Characterisation of *Xanthomonas campestris* pv. *campestris* isolates from South Africa using genomic DNA fingerprinting and pathogenicity tests

by

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ABSTRACT

Black rot caused by *Xanthomonas campestris* pv. *campestris* (*X. c* pv. *campestris*) is a major disease constraint to cabbage production. The control of black rot is difficult and resistant cultivars could play an important role in reducing the losses due to the disease. Information on the distribution and diversity of *X. c* pv. *campestris* is critical before any meaningful disease resistance screening can be done. However, little is known about the diversity and international significance of South African *X. c* pv. *campestris* strains. To assess the genetic diversity and international significance of *X. c* pv. *campestris* strains in South Africa, strains of the pathogen were obtained from cabbage growing districts in Gauteng, Mpumalanga and North West Provinces of South Africa in 2010. International strains were obtained from international culture collections. Isolates from South Africa were purified and race typed using differential sets of *Brassica* spp according to Nickerson-Zwaan protocols. Four races, race 1(14%), race 3 (7%), race 4 (68%) and race 6 (10%) of the pathogen were identified. Repetitive DNA polymerase chain reaction-based fingerprinting using Eric- and Box-primers were used to assess the genetic diversity. Polyacrylamide gel electrophoresis allowed clear and reproducible differentiation of the PCR products. Of the amplified loci for South African isolates 5 loci were present in at least 90 % of the isolates for Eric-profiles and 6 in at least 80% of the isolates for Box-profiles. Of these prominent loci, none had corresponding high presence in international isolates. While no loci had a presence greater than 51% and 61% for Eric- and Box- profiles in international isolates, respectively, several loci among South African isolates were unique to isolates from specific geographic origin. Generated fingerprints of *X. c* pv. *campestris* were similar for the South African isolates and distinguishable from those of *X. c* pv. *armoraciae* and *X. c* pv. *raphani* reference strains. However, when international *X. c* pv. *campestris* were considered, no profile

pattern was observed to be unique to international *X. c* pv. *campestris* isolates as was the case with South African isolates. Eric- and Box-PCR profiles of international isolates varied widely with some isolates having profile patterns similar to those of reference strains. Cluster analysis divided *X. c* pv. *campestris* into two major groups, the South African group and the international isolates group. The South African group could be divided into subgroups, which clustered according to the geographical origin of the isolates. The same was observed for international isolates, which generally clustered isolates according to country of origin. However, isolates from different countries also clustered together. A few *X. c* pv. *campestris* strains of international origin clustered with the South African isolates group. Furthermore, a few South African isolates were clustered in the international isolate group. Although *X. c* pv. *campestris* distribution may be unique to its geographical origin, our findings, based on the present data set, suggest wide spread of the pathogen both at national and international level. The existence of different races, genetic variability and international distribution of the pathogen should be considered when resistant crucifer cultivars are bred to control black rot of crucifers

Key words:

X. c pv. *campestris*, Black rot, pathogenicity, race typing, Eric-PCR, Box-PCR

DECLARATION

I declare that the dissertation for the degree of Master of Science (M. Sc) at the North-West University Potchefstroom Campus hereby submitted, has never 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.

……. …….

Lizyben Chidamba Date

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CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

Cabbage, a member of the cruciferous family that includes broccoli, mustard, cauliflower, Brussels sprouts, kale, kohlrabi and bok choy, is primarily valued as a fresh market vegetable and ranks fifth among the vegetable crops of the world (United States Department of Agriculture, Economic Research Service (ERS), 2002). World cabbage production was approximately 70 million tons in 2008, an increase of 40% over the last 10 years (ERS, 2008; Statistics Division, food and Agriculture Organization of the UN (FAO), 2010). The major disease constraint to commercial cabbage production in warm humid climates is black rot of crucifers caused by *Xanthomonas campestris* pv. c*ampestris (X. c* pv. *campestris)* (Alvarez, 2000). Black rot occurs worldwide and infects a large number of cruciferous plants, including weeds and agriculturally important crops such as broccoli, cabbage and cauliflower (Williams, 1980; Alvarez, 2000). The disease is characterised by V-shaped, chlorotic to necrotic lesions at the margin of leaves and blackened vascular tissues (Vicente *et al*., 2001). Diseased crops have a poor market value and are unsuitable for storage as they quickly rot after harvest and yield losses of up to 100% have been experienced (Massomo *et al*., 2003).

The black rot pathogen of crucifers has been isolated and identified unambiguously ((Ignatov *et al*., 1998; Vicente *et al*., 2001). However, information about occurrence and distribution of specific strains of *X. c* pv. *campestris* on commercial seed is scarce. Furthermore, even less is known about the biology of strains of *X. c* pv. *campestris* on wild crucifers which can serve as reservoirs of inoculum near seed production and fresh

produce fields (Williams, 1980; Ignatov *et al.* 2007). The existence of *X. c* pv. *campestris* strains unique to specific geographical origin has previously been reported and suggests the existence of pathogenic variants of this pathogen (Ignatov *et al*., 1998; Vicente *et al*., 2001; Taylor *et al*., 2002; Massomo *et al*., 2003). It is therefore imperative that knowledge about local genetic diversity of *X. c* pv. *campestris*, be considered whenever disease management strategies are determined.

Xanthomonas campestris pv. *campestris* is currently grouped into six races based on a gene-for-gene model postulated by Vicente *et al.* (2001). However, race grouping has no bearing on the pathogenic virulence, as strains belonging to the same race have been found to vary significantly in virulence (Ignatov *et al*., 2007).

Differentiation of *X. c* pv. *campestris* strains from closely related pathovars of *X. campestris* attacking other brassicas cannot be achieved by using morphological and biochemical characteristics and is often difficult by pathogenicity testing (Franken, 1992). There is, therefore, a need to develop and evaluate effective, accurate and rapid methods for differentiation of strains within *X. c* pv. *campestris* from closely related brassica pathovars of *X. campestris.* This is necessary in diagnosis, epidemiological studies and control of black rot.

The relative homogeneity of *X. campestris* pathovars associated with brassica has also been demonstrated (Minsavage and Schaad, 1983; Thaveechai and Schaad, 1986; Yang *et al*., 1993; Vauterin *et al*., 1995). Genomic fingerprinting methods such as repetitive-sequence-based Eric- and Box-PCR (de Bruijn, 1992; Versalovic *et al*., 1994), reveal sufficient genotypic and phylogenetic relationships of organisms. Eric- and Box-PCR

can therefore be used as rapid, highly discriminatory screening techniques to determine the taxonomic diversity and phylogenetic structure of bacterial populations.

The control of black rot is difficult and can only be achieved by the use of disease-free seeds and culture practices that limit the dissemination of the pathogen (Williams, 1980; Alvarez, 2000). Resistant cultivars could play an important role in reducing the losses due to the disease (Massomo *et al*., 2003). Studies on distribution and characterisation of *X. c* pv. *campestris* are critical before any meaningful disease resistance screening can be done as the *X. c* pv. *campestris* composition and distribution varies from region to region (Alvarez, 2000). However, information on the distribution and characterisation of South African strains of *X. c* pv. *campestris* is lacking.

Development and deployment of cultivars with durable resistance to black rot, whether through conventional breeding or using the transgenic approach, necessitates a detailed understanding of the genetic diversity in pathogen populations. The detection of variation in pathogen populations has traditionally relied upon use of phenotypic characteristics such as pathogenicity assays, morphological and biochemical tests. Since phenotyping is time-consuming and highly prone to error, several molecular techniques are used these days to examine pathogen diversity (Alvarez, 2000; Massomo *et al*., 2003). The goal of this study was to determine the distribution of *X. c* pv. *campestris* races/stains in South Africa and their potential impacts on *Brassica* production. Data generated from this study will be used to evaluate the international relevance of *X. c* pv. *campestris* races/strains from South Africa and to investigate its potential in rapid *X. c* pv. *campestris* identification and classification.

1.2 AIM

To characterise *Xanthomonas campestris* pv. *campestris* isolates from South Africa using genomic DNA fingerprinting and pathogenicity tests.

1.3 OBJECTIVES OF THIS STUDY WERE:

(i) to obtain, isolate and purify *X. c* pv. *campestris* from selected cabbage producing regions in South Africa.

(ii) to characterise *X. c* pv. *campestris* using pathogenicity tests and genomic DNA fingerprinting methods and to assess the data for its potential in rapid *X. c* pv. *campestris* identification and classification.

(iii) to compare various races and strains of *X. c* pv. *campestris* from South Africa to international races/strains.

(iv) to evaluate the international relevance of *X. c* pv. *campestris* races/strains from South Africa.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

This chapter gives a brief overview of the origin, history and uses of cabbage. The cabbage industry in South Africa and the world at large is described including production, consumption and exports. The genus *Xanthomonas* is described together with the role played by *X. c* pv. *campestris* in causing black rot of crucifers. Current methods in characterising *X. c* pv. *campestris* are also described and include, race typing and repetitive element polymerase chain reaction, repetitive extragenic palindromic (Rep) sequences, enterobacterial repetitive intergenic consensus (Eric) and Box-elements. The chapter ends with the current application of Eric- and Box-PCR in *X. c* pv. *campestris* as well as the numerical methods used in analyzing PCR profiles.

2.2 ORIGIN AND HISTORY OF CABBAGE

Cabbage, a member of the cruciferous family that includes broccoli, mustard, cauliflower, brussels sprouts, kale, kohlrabi and bok choy. These plants are thought to have evolved in north-western Europe during the early Middle Ages from leafy unbranched and thin-stemmed kales. The plants were introduced in Roman times, from the Mediterranean area where *Brassica oleracea* and related species occur naturally in coastal areas (Zohary and Hopf, 1994; ERS, 2002). However, wild *B. oleracea* is believed to have been cultivated for several thousand years. Its history, as a domesticated plant before Greek and Roman times is not certain. During the Greek and Roman times, *B. oleraceae* was known as a well-established garden vegetable (Tudge, 1996). Cabbage was originally valued by ancient Romans and Greeks as medicinal and used for a variety of ailments including gout, headaches and ingestion of poisonous

mushrooms (Thacker, 1979; ERS, 2002). Today cabbage is primarily valued as a fresh market vegetable. However, research continues into the value of the medicinal properties of cruciferous vegetables that have been found to aid in the prevention of cancer (Tannahill, 1988; Sumner, 2004).

2.2.1 Botanical and cultivar information of cabbage

The species *Brassica oleracea* has seven major cultivars grouped by their distinctive developmental forms. These include: broccoli (cultivar *Italica*); Brussels sprouts (cultivar *Gemmifera*), whose edible small green heads resemble diminutive cabbages; cauliflower (cultivar *Botrytis*) whose flower cluster is used as a vegetable; Chinese kale or Chinese broccoli (cultivar *Alboglabra*); kale or spring greens, a very hardy cabbage (cultivar *Acephala*) considered to be the original form of the cultivated cabbage that has curled, often finely cut leaves that do not form a dense head; collard greens, a type of kale; and kohlrabi (cultivar *Gongylodes*), having an edible stem that becomes greatly enlarged, fleshy and turnip-shaped. Hybrids include broccolini (cross breed between *Italica* and *Alboglabra*, cultivars), brocco flower (cross-breed between *Italica* and *Botrytis* cultivars) and choumoelliera or marrow cabbage (cross breed between cabbage, kohlrabi and kale) (Tsunoda *et al*., 1980; van der Vossen, 1993)

Brassica oleracea (headed cabbage) cultivar *capitata*, is subdivided into varieties, comprising white headed cabbage (with smooth white to green leaves), red headed cabbage (with red leaves) and variety *sabauda*, comprising Savoy headed cabbage (with curly green leaves) (Tindal, 1983). These three types of headed cabbage can best be considered as cultivar-groups and as such have been called white-headed cabbage group, red headed cabbage group and savoy headed cabbage group (Tsunoda *et al*.,

1980). However, a formal distinction into these groups at world level is often considered superfluous and confusing, although at a local level it may be relevant (Nieuwhof, 1969).

Hundreds of varieties of headed cabbage are grown worldwide. Early-maturing compact and round- or flat-headed F1 hybrids of white headed cabbage together with open-pollinated cultivars such as 'Golden Acre', 'Copenhagen Market', 'Glory of Enkhuizen', the flat-headed 'Drumhead' and the pointed 'Sugarloaf'are grown in the tropical regions. White-headed cabbage hybrids of Japanese and Taiwanese origin in particular, are often early maturing, heat toleranct and *Xanthomonas* and *Fusarium* resistant, making them suitable for the tropics (Nieuwhof, 1969). Red headed cabbage and Savoy headed cabbage are of economic importance mainly in Europe and America, but not common in tropical regions (Nieuwhof, 1969; Tindal, 1983; van der Vossen, 1993).

2.2.2 Nutritional information and uses of cabbages

For every 100g of edible white headed cabbage there is approximately 90g water, several minerals and essential metals (Ca 52mg, Mg 8mg, P 41mg, Fe 0.7mg, Zn 0.3mg), important organic compounds (protein 1.7g, fat 0.4g, carbohydrate 4.1g, dietary fibre 2.9g, carotene 385 µg, thiamine 0.15mg, riboflavin 0.02mg, niacin 0.5mg, folate 75 µg, ascorbic acid 49mg) (Holland *et al*.,1991; Rubatzky and Yamaguchi, 1997). Due the high levels of ascorbic acid, cabbage is considered an excellent source of vitamin C. This crop can be included in dieting programs, as it contains low calories but reasonable quantities of proteins and other nutrients. It also contains significant

amounts of glutamine, an amino acid that has anti-inflammatory properties (ERS, 2002).

Along with broccoli and other brassica vegetables, cabbage is a source of indole-3-carbinol, a chemical that boosts DNA repair in cells and appears to block the growth of cancer cells. The compound is also used as an adjuvant therapy for recurrent respiratory papillomatosis, a disease of the head and neck caused by human papilloma virus (Rajendra *et al*., 1995; Science Daily. 2010). Fresh cabbage juice has been shown to promote rapid healing of peptic ulcers. On the other hand, cabbage may also act as a goitrogen. It blocks organification in thyroid cells, thus inhibiting the production of the thyroid hormones thyroxin and triiodothyronine. The result is an increased secretion of thyroid stimulating hormone (TSH) due to low thyroid hormone levels. This increase in TSH results in enlargement of the thyroid gland causing goitre (Goodhart and Shils 1978; Balch and Balch, 1990).

Cabbage is used in a variety of dishes for its naturally spicy flavour. The so-called "cabbage head" is widely consumed raw, cooked, or preserved in a great variety of dishes It is the principal ingredient in coleslaw. Cabbage is often added to soups or stews, popular in Central and Eastern Europe. It is an ingredient in some kinds of borscht, garbure and kugel and many popular dishes in India (Fox, 1999; D'amico and Drummond, 2005). Cabbage rolls (dolma), are an East European and Middle Eastern delicacy (Ma, 1968). The dish ‘bubble and squeak’, consists of potatoes and cabbage or potatoes, cabbage and meat fried together. Other greens, boiled and mashed together make up a dish called colcannon, In the American South and Midland, corn dodgers were boiled as dumplings with cabbage and ham (Holland *et al*., 1991).

Cabbages can also be fermented and preserved as with the German sauerkraut. Chinese suan cai and Korean kimchi are produced using Chinese cabbage (Weaver, 2002). Cabbages are pickled by covering the freshly cut leaves with brine made of its own juice to which salt is added. It is left to ferment in a warm place for several weeks (Kaufmann and Schøneck, 2007). Sauerkraut was historically prepared as a way of storing food for the winter. Cabbage can also be pickled in vinegar with various spices. This can be done alone or in combination with other vegetables (Rubatzky and Yamaguchi, 1997; Weaver, 2002).

2.2.3 Cabbage cultivation

Cabbage varieties are placed in two groups, namely early or late maturing cultivars. The early varieties mature in about 50 days. They produce small heads that do not keep well and are intended for consumption while fresh. The late cabbage matures in about 80 days and produces a larger head (Tsunoda *et al*., 1980).

Cabbage is popular both for commercial production and for home gardens. For production, the crop requires a cool, humid climate (Nieuwhof, 1969). The length of the total growing period varies between 90 (spring-sown) and 200 (autumn-sown) days, depending on climate, variety and planting date. For good production, the growing period is about 120 to 140 days. Most varieties can withstand a short period of frost of -6°C, some down to -l0°C. Long periods (30 to 60 days) of -5°C temperatures and below are harmful. Optimum growth occurs at a mean daily temperature of about 17°C with daily mean maximum of 24°C and minimum of 10°C. Mean relative humidity should

be in the range of 60 to 90 percent (Nieuwhof, 1969; Tindal, 1983; van der Vossen, 1993).

2.2.4 Cabbage production and international trade

Cabbage ranks fifth among the vegetable crops of the world with an annual production of approximately 39 million metric tons. The leading cabbage producing countries are China, India and Russian Federation, South Korea, Japan, Poland and USA (FAO, 2010). As shown in Figure 2.1, world cabbage production between 1998 and 2008. World cabbage production totalled almost 70 million tons in 2008, 40% more than 10 years before (Figure 2.1). Asia was the top cabbage-producing continent, reaching 54 million tons in 2008 and accounting for 77% of international production. The Asian continent also reached higher growth of 60% between 1998 and 2008. Europe was the second-highest cabbage producer, representing 17% of entire worldwide market production. In 2008, the European continent totalled more than 11 million tons, which is a 3% decrease compared to 10 years earlier. The American continent reached the third position with more than 2 million tons, which represents only 3% of the world cabbage production. It also experienced a 25% drop during the 10-year period between 1998 and 2008. Following America, African cabbage production takes 3% of the world market share producing 2 million tons. Over the 10-year period, it showed a 20% increase. Oceania, on the other hand, is not a remarkable brassica producer. It accounted for 0.2% of world production in 2008.

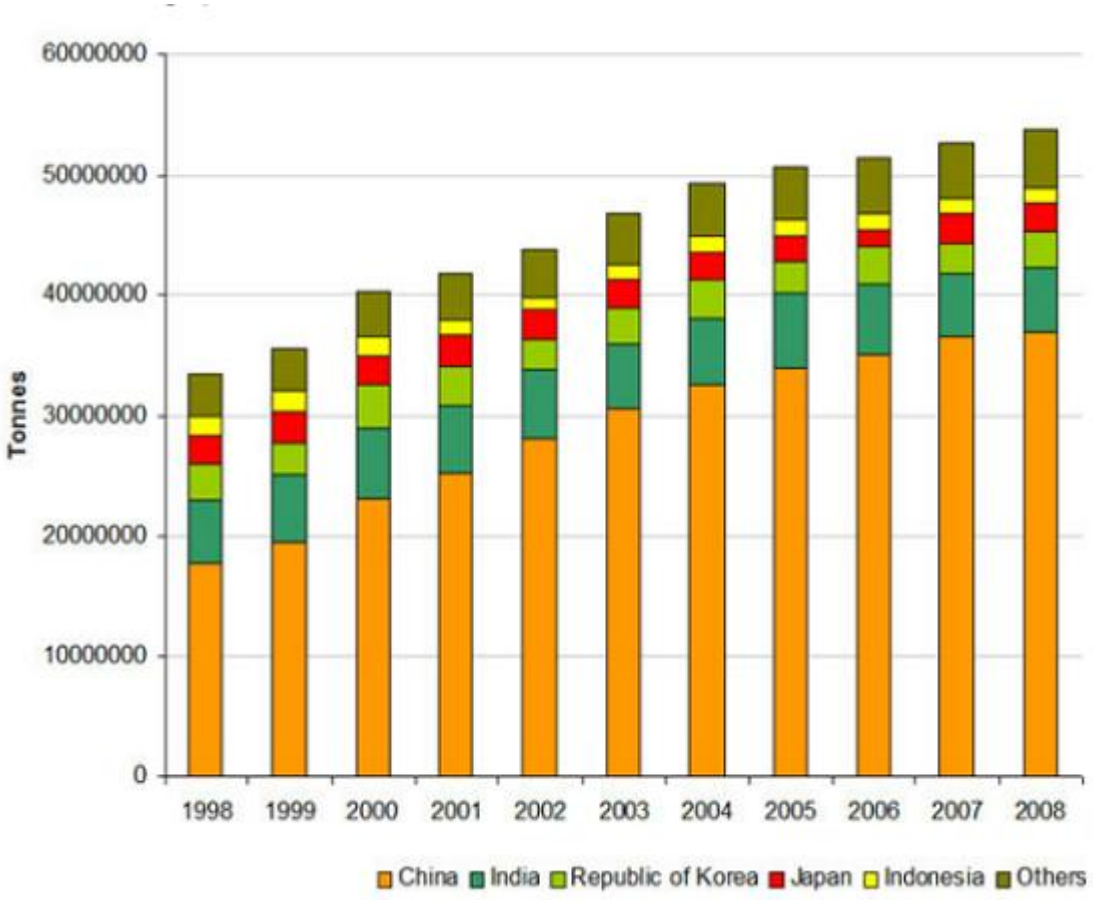

Figure 2.1: World cabbage production between 1998 and 2008 (FAO, 2010).

The area planted with headed cabbage worldwide in 2008 (Figure 2.2), was estimated at about 3 million ha in 124 countries producing some 52.5 million tons (Figure 2.1). Of these, 2 million ha were planted in Asia, 180,000 ha in the Americas and an estimate of only 100,000 ha in Africa. Reliable data on areas planted annually with headed cabbage are lacking for most countries in tropical Africa. Based on sales of commercial seed, at least 40,000 ha of white headed cabbage is grown in Kenya, Uganda and Tanzania. Furthermore, 10,000 ha are planted in the region covering Malawi, Zambia and Zimbabwe, 4000 ha in Ethiopia and 3000 ha in Cameroon. Mozambique imports considerable quantities of headed cabbage from South Africa and until recently did so also from Zimbabwe (Monfreda *et al*., 2008).

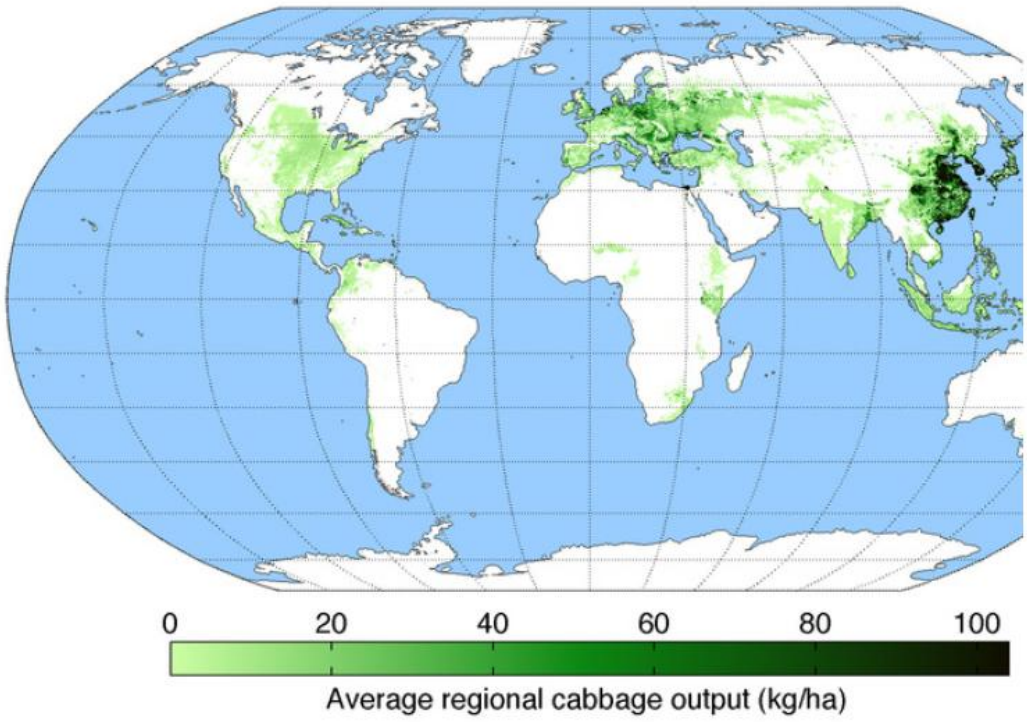

Figure 2.2: Map of cabbage production across the world (Monfreda *et al*., 2008).

2.2.5 Cabbage production in South Africa

Cabbages are produced in all provinces of South Africa but the production is concentrated in Western Cape, Kwazulu Natal, Eastern Cape, Gauteng, Free State and North West provinces. The cabbage industry contribution to the gross value of agricultural production from 1999 to 2003 increased steadily from R60 million to R110 million. There was a sharp decline in gross value in 2004 to R80 million. This was due to high production cost while the selling prices were not favourable for the producers. From 2005, the gross value increased steadily reaching a peak of R150 million in 2007. In 2008, there was a 10% decline in contribution due to decline in producer price in the same year. However, during the same period (2005 to 2010) cabbage production decreased from 200 million to 130 million metric tons per annum in 2008. The decline

in production can be attributed to increasing production input costs and unfavourable climatic conditions (Department of Agriculture, Forestry and Fisheries (DAFF), 2010).

2.2.6 South Africa cabbage exports

Most of the cabbages produced in South African are for domestic consumption. However, cabbage production is slightly higher than consumption and the surplus is exported. South Africa is not a major cabbage exporter, it represent 0.13% of world exports and is ranked number 36 in the world. In 2008, South African cabbage exports (Figure 2.3) were destined to United Kingdom, Netherlands, Angola, Mozambique, France, Mauritius and Democratic Republic of the Congo. Other exports markets for cabbage from South Africa exist in Switzerland, France, Angola and United Arab Emirates. However, if South Africa is to diversify its cabbage exports, the most lucrative markets exist in Congo and Mozambique as they have increased their cabbage imports from the world between 2004 and 2008 (Lambert, 2002; Monfreda *et al*., 2008; DAFF, 2010).

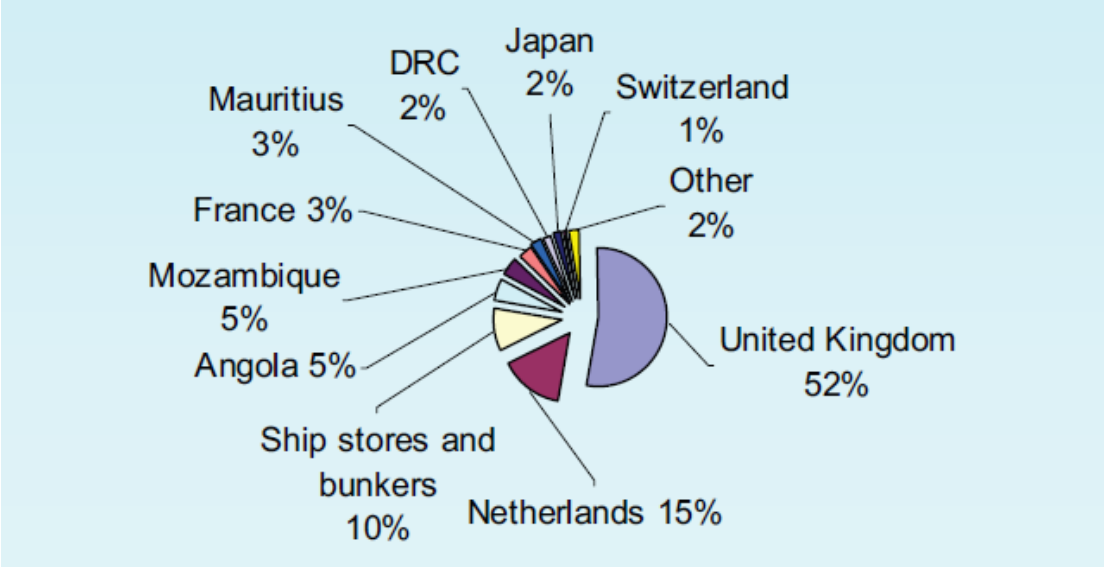

Figure 2.3: South Africa cabbage exports destinations in 2008 (DAFF, 2010).

2.2.7 Cabbage disease and pests

There a number important diseases of cabbages in tropical areas. These include: downy mildew caused by *Peronospora parasitica* that is important mainly at elevations above 1200 m) and grey leaf mould caused by *Alternaria brassicae*. Both of these fungal diseases can be controlled by fungicides and selection of tolerant cultivars. There is also bacterial soft rot caused by *Erwinia carotovora* under hot and humid conditions. Wire stem caused by *Rhizoctonia solaniis* is also a bacterial disease. It induces damping off and vein and leaf necrosis below the head. The disease clubroot is caused by *Plasmodiophora brassicae.* It is a serious threat at medium elevations (about 700 m). This disease (clubroot) can be prevented by extensive crop rotation, eradication of cruciferous weeds (alternative hosts of the pathogen). Liming and cultivation of soils with pH >7 also controls the disease by stimulating antagonistic fungi in the soil such as *Trichoderma* and *Mortierella* spp. (Voorrips, 1996). Another bacterial disease is black rot caused by *X. c* pv. *campestris.* This disease is controlled by disease -free seeds and seedlings (some cultivars have a good level of tolerance) and avoidance of overhead irrigation. Other diseases are: ring spot caused by *Mycosphaerella brassicicola*; cabbage yellows caused by *Fusarium oxysporum* f.sp. *conglutinans.* Of all diseases attacking cabbages, black rot caused by *Xanthomonas campestris* pv. *campestris* (*X. c* pv. *campestris*) is the major constraint to cabbage production in tropical areas. Diseased crops have a poor market value, and are unsuitable for storage as they quickly rot after harvest and yield losses of up to 100% have been experienced (Walangululu and Mushagalusa, 2000; Massomo *et al*., 2003).

2.3 THE GENUS *Xanthomonas*

The host range of the genus *Xanthomonas* extends over 66 genera of 9 monocotyledonous families and 160 genera of 49 dicotyledonous families (Leyns *et al*., 1984). The species *X. campestris*, formerly divided into 123 pathovars according to host specificity (Dye *et al*., 1980), was redefined using DNA/DNA homology (Vauterin *et al.*,1995). Dye *et al* (1980) proposed that the species *X. campestris* group bacteria that cause disease in cruciferous plants can be restricted to six pathovars: *X. c* pv. *aberrans*, *X. c* pv. *armoraciae*, *X. c* pv. *barbareae*, *X. c* pv. *campestris*, *X. c* pv. *incanae* and *X. c* pv. *raphani*. However, doubts about the accuracy of classification of certain pathovars (pv. *aberrans* and pv. *armoraciae*) of *X. campestris* have been expressed by Alvarez *et al.*, (1994), Vauterin *et al. (*1995) and Vicente *et al. (*2001). In a study by Fargier and Manceau (2007) *X campestris* was reclassified into three pathovars causing three different diseases: black rot of crucifers, caused by strains of *X. c* pv. *campestris* and *X. c pv. aberrans*, leaf spot of crucifers, caused by *X. c* pv. *raphani* and bacterial blight of garden stocks caused by *X. c* pv. *incanae.*

2.3.1 Black rot of crucifers

Black rot of crucifers, is considered the most important disease of crucifers worldwide (Williams, 1980). The typical symptoms of this disease are caused by strains of *X. c pv. campestris* and *X. c* pv. *aberrans* (Knösel, 1961), suggesting *X. c* pv. *aberrans* to belong to *X. c* pv. *campestris* as previously proposed by Vicente *et al.* (2001) and Fargier and Manceau (2007). The agriculturally most important host of *X. c* pv. *campestris* is *Brassica oleracea*, which includes cabbage, cauliflower, broccoli, brussels sprouts and kale. It also attacks other *Brassica* spp. and has been reported on a number of other cruciferous crops, weeds and ornamental plants (Bradbury, 1986).

Black rot often starts with infected seed as the initial inoculums. These infected seeds often appear healthy. There are strong indications that disease outbreaks are mainly caused by internal seed infections that moves up the plant systemically after germination (Köhl and Van der Wolf, 2005). Hence, the use of seed free from internal infections is one of the most important ways to avoid disease problems in agriculture, particularly organic agriculture.

The epidemiology of *X. c* pv. *campestris* should be known in order to identify critical control points (Cook *et al*., 1952). During the cropping period, it is established that inoculum is spread by water splashes, wind-driven rain, aerosols and by mechanical injury during cultivation. In particular, *X. c* pv. *campestris* will rapidly spread in misted seedbeds from infected seedlings (Köhl and Van der Wolf, 2005). The pathogen can survive for up to 5 days on flies and can be disseminated within a field. Flies can also migrate for a distance of more than 20 km, infecting cabbage plants at a more distant location (van der Wolf *et al*., 2006).

The primary mode of *X. c* pv. *campestris* entry into plants is through hydathodes at the leaf margins (Cook *et al*., 1952). The bacterium colonizes the vascular system. This restricts water flow and typically leads to the formation of V-shaped chlorotic-necrotic lesions on the margin of the leaves and blackened veins (Sutton and Williams, 1970; Williams, 1980). However, Cook *et al. (*1952) and Yuen and Alvarez (1985) reported *X. c* pv. *campestris* strains that induce leaf blight symptoms characterised by rapid necrosis of tissue with no vein blackening and others that induce a reaction intermediate between those typical of black rot and leaf blight. Closely related to *X. c* pv. *campestris*

is *X. c* pv. *armoraciae* identified by Black and Machmud (1983). This pathovar invades stomata and causes both leaf spot and hydathode necrosis on cabbage.

2.3.2 Races of the black rot phytopathogen

In addition to distinctions based on host range (pathovars), several *Xanthomonas* spp. and pathovars have been further differentiated into races based on their interaction with differential cultivars. More than 20 races were proposed for *X. oryzae* (Mew, 1987), 17 races for *X. c* pv. *malvacearum* (Brinkerhoff., 1970; Hunter *et al*., 1968) and eight races for *X. c* pv. *phaseoli* (Opio *et al*., 1996). For *X. c* pv. *vesicatoria*, eight races were defined based on their interactions with pepper cultivars and three races with tomato cultivars (Jones *et al*., 1998).

Initially, studies failed to recognize the existence of races of *X. c* pv. *campestris*. Variation between *X. c* pv. *campestris* isolates were generally considered to represent merely a difference in aggressiveness until Kamoun *et al*. (1992) separated the isolates of *X. c* pv. *campestris* into five different races (0 to 4) based on the response of certain cultivars of turnip (*B. rapa*) and a cultivar of mustard (*B. juncea*). Other studies indicated that some accessions of *B. napus* and *B. oleracea* have differential reactions to *X. c* pv. *campestris* isolates (Ignatov *et al*., 1998). Vicente *et al*. (1998) suggested that race 1 could be subdivided into three races (tentatively designated races 1a, 1b and 1c) based on their reaction on several accessions of *B. oleracea* and one of *B. carinata*. Similarly, Ignatov *et al*. (1998) separated a group of isolates formerly included in race 1 into two races (1 and 5) based on their reaction on two *B. oleracea* accessions.

Vicente *et al.* (2001) postulated a gene-for-gene model to explain the relationship between races and cultivars within *X. c* pv. *campestris* according to virulence on a range of differential cruciferous genotypes (Table 2.1). The hypothesis for model presented involved two matching gene pairs (avirulence genes in bacteria and resistance genes in plants) to explain the observed interactions. Although not tested, the hypothesis assumed gene homology for cultivars with the same pattern of reaction and six races of *X. c* pv. *campestris* were described by the Vicente *et al.* (2001) model. In a recent study, Fargier and Manceau (2007) reclassified six races in *X. c* pv. *campestris* into nine races after including *X. c* pv. *aberrans* in *X. c* pv. *campestris*. This race classification scheme was constructed with no addition of new avirulence genes in bacterial genotypes or resistance genes in plant genotypes to those proposed in the scheme designed by Vicente *et al.* (2001).

2.3.3 Race determination in *X. c* pv. *campestris*

When a plant is attacked by a pathogen, it can fend off the infection by recognizing the avirulence (*avr*) genes of the pathogens and mounting a battery of defence responses from the complementary resistance (*R*) genes (Staskawicz *et al*., 1995; Lamb *et al*., 1989). Recognition of the pathogen by the resistant plant triggers the activation of plant defence that results in the halting of pathogen ingress. Such host-pathogen recognition does not occur in the absence of either the *R* gene or the corresponding *avr* gene. The term 'avirulence' is commonly used in plant pathology to genetically define the inability of a pathogen to cause disease on a resistant host plant. A pathogen carrying a given avirulence gene is not impaired in its pathogenicity as it still causes disease on a host plant that lacks the corresponding resistance gene. This phenomenon of recognition is thought to have arisen during evolution as the host plant acquired the ability to specifically detect *avr* gene mediated molecules from the pathogen (Dangl, 1996).

Currently five avirulence and resistance gene pairs are recognised for *X. c* pv. *campestris* and *Brassica* spp. When a plant does not have the corresponding resistance gene to an *avr* a compatible reaction (susceptible) occurs. However, when a plant has the corresponding resistance gene to a particular avirulence gene an incompatible reaction (resistance) occurs (Staskawicz *et al*., 1995; Lamb *et al*., 1989). Race identification in *X. c* pv. *campestris* is carried out using Wirosa F1 (*B. oleracea*), Just Right Hybrid Turnip (*B. rapa*), Seven Top Turnip (*B. rapa*), PI 199947 (*B. carinata*), Florida Broad Leaf Mustard (*B. juncea*) and Miracle F1 (*B. oleracea*) with known resistance genes (Table 2.1). The combination of compatible and incompatible reactions are used to determine the *X. c* pv. *campestris* races as shown in Table 2.1.

Table 2.1: Postulated gene-for-gene model for *X. c* pv. *campestris* (Vicente *et al.*, 2001; Fargier and Manceau, 2007) .

						Races/Avirulence gene (A)								
						1	2	3	4	5	6	7	8	9
						A1	...	A1	A1?[a]	...	...	...	A1	A1
						...	A2	...	...	...	...	...	A2	...
						...	A3	A3	...	A3	...	...	A3	A3
						...	...	...	A4	...	...	...	...	A4
Differential cultivars	**Resistance genes (R)**					...	...	...	...	A5	...	A5	...	...
Wirosa F_1 (*B. oleracea*)	...	...	...	...	...	+[b]	+	+	+	+	+	+	+	+
Just Right Hybrid Turnip (*B. rapa*)	...	...	...	R4	...	+	+	+	–[c]	+	+	+	+	–
Seven Top Turnip (*B. rapa*)	...	R2	...	R4	...	+	–	+	–	+	+	+	–	–
PI 199947 (*B. carinata*)	R1	...	...	R4?[d]	...	–	+	–	–/(+)[e]	+	+	+	–	–
Florida Broad Leaf Mustard (*B. juncea*)	R1	...	...	R4?	R5	–	+	–	–	(+)	+	–	–	–
Miracle F_1 (*B. oleracea*)	...	...	R3	...	...	+	–	–	+	–	+	+	–	–
Number of strains						11	1	2	4	3	3	2	5	3

a A1? indicates possible presence of avirulence gene A1 (Vicente *et al.*, 2001).

b + compatible interaction (susceptibility).

c – incompatible interaction (resistance).

d R4? indicates possible presence of resistance gene R4 (Vicente *et al.*, 2001).

e (+) weakly pathogenic.

2.4 CHARACTERISATION OF *X. c* pv. *campestris*

The development and implementation of an integrated disease management program against Black rot includes the use of host-specific resistance in different production regions (Williams, 1980). However, the identification and deployment of such resistance would clearly depend on a detailed understanding of the genetic diversity of the pathogen (Massomo *et al*., 2003). Moreover, due to the preventive nature of such a control, sensitive and rapid methods of detection and discrimination are needed. However, differentiation of *X. c* pv. *campestris* strains from closely related pathovars of *X. campestris* attacking other brassicas is not possible on the basis of morphological and biochemical characteristics. It is often difficult to do this by pathogenicity testing as well (Franken, 1992). The relative homogeneity of *X. campestris* pathovars associated with brassica has also been demonstrated by DNA–DNA hybridization studies (Vauterin *et al*., 1995), fatty acid methyl ester (FAME) analysis (Yang *et al*., 1993) and SDS-PAGE protein patterns (Minsavage and Schaad, 1983; Thaveechai and Schaad, 1986). Other methods used to type the bacteria include phage typing, sero-typing, plasmid profiling and rRNA sequencing (Alvarez *et al*., 1994; de Bruijn *et al*., 1996). There is therefore a need to develop and evaluate effective, accurate and rapid methods for differentiation of strains within *X. c* pv. *campestris* from closely related brassica pathovars of *X. campestris*, necessary in diagnosis, epidemiological studies and control of black rot.

DNA-DNA homology methods have persisted as a dominant component of taxonomic analyses (Wayne *et al*., 1987; Murray *et al*., 1990). However, the complexity of DNA–DNA reassociation kinetics methods precludes the rapid analysis of large numbers of bacterial isolates. Methods that could analyse large numbers of representatives are

imperative for molecular microbial diversity studies. Alternatively, the analysis of 16S or 23S genes by DNA sequence analysis (Woese, 1987) or restriction enzyme digestion may be used (Heyndrickx *et al*., 1996; Moyer *et al*., 1996). However, the resolution of ribosomal DNA analysis resides at a high phylogenetic or taxonomic level. This is sufficient for classifying bacteria from the genus to kingdom level but insufficient to classify bacteria at the (sub) species or strain level (Woese, 1987; Fox *et al*., 1992; Stackebrandt and Goebel, 1994; Hauben *et al*., 1997).

Genomic fingerprinting methods such as repetitive-sequence-based (rep)-PCR (Versalovic *et al*., 1994; de Bruijn, 1992), random amplified polymorphic DNA (RAPD) or DNA amplification fingerprinting (DAF) (Caetano-Anolles and Gresshof, 1991) and amplified fragment length polymorphism (AFLP) (Vos *et al*., 1995) have been suggested as accurate approaches to determine taxonomic and/or phylogenetic relationships between bacteria (Janssen *et al*., 1996; Huys *et al*., 1996; Clerc *et al*., 1998). To validate this Rademaker *et al.* (2000) compared AFLP and rep-PCR genomic fingerprinting with DNA–DNA homology using *Xanthomonas* as a model system. These authors found a high correlation between the data sets, suggesting that genomic fingerprinting techniques reveal sufficient genotypic and phylogenetic relationships of organisms. Based on these studies, it was proposed that genomic fingerprinting techniques such as rep-PCR and AFLP could be used as rapid, discriminatory screening techniques to determine the taxonomic diversity and phylogenetic structure of bacterial populations.

Fingerprint patterns such as those obtained from black rot of crucifers, using a PCR-based method can be used to gain more insight into the causative agent. The

fingerprints can be compared to determine if severe symptoms are associated with a particular genotype or a group of closely related strains. Once the fingerprints are compiled into a dendograms, groups or clusters of isolates can be examined for common traits such as geographical location, races and symptom type. Attributes can be analyzed to determine if they occur in a specific group or cluster, or if they are randomly distributed throughout the population. Should a particular bacterial group or strain be more likely to cause severe signs or posses a particular virulence trait, efforts to further explore, classify and combat these strains can be focused to gain better insight into these organisms and their diseases (Anderberg, 1973; Ferligoj and Batagelj, 1982; Abel and Williams, 1985).

2.4.1 Repetitive-element polymerase chain reaction (rep-PCR)

Repetitive DNA are highly conserved, non-coding, naturally occurring sequences present in multiple copies in the genomes of most Gram-negative and several Gram-positive bacteria (Lupski and Weinstock., 1992; Versalovic *et al*., 1991). Three families, namely the 35 to 40bp repetitive extragenic palindromic (Rep) sequence (Gilson *et al*., 1984; Higgins *et al*., 1982), the 124 to 127bp, the enterobacterial repetitive intergenic consensus (Eric) sequence (Hulton *et al*., 1991; Sharples and Loyd, 1990) and the 154bp Box-element (Martin *et al*., 1992). Rep-, Eric- and Box-elements have the potential to form stem-loop structures and may play an important role in the organization of the bacterial genome (Krawiec, 1985; Krawiec and Riley, 1990; Lupski Weinstock, 1992).

Genome organization is thought to be shaped by selection, hence the dispersion of the Rep-, Eric- and Box-sequences maybe indicative of the structure and evolution of the

bacterial genome (Krawiec, 1985; Gilson *et al*., 1987; Krawiec and Riley, 1990; Lupski Weinstock, 1992). Based on repetitive sequence properties and knowledge about the clonal nature and population dynamics of pathogenic bacteria, each evolutionary specialized line, or pathovar of a pathogen should have a unique distribution or arrangement of repetitive sequences throughout the genome (Achtman and Pluschke, 1986; Denny *et al*., 1988). It should therefore be possible to generate genomic fingerprints that correlate with a specific lineage or pathovar.

Since the repetitive sequences are interspersed throughout the genome, rep-PCR is a method potentially capable of simultaneously surveying many DNA regions scattered in the bacterial genome. Rep-PCR has since been used successfully to characterise a large number of bacteria and differentiate closely related strains of bacteria (Louws *et al*., 1994; Versalovic *et al*., 1994; 1995; de Bruijn *et al*., 1996).

Repetitive element PCR based techniques are relatively fast, easy and inexpensive methods of fingerprinting bacteria. Single or multiple sets of primers can be used to obtain a variety of stable, complex results in order to differentiate closely related strains (Olive and Bean 1999). Fingerprints obtained with rep elements, such as *Eric- and Rep*-sequences, are also more robust, stable and reproducible than those obtained with RAPDs and produce less day-to-day variation (Liu *et al*., 1995; de la Puente- Redondo *et al*., 2000). Repetitive element PCR with Rep- Eric- and Box-primers has been successfully performed on numerous bacterial organisms including *E. coli* (Lam *et al*. 1996; Dopfer *et al*. 1999; de Moura *et al*. 2001), *Enterobacter* spp. (Georghiou *et al*., 1995; Zaher and Cimolai, 1997), *S. aureus* (Lipman *et al*., 1995) and *Salmonella* spp. (Bennasar *et al*., 2000).

2.4.1.1 Box elements

Box-PCR is a particular version of repetitive extragenic palindromic- PCR (rep-PCR) that uses the Box-A1R primer (Versalovic *et al*., 1991). The analysis is based on the Box dispersed-repeat motif, first identified in *Streptococcus pneumoniae*, but common in a number of bacterial species (Martin *et al*., 1992; Van Belkum *et al*., 1998). It has been shown to have a similar or even better strain differentiation power. This method is also easier to perform than ribosomal intergenic spacer analysis (RISA), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD) and other techniques (Niemann *et al*., 1997; Olive and Bean, 1999; Chmielewski *et al*., 2002). Box-PCR is quicker, cheaper and in many cases more discriminatory than pulsed field gel electrophoresis (PFGE) (Olive and Bean, 1999). The Box-PCR patterns are not affected by the culture age of the strain to be analyzed (Kang and Dunne, 2003). Furthermore fingerprinting output can be easily analyzed by computer-assisted methods (Ni Tuang *et al*., 1999). These features makeBox-PCR a frequently used tool in biogeography studies in environmental microbiology (Dombek *et al*., 2000; Singh *et al*., 2001; Landa *et al*., 2002; Cherif *et al*., 2003)

2.4.1.2 Repetitive extragenic palindromic (Rep) sequences

Repetitive extragenic palindromic (Rep) sequences, a type of rep-PCR, are occasionally referred to as palindromic units (PU) and are a 38 nucleotide consensus sequence that forms a palindrome (Gilson *et al*., 1984). Initially studied in *E. coli* and *Salmonella typhimurium* (Higgins *et al*., 1982; Glison *et al*., 1984), these Rep sequences are organized into clusters and can occur at up to 1000 locations. By nature, Rep sequences are capable of folding onto themselves and forming stable stem-loop structures with a

variable five base pair (bp) loop in the center (Stern *et al*., 1984). This ability has led many to examine the role of Rep sequences in DNA regulation (Yang and Ames, 1988; Gilson *et al*., 1990). Currently, Rep sequences are thought of as a form of 'selfish' DNA (Higgins *et al*., 1988; Lupski and Weinstock, 1992).

2.4.1.3 Enterobacterial repetitive intergenic consensus (Eric) sequences

Enterobacterial repetitive intergenic consensus (Eric) sequences are another type of highly conserved rep element. Also known as intergenic repeat units (IRU), Eric-elements are about 126bp long and located in extragenic (noncoding) regions of the genome (Sharples and Lloyd, 1990; Hulton *et al*., 1991). Compared with Rep sequences, Erics are a distinctly different group of repeated elements and are not related to Reps, though they both may have similar functions in the bacterial genome (Hulton, *et al*., 1991). It can be debated which primer set, Rep or Eric, will produce a more stable fingerprint with repeated reactions. Lipman *et al*. (1995) obtained more reproducible PCR fingerprints using Eric-primers compared to those obtained with Rep primers; they suspected this was attributed to the fact that Eric-primers are longer than those used for Rep sequences. However, Wong and Lin (2001) produced more stable fingerprints using Rep sequences rather than Erics.

2.4.2 *Xanthomonas campestris* and repetitive extragenic palindromic (rep) sequences characterisation

Several studies have shown the potential of repetitive DNA PCR-based fingerprinting (rep-PCR) to differentiate *Xanthomonas* pathovars (Scortichini and Rossi, 2003; Rademaker *et al*., 2005; Rademaker *et al*., 2006; Vicente *et al*., 2006) and strains within

pathovars (Louws *et al*., 1994; Rademaker *et al*., 2005). Rep-PCR could therefore be used as a diagnostic tool in pathology of plants affected by this pathogen. In a study of the diversity of *X. c* pv. *campestris* in commercial crops in Tanzania, seven genotypes were recognized among the 76 strains studied and the isolates tended to cluster within local geographical areas (Massomo *et al*., 2003). Similarly, Zhao *et al*. (2000) found that the majority of 45 local strains of *X. campestris* from Oklahoma belonged to a single BOX genotype similar to a known crop strain, PHW117, representative for the haplotype 1 (Alvarez *et al*., 1994; Tsygankova *et al*., 2004). Group B of *X. campestris* strains from Oklahoma was similar to the type strain NCPPB 528T of the haplotype 3 (Alvarez *et al*., 1994; Tsygankova *et al*., 2004). Based on Box-PCR, *X. c* pv. *campestris* strains from different countries have been found to cluster within larger geographical regions to some extent (Tsygankova *et al*., 2004). Results of rep-PCR (Rep-, Eric- and Box-PCR) fingerprinting allowed the separation of *X. c* pv. *campestris* from *X. c* pv. *raphani* strains and showed that, among *X. c* pv. *campestris* and *X. c* pv. *raphani* strains, there was a tendency for strains of the same race to cluster together (Vicente *et al*., 2006).

2.5 GEL ELECTROPHORESIS

The resolution and detection of DNA fragments are critical factors in the accuracy and sensitivity of DNA fingerprinting analysis. Agarose gel electrophoresis is the most commonly used method for the electrophoresis of Eric- and Box-PCR The advantages being simplicity, easy to perform and relatively good resolution. Polyacrylamide gel electrophoresis has been known to give better resolution of both low and high molecular weight than agarose and hence had been used in to resolve DNA fragments

from amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) (Huys *et al*., 1996; Janssen *et al*., 1996; Clerc *et al*., 1998).

The ability of polyacrylamide gels to resolve nucleic acids is influenced by electric field strength, gel buffer composition gel concentration and temperature (Kostichka *et al*., 1992). Different gel buffer systems are available for preparing polyacrylamide gels include TBE buffer and various denaturants to produce high resolution of banding patterns. The Laemmli (1970) gel buffer system normally used for protein electrophoresis has previously been reported to give high resolution of DNA fragments (Stellwagen, 2006). Polyacrylamide gel electrophoresis analysis of Eric- and Box-genomic fingerprinting have been reported for *Xanthomonas arboricola* pv. *juglandis.* (Scortichini *et al*., 2001). However, a detailed search in various databases could not yield any documents where sodium dodecyl sulphate Polyacryamide gel electrophoresis (SDS-PAGE) was used to resolve Eric- and Box-PCR products of *X. c* pv. *campestris.* Therefore SDS-PAGE may be evaluated against Tris-boric acid EDTA Polyacryamide gel electrophoresis TBE-PAGE and agarose gel electrophoresis on their electrophoretic resolution of Eric- and Box-PCR products for *X. c* pv. *campestris* strains.

2.6 NUMERICAL ANALYSIS OF ERIC- AND BOX-PCR GEL ELECTROPHORESIS PROFILES

Gel electrophoresis of both Eric- and Box-PCR products may yield a banding pattern that is unique to each bacterial strain. The occurrence or persistence of a particular strain can be determined by visually comparing elecrophoretic products. However, when dealing with a large number of isolates computer assisted pattern analysis

becomes necessary to interpret the numerous and complex fingerprints that arise from typing several isolates (Rademaker and de Bruijn, 1997; van Ooyen, 2001).

The similarity between two banding patterns can be expressed as a numerical value known as a similarity or proximity coefficient. This coefficient takes values ranging from 0, indicating no common trait, to +1, indicating the two strains are identical. Genetic similarity is proportional to the coefficients between 0 and +1, with more similar banding patterns having higher coefficients. Alternatively, a dissimilarity index can be constructed, resulting in inverse values. By comparing each strain to every other, the resulting similarity values can be compiled into a similarity or resemblance matrix.

To extrapolate the matrix into a dendograms second analysis, known as clustering, is performed, which expresses the similarity coefficients in a visual form (Romesburg, 1990; Rademaker and de Bruijn, 1997; van Ooyen, 2001). Characteristics from the rep-PCR gel electrophoresis pattern of each strain can be compared using band-based or curve-based characterisation to obtained similarity coefficients (van Ooyen, 2001).

2.6.1 Band-based similarity coefficients

In band-based coefficients, the selection of bands to include in the analysis can be performed manually by the researcher or by using computer software. However, manual band assignment is often tedious, labour intensive and subject to the viewer's interpretation. Additionally, temperamental alterations of the band's appearance may arise from variations in the staining and photographing of the gel. Multiple reviewers may have different interpretations of what should be included in a similarity analysis and faint bands may be overlooked or not included in the selection process. For these

reasons, a binary, band-based selection system may not be appropriate for fingerprints as complex as those obtained with rep-PCR (Rademaker and Bruijn, 1997; van Ooyen, 2001).

2.6.2 Curve-based similarity coefficients

The saturation of the pixels in a digital photograph of a gel gives the optical density (OD) of the bands and forms the basis of curve-based similarity coefficients. In this method, the banding pattern is converted into a transverse, linear graph of the band density running the length of the gel. The correlation coefficient of two strains is a direct comparison of the valleys and peaks in the graph, as well as the different ratios in peak height and width. The height of each band's peak, which corresponds to the intensity of the band, correlates to the quantity of the DNA in the band. As a result, the degree of band intensity can be quantified and factored into the comparison (van Belkum, 1998; Rademaker and Bruijn, 1997; van Ooyen, 2001).

Pearson's or product moment correlation coefficients are another measure of similarity, but, unlike Dice or Jaccard's method, use a curve based algorithm. With regard to rep-PCR, the similarity between two strains is calculated as the correlation between the densitometric values or optical density of the band(s). Pearson's coefficient is a more stable measurement of similarity than band-based methods because whole densitometric curves are compared, omitting subjective band scoring steps (Rademaker and Bruijn, 1997). Additionally, by applying Pearson's coefficient to a densitometric graph, artifactural differences between gels can be normalized and removed so that they do not alter the result (van Ooyen, 2001). Regardless of the method used, correlation analysis results in a similarity matrix of all the strains compared to each other. In

theory, a correlation coefficient can range from -1 to +1. However, due to the type of data used (ODs for Pearson's, 0 or 1 for Jaccard or Dice), the result is not less than zero. The data within the similarity matrix is then clustered to form a visual representation of the genetic relatedness of the isolates in a dendograms (van Ooyen, 2001).

2.6.3 Cluster analysis

Cluster analysis sequentially converts the similarity data into more inclusive groupings, combining like strains into clusters based on their similarity coefficient. Two very similar strains are grouped together and then joined with another cluster to form a new, large and more inclusive cluster. This is repeated until all strains and associated clusters are tied completely together. Several mathematical clustering methods exist, including the unweighted pair groups' method analysis (UPGMA), weighted pair groups method analysis (WPGMA), Ward, Complete Linkage and Single Linkage (Abel and Williams, 1985; Romesburg, 1990).

Ward's clustering method (Ward, 1963) is a hierarchical agglomerative method whose philosophy can be summarized as follows. Assuming that there are N elements to cluster, begin with N clusters consisting exactly of one entity, search the similarity matrix for the most similar pair of clusters and reduce the number of clusters by one through merger the most similar pair of clusters. Perform those steps until all clusters are merged. The Ward objective is to find at each stage those two clusters whose merger gives the minimum increase in the total within group error sum of squares (or distances between the centroids of the merged clusters) (Anderberg, 1973; Ferligoj and Batagelj,1982; Abel and Williams, 1985).

When comparing complex fingerprints of numerous strains, computer assisted analysis is essential. This is certainly the case when examining Eric-PCR fingerprints and calculating their similarity coefficients from densitometric curves using Pearson's product moment coefficients. Several commercial computer software packages with a variety of features exist to assist in microbial fingerprint pattern analysis, including, but not limited to, the AMBIS system (Scanalytics, Waltham, MA), GelCompar II, Bionumerics (Applied Maths, Inc., Austin, TX), Multi-Analyst and Molecular Analyst (Bio-Rad, Philadelphia, PA) (Vauterin and Vauterin, 1992; Rademaker and Bruijn, 1997), Phoretix 1D and Phoretix ID Pro software packages from (TotalLab Limited, UK). Digital images of the gel can be normalized to correct for inter and intra-gel variations. By comparing standard lane ladders, each lane can be adjusted to a standard size by elongating or shortening the lane. Background florescence can be removed so it is not factored into the similarity coefficient. Additionally, the greatest advantage to computer assisted analysis lies in high speed and great accuracy (Rademaker and Bruijn, 1997).

2.7 SUMMARY OF LITERATURE

In this literature review an overview of international cabbage production was presented. The origin, history of cultivation and available cabbage cultivar were discussed. Included are the critical factors to cabbage production which include breeding for resistance as diseases are the major threat to cabbage production.

Of all cabbage diseases, black rot of crucifers caused by *X. c* pv. *campestris* posses the worst threat to sustainable production especially in warm humid environment. The most

efective method of control for black rot is breeding for resistance. However, there is need for understanding of the variability and distribution of *X. c* pv. *campestris* before any meaningful breeding for resistance can be done.

In addition to distinctions based on host range, several *Xanthomonas* spp. and pathovars have been further differentiated into races based on their interaction with differential cultivars. Differentiation of *X. c* pv. *campestris* strains from closely related pathovars of *X. campestris* attacking other brassicas is not possible on the basis of morphological and biochemical characteristics and is often difficult to do this by pathogenicity testing as well.

Genomic fingerprinting methods such as repetitive-sequence-based (rep)-PCR have been suggested as accurate approaches to determine taxonomic and/or phylogenetic relationships between bacteria. Since the repetitive sequences are interspersed throughout the genome, rep-PCR is a method potentially capable of simultaneously surveying many DNA regions scattered in the bacterial genome. Repetitive-sequence-based (rep)-PCR with Eric- and Box-primers have been used to characterise X. campestris and groupings with relation to race, disease symptom type, geographical origin and the general variation within a geographical location have been established

CHAPTER 3

MATERIALS AND METHODS

3.1 SAMPLE COLLECTION

International samples were provided by Nickerson-Zwaan seed company (Netherlands). The host, source and country of origin details are provided in Table 3.1. South African strains were isolated from the East Rand and West Rand areas as shown in Figure 3.1. West Rand areas included; Brits and Vorentoe in North West Province; Magaliesburg, Tarlton, Randfontein and, Carltonville in Gauteng Province. While East Rand areas included Boksburg and Nigel in Gauteng Province, and Delmas and Witbank in Mpumalanga Province.

Leaf samples were collected from diseased cabbage. Collections were made in February 2010 from the Johannesburg Fresh Produce Market, which is the largest in South Africa and Africa at large. Johannesburg Fresh Produce Market receives fresh produce from the Gauteng province and the neighbouring provinces, including the North West and Mpumalanga provinces. Details of province of production, name of town and farm location were recorded during sample collection. Leaf samples with suspect black rot symptoms were placed in paper bags and kept at 4^{o}C before isolation of the causal bacteria associated with black rot symptoms.

Table 3.1: International *Xanthomonas campestris* isolates used in the study courtesy of Nickerson-Zwaan seed company (Netherlands).

ID	Host	Species	Source Where	ID	Host	Species	Source Where
254	cabbage	*Xcc*[1]	Portugal	559	Cabbage	*Xcc*	China
275	cabbage	*Xcc*	Portugal	560	Cabbage	*Xcc*	China
277	cabbage	*Xcc*	UK, Cornwall	561	Cabbage	*Xcc*	Chile
305	cabbage	*Xcc*	USA	562	Cabbage	*Xcc*	India 2006
306	cabbage	*Xcc*	UK	563	Cabbage	*Xcc*	Chile
307	cabbage	*Xcc*	Australia	564	Cabbage	*Xcc*	Australia
319	cabbage	*Xcc*	USA, FL	565	Cabbage	*Xcc*	Japan
320	cabbage	*Xcc*	Japan	566	-	*Xcc*	Italy
321	cabbage	*Xcc*	USA WI Coloma	567	-	*Xcc*	Germany
322	cabbage	*Xcc*	USA	80574.6-18	Cabbage	*Xcc*	Italy
323	cabbage	*Xcc*	Spain	80749.3-3	Cabbage	*Xcc*	Italy
324	cabbage	*Xcc*	Netherlands	81610-1.7	Cabbage	*Xcc*	Italy
325	cabbage	*Xcc*	Netherlands	82092-3.8	Cabbage	*Xcc*	Australia
326	cabbage	*Xcc*	Netherlands	81852-2.2	Cabbage	*Xcc*	Denmark
463	cabbage	*Xcc*	Netherlands	82504-1.1	Cabbage	*Xcc*	Chile
510	cauliflower	*Xcc*	Germany	82491-3.3	Cabbage	*Xcc*	New Zealand
511	cauliflower	*Xcc*	Germany	82451-5.21	Cabbage	*Xcc*	Chile
512	-	*Xcc*	South Africa	82450-5.3	Cabbage	*Xcc*	Italy
513	-	*Xcc*	South Africa	82695-2.8	Cabbage	*Xcc*	Italy
514	-	*Xcc*	North West-Bosnia	83163-1.5	Cabbage	*Xcc*	Chile
537	-	*Xcc*	Italy	83085-4.1	Cabbage	*Xcc*	Denmark
540	radish	*Xcr*[2]	-	83431-4.27	Cabbage	*Xcc*	Netherlands
550	Cabbage	*Xcc*	Chiba, Japan	83210-3.1	Cabbage	*Xcc*	USA
551	Cabbage	*Xcc*	Indonesia	09-3925.71	Cabbage	*Xcc*	Australia
552	Cabbage	*Xcc*	Chiba, Japan	84638-6.2	Cabbage	*Xcc*	Chile
553	Cabbage	*Xcc*	India	86221-2.2	Cabbage	*Xcc*	Italy
554	Cabbage	*Xcc*	Aichi, Japan	86547-3.2	Cabbage	*Xcc*	France
555	Cabbage	*Xcc*	Thailand	59867.3	Cabbage	*Xca*	Italy
556	daikon	*Xcc*	Chiba, Japan	2006-326	Cabbage	*Xcc*	German
557	Cabbage	*Xcc /Xca*[3]	Aude, France	2006-481	Cabbage	*Xcc*	Chile
558	Cabbage	*Xcc*	Spain				

[1]*X. c* pv. *campestris,* [2] *X. c* pv. *raphani,* [3]*X. c* pv. *armoraciae*

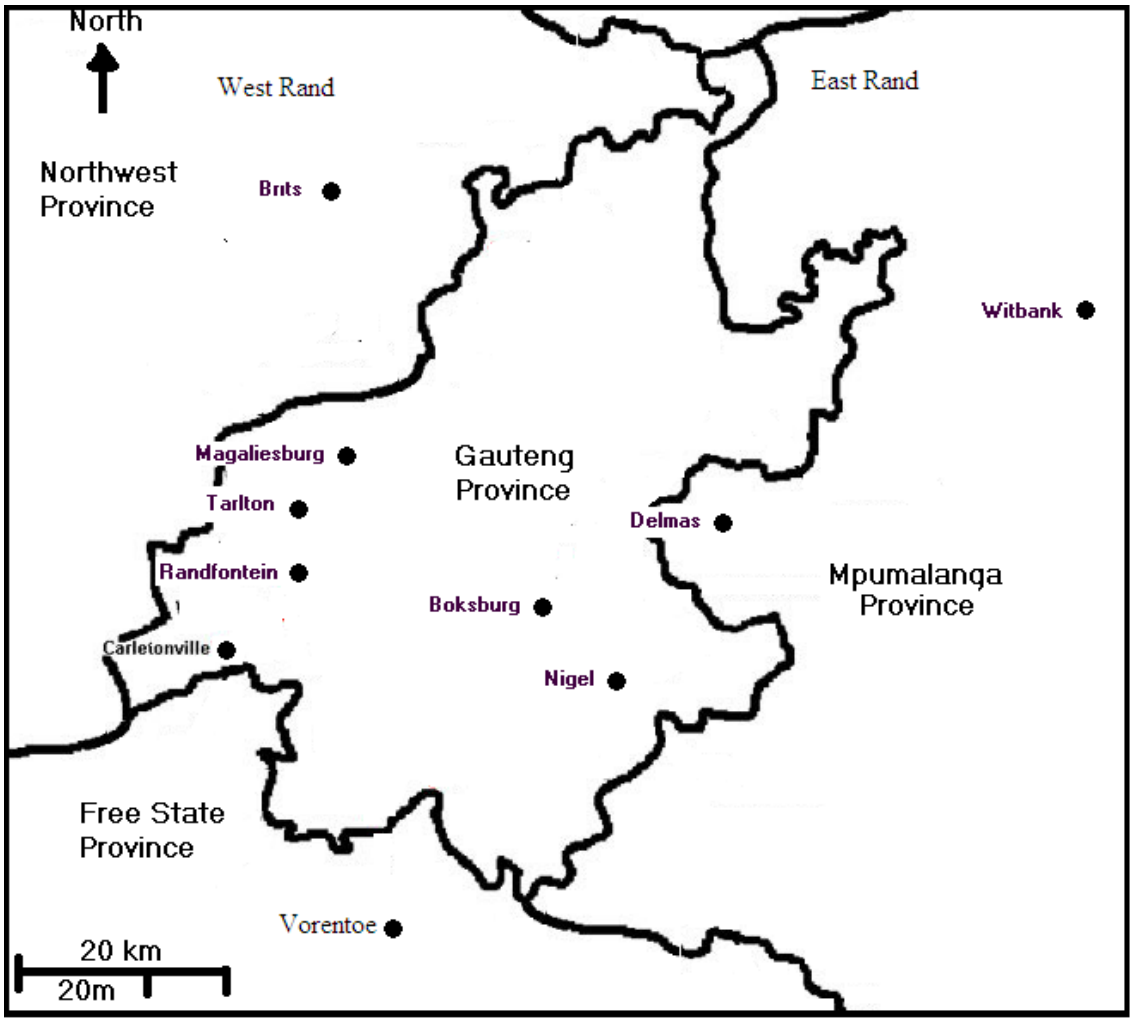

Figure 3.1: Map of Gauteng and neighbouring provinces showing sites from which strains of *X. c* pv. *campestris* were isolated.

3.2 METEOROLOGICAL CONDITIONS OF SAMPLING SITES

Ambient temperature and rainfall plays a role in the infection, spread and severity of the black rot symptoms on cabbages. It is thus notable that the prevailing meteorological conditions on the sites be described. Most of the samples represented the Gauteng area of which is the economic hub of the country. Figure 3.1 shows the average monthly rainfall values for Brits (540mm/year), Boksburg (588mm/year) Randfontein (571mm/year), Nigel (586/year), Witbank (533mm/year), Magaliesburg (546/year) and Carltonville (570mm/year). Boksburg and Nigel received the highest

rainfall per year whereas Brits, Witbank and Magaliesburg receive the least rainfall. For all the towns the highest rainfall in January whereas the least (0mm) is received in June and July and during mid summer (www.saexplorer.co.za/south-africa/climate/townname_climate.asp).

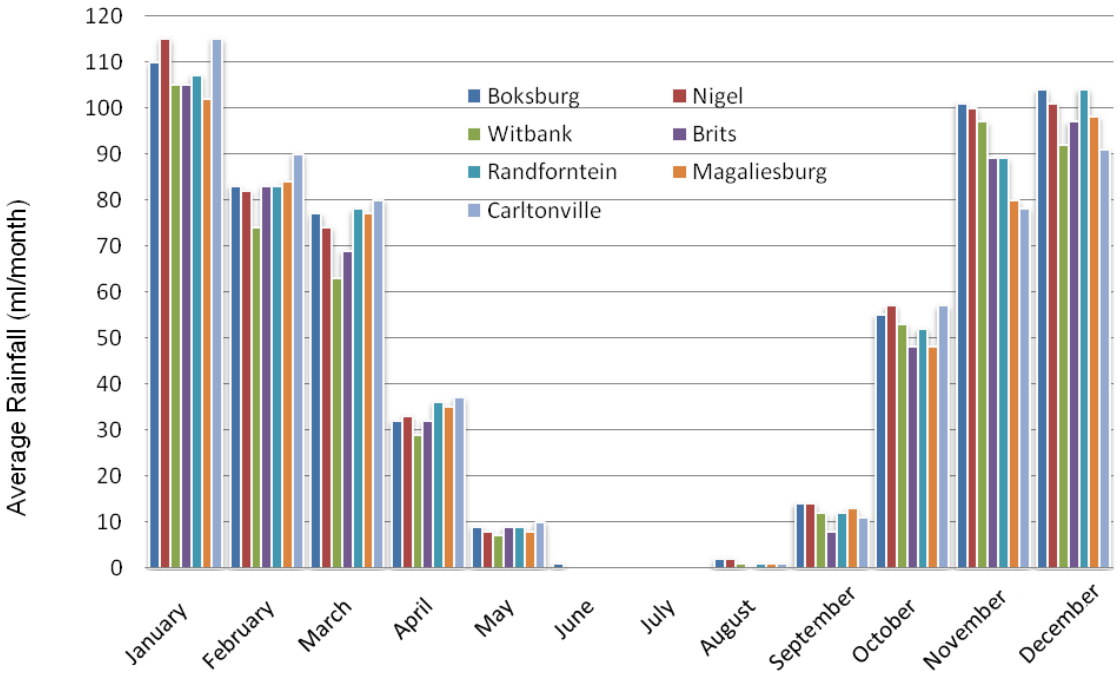

Figure 3.2: Average monthly rainfall for Brits, Boksburg, Randfontein, Nigel, Witbank, Magaliesburg and Carltonville.

Figure 3.3 below shows the monthly distribution of average mid day temperatures for Brits, Boksburg, Randfontein, Nigel, Witbank, Magaliesburg and Carltonville. Average mid day temperatures range from 16°C in June to 30°C in January. Brits, Magaliesburg and Carltonville are the warmest in summer with average midday temperatures ranging above 27°C. The temperatures are coldest in July when the temperatures range between 0°C to 3°C, on average during the night (Figure 3.4). Nigel, Randfontein and Boksburg are the coldest in winter with average night temperatures ranging from 0.1 to 0.2. (www.saexplorer.co.za/south-africa/climate/townname_climate.asp).

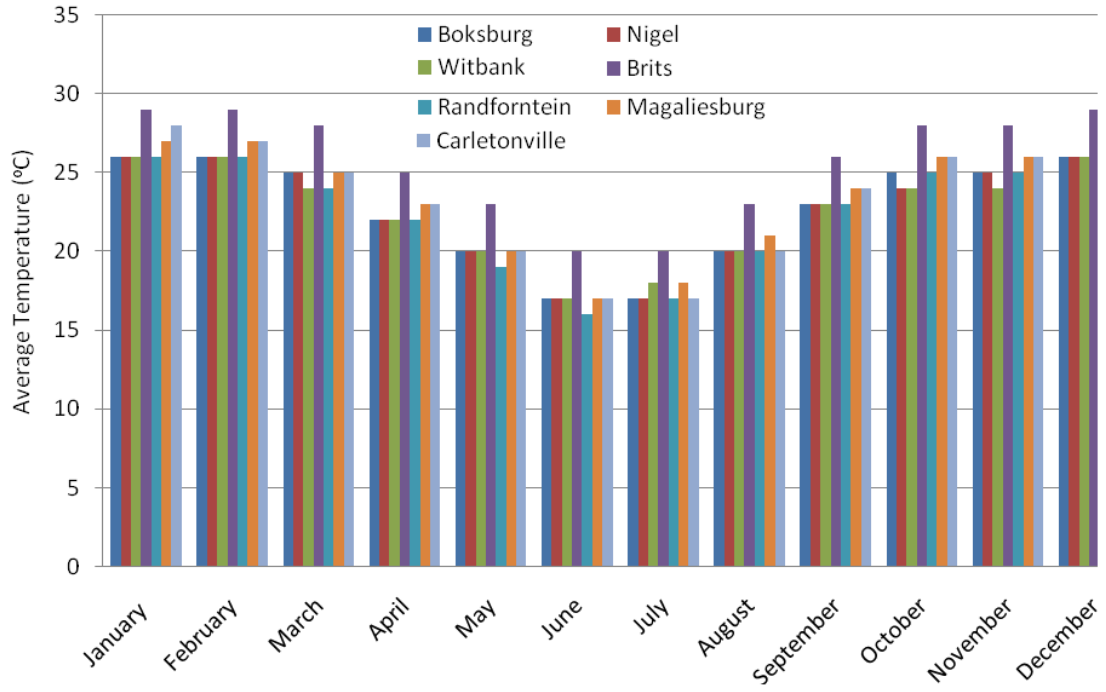

Figure 3.3: Average monthly, midday temperature for Brits, Boksburg, Randfontein, Nigel, Witbank, Magaliesburg and Carltonville.

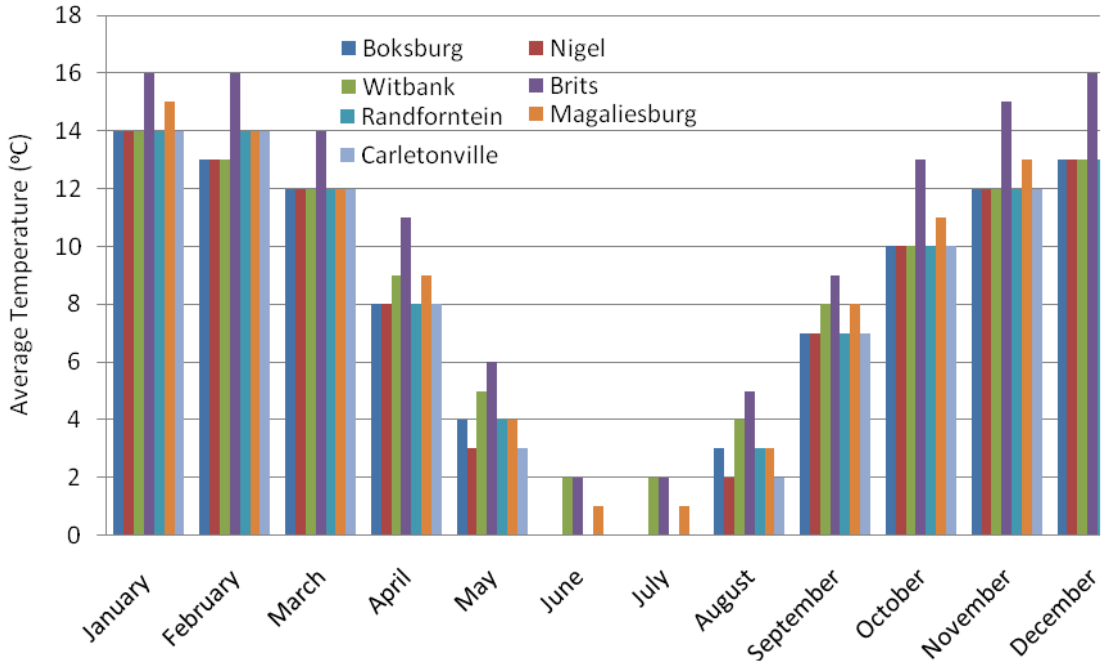

Figure 3.4: Average monthly night temperatures for Brits, Boksburg, Randfontein, Nigel, Witbank, Magaliesburg and Carltonville.

3.3 BACTERIAL ISOLATION

Isolations were made from leaves with black vascular veins surrounded by varying amounts of yellowing, as well as from leaves with black spots surrounded by yellow margins. Leaf surface contamination was removed by wiping the leaf with 75% alcohol. From each sample, leaf tissue segments of approximately 2×3 to 4mm were excised from lesion margins. These were cultured on pre-chilled plates of yeast extract-$CaCO_3$ (YDC) and Fieldhouse-Sasser (FS) agar media (Schaad *et al*., 2001). Plates were inspected for the presence of pale yellowish convex mucoid starch hydrolyzing bacterial colonies after two days of incubation at 28°C. The colonies were purified on yeast dextrose carbonate (YDC) agar and stored at -80°C in 80% glycerol. An average of 3 to 6 strains were selected per site and used for pathogenicity tests (Section 3.4).

3.4 IDENTIFICATION OF STRAINS

Presumptive *X. c* pv. *campestris* isolates and reference strains were tested for *X. c* pv. *campestris* determinative characteristics including Gram reaction, indirect ELISA using *X. c* pv. *campestris* specific monoclonal antibodies (Agdia, Inc. Elkhart, USA) and starch hydrolysis. Pathogenicity was confirmed by foliar spray inoculation of two to three leaf stage seedlings of two susceptible cabbage cultivars Burton F_1 and Frontier F_1 (Nickerson-Zwaan Seed Co, Netherlands) grown at 24 to 28°C in a greenhouse (Schaad *et al*., 2001).

Bacterial inoculums were grown on YDC agar for 48 h at 28°C, harvested and adjusted to 10^8 cfu/ml in 0.85% saline solution (Massomo *et al*., 2003). Plants were incubated in polyethylene humid chambers for 24 h in growth rooms maintained at 28°C with a 14/10 h light regime. Thereafter, they were removed from the humid chambers and kept

under the same light and temperature conditions. Types of symptoms induced by spray inoculation were recorded at 10 and 15 days after inoculation (DAI). Cultures were stored at –80°C. When cultures were needed, they were maintained by weekly transferring on to YDC agar.

3.5 RACE IDENTIFICATION

Races were identified using the differential cultivars described by Kamoun *et al*. (1992) and Vicente *et al*. (2001). The differential sets included, *B. rapa* L. 'Just Right Turnip Hybrid F_1, *B. juncea* L. 'Florida Broad Leaf mustard, *B. oleracea* L. 'Miracle' F_1 and *B. oleracea* 'Wirosa' F_1. These cultivars were provided by Nikerson-Zwaan Seed Co. (Netherlands). For race determination tests, 4-week-old seedlings of the differential cultivars (two- to three leaf stage) were inoculated. Two to four plants of each differential cultivar were evaluated per strain. Each experiment was repeated three times. Results were recorded 10 and 15 days after inoculation. Plants with 1 to 2mm diameter of black lesions were rated as being resistant to black rot. Plants with black lesions of 5 to 20 mm long surrounded by varying amounts of yellowing were scored as susceptible. Race identification followed the scheme of Vicente *et al*. (2001) (Table 2.1).

3.6 DNA ISOLATION

DNA was isolated from bacterial cells harvested from 48 hour old cultures grown on YDC agar at 28°C and suspended in 0.9 M NaCl by vigorous vortexing. The tubes were centrifuged at 10,000rpm for 15min to separate the cells from the polysaccharide, xantham gum. Cells were washed twice with sterile distilled water to reduce salt concentration and adjusted to 0.8-1.2 at OD_{600nm}. DNA was extracted using a

commercial DNA extraction kit (Nexttec, Germany) according to manufactures instruction. DNA concentration and purity were estimated by fluorescence intensity at 260 to 280nm using a Nanodrop ND-1000 (Wilmington, DE) spectrophotometer. The integrity of DNA was evaluated by electrophoresis on a 1% (w/v) agarose gel electrophoresis in 1X TAE buffer (40mM Tris, 20mM Acetic acid, 1mM EDTA, pH8.0, 25^{o}C). Ethidium bromide EtBr (lµg/ml) was added to the gel for visualization under UV light. Five microliters of DNA extraction product was mixed with 5µl loading buffer (6X Orange Loading Dye, Fermentas Life Sciences, US) and loaded into a gel slot. Three microliters of a lkb molecular weight marker (Fermentas Life Sciences, US) was also loaded into each gel to confirm the sizes of the extracted DNA. Electrophoresis was performed for 45min at 80V using 1X TAE buffer. Gel images were captured using a Gene Genius Bio-Imaging System (Syngene, Synoptics, UK) and GeneSnap (version 6.00.22) software. DNA concentration was adjusted to 50ng/µl by sterile nuclease-free water and stored at -20^{o}C.

3.7 ERIC- AND BOX-PCR AMPLIFICATION

Genomic fingerprinting was carried out using primer sets corresponding to Eric- and Box-elements (Versalovic *et al*., 1994). The Eric-1R (5'ATGTAAGCTCCTGGGGAT-3') and Eric-2 (5'AAGTAAGTGACTGGGGGT GAGC-3') as well as Box1A (5'CTACGGCAAGGCGACGCTGACG-3') were used to amplify putative Eric- and Box-elements, respectively. These primers were synthesized by Inqaba Biotech (Pretoria, South Africa). The PCR protocols were performed as previously described (Versalovic *et al*., 1994). PCR amplification reactions were performed using the following conditions: an initial denaturation step at 95°C for 5min followed by 30 cycles consisting of 94°C for 1min and annealing at 40 or 50°C for 1min with either

Eric- or Box-primers, respectively. Extension was at 65°C for 8min, this was followed by a single final polymerization step at 65°C for 15min before cooling to 4°C.

An ICycler thermal cycler (Bio-Rad, UK) was used to amplify the DNA. In both PCRs the final reaction volume of 25µl consisted of 12.5µl double strength PCR master mix (0.05U/µl *Taq* DNA Polymerase in reaction buffer, 0.4mM of each dNTP (dATP, dCTP, dGTP, dTTP), 4mM $MgCl_2$; (Fermentas Life Science, US), additional 1mM $MgCl_2$, PCR-grade water (Fermentas Life Science, US), 50ng sample DNA and 25pmole of the primer for Eric-PCR and 20pmol for Box-PCR.

3.8 GEL ELECTROPHORESIS OF PCR PRODUCTS

Agarose gel electrophoresis, Tris-boric acid EDTA (TBE-PAGE) and sodium dodecyl sulphate Polyacryamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) were evaluated for their potential to resolve amplified Eric- and Box-PCR products as described below. For all the gel systems, eight microliters (µl) of each PCR product of the amplified Eric- and Box-sequences were mixed with 2µl of 6X orange loading dye (10mM Tris-HCl (pH7.6, 25^{o}C), 0.15% orange G, 0.03% xylene cyanol FF, 60% glycerol and 60mM EDTA) and loaded onto gel wells. A molecular weight marker (Fermentas Technologies, Carlsbad, CA) was included in each of the gels.

Agarose gel (1.5% w/v) was prepared in 1X TBE (100mM Tris, 100mM boric acid, 2mM EDTA, pH8, 25^{o}C) gel buffer. The mixtures were heated in a microwave until complete melting. Ethidium bromide (1µg/ml) was added to the cooled mixture before casting. After complete polymerization, gels were electrophoresed in 1X TBE buffer at 45 V for 4 hours 30min.

Polyacrylamide gels consisted of the following; 1 to 1.5cm, 4% (w/v) staking gel and a 15cm, 7.5% (w/v) resolving gel. The TBE gels were buffered with 1X Tris-Borate-EDTA (TBE) (100mM Tris, 100mM boric acid, 2mM EDTA, pH8, 25^{o}C). While SDS-PAGE (Laemmli, 1970) staking gel was comprised of (4% (w/v) acrylamide in 0.5M Tri-HCl, (pH6.8, 25^{o}C) 0.4% w/v SDS) and the 7.5% (w/v) resolving gel were prepared in gel buffer containing 1.5M Tri-base (pH 8.8, 25^{o}C) and 0.4% SDS.

TBE- and SDS-PAGE gels were stacked for 40 min at 60V and resolved at 120V for 4 hours. This was done in a vertical electrophoresis unit (Protean II XL Bio-Rad, UK) with 1mm gel spacers. The gels were stained for 15min with EtBr (lμg/ml) and visualized using a Gene Genius Bio-Imaging System (Syngene, Synoptics, UK) and GeneSnap version 6.00.22 software.

3.9 ANALYSIS OF ERIC- AND BOX-PCR PROFILES

DNA profiles were analyzed using a trial version of Phoretix 1D and Phoretix ID Pro (version 10) software packages from TotalLab Limited (UK). The Pearson coefficient with Ward's algorithm was used to create the dendograms. Pearson's coefficient is a more stable measurement of similarity than band-based methods because whole densitometric curves are compared, omitting subjective band scoring steps (Rademaker and Bruijn, 1997). Additionally, by applying Pearson's coefficient to a densitometric graph, artefacts differences between gels can be normalized and removed so that they do not alter the results (van Ooyen, 2001). Ward's clustering method is a hierarchical agglomerative method whose objective is to create clusters that gives the minimum increase in the total within group error sum of squares (Ward, 1963) .

CHAPTER 4

RESULTS

4.1 INTRODUCTION

This chapter describes the findings obtained from the identification of bacterial strains, pathogenicity tests and race determination of isolated *X. c* pv. *campestris* strains. Findings from DNA extraction, Eric- and Box-PCR electrophoresis optimization, numerical as well as cluster analysis of electrophoresis results are described. The findings from cluster analysis are further used to discuss and establish the international relevance of South African *X. c* pv. *campestris* isolates and the international distribution of Eric- and Box-PCR profiles in *X. c* pv. *campestris*

4.2 IDENTIFICATION OF BACTERIAL STRAINS

Twenty eight *X. c* pv. *campestris* strains were isolated from cabbage leaf samples collected from diseased cabbage from farms in Gauteng, North West and Mpumalanga provinces. Leaf samples were treated as described in Section 3.3, *X. c* pv. *campestris* isolated and identified using methods described in Section 3.4. Monoclonal antibodies used reacted positively with all *X. c* pv. *campestris* strains distinguishing them from *X. c* pv. *raphani* and *X. c* pv. *armoraciae* reference strains. The latter two pathovars reacted negatively. In Table 4.1, a list of all the *X. c* pv. *campestris* obtained in this study is provided. In some cases cabbage samples were obtained from more than one farm representing a single geographical area, as a result, multiple isolates were collected per geographical area.

Table 4.1: List of South African *X. c* pv. *campestris* strains isolated during the study showing origin, host, species, Eric- and Box-cluster groups, symptom groups and race.

Isolate Number	Origin	Host	Species	Eric Profile	Box Profile	Symptom Group	Race
87	Boksburg	Cabbage	*Xcc*	4	12	c	4
88	Boksburg	Cabbage	*Xcc*	4	12	b	4
55	Brits	Cabbage	*Xcc*	3	13	b	4
59	Brits	Cabbage	*Xcc*	3	13	c	4
57	Brits	Cabbage	*Xcc*	3	13	a	4
103	Carltonville	Cabbage	*Xcc*	4	11	c	4
61	Delmas	Cabbage	*Xcc*	3	13	b	4
62	Delmas	Cabbage	*Xcc*	3	13	b	4
116	Delmas	Cabbage	*Xcc*	4	13	b	4
107	Magaliesburg	Cabbage	*Xcc*	5	9	b	3
105	Magaliesburg	Cabbage	*Xcc*	5	9	b	4
106	Magaliesburg	Cabbage	*Xcc*	4	9	a	6
64	Nigel	Cabbage	*Xcc*	3	11	b	4
65	Nigel	Cabbage	*Xcc*	3	11	c	4
66	Nigel	Cabbage	*Xcc*	3	11	c	4
97	Randfontein	Cabbage	*Xcc*	4	7	b	1
77	Randfontein	Cabbage	*Xcc*	10	11	c	1
78	Randfontein	Cabbage	*Xcc*	5	12	b	1
79	Randfontein	Cabbage	*Xcc*	5	12	c	1
80	Randfontein	Cabbage	*Xcc*	5	9	b	4
82	Randfontein	Cabbage	*Xcc*	5	12	b	4
81	Randfontein	Cabbage	*Xcc*	5	9	c	6
100	Tarlton	Cabbage	*Xcc*	5	6	b	3
69	Vorentoe	Cabbage	*Xcc*	3	11	a	4
94	Vorentoe	Cabbage	*Xcc*	4	12	a	4
72	Witbank	Cabbage	*Xcc*	5	12	a	4
73	Witbank	Cabbage	*Xcc*	4	11	a	4
74	Witbank	Cabbage	*Xcc*	4	11	a	6

4.3 PATHOGENICITY TESTS

Twenty eight South Africa *X. c* pv. *campestris* strains isolated from cabbage were tested for pathogenicity by foliar spray of two to three leaf stage seedlings of two susceptible cabbage cultivars Burton F_1 and Frontier F_1. Only those strains that gave the typical symptoms of the disease (Section 3.4) were considered to be *X. c* pv. *campestris*.

Black rot infection symptoms induced were arbitrarily categorized into three groups, ranging from characteristic to non-characteristic black rot symptoms (Figure 4.1). Twenty seven percent of the strains were in symptom group "a" and caused rapid, extensive chlorosis and collapse of vein-delimited mesophyll tissues. These symptoms occurred before vein blackening and wilting of plants. Forty six percent of the strains were in symptom group "b" and initially caused yellowing and small greyish spots on leaves followed by characteristic necrotic V-shaped lesions on the margins of leaves and chlorosis. Subsequently, vein blackening and wilting of leaves occurred. Twenty eight percent of the strains were in symptom group "c". These strains initially caused yellow halos around stomata that later turned into severe necrotic spots which afterwards coalesced and formed extensive necrotic blotches in the leaf lamina and distorted leaf margins.

All isolates from Witbank and Vorentoe displayed only the "a" symptom type. Those from Delmas and the one from Tarlton had only the "b" symptom type. The one representative from Carltonville belonged to the "c" symptom type. However, the representatives from Randfontein, Boksburg and Nigel displayed either "b" or "c"

symptom types. Those from Magaliesburg displayed either “a” or “b” and the isolates from Brits had all three-symptom types.

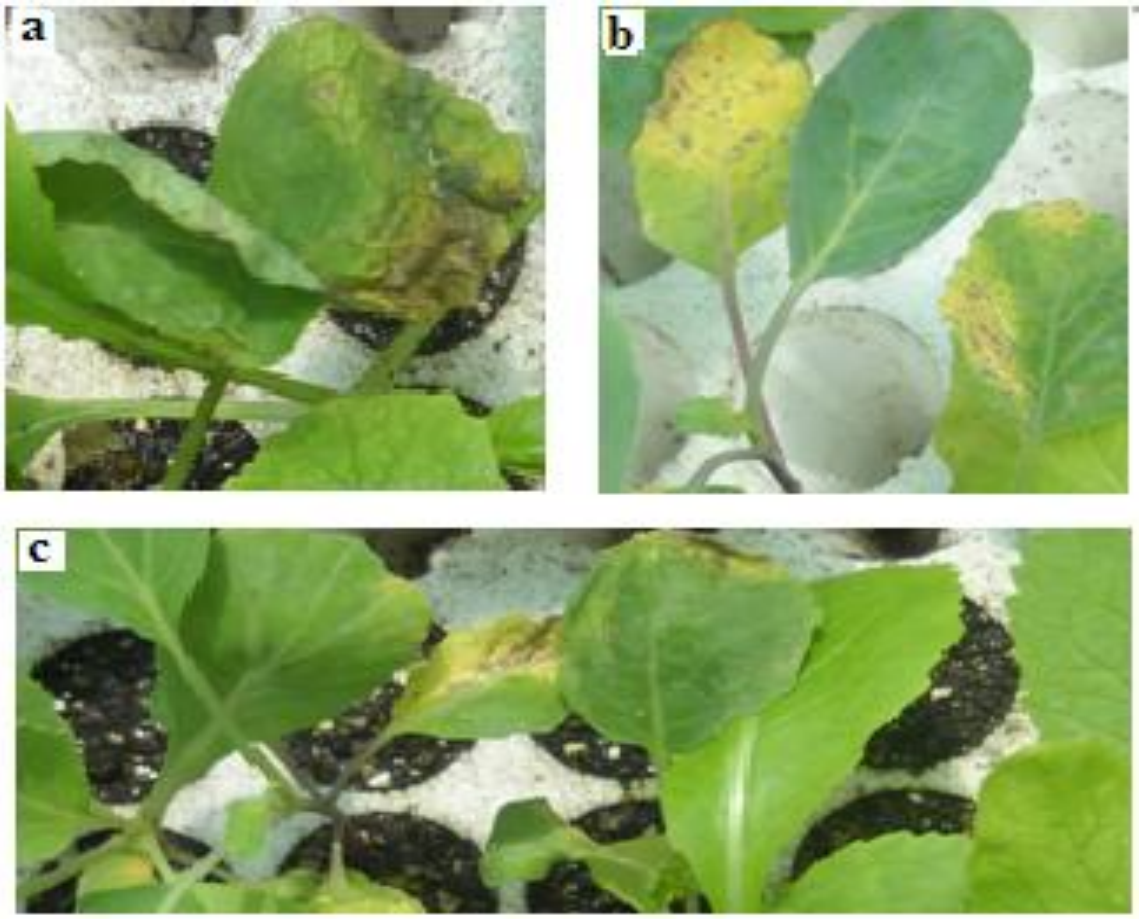

Figure 4.1: Symptom groups observed with Black rot infection. Strains in group “a” caused rapid, extensive chlorosis and sudden collapse of vein-delimited mesophyll tissues. Strains in symptom group “b” initially caused yellowing and small greyish spots on leaves followed by characteristic necrotic V-shaped lesions on the margins of leaves with chlorosis. Strains in group “c” initially caused yellow haloes around stomata that later turned into severe necrotic spots which later coalesced and formed extensive necrotic blotches in the leaf lamina and distorted leaf margins.

Calculation of the “a”, “b” and “c” symptom groups distribution indicate that in both East and West Rand regions the distribution was similar. Majority of the symptom types were from the “b” group (46.2%) in both the East and West Rand areas.

4.4 RACE DETERMINATION

Four races, namely 1, 3, 4 and 6, were identified. Of the 28 isolates, 4 (14%) were identified as race 1, 2 (7%) as race 3, 19 (68%) as race 4 and 8 (10%) were identified as race 6. Race 4 was the most common among the isolates and was found in all the sampling points except Tarlton (Table 4.2). Race 6 was identified among samples from Magaliesburg, Randfontein and Witbank. Race 3 was identified in samples from Tarlton and Magaliesburg and race 1 in samples from Randfontein only (Table 4.2).

Of the four races, race 4 was widely distributed both in the East Rand (53%) and West Rand (82%). The lower distribution of the other races might have been affected by the smaller sample size.

Table 4.2: Distribution of the *X. c* pv. *campestris* races isolated in February 2010.

Area	**Race 1**	**Race 3**	**Race 4**	**Race 6**
Boksburg			+	
Brits			+	
Vorentoe			+	
Carletonville			+	
Magaliesburg		+	+	+
Tarlton		+		
Randfontein	+		+	+
Kliprivier			+	
Nigel			+	
Delmas			+	
Witbank			+	+
Percentage	14%	7%	68%	10%

4.5 GENOMIC DNA EXTRACTION AND ANALYSIS

DNA was isolated as described in Section 3.6. Extracted DNA was analyzed by agarose gel (1%) electrophoresis to check for the integrity. Figure 4.2 is a negative image of an ethidium bromide stained agarose gel (1% w/v) after electrophoresis. The DNA was of high molecular weight and sufficient high concentrations. DNA concentration and purity were estimated by fluorescence intensity at 260 to 280nm using a Nanodrop ND-1000 spectrophotometer (Wilmington, DE) and ranged from 60 to 300ng/µl. Concentration of DNA used for PCR was adjusted to 50ng/µl by sterile nuclease-free water.

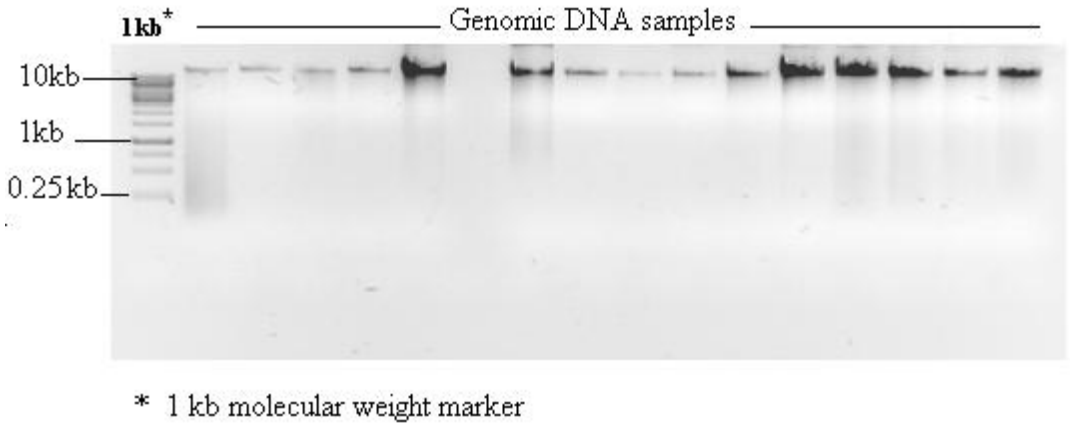

Figure 4.2: Negative image of ethidium bromide stained 1% (w/v) Agarose gel showing high molecular weight genomic DNA.

4.6 GEL ELECTROPHORESIS OPTIMIZATION

Agarose, TBE-PAGE and SDS-PAGE electrophoresis systems were evaluated for reproducibility and resolution of all fragments amplified by PCR. The PCR samples were randomly selected to test the different gel systems and their suitability in analysis and estimation of genetic diversity. Figure 4.3 shows Box-PCR profiles analysed by agarose gels (1.5%), TBE-PAGE (7.5%) and SDS-PAGE (7.5%) while Table 4.3 shows a summary of bands observed in the gels.

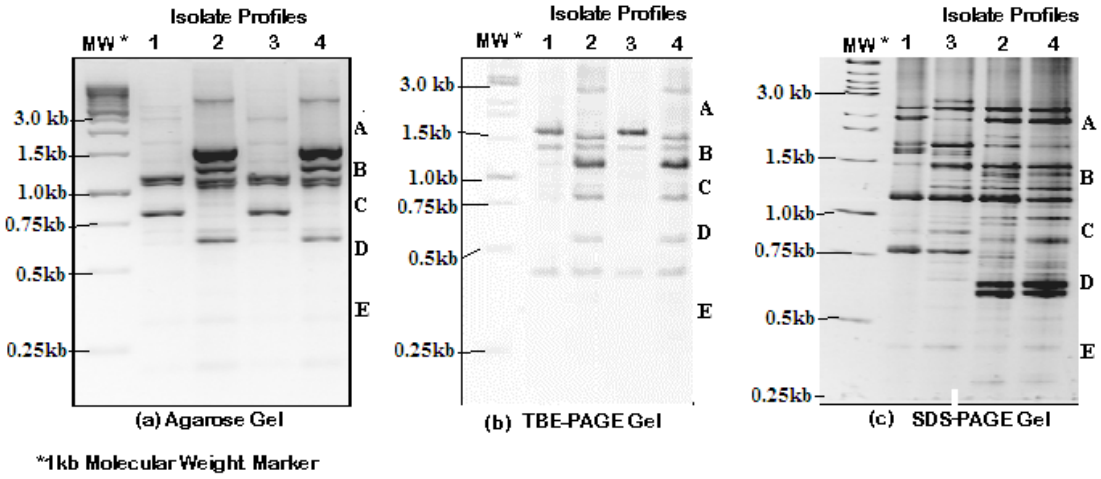

Figure 4.3: Gel images of Box-PCR of four representative PCR profiles obtained with agarose, TBE-PAGE and SDS-PAGE during gel electrophoresis optimization.

Table 4.3: Summary of bands present in five regions of Box-PCR elecrophoretic profiles obtained with agarose (AG), SDS-PAGE (SDS) and TBE-PAGE (TBE) during Gel electrophoresis optimization for four *X. c* pv. *campestris* isolates.

Region	**A**			**B**			**C**			**D**			**E**		
Size	**1.5- 3kb**			**1-1.5kb**			**0.75-1kb**			**0.5-0.75kb**			**0.25-0.5kb**		
Isolates	**AG**	**TBE**	**SDS**	**AG**	**TBE**	**SDS**	**AG**	**TBE**	**SDS**	**AG**	**TBE**	**SDS**	**AG**	**TBE**	**SDS**
1	2	1	4	2	2	3	1	2	2	0	0	2	1	1	1
2	0	0	5	4	3	4	2	2	4	2	1	6	1	1	4
3	3	2	6	3	3	4	1	1	3	2	0	3	0	0	1
4	0	1	6	4	3	6	1	2	3	2	1	6	0	1	3
Total	**5**	**4**	**21**	**13**	**11**	**17**	**5**	**7**	**12**	**6**	**2**	**17**	**2**	**3**	**9**

A total of 21 bands were observed in region “A” of the 4 isolates with SDS-PAGE gel system, whereas only 4 bands were observed for TBE-PAGE and 5 for agarose gels. Similar observations were made for all other regions with region “D” having the highest resolution difference between the gel systems. Seventeen bands were observed with SDS-PAGE and only 6 and 2 bands were observed in agarose gel and TBE-PAGE systems, respectively. Only in region “B” did agarose (13 bands) and TBE-PAGE (11

bands) have relatively similar resolution to SDS-PAGE (17 bands). However, the SDS-PAGE was still superior.

Problems associated with agarose (1.5%) and TBE-PAGE (7.5%) electrophoresis gel systems were observed and included non-uniform band resolution and masking of faint bands. These defects are usually attributed to uneven gel polymerization and inherent poor gel resolution efficiency. Such defects result in inaccurate band positioning which may affect cluster analysis. Consequently, the agarose and TBE-PAGE gel systems were not used for further DNA fingerprinting analysis. Most of the bands especially those of low molecular weight were not well defined and fragments of high molecular weight were not well separated in agarose and TBE-PAGE gel systems.

An improvement in separation and resolution of PCR amplified products was observed when the Laemmli (1970) polyacrylamide gel buffer system (SDS-PAGE) was used. As observed in Figure 4.3 and Table 4.3, there was co-migration of bands in agarose and TBE-PAGE gels systems resulting in fewer resolved bands, when compared to SDS-PAGE. The SDS-PAGE generated bands were well separated, sharp and straight for all samples tested. Consequently, the Laemmli (1970) protocol was employed for electrophoretic separations of PCR amplified DNA fragments for fingerprint analysis in this study. The highest number of well resolved bands for both larger sizes and smaller sizes were observed in SDS-PAGE gels. This illustrated the suitability of SDS-PAGE in electrophoretic separation of Eric- and Box-PCR products for analysis of genetic relationships between individuals.

4.7 ERIC- AND BOX-PCR PROFILES OF *X. c* pv. *campestris* ISOLATES

Electrophoretic patterns of Eric- and Box-PCR's are shown in Figure 4.4 and 4.5 for South African isolates and Figure 4.6 and 4.7 for international isolates, respectively. Electrophoretic profile patterns were analysed by a trial version of Phoretix 1D and Phoretix ID Pro (version 10) software packages from TotalLab Limited (UK). Summary of aligned and matched profiles with the respective molecular weight size are presented in Tables 4.4 to 4.11. For analysis purposes, Eric- and Box-PCR profiles were divided into five fragment size specific regions as follows "A" (1.500kb to 3.000kb), "B" (1.000kb to 1.500kb), "C" (0.750kb to 1.000kb), "D" (0.500kb to 0.750kb) and "E" (0.250kb to 0.500kb).

4.7.1 Eric- and Box-PCR profiles of South African *X. c* pv. *campestris* isolates

Figure 4.4 and 4.5 below shows a negative image of an ethidium bromide stained SDS-PAGE gels of South African *X. c* pv. *campestris* isolates for Eric- and Box-PCR profiles, respectively. A total of 32 South African *X. c* pv. *campestris* isolates were analysed. These included 28 isolates from our collection and 4 from previous collections. Generated fingerprint patterns were relatively similar for most isolates, compared to those of closely-related *X. c* pv. *armoraciae* and *X. c* pv. *raphani* pathovars. Several common prominent bands can easily be observed in the profiles. There were intensity variations among bands of the isolates. Five gel regions and their respective fragment size ranges (Section 4.7) are shown. While the figures do not show labels of the respective isolate profiles, labels were included in the Eric- and Box-PCR profile dendograms in Figure 4.8 and 4.9 for all the isolates.

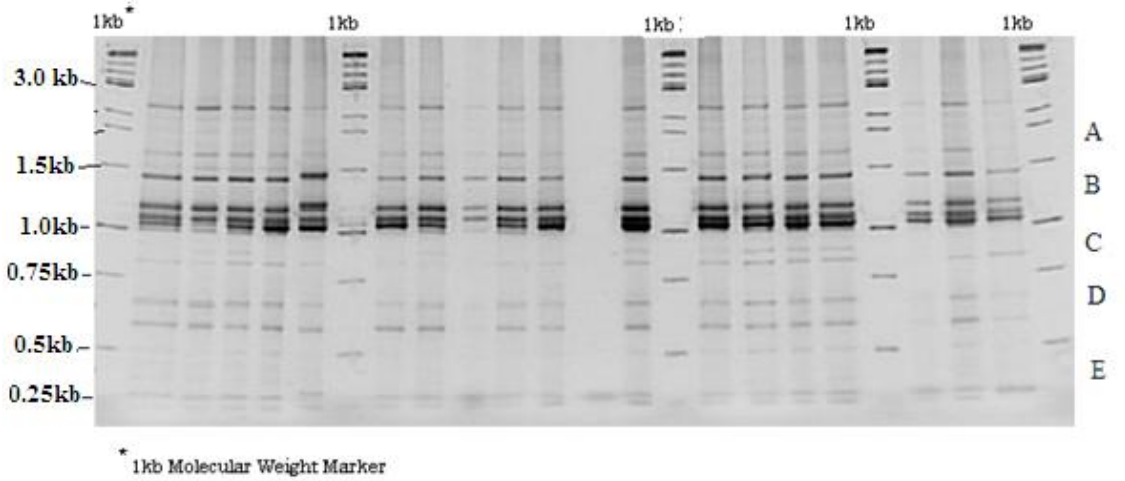

Figure 4.4: A negative image of an ethidium bromide stained SDS-PAGE gel showing Eric-PCR profiles of South African *X. c* pv. *campestris* isolates.

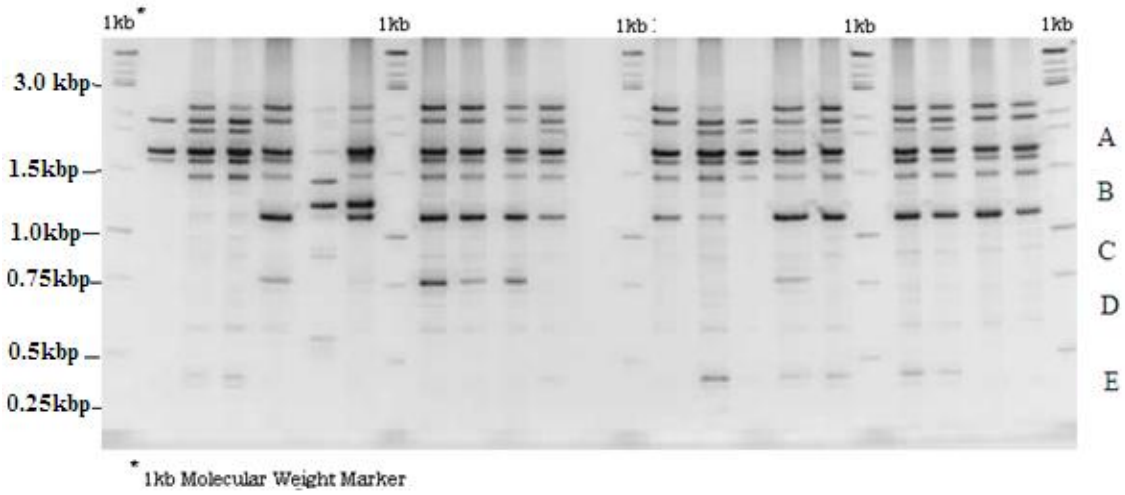

Figure 4.5: A negative image of an ethidium bromide stained SDS-PAGE gel showing Box-PCR profile of South African *X. c* pv. *campestris* isolates.

Eric-profiles in Figure 4.4 are different from the Box-profiles in Figure 4.5. All the profiles had bands present from 0.25kb to approximately 3.0kb. Bands with high intensity in Eric-profiles were concentrated in region "B", whereas those for Box-profiles were evenly spread in regions "A" and "B". The relative uniformity of profile patterns between isolates for both Eric- and Box-PCR were a characteristic of the South African *X. c* pv. *campestris* isolates. However, the intensities of common bands varied

among the isolates profiles patterns. The details of the profiles after analysis with mentioned software are presented below.

Table 4.4 shows the numbers of bands observed per isolate within the five fragment size specific regions “A” to “E” as previously described in Section 4.7. A total of 394 scorable bands were amplified for Eric-PCR profiles and 502 for Box-PCR profiles, for the 28 South African isolates from our collection and 4 (SA 482, SA 483, SA 512 and SA513) isolates from previous collections.

The highest number of bands were observed in region “A” (133 (33.8%) bands) and region “B” (99 (25.1%) bands) for Eric-profiles. A similar scenario was observed for Box-PCR profiles. In this case 163 (32.5%) bands were observed in region “A” and 151(30.1%) in “B”. Each band was assumed to correspond to one locus. The banding profiles, total number of bands as well as number of polymorphic bands were primer dependant. The number of amplified loci per individual varied from 6 to 19 for Eric-PCR profiles and 7 to 21 for Box-PCR profiles. Size range varied from 0.25kb to 3.0kb for both Eric- and Box-PCR profiles. Such size ranges of Eric- and Box-PCR profile bands have previously been reported (Rademaker *et al*., 2000; Massomo *et al*., 2003). The least number of bands were observed in regions “C” (0.75kb to 1kb)” with 54 (13.7%) of the bands, region “D” with 53 (13.5%) and region “E” with 55 (14%) of the bands for Eric-PCR. For Box-PCR profiles the regions “C” had 42 (8.3%) of the bands, “D” had 86 (17.1%) and “E” 60 (12%).

Table 4.4: Summary of band distribution among five regions of Eric- and Box-PCR elecrophoretic profiles for *X. c* pv. *campestris* isolates from South Africa.

(A) Eric PCR profile

Gel Region	Molecular Weight (kb	SA 482	SA 483	SA 512	SA 513	SA Delmas 116	SA Boksburg 87	SA Boksburg 83	SA Brits 55	SA Brits 57	SA Brits 59	SA Carltonville 103	SA Delmas 61	SA Delmas 62	SA Nigel 64	SA Nigel 65	SA Nigel 66	SA Magaliesburg 107	SA Magaliesburg 105	SA Magaliesburg 106	SA Randfomtein 77	SA Randfomtein 78	SA Randfomtein 79	SA Randfomtein 80	SA Randfomtein 81 ,	SA Randfomtein 82	SA Randfomtein 97..	SA Tarlton 100	SA Vorentoe 69	SA Vorentoe 94	SA Witbank 72	SA witbank 72	SA Witbank 74	Total
A	1.5-3.6	5	6	6	5	5	4	4	5	5	5	4	5	5	5	4	3	2	4	4	4	5	5	3	4	3	3	3	3	4	4	3	3	133
B	1-1.5	3	5	6	7	2	2	2	3	3	3	3	2	3	3	3	3	1	3	3	3	2	2	2	4	3	3	4	3	3	3	3	4	99
C	0.75-1	0	1	3	0	1	2	4	3	2	1	1	2	2	2	4	2	0	2	1	2	2	2	0	3	1	1	2	0	2	3	2	1	54
D	0.5-0.75	0	1	4	4	1	1	2	3	2	1	2	1	3	1	2	2	0	2	1	3	1	2	0	2	0	1	2	0	2	2	2	3	53
E	0.2-0.5	2	2	0	1	3	2	2	1	1	0	1	1	0	1	1	0	1	3	3	4	3	3	3	4	2	1	1	0	3	3	1	2	55
	Total	10	15	19	17	12	11	14	15	13	10	11	11	13	12	14	10	4	14	12	16	13	14	8	17	9	9	12	6	14	15	11	13	394

(B) Box PCR profile

Gel Region	Molecular Weight (kb	SA 482	SA 483	SA 512	SA 513	SA Delmas 116	SA Boksburg 87	SA Boksburg 83	SA Brits 55	SA Brits 57	SA Brits 59	SA Carltonville 103	SA Delmas 61	SA Delmas 62	SA Nigel 64	SA Nigel65	SA Nigel66	SA Magaliesburg 107	SA Magaliesburg 105	SA Magaliesburg 106	SA Randfomtein 77	SA Randfomtein 78	SA Randfomtein 79	SA Randfomtein 80	SA Randfomtein 81 ,	SA Randfomtein 82	SA Randfomtein 97..	SA Tarlton 100	SA Vorentoe 69	SA Vorentoe 94	SA Witbank 72	SA witbank 72	SA Witbank 74	Total
A	1.5-3.6	7	4	7	7	7	7	4	4	4	5	7	8	5	3	3	5	6	4	6	2	6	5	2	4	6	5	6	3	6	4	6	5	163
B	1-1.5	3	4	5	4	6	6	4	4	5	6	5	5	5	4	4	4	5	4	6	2	5	5	4	6	6	4	6	2	6	5	5	6	151
C	0.75-1	0	1	1	0	2	2	1	1	1	1	2	1	1	0	1	2	2	1	2	4	1	2	0	1	2	1	2	0	2	1	2	2	42
D	0.5-0.75	2	4	3	2	4	3	2	2	3	4	3	3	2	1	2	3	3	2	3	7	2	2	2	2	3	2	3	1	3	3	3	2	86
E	0.2-0.5	3	1	3	3	2	2	1	2	1	1	2	1	2	1	1	1	2	2	2	3	2	2	1	1	2	2	3	1	2	3	3	2	60
	Total	15	14	19	16	21	20	12	13	14	17	19	18	15	9	11	15	18	13	19	18	16	16	9	14	19	14	20	7	19	16	19	17	502

The details of percentage presence of various loci in profiles are provided in Table 4.4. This table provides a detailed record of the number of times a locus was recorded in the profiles. Data on percentage polymorphisms could thus be extracted from this table. If one considers that there were 32 individuals and if a locus was present in all 32 isolates, then the band was monomorphic and may be a potential marker for SA *X. c* pv. *campestris*. Loci were considered to be monomorphic if detected in more than 90% of the isolates, otherwise they were deemed polymophic. The monomorphic Eric-PCR loci were 2.709kb, 2.315kb, 1.697kb, 1.547kb and 1.136kb, whereas monomorphic Box-PCR loci were 1.184kb, 1.028kb, 0.661kb and 0.559kb.

4.7.1.1 Comparative analysis of Eric-PCR profiles of South African *X. c* pv. *campestris* isolates

Twenty nine loci were polymorphic for Eric-PCR, of which 17 (53.1%) were most polymorphic with band presence in 1 to 20% of the isolates. Of the 17 most polymorphic loci, five loci, 2.559kb, 1.422kb, 1.471kb, 0.413kb and 0.439kb had relatively unique distribution that could be correlated to the geographic origin of the isolates. Loci 0.413kb and 0.439kb were present in 5 isolates from Randfontein and 2 isolates from neighbouring Magaliesburg. These two areas are situated on the West Rand and are geographically close to each other (Figure 3.1). Locus 2.559kb was present in all 3 isolates from Brits on the West Rand, 2 isolates from Delmas and 1 from Nigel in the East Rand. Locus 1.471 was present in 5 isolates from Randfontein in West Rand and 1 isolate from Delmas on the East Rand. Similar observations were also made with locus 1.422kb which was present in seven isolates from the West Rand (3 isolates from Brits, 2 from Magaliesburg and individual isolates from Tarlton and Vorentoe) and three 3 isolates from Witbank in East Rand.

4.7.1.2 Comparative analysis of Box-PCR profiles of South African *X. c* pv. *campestris* isolates

Of the 36 polymorphic loci for Box-PCR only 3 loci, 3.04kb, 2.921kb had 1.563kb, had relatively unique distribution that could be correlated to the geographic origin of the isolates. There was spatial distribution of loci between the West Rand and East Rand isolates. Locus 3.04kb was present in 5 isolates from West Rand areas and included 3 isolates from Randfontein, individual isolates from neighbouring Magaliesburg, Carltonville and 3 isolates from previous collections (SA 482, 512 and 513). Locus 2.921kb was present in 4 isolates from Randfontein, individual isolates from Magaliesburg, Tarlton, Vorentoe and 3 isolates SA 482, 512 and 513 from previous collections. Loci were also shared between isolates from both the West rand and East Rand. Locus 1.563kb was present in 2 isolates from Brits and 2 from Magaliesburg in the West Rand and 2 isolates from Delmas, individual isolates from Boksburg and Nigel in East Rand and isolate SA 482 from previous collections. Some loci were unique to particular geographical areas whereas others were distributed throughout the region.

Table 4.5**:** Summary of band distribution among loci within the five regions of Eric- and Box-PCR electrophoretic profiles for *X. c* pv. *campestris* solates from South Africa.

(A) Eric Profiles

Gel Regions												
A			B			C			D			E		
Loci (mw)	Bands	% presence	Loci (mw)	Bands	% presence	Loci (mw)	Bands	% presence	Loci (mw)	Bands	% presence	Loci (mw)	Bands	% presence
2.709	29	90.6	1.547	31	96.9	0.717	16	50.0	0.973	12	37.5	0.439	12	37.5
2.599	8	25.0	1.471	6	18.8	0.675	4	12.5	0.867	23	71.9	0.413	9	28.1
2.315	31	96.9	1.422	13	40.6	0.650	4	12.5	0.809	8	25.0	0.290	2	6.3
2.069	12	37.5	1.357	6	18.8	0.599	8	25.0	0.774	2	6.3	0.258	11	34.4
1.947	2	6.3	1.323	6	18.8	0.585	18	56.3	0.755	9	28.1	0.216	21	65.6
1.775	4	12.5	1.266	3	9.4	0.557	1	3.1						
1.697	30	93.8	1.214	3	9.4	0.540	2	6.3						
1.596	17	53.1	1.136	29	90.6									
			1.004	2	6.3									

(B) Box Profiles

Gel Regions												
A			B			C			D			E		
Loci (mw)	Bands	% presence	Loci (mw)	Bands	% presence	Loci (mw)	Bands	% presence	Loci (mw)	Bands	% presence	Loci (mw)	Bands	% presence
3.632	1	3.1	1.459	28	87.5	0.992	2	6.3	0.749	1	3.1	0.432	1	3.1
3.490	24	75.0	1.410	16	50.0	0.923	3	9.4	0.733	3	9.4	0.369	3	9.4
3.040	10	31.3	1.331	6	18.8	0.861	11	34.4	0.707	4	12.5	0.353	12	37.5
2.921	13	40.6	1.299	7	21.9	0.808	26	81.3	0.661	30	93.8	0.320	7	21.9
2.751	23	71.9	1.184	32	100.0				0.589	30	93.8	0.295	12	37.5
2.567	19	59.4	1.090	13	40.6				0.545	1	3.1	0.264	8	25.0
2.365	12	37.5	1.062	17	53.1				0.523	1	3.1	0.251	17	53.1
2.075	9	28.1	1.028	32	100.0				0.504	16	50.0			
2.005	9	28.1												
1.666	7	21.9												
1.613	21	65.6												
1.563	10	31.3												
1.476	5	15.6												

4.7.2 Eric- and Box-PCR profiles of international *X. c* pv. *campestris* isolates

Figure 4.6 and 4.7 show typical, negative ethidium bromide stained SDS-PAGE gels of international *X. c* pv. *campestris* isolates for Eric- and Box-PCR profiles, respectively. A total of 53 *X. c* pv. *campestris* isolates from different parts of the world, 3 *X. c* pv. *armoraciae* and 1 *X. c* pv. *raphani* were analysed. Generated fingerprint patterns varied widely between isolates and no characteristic pattern could be designated for the *X. c* pv. *campestris* species. Five gel regions and their respective fragment size ranges (Section 4.7) are shown. While the figures do not show labels of the respective isolate profiles, they were included in the Eric- and Box-PCR profile dendograms in Figures 4.8 and 4.9 for all isolates.

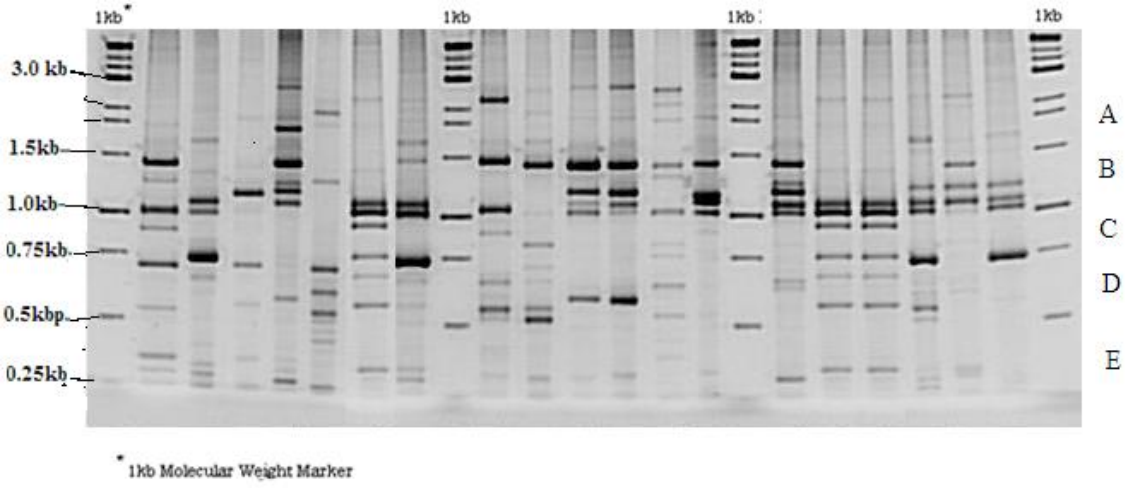

Figure 4.6: A negative image of ethidium bromide stained SDS-PAGE gel showing Eric-PCR profiles of *X. c* pv. *campestris* isolates of international origin.

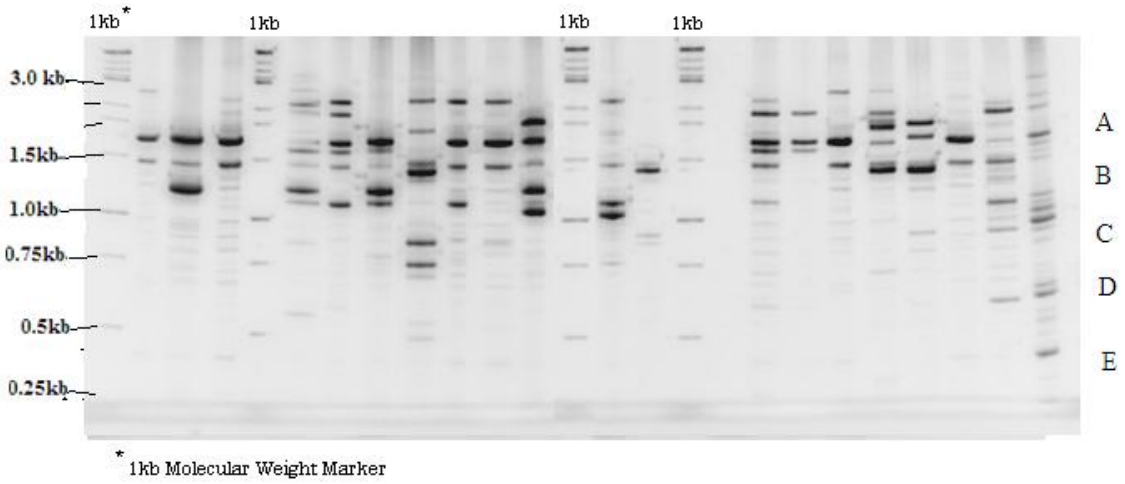

Figure 4.7: A negative image of ethidium bromide stained SDS-PAGE gel showing Box-PCR profiles of *X. c* pv. *campestris* isolates of international origin.

Eric-profiles in Figure 4.6 are different from the Box-profiles in Figure 4.7. The profiles had bands present from 0.25kb to approximately 3.0kb. Bands with high intensity in Eric-were in regions "B", "C" and "D" whereas those for Box-profiles were in regions "A" and "B". There was no relative uniformity of profile patterns between isolates for both Eric- and Box-PCR that could be designated as being characteristic of the *X. c* pv. *campestris* isolates. However, for the few isolates that had similar profile patterns, the intensities of common bands varied among the isolates. The details of the profiles, after analysis with mentioned software are presented below.

Table 4.4 shows the numbers of bands observed per isolate within five fragment size specific regions "A" to "E" as previously described (Section 4.7). A total of 662 scorable bands were amplified for Eric-PCR profiles and 708 for Box-PCR profiles, for the 53 isolates of international origin. The highest number of bands observe in Eric-PCR profiles were 277 (34%) bands in region "A" and 167 (25%) in "B". A similar scenario was observed for Box-PCR profiles. In this case 223 (28%) bands were observed in region "A" and 180 (23%) in region "D". Each band was assumed to

correspond to one locus. The banding profiles, total number of bands as well as number of polymorphic bands were primer dependant. The number of amplified loci per individual varied from 4 to 22 for Eric-PCR profiles and 7 to 23 for Box-PCR profiles with a size range varying from 0.21 to 3.6kb for both Eric- and Box-PCR.

The details (percentage presence) of various loci in profiles are provided in Table 4.7. This table provides a detailed record of the number of times a locus was recorded in the profiles. Data on percentage polymorphisms could thus be extracted from this table. If one considers that there were 57 individuals and if a locus was present in all 57 isolates, then the band was monomorphic and may be a potential marker for *X. c* pv. *campestris*. Loci were considered to be monomorphic if detected in more than 90% of the isolates, otherwise they were deemed polymorphic.

Table 4.6: Summary of band distribution among five regions of Eric- and Box-PCR elecrophoretic profiles for *X. c* pv. *campestris* isolates of international origin.

(A) Eric PCR profile

Gel Region	Molecular Weight (kb)	Australia 09-3925-71	Australia 307	Australia 564	Australia82092-3.8	Austria 322	Chile 2006-481	Chile 561	Chile 563	Chile 81610-1.7	Chile 82451-521	Chile 82504-1.1	Chile 83163-15	Chile 84638-6.2	China 559	China 560	Denmark 81852-2.2	Denmark 83085-4.1	France 86547-32	France Aude 557	German 2006-236	German 511a	German 567	India 553	India 562	Indonesia 551	Italy 61732-26	Italy 80574.6-18	Italy 80749-3.3	Italy 82450-5.3	Italy 82695-28	Italy 86221-22	Japan Chiba 556	Japan 320	Japan 565	Japan Aichi 554	Japan Chiba 550	Japan Chiba 552	Netherlands 272	Netherlands 325	Netherlands 326	Netherlands 463	Netherlands 83431-427	Newzealand 82491-3.3	Nothwest Bosnia 514	Portugal 254	Portugal 275	Spain 323	Spain 323	UK Cornwall 277	USA 276	USA 83210-3.1	USA Florida 319	USA WL Coloma 321	Total
A	1.5-3	6	5	4	6	4	4	5	3	6	7	5	5	7	5	3	2	4	6	1	4	3	7	3	4	2	6	5	3	0	7	7	5	3	4	3	1	3	2	5	3	3	6	3	6	7	3	5	5	4	5	5	3	2	225
B	1-1.5	4	4	5	3	2	2	4	3	5	2	4	3	4	3	0	2	3	2	2	2	2	3	4	4	4	6	3	5	2	6	3	3	3	5	3	3	3	4	2	1	1	4	3	5	3	3	4	4	4	2	2	3	1	167
C	0.75-1	3	3	2	4	2	1	3	2	4	4	2	4	3	3	0	1	1	3	2	4	0	3	3	0	2	3	3	0	1	2	1	3	1	3	2	3	3	1	2	0	1	2	0	2	3	3	2	2	2	0	0	3	0	107
D	0.5-0.75	3	4	1	0	2	0	1	1	2	5	3	2	4	3	0	2	1	1	0	1	0	5	2	1	3	2	1	1	0	1	1	0	1	3	2	2	2	1	1	0	0	2	0	1	3	2	1	1	2	0	0	0	0	77
E	0.25-0.5	1	2	3	1	3	0	3	2	1	0	0	2	2	3	2	0	0	2	2	3	1	4	3	3	2	3	2	2	3	3	1	2	0	2	2	2	3	2	0	0	0	2	2	1	0	1	2	2	0	3	0	0	1	86
	Total	17	18	15	14	13	7	16	11	18	18	14	16	20	17	5	7	9	14	7	14	6	22	15	12	13	20	14	11	6	19	13	13	8	17	12	11	14	10	10	4	5	16	8	15	16	12	14	14	12	10	7	9	4	662

(B) Box PCR profile

Gel Region	Molecular Weight (kb)	Australia 09-3925-71	Australia 307	Australia 564	Australia82092-3.8	Austria 322	Chile 2006-481	Chile 561	Chile 563	Chile 81610-1.7	Chile 82451-521	Chile 82504-1.1	Chile 83163-15	Chile 84638-6.2	China 559	China 560	Denmark 81852-2.2	Denmark 83085-4.1	France 86547-32	France Aude 557	German 2006-236	German 511a	German 567	India 553	India 562	Indonesia 551	Italy 61732-26	Italy 80574.6-18	Italy 80749-3.3	Italy 82450-5.3	Italy 82695-28	Italy 86221-22	Japan Chiba 556	Japan 320	Japan 565	Japan Aichi 554	Japan Chiba 550	Japan Chiba 552	Netherlands 272	Netherlands 325	Netherlands 326	Netherlands 463	Netherlands 83431-427	Newzealand 82491-3.	Nothwest Bosnia 514	Portugal 254	Portugal 275	Spain 323	Spain 323	UK Cornwall 277	USA 276	USA 83210-3.1	USA Florida 319	USA WL Coloma 321	Total
A	1.5-3	4	9	2	8	3	6	5	2	9	3	7	5	7	4	1	3	5	6	3	3	3	4	6	4	4	1	5	4	3	4	4	0	3	1	5	1	6	4	2	4	6	3	8	4	8	7	4	4	6	2	3	4	1	223
B	1-1.5	3	3	1	3	2	2	1	2	3	1	4	2	2	2	3	3	3	4	1	0	3	3	2	2	2	1	4	2	4	2	2	2	2	2	4	2	0	3	4	3	4	2	2	2	1	1	4	4	4	2	2	1	2	130
C	0.75-1	1	4	1	2	2	3	1	2	2	0	2	3	2	3	1	1	2	1	2	2	1	0	1	1	1	3	1	3	1	1	3	1	1	4	2	3	3	1	1	2	1	4	1	3	2	4	1	2	4	1	2	2	1	99
D	0.5-0.75	5	1	3	3	3	5	5	3	5	5	4	6	5	2	3	3	4	3	2	3	0	3	7	5	4	5	5	5	4	5	3	2	5	1	1	1	6	2	1	2	1	5	4	5	2	3	1	3	6	2	5	0	3	180
E	0.25-0.5	5	3	4	2	2	3	4	1	3	6	2	3	3	3	3	3	4	5	3	4	1	4	5	3	2	4	2	2	4	2	3	2	1	4	4	3	4	4	2	1	2	1	3	3	4	5	3	2	3	3	1	4	1	1	157
	Total	18	20	11	18	12	19	16	10	22	15	19	19	19	14	11	13	18	19	11	12	8	14	21	15	13	14	17	16	16	14	15	7	12	12	16	10	19	14	10	12	13	17	18	18	18	18	12	16	23	9	17	11	8	789

Table 4.7: Summary of band distribution among loci within the five regions of Eric- and Box-PCR elecrophoretic profiles for *X. c* pv. *campestris* isolates of international origin.

(A) Eric Profiles

Gel Regions												
A			B			C			D			E		
Loci (mw)	Bands	% presence	Loci (mw)	Bands	% presence	Loci (mw)	Bands	% presence	Loci (mw)	Bands	% presence	Loci (mw)	Bands	% presence
3.354	14	26.4	1.471	27	50.9	0.973	9	17.0	0.717	19	35.8	0.439	1	1.9
3.263	6	11.3	1.422	27	50.9	0.937	19	35.8	0.675	16	30.2	0.413	9	17.0
3.052	4	7.5	1.357	11	20.8	0.898	8	15.1	0.650	9	17.0	0.355	4	7.5
2.929	7	13.2	1.323	19	35.8	0.867	23	43.4	0.599	6	11.3	0.290	13	24.5
2.831	16	30.2	1.266	23	43.4	0.809	25	47.2	0.585	11	20.8	0.258	27	50.9
2.709	16	30.2	1.214	21	39.6	0.774	12	22.6	0.557	7	13.2	0.216	32	60.4
2.599	11	20.8	1.136	22	41.5	0.755	11	20.8	0.540	5	9.4			
2.433	11	20.8	1.072	15	28.3				0.502	4	7.5			
2.315	17	32.1	1.004	7	13.2									
2.069	15	28.3												
1.947	9	17.0												
1.864	11	20.8												
1.775	14	26.4												
1.697	11	20.8												
1.646	22	41.5												
1.596	20	37.7												
1.547	21	39.6												

(B) Box Profiles

Gel Regions												
A			B			C			D			E		
Loci (mw)	Bands	% presence	Loci (mw)	Bands	% presence	Loci (mw)	Bands	% presence	Loci (mw)	Bands	% presence	Loci (mw)	Bands	% presence
3.632	5	9.4	1.459	34	64.2	1.062	10	18.9	0.749	17	32.1	0.474	8	15.1
3.490	31	58.5	1.410	19	35.8	1.028	26	49.1	0.733	9	17.0	0.432	6	11.3
3.354	13	24.5	1.331	8	15.1	0.992	32	60.4	0.707	15	28.3	0.396	11	20.8
3.206	14	26.4	1.299	12	22.6	0.923	14	26.4	0.683	11	20.8	0.369	12	22.6
3.040	9	17.0	1.184	27	50.9	0.861	5	9.4	0.661	17	32.1	0.353	16	30.2
2.921	23	43.4	1.090	30	56.6	0.808	12	22.6	0.635	15	28.3	0.320	28	52.8
2.847	10	18.9							0.604	11	20.8	0.295	8	15.1
2.751	12	22.6							0.589	25	47.2	0.264	23	43.4
2.567	24	45.3							0.563	16	30.2	0.251	21	39.6
2.365	14	26.4							0.545	16	30.2	0.201	24	45.3
2.075	10	18.9							0.523	7	13.2			
2.005	11	20.8							0.504	21	39.6			
1.666	18	34.0												
1.613	13	24.5												
1.681	7	13.2												
1.563	9	17.0												

4.7.2.1 Comparative analysis of Eric-PCR profiles of international *X. c* pv. *campestris* isolates

A total of 47 loci were amplified using Eric-PCR of which only two loci, 1.471kb and 1.422kb were present in more than 50.9% of isolates. Of the 5 loci in South African Eric-PCR profiles that were present in more than 90% of isolates none had corresponding high band presence among international isolates. Loci 2.709kb, 2.315kb, 1.697kb, 1.547kb and 1.136kb were present in 16 (30.2%), 17(32.1%), 11 (20.8%), 21 (39.6%) and 22 (41.5%) of isolates, respectively.

Of the 47 polymorphic loci, 5 loci (3354kb, 2.079kb, 1.422kb, 1.755kb and 0.809kb) demonstrated some relative geographic distribution patterns by country of origin. Locus 3.354 was present in all 3 isolates from Chile, 3 from Netherlands, 2 from German, 2 from Portugal and 2 from Spain. Similar observations were made with locus 2.709kb which was present in 2 isolates from Australia, 2 from Chile and 4 from Japan. Locus 1.755kb was present in 5 isolates from Chile, while locus 1.422kb was present in all isolates from Denmark, France, India and 4 isolates from Japan. Locus 0.809 was present in two isolates from Australia, 6 from Chile and 2 from France.

While some loci were present in several isolates from one country, the same loci were shared by isolates from other countries on different continents. None of the loci were continent based, but were distributed in isolates from different continents. While certain loci were present in most isolates from the same country, they were absent in some isolates

sharing the same country of origin. This suggests wide variation and distribution of *X. c* pv. *campestris* in localised environments and the world at large.

4.7.2.2 Comparative analysis of Box-PCR profiles of international *X. c* pv. *campestris* isolates

Forty loci were amplified using Box-PCR and the highest band presence was in loci 1.459kb and 0.992kb which were present in 64.2% and 60% of the isolates, respectively. Of the 6 loci in South African Box-PCR profiles that were present in more than 90% of isolates none had corresponding high band presence among international isolates. Loci 1.459kb, 1184kb,1.028kb, 0.808kb, 0.661kb and 0.589kb were present in 34 (64.2%), 27(50%), 20 (49%), 12 (22%) and 17 (32%) of isolates, respectively.

Of the 40 polymorphic loci, 4 loci (2.921kb, 2.075kb, 1.410kb and 0.504kb) demonstrated the following distribution patterns. Locus 2.921kb was present in 4 isolates from Netherlands, 2 from USA, 5 from Chile and 2 from Australia. Locus 2.075kb was present in 5 isolates from Chile, 2 from Australia and individual isolates from USA and Italy. Locus 1.410kb was present in 3 isolates from Chile, 2 from Italy, 5 from Netherlands, 2 from USA and individual isolates from Spain, UK and Japan. Locus 0.504kb was present in 3 isolates from Australia, 8 from Chile, 5 from Italy and individual isolates from Denmark, France, Japan, New Zealand, Bosnia, Spain and UK. Locus 0.264kb was present in 4 isolates from Australia and six isolates from Chile

While loci were present in several isolates from one country, the same loci were shared by isolates from different countries in different continents. None of the loci were continent based, but were distributed in isolates from different continents. While certain loci were present in most isolates from the same country, they were absent in some of the isolates sharing the same country of origin. This suggests wide variation and distribution of *X. c* pv. *campestris* localised environments and the world at large. Eric-Loci distribution suggested a similar pattern

4.7.8 Comparative analysis of Eric- and Box-PCR of South African and international *X. c* pv. *campestris* isolates and reference strains of *X. c* pv. *armoraciae and X. c* pv. *raphani.*

Table 4.8 and 4.9 shows the numbers of bands observed in South African and international *X. c* pv. *campestris* isolates, and reference strains of *X. c* pv. *armoraciae* and *X. c* pv. *raphan* within the five fragment size specific regions "A" to "E" as previously described in Section 4.7. The highest number of bands were observe in regions "A" and "B" for Eric-profiles of South African isolates and region "A" and "D" for international isolates and *X. c* pv. *armoraciae* reference strains. For the reference strain *X. c* pv. *raphani,* the highest number of bands were observe in regions "B" and " E". A different scenario was observed for Box-PCR profiles. In this case the highest number of bands was observed in regions "A" and "B" for all *X. c* pv. *campestris* isolates including the reference strain of *X. c* pv. *armoraciae* (xca) and *X. c* pv. *raphani* except for the reference strain *X. c* pv. *armoraciae* (Italy 59863.3) which had the highest number of bands in regions "B" and"E" only.

Distribution of bands between these species is relatively similar in Box-PCR profiles as compared to Eric-PCR profiles. The similarity in distribution of Eric-PCR profile bands within the regions "A" to "E" was different between South African *X. c* pv. *campestris* isolates and reference strains. However, when international isolates are considered the case was different as the band distribution was similar to that of reference strains.

Table 4.8: Summary of bands present in five regions (A-E) of Eric-PCR profiles of South African and international *X. c* pv. *campestris* isolates, and reference strains of *X. c* pv. *armoraciae and X. c* pv. *raphani.*

Gel Region	South African *X. c* pv *campestris*		International *X. c* pv *campestris*		*X. c* pv *amoraciae* (xca)		*X. c* pv *amoraciae* (Italy 59863.3)		*X. c* pv *raphani* (xcr 540)	
	(n)	%	(n)	%	(n)	%	(n)	%	(n)	%
A	163	32.5	223	28.3	5	29	4	31	1	9.09
B	151	30.1	130	0.16	2	12	0	0	3	27.3
C	42	8.37	99	12.5	2	12	2	15	2	18.2
D	86	17.1	180	22.8	4	24	4	31	1	9.09
E	60	12	157	19.9	4	24	3	23	4	36.4
TOTAL	502	100	789	100	17	100	13	100	11	100

Table 4.9: Summary of band present in five regions (A-E) of Box-PCR profiles of South African and international *X. c* pv. *campestris* isolates, and reference strains of *X. c* pv. *armoraciae and X. c* pv. *raphani.*

Gel Region	South African *X. c* pv *campestris*		International *X. c* pv *campestris*		*X. c* pv *amoraciae* (xca)		*X. c* pv *amoraciae* (Italy 59863.3)		*X. c* pv *raphani* (xcr 540)	
	(n)	%	(n)	%	(n)	%	(n)	%	(n)	%
A	133	33.8	225	34	3	43	1	14.3	7	50
B	99	25.1	167	25.2	2	29	2	28.6	3	21.4
C	54	13.7	107	16.2	0	0	1	14.3	1	7.14
D	53	13.5	77	11.6	0	0	1	14.3	1	7.14
E	55	14	86	13	2	29	2	28.6	2	14.3
TOTAL	394	100	662	100	7	100	7	100	14	100

Forty seven loci were amplified in Eric-PCR. All loci were polymorphic for all the *X. c* pv. *campestris, X. c* pv. *armoraciae* and *X. c* pv. *raphani.* However, 5 loci were shared among the reference strains and at least 45% of the *X. c* pv. *campestris* isolates. One locus, 0.258kb was shared by all reference strains and, 46% of *X. c* pv. *campestris* isolates. Loci 0.216kb and 0.867kb was shared by *X. c* pv. *armoraciae, X. c* pv. *raphani* and 64% and 53% of *X. c* pv. *campestris* isolates, respectively. Locus 1.471kb was shared only between *X. c* pv. *armoraciae* and, 53% *X. c* pv. *campestris* isolates, while Locus 1.136kb was shared only between *X. c* pv. *raphani* and 53% *X. c* pv. *campestris* isolates. All loci present in *X. c* pv. *armoraciae* and *X. c* pv. *raphani* reference strains were shared by at least 7% of the *X. c* pv. *campestris* isolates.

Fifty one loci were amplified in Box-PCR. All loci were polymorphic for all the *X. c* pv. *campestris, X. c* pv. *armoraciae and X. c* pv. *raphani.* However, 7 loci were shared among

the reference strains and at least 45% of the *X. c* pv. *campestris* isolates. Two loci 0.589kb and 0.992 were shared by all reference strains and, 62% and 51% of *X. c* pv. *campestris* isolates, respectively. Loci 1.184kb and 1.459kb was shared by *X. c* pv. *armoraciae, X. c* pv. *raphani* and 66% and 64% of *X. c* pv. *campestris* isolates, respectively. Loci 0.661kb, 1.090kb and 3.490kb were shared only between *X. c* pv. *armoraciae* and, 48%, 48% and 60% of *X. c* pv. *campestris* isolates, respectively. All loci present in *X. c* pv. *armoraciae* and *X. c* pv. *raphani* reference strains were shared by at least 20% of the *X. c* pv. *campestris* isolates

The relative homogeneity in Box-PCR profile among *X. c* pv. *campestris, X. c* pv. *armoraciae* and *X. c* pv. *raphani* was relatively higher in Box-PCR profiles than Eric-PCR profiles. Only 5 loci were shared between individual reference strains and at least 45% of the *X. c* pv. *campestris* isolates in Eric-PCR. Whereas in Box-PCR more than 50% of *X. c* pv. *campestris* shared 2 loci with all reference isolates, 2 loci with *X. c* pv. *armoraciae* and *X. c* pv. *raphani and* 3 loci with *X. c* pv. *armoraciae* only. The percentages of shared bands between *X. c* pv. *campestris* and reference strains was generally higher in Box-PCR profiles than Eric-PCR profiles. The relative similarity between *X. c* pv. *campestris* and reference strains is higher with X. *c* pv. *armoraciae* than *X. c* pv. *raphani.* This suggests X. *c* pv. *armoraciae* to be more closely related to *X. c* pv. *campestris* than *X. c* pv. *raphani* (Black and Machmud, 1983; Alvarez *et al*., 1994; Tamura *et al*., 1994; Sahin and Miller, 1997; Vicente *et al*., 1998)

These findings suggest that Eric-PCR analysis has better resolution of differences between *X. c* pv. *campestris* and the closely related X. *c* pv. *armoraciae* and *X. c* pv. *raphani* than Box-PCR. While some bands were relatively dominant in Eric-PCR, serving as potential pathovar markers, the sample sizes of *X. c* pv. *armoraciae* and *X. c* pv. *raphani* used were too small to be conclusive. The diversity of *X. c* pv. *campestris* appears to be limited within localised geographical areas. However, when international populations are considered, the variation that exists is such that it will be difficult to distinguish between *X. c* pv. *campestris* from closely related pathovars.

Table 4.10: Summary of loci and band present in five regions (A-E) of Eric-PCR profiles of South African and international isolates, and reference strains of *X. c* pv. *armoraciae* and *X. c* pv. *raphani.*

Loci (mw)	South African and international *X. c* pv *campestris* isolates	*X. c* pv *amoraciae* (xca)	*X. c* pv *amoraciae* (Italy 59863.3)	*X. c* pv *raphani* (xcr 540)
A				
3.354	14	0	0	0
3.263	7	0	0	1
3.052	5	0	0	0
2.929	9	0	0	1
2.831	26	0	0	0
2.709	46	0	0	0
2.599	20	0	0	1
2.433	25	0	0	1
2.315	40	0	0	0
2.069	32	0	1	0
1.947	13	0	0	0
1.864	14	1	0	1
1.775	20	0	0	0
1.697	19	1	0	0
1.646	53	0	0	1
1.596	44	0	0	0
1.547	44	1	0	1

Loci (mw)	South African and international *X. c* pv *campestris* isolates	*X. c* pv *amoraciae* (xca)	*X. c* pv *amoraciae* (Italy 59863.3)	*X. c* pv *raphani* (xcr 540)
B				
1.471	53	1	0	0
1.422	41	0	0	1
1.357	20	0	1	0
1.323	28	0	1	0
1.266	31	1	0	0
1.214	28	0	0	1
1.136	53	0	0	1
1.072	25	0	0	0
1.004	11	0	0	0

Loci (mw)	South African and international *X. c* pv *campestris* isolates	*X. c* pv *amoraciae* (xca)	*X. c* pv *amoraciae* (Italy 59863.3)	*X. c* pv *raphani* (xcr 540)
C				
0.973	20	0	0	0
0.937	24	0	0	0
0.898	13	0	0	0
0.867	53	0	1	1
0.809	39	0	0	0
0.774	16	0	0	0
0.755	24	0	0	0

Loci (mw)	South African and international *X. c* pv *campestris* isolates	*X. c* pv *amoraciae* (xca)	*X. c* pv *amoraciae* (Italy 59863.3)	*X. c* pv *raphani* (xcr 540)
D				
0.717	41	0	0	0
0.675	24	0	0	0
0.65	15	0	0	0
0.599	16	0	0	0
0.585	34	0	0	0
0.557	9	0	0	1
0.54	8	0	1	0
0.502	5	0	0	0

Loci (mw)	South African and international *X. c* pv *campestris* isolates	*X. c* pv *amoraciae* (xca)	*X. c* pv *amoraciae* (Italy 59863.3)	*X. c* pv *raphani* (xcr 540)
E				
0.439	15	0	0	0
0.413	21	0	0	0
0.355	5	0	0	0
0.29	18	1	0	0
0.258	46	1	1	1
0.216	64	0	1	1

Note: The band presence for South African and international isolates are given as pecentages whereas those for the reference strains of *X. c* pv. *armoraciae* and *X. c* pv. *raphani* show the prence absence matrix.

Table 4.11: Summary of loci and band present in five regions (A-E) of Box-PCR profiles of South African and international isolates and reference strains of *X. c* pv. *armoraciae and X. c* pv. *raphani*.

Loci (mw)	South African and international *X. c* pv *campestris* isolates	*X. c* pv *armoraciae* (xca)	*X. c* pv *armoraciae* (Italy 59863.3)	*X. c* pv *raphani* (xcr 540)
	A			
3.632	8	0	0	0
3.49	60	0	1	0
3.354	15	0	0	0
3.206	21	1	0	0
3.04	15	0	0	0
2.921	39	1	0	1
2.847	17	0	1	0
2.751	34	0	1	0
2.567	46	0	0	0
2.365	29	0	1	0
2.075	21	1	0	0
2.005	21	0	0	0
1.666	28	1	0	0
1.613	37	1	0	0
1.681	7	0	0	0
1.563	21	0	0	0

Loci (mw)	South African and international *X. c* pv *campestris* isolates	*X. c* pv *armoraciae* (xca)	*X. c* pv *armoraciae* (Italy 59863.3)	*X. c* pv *raphani* (xcr 540)
	B			
1.476	7	0	0	0
1.459	66	1	0	1
1.41	42	0	0	0
1.331	15	0	0	1
1.299	24	0	0	0
1.184	64	1	0	1
1.09	48	1	0	0
1.062	30	0	0	1
1.028	51	0	0	0

Loci (mw)	South African and international *X. c* pv *campestris* isolates	*X. c* pv *armoraciae* (xca)	*X. c* pv *armoraciae* (Italy 59863.3)	*X. c* pv *raphani* (xcr 540)
	C			
0.992	51	1	1	1
0.923	19	0	1	0
0.861	18	0	0	0
0.808	43	0	0	0
0.749	20	0	1	0

Loci (mw)	South African and international *X. c* pv *campestris* isolates	*X. c* pv *armoraciae* (xca)	*X. c* pv *armoraciae* (Italy 59863.3)	*X. c* pv *raphani* (xcr 540)
	D			
0.733	12	0	0	0
0.707	21	1	0	0
0.683	13	0	0	0
0.661	48	1	1	0
0.635	20	0	0	0
0.604	12	0	0	0
0.589	62	1	1	1
0.563	18	1	0	0
0.545	19	0	1	0
0.523	9	0	0	0
0.504	42	1	1	0

Loci (mw)	South African and international *X. c* pv *campestris* isolates	*X. c* pv *armoraciae* (xca)	*X. c* pv *armoraciae* (Italy 59863.3)	*X. c* pv *raphani* (xcr 540)
	E			
0.474	9	0	0	0
0.432	7	0	0	0
0.396	12	0	0	0
0.369	16	0	0	0
0.353	31	1	0	1
0.32	38	1	1	1
0.295	22	0	0	0
0.264	35	0	0	1
0.251	42	1	1	0
0.201	30	0	0	1

Note: The band presence for South African and international isolates are given as pecentages whereas those for the reference strains of *X. c* pv. *armoraciae* and *X. c* pv. *raphani* show the prence absence matrix.

4.8 CLUSTER ANALYSIS OF SOUTH AFRICAN AND INTERNATIONAL *X. c* pv. *campestris* ERIC- AND BOX-PCR PROFILES.

Individual Eric- and Box-PCR profiles generated data sets were analyzed using the trial version of Phoretix 1D and Phoretix ID Pro (version 10) software packages from TotalLab Limited (UK). Pearson coefficient and Ward algorithm was used for the numerical analysis of these profiles and results displayed as dendograms. South African isolates Eric- and Box-PCR profile pattern of South African isolates (Figure 4.4 and 4.5) clustered separately at relatively lower Euclidian distance values as compared to international isolates (Figure 4.6 and 4.7) in both Eric-and Box-PCR dendograms (Figure 4.8 and 4.9). Most of the international isolates Eric- and Box-PCR profile patterns varied widely (Figure 4.6 and 4.7).

Two major clusters (I and II) were observed for both Eric- and Box-PCR profiles (Figure 4.8 and 4.9), respectively. Cluster I was mainly composed of South African isolates whereas cluster II was composed of international isolates. For discussion purposes the two major clusters were divided into 12 and 13 meaningful smaller clusters for Eric- and Box-dendograms, respectively. In the Eric based dendrogram cluster I was divided into clusters E1 to E6 and cluster II into E7 to E12 clusters (Figure 4.8). In Box-dendrogram, cluster I was divided into clusters B8 to B13 and cluster II into B1 to B7 clusters (Figure 4.8). In Box-dendrogram cluster I was further divided into clusters Ia and Ib. Cluster Ia was comprised of isolated from South African origin only while cluster Ib was composed of isolates of international origin and 5 South African isolates from the West Rand.

Some form of geographical separation was observed among South African isolates in both Figure 4.8 and 4.9. Group E4 was comprised of more than 50% isolates from East Rand, whereas at least 90% of group E5 isolates were from the West Rand (Figure 4.8). All isolates in cluster group B9, B13, at least 70% in cluster B12 and 50% in cluster B11 originated from East Rand (Figure 4.9).

Isolate of the same national origin generally clustered together as observed with two isolates from Netherlands in E9 and B6, Japanese isolates in E3 and B3, Italian isolates in E11, E10, B2 and B5, and Chinese isolates in B9 and E8. Nevertheless isolates of the same international origin were also clustered in different cluster groups with other isolates of different international origin as observed with isolates from the Netherlands which clustered in B2, B5, B6 and B10, Japanese isolates in E2, E3 and E6 and USA isolates in B1, B8 and B10. Cluster groups were also comprised of isolates originating from different countries, as observed with Cluster group E11 with isolates from Chile, German, Indonesia and Italy and Cluster group B10 with isolates from USA, Netherlands, Chile and Japan.

4.9 GEOGRAPHIC ORIGIN, SYMPTOM GROUP AND RACE CLASSIFICATION RELEVANCE TO *X. c* pv. *campestris* DISTRIBUTION

The distribution of different races and symptom group types among isolates from specific geographical areas within the East Rand and West Rand areas are shown in Figure A1 (Appendix 1). Eric- and Box-PCR profiles clustered South African isolates, mainly by their geographic origin as described above. Relative clustering according to races and symptom groups was also observed. Four of the seven isolates in symptom

group “a” were in cluster group E4, of the thirteen symptom group “b” isolates, four were in group E3 and B13, three in B9 and B12 and five were in E5. For Symptom group “c”, four of the isolates were in group B11 and three were in group E3. Five of the eight isolates in symptom group C were race 4, two race 1and 1 race 6. Race 4 was dominant in all the symptom groups nevertheless even minor races were distributed out within the symptom groups with no dominant race symptom group correlation. All isolates in group E3 were race 4 and all race 3 isolates were in group E5. Two of the three race 6 isolates were consistently grouped together in B9 and E5. The rest of the other isolates were clustered with no particular race grouping.

The observation of similar races and symptom group types clustering together appear to be coincidental as isolates of different races and symptom group types were equally grouped under the same clusters. Therefore it appears that race and symptom type grouping are independent of the Eric- and Box-elements distribution within the sampled *X. c* pv. *campestris* population. However the South African isolates Eric- and Box-elements seem to be geographical distributed within the West Rand and East Rand areas. There is also evidence of spread of the pathogen with the West Rand and East Rand, areas.

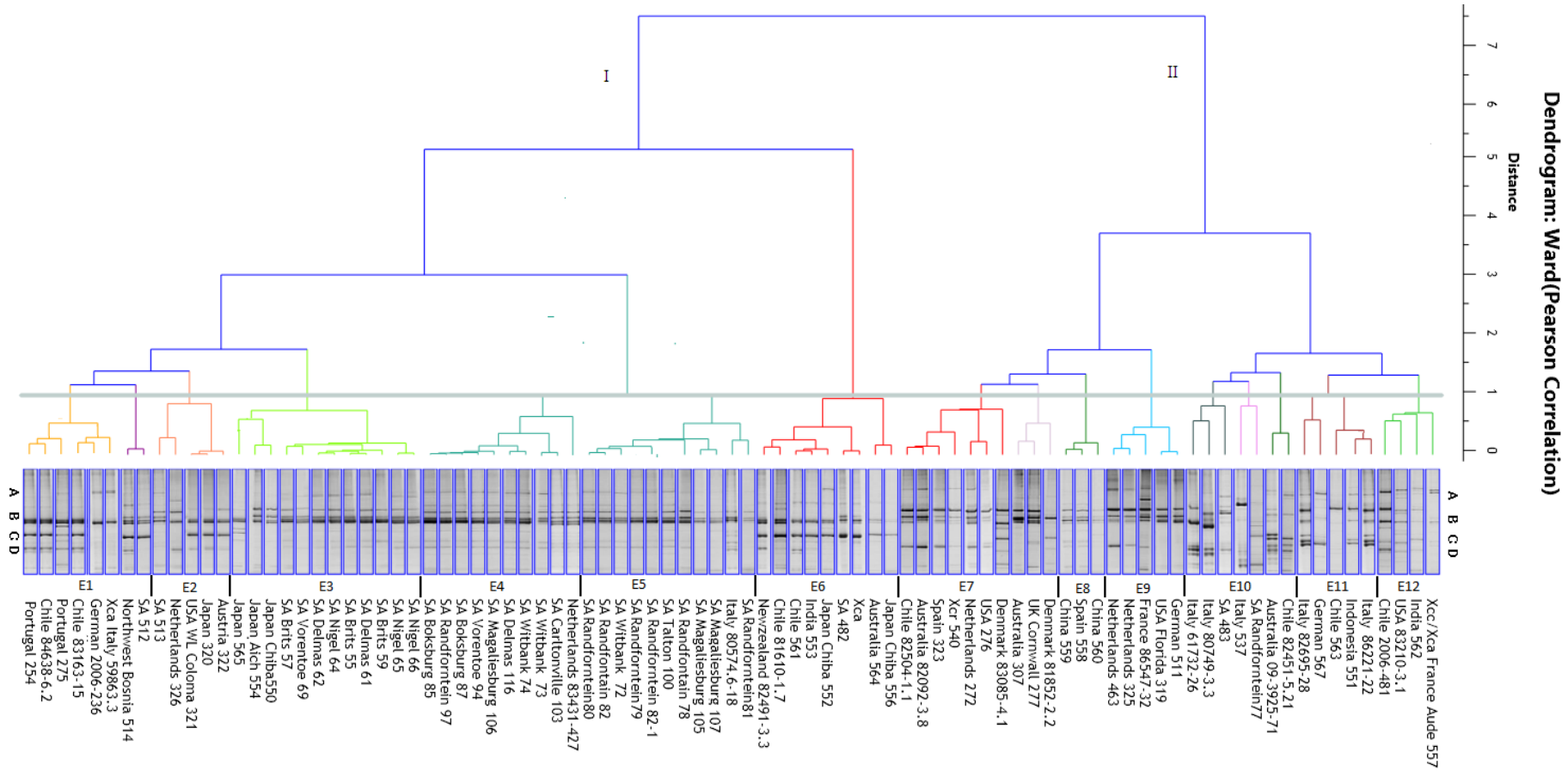

Figure 4.8: A dendrogram based on Ward's algorithm of Eric-PCR profiles of international representative and South African isolates *X. c* pv. *campestris*.

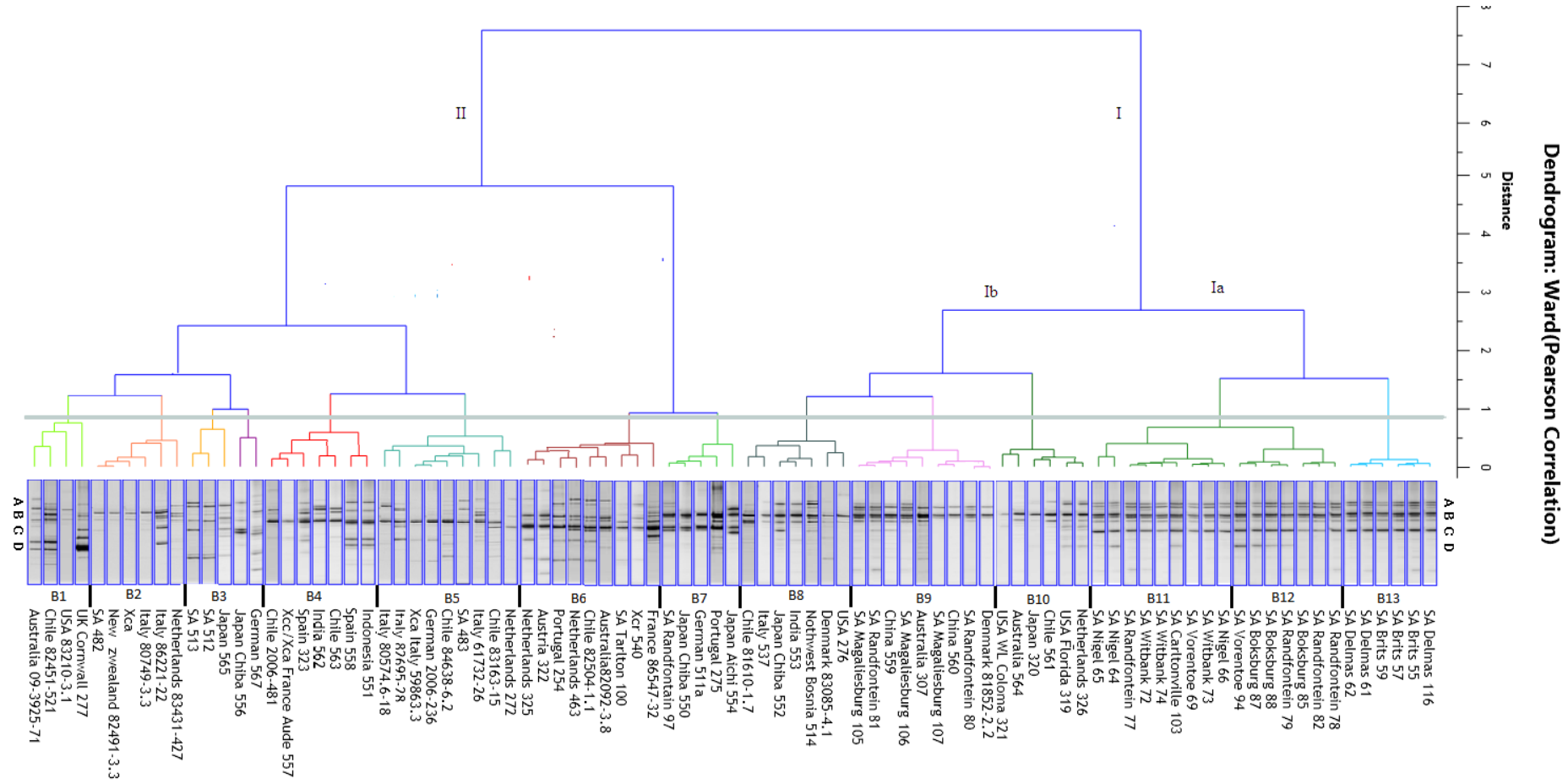

Figure 4.9: A dendrogram based on Ward's algorithm of Box-PCR of International representative and South African isolates *X. c* pv. *campestris.*

4.10 GLOBAL RELEVANCE OF SOUTH AFRICAN *X. c* pv. *campestris* ISOLATES

In the dendograms of Eric- and Box-PCR profiles, most South African, *X. c* pv. *campestris* isolates were grouped together in clusters, clusters E1 to E6 for Eric-PCR, (Figure 4.8) and B8 to B13 for Box-PCR (Figures 4.9). In both dendograms some clusters of South African isolates had international strains. Clusters E1 to E6 in Figure 4.8 consisted of 24 international strains, 10 of which were also found in cluster B8 to B13 of Figure 4.9 and included individual isolates from Italy, Netherlands, India, Italy, China and Chile. Although isolates from different international geographical origins gave unique Eric- and Box-profiles, some international isolates of different international origin gave similar Eric- and Box-profiles. This is evident in clusters being comprised of isolates from more than one country. Interestingly, international isolates including South African isolates from previous isolations, SA 512, SA 513 and SA 482 clustered with South African isolates in Eric-PCR dendrogram.

These findings suggest *X. c* pv. *campestris* distribution to have originated from limited, specific geographical areas and then spread around the world, or convergent evolution of the Eric- and Box-repetitive sequences within different geographical areas. Whichever the case, the diversity of *X. c* pv. *campestris* is great and local variations need to be taken into account if any measurers are to be successfully taken against its spread.

4.11 SUMMARY OF RESULTS

The pathogen, *X. c* pv. *campestris* responsible for the black rot of crucifers was isolated from cabbage leaf samples in the West Rand and East Rand areas of South Africa. All

the *X. c* pv. *campestris* isolates induced typical black rot symptoms that could be divided into three symptom groups “a”, “b” and “c”. Four races 1, 3, 4 and 6 of *X. c* pv. *campestris* were identified. Race 4 was dominant and widely distributed within the sampled area. Eric- and Box-PCR were used for the genomic fingerprinting of the isolates. Generated fingerprints of *X. c* pv. *campestris* were similar for the South African isolates and distinguishable from those of *X. c* pv. *armoraciae* and *X. c* pv. *raphani* reference strains. However, when international *X. c* pv. *campestris* were considered, no profile pattern was observed to be unique to international *X. c* pv. *campestris* isolates as was the case with South African isolates. Eric- and Box-PCR profiles of international isolates varied widely with some isolates having profile patterns similar to those of reference strains. Dendrograms of Eric- and Box- PCR grouped most South African and international *X. c* pv. *campestris* isolates in separate clusters. However, a few South African isolates were grouped with international isolates and a few international isolates were grouped with South African isolates.

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CHAPTER 5.

DISCUSSION

5.1 INTRODUCTION

This chapter describes and explains pathogenicity, race typing and Eric- and Box-PCR relevance to the distribution of *X. c* pv. *campestris* in south Africa and the significance of South African isolates to worldwide distribution of *X. c* pv. *campestris*. Findings from this research are also compared to previous research on *X. c* pv. *campestris* characterisation, distribution and clonal nature.

5.2 PATHOGENICITY TESTING AND RACE TYPING

Four races; 1, 3, 4 and 6 of *X. c* pv. *campestris* were found among the samples. Race 4 was dominant followed by race 1. A previous study (Vicente, 2004) also showed the presence and dominance of race 4, followed by race 1 and 6, among 51 strains from Portugal, mainly from *B. oleracea.* In that study, race 4 accounted for 53% of the strains, race 6 for 29% and race 1 for 18%. In contrast, race typing of 102 strains from the United Kingdom, mainly from *B. oleracea* showed that race 1 dominated (69%) followed by race 4 (30%). A single isolate of race 3 was also identified in the U.K. collection (Vicente *et al*., 2001). In the current study 4 races were identified and there were no significant differences in the race composition within the geographically isolated regions sampled, except for race 3 that was only found in West Rand and not on the east Rand

Races 1 and 4 of *X. campestris* are dominant among the strains from cultivated brassicas all over the world (Ignatov *et al*., 1998; Tsygankova *et al*., 2004). Races 5 and 6 (formerly race 0, designated by Kamoun *et al*., 1992) are found mostly in *B. rapa* and

mustard in locations with vast native populations of brassicas, such as Portugal (Vicente *et al*., 2001). It has been suggested that races 5 and 6 are the ancestral states for natural populations of *X. campestris.* Other races arose due to mutations or accumulation of additional avirulence genes via horizontal gene transfer. Races 5 and 6 are rare on widely cultivated crops of *B. oleracea* (Ignatov *et al*., 1998; Vicente *et al*., 2001). However, they become virulent on those plants after deliberate inoculation (Ignatov *et al*., 2002). It is possible that the suggested additional avirulence loci are responsible for adaptation to *B. oleracea*, the major cultivated species of brassicas (Castañeda *et al*., 2005).

Characteristic to non-characteristic black rot symptoms (Figure 4.1) were observed among South African *X. c* pv. *campestris* isolated. Black rot infection symptoms could be divided into three groups. Symptom group "a" caused rapid, extensive chlorosis and collapse of vein-delimited mesophyll tissues, before vein blackening and wilting of plants. Symptom group "b" initially caused yellowing and small greyish spots on leaves followed by characteristic necrotic V-shaped lesions on the margins of leaves and chlorosis. Subsequently, vein blackening and wilting of leaves occurred. Symptom group "c" isolates initially caused yellow halos around stomata that later turned into severe necrotic spots which afterwards coalesced and formed extensive necrotic blotches in the leaf lamina and distorted leaf margins.

Symptoms caused by *X. c* pv. *campestris* in cabbage depend upon several factors such as cultivar, plant age (Schaad and Alvarez, 1993), light and temperature (Williams *et al*., 1972), humidity, strain of the pathogen and even the method used for inoculation (Franken, 1992). However, in the true leaves, blackened vascular tissues and V-shaped

chlorotic to necrotic lesions along the leaf margins are characteristic symptoms of black rot (Cook *et al*., 1952; Williams, 1980; Onsando, 1992; Alvarez, 2000). Differences in the ability of *X. c* pv. *campestris* strains to cause disease in artificial inoculation tests have been previously reported (Yuen and Alvarez, 1985). Most of the *X. c* pv. *campestris* strains in that study caused systemic black rot symptoms. Blight symptoms from some *X. c* pv. *campestris* strains were observed and were characterised by necrosis and sudden collapse of large areas of mesophyll in advance of blackening of veins. Similar symptoms have been described by Alvarez *et al*. (1994). *X. c* pv. *campestris* strains that caused tan necrotic blotches, mesophyll collapse and severe blight-like symptoms were observed, similar to reports for two strains observed by Alvarez *et al*. (1994) who suggested that such strains were aggressive variants of *X. c* pv. *campestris.* However, field evaluation of cabbage varieties for resistance against *X. c* pv. *campestris*, revealed that these strains caused less severe black rot compared to the typical strains of *X. c* pv. *campestris* when plants were inoculated through the roots or foliage (Wulff, 2000; Massomo, 2003).

Findings from the present study on pathogenicity of South African *X. c* pv. *campestris* suggest similar distribution of pathological patterns of South African *X. c* pv. *campestris* to previous findings (Williams, 1980; Onsando, 1992; Alvarez *et al*., 1994; Alvarez, 2000). There appears to be a universal variation on pathological patterns despite differences in geographical origin. This allows for development of cultivars that are universally resistant to black rot, given the limited variation in pathogenicity within *X. c* pv. *campestris.*

A significant proportion of cabbage seed is imported to South Africa and it is important to ensure that such seed is tested and free of the pathogen to minimize the risk of introduction of additional new races or pathotypes. Selection of cultivars with disease resistance is another means of control, which should be considered in combination with sanitation schemes and crop rotation as the *X. c* pv. *campestris* pathogen can only survive up to four months in soil and crop debris (Schaad and White, 1974)

5.3 GEL ELECTROPHORESIS OPTIMIZATION

The sensitivity and accuracy of DNA fingerprinting analysis is influenced by the electrophoretic resolution and detection of DNA fragments. The ability of polyacrylamide to resolve nucleic acids is influenced by electric field strength, gel buffer composition, gel concentrations and temperature (Kostichka *et al*., 1992). Different gel buffer systems are available for preparing polyacrylamide gels and these include the use of various denaturants, to produce high resolution banding systems (Hames, 1981). Agarose, TBE-PAGE and SDS-PAGE were tested for their separation and resolution efficiency on Eric- and Box-PCR products. The Laemmli (1970) gel buffer system, normally used for protein separations gave well separated and resolved bands compared to TBE-PAGE and agarose gel electrophoresis. While the difference between agarose and polyacrylamide PCR profile resolution maybe explained by the difference in the different gel matrices, the difference between SDS-PAGE and TBE-PAGE can be explained by the differences in buffering capacities of TBE and SDS/Tris Buffer systems (Kostichka *et al*., 1992). From our findings in the present study it appears the SDS/Tris buffer (Laemmli, 1970) is far a superior buffer in preparing polyacryamide gels compared to TBE.

5.4 ERIC- AND BOX-PCR

PCR-based approaches have been used to study genetic diversity of phytopathogenic bacteria and generate evidence of their ecological distribution, dispersal and evolution (Leung *et al*., 1993; Adhikari *et al*., 1999; Kumar *et al*., 1994). The rationale behind Eric- and Box-PCR fingerprinting is that at sufficiently low temperature the primer anneals to many different sequences with a variety of mismatches. The number of bands in a fingerprint is an indication of the number of sequences that the primer anneals to in the initial PCR cycle. The number, reproducibility and intensity of bands are a function of several parameters including concentration of salts, primers annealing temperature, template concentration, primer length and sequence (Welsh and McClelland, 1990; Wieser and Busse, 2000).

Both Eric- and Box-PCR produced DNA fingerprints, conformed to a general pattern especially among South African isolates. The bands common to all individuals may serve as species specific markers and it is possible to use dominant and common bands for genetic mapping purposes (Wieser and Busse, 2000; Williams *et al*., 1990). However, the information content of Eric- and Box-PCR may be insufficient to define a genome but sufficient for definition of microbial clonal history (Schuenzel *et al*., 2005).

Many bands of varying intensities and sizes were observed in each of the DNA fingerprints patterns generated by Eric- and Box-primers. These bands made visual comparison of profiles difficult. However, the more bands are in a fingerprint the more statistically accurate is cbthe resulting dendograms (Kaemmer *et al*., 1992). The Pearson coefficient with Ward's algorithm was used to create the dendograms. Pearson's coefficient is a more stable measurement of similarity than band-based

methods because whole densitometric curves are compared, omitting subjective band scoring steps (Rademaker and de Bruijn, 1997). Additionally, by applying Pearson's coefficient to a densitometric graph, artefact differences between gels can be normalized and removed so that they do not alter the result (van Ooyen, 2001). Ward's clustering method is a hierarchical agglomerative method whose objective is to create clusters that gives the minimum increase in the total within group error sum of squares (Ward, 1963). The generated dendograms in the present analysis using Wards algorithm and Pearson's coefficient therefore provided an excellent analysis of Eric- and Box-profiles. Hence this analysis generated data are of greater accuracy and significance.

Data obtained from this study confirm previous findings describing the heterogeneity within *X. c* pv. *campestris* strains (Ignatov *et al*., 1998; Vicente *et al*., 2001; Taylor *et al*., 2002; Massomo *et al*., 2003). High similarity between South African isolates suggests endemism within the South African population. The high variability existing between isolates of different international origin and relative similarity between isolate of same international origin suggest endemism and clonal nature of palindromic sequences. Despite the high relative homogeneity within South African isolates, they could still be grouped into two major groups comprising the West Rand and East Rand groups.

Several independent studies of black rot pathogen have repeatedly demonstrated the existence of genetically narrow subgroups (Alvarez *et al*., 1994). These are distributed over the world on cultivated brassicas and more diverse wield populations with similarity to other pathovars of *X. campestris* (Massomo *et al*., 2003; Tsygankova *et al*. 2004; Jensen *et al.,* 2010). Tsygankova *et al*. (2004) grouped *X. campestris* strains from

cultivated plants collected all over the world by rep-PCR profile into clusters corresponding to two haplotypes of Alvarez *et al.* (1994). A third cluster was composed of leaf spot pathovars, exotic strains from Japan and strains from the United States. In a study of the diversity among strains of *X. campestris* in commercial crops in Tanzania, Massomo *et al*. (2003) observed considerable diversity based upon Box-PCR. They recognized seven genotypes among the 76 strains studied, with genotype 1 (70%) being the dominant. Despite the uniform Box-genotype, 21 of 53 strains of the group 1 (40%) were variable in biochemical properties (Biolog), 25 strains (47%) were different in rep-PCR profile and 33 strains (63%) were diverse in fatty acid methyl esterase (FAME) analysis (Massomo *et al*., 2003). Zhao *et al*. (2000) found that the majority of 45 local strains of *X. campestris* from Oklahoma belonged to a single Box-genotype similar to a known crop strain, PHW117, representative for the haplotype 1 (Alvarez *et al*., 1994; Tsygankova *et al*., 2004). Group B was similar to the type strain NCPPB 528T of the haplotype 3 (Alvarez *et al*., 1994; Tsygankova *et al*., 2004) and five strains belonged to "exotic" groups C, D and E from the United States and East Asia. Biolog results failed to correlate with pathovars or genotype grouping for the Oklahoma strains (Zhao *et al*., 2000).

The differences in Eric- and Box-Profile patterns observed between South African and international *X. c* pv. *campestris* isolates, suggest, the South African *X. c* pv. *campestris* pathogen to be native in South Africa. However, spatial variation observed might be accounted for by temporal variation assuming that South African *X. c* pv. *campestris* were introduced. There is therefore need to evaluate the temporal variation of Eric- and Box-PCR profiles. On the other hand, evaluation of other genetic fingerprinting

systems and their correlation to *X. c* pv. *campestris* genomic and pathotype distribution may give invaluable information

The present study not only supports genetic heterogeneity among *X. c* pv. *campestris* isolates, but also suggests that diversity within this pathovar is higher than previously estimated (Massomo *et al*., 2003) While all South African *X. c* pv. *campestris* isolates collected during the present investigation were relatively homogeneous, they significantly varied from isolates from previous collections suggesting temporal variation in distribution in addition to geographical variation. The high variation found in this study may be explained at least in part by the utilization of several isolates collected from different geographic locations. Whilst the presence of international isolates among South African isolates, suggest great capability of dissemination in comparison with other *X. c* pv. *campestris* isolates. The fact that a large number of isolates showed unique fingerprint patterns might be the result of adaptation of these isolates to particular habitats. Although isolates of the same geographical origin clustered together, clusters also included isolates of different geographical origin suggesting wide spread and distribution of the pathogen within the West Rand and East Rand areas of South Africa.

Localized spread of *X. c* pv. *campestris* can be accounted for by water splashes, wind-driven rain, aerosols and by mechanical injury during cultivation (Köhl and Van der Wolf, 2005). *X. c* pv. *campestris* can survive for up to 5 days on *Calliphora vomitoria* flies, which can migrate, for a distances of more than 20 km, infecting cabbage plants at a more distant location (van der Wolf *et al*., 2006). Besides, farm produce from surrounding provinces is supplied to the major fresh produce markets, which are in

Gauteng. Fresh produce which is distributed to several supermarkets around Gauteng create pathogen distribution channels for infected cabbages.

5.5 GEOGRAPHICAL ORIGIN SYMPTOM GROUP TYPE AND RACE TYPING

Using the Eric- and Box-PCR techniques, variations were observed among strains of *X. c* pv. *campestris* from brassica fields in East and West Rand areas of Gauteng, Mpumalanga and North West Provinces of South Africa (Figure 4.1a, Apendix 1). The spatial distribution of loci between the West Rand and East Rand isolates and grouping of West Rand isolates from Randfontein and neighbouring Magaliesburg in cluster groups B2, B3 and E5 suggests limited dissemination or adaptability of other *X. c* pv. *campestris* strains within these areas.

Linkage of rep-PCR profiles to geographical origin has been reported in *X. arboricola* pv. *arboricola* isolates from Persian walnut (*Juglans regia*) (Scortichini *et al*., 2001). Louws *et al*. (1994) considered that selection for a specialized niche could affect the distribution of repetitive sequences, leading to fingerprints unique to specific pathovars or strains. Sharples and Lloyd (1990) noted that evolution of bacterial genomes is often linked with repeated DNA elements. Therefore, the relative restricted nature of Eric- and Box-PCR profiles of strains from Randfontein and Magaliesburg may perhaps be due to the evolution of strains adapted in this locality.

In this study, some isolates obtained from the same source produced similar Eric- and Box-PCR profiles. Other isolates had different Eric- and Box-PCR profiles despite belonging to the same race. On the other hand, some isolates had similar profiles

despite belonging to different races. This suggests that Eric- and Box-PCR can be used for rapid initial screening of isolates to select non-identical ones for further analysis, including race typing and the relative independence of race determination factors from phylogenetic relationships in *X. c* pv. *campestris.*

In the present study, close relatedness was observed between the Eric- and Box-PCR profiles of *X. c* pv. *armoraciae* and South African *X. c* pv. *campestris* as compared to *X. c* pv. *raphani*. Alvarez *et al*. (1994) described the correlation between groups formed by RFLP and those formed by serology and pathogenicity tests. These authors inferred that *X. c* pv. *armoraciae* and *X. c* pv. *raphani* were synonymous in nature, a claim made earlier by Black and Machmud (1983). Tamura *et al*. (1994) reported that the host range of *X. c* pv. *armoraciae* was restricted to horse radish while Sahin and Miller (1997) reported the occurrence of a pathotype of *X. c* pv. *armoraciae* that attacked kale and radish (*Raphanus sativus*). In contrast, Vicente *et al*. (1998) could not confirm the existence of *X. c* pv. *armoraciae* and doubted the distinction between *X. c* pv. *campestris* and other brassica pathovars. Such differences may be an indication of the complex nature of *X. campestris* pathovars attacking brassica. Some of the problems associated with the differentiation of the pathovars of *X. campestris* are specifically related to their taxonomy and nomenclature.

The similarity between the profiles of South African isolates for both Eric- and Box-PCR suggest homogeneity within *X. c* pv. *campestris.* Strains of *X. c* pv. *campestris* that produced characteristic black rot symptoms were previously found to be highly similar in their membrane protein profiles (Minsavage and Schaad, 1983). However, the Eric- and Box-PCR fingerprinting pattern variability observed among international

isolates suggest that homogeneity within *X. c* pv. *campestris* is limited to localised geographical origins.

Linkage of repetitive elements to bacterial genome evolution has been reported by Sharples and Llyod (1990). Furthermore, Louws *et al*. (1994) considered that selection for a specialized niche could affect the distribution of repetitive sequences, leading to fingerprints unique to specific pathovars or strains. Therefore, in the present study the clustering of isolates of the same geographic origin under the same genotype may perhaps be due to the evolution of strains adapted to the specific geographical environments. Linkage of different Eric- and Box-PCR profiles genotypes to geographical origin have previously been reported for *X. c* pv. *campestris* (Massomo *et al*., 2003; Shakya *et al*., 2000) and *X. arboricola* pv. *arboricola* isolates from Persian walnut (*Juglans regia*) (Scortichini *et al*., 2001). In contrast, it was concluded that *X. c* pv. *raphani* and a selection of *X. c* pv. *campestris* strains clustered in accordance with races (Vicente *et al*., 2001).

X. c pv. *campestris* pathotypes groups could not be correlated to unique loci in both Eric- and Box-PCR profiles and cluster groups in Eric- and Box-PCR dendograms. Even in the case of strains from Randfontein and neighbouring Magaliesburg where unique loci in Eric- and Box-PCR fingerprinting patterns were dominant, pathotype groups could neither be correlated to specific loci nor cluster group. The clustering of the two or more *X. c* pv. *campestris* races under one Eric- or Box-PCR cluster group indicate a close relatedness between these races and therefore, it is possible that races may differ only in the presence of an avirulence (*avr*) gene. Mutants of *X. c* pv. *campestris* race 1 with insertions or deletions in *avr* gene have been shown to become

virulent on Florida Broad Leaf Mustard (Castañeda *et al*., 2005). This further supports the findings by Chen *et al*. (1994) which provided the evidence that pathovar status in *X. campestris* may in some cases, be determined by very few genes independent of phylogenetic background. This further suggests the need for detailed study on the variation between different fingerprinting protocols and pathovar status in *X. campestris.*

Findings in the present study suggest that Box-PCR profile reveal more variability within the *X. c* pv. *campestris* genome compared to Eric-PCR Profiles. The relative similarity between *X. c* pv. *campestris* isolates of different geographical origin was relatively higher in Eric-PCR profiles. This suggests that Eric-PCR profile may serve for *X. c* pv. *campestris* identification and at the same time contain enough variation to identify strains. Race typing of isolates using conventional methods based on differential plant lineages is time, space and labour intensive. This study has shown that fingerprinting of strains using the Eric- and Box-primer sets has potential to differentiate, *X. c* pv. *armoraciae* and *X. c* pv. *raphani* pathovars and strains of *X. c* pv. *campestris* within limited geographical areas. However, when isolates from diverse geographic origin are taken into account the high heterogeneity that exist greatly reduced the efficacy of Eric- and Box-PCR profile in distinguishing *X. c* pv. *campestris* isolates from closely related *X. c* pv. *armoraciae* and *X. c* pv. *raphani*. These findings may be due to small number of *X. c* pv. *armoraciae* and *X. c* pv. *raphani* isolates used

The variability between Eric- and Box-PCR profiles studies of other *Xanthomonas* spp and pathovars have shown grouping of strains affiliated with distinct symptom groups (Sahin *et al*., 2003), geographical origin (Scortichini *et al*., 2001) and year of isolations

(Scortichini and Rossi, 2003). This indicates that the potential to use Eric- and Box-PCR to differentiate pathogenic variants within a pathovar may depend on several parameters. Chen *et al*. (1994) provided evidence that the pathovar status in *X. campestris* may, in some cases, be determined by a very few genes independent of phylogenetic background. Hence, selective pressure of the host plant and environmental conditions might play a central role in selecting different populations of this pathogen.

Findings from the current study suggest South African *X. c* pv. *campestris* to be relatively distributed according to geographic origin independent of race and symptom group. The previous findings grouping *X. c* pv. *campestris* with year of isolations (Scortichini and Rossi, 2003) suggest that the genomic diversity of *X. c* pv. *campestris* population to be dynamic rather than static. This entails the need for further investigations into genomic diversity, population dynamics within specific geographical areas. Furthermore large sample sizes need to be considered so as to give a significant representation of the population.

CHAPTER 6

CONCLUSIONS AND RECOMENDATIONS

6.1 CONCLUSIONS

The aim of this study was to isolate and characterise *Xanthomonas campestris* pv. *campestris* isolates from South Africa using pathogenicity tests and genomic DNA fingerprinting. Four objectives were formulated and structured to achieve the aim of the study. These objectives could be divided into two main goals: (a) to establish the pathogenic potential and distribution of *X. c* pv. *campestris* isolates from selected cabbage producing regions in South Africa and, (b) to compare the various races and strains of *X. c* pv. *campestris* from South Africa to international races/strains of *X. c* pv. *campestris*.

(i) isolation and purification of *X. c* pv. *campestris* from South Africa

Yeast extract-$CaCO_3$ (YDC) and Fieldhouse-Sasser (FS) agar were successfully employed for the isolation of *X. c* pv. *campestris* from cabbage samples from all the 10 sample sites. Twenty eight isolates could be purified for further analysis. Thus black rot caused by *X. c* pv. *campestris* was present in all the sampled sites. The presence of black rot is a major risk factor to successful cabbage production especially during the rainy season. Hence farmers have to take caution on the extent to which their cabbage crops are able to resist black rot infections.

(ii) Pathogenicity and genomic DNA characterisation of *X. c* pv. *campestris*

All twenty eight of the isolates from South Africa were pathogenic to cabbage plants. Isolates produced typical black rot symptoms that could be associated with various *X. c*

pv. *campestris* races. Symptom groups could not be associated with specific loci, races and cluster groups in Eric- and Box-PCR dendograms

Twenty eight South African isolates had both similar Eric- and Box-PCR profiles which were distinct from those of isolates of international origin. Most South African isolates were found to cluster by their geographic origin. However, a few fingerprinting profile patterns were found to be widely distributed within West Rand and East Rand. The findings from this study further confirm the utility of Eric- and Box-PCR fingerprinting technique to be, reproducible, rapid and highly discriminatory in the study of *X. c* pv. *campestris* evolution and distribution in various specific ecological areas.

(iii) Comparison of *X. c* pv. *campestris* from South Africa to international isolates

In this study genetic diversity data, based on Eric- and Box-PCR profiles, were presented for thirty two *X. c* pv. *campestris* isolates of South African origin and sixty *X. c* pv. *campestris* isolates representing populations from Austria, Australia, Chile, China, Denmark, France, German, India, Indonesia, Italy, Japan, Netherlands, New Zealand, North West Bosnia, Portugal, Spain, United Kingdom and United States of America. From the results, it was evident that the genetic diversity within the South African population was relatively homogeneous as compared to the variability that existed in isolates from international representative populations.

South African isolates had unique Eric- and Box-PCR profiles that were distinct from those of isolates from international origin. Some isolates from international origin had

profiles that were similar to those of isolates from South African and thus were clustered together under both Eric- and Box-PCR generated dendrograms.

The relatively unique PCR profiles of South African isolates suggest the possibility of using Eric- and Box-PCR in rapid *X. c* pv. *campestris* determination. However, when international isolates are included the profile diversity was too great to assume a common profile for *X. c* pv. *campestris* identification. While the diversity was great on international scale, *X. c* pv. *campestris* still clustered relatively on wider geographical scales including the Asian, European, American and African geographical regions. Nevertheless clusters with isolates of mixed wider geographical origin were observed suggesting wide spread distribution of the black rot pathogen both on the local and international scale.

(iv) Global relevance of *X. c* pv. *campestris* races/strains from South Africa

South African isolates were observed to cause the three typical symptom types of black rot as previously described. Race 4 was the most abundant followed by race 1 while the other races were rare. These findings are in line with previous research which reported the dominance of race one and four. The South African *X. c* pv. *campestris* isolates may be unique to South Africa. However, the presence of international isolates among South African *X. c* pv. *campestris* cluster groups in Eric- and Box-dendograms suggest either spread of the disease to South Africa or convergent evolution of the Eric- and Box-genetic elements.

6.2 RECOMENDATIONS

1) Most cabbage seed is imported to South Africa. This may imply that the current *X. c* pv. *campestris* diversity has been impacted, with the pathogen being introduced through imported seed. However, many factors play a role in the occurrence and diversity of the pathogen and may together contribute to the occurrence and diversity of the pathogen. The presence of several races and genotypes of both local and international origin of *X. c* pv. *campestris* within South Africa, emphasizes the need for local surveys of *X. c* pv. *campestris* to gain insight into the population composition and change. The results may be of value in relation to regulatory issues, such as seed import, but also highlight the need to account for pathogenic diversity when breeding, introducing and cultivating resistant cultivars to control the disease.

2) There is need to re-evaluate other primers available for DNA fingerprinting on how they reveal the interrelatedness of worldwide *X. c* pv. *campestris* populations

3) There is need to further evaluate the distribution of *X. c* pv. *campestris* in relation to their races and symptom group as well as their severance under filed conditions in South Africa. Currently 4 races in West Rand and 3 races in East Rand are described. The scenario may be different if the study is expanded.

4) Plasmids are mobile genetic elements that can easily be spread between bacteria especially of the same species. They are known for the spread of drug resistance and virulence genes. It is thus possible that avirulence genes are transported between bacteria. Determining the diversity and distribution of plasmids as well as the information contained therein in *X. c* pv. *campestris* will thus be a useful study.

5) While the unique fingerprint profiles of *X. c* pv. *campestris* generated by Eric- and Box-PCR could be a useful tool in diagnosis and in differentiation of strains, without a database the utility of Eric- and Box-PCR for routine identification of *X. c* pv. *campestris* strains is limited.

This study has shown that there is a wide genomic diversity and distribution of *X. c* pv. *campestris* pathogen both locally and internationally. While most *X. c* pv. *campestris* isolates from South Africa (this study only) seem to be native, a few others appear to have been introduced. The pathogenicity and disease symptom types of South African *X. c* pv. *campestris* varies between and within races and the Eric- and Box-PCR profile seem to group *X. c* pv. *campestris* by geographical origin rather than races or symptom groups. Thus, the aim of this study i.e. to characterise *X. c* pv. *campestris* isolates from South Africa using genomic DNA fingerprinting was achieved by the successful execution of the four objectives.

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Appendix A

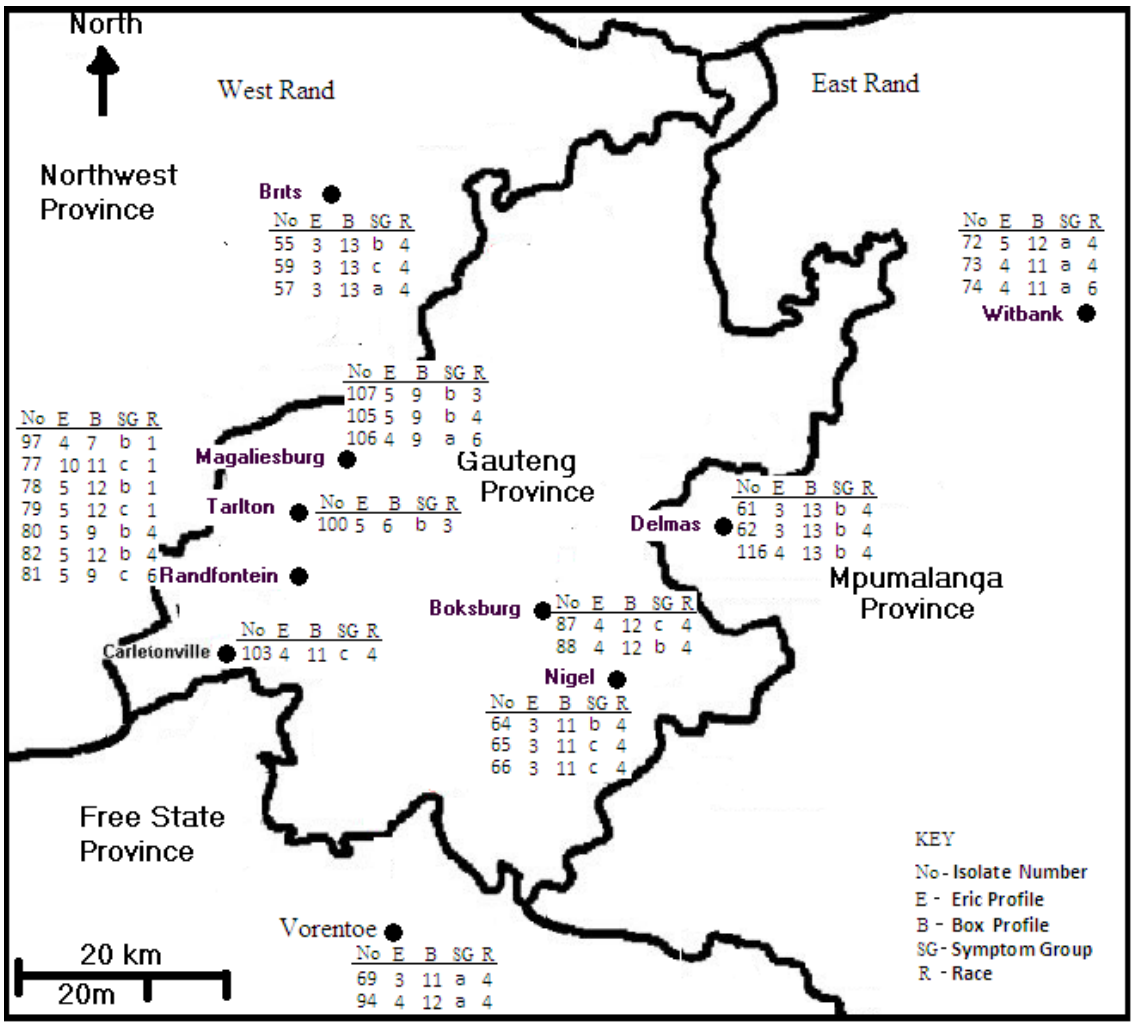

Figure A1: Map of Gauteng and neighbouring provinces showing sites from which strains of *X. c* pv. *campestris* were isolated, isolate number, Eric- and Box-cluster groups, symptom groups and race of respective individual isolates.