, 20(4):535-549.

COENYE, T., VANDAMME, P., GOVAN, J.R.W. & LIPUMA, J.J. 2001. Taxonomy and identification of the *Burkholderia cepacia* complex. *Journal of Clinical Microbiology*, 39:3427-3436.

COMAS, I., MOYA, A., AZAD, R.K., LAWRENCE, J.G. & GONZALEZ-CANDELAS, F. 2006. The evolutionary origin of Xanthomonadales genomes and the nature of the horizontal gene transfer process. *Molecular Biology and Evolution*, 23(11):2049-2057.

COMMURI, P.D. & JONES, R.J. 2001. High temperatures during endosperm cell division in maize. *Crop Science*, 41:1122-1130.

COMMURI, P.D., & JONES, R.J. 1999. Ultrastructural characterization of maize (*Zea mays L*.) kernels exposed to high temperature during endosperm cell division. *Plant, Cell and Environment*, 22:375-385.

CONVILLE, P.S. & WITEBSKY, F.G. 2001. Lack of usefulness of carbon utilization tests for identification of *Mycobacterium mucogenicum*. *Journal of Clinical Microbiology*, *39*(7): 2725-2728.

COOK, C., REASON, C.J.C. & HEWITSON, B.C. 2004. Wet and dry spells within particular wet and dry summers in the South African summer rainfall region. *Climate Research*, 26:17-31.

CORNELIS, G.R. & VAN GIJSEGEM, F. 2000. Assembly and function of type III secretory systems. *Annual Reviews in Microbiology*, 54(1):735-774.

COSTERTON, J.W., LEWANDOWSKI, Z., CALDWELL, D.E., KORBER, D.R. & LAPPIN-SCOTT, H.M. 1995. Microbial biofilms. *Annual Review of Microbiology*, 49:711-745.

COUTINHO, T.A. & WALLIS, F.M. 1990. Bacterial streak disease of maize (*Zea mays* L.) in South Africa. *Journal of Phytopathology*, 133(2):112-120.

CROWE, J.H., CROWE, L.M. & CHAPMAN, D. 1984. Preservation of membranes in anhydrobiotic organisms: the role of trehalose. *Science*, *223*(4637):701-703.

DAFF (Department of Agriculture, Forestry and Fisheries). 2012. Maize market value chain profile 2011/2012. http://www.nda.agric.za/docs/AMCP/MaizeMVCP2011.pdf. Date of access: 30 Oct. 2012.

DAMATA, F.M., GRANDIS, A., ARENQUE, B.C. & BUCKERIDGE, M.S. 2010. Impacts of climate changes on crop physiology and food quality. *Food Research International*, 43:1814-1823.

DE BAERE, T., VERHELST, R., LABIT, C., VERSCHRAEGEN, G., WAUTERS, G., CLAEYS, G., & VANEECHOUTTE, M. 2004. Bacteremic Infection with *Pantoea ananatis*. *Journal of Clinical Microbiology,* 42(9):4393–4395.

DE GROOTE, H., DEMAD, G., SONDAC, G.B. & GITONGAA, Z.M. 2013. Maize for food and feed in East Africa — the farmers' perspective. *Field Crops Research*. http://dx.doi.org/10.1016/j.fcr.2013.04.005 Date of access: 13 Dec. 2013.

DENNY, T.P. 1995.Involvement of bacterial polysaccharide in plant pathogenesis. *Annual Review of Phytopathology*, 33(1):173-197.

DI GIOVANNI, G.D., WATRUD, L.S., SEIDLER, R.S. & WIDMER, F. 1999. Comparison of parental and transgenic alfalfa rhizosphere bacterial communities using Biolog GN metabolic fingerprinting and enterobacterial repetitive intergenic consensus sequence-PCR (ERIC-PCR). *Microbial Ecology*, 37(2):129-139.

DICE, L.R. 1945. Measures of the amount of ecological association between species. *Ecology,* 26:297-307.

DIXON, M.T. & HILLIS, D.M. 1993. Ribosomal RNA secondary structure: compensatory mutations and implications for phylogenetic analysis. *Molecular Biology and Evolution*, 10(1):256-267.

DOA ***see*** South Africa. DOA.

DONLAN, R.M. 2002. Biofilms: microbial life on surfaces. *Emerging Infectious Diseases.*, *8*:881-890.

DOW, J.M. & DANIELS, M.J. 2000. *Xylella* genomics and bacterial pathogenicity to plants.*Yeast*, 17(4):263-271.

DOW, J.M., CROSSMAN, L., FINDLAY, K., HE, Y.Q., FENG, J.X. & TANG, J.L. 2003. Biofilm dispersal in *Xanthomonas campestris* is controlled by cell-cell signalling and is required for full virulence to plants. *Proceedings of the National Academy of Science USA*, 100(19):10995-11000.

DRANCOURT, M., BOLLET, C., CARLIOZ, A., MARTELIN, R., GAYRAL, J.P. & RAOULT, D. 2000.16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. *Journal of Clinical microbiology*, 38(10):3623-3630.

DU PLESSIS, J. 2003. Maize production. South African Department of Agriculture. http://www.nda.agric.za/publications. Date of access: 12 September 2011.

DU TOIT, A.S., PRINSLOO, M.A., DURAND, W. & KIKER, G. 2000. Vulnerability of maize production to climate change and adaptation assessment in South Africa. (*In* Kiker, G. *ed*. Climate Change Impacts in Southern Africa. Pretoria: Department of Environmental Affairs and Tourism.)

DU TOIT, W. 1997. Guide for the production of maize in the summer-rainfall area. Potchefstroom: Agricultural Research council - Grain Crops Institute.

DUBEY, R.S. 1997. Photosynthesis in plants under stressful conditions. (*In* Pessarakli, M., *ed.* Handbook of Photosynthesis. New York: Marcel Dekker. p. 859–875).

DUNGER, G., RELLING, V.M., TONDO, M.L., BARRERAS, M., IELPI, L., ORELLANO, E.G. & OTTADO, J. 2007. Xanthan is not essential for pathogenicity in citrus canker but contributes to *Xanthomonas* epiphytic survival. *Archives of Microbiology*, 188(2):127-135.

DURAND, W. 2006. Assessing the impact of climate change on crop water use in South Africa. Pretoria: University of Pretoria. (CEEPA Discussion paper, 28)

DUTTA, B., GITAITIS, R.D., LEWIS, K.J. & LANGSTON, D.B. 2013. A new report of *Xanthomonas cucurbitae* causing bacterial leaf spot of watermelon in Georgia, USA. *Plant Disease*, *97*(4):556-565.

DUVEILLER, E., BRAGARD. C.& MARAITE, H. 1997. Bacterial leaf streak and black chaff caused by *Xanthomonas translucens.* (*In* Duveiller, E., Fucikovsky, L., & Rudolph, K., *eds*. The Bacterial Diseases of Wheat: Concepts and Methods of Disease Management. Mexico: CIMMYT. p. 25-47).

DYE, D.W. & LELLIOT, R.A. 1974. Genus II: *Xanthomonas Dowson 1939*. (*In* Buchanan, R.E. & Gibbons, N.E. *eds*. Bergey's Manual of determinative bacteriology. 8th ed. London: Bailliére. p.243-249).

DYE, D.W. 1962. The inadequacy of the usual determinative tests for identification of *Xanthomonas* spp. *New Zealand Journal of Science*, 5(4):393-416.

DYE, D.W., BRADBURY, J.F., GOTO, M., HAYWARD, A.C., LELLIOT, R.A. & SCHROTH, M.N. 1980. International standards for naming pathovars of phytopathogenic bacteria and list of pathovar names and pathotype strains. *Review of Plant Pathology*, 59:153-168.

DYER, R.A. 1949. Botanical surveys and control of plant diseases. Farming in South Africa. *Annual Report of the Department of Agriculture, South Africa*, 275(24):119-121.

EGLI, T., GOTO, M. & SCHMIDT, D. 1975. Bacterial wilt, a new forage grass disease. *Journal of phytopatho*logy, 82(2):111-121.

ENGELEN-EIGLES, G., JONES, R.J. & PHILLIPS, R.L. 2000. DNA endoreduplication in maize endosperm cells. I: The effect of exposure to short-term high temperature. *Plant, Cell and Environment*, 23:657-663.

FAOSTAT. 2013. Food and Agriculture Organization of the United Nations, FAO Statistical Databases.

http://faostat.fao.org/site/562/default.aspxhttp://faostat.fao.org/sit e/562/ default.aspx Date of access: 13 October 2013.

FILLOUX, A., HACHANI, A., BLEVES, S. 2008. The bacterial type VI secretion machine: yet another player for protein transport across membranes. *Microbiology,* 154:1570-1583.

FISCHER, P.J., PETRINI, O. & SCOTT, H.L. 1992. The distribution of some fungal and bacterial endophytes in maize (Zea mays L.). *New Phytologist*, 122(2):299-305.

FREDERICKS, D.N. & RELMAN, D.A. 1996. Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates. *Clinical microbiology Reviews*, 9(1):18-33.

FUKUSHIMA, M., KAKINUMA, K. & KAWAGUCHI, R. 2002. Phylogenetic analysis of *Salmonella, Shigella*, and *Escherichia coli* strains on the basis of the *gyrB* gene sequence. *Journal of Clinical Microbiology*, 40(8):2779-2785.

GABRE-MADHIN, E. Z. & HAGGBLADE, S. 2004. Successes in African agriculture: results of an expert survey. *World Development*, *32*(5):745-766.

GALAN, J.E. & COLLMER, A. 1999.Type III secretion machines: bacterial devices for protein delivery into host cells. *Science*, 284(5418):1322-1328.

GARCÍA-MARTÍNEZ, J., ACINAS, S.G., ANTON, A.I. & RODRÍGUEZ-VALERA, F. 1999. Use of the 16S–23S ribosomal genes spacer region in studies of prokaryotic diversity. *Journal of Microbiological Methods*, *36*(1):55-64.

GARLAND, J.L. & MILLS, A.L. 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon source utilization. *Applied Environmental Microbiology*, 57:2351-2359.

GARRETT, K.A., DENDY, S.P., FRANK, E.E., ROUSE, M.N. & TRAVERS, S.E. 2006. Climate change effects on plant disease: genomes to ecosystems. *Annual Reviews of Phytopathology,* 44:489–509.

GAUMANN, E. 1950. Principles of plant infection. A text-book of general plant pathology for biologists, agriculturists, foresters and plant breeders. London: Crosby Lockwood.

GBETIBOUO, G.A. & HASSAN, R.M. 2004. Measuring the economic impact of climate change on major South African field crops: a Ricardian approach. *Global and Planetary Change,* 47:143-152.

GERLACH, R.G. & HENSEL, M. 2007. Protein secretion systems and adhesions: the molecular armoury of Gram-negative pathogens. *International Journal of Medical Microbiology*, 297(6):401-415.

GHINI, R., HAMADA, E. & BETTIOL, W. 2008. Climate change and plant diseases. *Scientia Agricola*, *65*:98-107.

GHOSH, P. 2004. Process of protein transport by the type III secretion system. *Microbiology and Molecular Biology Reviews*, 68(4):771-795.

GIESTER, L.J. & REES, J.M. 2004.Bacterial diseases of corn. St. Paul: Clay/Webster

GIRASE, M., BHOLAY, A.D. & PAWAR, K. 2012. Antimicrobial potential of *Lantana camara* against phytopathogens. *Journal of Industrial Research & Technology*, *2*(2):67-71.

GLEASON, C., HUANG, S., THATCHER, L.F., FOLEY, R.C., ANDERSON, C.R., CARROLL, A.J. & SINGH, K.B. 2011. Mitochondrial complex II has a key role in mitochondrial-derived reactive oxygen species influence on plant stress gene regulation and defense. *Proceedings of the national academy of sciences*, 108(26):10768-10773.

GÓMEZ-ZAVAGLIA, A., ABRAHAM, A., GIORGIERI, S. & DE ANTONIO, G. 1999. Application of polyacrylamide gel electrophoresis and capillary gel electrophoresis to the analysis of *Lactobacillus delbrueckii* whole-cell proteins. *Journal of Dairy Science*, 82:870-877.

GONZALES-VIGIL, E., BIANCHETTI, C.M., PHILLIPS, G.N. & HOWE, G.A. 2011. Adaptive evolution of threonine deaminase in plant defense against insect herbivores. *Proceedings of the National Academy of Sciences*, 108(14):5897-902.

GOSZCZYNSKA, T. & SERFONTEIN, J.S. 2000. Introduction to practical phytobacteriology. Pretoria: ARC-Plant Protection Research Institute,.

GOSZCZYNSKA, T., BOTHA, W.J., VENTER, S.N. & COUTINHO, T.A. 2007. Isolation and identification of the causal agent of brown stalk rot, a new disease of maize in South Africa. *Plant Disease*, 91:711-718.

GRACELIN, D.H.S, DE BRITTO, A.J. & KUMAR, B.J.R. 2012. Detection and identification of *Xanthomonas campestris* pv.*centellae* on leaves of *Centella asiatica* collected in Tamilnadu. *Asian Journal of Pharmaceutical and Clinical Research,* 5(1):111-113.

GRAYSTON, S.J., WANG, S., CAMPBELL, C.D. & EDWARDS, A.C. 1998. Selective influence of plant species on microbial diversity in the rhizosphere. *Soil Biology and Biochemistry*, *30*(3):369-378.

GREGORY, P.J., JOHNSON, S.N., NEWTON, A.C. & INGRAM, J.S.I. 2009. Integrating pests and pathogens into the climate change/food security debate. *Journal of Experimental Botany,* 60:2827-2838.

GRIMONT, F. & GRIMONT, P.A.D. 1986. Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. *Annales de l'Institut Pasteur/Microbiologie*, 137(1):165-175.

GUDESBLAT, G.E., TORRES, P.S., & VOJNOV, A.A. 2009. *Xanthomonas campestris* overcomes Arabidopsis stomatal immunity through a DSF cell-to-cell signal-regulated virulence factor. *Plant Physiology*, 149(2):1017-1027.

GURTLER, V. & STANISICH, V.A. 1996. New approaches to typing and identification of bacteria using the 16S–23S rDNA spacer region. *Microbiology*, 142:3-16.

HALLMANN, J., QUADT-HALLMANN, A., MAHAFFEE, W.F. & KLOEPPER, J.W.1997. Bacterial endophytes in agricultural crops. *Canadian Journal of Microbiology*, 43(10): 895-914.

HASHIMI, S.M. & BIRCH, R.G. 2010. Functional analysis of genes for benzoate metabolism in the albicidin biosynthetic region of *Xanthomonas albilineans. Applied Microbiology and Biotechnology*, 87:1475-1485.

HENDERSON, I.R., NAVARRO-GARCIA, F., DESVAUX, M. & FERNANDEZ, R.C. 2004. Type V protein secretion pathway: the autotransporter story. *Microbiology and Molecular Biology Reviews.*, 68*:* 692-744*.*

HILL, P.W., QUILLIAM, R.S., DELUCA, T.H., FARRAR, J. & FARRELL, M. 2011. Acquisition and assimilation of nitrogen as peptide-bound and D-enantiomers of amino acids by wheat. *PLOS ONE,* 6(4):e19220. doi:10.1371/journal.pone.0019220. Date of access: 3 Jan. 2012

HILLIS, D.M. & DIXON, M.T. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. *The Quarterly Review of Biology*, 66:411-453.

HORSFALL, J.G. & BARRAT, R.W. 1945. An improved grading system for measuring plant disease. *Phytopathology,* 35:655.

HOUNSA, C.G., BRANDT, E.V., THEVELEIN, J., HOHMANN, S. & PRIOR, B.A. 1998. Role of trehalose in survival of *Saccharomyces cerevisiae* under osmotic stress. *Microbiology*, 144(3):671-680.

HUANG, J., PRAY, C. & ROZELLE, S. 2002. Enhancing the crops to feed the poor. *Nature*, 418(6898):678-684.

HUECK, C.J. 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiology and Molecular Biology Reviews*, *62*(2):379-433.

HUGOUVIEUX, V., BARBER, C.E. & DANIELS, M.J. 1998. Entry of *Xanthomonas campestris* pv. *campestris* into hydathodes of *Arabidopsis thaliana* leaves: a system for studying early infection events in bacterial pathogenesis. *Molecular Plant-Microbe Interactions*, 11:537-543.

HUNT, D.E., KLEPAC-CERAJ, V., ACINAS, S.G., GAUTIER, C., BERTILSSON, S. & POLZ, M.F. 2006. Evaluation of 23S rRNA PCR primers for use in phylogenetic studies of bacterial diversity. *Applied and Environmental Microbiology*, 72(3):2221-2225.

IGNATOV, A.N., KORNEV, K.P., MATVEEVA, E.V., PEKHTEREVA, E.S., POLITYKO, V.A., BUDENKOV, N.I. & SCHAAD, N.W. 2007. Occurrence of bacterial spot and bacterial canker of tomato in the Russian Federation. (*In* II International Symposium on Tomato Diseases 808. p. 247-250).

JACOBS, M.J., BUGBEE, W.M. & GABRIELSON, D.A. 1985. Enumeration, location, and characterization of endophytic bacteria within sugar-beet roots. *Canadian Journal of Botany*, 63:1262-1265.

JANDA, J.M. & ABBOTT, S.L. 2007. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *Journal of Clinical Microbiology*, 45(9):2761-2764.

JANG, I.C., OH, S.J., SEO, J.S., CHOI, W.B., SONG, S.I., KIM, C.H. & KIM, J.K. 2003. Expression of a bifunctional fusion of the *Escherichia coli* genes for trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase in transgenic rice plants increases trehalose accumulation and abiotic stress tolerance without stunting growth. *Plant Physiology*, 131(2):516-524.

JAWF (Joint Agricultural Weather Facility). 1999. Major world crop areas and climate profiles. www.usda.gov/oce/waob/jawf/profiles/mwcacp2.html. Date of access: 7 Aug. 2013.

JHA, G., RAJESHWARI, R. & SONTI, R.V. 2005. Bacterial type two secretion system secreted proteins: double-edged swords for plant pathogens. *Molecular Plant-Microbe Interactions*, 18(9):891-898.

JI, P. & WILSON, M. 2002. Assessment of the importance of similarity in carbon source utilisation profiles between the biological control agent and the pathogen in biological control of bacterial speck of tomato. *Applied and Environmental Microbiology*, *68*(9):4383-4389.

JIMÉNEZ-HIDALGO, I., VIRGEN-CALLEROS, G., MARTÍNEZ-DE LA VEGA, O., VANDEMARK, G. & OLALDE-PORTUGAL, V. 2004. Identification and characterisation of bacteria causing soft-rot in *Agave tequilana*. *European Journal of Plant Pathology*, *110*(3):317-331.

JOGO, W., KARAMURA, E., TINZAARA, W., KUBIRIBA, J. & RIETVELD, A. 2013. Determinants of farm-level adoption of cultural practices for Banana *Xanthomonas* wilt control in Uganda. *Journal of Agricultural Science*, *5*(7):70.

JOHNSON, T.L., ABENDROTH, J., HOL, W.G. & SANDKVIST, M. 2006. Type II secretion: from structure to function. *FEMS Microbiology Letters*, 255(2):175-186.

JOLLIFFE, I.T. 2002. Principal Component Analysis. 2nd ed. New York: Springer.

JONES, J.B., BOUZAR, H., STALL, R.E., ALMIRA, E.C., ROBERTS, P.D., BOWEN, B.W. & CHUN, J. 2000. Systematic analysis of xanthomonads (*Xanthomonas* spp.) associated with pepper and tomato lesions. *International Journal of Systematic and Evolutionary Microbiology*, *50*(3):1211-1219.

JONES, J.B., CHASE, A.R. & HARRIS, G.K. 1993. Evaluation of the Biolog GN microplate system for identification of some plant pathogenic bacteria. *Plant Disease*, 77:553-558.

JONES, P.G. & THORNTON, P.K. 2003. The potential impacts of climate change on maize production in Africa and Latin America in 2055. *Global Environmental Change,* 13:51-59.

KAAKOUSH, N.O., DESHPANDE, N.P., WILKINS, M.R., RAFTERY, M.J., JANITZ, K. & MITCHELL, H. 2011. Comparative analyses of *Campylobacter concisus* strains reveal the genome of the reference strain BAA-1457 is not representative of the species. *Gut Pathogens,* 3:15.

KÄMPFER, P., BUCZOLITS, S., ALBRECHT, A., BUSSE, H.J. & STACKEBRANDT, E. 2003. Towards a standardized format for the description of a novel species (of an established genus): *Ochrobactrum gallinifaecis* sp. nov. *International Journal of Systemic Bacteriology,* 53:893-896.

KATZEN, F., FERREIRO, D.U., ODDO, C.G., IELMINI, M.V. & BECKER, A. 1998. *Xanthomonas campestris* pv. *campestris* gum mutants: effects on xanthan biosynthesis and plant virulence. *Journal of Bacteriology*, 180(7):1607-1617.

KHATRI, B., FIELDER, M., JONES, G., NEWELL, W., ABU-OUN, M. & WHEELER, P.R. 2013. High throughput phenotypic analysis of *Mycobacterium tuberculosis* and *Mycobacterium bovis* strains' metabolism using biolog phenotype microarrays. *PLOS ONE*, *8*(1):e52673.

KHOODOO, M.H.R., SAHIN, F., DONMEZ, M.F. & JAUFEERALLY FAKIM, Y. 2004. Molecular characterisation of *Xanthomonas* strains isolated from aroids in Mauritius. *Systematic and Applied Microbiology*, 28:366-380.

KIELAK, A.M., CRETOIU, M.S., SEMENOV, A.V., SØRENSEN, S.J. & VAN ELSAS, J.D. 2013. Bacterial chitinolytic communities respond to chitin and pH alteration in soil. *Applied and Environmental Microbiology*, *79*(1):263-272.

KIRALY, Z., EL-ZAHABY, H.M. & KLEMENT, Z. 1997. Role of extracellular polysaccharide (EPS) slime of plant pathogenic bacteria in protecting cells to reactive oxygen species. *Journal of Phytopathology*, 145(2-3):59-68.

KJER, K.M. 1995. Use of rRNA secondary structure in phylogenetic studies to identify homologous positions: an example of alignment and data presentation from the frogs. *Molecular Phylogenetics and Evolution,* 4:314-330.

KLAPPENBACH, J.A., Dunbar, J.M. & Schmidt, T.M. 2000. rRNA operon copy number reflects ecological strategies of bacteria. *Applied and Environmental Microbiology*, 66:1328-1333.

KLOEPPE, J. W., RODRIGUEZ-KABANA, R., ZEHNDER, A.W., MURPHY, J.F., SIKORA, E., & FERNANDEZ, C. 1999. Plant root-bacterial interactions in biological control of soilborne diseases and potential extension to systemic and foliar diseases. *Australasian Plant Pathology*, 28(1):21-26.

KLOPPERS, R. & TWEER, S. 2009. Maize diseases – bacterial leaf streak. http://www.plantwise.org/FullTextPDF/2011/20117800332.pdf. Date of access: 4 October 2011.

KOCKS, C.G., RUISSEN, M.A., ZADOKS, J.C. & DUIJKERS, M.G. 1998. Survival and extinction of *Xanthomonas campestris* pv. *campestris* in soil. *European Journal of Plant Pathology*, 104(9):911-923.

KOLBERT, C.P. & PERSING, D.H. 1999. Ribosomal DNA sequencing as a tool for identification of bacterial pathogens. *Current Opinion in Microbiology*, 2(3):299-305.

KONOPKA, A., OLIVER, L. & TURCO, R. 1998. The use of carbon substrate utilization patterns in environmental and ecological microbiology. *Microbial Ecology*, 35(2):103-15.

KORUS, K.A. 2011. Evaluating commercially available diagnostic tests for the detection of *Clavibacter michiganensis* subsp. *nebraskensis,* cause of Goss's bacterial wilt and leaf blight in corn. Nebraska: University of Nebraska. (Thesis – MSc).

KRAWCZYK, K., KAMASA, J., ZWOLINSKA, A. & POSPIESZNY, H. 2010. First report of *Pantoea ananatis* associated with leaf spot disease of maize in Poland. *Journal of Plant Pathology*, 92(3):807-811.

KRITTIDETCH, A., CHUABOON, W., & ATHINUWAT, D. 2013. Detection of *Acidovorax avenae* subsp. *avenae* in commercial corn seeds and its correlation with seedling transmission. *African Journal of Biotechnology*, 12(45): 6376-6381.

LAEMMLI, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227:680-685.

LAMKA, G.L., HILL, J.D., MCGEE, D.C. & BRAUN, E.J. 1991. Development of an immunosorbent assay for seedborne *Erwinia stewartii* in corn seeds. *Phytopathology*, 81:839-846.

LAPAGE, S.P., SNEATH, P.H.A., LESSEL, E.F., SKERMAN, V.B.D., SEELIGER, H.P.R. & CLARK, W.A., *eds*. 1992. International Code of Nomenclature of Bacteria (1990 Revision). Bacteriological Code. Washington: American Society for Microbiology.

LAWLOR, D.W. 2002. Limitation of photosynthesis in water-stressed leaves. Stomatal metabolism and the role of ATP. *Annals of Botany*, 89:871-885.

LEBLOND-BOURGET, N., PHILIPPE, H., MANGIN, I. & DECARIS, B. 1996. 16S rRNA and 16S to 23S internal transcribed spacer sequence analyses reveal inter- and intraspecific *Bifidobacterium phylogeny. International Journal of Systemic Bacteriology,* 46:102-111.

LEFFERS, H., KJEMS, J., OSTERGAARD, L., LARSEN, N. & GARRETT, R.A. 1987. Evolutionary relationships amongst archaebacteria. A comparative study of 23 S ribosomal RNAs of a sulphur-dependent extreme thermophile, an extreme halophile and a thermophilic methanogen. *Journal of Molecular Biology*, 195:43-61.

LEITE, R.P., MINSAVAGE, G.V., BONAS, U. & STALL, R.E. 1994. Detection and identification of phytopathogenic *Xanthomonas* strains by amplification of DNA sequences related to the *hrp* genes of *Xanthomonas campestris* pv. *vesicatoria*. *Applied Environmental Microbiology*, 60(4):1068-1077.

LEWIS, I.M.L., TUSIIME, G. & MILLER, S.A. 2010. A polymerase chain reaction assay for the detection of *Xanthomonas campestris* pv. *musacearum* in banana. *Plant Disease*, 94(1):109-114.

LEYNS, F., DE CLEENE, M., SWINGS, J. & DE LEY, J. 1984. The host range of the genus *Xanthomonas*. *Botanical Review*, 50(3):305-355.

LIGUORI, A.P., WARRINGTON, S.D., GINTHER, J.L., PEARSON, T., BOWERS, J., GLASS, M.B., MAYO, M., WUTHIEKANUN, V., ENGELTHALER, D., PEACOCK, S.J., CURRIE, B.J., WAGNER, D.M., KEIM, P. & TUANYOK, A. 2011. Diversity of 16S-23S rDNA internal transcribed spacer (ITS) reveals phylogenetic relationships in *Burkholderia pseudomallei* and its near-neighbors. *PLOS ONE*, 6(12):e29323.

LIMA, W.C., PAQUOLA, A.C.M., VARANI, A.M., VAN SLUYS, M.A. & MENCK, C.F.M. 2008. Laterally transferred genomic islands in Xanthomonadales related to pathogenicity and primary metabolism. *FEMS Microbiology Letters*, 281(1):87-97.

LOBELL, D. B., HAMMER, G. L., MCLEAN, G., MESSINA, C., ROBERTS, M. J., & SCHLENKER, W. 2014. Reply to'Temperature and drought effects on maize yield'. *Nature Climate Change*, *4*(4);234-234.

LODEWYCKX, C., VANGRONSVELD, J., PORTEOUS, F., MOORE, E.R., TAGHAVI, S., MEZGEAY, M. & DER LELIE, D.V. 2002. Endophytic bacteria and their potential applications. *Critical Reviews in Plant Sciences*, 21(6):583-606.

LÓPEZ, N.I., HAEDO, A.S. & MÉNDEZ, B.S. 1999. Evaluation of *Xanthomonas campestris* survival in a soil microcosm system. *International Microbiology*, 2(2):111-114.

LU, D., SUN, X., YAN, F., WANG, X., XU, R. & LU, W. 2013. Effects of high temperature during grain filling under control conditions on the physicochemical properties of waxy maize flour. *Carbohydrate Polymers*, 98(1):302-310.

LUDWIG, W. & SCHLEIFER, K.H. 1994. Bacterial phylogeny based on 16S and 23S rRNA sequence analysis. *Microbiology Reviews*, 15(2):155-173.

LUHUNGA, P.M. & MUTAYOBA, E. 2013. Variability of stability, momentum and heat fluxes in the stable boundary layer over highveld priority area, South Africa. *Applied Physics Research*, 5(4):23.

LYONS, M.M. & DOBBS, F.C. 2012. Differential utilization of carbon substrates by aggregate-associated and water-associated heterotrophic bacterial communities. *Hydrobiologia*, *686*(1):181-193.

MAGGI, R.G. & BREITSCHWERDT, E.B. 2005. Potential limitations of the 16S-23S rRNA intergenic region for molecular detection of *Bartonella* species. *Journal of Clinical Microbiology*, *43*(3):1171-1176.

MAHLASTI, T. A. 2012. Characterization of heavy metal tolerant bacterial plasmids isolated form a platinum mine tailings dam. Potchefstroom: NWU (Dissertation – M.Sc).

MANSOUR, M.M.F. 2000. Nitrogen containing compounds and adaptation of plants to salinity stress. *Journal of Plant Biology*. 43:491-500.

MASSOMO, S.M., NIELSEN, H., MABAGALA, R.B., MANSFELD-GIESE, K., HOCKENHULL, J. & MORTENSEN, C.N. 2003. Identification and characterisation of *Xanthomonas campestris* pv. *campestris* strains from Tanzania by pathogenicity tests, biolog, rep-PCR and fatty acid methyl ester analysis. *European Journal of Plant Pathology*, 109(8): 775-789.

MAUDE, R.B. 1996. Seedborne diseases and their control: principles and practice. Wallingford: CABI.

MAYEK-PEREZ, N., GARCIA-ESPINOSA, R., LOPEZ-CASTANEDA, C., ACOSTA-GALLEGOS, J.A. & SIMPSON, J. 2002. Water relations, histopathology and growth of common bean (*Phaseolus vulgaris* L.) during pathogenesis of *Macrophomina phaseolina* under drought stress. *Physiological and Molecular Plant Pathology*, 60:185-95.

MBEGA, E.R., MABAGALA, R.B., ADRIKO, J., MORTENSEN, C.N., LUND, O.S., & WULFF, E.G. 2011. Sampling-detection procedures: bacterial leaf spot pathogen of tomato. Copenhagen: University of Copenhagen.

MCINERNEY, M.J., STRUCHTEMEYER, C.G., SIEBER, J., MOUTTAKI, H., STAMS, A.J., SCHINK, B. & GUNSALUS, R.P. 2008. Physiology, ecology, phylogeny, and genomics of microorganisms capable of syntrophic metabolism. *Annals of the New York Academy of Sciences*, 1125(1):58-72.

MCINROY, J.A. & KLOEPPER, J.W. 1995. Survey of indigenous bacterial endophytes from cotton and sweet corn. *Plant and Soil*, 173(2):337-342.

MEASURES, J.C. 1975. Role of amino acids in osmoregulation of non-halophilic bacteria. *Nature*, 257, 398-400.

MEHTRE, R. 2010. Cloning of endochitinase genes from *Trichoderma* spp. and expression study in *Saccharomyces cerevisiae* INVSc1. Dharwad: University of Agricultural Sciences. (Thesis – Phd).

MENG, X.Q., UMESH, R.M., DAVIS, R.M. & GILBERTSON, R.L. 2004. Development of PCR-based assays for detecting *Xanthomonas campestris* pv.

carotae, the carrot bacterial leaf blight pathogen, from different substrates. *Plant Disease,* 88:1226-1234.

MERGAERT, J., VERDONCK, L. & KERSTERS, K. 1993. Transfer of *Erwinia ananas* (synonym, *Erwinia uredovora)* and *Erwinia stewartii* to the genus *Pantoea* emend. as *Pantoea ananas* (Serrano 1928) comb. nov. and *Pantoea stewartii* (Smith 1898) comb. nov., respectively, and description of *Pantoea stewartii* subsp. *indologenes* subsp. nov. *International Journal of Systemic Bacteriology,* 43:162-173.

MHEDBI-HAJRI, N., HAJRI, A., BOUREAU, T., DARRASSE, A., DURAND, K., BRIN, C., FISCHER-LE SAUX, M., MANCEAU, C., POUSSIER, S., PRUVOST, O., LEMAIRE, C. & JACQUES, M. 2013. Evolutionary history of the plant pathogenic bacterium *Xanthomonas axonopodis*. *PLOS ONE*, 8(3):e58474.

MIGNARD, S. & FLANDROIS, J.P. 2006. 16S rRNA sequencing in routine bacterial identification: a 30-month experiment. *Journal of Microbiological Methods*, 67(3):574-581.

MITCHELL, C.E., TILMAN, D. & GROTH, J.V. 2002. Effects of grassland plant species diversity, abundance, and composition on foliar fungal disease. *Ecology*, 83:1713-1726.

MOHAMMADKHANI, N. & HEIDARI, R. 2008. Drought-induced Accumulation of Soluble Sugars and Proline in Two Maize Varieties. *World Applied Sciences Journal* 3 (3):448-453.

MOLLET, C., DRANCOURT, M. & RAOULT, D. 1997. rpoB sequence analysis as a novel basis for bacterial identification. *Molecular Microbiology*, *26*(5):1005-1011.

MONDAL, K.K., VERMA, G. & MANI, C. 2013. Phylogenetic relatedness of *Xanthomonas axonopodis* pv. *punicae*, the causal agent of bacterial blight of pomegranate based on two loci, 16S rRNA and *gyrB*. *Annals of Microbiology*, 63(2):801-804.

MORGAN, M.C., BOYETTE, M., GOFORTH, C., SPERRY, K.V. & GREENE, S.R. 2009. Comparison of the Biolog OmniLog Identification System and 16S ribosomal

RNA gene sequencing for accuracy in identification of atypical bacteria of clinical origin. *Journal of Microbiological Methods*, 79:336-343.

MUKHALA, E. 1998. Radiation and water utilization efficiency by mono-culture and intercrop to suit small scale irrigation farming. Department of Agrometeorology. Bloemfontein: University of Orange Free State. (Thesis - Phd).

NDA. 2005. Maize profile. http://www.daff.gov.za/docs/FactSheet/maize.htm Pretoria: National Department of Agriculture. Date of access: 21 Aug. 2013

NICHOLSON, S.E., SOME, B. & KONE, B. 2000. An analysis of recent rainfall conditions in West Africa, including the rainy seasons of the 1997 E1 Nino and the 1998 La Nina years. *Journal of Climate* 13:2628-2640.

NIEMANN, N.J.J. 2014. Molecular characterisation of the causal agent of bacterial leaf streak of maize. Potchefstroom: NWU (Dissertation – M.Sc).

NOCHI, Z., SAHEBEKHTIARI, N., KHARAZIHA, P., CHIANI, M., MOTAVAZE, K., DABIRI, H. & NOORINAYER, B. 2009. Comparison of 16S rRNA, 23S rRNA and *gyrB* genes sequences in phylogenetic relationships of *Shigella* isolates from Iran. *Annals of Microbiology*, 59(3):615-622.

NOWBUTH, P., KHITTOO, G. & VENKATASAMY, S. 2005. Phenotypic diversity of *Xanthomonas* strains isolated from onion blight using metabolic fingerprinting (*MAS 375 - 381*). Mauritius: University of Mauritius.

NUNEZ, J.J., GILBERTSON, R.L., MENG, X. & DAVIS, R.M. 2002. First report of *Xanthomonas* leaf blight of onion in California. *Plant Disease*, 86(3):330-330.

OERKE, E.C. 2006. Crop losses to pests. *Journal of agricultural science*, 144:31-43.

OJANEN-REUHS, T., HELANDER, I.M., HAAHTELA, K., KORHONEN, T.K. & LAAKSO, T. 1993. Outer membrane proteins and lipopolysaccharides in pathovars of *Xanthomonas campestris*. *Applied and Environmental Microbiology*, 59(12):4143-4151.

OJHA, B., TIWARI, R. & CHANDRAVANSHI, S. 2012. Evaluation of some medicinal plant species for their antibacterial activity against *Xanthomonas campestris* pv. *campestris*, the black rot pathogen of cabbage. *Indian Phytopathology*, 57(3):308-311.

ORIO, A.G., BRÜCHER, E., PLAZAS, M. C., SAYAGO, P., GUERRA, F., DE ROSSI, R., DUCASSE, D.A., & GUERRA, G.D. 2012. First Report of Stewart's Wilt of Maize in Argentina Caused by *Pantoea stewartii*. *Plant Disease,* 96(12):1819-1819.

PARKINSON, N., COWIE, C., HEENEY, J. & STEAD, D. 2009. Phylogenetic structure of *Xanthomonas* determined by comparison of *gyrB* sequences. *International Journal of Systematic and Evolutionary Microbiology*, 59(2):264-274.

PATAKY, J.K., SUPARYONO, H.J.A., GARDINER, M.L. & PAULY, M.H. 1990. Associations between Stewart's wilt ratings and maturity of sweet corn hybrids. *Plant Disease*, 74:792-796.

PATEL, J.B. 2001. 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. *Journal of Molecular Diagnostics*, 6:313-321.

PATRIQUIN, D.G. & DOBEREINER, J. 1978. Light-microscopy observations of tetrazolium-reducing bacteria in endorhizosphere of maize and other grasses in Brazil. *Canadian Journal of Microbiology,* 24:734-742.

PÉCHY-TARR, M., BOTTIGLIERI, M., MATHYS, S., BANG LEJBØLLE, K., SCHNIDER-KEEL, U., MAURHOFER, M. & KEEL, C. 2005. RpoN (σ54) controls production of antifungal compounds and biocontrol activity in *Pseudomonas fluorescens* CHA0. *Molecular Plant-Microbe Interactions,* 8:260-272.

PETTI, C.A., POLAGE, C.R. & SCHRECKENBERGER, P. 2005. The role of 16S rRNA gene sequencing in identification of microorganisms misidentified by conventional methods. *Journal of Clinical Microbiology*, 43(12):6123-6125.

PIERETTI, I., ROYER, M., BARBE, V., CARRERE, S., KOEBNIK, R., COULOUX, A. & COCIANCICH, S. 2012. Genomic insights into strategies used by *Xanthomonas*

albilineans with its reduced artillery to spread within sugarcane xylem vessels. *BMC Genomics*, 13(1):658.

PLIKAYTIS, B.D., CARLONE, G.M. & PLIKAYTIS, B.B. 1986. Numerical analysis of normalized whole-cell protein profiles after sodium dodecyl sulphate-polyacrylamide gel electrophoresis. *Journal of General Microbiology*, 132:2653-2660.

POPLAWSKY, A.R., KAWALEK, M.D. & SCHAAD, N.W. 1993. A xanthomonadin encoding gene cluster for the identification of pathovars of *Xanthomonas campestris*. *Molecular Plant-Microbe Interactions*, 6(5):545-552.

POT, B., VANDAMME, P. & KERSTERS, K. 1994. Analysis of electrophoretic whole-organism protein fingerprints. (*In* Goodfellow, M. & O'Donnel, A.G., *eds*. Chemical methods in prokaryotic systematic. New York: Wiley. p.493-243).

POUSSIER, S., TRIGALET-DEMERY, D., VANDEWALLE, P., GOFFINET, B., LUISETTI, J. & TRIGALET, A. 2000. Genetic diversity of *Ralstonia solanacearum* as assessed by PCR-RFLP of the hrp gene region, AFLP and 16S rRNA sequence analysis, and identification of an African subdivision. *Microbiology*, *146*(7):1679-1692.

PRATHUANGWONG, S., JUTHWANTHANA, P., KETSUWAN, K., & KASEM, S. 2004. An outbreak of new bacterial disease of corn in Thailand. Paper presented at 1st Kasetsart University Corn and sorghum research program workshop, Ayutthaya, Thailand, 15-21 May 2004 www.lib.ku.th/KU?CR000320040030.pfd Date of access: 27 June 2014.

PRESTON, G.M., STUDHOLME, D.J. & CALDELARI, I. 2005. Profiling the secretomes of plant pathogenic *Proteobacteria*. *FEMS Microbiology Reviews*, 29(2):331-360.

PRESTON-WHYTE, R.A. & TYSON, P.D. 1993. The atmosphere and weather of Southern Africa. New York: Oxford University Press. p. 207-249.

QHOBELA, M., CLAFLIN, L.E. & NOWELL, D.C. 1990. Evidence that *Xanthomonas campestris* pv. *zeae* can be distinguished from other pathovars capable of infecting

maize by restriction fragment length polymorphism (RFLP) of genomic DNA. *Canadian Journal of Plant Pathology*, 12:183-186.

QUINET, M., NDAYIRAGIJE, A., LEFÈVRE, I., LAMBILLOTTE, B., DUPONT-GILLAIN, C.C. & LUTTS, S. 2010. Putrescine differently influences the effect of salt stress on polyamine metabolism and ethylene synthesis in rice cultivars differing in salt resistance. *Journal of Experimental Botany*, 61:2719-2733.

RADEMAKER, J.L.W., HOSTE, B., LOUWS, F.J., KERSTERS, K. & SWINGS, J. 2000. Comparison of AFLP and rep-PCR genomic fingerprinting with DNA-DNA homology studies: *Xanthomonas* as a model system. *International Journal of Systematic Evolutionary Microbiology*, 50(2):665-677.

RAHME, L.G., MINDRINOS, M.N. & PANOPOULOS, N.J. 1992. Plant and environmental sensory signals control the expression of *hrp* genes in *Pseudomonas syringae* pv. *phaseolicola. Journal of Bacteriology*, 174(11):3499-3507.

RAJENDHRAN, J. & GUNASEKARAN, P. 2011. Microbial phylogeny and diversity: small subunit ribosomal RNA sequence analysis and beyond. *Microbiological Research*, 166(2):99-110.

RANGASWAMI, G. & RANGARAJAN, M. 1965. A bacterial leaf-spot disease of banana. *Phytopathology,* 55:1035–1036.

REINHOLD-HUREK, B. & HUREK, T. 1998. Life in grasses: diazotrophic endophytes. *Trends in Microbiology*, 6(4):139-144.

RELMAN, D.A. 1999. The search for unrecognized pathogens. *Science*, *284*(5418):1308-1310.

REZZONICO, F., SMITS T., MONTESINOS E., FREY J.E. & DUFFY, B. 2009. Genotypic comparison of *Pantoea agglomerans* plant and clinical strains. *BMC Microbiology*, 9: 204.

RIJAVEC, T., LAPANJE, A., DERMASTIA, M. & RUPNIK, M. 2007. Isolation of bacterial endophytes from germinated maize kernels. *Canadian Journal of Microbiology*, 53(6): 802-808.

ROCAP, G., DISTEL, D.L., WATERBURY, J.B. & CHISHOLM, S.W. 2002. Resolution of *Prochlorococcus* and *Synechococcus* ecotypes by using 16S-23S ribosomal DNA internal transcribed spacer sequences. *Applied and Environmental microbiology*, 68(3):1180-1191.

ROGALL, T., WOLTERS, J., FLOHR, T. & BÖTTGER, E.C. 1990. Towards a phylogeny and definition of species at the molecular level within the genus *Mycobacterium*. *International Journal of Systematic Bacteriology*, 40(4):323-330.

ROOS, I.M. & HATTINGH, M.J. 1987. Pathogenicity and numerical analysis of phenotypic features of *Pseudomonas syringae* strains isolated from deciduous fruit trees. *Phytopathology*, *77*(6):900-908.

ROSENBLUETH, M. & MARTÍNEZ-ROMERO, E. 2006. Bacterial endophytes and their interactions with hosts. *Molecular Plant-microbe Interactions*, 19(8):827-837.

ROUMAGNAC, P., GAGNEVIN, L., GARDAN, L., SUTRA, L., MANCEAU, C., DICKSTEIN, E. R. & PRUVOST, O. 2004. Polyphasic characterization of xanthomonads isolated from onion, garlic and Welsh onion (*Allium* spp.) and their relatedness to different *Xanthomonas* species. *International Journal of Systematic and Evolutionary Microbiology*, 54(1):15-24.

RUHL, G., WISE, K., CRESWELL, T., LEONBERGER, A., & SPEERS, C. 2009. First report of Goss's bacterial wilt and leaf blight on corn caused by *Clavibacter michiganensis* subsp. *nebraskensis* in Indiana. *Plant Disease Notes,* 93:841.

RYAN, R.P., VORHÖLTER, F.J., POTNIS, N., JONES, J.B., VAN SLUYS, M.A., BOGDANOVE, A.J. & DOW, J.M. 2011. Pathogenomics of *Xanthomonas*: understanding bacterium-plant interactions. *Nature Reviews Microbiology*, 9(5):344-355.

SADEGHIFARD, N., GÜRTLER, V., BEER, M. & SEVIOUR, R.J. 2006. The mosaic nature of intergenic 16S-23S rRNA spacer regions suggests rRNA operon copy number variation in *Clostridium difficile* strains. *Applied and Environmental Microbiology*, 72(11):7311-7323.

SAILE, E., MCGARVEY, J.A., SCHELL, M.A. & DENNY, T.P. 1997. Role of extracellular polysaccharide and endoglucanase in root invasion and colonization of tomato plants by *Ralstonia solanacearum*. *Phytopathology*, 87(12):1264-1271.

SAIRAM, R.K. & TYAGI, A. 2004. Physiology and molecular biology of salinity stress tolerance in plants. *Current Science*, 86(3):407-421.

SAMANTA, J.N., & MANDAL, K. 2014. Assessment of intrapathovar variability of *Xanthomonas axonopodis* pv. *commiphorae* through phenotypic and molecular markers. *Forest Pathology*, 44:85-95.

SAMANTA, J.N., MANDAL, K. & MAITI, S. 2013. A novel pathovar of *Xanthomonas axonopodis* causes gumming of Guggal (Commiphora wightii). *European Journal of Plant Pathology*, 135(1):115-125.

SAUER, S. & KLIEM, M. 2010. Mass spectrometry tools for the classification and identification of bacteria. *Nature Reviews Microbiology*, 8(1):74-82.

SCHAAD, N.W. & DIANESE, J.C. 1981. Cruciferous weeds as sources of inoculum of *Xanthomonas campestris* in black rot of crucifers. *Phytopathology*, 71(11):1215-1220.

SCHAAD, N.W., KADO, D.R. 1975. Sumner, synonymy of *Pseadomonas avenae* Manns 1905 and *Pseadomonas alboprecipitans* Rosen 1922. *International journal of Systemic Bacteriology*, 25:133-137.

SCHAAD, N.W., POSTNIKOVA, E., SECHLER, A., CLAFLIN, L.E., VIDAVER, A.K., JONES, J.B., AGARKOVA, I., IGNATOV, A., DICKSTEIN, E. &RAMUNDO, B.A. 2008. Reclassification of subspecies of *Acidovorax avenae* as *A. avenae* (manns 1905) emend., *A. cattleyae* (Pavarino, 1911) comb. nov., *A. citulli* Schaad *et al.,*1978) comb. nov., and proposal of *A. oryzae* sp.nov. *Systemic and Applied Microbiology*, 31:434-446.

SCHÄGGER, H. & VON JAGON, G. 1987. Tricine-sodium dodecyl sulphate polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry*, 166(2):368-370.

SCHERM, H. & YANG, X.B. 1995. Interannual variations in wheat rust development in China and the United States in relation to the El Nino/Southern Oscillation. *Phytopathology*, 85(9):970-976.

SCHULTE, R. & BONAS, U. 1992. Expression of the *Xanthomonas campestris* pv. *vesicatoria hrp* gene cluster, which determines pathogenicity and hypersensitivity on pepper and tomato, is plant inducible. *Journal of Bacteriology*, 174(3):815-823.

SCHULZE, R.E. & MAHARAJ, M. 2007. Rainfall seasonality. (*In* Schulze, R.E., *ed*. South African Atlas of Climatology and Agrohydrology. Pretoria: Water Research Commission. WRC Report, 1489/1/06, Section 6.5).

SCHULZE, R.E. 1997. South African atlas of agrohydrology and -climatology. Pretoria: Water Research Commission, TT82/96.

SEGHERS, D., WITTEBOLLE, L., TOP, E.M., VERSTRAETE, W. & SICILIANO, S.D. 2004. Impact of agricultural practices on the *Zea mays* L. endophytic community. *Applied Environmental Microbiology,* 70:1475-1482.

SINTCHENKO, V., IREDELL, J.R. & GILBERT, G.L. 2007. Pathogen profiling for disease management and surveillance. *Nature Reviews Microbiology*, 5(6):464-470.

SMIDT, M. & VIDAVER, A.K. 1986. Population dynamics of *Clavibacter michiganensis* subsp. *nebraskensis* in field-grown dent corn and popcorn. *Plant Disease*, 70(11):1031-1036.

SONTAKKE, S., CADENAS, M.B., MAGGI, R.G., DINIZ, P.P.V. & BREITSCHWERDT, E. B. 2009. Use of broad range16S rDNA PCR in clinical microbiology. *Journal of Microbiological Methods*, 76(3):217-225.

SOUDI, M.R., ALIMADADI, N. & GHADAM, P. 2011. Minimal phenotypic test for simple differentiation of *Xanthomonas campestris* from other yellow-pigmented bacteria isolated from soil. *Iranian Journal of Microbiology*, *3*(2):84.

South Africa. DOA (Department of Agriculture). 2007. Strategic plan for the Department of Agriculture. Pretoria. p.12 -17.

STACKEBRANDT, E. 2011. Pitfalls of PCR-based rRNA gene sequence analysis: an update on some parameters. (*In* De bruijn, F.J., *ed.* Handbook of molecular microbial ecology I: metagenomics and complementary approaches. p. 129-133).

STARR, M.P. & STEPHENS, W.L. 1964. Pigmentation and taxonomy of the genus *Xanthomonas*. *Journal of Bacteriology*, 87(2):293-302.

STARR, M.P. 1981. The genus *Xanthomonas*, p. 742–763. (*In* Starr, M.P., Stolp, H., Truper, H.G., Balows, A., & Schlegel, H.G., *eds.* The prokaryotes, vol. I. Berlin: Springer-Verlag).

STEFANOWICZ, A. 2006. The Biolog plates technique as a tool in ecological studies of microbial communities. *Polish Journal of Environmental studies*, 15(5):669.

STOCK, I., GRUGER T. & WIEDEMANN, B. 2001. Natural antibiotic susceptibility of strains of the *Enterobacter cloacae* complex. *International Journal of Antimicrobial Agents*,18:537-545

STONE, B.A. & CLARKE, A.E. 1992. Chemistry and physiology of higher plant 1,3-*β* glucans (callose). (*In* Stone, B.A., & Clarke, A.E., *eds*. Chemistry and Biology of (1-3)-*β*-Glucans., Bundoora: La Trobe University Press. p. 365–429).

STRANGE, R.N. & SCOTT, P.R. 2005. Plant disease: a threat to global food security. *Annual Review of Phytopathology*, 43:83-116.

STUCKEY, E., NIBLACK, T.L., NYVALL, R.F., KRAUSZ, J.P. & HORNE, C.W. 1993. National corn handbook - Corn Disease Management.Iowa State University. http://corn.agronomy.wisc.edu/Management/pdfs/NCH10.pdf. Date of access: 4 July 2013.

STURZ, A.V., CHRISTIE, B.R. & NOWAK, J. 2000. Bacterial endophytes: potential role in developing sustainable systems of crop production. *Critical Reviews in Plant sciences,* 19:1-30.

SUN, L., LI, H. & WARD, N.M. 2007. Climate variability and corn yields in semiarid Ceara, Brazil. *Journal of Applied Meteorology*, 46:226-240.

SUPARYONO, H.J.A. & PATAKY, J.K. 1989. Relationships between incidence and severity of Stewart's and Goss's bacterial witls and yield of sweet corn hybrids. *Crop Protection*, 8:363-368.,

TADROSS, M., DAVIS, C., ENGELBRECHT, F., JOUBERT, A. & ACRCHER VAN GARDEREN, E. 2011. Regional scenarios of future climate change over Southern Africa. (*In* Climate risk and vulnerability: a handbook for Southern Africa. Davis, C.L., *ed.* Pretoria: CSIR).

TANG, X., XIAO, Y. & ZHOU, J.M. 2006. Regulation of the type III secretion system in phytopathogenic bacteria. *Molecular Plant-Microbe Interactions*, 19(11):1159-1166.

TECHATI, N. 2008. Identification and genetic diversity of bacterial leaf streak of corn. Bangkok – Kasetsart University (Thesis – M.Sc).

TESSO, T., PERUMAL, R., LITTLE, C., ADEYANJU, A., RADWAN, G., PROM, L. & MAGILL, C. 2012. Sorghum pathology and biotechnology-A fungal disease perspective: Part II. Anthracnose, stalk rot, and downy mildew. *The European Journal of Plant Science and Biotechnology*, *6*: 31-44.

THITISAKSAKUL, M., JIMÉNEZ, R.C., ARIAS, M.C. & BECKLES, D.M. 2012. Effects of environmental factors on cereal starch biosynthesis and composition. *Journal of Cereal Science,* 56(1):67-80.

THOMSON-CARTER, F.M. & PENNINGTON, T.H. 1989. Characterization of coagulase-negative *Staphylococci* by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblot analyses. *Journal of Clinical Microbiology*, 27:2199-2203.

TITTONELL, P., SHEPHERD, K.D., VANLAUWE, B. & GILLER, K.E. 2008. Unravelling the effects of soil and crop management on maize productivity in smallholder agricultural systems of western Kenya — an application of classification and regression tree analysis. *Agriculture, Ecosystems and Environment,* 123:137-150.

TOSIAH, S. 2013. Carbon utilization pattern of *Exserohilum monoceras* isolates from Malaysian rice fields. *Journal of Tropical Agriculture and Food Science,* 41(1):109-118.

VAN DEN MOOTER, M. & SWINGS, J. 1990. Numerical analysis of 295 phenotypic features of 266 *Xanthomonas* strains and related strains and an improved taxonomy of the genus. *International Journal of Systemic Bacteriology*, 40(4): 348-369.

VAN DIJK, K. & NELSON, E.B. 1998. Inactivation of seed exudate stimulants of *Pythium ultimum* sporangium germination by biocontrol strains of *Enterobacter cloacae* and other seed-associated bacteria. *Soil Biology and Biochemistry*, 30(2):183-192.

VAN DIJK, K., FOUTS, D.E., REHM, A.H., HILL, A.R., COLLMER, A. & ALFANO, J.R. 1999. The Avr (effector) proteins HrmA (HopPsyA) and AvrPto are secreted in culture from *Pseudomonas syringae* pathovars via the Hrp (type III) protein secretion system in a temperature- and pH-sensitive manner. *Journal of Bacteriology,* 181:4790-4797.

VAN TIENHOVEN, A.M., ZUNCKEL, M., EMBERSON, L., KOOSAILEE, A. & OTTER, L. 2006. Preliminary assessment of risk of ozone impacts to maize (*Zea mays*) in Southern Africa. *Environmental Pollution*, 140(2):220-30.

VAUTERIN, L., HOSTE, B., KERSTERS, K. & SWINGS, J. 1995. The relationship within genus *Xanthomonas* and a proposal for a new classification. *International Journal of Systematic Bacteriology*, 45(3):472-489.

VAUTERIN, L., RADEMAKER, J. & SWINGS, J. 2000. Synopsis on the taxonomy of the genus *Xanthomonas*. *Phytopathology*, 90(2):677-682.

VAUTERIN, L., SWINGS, J. & KERSTERS, K. 1991. Grouping of *Xanthomonas campestris* pathovars by SDS-PAGE of proteins. *Journal of General Microbiology*, 137(7):1677-1687.

VAUTERIN, L., SWINGS, J., KERSTERS, K., GILLIS, M., MEW, T.W., SCHROTH, M.N., PALLERONI, N.J., HILDEBRAND, D.C., STEAD, D.E., CIVEROLO, E.L., HAYWARD, A.C., MARAITE, H., STALL, R.E., VIDAVER, A.K. & BRADBURY, J.F.

1990. Towards an improved taxonomy of *Xanthomonas*. *International Journal of Systematic Bacteriology*, 40(3):312-316.

VAUTERIN, L., YANG, P., HOSTE, B., POT, B., SWINGS, J. & KERSTERS, K. 1992. Taxonomy of xanthomonads from cereals and grasses based on SDS-PAGE of proteins, fatty acid analysis and DNA hybridization. *Journal of General Microbiology*, 138:1467-1477.

VERNIERE, C., PRUVOST, O., CIVEROLO, E.L., GAMBIN, O., JACQUEMOUD-COLLET, J.P. & LUISETTI J. 1992. Evaluation of the Biolog substrate utilization system to identify and assess metabolic variation among strains of *Xanthomonas campestris* pv. *citri. Applied Environmental Microbiology,* 59(9):243.

VIDAVER, A.K. & MANDEL, M. 1974. *Corynebacterium nebraskense*, a new, orange-pigmented phytopathogenic species. *International Journal of Systematic Bacteriology*, 24(4):482-485.

VIDAVER, A.K. 1977. Maintenance of viability and virulence of *Corynebacterium nebraskense. Phytopathology*, 67:825-827.

VINCENTE, J.G., GUNN, N.D., BAILEY, L., PINK, DAC & HOLUB, E.B. 2012.Genectics of resistance to downy mildew in *Brassica oleracea* and breeding towards durable disease control for UK vegetable production. *Plant Pathology*, 61(3): 600-609.

VU, B., CHEN, M., CRAWFORD, R.J. & IVANOVA, E.P. 2009. Bacterial extracellular polysaccharides involved in biofilm formation. *Molecules*, 14(7):2535-2554.

WALKER, N.J & SCHULZE, R.E. 2008. Climate change impacts on agro-ecosystem sustainability across three climate regions in the maize belt of South Africa. *Agriculture, Ecosystems & Environment*, 124(1):114-124.

WALLIN, J.R., LOONAN, D.V. & GARDNER, A.C. 1979. Comparison of techniques for inoculating corn with *Erwinia stewartii. The Plant Disease Reporter,* 63:390-392.

WEI, Z.M., SNEATH, B.J. & BEER, S.V. 1992. Expression of *Erwinia amylovora hrp* genes in response to environmental stimuli. *Journal of Bacteriology*, 174:1875-1882.

WEISBURG, W.G., BARNS, S.M., PELLETIER, D.A. & LANE, D.J. 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology*, 173:697-703.

WILSON, W.J., & DILLARD, H.R. 1999. Assesment of Phenotypic Variability in *Erwinia stewartii* based on metabolic profiles. *Plant Disease,* 83(2):114-118.

WILSON, K. & WALKER, J. *eds*. 2000. Practical biochemistry: Principles and techniques. 5th ed. Cambridge: Cambridge University Press.

WINDING, A. 1994. Fingerprinting bacterial soil communities using Biolog microtitre plates. (*In* Ritz, K., Dighton, J., & Giller, K.E., *eds*. Beyond the biomass. London: Wiley-Sayce. p. 85-94).

WOO, P., LAU, S.K.P., TENG, J.L.L., TSE, H. & YUEN, K.Y. 2008. Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. *Clinical Microbiology and Infection*, 14(10):908-934.

WORLD BANK. 2007. World Development Report 2008 - Agriculture for Development. Washington

XIAO, Y., LU, Y., HEU, S. & HUTCHESON, S.W. 1992. Organization and environmental regulation of the *Pseudomonas syringae* pv. *syringae*61 *hrp* cluster. *Journal of Bacteriology*, 174:1734-1741.

XIE, W., YU, K., PAULS, K.P. & NAVABI, A. 2012. Application of image analysis in studies of quantitative disease resistance, exemplified using common bacterial blight–common bean pathosystem. *Phytopathology*, 102:434-442.

YAO, H., HE, Z., WILSON, M.J. & CAMPBELL, C.D. 2000. Microbial biomass and community structure in a sequence of soils with increasing fertility and changing land use. *Microbial Ecology*, 40:223-237.

YIN, H., CAO, L., XIE, M., CHEN, Q., QIU, G., ZHOU, J. & LIU, X. 2008. Bacterial diversity based on 16S rRNA and *gyrB* genes at Yinshan mine, China. *Systematic and Applied Microbiology*, 31(4):302-311.

YORDANOV, I., VELIKOVA, V. & TSONEV, T. 2003. Plant responses to drought and stress tolerance. *Bulgarian Journal of Plant Physiology*: 187-206.

YOUNG, J.M. 2008. An overview of bacterial nomenclature with special reference to plant pathogens. *Systematic and Applied Microbiology,* 31:405-424.

YOUNG, J.M., BRADBURY, J.F., DAVIS, R.E., DICKEY, R.S., ERCOLANI, G.L., HAYWARD, A.C. & VIDAVER, A.K. 1991. Nomenclatural revisions of plant pathogenic bacteria and list of names 1980–1988. *Review of Plant Pathology,* 70:211-221.

YOUNG, J.M., DYE, D.W., BRADBURY, J.F., PANAGO-POULOS, C.G. & ROBBS, C.F. 1978. A proposed nomenclature and classification for plant pathogenic bacteria, *New Zealand Journal of Agricultural Research,* 21:53-177.

YOUNG, J.M., PARK, D.C., SHEARMAN, H.M. & FARGIER, E. 2008. A multilocus sequence analysis of the genus *Xanthomonas*. *Systematic and Applied Microbiology*, 31(5): 366-377.

YU, C. & YU, Y. 2000. Evaluation of biodegradability by the reduction of Tetrazolium Violet in Biolog microplates. *Biotechnology Letters,* 22:909-913.

YU, Y., STREUBEL, J., BALZERGUE, S., CHAMPION, A., BOCH, J., KOEBNIK, R. & SZUREK, B. 2011. Colonization of rice leaf blades by an African strain of *Xanthomonas oryzae* pv. *oryzae* depends on a new TAL effector that induces the rice nodulin-3 Os11N3 gene. *Molecular Plant-microbe Interactions*, *24*(9):1102-1113.

YUN, M.H., TORRES, P.S., OIRDI, M., RIGANO, L.A., GONZALEZ-LAMOTHE, R., MARANO, M.R. & VOJNOV, A.A. 2006. Xanthan induces plant susceptibility by suppressing callose deposition. *Plant Physiology*, 141(1):178-187.

ZACCARDELLI, M., CAMPANILE, F., SPASIANO, A. & MERIGHI, M. 2007. Detection and identification of the crucifer pathogen, *Xanthomonas campestris* pv. *campestris*, by PCR amplification of the conserved Hrp/type III secretion system gene *hrcC*. *European Journal of Plant Pathology,*118:299-306.

ZHAO, Y., DAMICONE, J.P. & DEMEZAS, D.H. 2000. Bacterial leaf spot diseases of leafy crucifers in Oklahoma caused by pathovars of *Xanthomonas campestris*. *Plant Disease*, 848(9):1008-1014.

ZHOU, L.H. & JI, G.H. 2013. First report of bacterial leaf spot of Rieger Begonia caused by *Xanthomonas campestris* pv. *begoniae* in China. *Plant Disease*, *97*(2):282-282.

ZIERVOGEL, G., SHALE, M. & DU, M. 2010. Climate change adaptation in a developing country context: the case of urban water supply in Cape Town. *Climate and Development*, 2:94-110.

ZINSELMEIER, C., SUN, Y., HELENTJARIS, T., BEATTY, M., YANG, S., SMITH, H. & HABBEN, J. 2002. The use of gene expression profiling to dissect the stress sensitivity of reproductive development in maize. *Field Crops Research*, 75(2):111-121.

ZINSELMEIER, C., WESTGATE, M.E., SCHUSSTER, J.R. & JONES, J.R. 2005. Low water potential disrupts carbohydrate metabolism in maize (*Zea mays L.*) ovaries. *Plant Physiology,* 107:385-391.