PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF PECTOBACTERIUM AND DICKEYA SPECIES ASSOCIATED WITH BLACKLEG AND SOFT ROT OF POTATO IN KENYA

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Phenotypic and Molecular Characterization of *Pectobacterium* and *Dickeya* Species Associated With Blackleg And Soft Rot Of Potato in Kenya

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university

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DEDICATION

This work is dedicated to my immediate family, my husband, daughter and son for their patience and unfailing support during my studies.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABI	Applied Biosystems
CFU	Colony Forming Unit
CVP	Crystal Violet Pectate
DIECA	Diethyl dithiocarbamic acid
DNA	Deoxyribonucleic acid
DNTPs	Dinucleotide Triphosphates
EDTA	Ethylene diamine tetra acetic acid
ELISA	EnzymeLinked Immunosorbent Assay
FAS	Fluorescent antibody staining
FITC	Fluorescent isothiocyanate
GDE	Genetic Data Environment
GOLD	Genomes Online Database
IF	Immunofluorescence staining
IFC	Immunofluorescent colonies
IMS	Immunomagnetic separation
LAMP	Loop Mediated Isothermal Amplification
LPS	Lipopolysaccharides
MB	Mega Base pair
MgCl2	Magnesium chloride
ML	Milliliter
NA	Nutrient Broth
NAOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information

NGM	Nutrient agar with Glycerol Manganese chloride
nm	Nano mole
NCBI	National Center for Biotechnology Information
Pcb	*Pectobacterium carotovorum* subsp *brasiliense*
Pcc	*Pectobacterium carotovorum* subsp *carotovorum*
PCDWE	Plant Cell Wall Degrading Enzymes
PCR	Polymerase Chain Reaction
PL	Pectate lyase
Pwa	*Pectobacterium wasabiae*
rRNA	Ribosomal ribonucleic acid
SDS	Sodium Dodecyl Sulfate
SRE	Soft Rot *Enterobacteriaceae*
TE	Tris Editate
U	unit
Ul	microlitre
UPGMA	Unweighted Pair Group Method with Arithmetic mean

ABSTRACT

Blackleg and soft rot of potato, caused by *Pectobacterium* and *Dickeya* spp., are major production constraints in many potato-growing regions of the world. Despite advances in the understanding of the causative organisms, disease epidemiology and control, blackleg remains the principal cause of down- grading and rejection of potato seed in classification schemes across the world. Although symptom recognition is relatively straightforward and is applied universally in seed classification schemes, attributing disease to a specific organism is problematic and can only be achieved through the use of diagnostics. In Kenya *Pectobacterium* and *Dickeya* spp have not been characterized thus preventing early disease diagnosis and management. The purpose of this study was to isolate and characterize the pectolytic bacteria occurring in potato in Kenya. The methods included collection of symptomatic potato plants and tubers from four major potato production regions of Kenya which were: Nakuru, Nyandarua, Narok and Elgeiyo Marakwet. The samples were prepared and analysed using phenotypic methods which included plating on semi selective media(crystal violet polypectate(CVP) and nutrient agar with manganeseIIchlorite tetrahydrate(NGM), biochemical tests, pathogenicity tests and molecular based methods which included real time and conventional PCR as well as sequencing of 16S rDNA housekeeping gene using the sequencing primers. Sequence evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. Phylogeny was done using neighbor joining method with 1000 boostraps to determine the relationship of the species. Results of obtained from the sequences were used to compare the strains occurring in Kenya and those present in other countries. The results from phenotypic and molecular analysis identified 65 % isolates as *Pectobacterium carotovorum subsp. Carotovorum*, 19% as *Pectobacterium wasabiae* and 8% of the isolates as *Pectobacterium atrosepticum*. However, *Dickeya spp.* primer pair produced unexpected band size compared to the positive control, *Dickeya dianthicola* and therefore presented need for further investigation. The findings of this study confirmed presence of new pathogens; *Pectobacterium atrosepticum* and *Pectobacterium wasabiae* in potato fields in Nakuru and Elgeiyo Marakwet regions respectively for the first time in Kenya. There is therefore need for further studies to determine the origin and distribution of these two stains of *Pectobacterium* spp. in all potato growing regions of Kenya. There is also need for awareness creation to the potato farmers in order to understand economic importance of *Pectobacterium* and *Dickeya* spp. so as to initiate prevention and control measures.

CHAPTER ONE

INTRODUCTION

1.1 Background information about Pectobacterium and Dickeya spp.

The International Potato Centre (CIP) ranks potato to be the third most important food crop in the world after rice and wheat in terms of human consumption (Degefu, 2013),while in Kenya it is ranked second from maize(Wang'ombe & van Dijk, 2013). However, this important crop which holds a promise as a potential future food of the world is affected by about 160 different diseases and disorders of which 50 are fungal, 10 bacterial, 40 viral origin (Degefu, 2013) . Diseases affects the leaf, stems and tubers at all stages of development and can occur both in the field and storage(Rosenzweig *et al*., 2016). Among the pathogens affecting potato, bacteria are one of the most serious problems(Czajkowski *et al*., 2015). Blackleg and soft rot of potato caused by *Dickeya* and *Pectobacterium* species respectively are the most harmful and destructive diseases causing heavy losses in seed and table potato production.

For instance, in the Netherlands alone the annual losses in seed potato production due to *Dickeya* and *Pectobacterium* spp. is estimated to be up to 30 million euro (Degefu, 2013) .In Kenya, soft rot and blackleg of potato caused by *Pectobacterium* species is estimated to account for over 60% annual yield loss(E. M. Onkendi *et al*., 2014). Losses attributed to these complex bacteria occur due to poor emergence of the potato tubers, stem rots and lack of growth in the fields as well as decayed tubers in storages(Rosenzweig *et al*., 2016). Unfortunately, in Kenya, only two species of *Pectobacterium* have been reported to cause blackleg and soft rots in potato. These are *Pectobacterium carotovorum subsp carotovorum* and *Pectobacterium carotovorum subsp brasilience,* a highly aggressive species responsible for the blackleg and soft rot in potato reported in Mau Narok and Nyandarua regions of Kenya(E. M. Onkendi *et al*., 2014).The highest loss is experienced during long rainfalls between April to July (Muthoni & Nyamongo, 2009) possibly due to low temperatures which favor *Pectobacterium species*. However, *Dickeya species* infect

the crops in warm temperatures and therefore can be the cause of blackleg and soft rots during short rains(Czajkowski *et al*., 2015) ,but this has not yet been reported in Kenya.

Pectobacterium and *Dickeya spp*. have wide host range(Ma *et al*., 2007) among which are the frequently cultivated crops in Kenya. This wide host range complicates management of the disease due to the many plant species that act as reservoir hosts(Rosenzweig *et al*., 2016). This can be seen in the Kenyan agricultural sector where mixed cropping and/or rotational cropping is the normal farming practice in almost all major potato growing regions. Although a lot of study on this disease complex has been done in other parts of the world, in this country, no study has been carried out to determine the distribution and to quantify the effects of different bacterial species. This leaves the farmer with little knowledge on the cause, management and prevention of the disease. The purpose of this study was to determine occurrence, distribution and genetic diversity of *Pectobacterium* and *Dickeya* spp. in order to inform the agricultural sector on the disease status which can thereby play a role in enhancing food security in the country as well as help reduce or avoid disease spread in areas that are currently clean.

1.2 Statement of the problem

Despite the importance of potato crop in Kenya, it is constrained by bacterial diseases which are the major biotic production constraints. Symptoms of soft rot and blackleg disease in potato have been observed in major production areas in Kenya(E. M. Onkendi *et al*., 2014). However, there is very little literature on blackleg/soft rot disease complex and cataloguing the identity or genetic diversity of soft rot pathogens infecting potato or any other plant host. Research reports on causative agents for soft rot and blackleg of potato are very scanty. For instance, only recently *Pectobacterium* subsp *carotovorum* and subsp *brasilience* have been reported to be causative agents for blackleg and soft rot of potato in Mau Narok and Nyandarua regions of Kenya (E. M. Onkendi *et al*., 2014).While blackleg in the fields and soft rot of potato and in storages is increasing, it continues to impact negatively on food security in Kenya, little information is available on the possible

occurrence of other *Pectobacterium spp* and no information is available on occurrence of *Dickeya* spp infecting potato *in* Kenya.

In seed potato production, this disease complex is next in economic importance to bacterial wilt caused by *Ralstonia solanacearum* and is known to occur in many other countries including European countries some of which we are trading within potato sector (Degefu, 2013). However, very little studies have been conducted in Kenya to determine incidences and the prevalence of these species in the country especially given the recent approved trade of seed potatoes between Kenya and European countries. This disease complex has a wide host range(Ma *et al*., 2007) and can cause major losses in case of its occurrence across crops. Latently infected tubers are the major source of infection in a clean land while volunteer tubers are the major sources of subsequent infections if left in the land (Rosenzweig *et al*., 2016). Disease transmission is mainly through farming practices which include sharing farm machinery from infested land to a clean land as well as through water run offs. Disease knowledge and accurate identification tools are important for early detection. Following these research gaps, there was therefore need to find out the incidences, occurrence, distribution and genetic diversity of these bacteria in order to institute the appropriate phytosanitary and seed certification measures in Kenya and maximize potato production for trade and food security.

1.3 Justification of the study

Pectinolytic bacteria can cause significant yield loss on potato because the pathogen stays in the soil for long and prohibits subsequent production of potato in the same field. These phytopathogens may stay latent in the field with the consequence of high impact on the tuber yield in the upcoming season. Detections of latent infections by pectinolytic bacteria require sensitive diagnostic methods for identification of causative bacterial strains. As yet, the causal agent for blackleg disease and soft rot of potato has only been identified by Onkendi (2014) in two regions of Kenya, Nyandarua and Mau Narok which limits mitigation strategies to aid in decision making during phytosanitary certification processes for seed potato production. As

the crop is vegetatively propagated, the diseases can easily be transmitted through tubers and cause very high economic losses across wide geographical zones.

This study was carried out to fill the current knowledge gap on occurrence, distribution and genetic diversity of *Pectobacterium* and *Dickeya spp* associated with blackleg and soft rot of potato in major production regions of Kenya.

This information was important to achieve the following; 1) Detect the threat of *Pectobacterium* and *Dickeya* spp. in order to prevent their introduction and management if present or if they become present in the Kenya. 2)Support market access and trade in potato by providing surveillance information on the status of target pathogens. 3) Promote trading partners, confidence by ensuring the availability of current and reliable information on status of Pectobacterium and *Dickeya* spp in Kenya.

1.4 Research question

1. What regions of Kenya *Pectobacterium* spp. and/or *Dickeya* spp.are present?
2. What species of the genera *Pectobacterium* and *Dickeya* spp. occur in Kenya?

1.5 Hypothesis

1. There is no *Dickeya* spp.in Kenya.
2. There is only *Pectobacterium subsp.carotovorum* and *subsp brasiliense* in Kenya.

1.6 Broad Objective

To determine the occurrence, distribution and diversity of *Pectobacterium spp*. and Dickeya spp in major potato production regions in Kenya.

1.7 Specific Objectives

1 To determine the occurrence and distribution of *Pectobacterium* and *Dickeya spp* in Kenya

2 To determine the genetic diversity of *Pectobacterium* and *Dickeya* spp using molecular methods-: PCR, Sequencing of the PCR products and phylogenetics analysis

CHAPTER TWO

LITERATURE REVIEW

2.1 Importance of potato in Kenya

Potato is second most consumed produce in Kenya, after maize. It is used as a staple food as well as a cash crop for many rural and semi urban dwellers playing an important role in improving national food security and income generation for those involved in its value chain development (Wang'ombe & van Dijk, 2013). The crop is grown in Kenya by approximately 500,000 small-scale farmers on 120,000 hectares with an average yield of 7.7 tons per hectare. It is mostly grown in the highland areas where maize has low competitive advantage with over 70% of these potatoes being grown in areas located at around 2,100 M above sea level. Such areas include those surrounding Mt. Kenya namely, Meru, Embu and Kirinyanga, parts of Laikipia and both sides of the Aberdare ranges, which include parts of Nyeri, Muranga, Kiambaa and Nyandarua Districts. Other areas include Mau Narok, Molo, Tinderet, Nandi Escarpment and Cherangani hills. Kericho, Kisii and around Taita hills have also been reported to grow potato but in small acreages (Muthoni & Nyamongo, 2009). Potato is considered an important source of food, employment and income in developing countries (Muthoni & Nyamongo, 2009) and is therefore a potential focal crop in the country to attain food security (Wang'ombe & van Dijk, 2013). However, production of this crop is limited by a number of factors (Muthoni & Nyamongo, 2009). For instance, due to continuous production of potatoes in the same piece of land, soil degradation has been inevitable. Fertilizer application has been done below the recommended rates with the most common one being Di-Ammonium Phosphate (DAP) which is intended to increase soil fertility. Lack of certified seed whereby about 1% only of the planted area only has recorded use of certified seeds has also greatly contributed to potato production constraints (Wang'ombe & van Dijk, 2013). The other plantations are done using seeds raised locally through retention from previous harvest, which farmers obtain from their stores or buy from local markets, friends and relatives. Yield therefore reduces with each successive generation. Other factors contributing to low potato yields include:

high costs of inputs, poor marketing channel, poor farming practices, poor storage facilities and lack of enough capital for intensive production which can see their overall production increase as result of employing motorized machinery and diseases which are the major constraints.

The major potato diseases in Kenya are late blight (*Phytopthora infestans*) and bacterial diseases such as brown rot(*Ralstonia solanacearum*)(Muthoni & Nyamongo, 2009). Recently, new bacterial disease caused by *Pectobacterium* species has been reported to cause blackleg and soft rot of potato in Kenya thereby contributing to potato yield losses in both fields and storages(E. M. Onkendi *et al*., 2014)

2.2.1 Classification of Pectobacterium and Dickeya spp.

Pectobacterium and Dickeya spp are phytopathogens which belong to the soft rot *Erwinia* (SRE) group within the *Enterobacteriaceae* family (Humphris *et al*., 2015).They mainly consist of broad host range pathogens that cause wilts, rots, and blackleg disease on a wide range of plants and crops worldwide (Pritchard *et al*., 2013). Distinctively, the SRE are known to secrete different effective plant cell wall degrading enzymes (PCWDE) (Pritchard *et al*., 2013) which are responsible for tissue maceration and usually gives the characteristic water soaked, slimy and rotten appearance of the infected plant tissues(Rahman *et al*., 2012). In this regard, tissue maceration enables *Pectobacterium spp*. to colonize and establish in a wide range of host plants after infection (Pérombelon, 2002).

The Genus *Erwinia* belongs to the *Enterobacteriaceae* family whose most members cause diseases of plants (Kado, 2006). Recently 16S r DNA sequence comparisons have been proposed to delineate species originally classified in the Genus *Erwinia* into other genus including Pantoea ,*Enterobacter* and *Pectobacterium* (Kado, 2006). Species with distinct D-glucose metabolism of oxidizing glucose into various forms of glucose have been moved from genus *Erwinia* into genus Pantoea.All of the genera possess similar phenotypic characteristics of the *Enterobacteriaceae*(Kado, 2006). They produce acid from sugars, they are gram negative rods with peritrichous flagella and ferments substrates anaerobically. The phylogenetics of this genus

Erwinias have been explored using 16S rDNA nucleotide sequence comparisons. Based on the results, four groups have been discovered(Kado, 2006).

The first group represents *Erwinia amylovora, Erwinia rhapontici, Erwinia persicina, Erwinia psidii, Erwinia pyrifoliae, Erwinia maltotivora and Erwinia tracheiphila*. The second group represents *Erwinia carotovorum* sub species, *Erwinia chrysanthemi* and *Erwinia cypripedii*. This genus was moved to *Pectobacterium* because the species primarily produce pectinolytic enzymes for pathogenesis. They cause rapid necrosis of plants affected, progressive tissue maceration called soft rot, occlusion of vessel elements called vascular wilt and hypertrophy leading to gall or tumor formation.

The third group represents *Erwinia alni*, *Erwinia nigrifluens*,*Erwinia paradisiacal*,*Erwinia quercina* and *Erwinia salicia*. This group was moved into genus *Brenneria*.

The fourth group comprises members of *Erwinia* which display unusual oxidative metabolism of D-glucose and produce yellow to mauve colonies on nutrient agar and was reclassified in genera Pantoea(Kado, 2006).Other research on *Erwinia* are still being done and revisions in classification is widening. For instance these bacterial complex was revised into biovar and pathovars based on biochemical classification by Garden *et al.,* and called these pathogens as soft rot *Pectobacterium spp*. (Czajkowski *et al*., 2015).In his study, he discovered six different strains of *Pectobacterium spp.* These were: *Erwinia carotovorum subsp carotovorum* was reclassified as *Pectobacterium carotovorum* subsp *carotovorum(Pcc), Erwinia carotovorum* subsp *atrosepticum* was reclassified as *Pectobacterium atrosepticum(Pa)* and *Erwinia carotovorum* subsp *wasabiae* was reclassified as *Pectobacterium wasabiae (Pwa)* (Czajkowski *et al*., 2015)

Soft rot *Dickeya* was discovered by Samson *,* in 2005 as another class of *Erwinia* soft rot(Czajkowski *et al*., 2015). Based on biochemical and molecular identification, these pathogens were classified as *Erwinia chrysanthemi* then into *Pectobacterium chrysanthemi* and *later the Dickeya spp.* (Czajkowski *et al*., 2015) This means that the taxonomic position of pectinolytic bacteria has undergone several revisions on

the basis of not only molecular, biochemical but also host range studies(Ngadze, 2012).

Strains formerly described as *Erwinia carotovorum* have been re-classified into the genus *Pectobacterium* and strains classified as *Erwinia chrysanthemi* are now assigned to the genus *Dickeya* (Ngadze, 2012; AHDB-Potato Council.,, 2007). The genus *Pectobacterium* is currently clustered into six distinct clades and they include *Pectobacterium carotovorum subsp.carotovorum (Pcc), Pectobacterium atrosepticum* (Pa), *Pectobacterium wasabiae (Pw), Pectobacterium betavasculorum(Pbv), Pectobacterium carotovorum subsp. brassiliences* (*Pcb*), and *Pectobacterium carotovorum subsp. Oderiferum* ((Dickey, 1978;Dahaghin and Shams-Bakhsh, 2014); Onkendi *et al*., 2014).

The major pathogenecity determinant of these bacteria is their copious production of plant cell wall-degrading enzymes (PCWDE) (Tsuyumu *et al.,* 2014) including pectinases, cellulases, and proteases, which macerate host tissue (Prajapat *et al.,* 2013) . Subsequent molecular study of these taxa demonstrated that the cause of blackleg, tuber soft rot, stem wilt and rot in potato can also be *D. dadantii*, *D. dianthicola, D. solani*, *Pectobacterium. atrosepticum, Pectobacterium. wasabiae* and *Pectobacterium. carotovorum* subsp. *carotovorum* and *Pectobacterium. carotovorum.* subsp. *brasiliensis* (Baghaee Ravari, Moslemkhani and Khodaygan, 2013 ;Dahaghin and Shams-Bakhsh, 2014: Onkendi., 2016) .

Globally, blackleg and soft rot of potato are the most important bacterial disease of potatoes in many production systems (Czajkowski *et al*., 2015). Blackleg usually occurs in the field while soft rot affects stored potatoes. The pathogens associated with blackleg/soft rot complex are *Pectobacterium species and* subspecies formally *Erwinia spp* and *Dickeya spp* formally *Erwinia chrysanthemi* complex (Czajkowski *et al*., 2015; Pérombelon 2002). These phytopathogens cause tuber soft rot, stem wilt and blackleg diseases in potatoes and on a wide range of plants (Pérombelon, 2002). The loss attributed to *Pectobacterium spp*. can occur during crop growth, transportation of crop produce or even at storage (Muponda *et al.*, 2016;Rosenzweig *et al.*, 2016). *P. atrosepticum* and *P. carotovorum* subsp. *carotovorum* the most

significant production constraints. *P. carotovorum* subsp. *carotovorum* has a broad host range and a world-wide distribution in contrast to *P. atrosepticum* which is largely restricted to potato and is principally only found in temperate regions (Czajkowski *et al*., 2015). Disease spread is thought to be largely due to movement of latently infected potato seed tubers which can lead to the introduction of highly pathogenic *Pectobacterium* and *Dickeya* spp. into different countries resulting in new disease outbreaks(Brierley *et al.*, 2008: Baghaee Ravari *et al.,* 2013 : Waleron *et al.,* 2013) and also using home saved seed in a year to year potato production giving many other chances of spread.

Pectobacterium carotovorum (Pc) is widely distributed globally as compared to *Pectobacterium atrosepticum* (Pa) which is usually associated with plant hosts in temperate regions (Czajkowski., 2011). In the tropics and the sub-tropics, *Pectobacterium carotovorum subsp. brasiliense, P.wasabiae, P.carotovorum subsp. Carotovorum*, *Dickeya zeae* and *D. dadanti* have been shown to be responsible for significant potato crop losses annually due to soft rot and blackleg diseases (Ngadze *et al*., 2012). To date, *Pectobacterium* and *Dickeya spp*. and other soft rot bacteria have been reported from potato and other hosts in many parts of the world including Europe and in Africa (Ngadze *et al*., 2012). *Dickeya spp* associated with soft rot and blackleg of potato include; *Dickeya solani*, *Dickeya dianthicola* and *Dickeya dadantii* (Ngadze *et al*., 2012).

Table 2.1 below shows some major cultivated host plants of *Pectobacterium* and *Dickeya* spp. Most of these crops are cultivated in Kenya and are used for crop rotations.

Table 2.1: The major cultivated host plants of *Pectobacterium* and *Dickeya spp.*

Scientific Name	Type/Common Name	References
Musa spp	Banana	Dickey ,1978
Dieffenbachia spp.	Dieffenbachia, Dumb Cane	Dickey, 1978
Euphorbia pulcherrima	Poinsettia	Dickey, 1978
Kalanchoe blossfeldian	Kalanchoe, Flaming Katy	Dickey, 1978
Zea mays	Maize	Ma *et al*., 2007
Solanum tuberosum	Potato	Ma *et al*., 2007
Ipomea batatas,	Sweet Potato	Ma *et al*., 2007
Saintpaulia ionantha	African Violet	Dickey, 1978
Syngonium podophyllum	Arrowhead Vine	Dickey, 1978
Brassica spp,	Cabbages	Ma *et al*., 2007
Capsicum spp,	Bell Pepper	Ma *et al*., 2007
Daucus carota	Carrots	Dickey, 1978
Alium cepa	Onion	Ma *et al*., 2007
Solanum lycopersicum	Tomato	Ma *et al*., 2007
Ananas comosus	Pineapple	Ma *et al*., 2007
Oryza sativa	Oriental	Dickey, 1978
Saccharum officinarum	Sugarcane	Dickey, 1978

2.2.2. Infection cycle

Infection with pectinolytic bacteria may start when temperatures rise at planting time from latently infected planting material (plantlets, cuttings, tubers) or soil, and when also wounding of plant parts take place due to cultural practices or weather conditions (Rosenzweig *et al*., 2016). The bacteria remain and tend to survive in surface water, low-oxygen environments and on roots of plants including weeds. Infected seed of volunteer potatoes are mostly a source of new infection in

subsequent growing seasons (Rosenzweig *et al*., 2016). These pathogens can survive for long periods in plant debris, including potato tissue, and they may be spread by contaminated equipment or human activities, surface runoff and irrigation water. Secondary infection occurs through wounds and natural openings in the raised parts of the potato plant and also tubers and spread further from tubers to stems following rain/hail events. Saturated soil impacts enlargement of tuber lenticels which serves as the bacterial infection sites. High temperature and moisture elevate rates of disease onset and disease development. A thin layer of water or fluid surrounding the potato tuber or stems causes a low-oxygen environment, thereby increasing the rate of bacterial diseases in the field or storage. During storage, rotting tubers release moisture by cell leakage, a bacterial pocket with some anaerobiosis develops and the leaking of plant sap may further spread the bacteria, causing further progress of the disease and contamination of healthy tubers in storage (Rosenzweig *et al*., 2016) and the new disease cycle begins during planting.

2.2.3 Symptoms of potato blackleg and soft rot disease

Usually, soft rot symptoms manifests as water-soaked lesions on tubers, which gradually become soft, mushy, disintegrated, depressed and discolored (Rahman *et al*., 2012). The tissue within the affected region becomes creamy and slimy in color and gradually become disintegrated and mushy mass of disorganized cells. Sometimes the tuber turns into a soft, watery, decayed mass within a very short time, usually 3-5 days. Black rots or lesions spreading from the rotting mother tuber up the stems with black coloration is considered a diagnostic symptom of the disease. Seed piece decay, stem and storage rot all contribute to economic losses (Baghaee Ravari *et al*., 2013). Some of the diseases predisposing factors are poor storage due to inadequate storage facilities, improper cultural practices (such as careless desprouting, cutting of seed, rough harvesting under wet conditions, use of home saved seed, poor farming practices, insufficient removal of weed hosts and volunteers (Czajkowski *et al*., 2011; Pérombelon, 2002). Infected seed crops can be rejected from the market due to poor quality(Czajkowski *et al*., 2011; Pérombelon, 2002). Farmers are affected by low yields (Muponda *et al*., 2016) and are therefore limited in potato trade.

Symptoms of soft rot disease on potato tubers are similar whenever caused by either *Dickeya* or *Pectobacterium* spp. In the field, disease develops following movement of pathogen from the seed tuber to the stem base. However, there are many other ways of spread in the field, especially also for the more soil-surviving species such as *Dickeya* and *Ecc* /*Ecb*. Blackleg, caused by *Pectobacterium* and *Dickeya* spp. is characterized by the production of a slimy, wet, black rot lesion spreading from the rotting mother tuber up the stems, especially under wet conditions (Pritchard *et al*., 2013). In dry conditions, symptoms tend to lead to stunting, yellowing, wilting, and desiccation of stems and leaves. Under warm wet conditions, blackleg and soft rot symptoms in potato are similar whether caused by *Pectobacterium* or *Dickeya* spp. which makes it almost impossible to identify the causal agent by visual assessment alone. It is therefore important to be able to rapidly and reliably detect and identify these bacterial species and subspecies.

2.2.4 Biology and dissemination of the pathogen

Factors influencing disease development on potato caused by *Dickeya* spp. are similar as for *Pectobacterium spp.*(Czajkowski *et al*., 2015), with the exception of temperature, where a warmer spring and summer favours disease development by *Dickeya* spp. In contrast, *Pectobacterium* spp. causes typical blackleg symptoms at 22 ºC but does not cause disease at 27 ºC. It may also be the case that *Dickeya* spp. may cause significant disease from lower inoculum levels than required for *Pectobacterium* spp. These bacteria exist in many areas especially tropical and subtropical countries where they are endemic (Czajkowski *et al*., 2015). In plant-free soil, survival of *Dickeya* spp. is less than 6 months and over-wintering in temperate climate is therefore unlikely, although wild host plants could potentially play an important role in survival. *Dickeya* spp. have been identified in watercourses (Dahaghin & Shams-Bakhsh, 2014) in several countries and in one case in Sweden on the riparian weed *Solanum dulcamara*. *D. zeae* has been previously found in river water and infected potatoes in Australia and in maize in Italy (AHDB-Potato Council, 2009)– and most likely this *D. zeae* and also *D. dadanti* are widely present in Kenya.

2.2.5. Control

The blackleg-affected plant is thought to be the main source of the pathogen. Therefore, disease control can be achieved by an indexing (certification) system of seed obtained from disease-free crops. In Europe, plant inspection services under national jurisdiction are responsible for certification of seed potato (Czajkowski*.,* 2011).The European Plant Protection Organization provides standardized protocols and guidance for certification of plant material. Inside the European Union, the Phytosanitary Directive (2000/29/EG) describes general regulation on crop production requirements and guidance for member states in respect of good cultivation practices.

However, the degree of control achieved is erratic and heavily dependent on the weather prevailing during growth of the seed crop. These measures cannot detect widespread latent infection of progeny tubers (the next generation of seed) from symptomless plants. Moreover, depending on weather conditions, heavily contaminated seed can give rise to little or no disease, and the converse is also true (Rosenzweig *et al*., 2016). Despite this, the measures can do some good. For example, roguing at an early stage of crop growth, which entails the removal of diseased plants, including daughter tubers, no doubt contributes to reducing an important source of the pathogen. Rotting progeny tubers are common on plants with symptoms, from which bacteria can spread during mechanical handling at harvest and postharvest (Rosenzweig *et al*., 2016).

Seed potato crops are classified into different seed grades according to several criteria, including the level of rouging and blackleg, from nil to a given percentage, depending on national certification standards. Seed crops are usually subjected to field inspections twice during the growing season in most seed-producing countries including Kenya. Infected crops can be downgraded to a lower seed category or rejected from the market. Harvested progeny tubers (future-generation seed) can be tested also with molecular and serological methods to detect latent infections (Rosenzweig *et al*., 2016). As tubers from disease-free crops are frequently contaminated, laboratory testing can help to detect latently infected tubers. In

contrast to other bacterial diseases of potato where there is often zero tolerance, some contamination of seed tubers can be allowed, in particular in low-grade seed (Rosenzweig *et al*., 2016). Therefore, the use of a detection procedure which allows estimation of both the density of bacteria and the incidence of contaminated tubers is considered (Perombelon, 20002). Good storage is of importance, not only to prevent tuber decay, but also to avoid increasing the tuber inoculum load, which would result in greater subsequent disease risks.

2.2.6 Diagnostic methods for Pectobacterium and Dickeya spp.

2.2.6.1 Isolation from plant material

Isolation of Pectobacterium and Dickeya spp. from diseased plant materials is achieved from symptomatic tissues, where bacterial densities are often greater than 10^6 cells/ul. It is best to sample from the advancing front of the rot or from newly diseased tissue to avoid interference and growth suppression by contaminating saprophytes. In potato tubers, they are more commonly present in the stolon end while in stems they are concentrated on the vascular tissues (Costa *et al.*, 2006: Lichens-park, Dennis C Gross Ann, 2014). Extraction of the bacteria from tubers varies and is either from ground peel strips encompassing the stolon end or the stolon end tuber section of individual or replicated bulked tuber lots (Czajkowski *et al.*, 2015). The sample is usually suspended and diluted in sterile water or buffer and a loopful streaked on a growth medium selective for SRE. Plates are incubated at different temperatures as the pathogens have different optimal growth temperatures (Czajkowski *et al*., 2015). Depending on the medium, bacterial colonies appear after 24–48 h at 21–37°C. This method is however limited due to the introduction of the saprophytic bacteria which can lead to false results.

2.2.6.2 Artificial media used for isolation and identification of Pectobacterium and Dickeya species

Different agar media have been developed for the isolation of soft rot Erwinia (SRE) from plant tissue, soil and water; however, till present only one medium –CVP– has been used extensively worldwide (Hélias *et al*., 2012). Detection of SRE on CVP

depends on the formation of characteristic deep cavities by the bacterial colonies. The selectivity of the CVP medium is based on the presence of crystal violet, which inhibits growth of most Gram-positive bacterial species, and the use of polypectate as the sole carbon source. At present, only one source appears to be available commercially (Hélias *et al*., 2012) that can be used also in single and double layer forms (Czajkowski *et al*., 2015). Crystal violet pectate remains the preferred diagnostic selective medium for the isolation of SRE from plants and the environment (Hélias *et al*., 2012).

Another medium that has been used for isolation of SRE is the NGM medium (Y. A. Lee & Yu, 2006). This media contains glycerol and manganese chlorite, on which Dickeya spp. form blue colonies. Other SRE do not produce the blue pigment (D. H. Lee *et al*., 2013). The medium was used successfully to differentiate D. solani and D. dianthicola from Pectobacterium spp. isolated from symptomatic plants ((Pierce & McCain, 1992)). In addition strains of *E. chrysanthemi* grow well on the NGM medium, developing dark brownish to blue colonies which are easily distinguishable from other *Erwinia* spp. The results indicate that pigment production on the NGM medium is a very stable property and can be used as a phenotypic property to differentiate *E. chrysanthemi* from other *Erwinia* spp. However, not all strains of Dickeya spp. produce the blue pigment when grown on this medium (Czajkowski *et al*., 2015), therefore its results must be confirmed by another method e.g. by use of biochemical, serological and molecular methods.

2.2.6.3 Biochemical characterizatio of Pectobacterium and Dickeya species

Biochemical methods are often based on a restricted number of characters and by ignoring the natural variability within taxa, run the risk of closely related species or subspecies being missed (Dickey, 1978). The biochemical approach is mainly used to confirm results obtained with other less cumbersome methods(Rahman et al., 2012). The restricted biochemical tests involve catalase, oxidase activity and oxidation/fermentation tests (oxidative/fermentative metabolism of carbohydrates;(Czajkowski *et al*., 2015)). Pectobacterium and Dickeya species possess catalase and oxidase activity and are able to utilize carbohydrates both via

fermentation and oxidation. Biochemical tests however, can give rise to ambiguous results, which can be avoided by using freshly grown, pure cultures of the test bacteria. At the moment there are no reliable biochemical tests available for distinguishing species within a genera due to their high strain variation (Hibar, 2007).

2.2.6.4 Immunofluorescence staining and immunofluorescence colony staining

Immunofluorescence staining (IF) or fluorescent antibody staining (FAS) method is based on the application of the antibodies conjugated to a fluorophore (e.g. fluorescein and its derivatives – isothiocyanate FITC; rhodamin) which bind to bacterial cell walls (e.g. proteins, exopolymeric substances, lipopolysaccharides) hence facilitating recognition (Bertheau Y, Fréchon D, Toth IK, 2002).

Immunofluorescence staining is used in soft rot plant pathogenic bacteria, such as *Pba* and *Pcc*. Its sensitivity is not high, but it allows detection of SRE in mixed populations, 10^4 cells mL^{-1} of *Pcc* and 4×10^3 cells cm^{-2} of *Pba* on potato plant leaf surface(Bertheau Y, Fréchon D, Toth IK, 2002). The major problem of applying IF and IFC methods for diagnostic purposes is that antibodies used for detection of Pectobacterium and Dickeya species may not be sufficiently specific, thereby they allow the detection of non-target (saprophytic) bacteria present in the same environment or fail to detect serological variants of the bacterial species and is advisable to confirm the results using non serological tests(Bertheau Y, Fréchon D, Toth IK, 2002).

2.2.6.5 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) has been mainly used for detection of SRE in a double antibody sandwich format. More complex ELISA systems (e.g. with IF) have been developed but they all suffer from low sensitivity, with a detection lower limit of 10^5–10^6 cells g^{-1} of plant material (Bertheau Y, Fréchon D, Toth IK, 2002) . In addition, ELISA assays fail to distinguish between viable and nonviable Pectobacterium and Dickeya species cells and, as in case of IF and IFC, they are also

and even more prone to cross-reactions with non-target bacteria(Bertheau Y, Fréchon D, Toth IK, 2002).

2.2.6.6. Nucleic acid-based molecular detection methods

Molecular detection methods based on the analysis of bacterial genomic DNA have become most frequently used to detect and differentiate tuber soft rot and blackleg pathogens in environmental samples (Potrykus et al., 2014). They consist of amplification of target-specific sequences by PCR assays of different formats (Czajkowski *et al*., 2015) The PCR-based assays enable faster and cheaper detection of the pathogen than the standard plating methods, but they require specific and often expensive equipment and reagents (Pritchard *et al*., 2013). In comparison with serological assays they are more specific as PCR primers can be designed with specificity for the target genus, or subspecies level (Pritchard *et al*., 2013). There are, however, well-reported limitations. Firstly, the procedures are based on the detection of nucleic acids that can be often relatively more stable in the environment than viable bacterial cells (Czajkowski *et al*., 2015). Consequently, viable cells cannot be reliably distinguished from non-viable cells (Czajkowski *et al*., 2015). Furthermore, variation in the bacterial population genome may result in false-negative results in the case where 16s is the gene of interest (Darrasse *et al*., 1994). Despite these drawbacks, DNA-based amplification methods are still now the most widely used in modern laboratories for detection and differentiation of Pectobacterium and Dickeya species (Darrasse *et al*., 1994). New assays for specific detection of important species and subspecies of plant pathogenic SRE are continuously being developed. Some Molecular based detection methods include: PCR-based detection with species-specific primers, Multiplex PCR, Real-time PCR, Loop-mediated isothermal amplification, Padlock probes, Molecular fingerprinting methods, Repetitive sequence-based PCR which is used for characterization of Pectobacterium and Dickeya species strains(Czajkowski *et al*., 2015).

2.2.6.7. Multi locus sequence tagging

Despite the amount of available genes in public databases, the usual method to place genomes in a taxonomic context is based mainly on the 16S rDNA or housekeeping

genes (Neto *et al*., 2015). Multi locus sequence tagging has been applied to differentiate isolates of Pectobacterium and Dickeya species and it is recognized as a useful technique for this purpose (Duarte *et al*., 2004). The genes that are more commonly utilized in this assay are the housekeeping genes (Neto *et al*., 2015).

This study used the combination of both the phenotypic isolation of Pectobacterium spp and Dickeya spp. on semi-selsctive medium, biochemical tests and pathogenicity tests) and molecular based techniques (conventional PCR and sequencing) to isolate, identify and characterize the pectinolytic bacteria occurring in Kenya.

2.3 Summary and research gaps

It is clear that a lot of study on *Pectobacterium* and *Dickeya spp.* has been carried out in many parts of the world. Research result shows that there is a lot of discoveries of new strains and their reclassification depending on the outcomes of the tests carried out as well as the host range. The genus *Erwinia* is under continuous review including full genome sequencing in order to confirm the species found in the particular genus. However, not much research on these phytopathogens has been carried out to draw a base line of what species are present in potato or any other crops in Kenya. This knowledge gap has led to increased blackleg and soft rot disease of potato in the country impacting negatively on food security and Kenyan economy at large. This study was therefore a critical evaluation of the current potato disease status associated with pectinolytic bacteria. The results of this work will be used by the Kenya's agricultural sector to create awareness about the new potato bacterial pathogens and look for ways of controlling the disease spread hence improve on food security in the country.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Objective 1: Occurrence and Distribution of Pectobacterium and Dickeya spp. in Kenya by phenotypic methods.

3.2 Experimental design

This being a detection survey, cross-sectional design was used for the study. The survey was undertaken in four major potato production areas: Nyandarua, Nakuru, Narok and Elgeiyo Marakwet. The potato production capacity of each county was obtained from the regional county extension officers. This was used to calculate the sample size based on each county's production capacity. A 30% sample size (number of farms as described in ISPM 31 and ISPM 6 for the surveillance and samples collection).

Each farm had equal chance of being included in the sample. Sampling was done using simple random technique as well as with convenient sampling where symptomatic samples were seen. A descriptive questionnaire was used to obtain information on farmer details, disease symptoms for *Pectobacterium* and *Dickeya* spp. and crop details.

Table 3.1: Sampling of infected plants and tubers

County	Number of potato farms as given by extension officer	Expected sample size (30% of total population as per ISPM 31 guideline)	Actual sample size
Nyandarua	50	15	19
Nakuru	70	21	29
Narok	40	12	20
Elgeiyo Marakwet	60	18	23

3.2.1 Sample collection

A field survey was conducted during September to October 2016 potato growing season. Sampling for symptomatic plants and tubers was done depending on the distribution of the crop in the respective administrative locations surveyed following simple random sampling techniques and by moving diagonally across each field.

At the time of sampling crops were at flowering-tuber setting stage. Fields were checked for the occurrence of typical soft rot and stem rot (blackleg) symptoms. Data related to crop variables such as growth stage and variety, disease symptoms, disease incidences, purpose of production (ware, seed or research) altitude of each location and their corresponding geographical position using the geographical positioning system (GPS) were collected. Potato plants with blackleg and soft rot symptoms were collected by uprooting the diseased plant and placement into sample bags. The samples were labeled accordingly and submitted to the Plant Quarantine and Biosecurity Station laboratory for analysis. A total of 91 symptomatic samples were collected (Table 3.1) for screening using phenotypic and molecular detection methods. Reference cultures of the different species were used as positive controls. Tested Healthy potato tubers grown in Kenya were used as negative controls.

3.2.2 Sample preparation and bacterial isolation

The samples were washed in tap water to remove soil and plant debris. Surface sterilization was then done using 1% sodium hypochlorite for five minutes followed by triple rinsing using sterile distilled water. From symptomatic tuber tissues, a sample was picked using sterile scalpel from the advancing front of the rot and from newly diseased tissue to avoid interference and growth suppression by contaminating saprophytes. For the stems, a separate disposable scalpel was used to cut small pieces of vascular tissue after removing the epidermis about 2-3cm in length. The sample tissues were extracted by crushing in a polyvinyl plastic bag containing 2ml sterile phosphate buffer solution P^H 7.4 and allowed to stand for 30 minutes to release bacteria. 20ul of about 10.7 cells/ul of bacterial suspension was spread plated on the surface of the selective medium (CVP) in a plate and on NGM medium as described

by (Hélias et al., 2012) and (Y. A. Lee & Yu, 2006). The plates were incubated at 27^{o}C for 24-48hours and then checked for typical (pectolytic) colonies and indigoin pigment production. Characteristic pit cavities that appeared on CVP and those that gave pigment on NGM medium. All present colonies were picked up using a sterile bacterial loop and transferred to another plate of CVP and NGM medium respectively. Purification of bacterial colony was done by re-streaking of single colony in sterile nutrient ager plates and about 10.7 cells/ul of each isolate was stored at -20 ^{o}C in phosphate buffer saline with 10% glycerol.

Table 3.2. Isolates used in this study as positive controls

Strain Name	**Strain Number**	**Source (Isolated from)**	**Origin**
Erwinia chrysanthemi	PD 97	*Chrysanthemum* sp	Netherlands
Pectobacterium carotovorum* subsp. *carotovorum	PD 1006	*Solanum tuberosum*	Netherlands
Dickeya dianthicola	GBBC 322	*Solanum tuberosum*	Belgium
Pectobacterium carotovorum* subsp. *brasilinse	GBBC 1819	*Solanum tuberosum*	Belgium
Pectobacterium carotovorum* subsp. *wasabiae	GBBC 1814	*Solanum tuberosum*	Belgium
Pectobacterium carotovorum* subsp. *atrosepticum	GBBC 1794	*Solanum tuberosum*	UK
Pectobacterium carotovorum* subsp. *brasilience	GBBC 1819	*Solanum tuberosum*	Belgium

3.3 Biochemical tests

The isolated bacterial strains were characterized according to biochemical testing method as described by Perombelon, 2002.The tests performed were oxidative fermentation metabolism of glucose, oxidase reaction, catalase test with 6% hydrogen peroxide, reduction of sugars from sucrose, and ability to produce indole from tryptophan.

3.3.1 Oxidative fermentation test (O/F)

The test was done to differentiate fermentative from oxidative metabolism of carbohydrates**.** The test determines if the bacteria is able to metabolize glucose by fermentation or by respiration (oxidatively). At the time of test, a loopful of a 48-hour culture on nutrient agar was stab inoculated in the prepared media with a straight wire inoculating needle. A1cm depth of sterile liquid paraffin oil was poured on tubes to induce anaerobic conditions. The samples were incubated at 27^0C for 48 hours. Color formation resulting from acid production in the tube indicated fermentative bacteria ($_{+/+}$.) Color formation from the tube without the oil cover (Control) indicated oxidative bacteria (+/-).As controls tube with inoculated medium without glucose and tubes with non-inoculated medium and with and without oil over layer were included.

3.3.2 Production of reducing substances from sucrose

This test was done to determine ability of bacteria to decompose simple carbohydrates. The test identifies sugars which have free ketone or aldehyde functional groups. The test utilizes Benedict's reagent as the reactant. When Benedict's reagent and simple carbohydrates are heated, the solution changes from green to brick red. This reaction is caused by the reducing property of simple carbohydrates. The copper (II) ions in the benedict's reagent is reduced into copper (I) ions which causes the colour change. Different strains of pectinolytic bacteria give varying results following this test. At the time of test, a loopful of a 48-hour culture on nutrient agar was inoculated on the sucrose medium and incubated at 27^oC for 48 hours.

An equal volume of Benedict's reagent was added and heated in a boiling water bath for 10 minutes. Production of a yellow-orange/ brown to brick red colour (with or without precipitate) indicated a positive reaction. A green colour was considered negative.

3.3.3 Oxidation test

Oxidase test was carried out to detect presence of cytochrome oxidase system that catalyzes transport of electrons between electron donors in the bacteria and a redox dye-tetramethyl-p-phenylene-diamine. The dye is reduced to deep purple color in absence of the above enzyme. Cytochrome containing organisms produce an intracellular oxidase enzyme which catalyzes the oxidation of cytochrome c. Pectinolytic bacteria however, do not have this cytochrome c system hence are oxidase negative.

A loopful of a 48-hour culture on nutrient agar was rubbed on a filter paper impregnated with a drop of 1% (w/v) freshly prepared aqueous tetramethyl-p-phenylediamine dihydrochloride solution. Development of a purple coloration within 10 seconds indicates a positive reaction. Color development within 10-60 seconds indicated a delayed reaction also considered to be negative. Reaction after 60 seconds indicated negative result.

3.3.4 Catalase test

Catalase is an enzyme produced by microorganisms that live in oxygenated environments to neutralize toxic forms of oxygen metabolites; hydrogen peroxide (H_2O_2). The enzyme neutralizes bactericidal effects of hydrogen peroxide and protects them.

A loopful of a 48-hour culture on nutrient agar was mixed with a drop of 6% H_2O_2 on a glass slide. Formation of gas bubbles at either concentration indicated a positive reaction.

3.5 Production of indole from tryptophan

This test was used to demonstrate the ability of bacteria to decompose amino acid tryptophan to indole; which accumulates in the medium.

Tryptophan+water in presence of tryptophanase=indole+pyruvate+ammonia.When the indole reacts with Kovac's reagent (which contains HCL and p-dimethylaminobenzaldehide in amyl alcohol) the solution turns from yellow to cherry red. Because amyl is not water soluble, the red coloration will form in an oily layer at the top of the broth. *Pectobacterium spp*. are indole negative while *Dickeya spp*. are indole positive. A loopful of a 48h culture of the bacterium on nutrient agar was inoculated in the test medium and incubated at 27 ºC for 3 days. 1 ml of Kovac's reagent was added, shaken gently and allowed to stand for 5 minutes at room temperature. A positive reaction was indicated by the presence of a dark red color ring in the surface layer of the broth.

3.5.6 Pathogenicity Testing

All bacterial isolates were tested for their ability to cause tuber soft rot on potato discs and blackleg symptoms in potato plant as per the method described by Naqqash et al. 2016 and Ismail & Mostafa 2012 respectively. Healthy potato tubers were sterilized with 70% alcohol, rinsed with sterile distilled water and aseptically cut into slices (about 1cm width). The potato slices were put in sterile petri dishes containing sterilized filter paper impregnated with 2ml of sterile distilled water. The soft rot test was done in duplicate. The potato slices were inoculated by spreading a bacterial colony on the surface using a sterile inoculating loop. The inoculated slices were maintained in moist conditions and were incubated at 27^{o}C for five days. The bacterial cultures that produced characteristic symptoms of soft rot on potato slices were selected and recorded as positive. For the blackleg and stem rot test, the healthy potato tubers with 3-5 buds were sterilized with 70% alcohol and rinsed with sterile distilled water. The tubers were inoculated with needle pricking method. The wounded tubers were soaked for 15 minutes in bacterial suspension containing 10^{6}-10^{7} cells per ml determined by serial dilutions using sterile distilled water to allow tuber infection. Inoculated tubers were planted in pots containing sterile soil

containing di ammonium phosphate (Mavuno®) fertilizer and were maintained in greenhouse. The temperatures were maintained at 25-30 ^{o}C for 90 days. Watering was done for 3 days in a week and observations were made for three months.

3.4 Objective Two: Genetic diversity of Pectobacterium and Dickeya spp using molecular methods-: PCR, Sequencing of the PCR products and phylogenetics analysis

3.4.1 Bacterial DNA Extraction

Genomic DNA was extracted from bacterial cells using standard DNA extraction method as described by Sambrook and Russell, (2001). Total genomic DNA of each isolate was extracted from cultures grown for 24 h at 27°C in nutrient agar. A third loopful of bacterial colony containing about 10^6-10^8 cells was picked and resuspended in molecular grade water in 1.5ml micro centrifuge tubes. The suspension was centrifuged at 13,000g for 10 minutes, and the supernatant discarded. The pellet was re-suspended in 0.3 ml extraction buffer (0.5 M Tris-HCl, pH 7.5, 0.4 M EDTA, 1% SDS, 0.2 mg ml^{-1} proteinase K) and incubated at 65°C in a water bath for 45 minutes. Equal volumes of phenol-chloroform (1:1) was added to the tube, aqueous and organic phases were mixed by gentle inversion until the phases were completely mixed. The samples were subsequently centrifuged at 10,000 *g* for 15 minutes at room temperature to separate the aqueous phase from the organic phase. The upper aqueous phase was transferred to a clean tube, and then an equal volume of chloroform was added to it, mixed and centrifuged as above. The upper phase was transferred to a new tube and DNA was precipitated with 0.1 vol. of 3 M sodium acetate pH 5.2 and 0.7 vol. of cold isopropanol. After precipitation, tubes were kept at -20°C for 30 min and subsequently centrifuged at 14,000 *g* for 10 minutes. Supernatant was discarded and DNA washed with 70% ethanol, air-dried, resus-pended in 50 ml TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and stored at -20°C for further use.

3.4.2 DNA quantification and purity check

The DNA quantity and purity were analysed using Nano Drop and 0.8% Agarose gel. For the nano drop, 1µL of the sample was analysed at Absorbance 260 and 280 with

the lid factor 50. For agarose gel,1µL of DNA sample was dissolved in TE and pipetted on to a Para film then mixed well with 3 µL of 10X loading dye by pipetting up and down several times. The sample was then carefully loaded into the wells in the gel. The gel was run at 80v/cm for 1hour and bands visualized and documented using gel documentation system (Molecular Imager®, Gel doc TM XR system 170-8170, BIO-RAD, USA).

3.4.3 PCR amplifications

Conventional PCR was performed to further identify specific strains of pectinolytic bacteria isolate from the samples that turned positive by phenotypic methods. Reactions were performed with final concentrations consisting of 50ng/ul of template DNA, 0.2 µM dNTPs, 1.5 µM $MgCl_2$, 0.4 µM each forward and reverse primer, 0.5 U Dream Taq DNA polymerase (Fermentas), 1 × Taq DNA polymerase reaction buffer (Fermentas®) in a total volume of 25µl. Amplifications were done with SR3F and SR1cR(for *Pectobacterium* and *Dickeya* spp), ECA1f/ECA2r(for *Pectobacterium atrosepticum*) ,EXPCCF/EXPCCR(for *Pectobacterium carotovorum*), PW7011F/PW7011R (for *Pectobacterium wasabiae*), BRIf /LIr (*For Pectobacterium brasiliense* and ADE1/ADE2 (for *Dickeya* spp) primer pairs (Pritchard *et al*., 2013). All amplification reactions were done using ABI GENE Thermocycler ® while all primers were sourced from Inqaba Biotech, South Africa. An aliquot of 5µl from the amplified PCR products was stained with GelRed® and the mixture size separated on 1.5 % (w/v) agarose gel in 1 × TBE buffer. Electrophoresis was run at 80 V for 60 min and products visualized under Gel Doc-It Imaging system® (800)452-6788, Upland-CA, USA, gel documentation system. Amplicon sizes were established by comparing to a standard 1 kb plus molecular ladder (Fermentas®).

3.4.4 Genetic diversity of Pectobacterium and Dickeya spp.

Sequencing of genomic DNA was done by partial amplification of 16S rDNA genes(Frank *et al*., 2008) by PCR using sequencing primers as shown in table 3.3. Amplicons were purified using the Wizard SV gel and PCR clean-up system (Promega A9280) and sequenced at Inqaba Biotech Laboratories (South Africa).

Sequence evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ (Frank *et al*., 2008) algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 13 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 75 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Table 3.3 Sr DNA Primers sequences that were used for sequencing of PCR products(Frank *et al.*, 2008).

Name of Primer	Target	Sequence (5' to 3')
16S-27F	16S rDNA sequence	**AGAGTTTGATCMTGGCTCAG**
16S-1492R	16S rDNA sequence	**CGGTTACCTTGTTACGACTT**

3.5 Data analysis

Both comparative and descriptive statistics were used to analyze obtained data. Chi-square test of association was used to test if there was any relationship between origin of the samples and presence of disease of interest. The null and alternative hypothesis were as follows:

H_O: These is no *Dickeya* spp. in Kenya

H_1: There are only *Pectobacterium carotovorum* and *Pectobacterium brasilience* subsp. in Kenya.

The null hypothesis was tested at 0.05 level of significance and the rule was to reject the null hypothesis if the P Value(asymp.sig.2-sided) is less than 0.05, or accept the hypothesis if otherwise.

CHAPTER FOUR

RESULTS AND INTERPRETATION

4.1 Objective 1: Occurrence and distribution of Pectobacterium and Dickeya spp in Kenya by phenotypic (Isolation on media) biochemical tests and pathogenicity tests

A total of 91 samples showing typical symptoms of blackleg, tuber soft rot or stem rot were collected in the field showing actions of pectinolytic bacteria secreted effective cell wall degrading enzymes responsible for tissue maceration which gave characteristics water soaked, slimy and rotten appearance of the infected plant as well as black colouration from the mother tuber up the stem. Infected tubers were soft, watery with decayed mass.

Figure 4.1: Blackleg symptoms on stems of potato plants in the fields where black colouration from the mother tuber up the stem was observed.

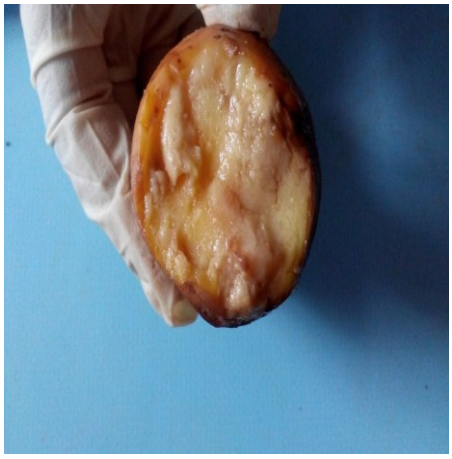

Figure 4.2: Soft rot symptoms observed on tubers in potato fields where soft, watery with decayed mass were observed.

Following isolation on CVP medium, 79 samples produced pit cavities.

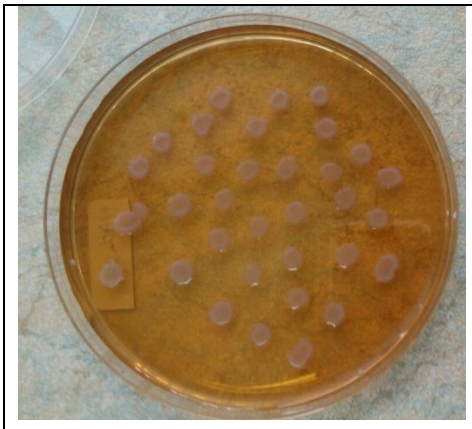

Figure 4.3 Sample origin: Nakuru
Samples produced pit cavities as an indication of action of the cell wall degrading enzymes digestion the pectin as the source of carbon.

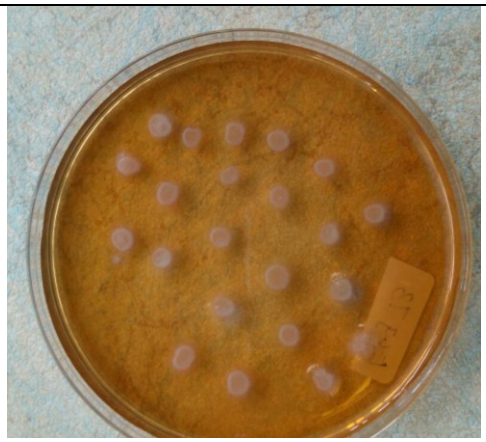

Figure 4.4 Sample origin: Elgeyo Marakwet.
with pit cavities. Samples produced pit cavities as an indication of action of the cell wall degrading enzymes digestion the pectin as the source of carbon

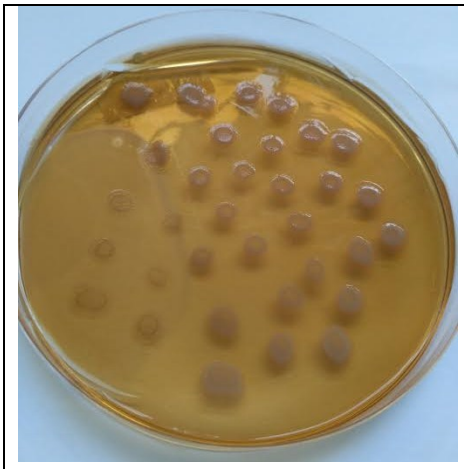

Figure. 4.5: Sample origin: Nyandarua with pit cavities

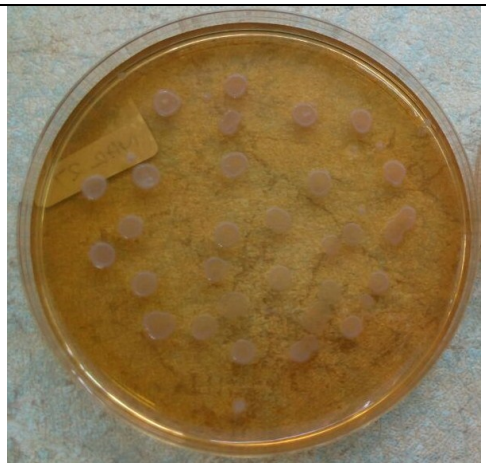

Figure 4.6 Sample origin: Narok with pit cavities

Each region had pectinolytic bacteria indicated by formation of pit cavities on media after incubation at 27^{o}C for 48hours as shown below.

When these isolates were tested on tubers for pathogenesis, 26 of them showed rotting on potato slices (Fig. 4.7) and 12 out of the 26 showed blackleg and stem rot symptoms (Fig. 4.8).

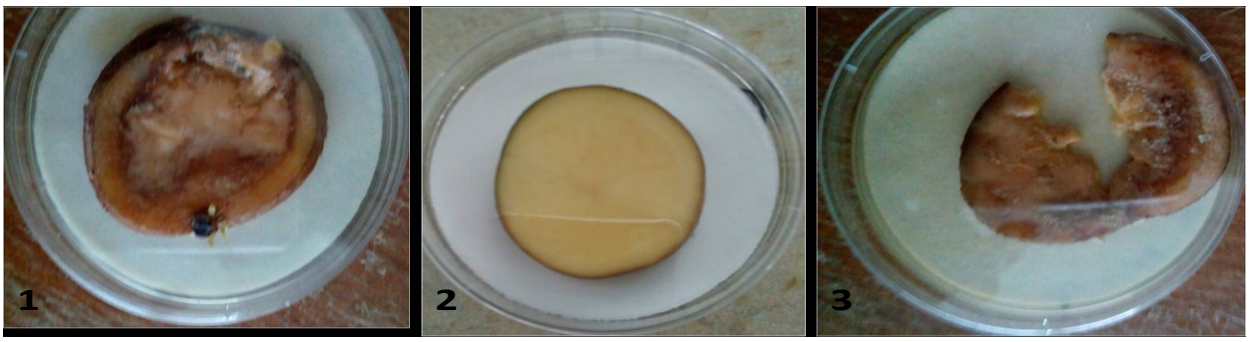

Figure 4.7: Slice rots trials.

1, seed tuber infected with a pure culture colony from the Elgeiyo market samples.

2, healthy seed tuber used as the negative control.

3, infected seed tuber with *Pcc* positive control strain number PD 1006. Rotting was observed within 72 h.

Figure. 4.8: Pot trials.

1, uninfected plants serving as the negative controls; the plants remained healthy for the entire 90 days.

2, seed tuber infected with positive control PD 1006; the plant died within 21 days.

3, 4, 5, seed tubers infected with pure culture colonies from Elgeiyo Marakwet.; the plants showed soft rot and black rot symptoms from the base upwards within 21 days.

Biochemical tests were done to further characterize the isolates and to separate the putative *Pectobacterium* spp. and the *Dickeya* spp. The results showed that nine isolates did not produce indole and might belong to the genus *Pectobacterium* and 12 isolates were indole positive, indicative to the genus *Dickeya* although *Pectobacterium carotovorum* subsp. *brasiliense* strains may also be indole positive (Lee *et al*., 2014). According to Dickey (1978), pectinolytic bacteria are oxidase negative because they lack cytochrome oxidase enzyme that catalyzes transport of electrons between donors in the bacteria and a redox dye-tetra-metthyl-p-phenelyne-diamine to produce purple color, but may vary on indole and other tests. Most *Pectobacterium* spp. and strains are indole negative. They lack the enzyme tryptophanase which decomposes the amino acid tryptophan to indole. *Dickeya* spp. and some *P.c.* subsp. *brasiliense* strains are indole positive because they contain the

enzyme tryptophanase (Ma *et al.*, 2007; Lee and Yu, 2006; Lee *et al*., 2014). Other biochemical reactions may vary among species of the same genus.

Figure 4.9 : Oxidative fermentation:

Pectinolytic bacteria were positive for the test as shown by color change from blue to yellowish brown which means they were able to ferment and oxidize glucose. The controls without sample and that without glucose were negative as was expected.

Figure 4.10: Reducing substances from sugars:

Results for reducing substances from sugars

The color turned from green to brownish brick red for most of the samples indicating positive results for pectinolytic bacteria. Other isolates were negative in these test phenomena, expected for some species like *Pectobacterium wasabiae*. The controls without sample remained green while that with a known positive isolate changed color from green to brick red as was expected.

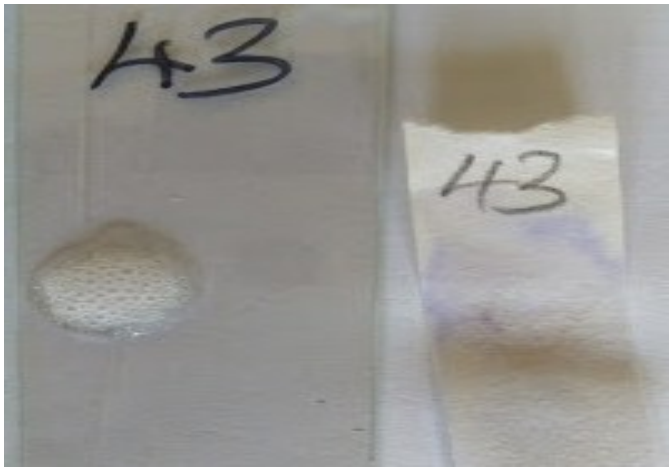

Figure 4.11: Catalytic and Oxidase tests

For catalytic tests, there was bubble formation positive for pectinolytic bacteria.
For oxidative tests, there was no color change since the pectinolytic bacteria are oxidase negative.

Figure. 4.12: Indole tests:

Figure. 4.12 Indole tests: color change on media upon decomposition of tryptophane by tryptophanase for *Dickeya* spp. *Pectobacterium spp*. are indole negative and therefore did not have red ring on the surface of the medium while the *Dickeya spp*. are indole positive hence formed the red ring upon reacting with kovac's reagent as compared to the positive control

Table 4.1: Summary of biochemical tests results

Isolate number	Origin	Catalase test	Oxidase test	Reduction of sugar from sucrose	Oxidative fermentative tests (OF)	Indole test	Remarks
1	Nyandarua	+ve	+ve	+ve	+ve	-ve	Doubtful
2	Nyandarua	+ve	+ve	+ve	+ve	-ve	Doubtful
13	Nyandarua	+ve	+/-ve	+ve	+ve	-ve	*Pectobacterium*
19	Nyandarua	+ve	-ve	+ve	+ve	+ve	*Dickeya*
20	Narok	+ve	-ve	+ve	+ve	+ve	*Dickeya*
26	Narok	+ve	-ve	+ve	+ve	+ve	*Dickeya*
27	Narok	+ve	+/-ve	+ve	+ve	-ve	*Pectobacterium*
28	Narok	+ve	+/-ve	+ve	+ve	-ve	*Pectobacterium*
31	Narok	+ve	+ve	+ve	+ve	-ve	Doubtful
34	Narok	+ve	-ve	+ve	+ve	+ve	*Dickeya*
43	Nakuru	+ve	-ve	+ve	+ve	+ve	*Dickeya*
47	Nakuru	+ve	-ve	-/+ve	+ve	-ve	*Pectobacterium*
50	Nakuru	+ve	-ve	-ve	+ve	-ve	*Pectobacterium*
53	Nakuru	+ve	-ve	-/+ve	+ve	-ve	*Pectobacterium*
57	Nakuru	+ve	-ve	-/+ve	+ve	+ve	*Dickeya*
58	Nakuru	+ve	-ve	+ve	+ve	-ve	*Pectobacterium*
59	Nakuru	+ve	-ve	+ve	+ve	+ve	*Dickeya*
66	Nakuru	+ve	-ve	+ve	+ve	+ve	*Dickeya*
71	Elgeiyo M	+ve	-ve	+ve	+ve	+ve	*Dickeya*

74	Elgeiyo M	+ve	-ve	+ve	+ve	+ve	*Dickeya*
76	Elgeiyo M	+ve	-ve	+ve	+ve	+ve	*Dickeya*
81	Elgeiyo M	+ve	-ve	-/+ve	+ve	+ve	*Dickeya*
84	Elgeiyo M	+ve	-ve	-/+ve	+ve	-ve	*Pectobacterium*
86	Elgeiyo M	+ve	+ve	-/+ve	+ve	-ve	Doubtful
87	Elgeiyo M	+ve	+ve	-/+ve	+ve	-ve	Doubtful
91	Elgeiyo M	+ve	-ve	-/+ve	+ve	-ve	*Pectobacterium*
PD 1006	Netherlands	+ve	-ve	+ve	+ve	-ve	*Pectobacterium*
PD 97	Netherlands	+ve	-ve	+ve	+ve	+ve	*Dickeya*
NEG	Sterile phosphate buffer	-ve	-ve	-ve	-ve	-ve	

Key: + (positive), - (negative), -/+ve (doubtful, delayed reaction, regarded as negative).

Table 4. 2: Summary of phenotypic test results

County	Total number of symptomatic samples	Samples positive on CVP medium	Samples positive on slice rot tests	Samples positive on stem rot test	Samples positive for *Pectobacterium* spp. Using biochemical test	Samples positive for *Dickeya* spp. Using biochemical test	Remarks
Nyandarua	19	16	4	2	1	1	**Positive for *Pectobacterium* and *Dickeya* spp.**
Narok	20	18	6	1	3	3	**Positive for *Pectobacterium* and *Dickeya* spp.**
Nakuru	29	25	8	4	3	3	**Positive for *Pectobacterium* and *Dickeya* spp.**
Elgeyo Marakwet	23	20	8	5	2	5	**Positive for *Pectobacterium* and *Dickeya* spp.**
Total samples	91	79	26	12	9	12	

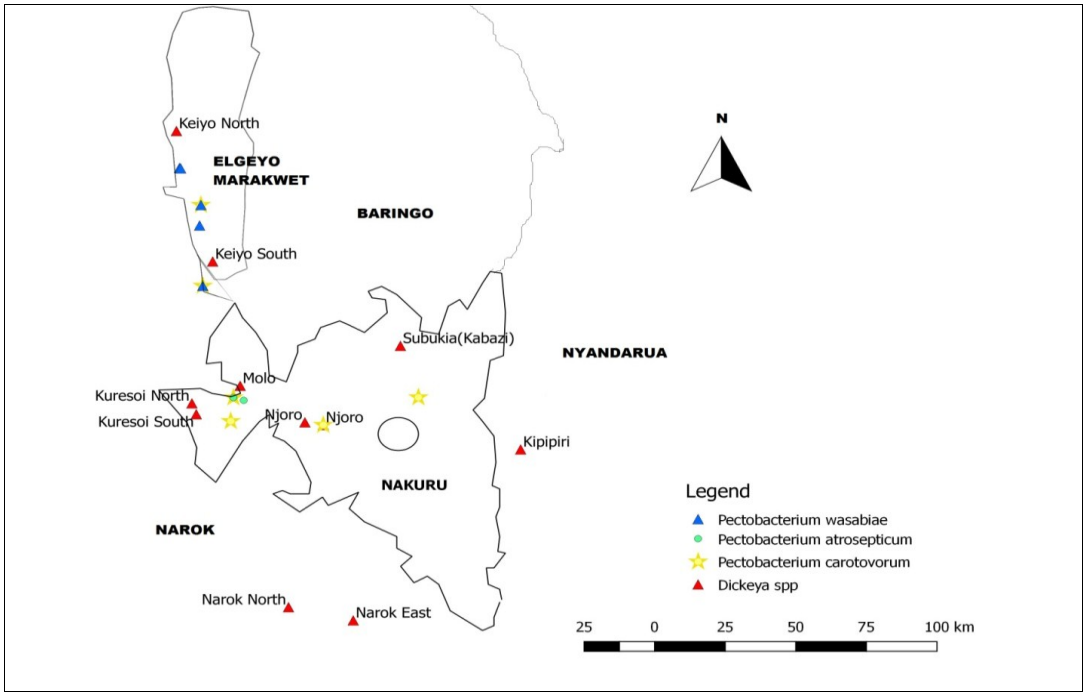

Figure 4.13: The Map showing distribution of *Pectobacterium* and *Dickeya* spp in the four sampled regions.

4.2 Objective two: Genetic diversity of Pectobacterium and Dickeya spp using molecular methods-: PCR, Sequencing of the PCR products and phylogenetics analysis

4.2.1 Molecular characterization of Pectobacterium and Dickeya spp.

Results of bacterial DNA quantification and qualification using UV spectrophotometer are given in annex V**.** Out of 91 samples, 85 were pure giving absorbance of between 1.7-2.0 at A260/280 and concentration above 50ng/ul. The DNA for all the samples was intact and without any shears. It was also at the right concentration of about 20,000bp in size.

4.2.2 PCR amplification using generic and species-specific primers.

The 26 isolates that were positive on slice rot tests including the 12 which showed blackleg and stem rot symptoms were subjected to conventional PCR for further identification.15 isolates were amplified with generic primer SR3F and SR1cR

(Fig.4.13) to give expected size of 119bp indicating that they were pectinolytic. Six isolates (23%) were successfully amplified with the EXPCCF/EXPCCR primer pair to produce the expected 550 bp amplicon (Fig.4.14) of *P.c.* subsp. *carotovorum* while five (19%) amplified with PW7011F/PW7011R primers (Fig.4.15) to produce the expected amplicon of 140 bp for *P.c.* subsp. *wasabiae* and 2 samples (8%) amplified with primer pair ECAf /ECA2r (Fig.4.16) to give expected size 690bp for *Pectobacterium atrosepticum* for only Nakuru samples. However, we could not confirm by using the available primer set the group of 12 isolates which were indole positive as potential *Dickeya* spp. (*E. chrysanthemi*) or *Pectobacterium carotovorum* subsp. *brasiliense*. Conventional PCR was ran for this group of isolates using *Dickeya* generic primer set ADE1 / ADE2 and BRIf /LIr specific for *Pectobacterium carotovorum* subsp. *brasiliense (*Pritchard *et al*., 2012*).* Apart from the positive *control (Dickeya dianthicola* strain GBBC 322*), none* of the isolates in this group gave the expected product size of 420 bp for *Dickeya* spp., instead, the isolates produced bands between 1000 and 1500 bp.(Fig.4.17) On the test for *Pectobacterium carotovorum* subsp. *brasiliense* all the twelve isolates were negative too. Re isolation of the pathogens from the host plants and potato slices which had turned positive for *P. Carotovorum, P. atrosepticum and P. wasabiae* was done to identify the specific causative agent of blackleg and soft rot disease in these potato samples. All the re-isolated bacteria were tested by conventional PCR as described by Pritchard et al. (2012) and biochemical tests as described by Perombelon (2002). The six samples which had previously turned positive for *P. carotovorum* remained positive for this test while the five which had turned positive for *P. wasabiae* remained positive too for this test. The tow samples which were previously positive for *P.atrosepticum* remained positive too. These isolates were also positive on O/F tests but negative on indole tests, and oxidase tests.

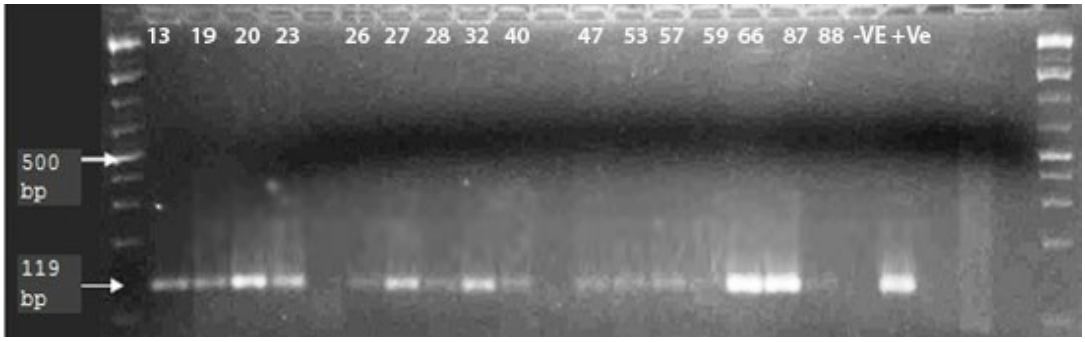

Figure 4.14: PCR for Dickeya spp and Pectobacterium spp.

Fig. 4.14: PCR for *Dickeya spp* and *Pectobacterium spp*. using generic primers SR3F and SR1cR. This primer pair targets 16S and rRNA gene *Pectobacterium* and *Dickeya* species. Only 15 samples from all regions gave an amplification to produce the expected band size of 119bp as shown in the figure above.

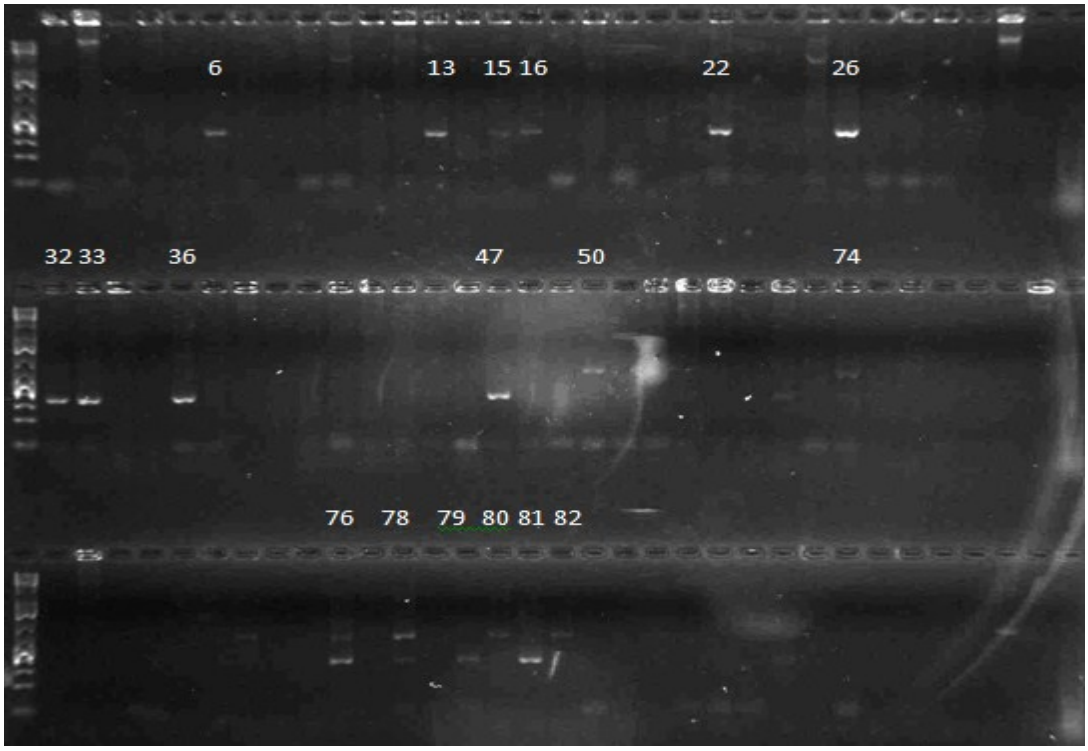

Figure 4.15: PCR amplification for Pectobacterium carotovorum using specific primers EXPCCF/EXPCCR.

This primer targets the genome of *Pectobacterium subsp carotovorum.* A total of 18 samples out of 91 from all the four regions as shown in the figure above gave

expected product of 550bp as compared to the molecular marker. From the 26 samples that caused rots on tubers during pathogen city testing, 6 of them gave expected band size of 550bp.

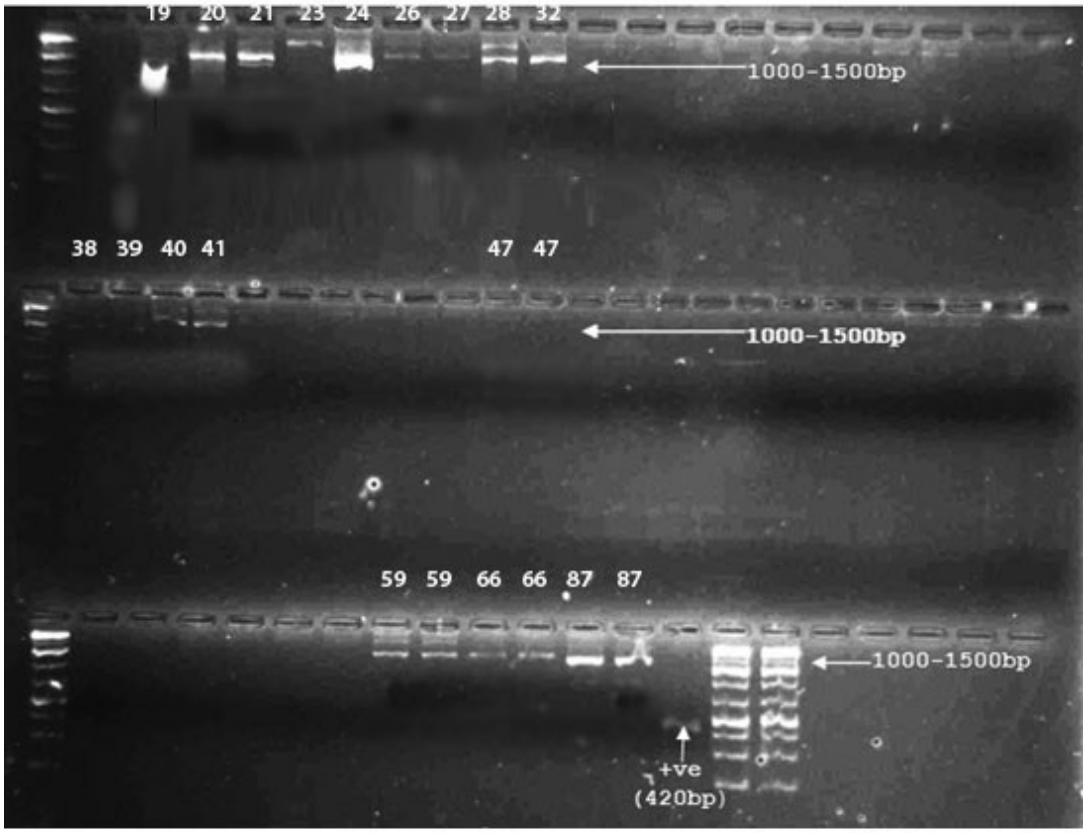

Figure 4.16 Gel image for Dickeya spp

Figure 4.16 Gel image for *Dickeya* spp. using ADE1/2 primer pair. *Dickeya Dianthicola* (strain GBBC 322) was used as the positive control. This primer targets 16S pel gene for *Dickeya spp*. None of the samples gave the product of 420 bp as was expected apart from the positive control. However, 16 out of 26 samples from all regions gave unexpected product of between 1000-1500bp with others showing multiple bands as shown in figure above.

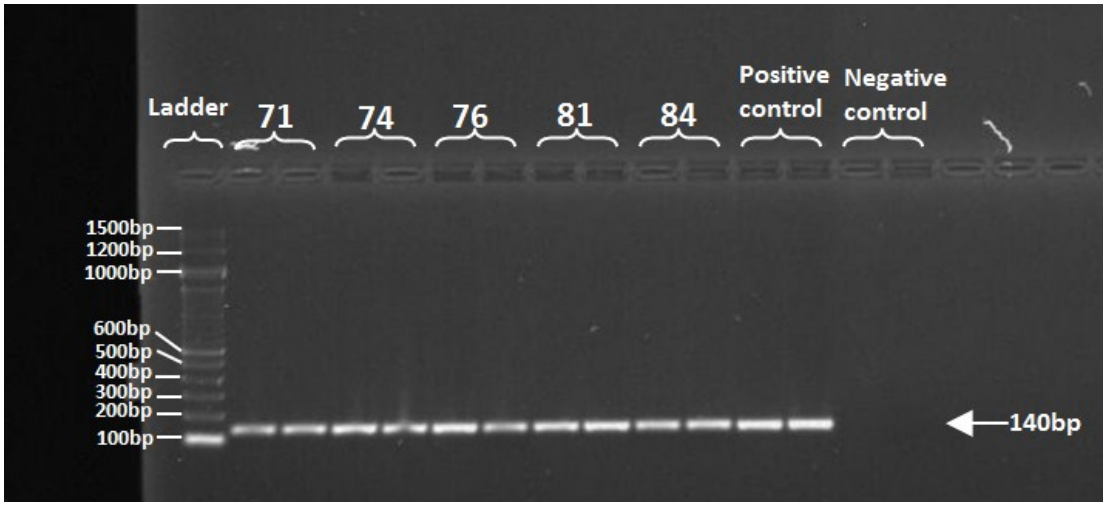

Figure 4.17: Results of PCR amplification using generic primers PW7011F/PW7011R for Pectobacterium wasabiae.

This primer pair targets TYD protein of the bacteria. Only 5 samples from Elgeiyo Marakwet gave the expected product of 140 bp as compared to the molecular marker,1kb plus ladder as shown in the figure above. This strain has now detected for the first time in Kenya during this work. PCR amplification of *Pectobacterium carotovorum* subsp.*wasabiae*; 71, 74, 76, 81, 84 are samples from Elgeiyo Marakwet. Band sizes compared against 100 bp ladder. The isolates gave the expected size of 140 bp.

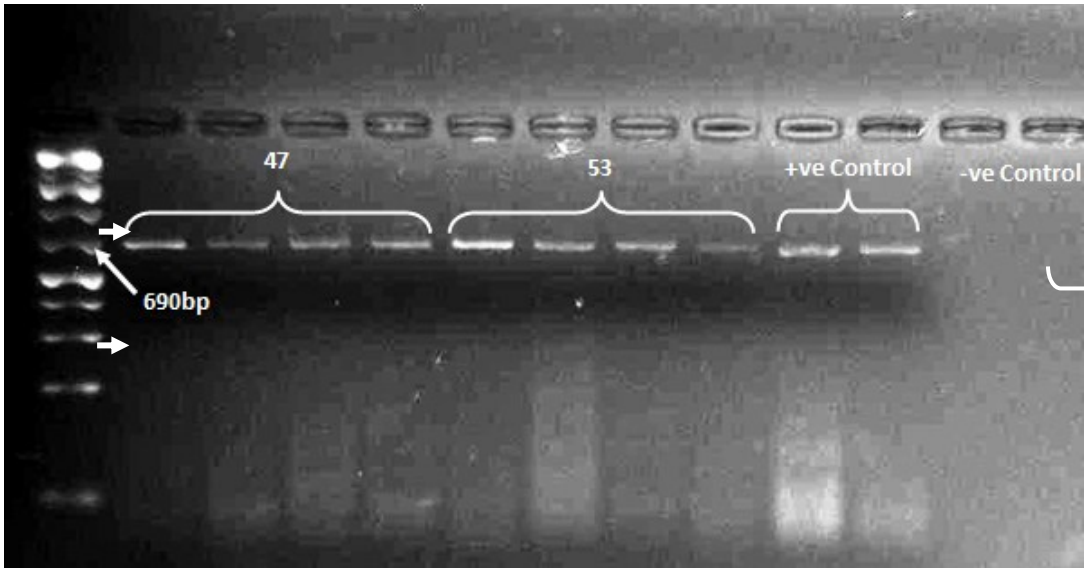

Figure 4.18: Results of PCR amplification using generic primers ECA1f /ECA2r for Pectobacterium subsp atrosepticum.

This targets the genome of Pectobacterium subsp atrosepticum. Only two samples from Nakuru region gave the expected product of 690bp as compared to the positive control. Again, this strain was detected for the first time in Kenya during this work.

2 samples from Nakuru amplified at 690bp indicating that they were positive for *P.atrosepticum*.

4.2.3 Genetic diversity for Pectobacterium and Dickeya SPP.

Genomic DNA was amplified and sequenced using universal primer pairs used for the barcoding of bacteria. Only samples with single band were considered for further analysis. From the phenotypic results, a total of 11 samples from the four regions were entered for sequencing. These samples were chosen based on their special characteristics evidenced from the phenotypic result. For instance, sample no 1 and 86 were oxidase positive and indole positive giving doubtful results. These samples also did not give amplification with SR3F and SR1cR too. All pectinolytic bacteria are oxidase negative. Sample no. 4 and 76 were oxidase negative but were positive on indole tests and reduction of sugars from sucrose indicating that they could be any pectinolytic bacteria. Sample no.47,53 were oxidase negative and indole negative indicating that they were positive for *Pectobacterium* spp. These samples also gave amplification with SR3F and SR1cRprimer. Sample no.57 was negative on oxidase test and reduction of sugars from sucrose but indole positive which meant it could be any pectinolytic bacteria and gave amplification with SR3F and SR1cR primer. Sample 26, 34 and 59 were oxidase negative, indole positive and positive on reducing sugars from sucrose indicating that they were *Dickeya* spp. These samples too gave amplification with SR3F and SR1cR.Sample number 91 was indole negative, oxidase negative and negative on reducing sugars from sucrose suggesting need for further investigation. This sample also did not give amplification with SR3F and SR1cR.

Amplification of samples with the sequencing primer 16S-27F/16S-1492R was observed at 1400bp as expected and as shown in the sample figure 4.18 below.

Amplicons were purified using the Wizard SV gel and PCR clean-up system (Promega A9280) then sequenced at Inqaba Biotech Laboratories (South Africa) .

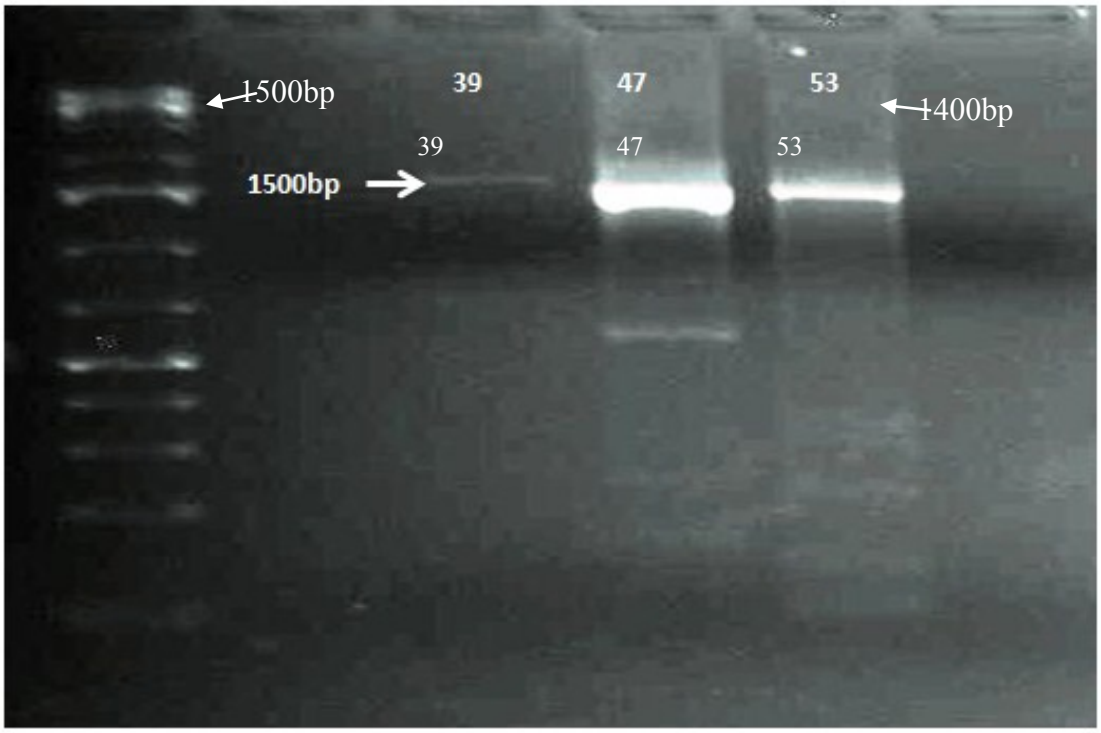

Figure 4.19: Results of conventional PCR for genetic diversity.

Figure 4.19: this figure above shows results of conventional PCR for genetic diversity. Samples positive are 39, 47(with multiple bands) and 53 showing bands of 1400bp in size as compared to molecular marker.

Blast search was performed on National Center for Biotechnology Information (NCBI) and nucleotides sequences obtained in after analysed using maximum likelihood method.

Multiple sequence alignment was performed in Mega 6 software and consensus sequences obtained in fasta format. Phylogenetic tree was constructed to show the identity and evolutionary relationship of the pathogens determined.

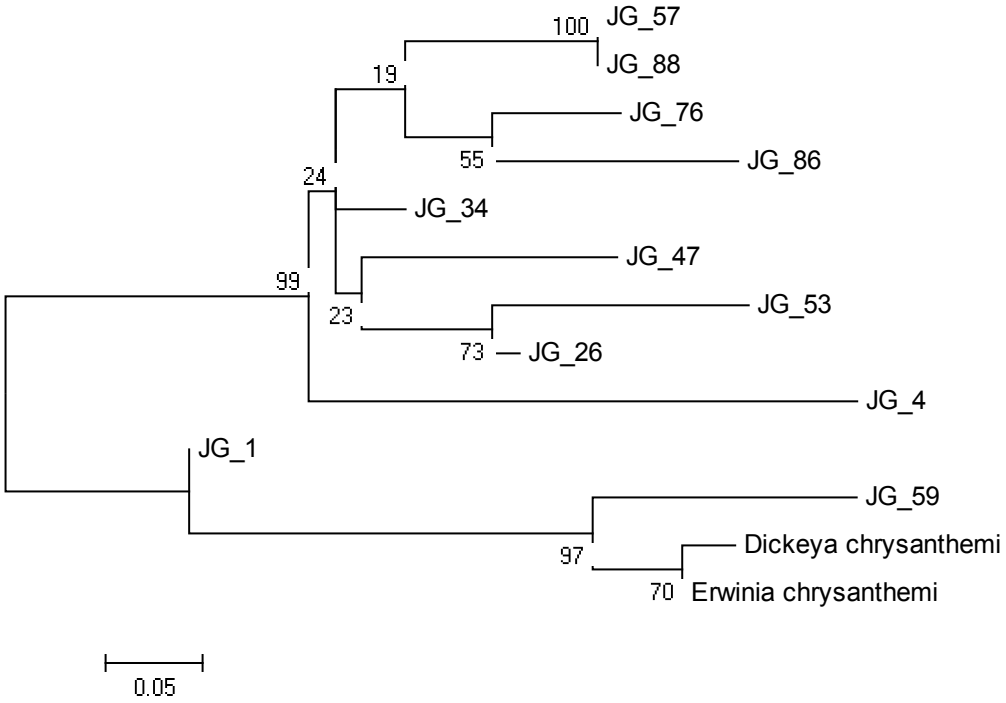

Figure 4.20 Phylogenetic tree of *Pectobacterium* and *Dickeya* isolates done in Mega 6.

Nine isolates:4,26,34,47,53,57,76,86 and 88 clustered together with an evolutionary relationship of 99% while two isolates sample no.1 and 59 clustered with *Erwinia chrysanthemi.* Isolate 59 gave an evolutionary relationship of 97% with *Erwinia chrysanthemi.*

Table 4.3: Summary of amplification results per county

County	Total number of samples positive on slice rot test	Samples positive on SR3F	Samples positive on EXPCCF/EXPCCR	Samples positive on ADE1/2	Samples positive PW011F/R	Samples positive on ECA1f/ ECA2r	Remarks
Nyandarua	4	2	4		0	0	
Narok	6	6	5	0	0	0	**Positive for *Pectobacterium sub spp.carotovorum***
Nakuru	8	5	2	0	0	**2**	**Positive for *Pectobacterium sub sp.carotovorum and subsp atrosepticu***
Elgeyo Marakwet	8	2	7	0	**5**	0	**Positive for *Pectobacterium sub sp.carotovorum and subsp wasabiae***
Total samples	26	15	18	0	5	0	
Percentage incidence	**26**	**5/26X100=57%**	**18/26X100=69%**	**0**	**5/26X100=19%**	**2/26X100=8 %**	

4.2.4 Data analysis

Table 4.4. Chi-Square Test

Chi-Square Tests			
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	1.099[a]	3	0.777
Likelihood Ratio	1.114	3	0.774
Linear-by-Linear Association	0.635	1	0.426
N of Valid Cases	91		

A. 0 cells (.0%) have expected count less than 5. the minimum expected count is 5.22.

Chi-square test of association was used to test if there was any species of *Pectobacterium* or *Dickeya* in any of the counties from which the samples were collected and presence of the disease of interest. The null and the alternative hypothesis were as follow:

H_0: There is no Dickeya spp in Kenya.

H_1: Only *Pectobacterium* subsp *carotovorum* and subsp *brasilience* are present in Kenya.

The null hypothesis was tested at 0.05 level of significance and the rule was to reject the null hypothesis if our P value (asymp. sig. 2-sided) is less than 0.05, or accept the null hypothesis if otherwise. From our Chi-Square tests table, our P value is 0.777, which is greater than 0.05. Therefore, we accept the null hypothesis and conclude that there is no *Dickeya* spp.in Kenya and there are other subspecies of *Pectobacterium* spp.

CHAPTER FIVE

DISCUSSION

5.1 Objective 1: Occurrence and distribution of Pectobacterium and Dickeya spp in Kenya using phenotypic methods.

Soft rot and blackleg diseases form one of the most devastating disease complexes of potato in Kenya. It accounts for as much as 1/4 of the annual potato losses in Kenya (E. M. Onkendi *et al*., 2014) . In Kenya *Pectobacterium subsp carotovorum* was first detected from *Ornithogalum arabicum* strain code 1120, studies carried out in Portugal in the year 2006 (Costa *et al*., 2006) and later in potato in 2013-2014(E. M. Onkendi *et al*., 2014) . The disease is characterized by foul-smelling rot and collapse of the potato tubers (Rahman *et al*., 2012).Identification of these pathogens by phenotypic methods is cumbersome and time consuming. Phenotypic methods also have low specificity. For instance, not all pectinolytic bacteria grow or produce pit cavities on CVP medium (Czajkowski *et al*. 2014) a phenomenon that is not fully understood. On the other hand, *Dickeya* spp is the only species in the genus *Erwinia* that is able to produce a water insoluble blue pigment known as indigoidine (Y. Lee & Yu, 2006) which impacts a dark brown to bluish pigment to bacterial colonies and thus can be used as a chemotaxonomic trait for rapid identification of *E.chrysanthemi (Dickeya spp.)* (Y. Lee & Yu, 2006). However, not all *Dickeya spp* do produce a blue pigment on NGM medium(Czajkowski *et al*. 2014), also a phenomenon that is not fully understood. For instance, none of our isolates gave blue pigment on NGM medium, instead they produced dark brown pigments. On biochemical tests according to Dickey, 1978 and Perombelon,2002, pectinolytic bacteria are oxidase negative but may vary on indole and other tests. The *Pectobacterium spp* are indole negative while the *Dickeya spp* are indole positive (Rahman *et al.*, 2012: Dickey 1978). Moreover, *P. atrosepticum* produces pit cavities on CVP medium, does not produce pigment on NGM medium, is positive on catalase tests, reducing substances from sugars but is negative on oxidation and indole tests. On the other hand, *P.wasabiae* produces pit cavities on CVP medium, does not produce pigment on NGM medium, is positive on catalase tests, but is negative on reducing substances

from sugars, oxidation and indole tests. All these detection limitations warrant the need to apply broader detection methods in order to accurately identify the causative agents of the potato disease.

The main objective of this study was to determine occurrence, distribution and genetic diversity of pectinolytic bacteria occurring in potato in Kenya using phenotypic and molecular based methods. As shown by other studies, strains of this bacterium have significant genomic differences but the tests used till now do not reveal a clear-cut relationship between this variability and factors such as geographical origin, sampling season, host species, environmental condition and soil type (Baghaee Ravari *et al*., 2013). Knowledge on the existence of variability in pathogen populations is important for early disease detection (Czajkowski *et al*., 2015). The bacterial strains included in this study came from four different regions of Kenya and were collected in the same season and from seven (Rudolph, Markies, Kenya Mpya, Arizona, Desiree, Kenya Karibu and Shangi) different potato cultivars but Shangi cultivar was the most cultivated in all the four regions. The isolate type did not influence the phenotype and molecular traits. Pathogenicity results were in agreement with previous reports, as variable levels of aggressiveness were observed among isolates recovered in the same season. All isolates tested were confirmed by specific PCR and phenotypic tests as *Pcc,pa,pwa* and *Dickeya spp.*

5.2 Objective 2: Genetic diversity of Pectobacterium and Dickeya spp. by molecular methods.

The results demonstrated that 23% of the isolates were *P.carotovorum subsp carotovorum (Pcc)*, 19% were *P.carotovorum subsp wasabiae(Pwa)*, and 8% were identified as *P.carotovorum subsp atrosepticum(Pca)*. On the other hand, phenotypic results also revealed 46% of pectinolytic bacteria to be *Dickeya spp*.

Although some research have shown clearly the possibility of differentiating between *Pectobacterium* and *Dickeya* spp by phenotypic methods (Dickey, 1978), several studies have shown that phenotypic methods on their own are unable to accurately discriminate soft rot *Erwinia* species particularly those closely related to C*arotovorum* subsp *carotovorum* Pitman,2008). Molecular diagnostic tools such as

species-specific primers have been used successfully to resolve identities of soft rot *Erwinia* that could not be resolved using biochemical and physiological characteristics *(*Pitman, 2008). In this study, there was clear difference between *Pectobacterium* spp and *Dickeya* spp by phenotypic methods. The *Pectobacterium* spp were indole negative while the *Dickeya* spp were indole positive. The results showed that these isolates were pectinolytic hence prompted need to further confirm the results using conventional PCR. Thus, the *pel* gene generic primer SR3F and SR1cR and specific primer pair, EXPCCF/EXPCCR, ECA1f/ECA2r, ADE1/2 and PW7011F/PW7011R were used to further resolve the identities of these isolates. Out of 26 isolates that were positive for pectinolytic bacteria using phenotypic methods, 6 of them were successfully amplified with the EXPCCF/EXPCCR primer pair to produce the expected 550 bp amplicon and were therefore considered positive for *P. carotovorum subsp carotovorum*. 2 other isolates amplified with ECA1f/ECA2r and were considered positive for *P. atrosepticum* to produce 690 bp while 5 strains amplified with PW7011F/PW7011R primers to produce 140 bp hence considered to be positive for *P. wasabiae*. Further, other samples which were positive for *Pectobacterium* spp by molecular methods also turned positive for *Dickeya spp*. using phenotypic methods, a phenomenon which was understood since soft rot and blackleg disease can be caused by more than one species. Examples are the samples from Elgeiyo Marakwet which were positive for *P. wasabiae* using primer pair PW7011F/PW7011R. The identified isolates were able to elicit a form of tissue collapse on potato discs and in potato plants in pathogenicity tests. These results are in keeping with earlier observations that *P. atrosepticum* , *P. Wasabiae* and *Dickeya spp.* are able to cause tissue maceration leading to wilting and blackleg on potato plants and decay on potato tubers thereby causing soft rot and blackleg disease of potato (Rahman *et al.*, 2012). However, genomic DNA from all Kenyan Potato SRE isolates previously identified as *Dickeya spp*. on the basis of physiological and biochemical techniques were specifically amplified using ADE1/ADE2 primers, but could not be identified by PCR using these generic primers. It was not possible to exactly explain the failure of amplification of these isolates other than to consider variation in the pectate genes or non-specificity of the primers. The size of the produced bands were between 1000-1500 bp while the Netherlands isolate (strain

GBBC 322) which was used as the standard produced band of 420bp. Based on the distribution of these pathogens in different regions of Kenya, we decided to evaluate the genetic variation of *Pectobacterium and Dickeya spp* in the country with 16S rDNA sequencing. Phylogenetic analysis (which involved eleven isolates) followed by clustering methods was performed using partial sequences of 16S rDNA for Kenyan pectinolytic isolates and a collection of strains belonging to the genus *Pectobacterium* and *Dickeya*. The topologies of the trees with both neighbours joining and maximum likelihood algorithms were identical, 9 Kenyan isolates grouping on their own as previously indicated by banding patterns using ADE1/ADE2 primer pair while two isolates with the *Dickeya chrysanthemi* strain CMJXGZ-13 16S ribosomal DNA gene. Whereas the position of interspersed repeated sequences in *Enterobacteriales* genomes varies even between different strains, we applied both phenotypic and molecular detection techniques and found a great diversity among *Pectobacterium isolates and Dickeya spp* isolates. Based on this study, the discrimination ability of these detection methods among pectinolytic strains confirmed the results. The generic primers, specific primers and sequence analysis of 16S rDNA showed more diversity than the phenotypic methods. Cluster analysis of the pairwise similarity values of analysed sequences combined using UPGMA, categorized Kenyan isolates in two clusters. Group one isolates were isolated from all the four regions, but showed the same clustering and banding profile. The remaining two isolates from Kenya were Nyandarua and Nakuru and were 97 % identical with *Dickeya chrysanthemi* in the 16S rDNA sequence as shown in figure 4.20 above for Phylogenetic tree done in Mega 6 .Differences among pectinolytic bacterial isolates are most probably due to some insertions and deletions in their genomes, although this needs to be determined and verified(Darrasse *et al*., 1994). Altogether, the results of this study are largely in agreement with other techniques used in other studies (Baghaee Ravari *et al*., 2013). This means phenotypic and molecular detection methods can be applied successfully for the identification of *Pectobacterium and Dickeya spp.* isolated from potato plants and tubers.

CHAPTER SIX

CONCLUSION

6.1 Objective 1: Occurrence and distribution of Pectobacterium and Dickeya spp in Kenya using phenotypic methods.

This study revealed *P.carotovorum subsp carotovorumm(Pcc*), *P.carotovorum subsp wasabiae (Pwa)*, and *P.carotovorum subsp atrosepticum(Pca)* strains causing potato blackleg and soft rot symptoms of potato in Kenya using a combination of phenotypic and molecular methods. *P.carotovorum subsp carotovorumm(Pcc*) was found in four counties :Nyandarua, Nakuru, Narok and Elgeiyo Marakwet. *P.carotovorum subsp atrosepticum (Pca)* was found in Nakuru County while *P.carotovorum subsp wasabiae (Pwa)*, was found in Elgeiyo Marakwet. The isolates remained aggressive on potato stems and tubers as revealed during pathogenicity tests. Survival and virulence mechanisms of these strains warrants further research. From the results obtained in this study, this is the first report of *P. subsp wasabiae (Pwa)*, and *P. atrosepticum* causing blackleg and soft rot on potato in Kenya. *P.carotovorum subsp carotovorumm (Pcc*) was first detected in potato and reported in 2014 in Nyandarua and Mau Narok, and now is detected for the second time in Nyandarua, Nakuru, Narok and Elgeiyo Marakwet.

6.2 Objective 2: Genetic diversity of Pectobacterium and Dickeya spp. by molecular methods.

The *pel* gene generic primer SR3F and SR1cR and specific primer pair, EXPCCF/EXPCCR, ECA1f/ECA2r, ADE1/2 and PW7011F/PW7011R were used to confirm the above phenotypic. Out of 26 isolates that were positive for pectinolytic bacteria using phenotypic methods, 6 of them were successfully amplified with the EXPCCF/EXPCCR primer pair to produce the expected 550 bp amplicon and were therefore considered positive for *P. carotovorum subsp carotovorum*. 2 other isolates amplified with ECA1f/ECA2r and were considered positive for *P. atrosepticum* to produce 690 bp while 5 strains amplified with PW7011F/PW7011R primers to produce 140 bp hence considered to be positive for *P. wasabiae*. Indole positive

isolates (*Dickeya spp*.) identified by phenotypic methods and clustered on their own after phylogenetic analysis were found in all the four counties.

In this study, both objectives on determining the occurrence, distribution and genetic diversity of *Pectobacterium* and *Dickeya* spp. in Kenya by phenotypic and molecular methods were well achieved. Therefore, we accept the null hypothesis and conclude that there is no *Dickeya* spp.in Kenya and that there are other subspecies of *Pectobacterium* spp.

However, there was limitation in finances since the research was partially supported by Kenya Plant Health Inspectorate Service hence not all samples from all the regions could be sequenced. There was also the limitation on time since i was not on study leave and therefore had to use my little time majorly to run the analysis to achieve the goals.

CHAPTER SEVEN

RECOMMEDATION FOR FURTHER WORK

7.1 Recommendation for Further Work

Diverse environmental conditions in Kenya with differences in cropping seasons, provide niches for different bacterial species and subspecies causing blackleg and potato tuber soft rot. Further studies on the origin, diversity and contamination pathways for *Pectobacterium* and *Dickeya* spp may assist in effective control strategies, especially at postharvest steps.

Considering all aspects of this issue, significant effort should be applied for the efficient detection of latent infections in potato tubers in order to provide better understanding of the pectinolytic bacterial population structure in Kenya and can be useful for early disease detection and controls.

The isolates that could not be resolved using ADE1/ADE2 primer pair suggest need for further investigation.

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APPENDICES

Appendix I: List of potato stem and tuber samples collected and used in this study

S. No	Variety	S.part	County	Sub-county	GPS	Symptoms in the field
1	Shangi	Tuber	Nyandarua	Olkalau	Lat;-0.555363 Long;36.5431939 Alt;2422m	Rotting
2	Shangi	Tuber	Nyandarua	Olkalau	Lat;-0.5847892 Long;36.5625572 Alt;2449m	Rotting
3	Shangi	Tuber	Nyandarua	Olkalau	Lat;-0.232915 Long;36.394186 Alt;2403.4m	Rotting
4	Shangi	Stem	Nyandarua	Olkalau	Lat;-0.37681 Long;36.3767 Alt;2403.4m	Wilting,blackening stem from the roots,rotten roots
5	Shangi	Stem	Nyandarua	Olkalau	Lat;-0.355136 Long;36.2441m Alt;2441m	Wilting,blackening stem from the roots,rotten roots
6	Shangi	Tuber	Nyandarua	Olkalau	Lat;-0.2331 Long;36.4039m Alt;2368m	Rotting
7	Shangi	Stem	Nyandarua	Olkalau	Lat;-0.1894 Long;36.2211m Alt;2626m	Wilting,blackening stem from the roots,rotten roots
8	Shangi	Tuber	Nyandarua	Olkalau	Lat;-0.3315 Long;363773m Alt;2322m	rotting

9	Shangi	Tuber	Nyandarua	Olkalau	Lat;-0.37234 Long;36.3572 m Alt;2254m	rotting
10	Shangi	Tuber	Nyandarua	Olkalau	NA	rotting
11	Shangi	Stem	Nyandarua	Olkalau	Lat;-0.2186 Long;36.2351 m Alt;2519m	Wilting,blackening stem from the roots,rotten roots
12	Shangi	Stem	Nyandarua	Olkalau	Lat;-0.232915 Long;36.394186 Alt;2403.4m	Wilting,blackening stem from the roots,rotten roots
13	Shangi	Stem	Nyandarua	Kipipiri	Lat;-0.2744 Long;36.2507 Alt;2571m	Wilting,blackening stem from the roots,rotten roots
14	Shangi	Stem	Nyandarua	Kipipiri	Lat;-0023934 Long;0023.934 Alt;8356m	Wilting, blackening stem from the roots,rotten roots
15	Shangi	Stem	Nyandarua	Kipipiri	Lat;-S 00 25.263 Long;36.35209 Alt;8356m	Wilting,blackening stem from the roots,rotten roots
16	Shangi	Stem	Nyandarua	Kipipiri	Lat;-S 0023.025 Long;36.3208 Alt;8924m	Wilting,blackening stem from the roots,rotten roots
17	Shangi	Stem	Nyandarua	Kipipiri	Lat;-02048.9376 Long;36.265458 Alt;2329m	Wilting,blackening stem from the roots,rotten roots
18	Shangi	Stem	Nyandarua	Kipipiri	Lat;-S00 26.293 Long;36.35954 Alt;9206m	Wilting,blackening stem from the roots,rotten roots
19	Shangi	Stem	Nyand	Kipipir	Lat;-	Wilting,blac

			arua	i	0023.7413 Long;36.32453 Alt;9123m	kening stem from the roots,rotten roots
20	Shangi	Stem	Narok	Narok East	Lat;-1.0616133 Long;36.1179617 Alt;2217m	Wilting,blackening stem from the roots,rotten roots
21	Shangi	Stem	Narok	Narok East	Lat;-1.020365000 Long;36.1372999 Alt;2468.5m	Wilting,blackening stem from the roots,rotten roots
22	Shangi	Stem	Narok	Narok East	Lat;-0.8980917 Long;36.1165617 Alt;2846.6m	Wilting,blackening stem from the roots,rotten roots
23	Shangi	Stem	Narok	Narok East	Lat;-0.911028333 Long;36.129515 Alt;2923.2m	Wilting,blackening stem from the roots,rotten roots
24	Shangi	Stem	Narok	Narok North	Lat;-0.615835 Long;35.84951 Alt;2814.2m	Wilting,blackening stem from the roots,rotten roots
25	Shangi	Tuber/stem	Narok	Narok East	Lat;-0.8980917 Long;36.1165617 Alt;2846.6m	Wilting,blackening stem from the roots,rotten roots
26	Shangi	Stem	Nakuru	Njoro	Lat;-0.6498517 Long;35.9830867 Alt;2865.7m	Wilting,blackening stem from the roots,rotten roots
27	Shangi	Stem	Narok	Narok East	Lat;-0.9289833 Long;36.1231483 Alt;2810.3m	Wilting,blackening stem from the roots,rotten roots
28	Shangi	Stem	Narok	Narok North	Lat;-0.83485 Long;35.89392666 Alt;2470.4m	Wilting,blackening stem from the roots,rotten

						roots
29	Shangi	Stem	Narok	Narok North	Lat;-0.740248333 Long;35.889971666 Alt;2645m	Wilting,blackening stem from the roots,rotten roots
30	Shangi	Stem	Narok	Narok North	Lat;-0.8892667 Long;36.1560817 Alt;2735.5m	Wilting,blackening stem from the roots,rotten roots
31	Shangi	Stem	Narok	Narok East	Lat;-1.0258517 Long;36.1733283 Alt;2202.3m	Wilting,blackening stem from the roots,rotten roots
32	Shangi	Stem	Narok	Narok East	Lat;-0.9942017 Long;36.1248433 Alt;2552.9m	Wilting,blackening stem from the roots,rotten roots
33	Shangi	Stem	Narok	Narok North	Lat;-0.8067883 Long;35.8984.533 Alt;2508m	Wilting,blackening stem from the roots,rotten roots
34	Shangi	Stem	Narok	Narok North	Lat;-0.67008 Long;35.9392383 Alt;2753.6m	Wilting,blackening stem from the roots,rotten roots
35	Shangi	Stem	Narok	Narok North	Lat;-0.9564517 Long;36.1556783 Alt;2565.1m	Wilting,blackening stem from the roots,rotten roots
36	Shangi	Stem	Narok	Narok North	Lat;-0.8637716666 Long;36.16480666 Alt;2856m	Wilting,blackening stem from the roots,rotten roots
37	Shangi	Stem	Narok	Narok North	Lat;-0.6858333 Long;35.874225 Alt;2709.7m	Wilting,blackening stem from the roots,rotten roots
38	Shangi	Stem	Narok	Narok	Lat;-	Wilting,blac

				North	0.86219933 Long;36.1357 Alt;2639m	kening stem from the roots,rotten roots
39	Shangi	Stem	Narok	Narok North	Lat;-0.78829333 Long;35.89051 Alt;2565.3m	Wilting,blackening stem from the roots,rotten roots
40	Shangi	Stem	Nakuru	Njoro	Lat:-0.5127602 Long:35.9831852 Alt:2975m	Wilting,blackening stem from the roots,rotten roots
41	Shangi	Stem	Nakuru	Njoro	Lat:-0.5027603 Long:35.98481 Alt:2555m	Wilting,blackening stem from the roots,rotten roots
42	Shangi	Tuber	Nakuru	Njoro	Lat:-0.4927601 Long:35.96388 Alt:2475m	rotting
43	Shangi	Stem	Nakuru	Njoro	Lat:-0.5227600 Long:35.8848852 Alt:2585m	Wilting,blackening stem from the roots,rotten roots
44	Shangi	Stem	Nakuru	Kabazi	Lat:0.0825616 Long:36.1808183 Alt:2284m	Wilting,blackening stem from the roots,rotten roots
45	Shangi	Stem	Nakuru	Njoro	Lat:-0.5627602 Long:35.9848852 Alt:2575m	Wilting,blackening stem from the roots,rotten roots
46	Shangi	Stem	Nakuru	Kabazi	Lat:0.0825616 Long:36.1808183 Alt:2284m	Wilting,blackening stem from the roots,rotten roots
47	Shangi	Stem	Nakuru	Kuresoi n sirikwa	Lat:0.2750233 Long:35.6620927	Wilting,blackening stem from the roots,rotten

					Alt:2725m	roots
48	Shangi	Stem	Nakuru	Molo-turi	Lat:0.2712989 Long:35.7679348 Alt:2437m	Wilting,blackening stem from the roots,rotten roots
49	Shangi	Stem	Nakuru	Molo	Lat:-0.5627602 Long:35.9848852 Alt:2575m	Wilting,blackening stem from the roots,rotten roots
50	Shangi	Stem	Nakuru	Elburgon	Lat:-0.2846781 Long:35.8355235 Alt:2288m	Wilting,blackening stem from the roots,rotten roots
51	Shangi	Stem	Nakuru	Subukia-arash	Lat:-0.0156357 Long:36.2262652 Alt:2048m	Wilting,blackening stem from the roots,rotten roots
52	Shangi	Stem	Nakuru	Kuresoi n Kamara ward	Lat:0.2470596 Long:35.6697937 Alt:2737m	Wilting,blackening stem from the roots,rotten roots
53	Shangi	Stem	Nakuru	Kuresoi n sirikwa	Lat:0.2848661 Long:35.6947585 Alt:2632m	Wilting,blackening stem from the roots,rotten roots
54	Shangi	Stem	Nakuru	Molo-turi	Lat:0.2712989 Long:35.7679348 Alt:2437m	Wilting,blackening stem from the roots,rotten roots
55	Shangi	Stem	Nakuru	Kuresoi n	Lat:0.240975 Long:35.6842806 Alt:2681m	Wilting,blackening stem from the roots,rotten roots
56	Rudolph,markies,arizona	Tuber	Nakuru	Rongai	Lat:-0.1762004 Long:36.0373496 Alt:1982m	No symptoms observed
57	Rudolph,markies,arizona	Stem	Nakuru	Molo	Lat:-0.1762004	No symptoms

					Long:36.0373496 Alt:1982m	observed
58	Tigoni,shereheke a	Tuber	Nakuru	ADC sirikwa	Lat:-0.3585743 Long:35.6535447 Alt:2737m	rotting
59	Shangi	Tuber	Nakuru	Subikia -kabazi	Lat:-0.092313 Long:36.1935036 Alt:2373m	rotting
60	Shangi	Tuber	Nakuru	Kureso i n-nyakin ywa	Lat:-0.2438833 Long:35.6804632 Alt:2701m	rotting
61	Shangi	Tuber	Nakuru	Kureso i s Mchor we	Lat:-0.3252346 Long:35.6748081 Alt:2724m	rotting
62	Shangi	Tuber	Nakuru	Njoro	Lat:-0.5627602 Long:35.9848852 Alt:2575m	rotting
63	Shangi	Tuber	Nakuru	Molo-Turi	Lat:0.2712989 Long:35.7679348 Alt:2437m	rotting
64	Shangi	Tuber	Nakuru	Rongai	Lat:-0.245052 Long:36.0069443 Alt:1996m	rotting
65	Shangi	Tuber	Nakuru	Kureso i n Nyota	Lat:-0.3511099 Long:35.6584448 Alt:2732m	rotting
66	Shangi	Tuber	Nakuru	Kureso i south	Lat:-0.3511099 Long:35.6584448 Alt:2732m	rotting
67	Shangi	Tuber	Nakuru	Kureso i south	Lat:-0.2910884	rotting

					Long:35.6921248 Alt:2681m	
68	Shangi	Tuber	Nakuru	Kuresoi north-Sirikwa	Lat:0.240975 Long:35.6842806 Alt:2681m	rotting
69	Shangi	Stem	E Marakwet	Keiyo North	Lat:0.7340009 Long:35.4898021 Alt:2235m	Wilting,blackening stem from the roots,rotten roots
70	Shangi	Stem	E Marakwet	Keiyo North	Lat:0.5365277 Long:35.4904184 Alt:2455m	Wilting,blackening stem from the roots,rotten roots
71	Shangi	Stem	E Marakwet	Keiyo North	Lat:0.5365277 Long:35.4904184 Alt:2455m	Wilting,blackening stem from the roots,rotten roots
72	Shangi	Stem	E Marakwet	Keiyo South	Lat:0.397973 Long:35.5533644 Alt:2614m	Wilting,blackening stem from the roots,rotten roots
73	Shangi	Stem	E Marakwet	Keiyo South	Lat:0.518578 Long:35.513327 Alt:2496m	Wilting,blackening stem from the roots,rotten roots
74	Shangi	Stem	E Marakwet	Keiyo South	Lat:0.4056407 Long:35.558556 Alt:22657m	Wilting,blackening stem from the roots,rotten roots
75	Shangi	Stem	E Marakwet	Keiyo South	Lat:0.397973 Long:35.5533644 Alt:2614m	Wilting,blackening stem from the roots,rotten roots
76	Shangi	Stem	E Marakwet	Keiyo South	Lat:0.1198137 Long:35.564483 Alt:2591m	Wilting,blackening stem from the roots,rotten roots
77	Shangi	Tuber	E	Keiyo	Lat:0.119813	Wilting,blac

			Marak wet	South	7 Long:35.564483 Alt:2591m	kening stem from the roots,rotten roots
78	Shangi	Stem	E Marak wet	Keiyo North	Lat:0.7330267 Long:35.5115046 Alt:2292.7m	Wilting,blac kening stem from the roots,rotten roots
79	Shangi	Stem	E Marak wet	Keiyo South	Lat:0.4716784 Long:35.5529462 Alt:2611m	Wilting,blac kening stem from the roots,rotten roots
80	Shangi	Stem	E Marak wet	Keiyo South	Lat:0.1788633 Long:35.6284129 Alt:2622m	Wilting,blac kening stem from the roots,rotten roots
81	Shangi	Stem	E Marak wet	Keiyo South	Lat:0.333124 Long:35.5543193 Alt:2646m	Wilting,blac kening stem from the roots,rotten roots
82	Shangi	Tuber	E Marak wet	Keiyo South	Lat:0.3670454 Long:35.5614402 Alt:2630m	rotting
83	Shangi	Stem	E Marak wet	Keiyo South	Lat:0.3670454 Long:35.5614402 Alt:2630m	Wilting,blac kening stem from the roots,rotten roots
84	Shangi	Stem	E Marak wet	Keiyo North	Lat:0.5351421 Long:35.493941 Alt:2455m	Wilting,blac kening stem from the roots,rotten roots
85	Shangi	Stem	E Marak wet	Keiyo North	Lat:0.5216104 Long:35.491544 Alt:2470m	Wilting,blac kening stem from the roots,rotten roots
86	Shangi	Stem	E Marak wet	Keiyo North	Lat:0.5365277 Long:35.4904184	Wilting,blac kening stem from the roots,rotten

					Alt:2455m	roots
87	Shangi	Tuber	E Marakwet	Keiyo North	Lat:0.7330267 Long:35.5115046 Alt:2292.7m	Rotting
88	Shangi	Tuber	E Marakwet	Keiyo North	Lat:0.7496991 Long:35.4995373 Alt:2249m	Rotting
89	Shangi	Stem	E Marakwet	Keiyo North	Lat:0.5303273 Long:35.508466 Alt:2458m	Wilting,blackening stem from the roots,rotten roots
90	Shangi	Stem	E Marakwet	Keiyo North	Lat:0.5206077 Long:35.4895508 Alt:2474m	Wilting,blackening stem from the roots,rotten roots
91	Tigoni	Stem	E Marakwet	Keiyo South	Lat:0.4637115 Long:35.5583873 Alt:2635m	Wilting,blackening stem from the roots,rotten roots

Appendix II: Media preparation procedures

General media

Phosphate buffer solution used samples homogenisation.

Nutrient agar for purification of the isolated pathogens and storage

Selective media

Crystal Violet Pectate medium(Himedia, 2015)

Nutrient agar with Glyceral and Manganese II chloride tetrahydrate(Czajkowski et al., 2014)

Phosphate buffer solution to release the pathogen

The purpose of this media was to enrich the pathogens from the asymptomatic samples in order to release the bacteria in case it was present so as to detect latent infections.

This media was prepared by dissolving 8.0grams sodium chloride (NaCl),0.2grams of potassium di-hydrogen phosphate(KH_2PO_4),1.15grams of disodium hydrogen phosphate(Na_2HPO_4) and 0.2grams of potassium chloride(KCl) with 1000ml distilled water. Heating while stirring on magnetic plate was done to dissolve the powder. The media was sterilized by autoclaving at 121 °C for 15 min. 500ul of this media was dispensed in individual 1.5 sterile eppendorf tubes under lamina air flow prior to use.

Media preparation for bacterial isolation on Selective media

Crystal Violet Pectate Medium

CVP mono- layer Media-two step

MIX A

Tryptone (Oxoid L42)	1.0g
Tri sodium citrate	5.0g
Sodium nitrite	2.0g
10%w/v Calcium chloride hydrated (Cacl2.H2O)	10.2ml
0.075%Crystal Violet	2.0ml
Bacteriological Agar	4.0g
Distilled water	500ml

MIX B

5M sodium hydroxide	2.5ml
Polygalacturonic acid sodium salt	18.0g
Distilled water	500ml

The above two mediums were prepared separately and were sterilized at 121°C for 15 minutes. The pressure in the autoclave was restored slowly to avoid bubble formation.Mix A was mixed into mix B while still hot and shook gently by rotating the flask.The PH was confirmed at 7.0.The media was then dispensed 18ml per petri dish of 9cm under lamina airflow.The media was allowed to dry for 24hours and then stored at 4°C awaiting analysis.The CVP media was for isolation of all the pectolytic bacteria.

Nutrient Glyceral Manganese Chlorite (NMG) Media

Nutrient agar was supplemented with 1% glycerol that induces pigment production. 2mM MnCl2*4H2O was prepared and added to the solution to further enhance colour development. The media was sterilized at 121°C for 15 minutes. This was then dispensed 18ml per petri dish of 9cm under lamina airflow. The media was allowed to dry for 24hours. and was then stored at 4°C awaiting analysis. This NGM media was for the isolation *of Dickeya* spp.

Requirements for Sample Preparation

1. 70 % (aq., v/v) ethanol.
2. 10 % (aq., v/v) sodium hypochlorite.
3. Disposable scalpel.
4. Universal long Extraction bags (Bioreba).
5. Distilled water and sterilize by autoclaving.
6. Rolling pins for sample homogenization.

Appendix III: Biochemical tests procedures

Oxidative fermentation test

The test was done to differentiate fermentative from oxidative metabolism of carbohydrates**.**

Bacto peptone (Oxoid L37)	2.0g
NACL	5.0
K2HPO4	0.3g
Bromothymol blue	0.03g
Agar	8.0g
Distilled water	1000ml

The ingredients were dissolved and the PH adjusted to 7.0. About 5ml of the media was dispensed in vials and sterilized at 1210c for 15 minutes. The media was then cooled to about 45-50^{o}C and filter sterilized (0.45um filter) 10 % glucose solution added to each tube.The solution was mixed and allowed to cool .A loopful of a 48-hour culture on nutrient agar was stab inoculated in the prepared media with a straight wire inoculating needle .A1cm depth of sterile liquid paraffin oil poured on tubes to induce anaerobic conditions. The samples were incubated at 27 ^{0}C for 48hours. Colour formation resulting from acid production in the tube indicated fermentative bacteria ($_{+/+}$.) Colour formation from the tube without the oil cover (Control) indicated oxidative bacteria (+/-).As controls tube with inoculated medium without glucose and tubes with non inoculated medium and with and without oil overlayer were included.

Oxidation

A loopful of a 48-hour culture on nutrient agar was rubbed on a filter paper impregnated with a drop of 1% (w/v) freshly prepared aqueous tetramethyl-p-phenylediamine dihydrochloride solution.

Development of a purple coloration within 10seconds indicated a positive reaction.

Colour development within 10-60 seconds indicated a delayed reaction. Reaction after 60 seconds indicated negative result.

Catalase test

A loopful of a 48-hour culture on nutrient agar was mixed with a drop of 6% H_2O_2 on a glass slide. Formation of gas bubbles indicated a positive reaction.

Production of reducing substances from sucrose reduction

Media

Bacto peptone (Oxoid L37) 10.0g

NACL 5.0

Sucrose 40g

Distilled water 1000ml

The pH was adjusted to 7.3.and aliquots of 3ml dispensed per test tube then sterilized at 121^{o}C for 15 min.

Benedicts reagent

Sodiun citrate 173.0g

NA_2CO_3.H2O 100g

CuSO4.5H2O 17.3g

Distilled water 1000ml

The first two salts were dissolved by stirring and heating in 800ml distilled water. The salts were filtered through whatman paper no.1 with the aid of a vacuum pump and the filtrate adjusted to 850ml with distilled water. While stirring the 3rd salt was added and when dissolved, the volume was adjusted to 1000ml.The reagent was covered with aluminium foil.

At the time of test, a loopful of a 48-hour culture on nutrient agar was inoculated on the sucrose medium and incubated at 27^{o}Cfor 48hours.

An equal volume of Benedict's reagent was added and heated in a boiling water bath for 10 min.

Production of a yellow-orange/ brown colour (with or without precipitate) indicated a positive reaction. A green colour was considered doubtful and the test needed to be repeated. A negative reaction is when the initial blue coloration remained.

Production of indole.

Test medium:

Tryptone (Oxoid, L42) 10.0 g

L-tryptophan 1.0 g

Distilled water to 1000 ml

pH was adjusted to 7.4, dispense 5 ml per test tube and sterilized at 121 ºC for 15 min

Kovac's reagent*:*

p-dimethylaminobenzalaldehyde 5.0 g

Pure amyl, iso-amyl or butyl alcohol 75 ml

HCl (concentrated) 25 ml

The aldehyde was dissolved in alcohol in a water bath at 50-55 °C, cooled and slowly the acid was added.

Appendix IV: Results of DNA quantification and purity by UV spectrophotometry.

Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
P1	101.3	ng/µl	2.026	1.1	1.84	1.27	DNA	50
P2	242.4	ng/µl	4.847	2.672	1.81	1.62	DNA	50
P3	114.8	ng/µl	2.297	1.262	1.82	1.05	DNA	50
P4	316.4	ng/µl	6.329	3.195	1.98	2.02	DNA	50
P5	371.3	ng/µl	7.425	3.942	1.88	1.64	DNA	50
P6	80.4	ng/µl	1.609	0.85	1.89	1.4	DNA	50
P7	87	ng/µl	1.74	0.973	1.79	1.6	DNA	50
P8	92.3	ng/µl	1.847	1.065	1.73	1.44	DNA	50
P9	179.8	ng/µl	3.596	1.939	1.85	1.57	DNA	50
P10	211.7	ng/µl	4.234	2.298	1.84	1.53	DNA	50
P11	48.4	ng/µl	0.968	0.63	1.54	0.63	DNA	50
P12	160.2	ng/µl	3.205	1.753	1.83	1.3	DNA	50
P13	72.3	ng/µl	1.446	0.81	1.79	1.13	DNA	50

P14	162.7	ng/µl	3.254	2.068	1.57	0.64	DNA	50
P15	120.4	ng/µl	2.408	1.328	1.81	1.18	DNA	50
P16	64.1	ng/µl	1.282	0.728	1.76	1.21	DNA	50
P17	82.3	ng/µl	1.647	0.91	1.81	1.5	DNA	50
P18	64.8	ng/µl	1.296	0.783	1.65	0.76	DNA	50
P19	478.5	ng/µl	9.57	5.17	1.85	1.78	DNA	50
P20	54.9	ng/µl	1.098	0.636	1.73	1.02	DNA	50
P21	80.4	ng/µl	1.608	0.922	1.74	1.1	DNA	50
P22	113.2	ng/µl	2.264	1.306	1.73	0.92	DNA	50
P23	35.8	ng/µl	0.716	0.385	1.86	0.26	DNA	50
P24	42.9	ng/µl	0.858	0.473	1.81	0.19	DNA	50
P25	134.8	ng/µl	2.696	1.416	1.9	0.72	DNA	50
P26	57.6	ng/µl	1.151	0.625	1.84	0.87	DNA	50
P27	84.7	ng/µl	1.693	0.9	1.88	1.21	DNA	50
P28	183.8	ng/µl	3.677	2.35	1.56	0.53	DNA	50
P29	103.1	ng/µl	2.061	1.279	1.61	0.74	DNA	50

P30	59.3	ng/µl	1.186	0.669	1.77	1.15	DNA	50
P31	83	ng/µl	1.66	0.917	1.81	1.14	DNA	50
P32	174.8	ng/µl	3.496	1.87	1.87	1.55	DNA	50
P33	84.7	ng/µl	1.693	0.98	1.73	0.74	DNA	50
P34	121.7	ng/µl	2.434	1.369	1.78	1.02	DNA	50
P35	102.8	ng/µl	2.057	1.188	1.73	1.02	DNA	50
P36	306.8	ng/µl	6.135	3.138	1.95	1.73	DNA	50
P37	103.8	ng/µl	2.077	1.171	1.77	1.1	DNA	50
P38	76.2	ng/µl	1.524	0.814	1.87	1.6	DNA	50
P39	110.1	ng/µl	2.202	1.326	1.66	0.89	DNA	50
P40	137.3	ng/µl	2.746	1.417	1.94	1.11	DNA	50
P41	96.8	ng/µl	1.936	1.117	1.73	1.01	DNA	50
P42	76.6	ng/µl	1.533	0.902	1.7	1.07	DNA	50
P43	66.9	ng/µl	1.338	0.855	1.56	0.66	DNA	50
P44	105.4	ng/µl	2.109	1.219	1.73	1.13	DNA	50
P45	103.9	ng/µl	2.079	1.128	1.84	1.38	DNA	50

P46	101.3	ng/µl	2.025	1.098	1.84	1.39	DNA	50
P47	94.8	ng/µl	1.897	1.035	1.83	1.58	DNA	50
P48	104.1	ng/µl	2.082	1.198	1.74	1	DNA	50
P49	144.7	ng/µl	2.895	1.604	1.8	1.14	DNA	50
P50	59.8	ng/µl	1.195	0.665	1.8	1.18	DNA	50
P51	53.7	ng/µl	1.074	0.625	1.72	1.28	DNA	50
P52	78.1	ng/µl	1.563	0.913	1.71	1.01	DNA	50
P53	76.9	ng/µl	1.539	0.881	1.75	1.19	DNA	50
P54	627.9	ng/µl	12.557	7.128	1.76	0.92	DNA	50
P55	165.1	ng/µl	3.303	1.808	1.83	1.35	DNA	50
P56	126.8	ng/µl	2.536	1.401	1.81	1.05	DNA	50
P57	183	ng/µl	3.661	1.866	1.96	0.71	DNA	50
P58	79.6	ng/µl	1.592	0.955	1.67	1.11	DNA	50
P59	1104.7	ng/µl	22.094	12.926	1.71	0.86	DNA	50
P60	177.1	ng/µl	3.542	1.878	1.89	1.6	DNA	50
P61	6.8	ng/µl	0.136	0.118	1.15	0.55	DNA	50

P62	34.7	ng/µl	0.695	0.438	1.59	1.19	DNA	50
P63	39.6	ng/µl	0.792	0.51	1.55	1.38	DNA	50
P64	103.7	ng/µl	2.074	1.174	1.77	1.39	DNA	50
P65	686.7	ng/µl	13.734	6.875	2	1.12	DNA	50
P66	231.4	ng/µl	4.629	2.332	1.98	0.64	DNA	50
P67	12.5	ng/µl	0.251	0.151	1.66	0.27	DNA	50
P69	164.4	ng/µl	3.287	1.847	1.78	0.49	DNA	50
P70	140.7	ng/µl	2.814	1.728	1.63	0.44	DNA	50
P71	207.9	ng/µl	4.158	2.365	1.76	0.55	DNA	50
P72	158.8	ng/µl	3.177	1.808	1.76	1.03	DNA	50
P73	258.2	ng/µl	5.165	2.638	1.96	0.61	DNA	50
P74	232.5	ng/µl	4.65	2.368	1.96	0.57	DNA	50
P75	233.2	ng/µl	4.663	2.386	1.95	0.57	DNA	50
P76	191.1	ng/µl	3.822	1.899	2.01	0.87	DNA	50
P77	163.3	ng/µl	3.266	1.753	1.86	0.5	DNA	50
P78	259.1	ng/	5.182	2.636	1.97	0.87	DNA	50

		µl						
P79	218.6	ng/µl	4.371	2.201	1.99	1.91	DNA	50
P80	210.1	ng/µl	4.202	2.132	1.97	0.55	DNA	50
P81	82.6	ng/µl	1.651	0.887	1.86	1.74	DNA	50
P82	58.4	ng/µl	1.169	0.551	2.12	0.28	DNA	50
P83	161.1	ng/µl	3.223	1.677	1.92	0.47	DNA	50
P84	428.1	ng/µl	8.562	4.387	1.95	0.98	DNA	50
P86	165.7	ng/µl	3.315	1.784	1.86	0.49	DNA	50
P87	216.8	ng/µl	4.336	2.322	1.87	0.51	DNA	50
P88	182.4	ng/µl	3.648	1.773	2.06	0.49	DNA	50
P89	223.1	ng/µl	4.463	2.487	1.79	0.46	DNA	50
P90	251.9	ng/µl	5.039	2.642	1.91	0.67	DNA	50
P91	185.7	ng/µl	3.714	2.16	1.72	0.48	DNA	50
POSITIVE	172	ng/µl	3.441	2.102	1.64	0.66	DNA	50
NEGATIVE	-0.3	ng/µl	-0.005	0.009	-0.6	0.81	DNA	50

Appendix V: PCR amplification primers and their cycling conditions.

PCR amplification primers(Pritchard et al., 2013)

Target Organism	**Gene target**	**Primer name**	**Primer sequence (5′-3′)**	**Amplicon size (bp)**
Pectobacterium and *Dickeya spp.*	16S rRNA	SR3F SR1cR	GGT GCA AGC GTT AAT CGG AAT G AGA CTC TAG CCT GTC AGT TTT	119
Pectobacterium atrosepticum	Genome	ECA1f ECA2r	CGG CAT CAT AAA AAC ACG GCA CAC TTC ATC CAG CGA	690
P. carotovorum subsp. carotovorum	Genome	EXPCCF EXPCCR	GAA CTT CGC ACC GCC GAC CTT CTA GCC GTA ATT GCC TAC CTG CTT AAG	550
P. wasabiae	YD protein gene	PW7011F PW7011R	CTATGACGCTCGCGGGTTGCT GTT CGGCGGCGTCGTAGT GGAAAGTC	140
P. carotovorum subsp. brasiliensis	*16S-23S rRNA*	*BR1f* *L1r*	*GCG TGC CGG GTT TAT GCA CT CAA* *GGC ATC CAC CGT*	*322*
Dickeya spp	*pectate lyase gene*	*ADE1* *ADE2*	*GAT CAG AAA GCC CGC AGC CAG AT CTG TGG CCG ATC AGG ATG GTT* *TTG TCG TGC*	*420*

PCR cycling conditions (Pritchard et al., 2013)

Target organism	**Step 1**	**Step 2**	**Step 3**
Pectobacterium and Dickeya spp	*94° for 5 min*	*40 cycles: 94 °C for 30 s, 68 °C for 45 s, 72 °C for 45 s*	*72 °C for 7 min*
Pectobacterium atrosepticum	*94° for 5 min*	*36 cycles: 94 °C for 30 s, 62 °C for 45 s, 72 °C for 45 s*	*72 °C for 7 min*
P. carotovorum subsp. carotovorum	*94° for 5 min*	*30 cycles: 94 °C for 60 s, 60 °C for 1 min, 72 °C for 2 min*	*72 °C for 7 min*
"P. carotovorum subsp. brasiliensis"	*94° for 5 min*	*25 cycles: 94 °C for 45 s, 62 °C for 45 s, 72 °C for 90 s*	*72 °C for 7 min*
Dickeya spp	*94° for 5 min*	*25 cycles: 94 °C for 60 s, 72 °C for 2 min*	*72 °C for 7 min*

Appendix VI: Sequences obtained in NCBI after blast search during barcoding of sequenced bacterial DNA

>EF530560.1 *Erwinia chrysanthemi* strain ICMP 4649 16S ribosomal DNA gene, partial sequence

CGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGG
GATAACTACTGGAAACGGTAG
CTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTC
TTGCCATCGGATGTGCCCAGA
TGGGATTAGCTAGTAGGTGGGGTAAAGGCTCACCTAGGCGACGATCCCT
AGCTGGTCTGAGAGGATGACC
AGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAG
TGGGGAATATTGCACAATGGG
CGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTT
GTAAAGCACTTTCAGCGGGGA
GGAAGGGAGCAGGGTTAATAACCCTGTTCATTGACGTTACCCGCAGAAG
AAGCACCGGCTAACTCCGTGC
CAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATGACTGG
GCGTAAAGCGCACGCAGGCGG
TCTGTTAAGTTGGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTC
AAAACTGACAGGCTAGAGTC
TCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGAT
CTGGAGGAATACCGGTGGCGA
AGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGA
GCAAACAGGATTAGATACCCT
GGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTGGTCTTGAACC
GTGGCTTCCGGAGCTAACGC
GTTAAATCGACCCCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATG
AATTGACGGGGGCCCGCACAA
GCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTAC
TCTTGACATCCAGAGAAGCC

TGCAGAGATGCGGGTGTGCCTTCGGGAGCTCTGAGACAGGTGCTGCATG
GCTGTCGTCAGCTCGTGTTGT
GAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGC
CAGCACGTAATGGTGGGAAC
TCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCA
AGTCATCATGGCCCTTACGAG
TAGGGCTACACACGTGCTACAATGGCGTATACAAAGAGAAGCGACCTCG
CGAGAGCAAGCGGACCTCATA
AAGTACGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCG
GAATCGCTAGTAATCGTAGA
TCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTC
ACAGCCATGGGAGTGGGTTG
CAAAAGAAGTAGGTAG

>KX836383.1 *Dickeya chrysanthemi* strain CMJXGZ-13 16S ribosomal DNA gene, partial sequence

ATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAGCACA
AGGGAGCTTGCTCCCTGGGTG
ACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGG
GGATAACTACTGGAAACGGTA
GCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCT
CTTGCCATCGGATGTGCCCAG
ATGGGATTAGCTAGTAGGTGGGGTAAAGGCTCACCTAGGCGACGATCCC
TAGCTGGTCTGAGAGGATGAC
CAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCA
GTGGGGAATATTGCACAATGG
GCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGT
TGTAAAGCACTTTCAGCGGGG
AGGAAGGGGGCAGGCTTAATACGTCTGTTCATTGACGTTACCCGCAGAA
GAAGCACCGGCTAACTCCGTG
CCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATGACTG
GGCGTAAAGCGCACGCAGGCG

GTCTGTTAAGTTGGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATT
CAAAACTGACAGGCTAGAGT
CTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGA
TCTGGAGGAATACCGGTGGCG
AAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGG
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CCAGCACGTAATGGTGGGAA
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GCGAGAGCAAGCGGACCTCAT
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CACACCATGGGAGTGGGTTG
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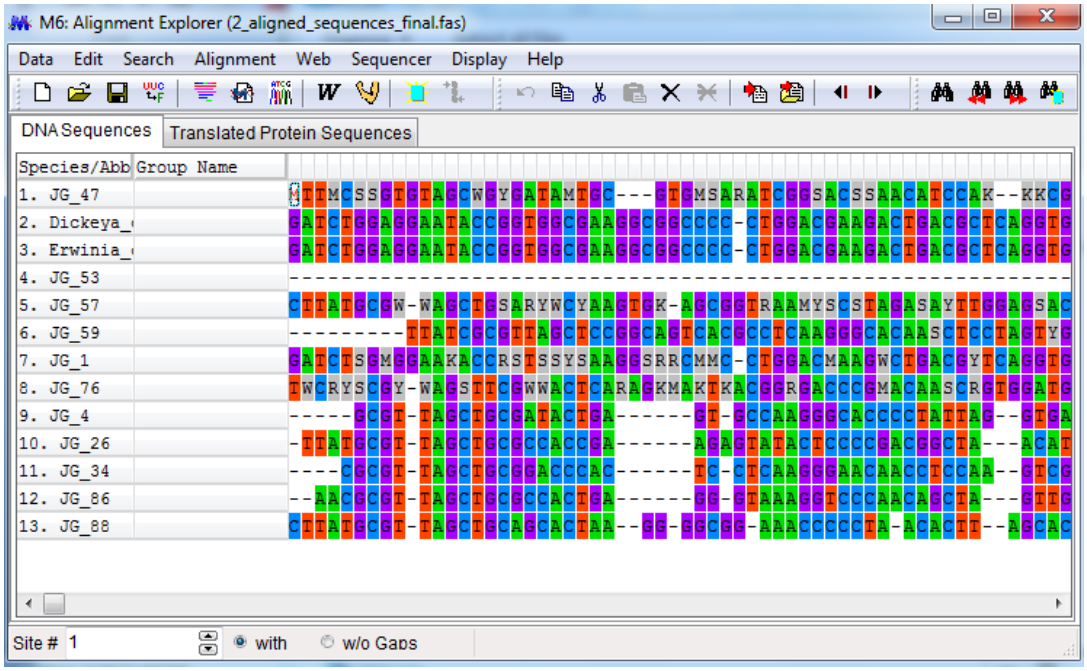
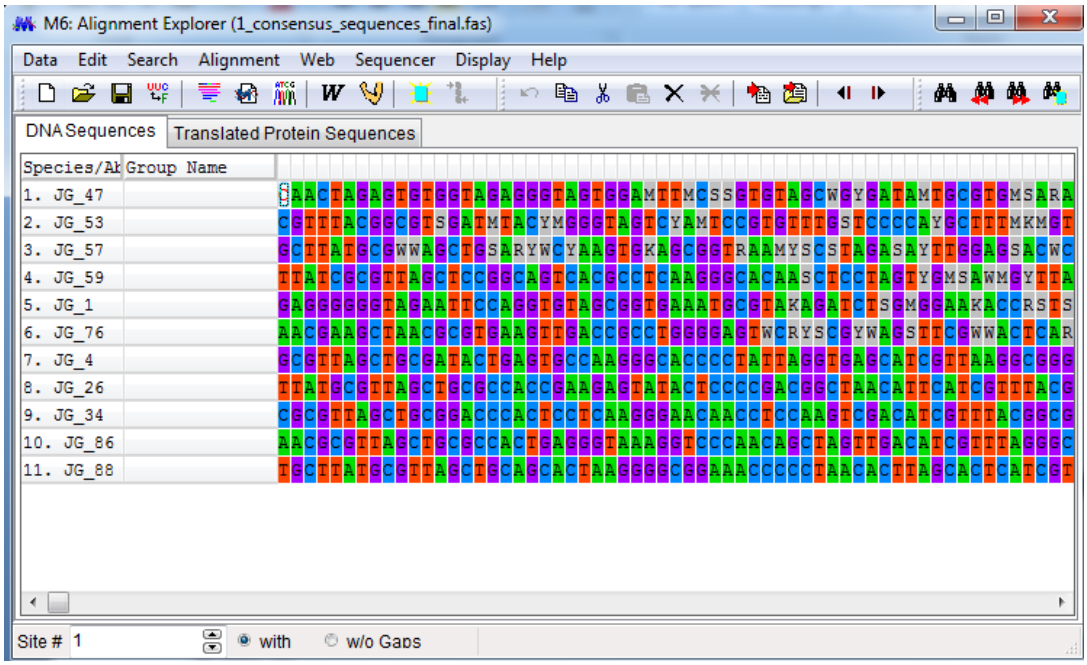
Appendix VII: Multiple sequence alignment in fasta format





Appendix VIII: Blackleg and Soft Rot Sampling Form

Date	**Interview start time**	**Survey Form No.**
GPS Coordinates		**Administrative location**
Latitude		County
Longitude		Sub-County or Constituency
Altitude (m)		Location or Ward
Nearest town or shopping centre		Sub-Location or Village
Interviewee's Name		
Relationship with farm		
Cell Phone number (or other contact)		

Which crops Solanaceous crops are normally grown on this farm?				
Tick in appropriate space	Potato	Tomato	Capsicum	Others (write the name of the specifics crop)
Yes				
No				

If potatoes are normally grown on this farm, state the following:			
Main varieties grown (list names)	Area normally planted (acres)	Source(s) of planting material	Other comments
1.			
2.			
3.			

Does the interviewee do crop rotation in potato fields	Yes (tick) ----	No (tick) ----

If Yes, give length of rotation period (No. of crop seasons)		State crops used in rotation with potato	

Has the interviewee ever seen symptoms/signs Blackleg and soft rot of potato? (show photographs)				
	Yes (tick)	No (tick)	If yes, when were the symptoms or signs seen for the first time?	What effects are attributable to the symptoms or signs
On potato				
On capsicum				
On tomato				
Others (specify)				

	No.	Description of sampled land	Other notes (e.g. photos take, drawing a sketch map of the sampled land overleaf, etc)
Tuber samples taken for blackleg and soft rot disease			
Plant samples taken for blackleg and soft rot disease			

State the number of samples taken			
Interview end time		**Name of interviewer**	