THE USE OF PCR BASED COMMUNITY PROFILING TECHNIQUES TO DETERMINE CHANGE IN COMMUNITY STRUCTURE AND qPCR QUANTIFICATION OF POTATO PATHOGENS IN SOIL UNDER DIFFERENT TEMPERATURE-MOISTURE CONDITIONS OVER TIME

BY

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Dedication

To all the African children

who are malnourished and are

dying of hunger

Abstract

Monitoring of pathogens levels in soil is an important tool for disease management and understanding pathogen ecology. Rapid pathogen detection methods that can quantify the pathogen are desired for routine pathogen detection. *Rhizoctonia solani* (*R. solani*), a soil borne pathogen that has 13 anastomosis groups (AGs) of which AG3-PT is the group frequently associated with disease in potatoes. A qPCR method described by Woodhall *et al.*, (2013) was used to detect the quantity of *R. solani* in soil from a diseased potato field, kept under different moisture-temperature conditions in the greenhouse for 18 weeks. Another problematic pathogen in all the potato growing regions of the world is *Pectobacterium*, and in South Africa *Pectobacterium carotovorum subsp. brasiliensis* (*Pcb*) is the main causal agent for blackleg and tuber soft rot on potatoes. Using primers and probes designed by Woodhall (unpublished), a qPCR assay was designed for *Pcb*, and was used to detect *Pcb* in soil from a diseased field. Results showed that the qPCR protocols for *R. solani* and *Pcb* were able to detect very low quantities of the pathogens in the soil. ANOVA analysis for *R. solani* and *Pcb* showed that there was no statistical difference in the quantities of pathogens detected for the different moisture temperature combinations over time. However, at optimum growth temperatures (20^{o}C) for *R. solani* and *Pcb*, more quantities of the pathogens were detected. Terminal restriction fragment length polymorphism was used to monitor change in microbial community structure for the soil under different moisture temperature combinations over 18 weeks. T-RFLP analysis results showed a dominant OTU of 71 base pairs that was present in all soil samples from the different moisture temperature combinations for all sampling times.

Some OTUs were gained and some lost over time reflecting the role of environmental effect on different bacteria in shaping the community structure.

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Abbreviations

AG	Anastomosis Group
AG3-PT	Anastomosis Group 3 Potato subtype
AG3-TB	Anastomosis Group 3 Tobacco subtype
AG3-TM	Anastomosis Group 3 Tomato subtype
ARDRA	Amplified Ribosomal DNA Restriction Analysis
Ct	Threshold cycle
DGGE	Denaturation Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
FAO	Food and Agricultural Organisation
FRET	Fluorescence resonance energy transfer
IPTG	Isopropylthio-β-galactoside
LB	Luria Bertani
NAMC	National Agricultural Marketing Council
NCBI	National Center for Biotechnology Information
nMDS	non –metric multidimensional scaling

nrDNA	Nuclear ribosomal deoxyribonucleic acid
OTU	Operational taxonomic unit
Pa	*Pectobacterium atrospectica*
Pcb	*Pectobacterium carotovorum* subsp. *brasiliensis*
Pcc	*Pectobacterium carotovorum* subsp. *carotovorum*
PCR	Polymerase chain reaction
Pw	*Pectobacterium wasabie*
qPCR	Quantitative Polymerase chain reaction
RAPD	Randomly amplified polymorphic DNA
rDNA	Ribosomal Deoxyribonucleic acid
RISA	Ribosomal Intergenic spacer analysis
RNA	Ribonucleic acid
SSCP	Single strand
TRF	Terminal restriction fragment
T-RFLP	Terminal Restriction Length polymorphism
Xgal	5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside

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Chapter 1

INTRODUCTION

1.1 Food security

Food security was defined by the World Food summit of 1996 as existing when all people, at all times, have physical and economic access to sufficient, safe and nutritious food to meet their dietary needs and food preferences for an active and healthy life . Sub Saharan Africa region has the highest prevalence of undernourishment with more than one in five people estimated to be undernourished. Food security in the region is threatened by a number of factors which include economic, political, droughts and agricultural under-production. (FAO, IFAD and WFP (2013).

Agriculture plays a critical role in food security worldwide. Land degradation due to increasing population pressure, erosion, and water scarcity are common problems in developing countries (Salami and Popoola, 2007). Reduced land for crop production requires that agriculture be intensified on the available arable land. Food crops that yield more tonnage per hectare of land such as potatoes become favorable in promoting food security (FAO, 2008). However, food crops such as potatoes are under threat of many plant diseases caused by plant pathogens such as bacteria, viruses, nematodes, fungi and parasitic plants (Salami and Popoola, 2007). These reduce productivity which in turn contributes to food insecurity.

1.2 Potatoes

After rice (*Oryza sativa*), maize (*Zea Mays*) and wheat (*Triticum aestivum*), potatoes (*Solanum tuberosum*) rank as the fourth major food crop in the world (Fiers *et al*., 2012); Ngadze *et al*., 2012; Czajkwoski *et al*., 2009; FAO, 2008), when both crop production and cultivated area are considered. Popularity of potato can be attributed to adaptations of this crop to different environmental conditions; as it can be grown in both warm and temperate regions (Czajkwoski *et al*., 2011).

1.2.1 Potato Production in South Africa

Potatoes are one of the most common foods in South Africa (NAMC, 2007) and the major potato producing regions are Limpopo (21%), Sandveld (15%), Western Free State (13%), Eastern Free State (11%), KwaZulu Natal (8%) Mpumalanga (7%) and North West (4%) (Joubert *et al*., 2010), all of them having different climatic conditions. There are a variety of uses for potatoes, which include, for example, French fries, crisps, vegetable relish/salad, canning and livestock feeding (Ngadze et al, 2012).

1.2.2 Nutritional Value of Potatoes

Potatoes are a quick source of more food on less land than any other major crops (FAO 2008). Potatoes have relatively low calories/energy per gram of food and can contribute significant amounts of dietary fiber and micronutrients such as potassium, magnesium, folate, vitamin C, vitamin B6, as well as unsaturated fatty acids, which contribute to lowering of dietary concentration of saturated fat (Gibson and Kurilich, 2013).

There has been an increase in potato consumption and utilization globally, and consumers have also begun to take into consideration the tuber quality in terms of appearance and palatability (Olanya, *et al.*, 2010), hence the need for increased production of high quality potatoes.

1.2.3 Potato pests and diseases

Potatoes are susceptible to over forty pest and diseases caused by a range of viruses, bacteria, nematodes, insects and fungi, these can be transmitted via soil, water, air, seed, contaminated equipment, and vectors (Fiers *et al*, 2012). Some of the most common soil-borne potato diseases include: stem canker and black scurf, caused by *Rhizoctonia solani*; common scab, caused by *Streptomyces scabiei*; powdery scab, caused by *Spongospora subterranean* f. sp. *subterranea*; pink rot caused by *Phytophthora erythroseptica*; and Verticillium wilt caused by *Verticillium dahlia (*Larkin *et al*., 2011). Distribution of these pathogens varies in the different potato growing regions worldwide.

1.2.4 Seed borne potato pathogens

Potato tubers are a source of inoculum during the growing season, and infected tubers will also contaminate the soil. (Tsror *et al*., 1999). The most common tuber borne diseases of potato are; tuber soft rot and blackleg caused by *Pectobacteria* and *Dickeya* species, bacterial wilt caused by *pseudomonas solanacearum* and common scab caused by *streptomyces scabiei* (French *et al*., 1996)

This study focused on *Pectobacterium carotovorum* subsp. *brasiliensis,* a seed-borne bacterial pathogen of potato, which causes soft rot and blackleg in South Africa. The other pathogen investigated in this research was the soil-borne fungus *Rhizoctonia solani*, which is the causal agent of both black scurf and stem canker in most potato growing regions of South Africa.

1.2.5 Potato pathogen detection

In order to actively monitor potato plant diseases, there is a need for rapid diagnostic tests to provide early warnings on the presence and quantity of pathogens in soil and tubers (Crump, 2005; De Boer and López, 2012). PCR technologies have been widely used in routine diagnostics of plant and soil pathogens and qPCR provides both quantification and identification of the inoculum present in samples (Crump, 2005). Other methods, such as terminal restriction length polymorphism (T-RFLP), have also been applied in studying the change in community structure of infected soil over time and different environmental conditions.

Routine quantification of the pathogen community in soil results in better understanding of pathogen ecology. This will lead to development of improved disease control strategies (Crump, 2005) and provide assistance to growers in selection of seed lots and fields to be cropped with potato (Tsror, 2009). Active management of soil microbial communities' allows for development of natural suppression of soil borne diseases (Larkin, 2007).

Monitoring of survival of potato pathogens in the soil in the absence of a host is an important aspect in potato growing. Farmers need to know how long the pathogens survive in the soil under different climatic conditions. This will help farmers to apply relevant control measures before planting their next crop, thus preventing further losses that may arise from infection of new crop.

1.3 Soft rot and Blackleg-causing bacteria

Pectobacterium and *Dickeya* species are the main bacteria causing blackleg of potatoes, which affects the growing plant, and tuber soft rot; as well as soft rot of a variety of other vegetable crops worldwide (Slawiack *et al*., 2013,Czajkowski *et al*., 2011). Blackleg and soft rot are economically important diseases that cause heavy losses to potato crop production, both in the field and during storage. These two microorganisms were previously classified under the genus *Erwinia* and through recent phylogenetic analysis they have been reclassified into two different genera (Nykyri *et al*., 2012).

Pectobacterium and *Dickeya* are opportunistic genera that switch from an asymptomatic latent phase to a virulent phase when environmental conditions are conducive. The virulent phase is induced by anoxic conditions, where oxygen-radical dependent plant defense mechanisms are reduced, thus permitting bacterial multiplication resulting in cell wall degradation (Nykyri *et al*., 2012).

1.3.1 Pectobacterium

Pectobacterium is a Gram negative, non-sporing, facultative anaerobic enterobacterial plant pathogenic genus (Marquez- Villaviencio *et al.,* 2011), which produces cell wall degrading enzymes such as pectinases, polygalacturonase, cellulase (Lee *et al*., 2014), xylanases and proteases (Lee *et al*., 2013). These enzymes produced by *Pectobacterium* degrade cell walls resulting in infiltration and maceration of plant organs and tissues. (Khettani-halabi*, et al.*, 2013; Czajkowski *et al*., 2011).

1.3.1.1 *Pectobacterium* taxonomy

Pectobacterium spp. is a complex taxon consisting of strains with a range of different phenotypic, biochemical, host range and genetic characteristics (Khettani-halabi *et al.*, 2013). *Pectobacterium carotovorum (Pc)*, *Pectobacterium atrosepticum* (*Pa*), *Pectobacterium betavasculorum (Pb)*, and *Pectobacterium wasabiae* are the four major *Pectobacterium* species that have been described to date (Slawiack *et al*., 2013; Kim *et al*., 2009; Marquez-Villavicencio *et al*., 2011). *Pa* has a narrow host range, it infects almost exclusively potatoes (Pitman *et al.*, 2008) in temperate regions where it causes blackleg of stem and tuber soft rot of potato (Panasen *et al.*, 2013). However, some strains of *Pa* have been isolated from sunflower and pepper (Marquez-Villavicencio *et al*., 2011). *Pc* has a broad host range and has been isolated from a wide range of plant species (Marquez-Villavicencio *et al*., 2011) causing soft rot disease to crops such as potato, carrot, capsicum and calla lily (Pitman *et al*., 2008).

1.3.1.2 *Pectobacterium carotovorum*

The species *P. carotovorum* is divided into different subspecies, with *P. carotovorum* subsp. *carotovorum* (*Pcc*) and *P. carotovorum* subsp. *brasiliensis* (*Pcb*) being the most common (De Boer *et al*., 2012). *Pcb* was first described in Brazil (Duarte *et al*., 2004), and later on in South Africa (Van der Merwe et al., 2010) Zimbabwe (Ngadze *et al*., 2012), New Zealand (Panda *et al*., 2012) and Netherlands (Leite *et al*, 2014). 82% of *Pcb* genes are shared with *Pa*, and 84% of the genes are shared with *Pcc* while 13% of the genes are found in neither *Pa* nor *Pcc* (Kim *et al*., 2009). *Pcc* is found worldwide (Kim, et al., 2009), although it is not a major potato blackleg pathogen in temperate zones (Czajkowski *et al*., 2009).

Pcc is associated with stem and tuber soft rot (Marquez-Villavicencio *et al*., 2011; Laurila *et al*., 2008) whilst *Pcb* causes stem rot, tuber soft rot, and blackleg (Marquez-Villavicencio *et al*., 2011). However, *Pcc* is also able to cause blackleg-like symptoms on potato stems, which is often interpreted as stem rot caused by air-borne infection in the stems (P´erombelon, 2002). *Pcb* is the most important causal agent of blackleg in South Africa, and it is widely spread throughout the potato production regions in the country (Van der Merwe *et al*., 2010).

1.3.2 Sources of inoculum of tuber soft rot and blackleg

Infected potato seed tubers are the major source of inoculum, contamination and long distance dispersal of *Pectobacterium* (Anajjar, *et al*., 2014; Czajkowski *et al*., 2011). The major source for blackleg infection is latently infected seed tubers which rot and release bacteria into the soil (Czajkowski *et al*., 2011). Bacteria in soil can colonize potato roots and move via the vascular system into progeny tubers. When the bacteria reach the stems, they can survive in the stems in a latent form (Czajkowski *et al*., 2010).until the bacteria starts multiplying in tuber tissue after overcoming inhibiting factors such as water, nutrient availability and host resistance (P´erombelon, 2002). Soft rot bacteria can be also carried by insects from a diseased plant to a healthy one. *Pcc* and *Pa* are the most common *Pectobacterium* subspecies that have been found in air samples (Czajkowski *et al*., 2011).

1.3.2.1 Symptoms of soft rot and blackleg

During pathogen infection, extracellular pectinolytic enzymes degrade plant structures composed of pectin, cellulose and hemicellulose fibers, leading to plant cell necrosis and tissue maceration (Lee *et al*., 2013).

1.3.3 Blackleg

Figure 1 : Potato stem showing symptoms of blackleg.

Photograph courtesy of Prof Jacquie Van der Waals (Department of Microbiology and Plant Pathology, University of Pretoria).

Under wet conditions, *Pectobacterium* moves out of the xylem and macerates parenchymatous tissues resulting in blackleg symptoms (Czajkowski *et al*., 2011). Blackleg symptoms under wet conditions are characterized by a slimy, wet, black rot lesion that spreads from the rotting mother tuber up the stems (Czajkowski *et al*., 2011). When there is persistent rainfall, extensive stem rot from the top progressing downwards to the base occurs, usually caused by *Pcc* but is often confused with black leg symptoms (Czajkowski *et al*., 2011). Under dry conditions wilting, chlorosis, desiccation, yellowing and stunting of stems and leaves occur (Anajjar *et al*., 2014; Czajkowski *et al*., 2011). Generally, humid conditions favor disease development and temperature plays a major role in determining the dominant pathogen (Pasanen *et al*., 2013).

1.3.3.1 Tuber soft rot

Figure 2: Potato tubers showing symptoms of soft rot.

Photograph courtesy of Prof Jacquie Van der Waals (Department of Microbiology and Plant Pathology, University of Pretoria)

Tuber soft rot is initiated at lenticels, the stolon end and ⁄ or in wounds under wet conditions. During storage bacteria can spread to the rest of the tuber and to neighboring tubers especially when ventilation is inadequate. Maceration of tuber tissues results in a creamy consistency of tuber tissues which turns black in the presence of air, developing an odor after invasion by secondary organisms. Blanking occurs when seed tubers start rotting in the field (Czajkowski *et al*., 2011). Post-harvest and storage tuber rotting is as a result of *Pectobacterium* carried as a latent infection in tubers (Anajjar *et al*., 2014).

1.3.4 Overwintering of *Pectobacterium*

Pectobacterium is not known to overwinter in soil as its survival in soil in the absence of a host is limited to one week to six months. Its survival in soil is dependent on soil environmental conditions such as moisture, temperature, pH (Czajkowski *et al*., 2011) and soil microbial composition ((P´erombelon, 2002). Bacteria such as *Pseudomonas* species have been found to reduce populations of *Pectobacterium* in soil. However, where there are other plants that act as alternative hosts, *Pectobacterium* is capable of overwintering for long periods in soil (Czajkowski *et al*., 2011). Alternative hosts for *Pectobacterium* vary and depend on the subspecies, most common alternative hosts for *Pectobacterium* are weeds.

1.4 *Rhizoctonia solani*

Rhizoctonia solani (teleomorph *Thanatephorus cucumeris*) is a soil-borne plant pathogenic fungus (Das *et al*., 2013; Ritchie *et al*., 2009; Lees *et al*., 2002; Kuninaga *et al*., 1997). *Rhizoctonia solani* has a worldwide distribution and has a wide range of hosts under different environmental conditions (Orozco-Avitia *et al*., 2013) including several important food crop species (Das *et al*., 2013) such as potatoes, sugarbeet, cereals, lettuce and bean (Gonzalez- Garcia *et al*., 2006). It is one of the most economically important pathogens of potato, causing significant production losses as a result of reduced tuber size, number and quality.

The fungus *R. solani* does not produce sexual spores; asexual production of sclerotia and/or vegetative mycelium is its major means of dispersal (Rosewhich *et al*., 1997). Sclerotia are encapsulated, tight hyphal clumps that protect and preserve the fungus over non-optimal times (Lehtonen *et al*., 2008).

1.4.1 Grouping of Rhizoctonia solani

Rhizoctonia solani is a species complex composed of individuals that show differences in morphological, physiological and genetic characteristics. They are grouped according to their compatibility reaction in hyphal anastomosis (Orozco-Avitia *et al*., 2013). Anastomosis is the mechanism of genetic exchange in Rhizoctonia, which occurs by fusion of hyphae of genetically compatible individuals (Budge *et al*., 2009). Based on the hyphal anastomosis, *R. solani* is divided into 13 anastomosis groups (AGs) AG1-AG13, (Das *et a*l., 2013; Miles *et al*., 2013; Wibberg *et al*., 2013; Budge et al., 2009; Ritchie *et al*., 2009; Lehtonen *et al*., 2008). There have been also further divisions of AGs into subgroups, and subgroups into subsets based on culture morphology, pathogenicity, host range, nutritional requirements, biochemical and genetic properties (Das *et al*., 2013). For example AG2 is divided into AG2-1 and AG2-2 (Ferrucho *et al*., 2012).

1.4.1.1 AG3

AG3 is predominantly responsible for infection of potato plants around the world (Miles *et al*., 2013; Ritchie *et al*., 2009), causing black scurf and stem canker (Das *et al*., 2013). AG3 has subgroups AG3-PT for potato, AG3-TM for tomato and AG3-TB for tobacco which are based on the variation in nuclear ribosomal rDNA internal transcribed spacer (ITS) sequences (Das *et al*., 2013; Kunianga *et al*., 2000). Potato Subgroup AG3-PT is frequently associated with disease in potatoes (McCormack *et al*., 2013; Woodhall *et al*., 2013).

AG3 was originally known to cause disease only on potato. However, it has been shown that members of AG3 can also infect aubergine, tomato and tobacco (Das *et al*., 2013). There are also other AGs that have been associated with potato disease in different parts of the world such as AG-2, AG-4, AG-5, AG-7, AG-8 and AG-9d, but they cause less damage than AG-3 (Das *et al*., 2013).

1.4.2 Disease symptoms of black scurf and stem canker

Symptoms of Rhizoctonia disease complex are observed on below and above ground parts of the plant in two phases: infection of growing plants (stem canker) and infection of tubers due to formation of sclerotia (black scurf) (Tsror, 2009).

1.4.2.1 Stem canker

Stem canker symptoms occur early in the season and include necrotic lesions on sprout tips. These lesions may inhibit or delay emergence of shoot, causing poor and uneven stands with weakened plants (Tsror, 2009). Dark brown, dry and usually sunken lesions on the underground stems, stolons and roots are also a characteristic of stem canker which develops prior to shoot emergence (Tsror, 2009; Lees *et al*., 2002).

Brown cankers formed on stem bases girdle the stems and cause stunting. If severe infection is present, small, green aerial tubers may be formed on the stem above the soil

(Tsror, 2009). Tuber-borne inoculum is the predominant cause of the disease (Lees *et al*., 2002) and infection occurs soon after planting, delaying plant emergence (Lehtonen *et al*., 2008). However some studies have shown that soil borne disease is more important in the disease development.

Figure 3: Stem canker on potato stems caused by *Rhizoctonia solani* AG3. (*Adapted from Miles et al., 2013)*

1.4.2.2 Black scurf

The most noticeable sign of *Rhizoctonia* disease is black scurf which occurs later on in the growing season (Lehtonen *et al*., 2008). Black scurf is characterized by the formation of black, irregular sclerotia of different sizes on the tuber. Development of lesions on stolons is as a result of soil-borne inoculum (Lehtonen *et al*., 2008) and stolon infection can influence number and size distribution of tubers (Lees *et al*., 2002). In severe infections, tubers can be deformed and cracked. *R. solani* that develops on progeny tubers results in the tuber blemish disease black scurf, which affects tuber quality and acts as a source of inoculum together with soil inoculum for subsequent crops (Lees *et al*., 2002). Black scurf does not physically harm tubers but reduces their market value due to the blemishes (Das *et al*., 2013).

Figure 4 : Potato tuber with black scurf symptoms.

Photograph courtesy of Prof Jacquie Van der Waals (Department of Microbiology and Plant Pathology, University of Pretoria)

1.4.2.3 *Rhizoctonia solani* inoculum sources

Sources of *R. solani* are seed tubers and soil and these two are capable of harboring mycelium and sclerotia (Ritchie *et al*., 2009). Soil-borne inoculum of *R. solani* is mainly responsible for black scurf whilst tuber borne inoculum is responsible for stem canker (Lehtonen *et al*., 2008). Sclerotia are however, the main form of the fungus responsible for dispersal by wind, water or agricultural practices such as tillage and seed transportation. As a saprophyte, the fungus can be retained in the soil for long periods by obtaining leaking nutrients from actively growing plant cells or decaying plant material (Lehtonen *et al*., 2008).

1.4.2.4 Infection process of *Rhizoctonia solani*

Infection by *R. solani* is initiated by growth of mycelia or hyphae from a germinating sclerotium towards a suitable host as a result of chemotaxis by plant components such as amino acids, sugars, organic acids and phenols. After the first contact, loose and unattached hyphae grow over the plant and directional growth over epidermal cells is initiated. The fungus actively enters the plant by locating weak spots on the surface to break down the protective layer. Swollen hyphal tips are used for penetration of host cuticle and epidermal cells in order to access the epidermal tissues and the outer cortex layer (Lehtonen *et al*., 2008). Hydrostatic pressure is used to achieve penetration with the aid of pectinolytic and cellulolytic enzymes (Wibberg *et al*., 2013). Inside the host, the fungus grows inter- and intracellularly degrading the tissue, most commonly observed as necrotic lesions on epidermal tissue of shoots, roots and stolons or as damping-off of the young seedlings (Lehtonen *et al*., 2008).

1.4.3 Effect of environmental conditions on *Rhizoctonia solani*

Orozco-Avitia *et al*. (2013) were able to show that metabolism of *R. solani* was more efficient between 15˚C and 25˚C despite growth rate being favored between 15˚C and 30˚C. Orozco-Avitia *et al*. (2013) was able to show that metabolic efficiency is reduced at temperatures above 30˚C and that optimum temperature was different for *R. solani* isolates.

1.5 Soil microbial communities

The microbial communities of soils are very diverse and a gram of soil has been estimated to contain up to 10 billion microorganisms and thousands of different species (Delmont *et al*., 2011). Soil microbial communities develop in response to limitations which could be physical, chemical or biological (Chau *et al*., 2011). Water and nutrients have a powerful control on the metabolism and the survival of soil microorganisms. Surfaces of soil environments are exposed to cyclic changes in water content, ranging from water saturation to extreme dryness. Part of the microbial community dies due to inability to adapt to the drying-and-wetting cycles resulting in change of community structure (Daniel 2005).

Soil microorganisms adsorb strongly onto soil particles such as sand grains or clay-organic matter complexes. Microhabitats for soil microorganisms include the surfaces of the soil aggregates, and the complex pore spaces between and inside the aggregates (Daniel, 2005).

Microbial community analysis

Less than 1% of soil microorganisms are considered to be culturable (Delmont *et al*., 2011; Riesenfeld *et al*., 2004) and this is dependent on the reproducibility of their metabolic and physiological requirements *in vitro* (Thomas *et al*., 2012; Rondon *et al*., 2000). Culture methods take several days to give results hence they become undesirable where rapid diagnostic results are required (Thomas *et al*., 2012). Metagenomic approaches have been applied to study a range of soil environments (Delmont *et al*., 2011), and can access 100% of the genetic resources of an environmental sample (Traversi *et al*., 2012). Metagenomics is the direct genetic analysis of collective microbial genomes contained within an environmental sample (Thomas *et al*., 2012; Riesenfeld *et al*., 2004; Rondon *et al*., 2000).

Molecular techniques

Microbial analysis to determine abundance of microorganisms in an environment is achieved by molecular genetics techniques of extracted genomic or ribosomal nucleic acids (Piterina &Pembroke, 2013; Thomas *et al*., 2012). Most molecular methods involve separation of PCR amplicons of taxonomic (16S rDNA for bacteria and 18S rDNA for eukaryotes) or functional genes (Piterina &Pembroke, 2013). These molecular techniques include: denaturing gradient gel electrophoresis (DGGE), ribosomal intergenic spacer analysis (RISA), single-strand conformation polymorphism (SSCP), amplified ribosomal DNA restriction analysis (ARDRA), terminal restriction fragment length polymorphism (T-RFLP) (Thomas *et al*., 2012) and Quantitative PCR (qPCR) (Smith & Osborn, 2009). Molecular methods for microbial community analysis can be divided into two, namely partial community analysis approaches and whole community analysis approaches (Rastogi and Sani, 2011).

1.5.1.1 Partial community analysis

Partial community analyses include polymerase chain reaction (PCR)-based methods, which involve total DNA/RNA extraction from an environmental sample and use of the nucleic acids as templates for the characterization of microorganisms. The PCR products generated reflect a mixture of microbial genes from all organisms present in the sample. PCR products amplified from environmental DNA are analyzed by clone library methods, genetic fingerprinting, DNA microarrays, or by a combination of these techniques (Rastogi and Sani, 2011).

1.5.1.2 Genetic fingerprinting techniques

Genetic fingerprinting techniques include DGGE, SSCP, ARDRA, T-RFLP, and RISA and they produce a community fingerprint based on either sequence polymorphism or length polymorphism. Genetic fingerprinting techniques are rapid and amenable to simultaneous analyses of multiple samples. Fingerprinting techniques demonstrate the effect of environmental pressures on microbial communities or differences between microbial communities but do not provide direct taxonomic identities (Rastogi and Sani, 2011). This study focuses on one partial community fingerprinting techniques T-RFLP to track changes in whole community structure over time and different temperature conditions.

A comprehensive view of genetic diversity of microbial community can be obtained from whole-genome molecular techniques. Analysis of all the genetic information present in total DNA extracted from an environmental sample or pure culture is a major strength of these techniques (Rastogi and Sani, 2011).

16S rRNA gene in microbial community studies

PCR amplification of 16S rRNA which is a conserved gene, from environmental samples has been used extensively in microbial community studies. Characteristics of the 16S rRNA gene such as its presence in all prokaryotes, its conserved structural and functional properties, presence of variable and highly conserved regions along the gene, suitable gene size (~1,500 bp) and the increasing number of 16S rRNA sequences available for comparison in sequence databases have resulted in the wide use of the gene (Rastogi and Sani, 2011).

1.6 Quantitative –Polymerase Chain Reaction (qPCR)

Definition

Real time quantitative Polymerase Chain Reaction) also referred to Quantitative PCR (qPCR) (Postollec *et al*., 2011) is defined as the reliable detection and measurement of products generated during each cycle of the PCR process and these products are directly proportional to the initial amount of template at the start of PCR (Araya *et al*., 2005). It can also be defined as the technique of collecting data throughout the PCR process, therefore combining amplification and detection into one step (Wong *et al*., 2005).

Use of qPCR in microbial ecology studies

qPCR is a molecular technique now widely applied in microbial ecology to determine copy number of a specific taxonomic and functional gene marker present in the DNA extracted from an environmental sample (Smith & Osborn, 2009). It is applicable to both culturable and non-culturable microorganisms (Ahn *et al*., 2013) and can detect dead or viable cells (Postollec *et al*., 2011). It is a highly sensitive and specific method (Attallah *et al*., 2007) for quantitation of PCR products formed during the exponential phase of the reaction (Ahn *et al*., 2013).

Principles of qPCR

q PCR works in essentially the same way as conventional PCR (Smith and Osborn, 2009) but differs in that conventional PCR detects end point amplification whilst qPCR detects amplicons in each cycle. It consists of multiple amplification cycles in which a DNA template is denatured and an oligonucleotide complementary to a specific sequence is annealed to the target, followed by extension of a complementary strand from each annealed primer by a thermostable DNA polymerase such as *Taq polymerase*. This results in an exponential increase in the number of amplicons generated during the PCR. In qPCR, the increase in amplicon numbers is recorded in 'real-time' in every cycle during the PCR by detection of a fluorescent reporter that indicates amplicon accumulation (Smith and Osborn, 2009; Mackay *et al*., 2002).

qPCR reporter systems

Two reporter systems are commonly used in qPCR; the intercalating SYBR green assay and the TaqMan probe system (Smith and Osborn, 2009; Araya *et al*., 2005). Of these two, the TaqMan system is the most popular.

1.6.1.1 TaqMan Probe method

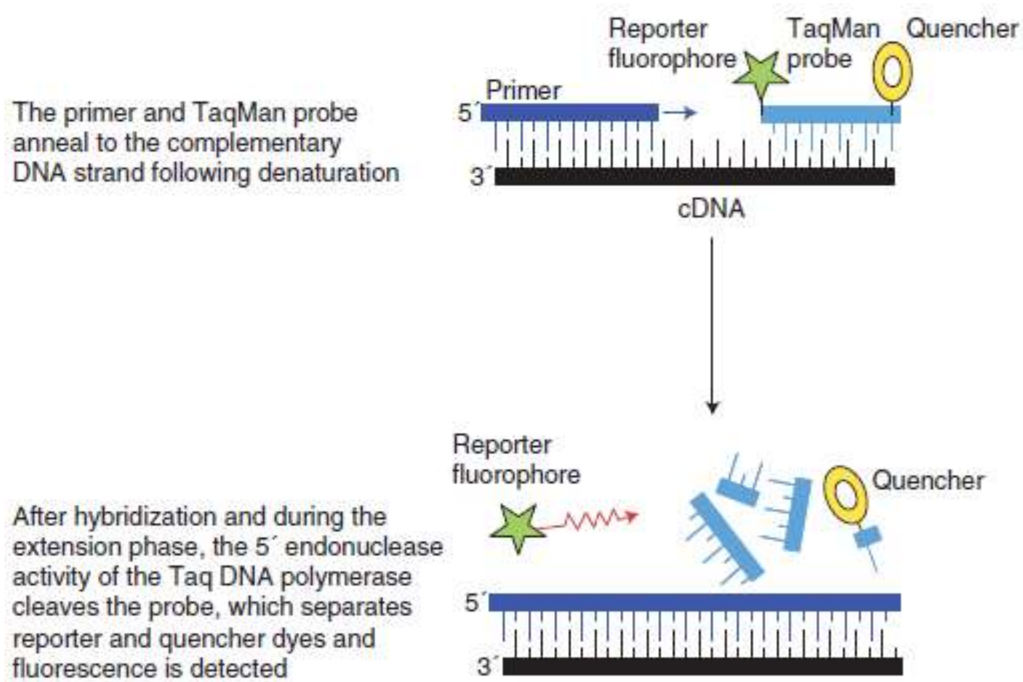


Figure 5: TaqMan Probe assay. Adapted from Arya *et al*., 2005

The TaqMan probe method utilizes a fluorogenic non extendable TaqMan labelled probe that has a fluorescent reporter dye attached to its 5' end and a quencher dye attached to the 3' end (Smith and Osborn, 2009; Araya *et al*., 2005). Close proximity of the quencher molecule to the fluorophore on the probe prevents excitation of the fluorophore by resonance energy (Smith and Osborn, 2009). On an intact probe Fluorescence Resonance Energy Transfer (FRET) occurs and the fluorescence emission of the reporter dye is absorbed by the quencher molecule (Smith and Osborn, 2009; Araya *et al*., 2005; Nadkarni et al., 2002). The probe anneals to the template downstream of the primer, and is cleaved by 5' nuclease activity of Taq polymerase (Araya *et al*., 2005). Cleavage of the probe separates the reporter dye and the quencher dye resulting in fluorescence being detected (Smith and Osborn, 2009; Araya *et al*., 2005; Nadkarni *et al*., 2002). Annealing of probe to template does not interfere with the exponential increase of PCR product, as the Taq polymerase enzyme continues with primer extension after cleavage of probe (Araya *et al*., 2005). Release and accumulation of the fluorophore during the extension stage of each PCR cycle is used to measure template amplification (Smith and Osborn, 2009) thereby allowing rapid detection and quantification of DNA without the need for post-PCR processing, such as gel electrophoresis and radioactive hybridization (Nadkarni *et al*., 2002).

qPCR Phases

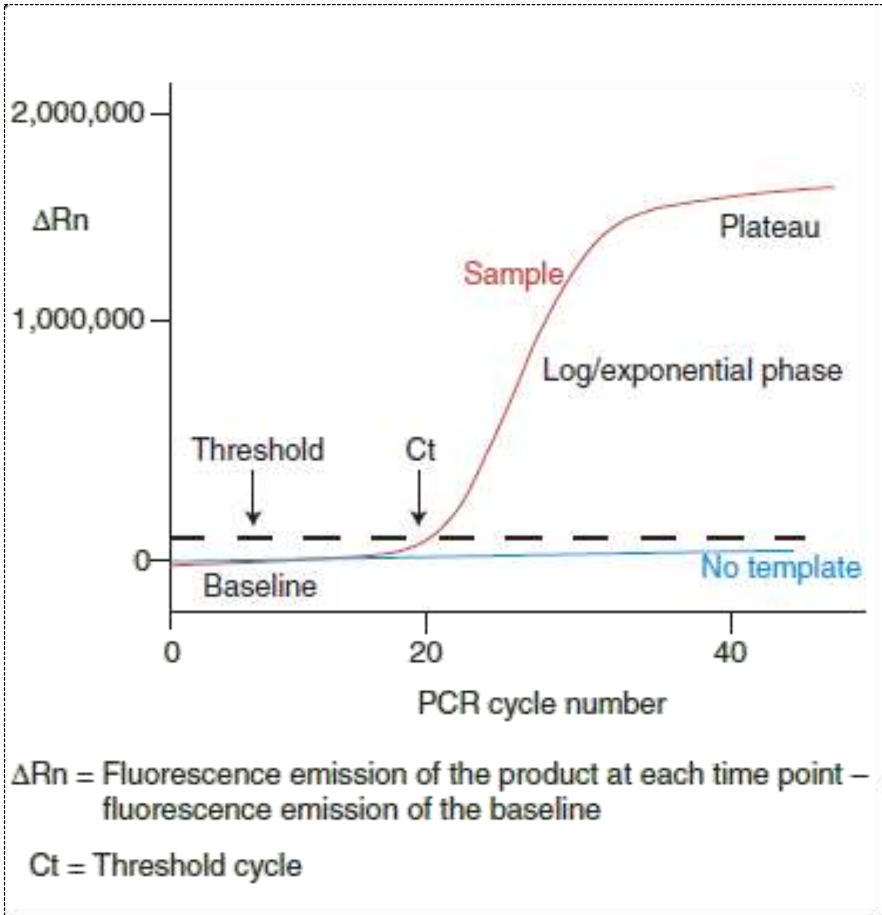


Figure 6 : qPCR Phases as signified by plot of fluorescence emission of the product against PCR cycle number.

qPCR is made up of four major phases: the linear ground phase, early exponential phase, exponential phase, and plateau phase (Wong *et al.,* 2005). During the linear ground phase (usually the first 10–15 cycles), PCR is just beginning, and fluorescence emission at each cycle is still below background level, and baseline fluorescence is calculated at this point. At early exponential phase, the amount of fluorescence reaches a threshold where it is significantly higher (usually 10 times the standard deviation of the baseline) than background levels. The cycle at which this occurs and the product is first detected is known as threshold cycle (Ct). The higher the concentration of target DNA in the starting material, the earlier the product is detected, resulting in a lower Ct (Wong *et al.*, 2005; Guilietti *et al*., 2001). The Ct value is representative of the starting copy number in the original template and is used to calculate experimental results. During exponential phase, PCR reaches its maximum efficiency in amplification with the PCR product doubling after every cycle under ideal reaction conditions. PCR reaches plateau phase when reaction components become limiting and fluorescence can no longer be used for data calculation (Wong *et al.*, 2005).

qPCR quantification

qPCR quantification can either be relative quantification or absolute quantification. Relative quantification uses mathematical equations to calculate relative amounts of target compared to a relative control such as an untreated sample. In absolute quantification, the amount of template in the sample is quantified by using a standard curve to determine initial copy number (Arya *et al*., 2005).

1.6.1.2 Absolute quantification

A standard curve in absolute quantification is generated by five- or ten-fold serial dilution for a sample with known concentration or copy number. Unknown samples have their Ct measured and extrapolated on the standard curve to determine initial copy number. A plasmid containing an insert of the gene of interest is a common source of a known sample (Yun *et al*., 2006). A standard curve is generated using serial dilutions of the plasmid and plotting log Ct against log amount. A synthetic single stranded sense oligonucleotide of known copy number can also be used to generate a standard curve (Yun *et al*., 2006; Arya *et al*., 2005). The latter option is easier to use but expensive.

1.7 Terminal Restriction Fragment length polymorphism (T-RFLP)

Definition

Terminal restriction fragment length polymorphism (T-RFLP) analysis is a high throughput genetic fingerprinting technique used to monitor changes in the structure and composition of microbial community (Schutte *et al.,* 2008).

Applications

The T-RFLP method can be used to analyze communities of bacteria, archaea or fungi (Thies, 2007), to compare and contrast microbial community structure (Kent *et al*., 2003) in different environmental conditions over a period of time (Thies, 2007). The different environments where microbial communities have been studied include soil, water, marine, and lacustrine sediments, biofilms, faeces, in and on plant tissues, and in the digestive tracts of insects and mammals (Thies, 2007). The relative abundance of dominant bacterial species (relatively quantitative) and the pattern shift (in terms of dominant bacterial groups and their relative abundance) of bacterial community structures can be studied and estimated by T-RFLP (Thiyagaranjan *et al*, 2005). T-RFLP method is capable of identifying specific organisms in a microbial community based on their T-RF length and comparing with existing T-RF databases (Kent *et al*., 2003).

T-RFLP Technique

Discrimination of bacterial populations by T-RFLP analysis relies on detecting sequence polymorphisms of 16S rRNA by using restriction enzymes (Liu *et al*. 1997). In bacterial and archeal community comparisons, the most widely amplified DNA targets are the 16S rRNA genes whilst the 18S rRNA genes are less frequently used for characterizing eukaryotic communities, such as fungi. The

18S rRNA genes lack sequence variation across the major fungal taxa that are needed for differentiation thus making the 18S rRNA genes undesirable for fingerprinting in T-RFLP. The internal transcribed spacer (ITS) region or the nuclear ribosomal DNA (nrDNA) is more commonly used for comparisons of fungal community composition (Thies, 2007) because of its hypervariable nature. Enzymes that have four base-pair recognition sites are used as their recognition sites in the sequences have high frequency of occurrence. Use of multiple restriction enzymes increases specificity and the resolution of bacterial communities (Kent *et al*., 2003; Liu et al. 1997).

T-RFLP Procedure

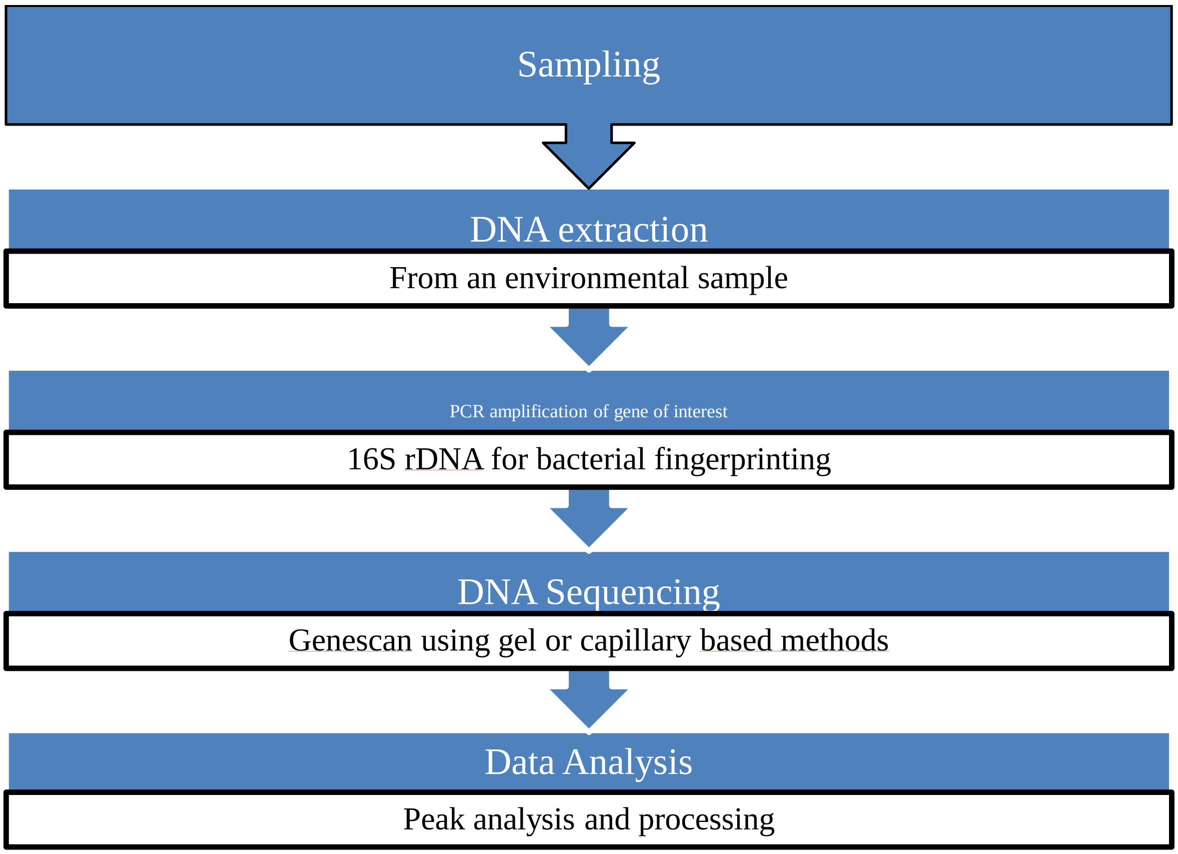


Figure 7: Flow diagram of T-RFLP process from sampling to data analysis (Marsh, 2005)

Extraction of community DNA or RNA from environmental samples is the first step in the T-RFLP analysis. Optimised PCR conditions are used to amplify selected genes from the sample DNA (or cDNA) (Thies, 2007; Thiyagaranjan *et al*, 2005). The PCR reactions use either reverse or forward primers labeled with a fluorophore. Primers labeled at the 5’ end are commonly used since the 5’ end contains hyper variable regions V1, V2 and V3 (Suzuki *et al*., 1998) which provides much more polymorphism than the 3' end. Different fluorophores are available for use, including HEX, FAM, and ROX dye chemistries. Fluorophores for primer end-labeling are chosen based on the fluorophore specifications of the automated sequencer to be used for fragment sizing. Amplification of highly conserved DNA sequences results in PCR products of similar size (Thies, 2007). Separation of the PCR products to obtain a fingerprint is done by hydrolysis by one or more restriction enzymes that have four base pair recognition sites in separate reactions (Schutte *et al*, 2008). Different sizes of labeled terminal restriction fragments (TRFs) from amplified DNA of different organisms result from restriction digest. A fingerprint characteristic of the community under study is obtained by sizing the T-RFs on an automated DNA sequencer using either gel-based or capillary electrophoresis methods (Thies, 2007), which allows precise and accurate size resolution and quantification of T-RF abundance (Waldron *et al*., 2009).

T-RFLP Data Analysis

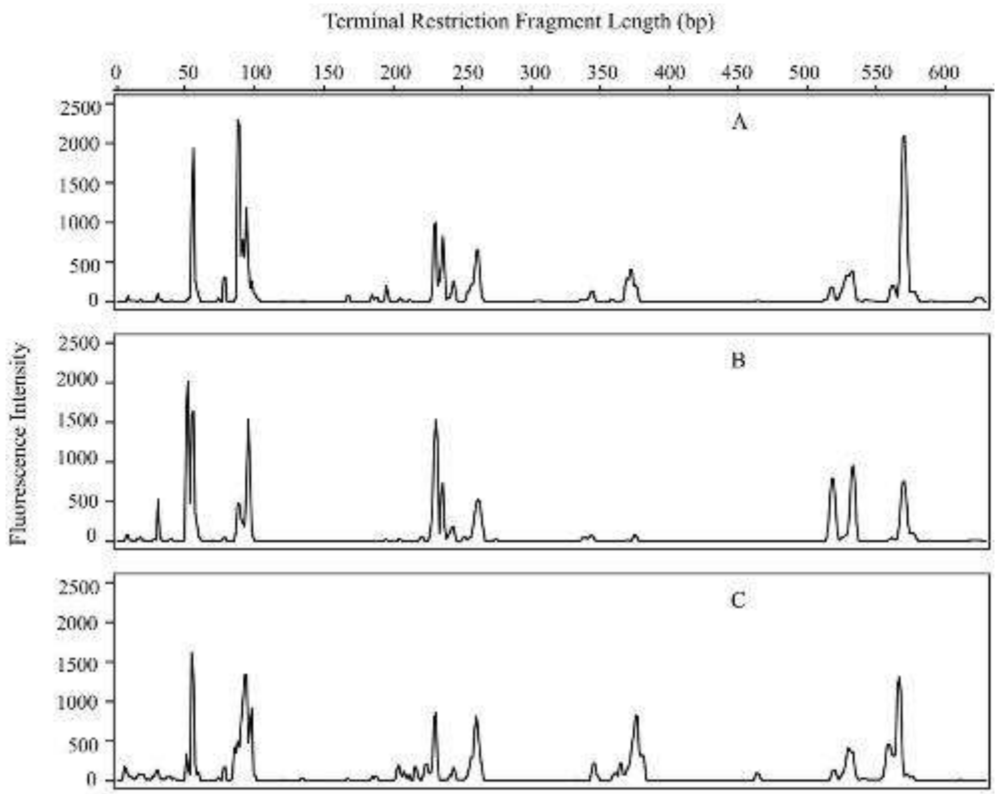


Figure 8: An example of T-RFLP profiles after analysis of fluorescence for samples A, B, C showing peaks and their Fluorescence intensity. Adapted from, Mou *et al*, 2005

1.8 Aims of study

This study aims to develop rapid and effective diagnostic methods for detection of *Rhizoctonia solani* (AG3-PT) and *Pectobacterium carotovorum* subsp. *brasiliense* from soil, as a tool for monitoring levels of pathogenic microbial communities in potato fields. In this study the survival period of the two pathogens in soil in the absence of a host under different temperature and moisture conditions will be determined. The study also monitors changes in microbial community of soil from potato fields over time under different temperature-moisture conditions.

1.9 Specific Objectives

1. To set up pot trials of infested field soil at the following moisture-temperature conditions; constant watering at temperatures 10, 20, 30˚C and Dry at 10, 20, 30˚C. These conditions resemble the different climatic regions of potato production in South Africa.
2. Optimise and test specificity of unpublished *Pcb* primers
3. To quantify *R. solani* AG3-PT and *Pcb* in soil at six-week intervals (six weeks, 12 weeks and 18 weeks after setting up pot trials by qPCR.

4. To determine change in general microbial community structure of the soil using T-RFLP at six week intervals up to eighteen weeks.

Chapter 2

Materials and Methods

1.10 Soil sample collection and setting up of pot trials

In this study, soil was collected from an infested field in which potatoes with symptoms of common scab, soft rot, blackleg and stem canker were recently harvested. Random sampling of the field plot was done to collect soil at approximately 10 cm deep. Pot trials (10 cm pots) with the soil were set up at three different temperatures, 10, 20 and 30˚C with three replicates for each treatment. The soil in each of the temperature treatments was maintained at two different moisture conditions i) dry and ii) wet (constant watering). Since soil was collected during the wet season, soil samples for dry conditions were air dried for 24 hours in order to maintain the physiochemical and microbial properties of the soil as close to the natural environment as possible. The six moisture- temperature treatments represented the different seasons and environmental conditions experienced in the potato growing regions of South Africa. Soil samples were taken at six-week intervals up to eighteen weeks after trial establishment for analysis by Q-PCR and T-RFLP.

1.11 qPCR Primer Design

1.11.1 Rhizoctonia solani

A set of primers and probes described by Woodhall et al. (2013) and designed for amplification of the AG3 -PT internal transcribed sequence ITS region were used for qPCR The sequences were as follows; forward (AG3-PT_F) 5'-ATG AAG AGT TTG GTT GTA GCT GGT CT-3', reverse (AG3-PT_R) 5'-TAT TAC AAW AAA TAA CAA ATA AAT TCC CCA A-3' and probe (AG3-PT_P) 5'-CCC TCT TTC ATC CCA CAC ACA CCT G-3'. Primers were modified by removing the 5' fluorophore label and the probe label was modified to a double quenched probe HEX™: ZEN™/Iowa Black® FQ.

1.11.2 *Pectobacterium carotovorum* subsp. *brasiliense*

Primers and probes for *Pectobacterium carotovorum* subsp. *brasiliense* were designed by Woodhall (unpublished) targeting the ITS and the t-glu gene and the sequences were as follows: forward primer 5'-CCTTACCAAGAAGATGTGTGTTGC-3', reverse 5'-CATAAACCCGGCACGCT-3' and probe 5′-

CAAGCGCACCTGTTGATGTCATGAGTG-3’. The probe was labeled with FAM™: ZEN™/Iowa Black® FQ.

All primers and probes were ordered from Integrated DNA Technologies (IDT, USA).

1.12 Soil sampling and DNA extraction

A total of 0.25g of soil from each pot was used for DNA extraction using ZR soil microbe DNA mini Prep™ kit (Zymo Research). The same kit was used for extraction of DNA from pure cultures for use in positive controls of PCR reactions. DNA was stored at -20˚C for downstream analyses.

1.13 Quantitative detection of pathogens in soil by Q-PCR

1.13.1 *Pcb* primer specificity verification

Pcb 16S rDNA has a few base pair differences with other species and subspecies of *Pectobacterium*, hence the designed primers were verified for specificity. Three other *Pectobacterium* species, *Pectobacterium wasabiae* (*Pw*), *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) and *Pectobacterium atrosepticum* (*Pa*) were amplified using the *Pcb* primers. Increasing annealing temperature (from 54 ˚C to 58˚) increased the specificity of the primers. PCR reactions in final concentrations of 25 µl were set up as follows: Primers (0.02 µM), dNTPS (0.04 mM), Dream Taq buffer with Mg (1X), BSA (0.004%), Dream *Taq polymerase* (1U) (Thermoscientific), 4 µl DNA and double distilled water to 25 µl. The following cycling conditions were used: 95˚C for 3 minutes followed by 30 cycles of 95˚C for 30 seconds, 54˚C for 30 seconds, 72˚C for 45 seconds and final extension of 72˚C for 5minutes with a final hold of 12˚C in a BIORAD T100 thermocycler. Gel electrophoresis in 1.5% agarose at 90 V for 45 minutes was done to confirm the PCR products.

1.13.2 Standard Curve

A standard curve is an important qPCR tool for quantification. To obtain a standard curve, samples of DNA from pure cultures of the pathogens (*Pcb* and *R. solani*) extracted using ZR soil microbe DNA mini Prep™ kit (Zymo Research) were amplified using respective designed qPCR primers. The amplicons were purified and cloned in the pGEMT Easy vector (Promega, USA).

1.13.2.1 Cloning of PCR amplicons

1.13.2.1.1 Conventional PCR

Conventional PCR using qPCR primers was used to generate amplicons for cloning. The PCR reactions for each pathogen were optimized. Primers (0.02 µM), dNTPS (0.04 mM), Dream *Taq* buffer with Mg (1X), BSA (0.004%), Dream *Taq polymerase* (1U), 4 µl DNA and double distilled water to 25 µl were used for the PCR reaction. The following cycling conditions were used: 95˚C for 3 minutes followed by 30 cycles of 95˚C for 30 seconds, 54˚C for 30 seconds, 72˚C for 45 seconds and final extension of 72˚C for 5 minutes with a final hold of 12˚C in a BIORAD T100 thermocycler. Gel electrophoresis in 1.5% agarose at 90 volts for 45 minutes was done to confirm the PCR products.

1.13.2.1.2 Purification

PCR products of *Pcb*, and *R. solani* were purified using Nucleospin® Gel and PCR clean up kit (Macherey-Nagel, France). Briefly, PCR products were topped to 100 µl using double distilled water. Double volume (200 µl) of DNA binding buffer NT1 was mixed with 100 µl of PCR products. The contents were added to the column and centrifuged in an Eppendorf 2020 centrifuge for 30 seconds at 11 000 rpm. The flow through was discarded and 700 µl of wash buffer NT3 was added to the column, centrifuged at 11 000 rpm for 30 seconds, the flow through was discarded and the wash was repeated. Another centrifugation was done at 11 000 rpm for 1 minute to remove excess buffer. DNA was eluted using 30 µl of NE buffer and incubated at room temperature for 5 minutes and centrifuged at 11 000rpm for 1 minute. The cleaned products were quantified and assessed for quality using 260/280 ratios using a NanoDrop® ND-2000 UV/Vis Spectrophotometer (NanoDrop Technologies, USA) and stored at -20˚C.

1.13.2.1.3 Electro-competent cells

Electro-competent cells were made in sterile and cold conditions. JM109 pre-inoculum cells were grown overnight in LB broth at 37˚C in a shaking incubator (Stuart Orbital SI500, UK). The cells were re-inoculated in fresh LB (35 ml) and grown until an optical density of 0.5 (at 600 nm) was reached. The cells were then preserved on ice and pelleted at 5000rpm for 5 min in a bench top Eppendorf 2020 centrifuge and the supernatant was discarded. The cells were washed in 3.5 ml of 10% sterile cold glycerol. Cells were pelleted, the supernatant discarded and the washing was done twice. 350 µl of 10% glycerol was used to re-suspend the cells and aliquots of 50 µl were transferred to a clean sterile 1.5 ml Eppendorf tubes and stored at -80˚C for further use.

1.13.2.1.4 Ligation into pGEM-T Easy Vector

A pGEM-T Easy vector was used to clone PCR products. pGEM-T Easy vector and control insert DNA tubes were briefly centrifuged to collect contents to the bottom. Ligation reactions were set up for the standard reaction, positive control and background control. Each reaction contained 2X Rapid Ligation buffer T4 DNA ligase;

50ng pGEM- µl T Easy vector; 3neiss/µl DNA Ligase and Deionised water to a total volume of 10 µl. For the standard reaction 6 ng of DNA was added, and 6ng of control insert was added to the positive control reaction. Deionised water was used for the background control. DNA of purified PCR products was diluted to 6 ng per µl. The amount of insert DNA needed for the ligation reaction at an insert: vector Molar ratio of 5:3 was calculated using the following formula as described by manufacturer:

$$\frac{\text{ng of vector X *kb of insert}}{\text{kb of vector}} \text{ X insert : vector molar ratio}$$

$$= \frac{\text{50ng X kb of insert}}{\text{3kb}} \text{ X } \frac{5}{3}$$

=ng of insert DNA

*inserts of *R. solani* and *Pcb* were 122 and 120 respectively hence 6 ng of each was used in the ligation reactions.

Ligation was carried out overnight at 16 ˚C in a BIORAD T100 thermocycler and verified by gel electrophoresis by running 4 µl of ligation mixture in 1% agarose at 90 V for 45 minutes.

1.13.2.1.5 Transformation

LB Agar plates containing 100 µg/ml ampicillin, 100 mM IPTG and 50 mg/ml Xgal were prepared in duplicate for each ligation reaction. The plates were equilibrated at room temperature prior to use. Tubes containing ligation reactions were centrifuged briefly to collect contents at the bottom. 2 µl of each ligation mixture was added to sterile 1.5 ml micro centrifuge tubes on ice. Tubes of frozen electro-competent JM109 cells were thawed on ice for 5 minutes and mixed by gently flicking the tube. 50 µl of the cells were transferred to the 2 µl of ligation reactions and the tubes were flicked to mix. 52 µl of the transformation mixture was transferred to ice cold electroporation cuvette and 1.6 V of electrical pulse was delivered to the chamber. Once the pulse was delivered, 950 µl of SOC medium was added to the electroporation cuvette. The cells were transferred to 15 ml Falcon tubes and incubated in a shaking incubator (Stuart Orbital Incubator SI500) at 37˚C for 1 hour 30 minutes. 100 µl of the transformation culture was plated onto duplicate LB Agar/Ampicillin/IPTG/Xgal plates. Plates were left at room temperature until the liquid had been absorbed and incubated at 37˚C overnight in a static incubator. Colony PCR was done on the white colonies using specific primers for the pathogen (AG3-PT for *R. solani* and *Pcb* primers for *Pcb*).

1.13.2.2 Colony PCR

Six colonies for each of the pathogens (*Pcb* and *R. solani*) were selected using a sterile toothpick which was used to grow the colonies onto new Ampicillin-LB-Agar Plates and dipped in PCR tube containing: primers (Pcb/ AG3-PT) forward and reverse (0.02 µM) each, dNTPS (0.04 mM), Dream Taq buffer with Mg (0.02 mM) (1X), BSA (0.004%), Dream *Taq polymerase* (1U) and double distilled water to 25 µl. The following cycling conditions were used: 95˚C for 3 minutes followed by 30 cycles of 95˚C for 30 seconds, 54˚C for 30 seconds, 72˚C for 45 seconds and final extension of 72˚C for 5 minutes with a final hold of 12˚C in a BIORAD T100 thermocycler. Gel electrophoresis in 1.5% agarose at 90 volts for 45 minutes was done to confirm amplifications. Cleaned PCR products used for cloning were used as the positive control in gel electrophoresis. Ampicillin-LB-Agar plates were incubated at 37˚C overnight in a Stuart Incubator.

1.13.2.3 Plasmid Mini Prep

The confirmed colonies were grown overnight in Ampicillin-LB broth at 37˚C in a shaking Stuart Orbital Incubator SI500. The cultures were pelleted by centrifugation at 8000 rpm for 3 minutes at room temperature. QIAprep® Spin Miniprep Kit (Qiagen, Netherlands) was used to extract the plasmids. Pelleted bacterial cells were re-suspended in 250 µl of Buffer P1 and transferred to a micro-centrifuge tube. 250 µl of Buffer P2 was added and mixing was done by inverting the tube 6 times until the solution was clear. 350 µl Buffer N3 was added to the reaction and mixing was done immediately by inverting the tube six times. The tubes were centrifuged for 10 minutes at 13 000 rpm in a bench top Eppendorf 2020 micro-centrifuge. The resulting supernatant was added to QIAprep spin column by pipetting. The columns were centrifuged at 13 000 rpm for 60 seconds and the flow through was discarded. 500 µl of Buffer PB was used to wash the QIAprep column, centrifugation at 13 000 rpm was done and flow through was discarded. 750 µl of Buffer PE was used to wash the QIAprep spin column, centrifugation was done at 13 000 rpm for 60 seconds and the flow through was discarded. To dry the column and remove residual buffer, QIAprep column was centrifuged at 13 000 rpm for 1 minute. QIAprep column was placed in a clean 1.5 ml micro-centrifuge tube and DNA was eluted with Nuclease free sterile water and centrifuged at 13 000 rpm for 1 minute.

The DNA was stored at -20˚C until needed.

1.13.2.4 Sanger Sequencing of the plasmid

1.13.2.4.1 Sequencing PCR

Sequencing PCR reaction was carried out with the following conditions, 2.4 µl sequencing buffer, 0.5 µl Big Dye, 4 µl purified DNA product (plasmid mini prep), 1 µl M13F (10 µM) forward primer (for forward reaction tube) and 1 µl M13R (10 µM) Reverse primer (for reverse reaction tube) and nuclease free sterile water to 12 µl. The cycling conditions were as follows; 94˚C for 5seconds, 25 cycles of 94˚C for 10 seconds, 54˚C for 10 seconds and 60˚C for 4 minutes and a final hold of 4˚C.

1.13.2.4.2 Sodium acetate precipitation

12 µl of sequencing reaction was added to 16µl of 100% ice cold ethanol in a 600 µl sequencing Eppendorf tube and 2 µl of sodium acetate (3M) was added to the mixture. The resulting mixture was centrifuged at 14 000 rpm for 30 minutes. Without touching the sides of the Eppendorf tube, the supernatant was pipetted and discarded. 150 µl of 70% ice cold ethanol was added to the tube and centrifuged at 14 000 rpm for 5 minutes. The supernatant was pipetted off and discarded. Washing with 70% ice cold ethanol was done twice. The sequencing Eppendorf was heated on a heating block at 90˚C for 3 minutes with the lid open to remove excess liquid. Eppendorf tubes were stored at -20˚C until they were sent to the sequencing facility at ACGT, University of Pretoria.

1.13.2.4.3 BLAST searches

The plasmid + insert sequence obtained from the sequencing were blasted in NCBI nucleotide blast software engine and the positions of the inserts were located. These were also confirmed by blasting the insert sequences only in NCBI nucleotide blast software engine to confirm the specific sequences.

1.13.3 Plasmid Digestion

Using NEbcutter V.2.0 (BioLabs), the vector restriction sites were analysed, and restriction enzymes that cut the vector sequence once were selected. From this list, a restriction enzyme that did not cut the sequences of the inserts was selected and used to linearise the plasmid. 2 µl of fast digest NCoI restriction enzyme (Thermoscientific, USA), 2 µl of 10x fast digestion buffer, 1µg of plasmid and double distilled water to 20 µl were added in a PCR tube. The reaction mixture was incubated in a BIORAD T100 Thermocycler for 15 minutes at 37˚C. Digested products were run on 1% agarose gel in for 40 minutes at 90 V together with undigested product. The digested products were cleaned with PCR Nucleospin® Gel and PCR clean up kit (Macherey-Nagel, USA) and the products were quantified using Thermoscientific Nanodrop 2000 and stored at -20˚C until needed.

1.13.4 qPCR

Eight serial dilutions of the purified plasmids were done from 1/10 to 1/100 000 000.

qPCR was performed in a PikoReal 96 thermocycler (Thermoscientific) and the 10 µl reaction consisted of 300 nM each of the primers (reverse and forward primers); 200 nM of probe; 75 ng of genomic DNA template; 2X Maxima Probe qPCR Master Mix

(Thermoscientific). All samples and the standards were run in duplicate in a white 96 well plate which was sealed with a transparent adhesive seal. Cycling conditions were 50˚C for 2 minutes, 95˚C for 10 minutes and 40 cycles of 95˚C for 15 seconds and 60˚C for 1 minute. Data analysis was done during the extension step and melting curve analysis was done from 60˚C to 95˚C. Standards were included with each run, and the obtained standard curve was used to quantify the pathogens in the samples.

1.14 T-RFLP

1.14.1 PCR Amplification

PCR reactions were carried out using the following reagents (25 µl final volume): BSA 0.2 mg/ml, dNTPs 0.1 mM, forward primer e9F and reverse primer u1510 (0.5 µM). Dream Taq Buffer with 0.02 Mm Mg 1X, Dream Taq 2U (Thermoscientific), 6 µl of DNA and double distilled water was used top up to 25 µl final volume. PCR reactions were carried out in BIORAD T100 Thermocycler using the following conditions 95˚C for 5 minutes, and 30 cycles of 95˚C for 30 seconds, 55˚C for 30 seconds, 72˚C for 90 seconds and final extension at 72˚C for 10 minutes and a final hold of 16˚C. PCR products were run in 1% agarose at 90 V for 45 minutes to check for amplification.

A nested PCR was performed using primers pair 341F (FAM labeled) and 907RSamples were run in duplicate. Reagent final concentrations were as per first PCR reaction as well as equipment and cycling conditions, except the annealing time was extended to 1minute. PCR reactions were carried out in a final volume of 50 µl.Duplicate PCR products were pooled and an aliquot run in 1% agarose gel at 90 V for 45 minutes to check for amplification. Samples with Amplicons showing the expected size (approximately 600 bp) were purified.

1.14.2 Purification of PCR products

Nucleospin® Gel and PCR clean up kit (Macherey-Nagel, France) was used to clean up the PCR products. PCR products were topped to 100 µl using double distilled water. Double volume (200 µl) of DNA binding buffer NT1 was mixed with 100 µl of PCR products. The contents were added to Nucleospin gel and PCR clean-up column and centrifuged in Ependorf 2020 centrifuge for 30 seconds at 11 000 rpm. The flow through was discarded, 700µl of wash buffer NT3 was added, centrifuged at 11 000 rpm for 30 seconds and the flow through was discarded and the wash was repeated. Another centrifugation was done at 11 000 rpm for 1 minute to remove excess buffer. DNA was eluted using 20 µl of NE buffer and incubated at room temperature for 5 minutes and centrifuged at 11 000 rpm for 1 minute.

1.14.3 Quantification

The cleaned PCR products were quantified and assessed for quality at 260/280 ratio using Thermoscientific Nanodrop 2000 and readings were taken in duplicate and average value obtained.

1.14.4 Restriction digestion

Restriction digestion was done with *Hae*III in a reaction mix of 20 µl. The reaction mix consisted of 1 µl of enzyme, 2 µl of restriction digest buffer, 200 ng of DNA and was made up to 20 µl with double distilled nuclease free water. The digestion mixture was incubated at 37˚C in a BIORAD T100 thermocycler overnight with lid temperature of 40˚C.

1.14.5 Purification of digestion products

Nucleospin® Gel and PCR clean up kit (Macherey-Nagel, France) was used for clean-up. The volume of the digestion mix was adjusted to 50 µl using double distilled water. 100 µl of NT1 buffer was added to the digested products, the mixture was added to Nucleospin column and centrifuged at 11 000 rpm for 30 seconds. 700 µl of wash buffer NT3 was added and centrifuged at 11 000 rpm for 30 seconds. This washing step was repeated and the columns were dried by removing excess wash buffer by centrifugation at 11 000 rpm for 1 minute. DNA was eluted with 15 µl of NE (EB) elution buffer, incubated at room temperature for 5 minutes and centrifuged at 11 000rpm for 1 minute.

1.14.6 ABI Electrophoresis

The cleaned restriction digest product (4 µl) DNA was mixed with 0.25 µl LIZ600 size standard and 6.75 µl of formamide in a 96 well plate. The plate was covered with plastic film and incubated in a BIORAD T100 thermocycler at 95˚C for 5 minutes to denature the DNA. Spinning of the plate was done in a centrifuge to remove bubbles and submitted to the African Center for Gene Technologies (ACGT) for gene scanning.

1.14.7 Data Analysis

Terminal restriction fragments (T-RFs) generated by Gene mapper software v4.0.1 (Applied Biosystems) were filtered and binned by the method developed by Abdo *et al*. (2006) Statistical analyses were performed using Paleontological Statistics 3 (PAST3) software package (http://folk.uio.no/ohammer/past/).

Chapter 3

3.0 Results and discussion

3.1 T-RFLP

T-RFLP is a fingerprinting technique for assessing change in microbial community structure on temporal and spatial scales through monitoring of gain/loss of specific labeled terminal fragments from the profiles obtained (Schutte *et al*., 2008).

3.1.1 DNA Extraction

Extraction of nucleic acids from an environmental sample is usually the first step in molecular microbial analysis and for T-RFLP analysis DNA is the nucleic acid of choice (Otlewska *et al*., 2014). DNA extraction in this study was done using the Zymo DNA extraction kit and yielded low DNA quantities. Nanodrop measurements of the extracted DNA averaged 23ng/µl and the extracted genomic DNA could not be viewed in 1 % agarose when gel electrophoresis was performed. However, amplification of the extracts in PCR reactions with e9f/u1510 bacterial 16S rDNA generic primers produced expected bands of 1500 bp in 1% agarose gels, confirming the presence of bacterial DNA in the soil.

Low DNA yields can be investigated by carrying out experiments using the same DNA extraction protocol and soil from different environments and comparing the DNA yield, and use of different extraction protocols for the soil of interest and comparing the DNA yields. In cases where high throughput routine testing is done, the Kingfisher method based on the automated magnetic processor has been found to be effective for DNA extraction of high quality and purity of DNA (Budge *et al*., 2009).

In this study, DNA extraction was done using a bead-beating matrix which is a moderate DNA extraction method. The DNA extraction method used also affects the microbial diversity of the community; harsh cell lysis methods increase the abundance of gram positive bacteria but results in shearing of DNA from gram negative bacteria resulting in bias of the T-RFs sizes (Thomas *et al*., 2012). The final step of DNA extraction involved a purification step aimed at removing contaminants such as humic acids by filtering through a silica matrix. DNA extracted from soil is mostly co-extracted with contaminants such as humic acids, phenolic compounds (Daniel, 2005) and fulvic acids (Schneegurt *et al*., 2003). Humic acids act as inhibitors of PCR and inhibit the enzymatic action of DNA *Taq Polymerase* (Thomas *et al*., 2012) and restriction enzyme digestion (Daniel, 2005).

3.1.2 PCR

PCR is the second stage of T-RFLP analysis and requires optimization for the sample type and origin. PCR with Primer set 341/908 (Ishii and Fukui, 2001) produced little amounts of the PCR product which had faint bands when viewed in 1% agarose gel as shown in **Figure 9**. After quantification of DNA using Nanodrop, results s howed that average DNA quantity was 11ng/µl. These quantities were inadequate to carry out restriction digest. Optimisation and troubleshooting resulted in the use of nested PCR with primer set e9F/u1510 (Smith *et al*., 2006) as the outer primers and 341F/908 as the internal primers yielded a positive result. The gel electrophoresis for the final PCR with 341/908 is shown in **Figure 10**. Use of one set of fluorescently labeled primers may result in the underestimation of microbial diversity in a sample. Different bacterial populations can share the same terminal restriction fragment length for a set of primer/enzyme combination hence produce the same TRFs. Using two fluorescently labeled primers increases the resolution of community diversity and use of three primers further increases the resolution although it is expensive (Osborne, 2000).

PCR is a source of bias in T-RFLP analysis; to minimize PCR bias 25 cycles of PCR were done as suggested by Suzuki and Giovannoni (1996). Variation resulting from pipetting was reduced by preparing master mixes for reagents and enzymes used for PCRs and restriction digestions.

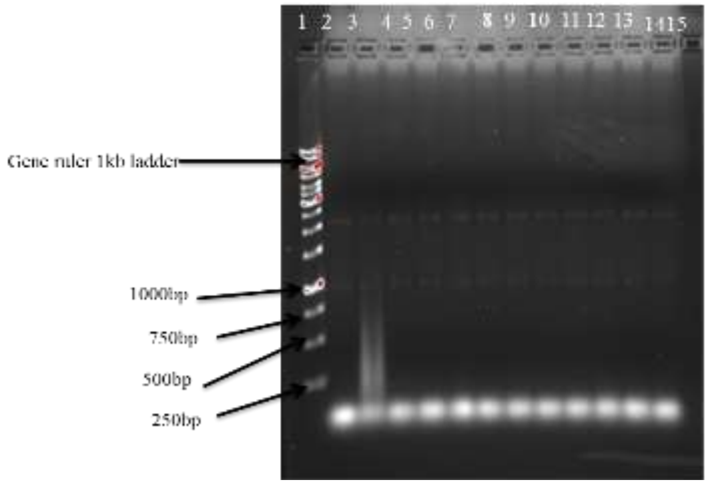


Figure 9: Gel electrophoresis for PCR products amplified using 341/908 primer set. Lane 1 is the ladder (gene ruler 1kb ladder) and lanes 2-14 are soil samples extracted from six weeks sampling point. Lane 15 is the no template control.

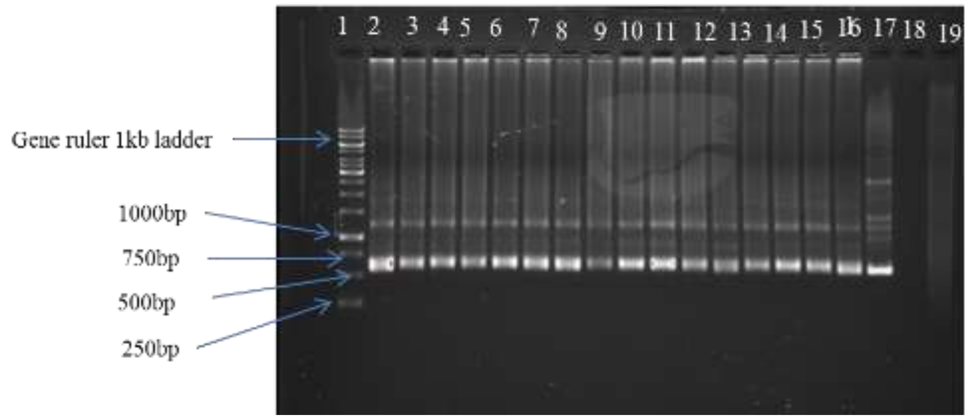


Figure 10: Gel electrophoresis for PCR products amplified using e9f/u1510 as external primers followed by amplification with FAM341/908 as internal primers. The resulting fragments were 567bp. Lane 1 is the ladder (gene ruler 1kb ladder) and lanes 2-17 are soil samples extracted from six weeks sampling point. Lane 18 is an empty lane and lane 19 is the no template control.

3.1.3 Restriction digest

Determination of microbial diversity of a given community using TRFLP analysis relies on detecting 16S rRNA gene sequence polymorphisms using restriction enzymes and the choice of enzyme used plays a major role in resolving the diversity of the community (Marsh, 2005). Using two or more restriction enzymes has been shown to increase the resolution of a bacterial community as microbial communities may have members sharing the same T-RF for the same enzyme-primer combination (Schutte *et al*., 2008).

3.1.4 Genescan Sequencing

The pattern of T-RFs generated reflects the composition of numerically dominant population in a community. Differences in the size of T-RFs reflect differences in the sequences of 16rRNA gene polymorphism (Schutte *et al.*, 2008).

Preferential injection of smaller molecules such as salts, primer dimers and restriction products occurs in injection of T-RFLP samples (Schutte *et al*., 2008). In this study, clean-up of PCR and restriction digestion products was done to ensure efficient injection of samples.

Table 1: Presence of most frequent OTUs across moisture-temperature conditions over time

	6weeks						12weeks						18 weeks					
	Dry			Wet			Dry			Wet			Dry			Wet		
OTU	10^{o}C	20^{o}C	30^{o}C	10^{o}C	20^{o}C	30^{o}C	10^{o}C	20^{o}C	30^{o}C	10^{o}C	20^{o}C	30^{o}C	10^{o}C	20^{o}C	30^{o}C	10^{o}C	20^{o}C	30^{o}C
22														X	X		X	X
23			X										X	X	X		X	X
25		X	X				X	X	X									
55	X	X	X		X	X	X	X	X	X	X	X						
71	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
72									X				X	X	X			
110														X		X		
111													X	X				X
242						X												
245							X		X									
556													X	X	X			
557	X	X	X	X	X			X	X		X		X	X	X	X	X	X
575	X	X	X	X	X	X		X			X			X	X	X	X	X
581		X				X							X	X	X	X	X	X
583	X	X	X		X	X	X	X	X	X	X	X						

X denotes presence of OUT.

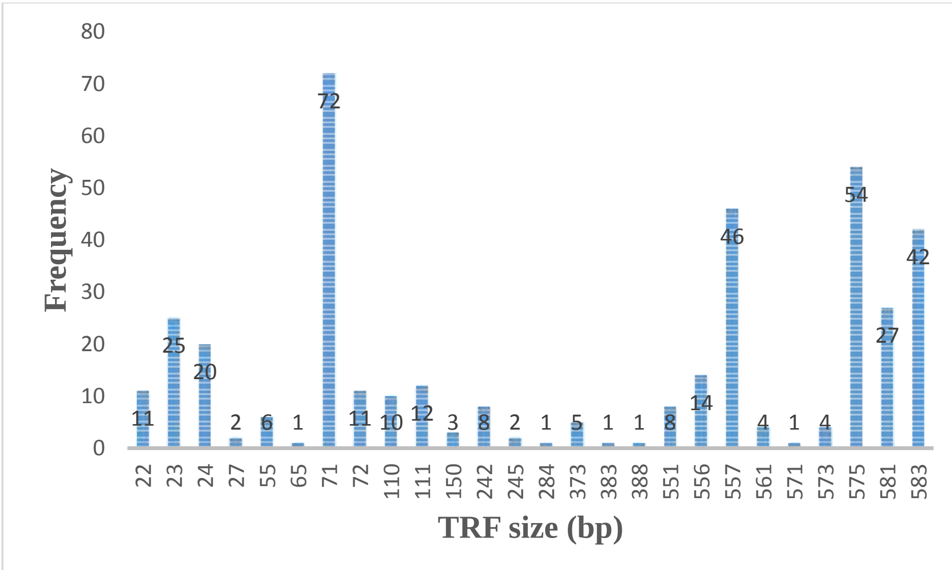


Figure 11: Frequency of TRFs observed in TRFLP analysis. Figures on the bars show the frequency of the specific TRF in the 72 samples as observed in the abundance matrix.

Results from a gene scan automated sequence analyser produced TRF profiles of a minimum of 14 and a maximum of 63 operational taxonomic units (OTUs) per sample. The average number of TRFs for the 72 samples was 44. When these samples were programed in R software package an average of 10 OTUs were recorded as true peaks per sample. Peaks that were less than 50 fluorescence units (FUs) were removed as they could not be differentiated from background noise and peaks that were within 1basepair apart were taken as the same OTU as previously described by Shutte *et al*. (2008). Low number of OTUs in the samples could have been due to the overshadowing of low abundance taxa in PCR amplification. T-RFLP technique is unable to detect low abundance taxa (Makhalanyane *et al*., 2013).

Results showed that the OTU of 71 bp was the most abundant in all the samples as shown in **Figure 11**, with average relative abundance of 60% across all samples for the different moisture-temperature conditions, and the only one that was present in all samples. The abundance of this OTU did not change much across different moisture-temperature conditions over time. If we assume the likely hypothesis that states that the dominant microorganisms in a sample are those that play the most important functional role under normal conditions (Grime, 1998); this might be a consequence of the importance of this OTU in the microbial communities under study.

TRFs 23bp, 557bp, 575bp, 581bp and 583bp also had higher frequency of occurrence in the samples as shown in **Figure 11** above, which could have obscured the detection of other low occurrence species of bacteria.

The 25bp OTU was present in 20^{o}C dry samples at 6 weeks, and dry samples 20^{o}C at 12 weeks but absent in wet samples for both sampling times. However this OTU was not present at 18 weeks for all temperature-moisture conditions. The species with this OTU could have been out-competed with time and became less abundant. Results also show that the 25bp OTU was absent in all wet samples at all temperatures which could mean that this species or group of species does not grow under wet conditions. The 23bp OTU was gained at 18 weeks, it was never observed at 6 weeks and 12 weeks. At 18 weeks, the 23bp OTU was absent in the wet samples at 10^{o}C but present at 20^{o}C wet and dry, 30^{o}C wet and dry and 10^{o}C dry. Bacteria with the OTU 23bp could have existed as low abundance taxa at 6 and 12 weeks and hence could not be detected in T-RFLP. After adapting to the environment, or due to a loss of competitive taxa, the bacteria became more abundant and were detected in T-RFLP. 10°C wet conditions did not favor growth of bacteria with the OTU of 23bp.

The OTU 557bp was absent in 30°C dry at 12 weeks, 20°C dry at18 weeks, 30°C dry 18 at weeks and all wet samples at 18 weeks. The wet samples at 6 and 12 weeks had this 557bp OTU which was lost at 18 weeks. The 583bp OTU was present in almost all samples in 6 and 12 weeks was lost at 18 weeks. The gain and loss of TRFs could have been due limiting resources with time since the samples were isolated in greenhouse and no plants or insects were part of the environment to provide energy. Strong competitors that adapted to the environment survived and were detected in T-RFLP. Results showed that temperature, moisture condition and time all affected the microbial diversity of the community by gain or loss of TRF.

Using the PAST software, non-metric multi-dimensional scaling (nMDS) was performed resulting in **Figures 12, 13, 14** and **15**. The samples were depicted according to moisture, temperature and sampling time using different colors and symbols as denoted in **Table 3**.

Table 1: Legend for symbols used in **Figures 12, 13, 14** and **15.**

	6 weeks			**12 Weeks**			**18 weeks**
□	20^{0}C dry		□	20^{0}C dry		□	20^{0}C dry
■	20^{0}C dry control		■	20^{0}C dry control		■	20^{0}C dry control
△	30^{0}C dry		△	30^{0}C dry		△	30^{0}C dry
▲	30^{0}C dry control		▲	30^{0}C dry control		▲	30^{0}C dry control
●	10^{0}c dry		●	10^{0}c dry		●	10^{0}c dry
○	10^{0}c dry control		○	10^{0}c dry control		○	10^{0}c dry control
□	20^{0}C wet		□	20^{0}C wet		□	20^{0}C wet

■	20^{0}C wet control		■	20^{0}C wet control		■	20^{0}C wet control
△	30^{0}C wet		△	30^{0}C wet		△	30^{0}C wet
▲	30^{0}C wet control		▲	30^{0}C wet control		▲	30^{0}C wet control
●	10^{0}C wet		●	10^{0}C wet		●	10^{0}C wet
○	10^{0}C wet control		○	10^{0}C wet control		○	10^{0}C wet control

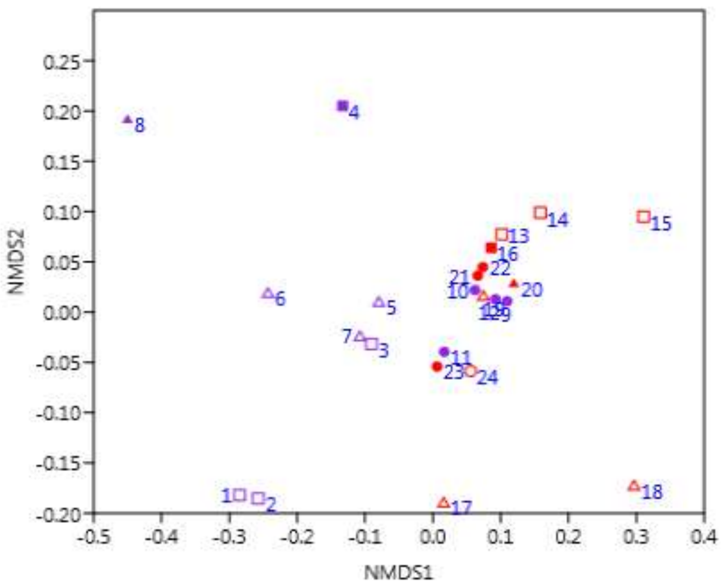


Figure 12: Non Metric Multidimensional Scaling Bray-Curtis Ordination plot for six week sampling, samples 1-24, (1-24 are sample numbers).

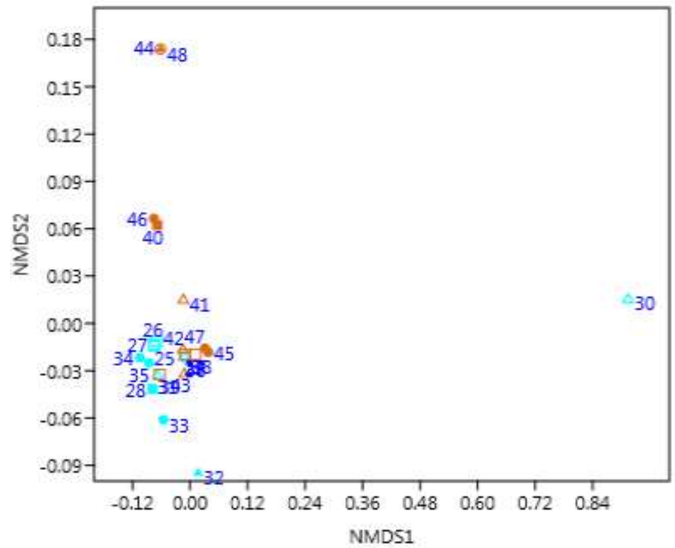


Figure 13: Non Metric Multidimensional Scaling Bray-Curtis Ordination plot for 12 week sampling, samples 25-48, (25-48 are sample numbers).

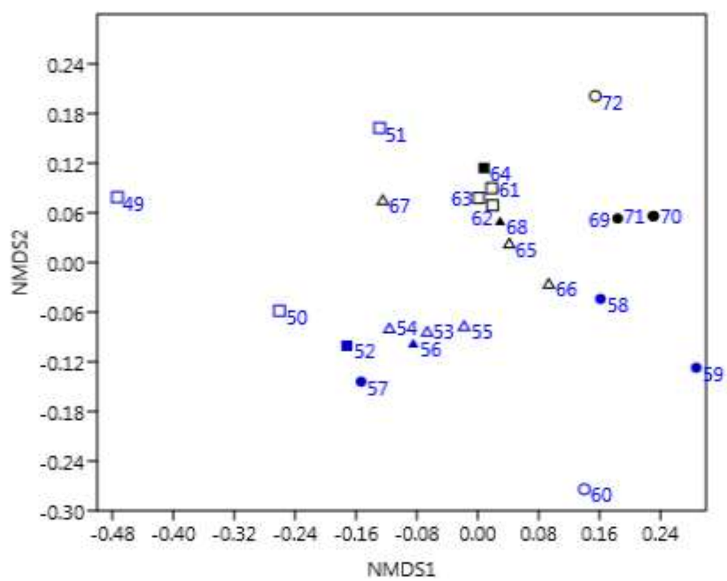


Figure 14: Non Metric Multidimensional Scaling Bray-Curtis Ordination plot for 18 week sampling, samples 49-72, (49-72 are sample numbers) .

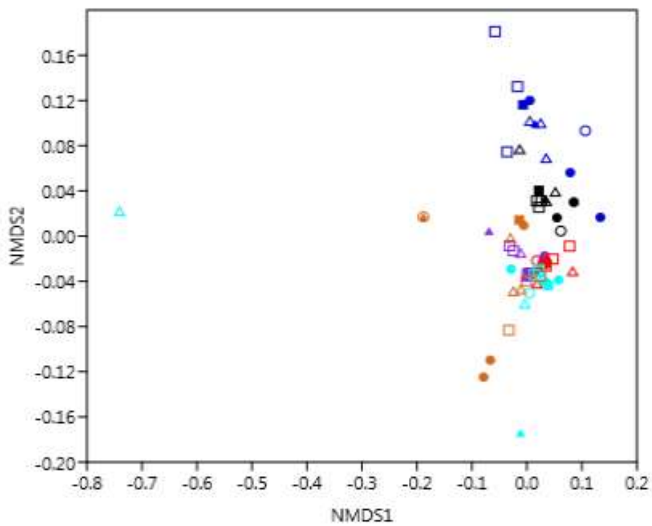


Figure 15: Non metric multidimensional scaling (based on Bray-Curtis similarities) ordination plot for the 72 (1-72) samples that were analysed by T-RFL. Each moisture-temperature combination had three replicate pots.

nMDS Bray-Curtis ordination plot for 6 weeks **(Figure 12)** shows that 10^0C wet and 10^0C dry communities clustered together, showing that temperature was an important environmental factor in profiling of bacterial community structures. The moisture condition did not affect the diversity and distribution of microbial communities at temperature 10^0C in the first six weeks of the pot trials. **Figure 13** shows nMDS Bray-Curtis ordination plot for 12 weeks reflecting similarity of samples as observed in the clustering of the ordination points. The microbial community might have been adapted to the environment after selection pressure due to change in environmental conditions producing a similar bacterial community profile. The microbial diversity pattern could have been created by bacteria that are better adapted to the temperature-moisture combinations of exposure in the absence of plants. The organic matter in the soil, which is the main source of energy for the bacteria was a limiting resource and thus only better competitors survived. Samples 30, 32, 40, 44, 46, 48 were dissimilar to the rest of the samples as their points are further from the main cluster. These could be experimental outliers due to errors and biases introduced by DNA extraction, PCR and pipetting errors. Experimental errors were also evident in **Figure 12** where samples 1, 2 and 3 were replicates and sample 3 was further away from samples 1 and 2 showing dissimilarity between replicate.

Figure 14 for 18weeks nMDS ordination plot shows spatial distributions of samples, wet samples are clustered different from the dry samples. However, for 20^{0}C and 30^{0}C wet and dry samples were similar as the points were close to each other whilst the 10^{0}C samples were dissimilar as points were further from each other.

Results for all the 72 samples plotted in an nMDS Bray-Curtis ordination plot in **Figure 15** showed temporal distribution of the 72 samples as reflected by ordination points close to each other showing close similarity of the samples. Points represent the composition of a community in multidimensional space, and the distance between two points represents the similarity between those two communities. Communities that are closer together are more similar in bacterial composition (Makhalanyane *et al*., 2013). Samples were from the same field and were bound to have the same microbial composition. Exposure to different moisture-temperature combinations resulted in little variation in the overall microbial composition. The period of exposure could have been too short to observe change in microbial diversity of the different moisture temperature conditions.

3.2 qPCR

3.2.1 *Pcb* Primer specificity

Pcb has been reported to share 82% of its genes with *Pa*, while 84% of the genes are shared with *Pcc* (Kim *et al*., 2009). Moreover, there is a very small difference in the 16S rDNA region and known functional genes of these organisms, which makes primer design difficult. PCR amplification of *Pcb*, *Pwa*, *Pcc* and *Pa* at 54˚C, 56˚C and 58˚C proved that the designed primers were specific for *Pcb*. Observation in 1.5% agarose gel electrophoresis showed that only *Pcb* had the expected 120bp fragment as shown in **Figures 16, 17** and **18**. At 54˚C, unspecific amplification was observed for *Pcc* and *Pwa* which produced fragments that were greater than 1Kb in size. *Pa* had one band that was also greater than 1kb which could suggest that one of the primers amplified the whole region of the 16s rRNA gene. Increasing annealing temperature increases the specificity of the primers. Since the primers were used with a specific Probe in qPCR and the annealing temperature used was 60˚C, the specificity of the reaction was greatly enhanced for *Pcb*. The qPCR procedure used in this study was the two-step protocol that combined annealing and extension step at 60˚C.

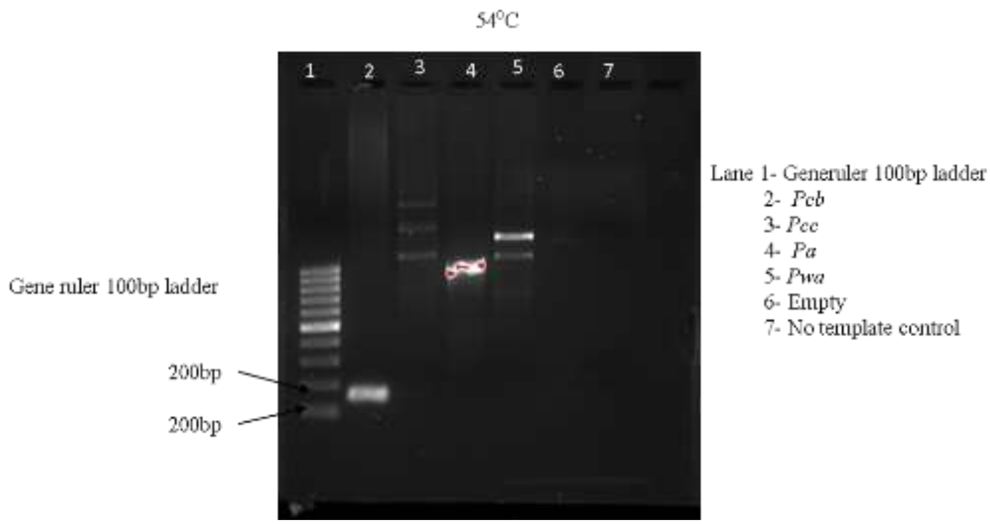


Figure 16: Specificity of designed *Pcb* primers, when used to amplify *Pectobacterium* species and subspecies that are closely related to *Pcb* at 54^{0}C, namely *Pcc*, *Pa*, *Pwa*.

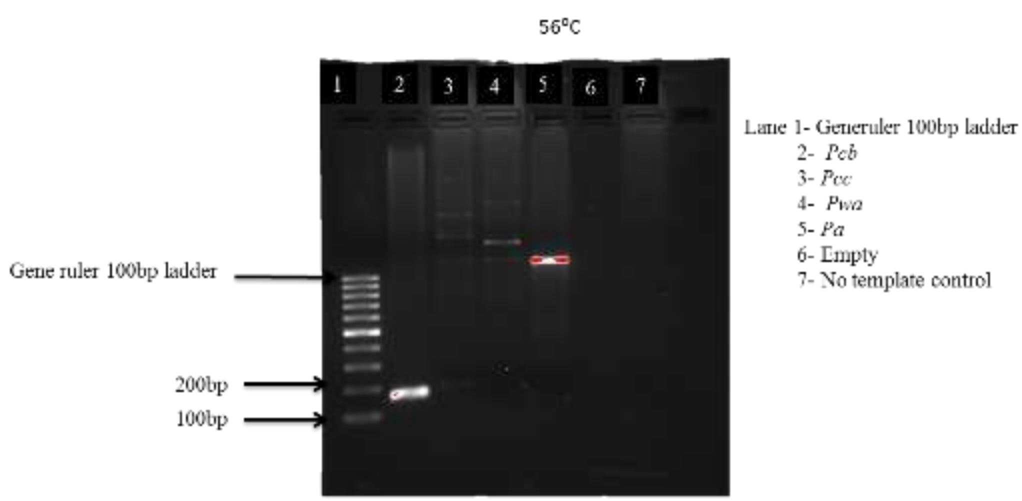


Figure 17: Specificity of designed *Pcb* primers, when used to amplify *Pectobacterium* species and subspecies that are closely related to *Pcb* at 56^{0}C, namely *Pcc, Pa, Pwa*.

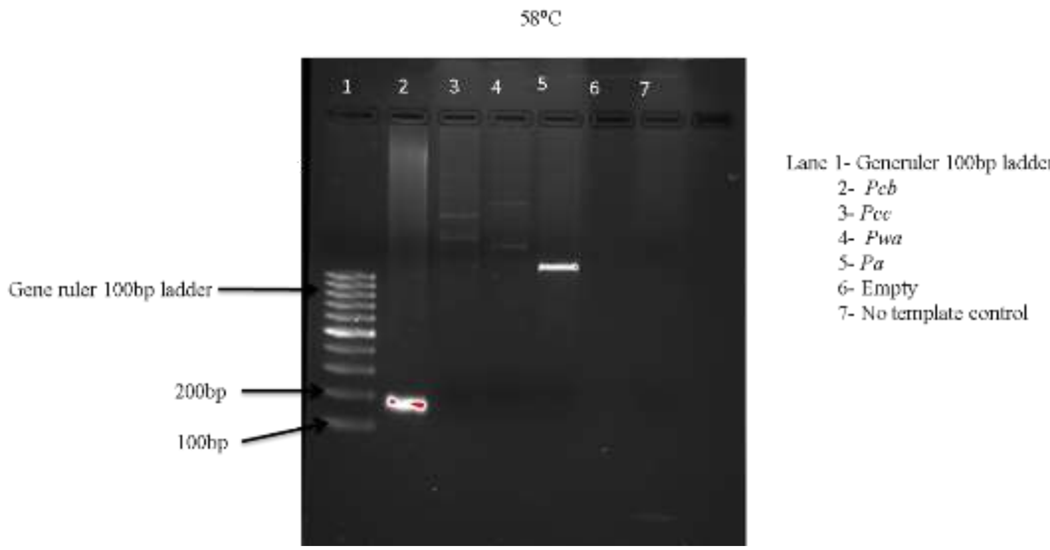
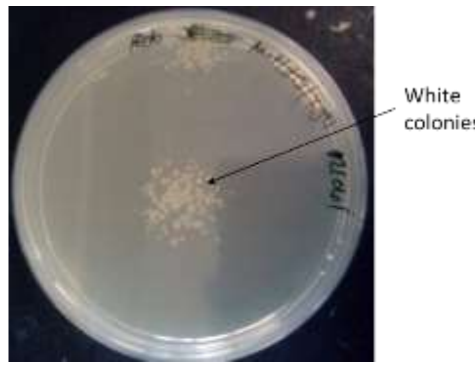


Figure 18 : Specificity of designed *Pcb* primers, when used to amplify *Pectobacterium* species and subspecies that are closely related to *Pcb* at 58^{0}C, namely *Pcc, Pa, Pwa*.

3.2.2 Cloning of *Pcb* and *R. solani* AG3-PT

Purified PCR products for *Pcb* and *R. solani* were used for ligation into pGEM-T Easy vector. pGEM-T Easy vector has 3'Thiamine (T) overhang that ligates to the 3' Adenine (A) of the PCR product added by *Taq polymerase* during extension step by its deoxynucleotidyl transferase activity. Ligation was optimum at 16˚C overnight instead of 4˚C overnight, and the vector: insert ratio was optimized at 5:3 to produce optimum ligation of the insert into the vector. The transformation produced more white colonies than blue colonies although the white colonies were smaller than the blue ones. The pGEM-T Easy vector used for cloning is equipped with the Lac Z gene and ampicillin resistance gene for selection and results for blue and white colonies are shown in **19.**



A **B**

Figure 19: Agar plate showing blue and white transformed colonies **A** for *R. solani* and **B**, for *Pcb*. White colonies are assumed to carry *R. solani* AG3-PT cloned fragments in pGEM-T Easy vector whilst blue colonies were transformed with the vector without the insert.

Colony PCR confirmed the presence of the insert in the clones and 1.5% agarose gel electrophoresis showed bands of around 120bp for both *Pcb* and *R. solani* AG3-PT as shown in **Figure 20** and **21** respectively. To confirm the expected sequence of the inserts, clones were grown on LB and plasmid mini prep were done and plasmids sequenced. Blasting in NCBI nucleotide blast confirmed the sequences of the inserts.

Pcb sequence in vector for forward sequence was located from position 91 to 214. *R. solani* AG3-PT ITS region was located from position 95 to 217 in forward sequence. *NcoI* was selected as the ideal restriction enzyme for linearization of the vectors as it cuts the vector once without cutting the insert sequence. Linearization of the vector increases the exposure of the target insert sequence for qPCR reaction.

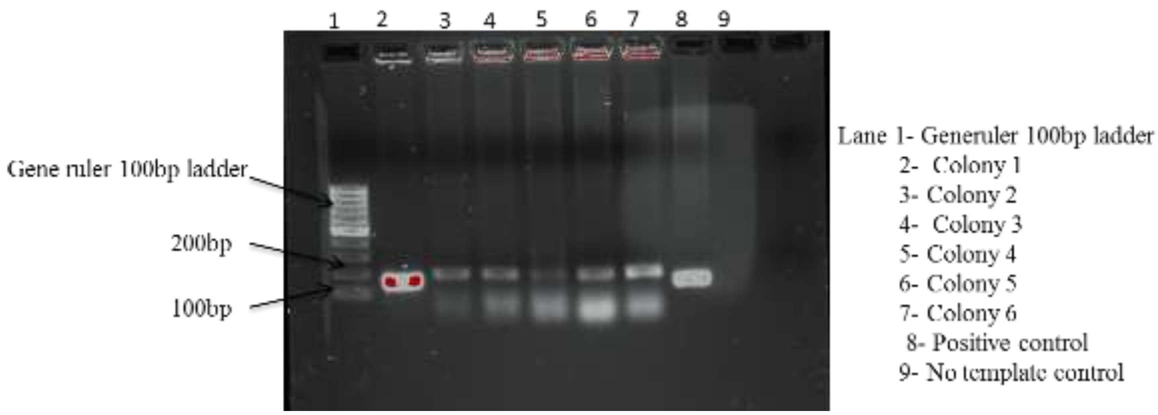


Figure 20: Colony PCR for *Pcb*

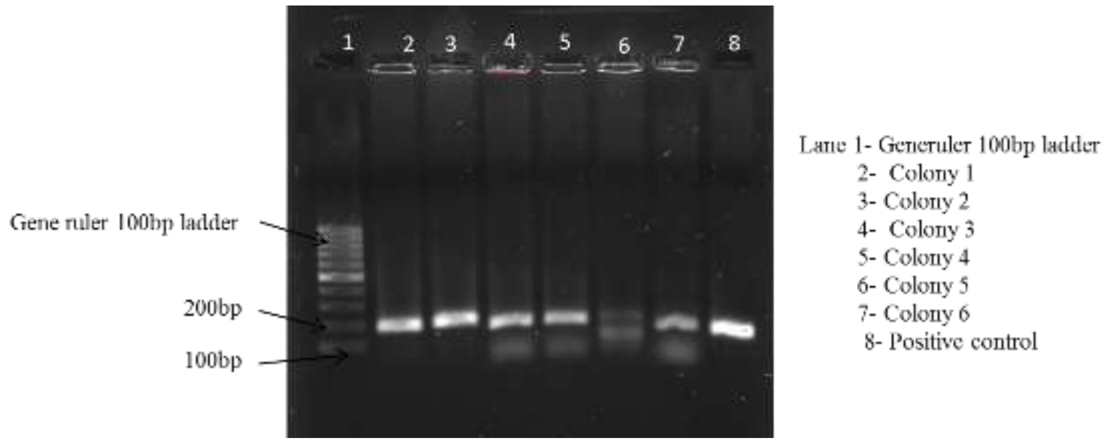


Figure 21: Colony PCR for *R. solani* AG3-PT

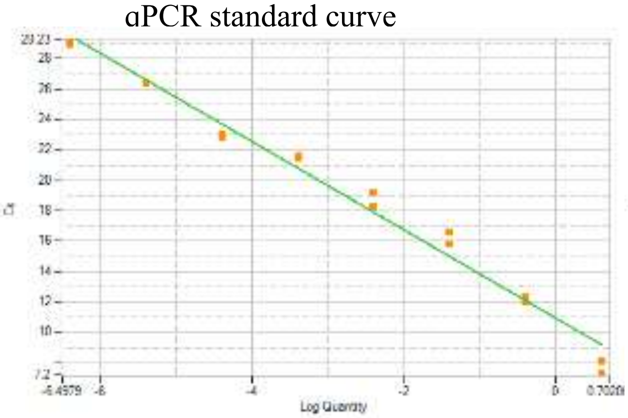
3.2.3 qPCR Analysis

3.2.3.1 R. solani qPCR analysis

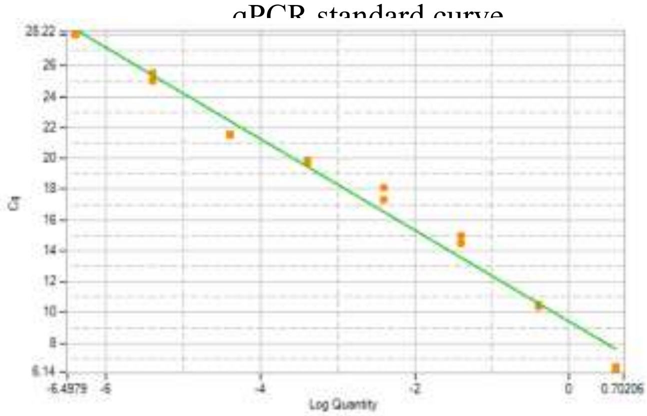
Soil-borne pathogens such as *R. solani*, which affect yield and quality of potatoes, need to be diagnosed at an early stage in seed and/or in soil. A rapid, sensitive, specific diagnostic which quantifies the amount of pathogen is a strength of qPCR as a tool in diagnostics and disease epidemiology (Budge *et al*., 2009). Inoculum density levels in soil can be used for risk-prediction to decide control actions of *Rhizoctonia* diseases (Tsror, 2010) on potatoes hence the need to quantify the amount AG3-PT in soil from potato fields.

Soil samples from the field were sampled randomly and were taken up to 10cm soil depth. Sampling of soil in the field plays a major role in the detection of the amount of pathogen in the area of study. A study carried out by Budge and coworkers in 2009 revealed that more AG2-1 was significantly detected in soil sampled from shallower depths than in deeper soil samples from cauliflower fields. These findings also support work done by Papavizas *et al*. (1975) who found no viable *R. solani* below 10cm depths in field samples as *R. solani* spread faster and further along surfaces than within soils

A: *R. solani* AG3-PT **6 weeks** qPCR standard curve



B: *R. solani* AG3-PT **12 weeks** qPCR standard curve



C: *R. solani* AG3-PT **18weeks** qPCR standard curve

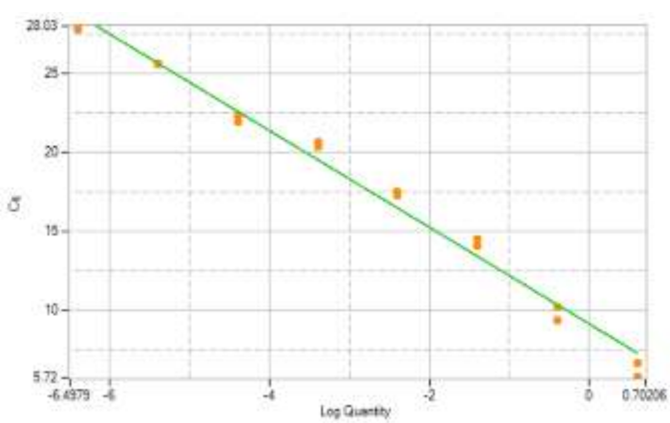


Figure 22: qPCR Standard curves for *R. solani* AG3-PT with the R^2 values and y intercepts for A; 6weeks, B; 12 weeks and C; 18weeks sampling times. The standard curves are plotted Cq against log quantity**.**

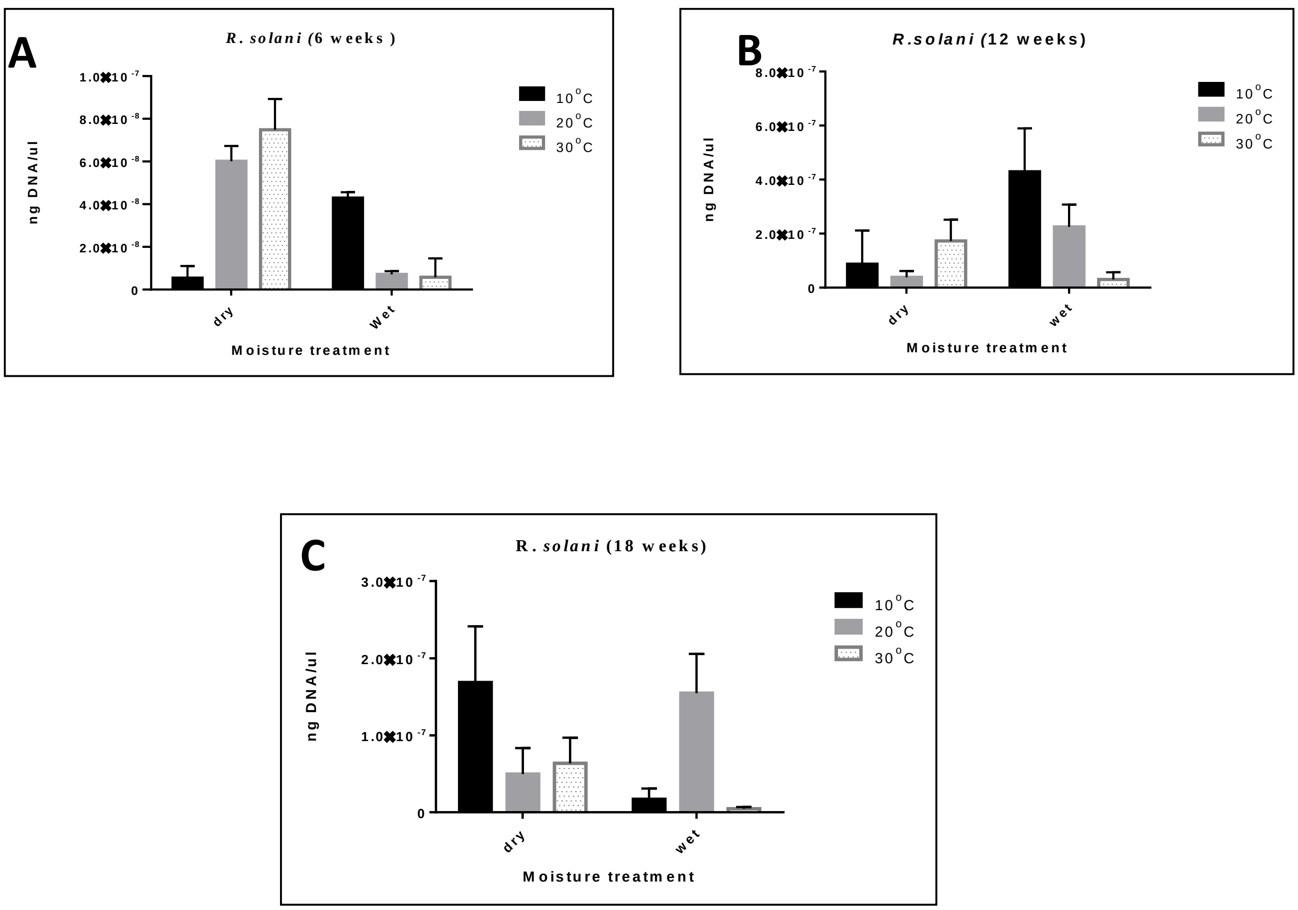


Figure 23: *R. solani* AG3-PT qPCR results for moisture-temperature conditions at each sampling point. A shows quantity of *R. solani* AG3-PT detected at 6 weeks sampling time, B shows quantities of *R. solani* AG3-PT detected at 12 weeks sampling time and lastly C shows *R. solani* AG3-PT quantities detected at 18 weeks sampling time.

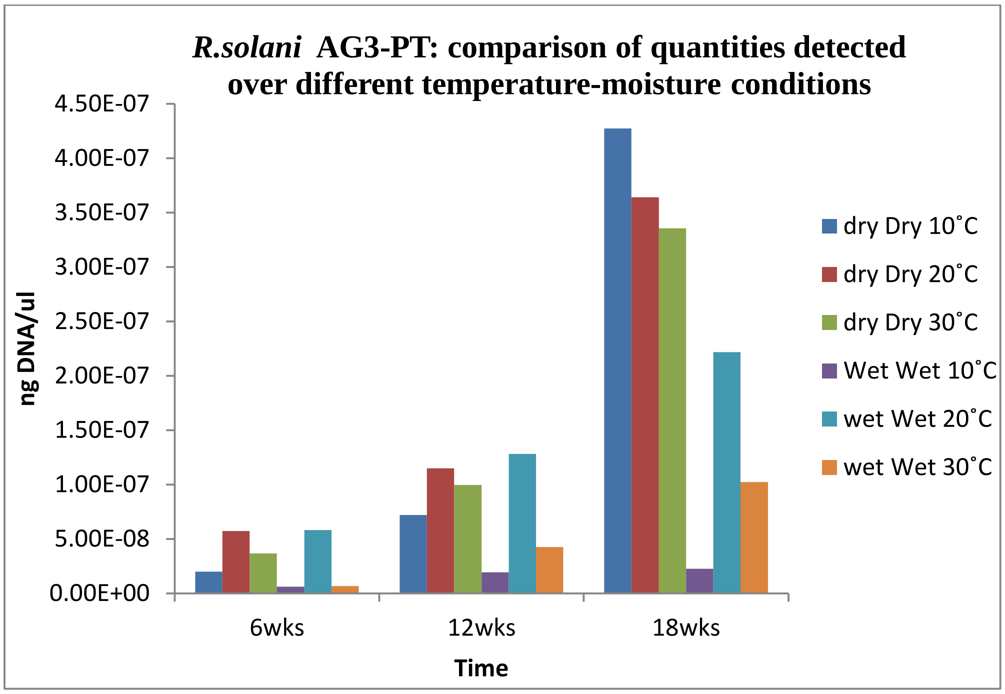


Figure 24: *R. solani* AG3-PT qPCR quantification showing comparison of different moisture-temperature combinations for each of the sampling time (6 weeks, 12 weeks and 18 weeks). Quantities of DNA are in ng DNA/ul as extrapolated from the standard curve in **Figure 22**.

Figure 23 shows the amount of *R. solani* AG3-PT detected in each moisture-temperature combinations for the three sampling times, 6 weeks, 12 weeks and 18 weeks. The results are expressed as the mean + standard deviation of the biological replicates (3 pot trials for each temperature-moisture condition) and experimental replicate (2 replicates for each qPCR run). For all the samples in **Figure 23**, mean *R. solani* AG3-PT quantities were between 10^{-7} and 10^{-8} ng DNA/µl. Comparison of results in **Figure 23** and **Figure 26** shows that there was generally more Pcb detected than *R. solani* AG3-PT in the soil samples. Lower quantities of *R. solani* AG3-PT detected can be as a result of low pathogen concentration in the naturally infested soil (Henis *et al*., 1978) or inefficient DNA extraction method.

As previously described earlier under T-RFLP section on DNA extraction, the extraction kit might not have been suitable for extraction of *R. solani*. *R. solani* survives in soil mainly as sclerotia which are dormant structures, that are hard and resistant to unfavourable conditions and chemical and biological degradation (Shu *et al*., 2014). These tough structures of sclerotia require more harsh methods of DNA extraction especially the cell lysis step to yield more DNA.

Experiments for field-plot burial by Ritchie *et al*., 2013 confirmed that prolonged burial in soil reduces the survival of sclerotia produced by *R. solani* AG3PT. This could also explain the reason for low levels of *R. solani* AG3-PT detected in the soil samples in this study. *R. solani* is considered as a surface pathogen, it thrives better on the surface than deep in soil (Otten and Gilligan, 1998).

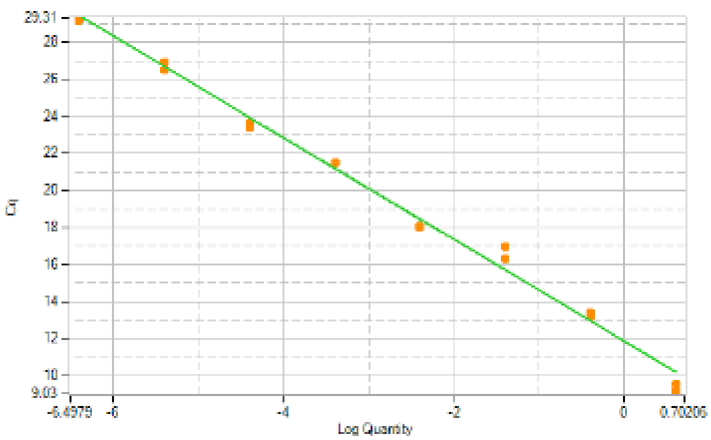
Results from these greenhouse pot trials only give an indication of survival of pathogen under different field conditions were the field is constantly cleared of weeds. In the field *R. solani* is known to survive longer periods in the presence of alternative hosts such as weed species; couch grass (*Elytrichia repens*) and black nightshade (*Solanum nigrum*) (Ritchie *et al*., 2013; Woodhall *et al*., 2008). *R. solani* AG3 has also been isolated from roots of other commercial crops such as barley and sugar beet (Tsror, 2010). The wide spectrum of *R. solani* alternative hosts plays an important role in survival of the pathogen in soil in potato fields and should be considered in integrated diseases management (Tsror, 2010). The nutritional status of soil also contributes to the survival of *R. solani* in soil as the pathogen survives as a saprophyte, obtaining its energy from dead plant material in the absence of host. The soil used in this sample could have had limited amount of dead plant material hence the low quantities of *R. solani* AG3-PT detected in the study. Previously Ritchie *et al*, 2009 showed that *R. solani* produces few or no sclerotia in poor nutrient soils or harsh environmental conditions.

Figure 24 shows a general increase in the amount of *R. solani* AG3-PT detected from 6 weeks to 18weeks with a marked increase observed between 12 and 18 weeks. Increase could be adaptation of the fungus to adjust to environmental conditions. A decrease in the amount of pathogen was more likely to be observed if the experiments were continued beyond 18 weeks as the survival of the pathogen will be reduced. There was no increase/minimal increase in the amount of *R. solani* AG3-PT detected at 10˚C wet and 30˚C dry.

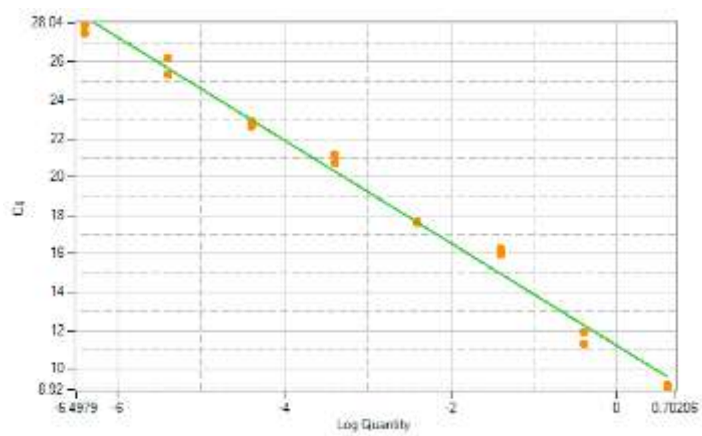
ANOVA analysis of the quantities of *R. solani* AG3-PT detected showed no statistically significant difference between moisture-temperature combinations over time as P value at 95% confidence interval was 0.138. The absence of statistical difference could be as a result of the extraction protocol used as described earlier in **3.1.1**. The potatoes harvested from diseased field were not assessed for disease severity; therefore conclusions cannot be made on whether the low amounts of pathogen were due to low incidence or poor extraction protocol. From this study it can be concluded that the qPCR method designed by Woodhall *et al*. (2013) is an effective diagnostic tool in detecting very low levels of *R. solani* AG3-PT in South African soil.

3.2.3.2 Pcb qPCR Analysis

A: *Pcb* **6weeks** qPCR standard curve



A: *Pcb* **12weeks** qPCR standard curve



A: *Pcb* **18weeks** qPCR standard curve

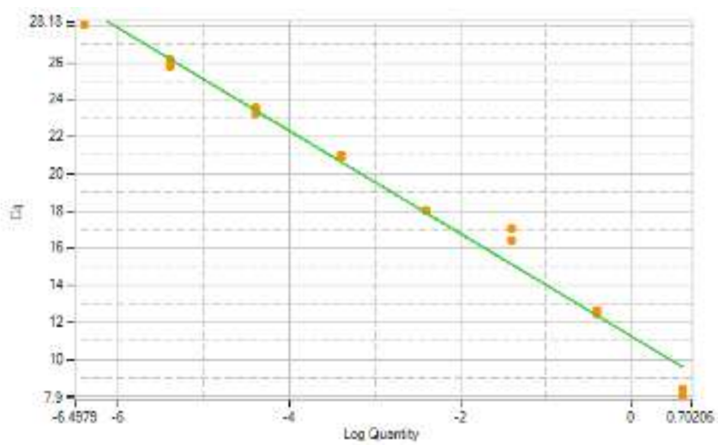


Figure 25: qPCR Standard curves for *Pcb* with the R2 values and y intercepts for A; 6weeks, B; 12 weeks and C; 18weeks sampling times. The standard curves are plotted Cq against log quantity.

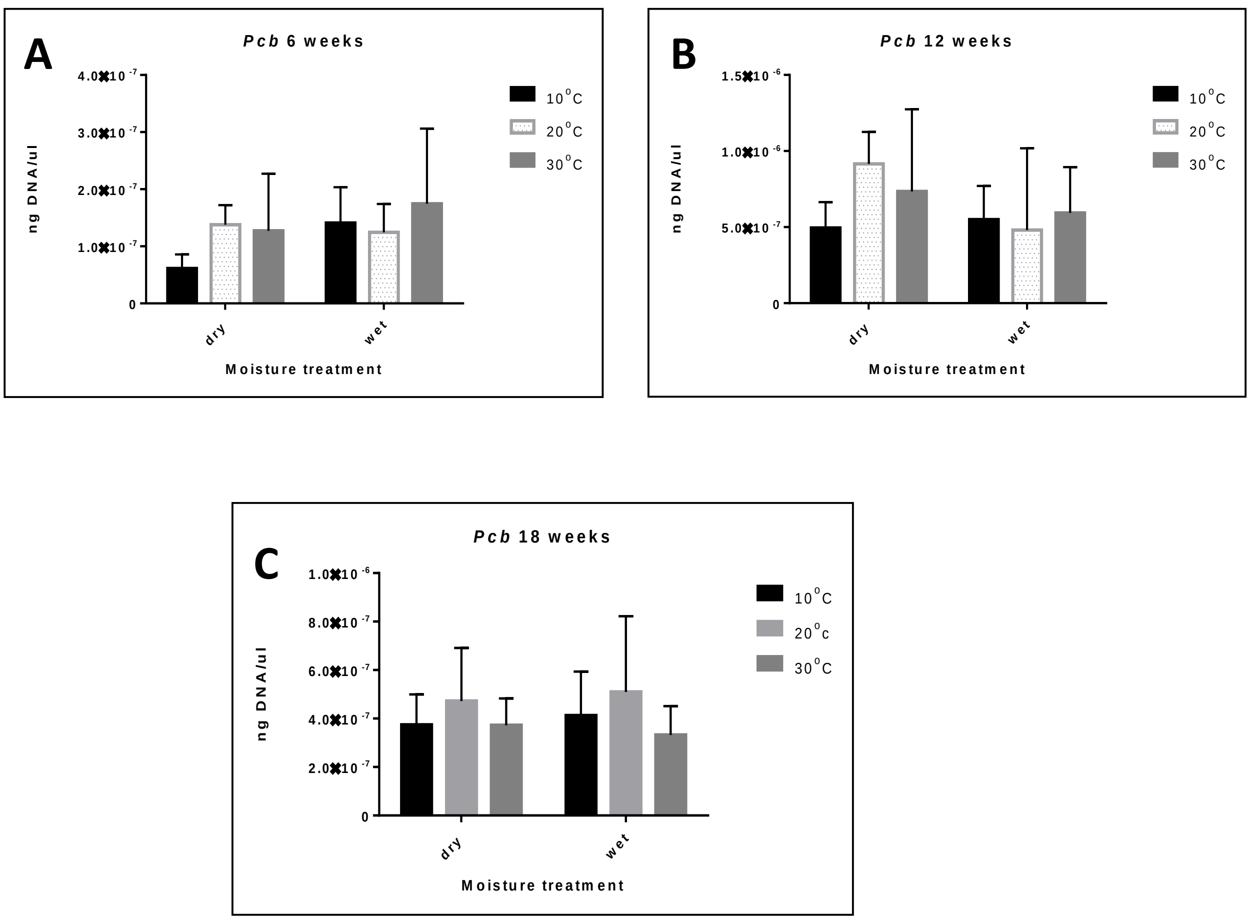


Figure 26: *Pcb* qPCR results for moisture-temperature combinations at each sampling time. **A** shows quantity of Pcb detected at 6 weeks sampling time, **B** shows quantities

of *Pcb* detected at 12 weeks sampling time and lastly **C** shows *Pcb* quantities detected at 18 weeks sampling time.

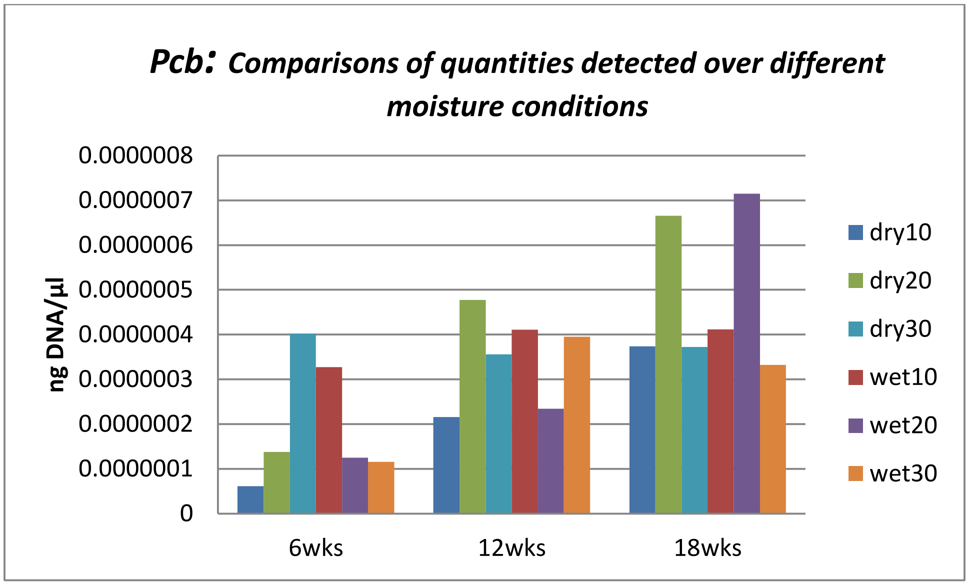


Figure 27: *Pcb* qPCR quantification showing comparison of different moisture-temperature combinations for each of the sampling times (6 weeks, 12 weeks and 18 weeks). Quantities of DNA are in ng DNA/ul as extrapolated from the standard curve in **Figure 25.**

Figure 25 shows the qPCR standard curves for *Pcb* for 6, 12 and 18 weeks as denoted by the letters A, B and C respectively. The standard curves in **Figure 25** had an R^2 value of 0.99 showing close relatedness of the plotted standard curve to the true value. Using the Thermo Scientific PikoReal 96 qPCR machine and software, the values of the unknown samples were extrapolated from the standard curves and were used to plot graphs in **Figure 26** and **Figure 27**. **Figure 26** shows the amount of *Pcb* detected in each temperature-moisture combination for the three sampling times, 6 weeks, 12 weeks and 18 weeks. The results are expressed as the mean + standard deviation of the biological replicates (3 pot trials for each temperature-moisture condition) and experimental replicate (2 replicates for each biological replicate). For all the samples in **Figure 26**, mean *Pcb* quantities were between 10^{-6} and 10^{-7} per ul of the extracted DNA.

ANOVA statistical analysis of *Pcb* qPCR data showed that there was no statistical difference (P= 0.108 at 95% confidence interval) in the amount of *Pcb* detected in the different moisture-temperature conditions. The soil for pot trials was from the same field and same sample hence this could attribute to the similar quantities. qPCR is unable to determine whether cells are viable or dead hence DNA detected in this study could be from dead *Pcb* cells that could have died a few weeks after harvesting of the potato crop. *Pectobacterium* is not known to survive in soil for prolonged periods in the absence of suitable host or alternative host (Czjkwoski *et al*., 2011). Survival of *Pectobacterium* is restricted to one week to 6 months in soil and results from this study concur with these earlier studies. Growth of *Pectobacterium* in soil is also restricted by the presence of other pectinolytic bacteria in soil such as *Clostridium* spp., *Bacillus* spp., and *Pseudomonas* spp. which outcompetes *Pcb* and other *Pectobacterium* in an unfavorable environment (P’erombelom, 2002).

Despite the statistical insignificance in the quantities of *Pcb* detected, the graph in **Figure 27** shows some differences in the amount of *Pcb* pathogen detected under different temperature-moisture conditions which is not necessarily statistically different. **Figure 27** show that there was a general increase in the amount of pathogen detected from 6 weeks to 18 weeks for all temperature-moisture combinations used in this study. At 12 weeks and 18 weeks the quantity of *Pcb* detected did not change much for all the other moisture-temperature combinations except for 20˚C dry and 20˚C wet where there was an increase in the quantity of pathogen detected. 20ºC might be optimum temperature for the growth of the pathogen, *Pcb.* In Brazil, *Pcb* has been shown to cause disease in relatively cool temperatures (17–20˚C) during the growing season (Duarte *et al*. 2004). Optimum temperature for multiplication of *Pcb* could be around this temperature hence the observed increase at 20˚C in pot trials used in this study.

More *Pcb* was detected for 20˚C wet than for 20˚C dry at 18 weeks sampling time. *Pectobacterium* has been shown to require water which creates anaerobic conditions, in turn promoting multiplication of the bacteria (P'erombelom 2002). If this is true for all *Pectobacterium* spp., then from the study constant watering of the pot trial at 20˚C promoted multiplication of the *Pcb* although cell growth had a low turnover.

In this study soil was not sampled immediately after harvesting of infected crop, sampling of soil for pot trials was done approximately 4 weeks post-harvest. There were rains in the period between harvest and sampling which could mean some of the bacteria, *Pcb*, was washed away or leached deeper into the ground below the sampling depth. There have been few studies to show how *Pcb* or any other *Pectobacterium* exist in soil, whether they are bound to soil particles or as aggregates of the bacteria conditions, which could affect the amount of *Pcb* extracted and detected in soil.

Pectobacterium is not known to overwinter in soil especially in the absence of host and this was supported by the low quantities of *Pcb* detected in this study. Moisture and temperature conditions affect survival of *Pcb* in soil and 20^{o}C was shown to be the optimum growth temperature for *Pcb* in this study which confirms the 17-20oC previously reported by Duarte *et al*., (2004). qPCR detection of *Pcb* from soil showed that using the designed primers, probe and protocol described here, very low quantities of *Pcb* can be detected in soil.

Pectobacterium is known to exhibit some dormancy under unfavorable conditions; where low quantities of *Pcb* below infective limits are detected by qPCR, results should be interpreted with care when advising farmers on cropping in such a field. Under favorable conditions in the presence of a susceptible host, *Pcb* in soil can cause blackleg and soft rot, causing some economic losses to the farmer.

Chapter 4

Conclusions and Recommendations

Molecular techniques have contributed much in the area of microbial ecology and disease epidemiology and control. Results from this study have shown that qPCR is a very sensitive method for detecting minute quantities of *Rhizoctonia solani* AG3-PT as low as 10^{-8} ng DNA/ul and as low as 10^{-7} ngDNA/ul for *Pectobacterium carotovorum subp. Brasiliensis* in soil. Low quantities of these pathogens detected by qPCR enable pre-symptom detection in seed tubers and monitoring of inoculum levels in soil. Pcb primers used in this study were specific to *Pectobacterium carotovorum subp. Brasiliensis* Survival of pathogens in soil is affected by presence of other microbial communities in that soil hence T-RFLP was used to assess change of microbial communities under different cropping environmental conditions. From the results, it can be concluded that, time, moisture content and temperature all affect composition of microbial communities.

Future studies will however be needed to establish how the pathogens, *Rhizoctonia solani* AG3-PT and *Pectobacterium carotovorum subp. brasiliensis* exist in soil particles in order to develop a suitable and effective DNA extraction protocols. Field experiments of survival of the pathogens in alternative hosts are also necessary to establish the levels of pathogen in soil in the presence of alternative host. Pyrosequencing of the abundant OTUs to identify the species or group of species represented by the OTUs need to be done. Studies to establish the microbial interaction of the pathogens and the OTUs in soil are important in order to develop biological control methods of the pathogens in soil.

Chapter 5

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