IDENTIFICATION OF SOFT ROT SPECIES FOUND IN THE HARARE AREA OF ZIMBABWE AND AN EVALUATION OF THE SUSCEPTIBILITY OF SELECTED POTATO CULTIVARS TO THE PATHOGENS

by

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DEDICATION

To my beloved son Tavonga

ABSTRACT

Experiments were conducted at the University of Zimbabwe, Crop Science Department from 2012- 2014 with the overall aim of identifying soft rot pathogens in the peri-urban Harare area and to screen some of the locally grown potato cultivars for resistance to these pathogens. In the first experiment, potato tubers and stems showing soft rot and blackleg symptoms respectively were purposely collected from eight commercial potato farms within a 60 km radius of Harare. Using standard biochemical tests, a polymerase chain reaction and sequencing, the predominant soft rot pathogen in the area was found to be *Dickeya* species.

In experiment II, five potato cultivars namely Amethyst, BP1, Jasper, Montclaire and KY20 were evaluated for their response to blackleg caused by *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pcb*) in a potted experiment. A randomised complete block design with four blocks was used. Six-week old plants were inoculated in the stem with 6×10^8 cfu ml^{-1} *Pcb* suspension. Data on disease incidence was collected after one week. Analysis of variance on $\log_{10}$ transformed disease incidence data showed no significant differences among cultivars ($P > 0.05$). All the cultivars showed typical blackleg symptoms and were susceptible to blackleg caused by *Pcb*.

The objective of experiment III was to evaluate the biochemical defence mechanisms of the five cultivars by analysing the activities of two defence enzymes, polyphenol oxidase (PPO) and peroxidase (POD) in response to pathogen infection and mechanical damage. The experiment was arranged in a Completely Randomised Design with a 5*6 factorial treatment structure replicated twice. The moderately resistant cultivar Montclaire was used as the control. Six week old plants were stem inoculated with 6×10^8 cfu ml^{-1} *Pcb* or wounded by cutting off the plant's apical bud. Enzyme assays were conducted on leaf samples taken at 0, 12, 24, 48, 84 h and one week after treatments. Four leaves from the middle of the plant were excised using the destructive sampling method. Results showed there was no significant difference on PPO and POD activity in inoculated and wounded plants for both seasons ($P > 0.05$). The interaction between time and cultivar had a significant effect on enzyme activity ($P < 0.05$) with differences in POD activity observed at 12, 24 and 84 h at which Montclaire showed the highest enzyme activity, Amethyst, BP1 and Jasper were intermediate with KY20 exhibiting the lowest enzyme activity. For PPO, the interaction between cultivar and time was significant in 2012 only with Montclaire showing a higher activity than the other cultivars at 12, 24 and 84 h. These results suggest that both pathogen infection and mechanical damage can induce PPO and POD although the level of induction depends on other factors such as cultivar and time after treatment.

Experiment IV was carried out find out if foliar sprays of salicylic acid (SA) and acibenzolar-s-methyl (ASM) can cause an induction of PPO and POD. The above mentioned five cultivars were used. The experiment was a Completely Randomised Block Design with a 5*3*3 factorial treatment structure. Replication was done across blocks. In the three treatment groups, four week old plants were sprayed with 1.5 mM SA; 100 mg active ingredient/litre (a.i/l) ASM and water (control) till run-off. Sampling was carried out as in experiment III and assays were done at 0, 3 and 6 days after treatments. Results showed that cultivars had a significant effect on PPO and POD activity ($P < 0.05$). The interaction between treatment and time showed a significant effect on enzyme activity ($P < 0.05$). The control treatment exhibited enzyme activities higher or equal to SA and ASM treatments. Results suggest that 1.5 mM SA and 100 mg a.i/l ASM applied as foliar sprays were not effective in enhancing PPO and POD activity.

Control of soft rot pathogens using on-farm cultural practices still remains an effective management strategy to reduce losses from soft rots. Defence enzymes alone were not enough to protect the plants as all the cultivars proved susceptible to blackleg caused by *Pcb*. With *Dickeya* species now the predominant soft rot pathogen in the area under study, an understanding of these plant-pathogen systems enables the implementation of appropriate control measures in order to minimise losses.

LIST OF FIGURES

Figure 1: The location of farms where samples were collected..... 21

Figure 2: Characterisation of strains isolated on farms with PCR amplification using *Dickeya* spp specific primers ADE 1 and ADE 2. Lanes M, DNA molecular marker; C, negative control (water); 1-8, isolates. 26

Figure 3: Blackleg symptoms on six-weeks old potato plants after artificial inoculation of stems with *P. carotovorum* subsp. *brasiliensis* in cv.Amethyst (a); cv. BP1 (b); cv. Jasper (c); cv. KY20 (d); cv. Montclaire (e). 33

Figure 4: The effect of treatments on polyphenol oxidase (PPO) activity assayed in the leaves of wounded and inoculated potato cultivars..... 39

Figure 5: The effect of treatments on peroxidase (POD) activity assayed in the leaves of inoculated and wounded potato cultivars. 40

Figure 6: The interaction effect between time and cultivar on peroxidase activity assayed in the leaves of five potato cultivars harvested at different time intervals in 2012 and 2013. 41

Figure 7: The interaction effect between time and cultivar on polyphenol oxidase activity assayed in the leaves of five potato cultivars harvested at different time intervals in 2012 and 2013..... 42

Figure 8: Time course of polyphenol oxidase in potato plants following treatments with water (control), salicylic acid (SA) 1.5 mM and acibenzolar-s-methyl (ASM) 100 mg a.i/l at 0, 3 and 6 days after foliar sprays 49

Figure 9: Time course of peroxidase activity in potato plants following treatments with water (control), salicylic acid (SA) 1.5 mM and acibenzolar-s-methyl (ASM) 100 mg a.i/l at 0, 3 and 6 days after foliar sprays. 50

LIST OF TABLES

Table 1: List of primers used in the study..... 23

Table 2: Biochemical tests for the identification of soft rot pathogens isolated from farms around Harare..... 24

Table 3: Blast search results for gyraseB and recA gene sequences of isolates from potato 26

Table 4: The susceptibility of five potato cultivars to blackleg measured based on disease incidence (log_{10} transformed data) caused by the inoculated soft rot pathogen *P. c.* subsp. *brasiliensis* 34

Table 5: The levels of peroxide (POD) and polyphenol oxidase (PPO) enzymes measured in the leaves of different potato cultivars after spraying with salicylic acid and acibenzolar-s-methyl 48

LIST OF APPENDICES

Appendix 4.1: Analysis of variance on the response of different cultivars to inoculation with *Pectobacterium carotovora* subsp. *brasiliensis* based on blackleg disease incidence (log_{10} transformed data) (Experiment 1)……..68

Appendix 4.2: Analysis of variance on the response of different cultivars to inoculation with *Pectobacterium carotovora* subsp. *brasiliensis* based on blackleg disease incidence (log_{10} transformed data) (Experiment 2)……..... 68

Appendix 5.1: Analysis of variance on the effect of pathogen inoculation and mechanical damage on polyphenol oxidase activity assayed in different potato cultivars at different time intervals (2012)…….……69

Appendix 5.2: Analysis of variance on the effect of pathogen inoculation and mechanical damage on polyphenol oxidase activity assayed in different potato cultivars at different time intervals (2013)……..……..……..69

Appendix 5.3: Analysis of variance on the effect of pathogen inoculation and mechanical damage on peroxidase activity assayed in different potato cultivars at different time intervals (2012)..……..……70

Appendix 5.4: Analysis of variance for peroxidase activity assayed in different potato cultivars at different time intervals following pathogen inoculation and mechanical damage (2013)……70

Appendix 6.1: Analysis of variance on the effect of salicylic acid and acibenzolar-S-methyl treatments on polyphenol oxidase assayed in different potato cultivars at different time intervals (Experiment 1)…….…….……..71

Appendix 6.2: Analysis of variance on the effect of salicylic acid and acibenzolar-S-methyl treatments on polyphenol oxidase assayed in different potato cultivars at different time intervals (Experiment 2)……..……..71

Appendix 6.3: Analysis of variance on the effect of salicylic acid and acibenzolar-S-methyl treatments on peroxidase assayed in different potato cultivars at different time intervals (Experiment 1)…….....72

Appendix 6.4: Analysis of variance on the effect of salicylic acid and acibenzolar-S-methyl treatments on peroxidase assayed in different potato cultivars at different time intervals (Experiment 2)…….…..72

LIST OF ABBREVIATIONS AND SYMBOLS

ANOVA	Analysis of variance
ASM	Acibenzolar-s-methyl
Avr	Avirulence
ABA	Aminobutyric acid
CVP	Crystal Violet Purple
Dd	*Dickeya dadantii*
DNA	Deoxyribonucleic acid
ET	Ethylene
INA	Isonicotinic acid
JA	Jasmonic acid
LSD	Least significant difference
NA	Nutrient Agar
NaCl	Sodium chloride
Pa	*Pectobacterium atrosepticum*
PAL	Phenylalanine ammonia-lyase
Pcb	*Pectobacterium carotovorum* subsp. *brasiliensis*
Pcc	*Pectobacterium carotovorum* subsp. *carotovorum*
PCR	Polymerase chain reaction
PG	Polygalacturonase

PL	Pectate lyase
PME	Pectin methyl esterase
PnL	Pectin lyase
POD	Peroxidase
PPO	Polyphenol oxidase
PPP	Phenylpropanoid pathway
PR	Pathogenesis-related
PVP	Polyvinylpyrrolidone
QTL	Quantitative trait loci
R-gene	Resistance gene
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
YDC	Yeast extract-dextrose-$CaCO_3$ medium
a.i/l	active ingredient per litre
cfu ml^{-1}	colony forming units per millilitre
h	hour
mg	milligram
mM	millimolar
nm	nanometer

TABLE OF CONTENTS

ACKNOWLEDGEMENTS i

DEDICATION ii

ABSTRACT iii

LIST OF FIGURES iv

LIST OF TABLES v

LIST OF APPENDICES vi

LIST OF ABBREVIATIONS AND SYMBOLS viii

TABLE OF CONTENTS x

CHAPTER 1: INTRODUCTION 1

1.1 Background 1

1.2 Justification 2

1.3 Main objective 3

1.3.1 Specific objectives 3

1.3.2 Research hypotheses 4

1.4 Chapter outline 4

CHAPTER 2: LITERATURE REVIEW 5

2.1 Introduction 5

2.2 Ecological and epidemiological characteristics 6

2.3 Host range and disease expression 7

2.4 Pathogenesis of soft rot pathogens 8

2.5 The role of cell wall degrading enzymes 8
2.6 Identification and detection of soft rot pathogens 9
2.7 The polymerase chain reaction technique 9
2.8 Control of soft rot pathogens 10
2.9 Genetic resistance as a tool of disease control 11
2.10 The nature of resistance in the *Solanaceae* family to soft rot bacteria 12
2.11 Current status of resistance of potato cultivars to *Pectobacterium* and *Dickeya* species . 12
2.12 Why screening for soft rot resistance across potato cultivars is difficult 13
2.13 How plants respond to infections 13
2.14 The role of the phenylpropanoid pathway in plant defence systems 14
2.15 The important enzymes in the phenylpropanoid pathway 14
2.16 Factors of resistance within potato cultivars 16
2.16.1 The cell wall structure……16
2.16.2 Pectin methylation…… 16
2.17 Distinguishing between resistant and susceptible cultivars 17
2.18 Resistance inducing chemicals as a tool for disease control 17

CHAPTER 3: Identification of soft rot pathogens causing tuber soft rot and blackleg symptoms in potatoes grown on farms in peri-urban Harare area 19
ABSTRACT 19
3.1 INTRODUCTION 20
3.2 MATERIALS AND METHODS 20
3.2.1 Collection of samples 20
3.2.2 Bacterial isolations 21
3.2.3 Biochemical identification and classification of strains 22
3.2.4 Pathogenicity tests 22

3.2.5 Isolation of bacterial DNA for PCR identification 22
3.2.6 PCR amplification 22
3.2.7 Sequencing and phylogenetic analysis 23
3.3 RESULTS 24
3.3.1 Results of biochemical tests 24
3.3.2 Pathogenicity tests 24
3.3.3 PCR test results 25
3.3.4 Sequencing 26
3.4 DISCUSSION 27
3.5 CONCLUSION AND RECOMMENDATION 29

CHAPTER 4: The response of different potato cultivars to *Pectobacterium carotovorum* subspecies *brasiliensis* causal agent of blackleg 30
ABSTRACT 30
4.1 INTRODUCTION 31
4.2 MATERIALS AND METHODS 31
4.2.1 Pathogenicity tests 31
4.2.2 Experimental design and analysis 32
4.3 RESULTS 32
4.3.1 Blackleg disease expression 32
4.3.2 Disease incidence across cultivars 33
4.4 DISCUSSION 34
4.5 CONCLUSION AND RECOMMENDATION 35

CHAPTER 5 Induction of polyphenol oxidase (PPO) and peroxidase (POD) enzymes in different potato cultivars (*Solanum tuberosum*) as a result of pathogen inoculation and mechanical ……36
ABSTRACT 36

5.1 INTRODUCTION 36
5.2 MATERIALS AND METHODS 37
5.2.1 Host plants 37
5.2.2 Bacterial culture 38
5.2.3 Treatments 38
5.2.4 Enzyme assays 38
5.2.5 Statistical analysis 39
5.3 RESULTS 39
5.3.1 Effect of treatments on enzyme activity 39
5.3.2 The effect of cultivar and time on enzyme activity 40
5.3.3 Peroxidase activity 40
5.3.4 Polyphenol oxidase 41
5.4 DISCUSSION 42
5.5 CONCLUSION 45

CHAPTER 6: The use of salicylic acid and acibenzolar-s-methyl to induce resistance in different potato cultivars through the activation of polyphenol oxidase and peroxidase 46
ABSTRACT 46
6.1 INTRODUCTION 46
6.2 MATERIALS AND METHODS 47
6.2.1 Enzyme assays 47
6.3 RESULTS 48
6.3.1 Enzyme activity in different cultivars 48
6.3.2 Effect of time and treatments on enzyme activity 49
6.4 DISCUSSION 50
6.5 CONCLUSION AND RECOMMENDATION 52

CHAPTER 7 53

GENERAL DISCUSSION 53

REFERENCES 56

APPENDICES 72

CHAPTER 1: INTRODUCTION

1.1 Background

The potato (*Solanum tuberosum* L.) has a significant global importance. It is the most important non-grain food crop in the world (Potato Genome Sequencing Consortium, 2011; Alyokhin et al., 2012; DeFauw et al., 2012) and is currently the fourth main food crop globally after rice (*Oryza sativa* L.), maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) with regards to both total production volumes and area cultivated (Krauss, 2008; King and Slavin, 2013). Before the 1990s, potato cultivation was mainly concentrated in Europe, North America and some parts of the former Soviet Union. In recent years though, there has been a big change in the geographical areas of production. As its worldwide importance continues to increase, global output increased from 30 million tonnes recorded in 1960 to 320 million tonnes in 2008 (Krauss, 2008) and exceeded 329 million tonnes in 2009 (Potato Genome Sequencing Consortium, 2011; DeFauw et al., 2012). There has been a tremendous increase in demand and production in Asia, Africa and Latin America with half of the global production coming from these developing countries (Alyokhin et al., 2012; DeFauw et al., 2012). Average production statistics from 2012 to 2013 indicate that Asia is now the highest producer accounting for 49.1% of global production, with Europe producing 30.9%, North and South America 11.6% and Africa 8%. Currently, China is the largest single producer (94 million tonnes), followed by India (43 million tonnes) and Russia (30 million tonnes) (FAOSTAT, 2015). Several factors have contributed to this rise in potato cultivation especially in the developing world.

Within the global food system, the potato forms an integral part which is central to food security (Potato Genome Sequencing Consortium, 2011). In developing countries, potatoes are an important crop for both food security and poverty alleviation (Krauss, 2008; King and Slavin, 2013). It is highly nutritious, containing more starch per hectare than any other crop and is second to soybeans in protein content (Oerke and Dehne, 2004). The potato has several cultivars that vary in many aspects making them suitable for cultivation in a wide range of climates and conditions and this has made its production by low income farmers possible. The high yielding capacity of the potato per given area also ensures that even small scale farmers can achieve enough yields to satisfy their own food requirements to meet subsistence and income goals (Krauss, 2008). Even

the Zimbabwean government acknowledged the potential of tuber crops in complimenting grain as a food source to meet both national and household food security (Agricultural Research Extension, 2007). Given its many attributes, it is not surprising that potato production has continued to increase considerably although constraints such as pests and diseases remain unavoidable.

Among the potato pathogens, the soft rot bacterial pathogens are the most important bacterial pathogens of potatoes and vegetables (Zolobowska and Pospieszny, 1999; Czajkowski et al., 2011) mainly because they are virulent under all climatic conditions (Pérombelon, 1988). Soft rot pathogens fall under the genera *Pectobacterium* and *Dickeya*. They cause rotting by producing cell wall degrading enzymes which cause tissue maceration (Pitman et al., 2008), resulting in economic losses during the various stages of crop production (Reeves et al., 1999; Ren et al., 2000; Duarte et al., 2004; Diallo et al., 2009). Chemical control of soft rot pathogens has not yet been achieved (Bhat et al., 2010). Reduction of losses over the past years can be attributed to improved crop management systems. Soft rot pathogens are best managed using an integrated approach, using cultural methods such as clean seed, good handling and sanitary practice and planting tolerant varieties (Snijder and van Tuyl, 2002; Pérombelon, 2002; Smid et al., 1995). Another approach that has been indicated as promising for controlling post-harvest losses is using resistance inducing chemicals such as salicylic acid (SA) and acibenzolar-S-methyl (ASM) which induce resistance in plants against a wide range of pathogens (Baysal et al., 2005; Oostendorp et al., 2001).

1.2 Justification

Climate change has caused a shift in food systems in Southern Africa. Decreased annual rainfall has caused a decline in maize crop production in Lesotho, South Africa, Mozambique and Zimbabwe. This reduction in food security in Southern Africa has caused African policy makers to look elsewhere (Scott et al., 2013). The potato has captured great attention as a crop that can contribute to enhanced food security and poverty reduction both at the national and household level. This work is motivated by the threat posed to potato production in Zimbabwe and the region in general, by soft rots. Although blackleg and tuber soft rot used to be predominantly important diseases in the temperate regions, they have increased in their incidences in the tropics and sub-tropics since the climate in these regions favour bacterial growth and multiplication. The need for more food has led to the high demand of potatoes in the region, with Zimbabwe processors and

retailers importing potato from countries such as South Africa to make up for the local deficit (Makoni et al., 2014). This movement of agricultural produce has implications in the spread of diseases. Soft rot pathogens are already a major challenge in the region. In South Africa, potato blackleg caused severe economic losses in commercial potato production during the 2006/2007 potato growing season (van der Merwe et al., 2009) and losses due to *Dickeya dadantii* in Zimbabwe can reach up to 60% (Ngadze et al., 2010). This calls for more research and development of potato based systems to prevent increased losses in the future.

The focus of this study was to add to the knowledge of the use of cultural methods to manage these pathogens since there is no chemical control. Experiments were done firstly, to identify the soft rot species present in the area under study. Different locally grown cultivars were screened for resistance to soft rot pathogens and the level of defence enzymes in these cultivars measured to establish if there is any correlation between the level of defence enzymes in a cultivar and the overall resistance of that cultivar to the pathogen. Lastly, experiments were conducted to assess how application of resistance inducing compounds could influence the production of plant defence enzymes.

1.3 Main objective

The main objective of this study was to evaluate the resistance of different potato cultivars to soft rot pathogens.

1.3.1 Specific objectives

The specific objectives of this study were to:

1. Identify, using standard biochemical tests, a polymerase chain reaction and sequencing, the soft rot species presumed to be the cause of potato soft rot and blackleg affecting potatoes in commercial farms around the Harare area in Zimbabwe.
2. Assess the variation in blackleg resistance among local potato cultivars currently available to farmers namely Amethyst, BP1, Jasper, KY20 and Montclaire using the stem inoculation method.
3. Evaluate the defence mechanisms of five potato cultivars namely Amethyst, BP1, Jasper, Montclaire and KY20 in response to pathogen inoculation and mechanical damage by analysing the changes in the activities of two defence-related enzymes, polyphenoloxidase (PPO) and peroxidase (POD).

4. Evaluate the potential of acibenzolar-s-methyl (ASM) and salicylic acid (SA) in protecting locally grown potato cultivars against soft rot by analysing the induction of two defence related enzymes, PPO and POD to assess a possible relationship between the activation of these enzymes and the use of ASM and SA.

1.4 Research hypotheses

1. The soft rot pathogen species associated with soft rot and blackleg diseases can be isolated from plants showing soft rot and blackleg symptoms.
2. All cultivars will show different levels of resistance to the inoculated soft rot pathogen.
3. Wounding and pathogen inoculation will increase the activities of PPO and POD in potato.
4. Acibenzolar-s-methyl and salicylic acid will cause increased levels of PPO and POD activity in the treated plants.

1.5 CHAPTER OUTLINE

Chapter 2 Reviews current knowledge on potato soft rot. This chapter gives the classification and identification of the different soft rot pathogen species, how their ecological and epidemiological characteristics differ with species and factors that lead to disease development. Since there is no chemical control of these pathogens, control is through cultural practices. In this chapter, genetic resistance as found in different cultivars and the use of resistance inducing chemicals are highlighted as some of the control strategies.

Chapter 3 The objective of this chapter is to explain how the identification of the soft rot pathogens found in the area under study was carried out, detailing experiments carried out to isolate the bacteria, and the various biochemical and molecular tests used to identify the bacterial species.

Chapter 4 Planting tolerant cultivars can reduce losses due to soft rot pathogens. An experiment to screen five locally grown cultivars for resistance to blackleg using the stem inoculation method is described in this chapter.

Chapter 5 Plant defence enzymes are produced by plants in response to biotic and abiotic stresses as part of the plant's natural defence system. This chapter outlines how

enzyme activity changes in different cultivars as they respond to mechanical damage and inoculation with a pathogen.

Chapter 6 The focus of this chapter was on how two resistance inducing compounds, acibenzolar-s-methyl and salicylic acid can alter the activity of plant defence enzymes and add to the overall resistance of the plant.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Pectobacterium and *Dickeya* pathogens are pectinolytic enterobacteria, formerly belonging to the genus *Erwinia* before their reclassification based on 16S-23S DNA sequences into several species and subspecies (Pitman et al., 2008; Laurila et al., 2008). They are major pathogens throughout

the world (Diallo et al., 2009). Both species have several subspecies, with different hosts and climatic requirements favouring their growth and pathogenicity and this has led to their presence in a large geographical area (Elphinstone, 1988). Resultantly, these pathogens can be found anywhere where potatoes are grown, hence their world-wide distribution (Abo-Elyousr et al., 2010; Carputo et al., 1997).

2.2 Ecological and epidemiological characteristics

The *Pectobacterium* genus (formerly *Erwinia carotovorum*, Winslow et al., 1920) was divided into five species; *atrosepticum*, *carotovorum*, *betavasculorum*, *odorifera* and *wasabie* (Duarte et al., 2004). These species show host specificity and geographical preference (De Boer, 2003). *Pectobacterium atrosepticum* (*Pa*), is found almost exclusively on potato in temperate regions (Ma et al. 2007; Czajkowski et al., 2011; Duarte et al. 2004) and its optimum temperature is around 15°C (Pérombelon, 1988). *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) has a broad host range, occurring on many plant species and is pathogenic across all temperature ranges (Peltzer and Sivasithamparam, 1985) with an optimum of 25°C (Pérombelon, 1988). *Pectobacterium*. *betavasculorum*, *P*. *wasabie* and *P*. *odiferum* are found on sugar beet, horseradish and chicory respectively although *P*. *wasabie* has also been isolated from potato (Pitman et al., 2008). Another *P*. *carotovorum* strain, *P*. *carotovorum* subsp. *brasiliensis* (*Pcb*), which is more virulent than *Pcc* and *Pa*, was isolated from potato (Marquez-Villavicencio et al., 2011). It has been isolated in other countries such as Brazil (Duarte et al., 2004), South Africa (van der Merwe et al., 2009) and Zimbabwe (Ngadze et al., 2012).

The *Dickeya* genus (formerly *E*. *chrysanthemi*, Buckholder et al., 1953) was divided into six species; *dieffenbanchiae*, *chrysanthemi*, *dianthicola*, *zeae*, *paradisiaca* and *dadantii* (Tsror (Lahkim) et al., 2008; Toth et [1]al., 2011). *Dickeya* pathogens affect a wide range of crops including ornamentals, banana, tomato and potato (Toth et al., 2011). These species are pathogenic in tropical, subtropical and warm climates (Laurila et al., 2010; Tsror (Lahkim) et al., 2008) and their optimum temperatures are >30°C (Pérombelon, 1988). The important *Dickeya* species which cause disease on potatoes in warm climates are *D*. *dadantii* and *D*. *zea* (Tsror (Lahkim) et al., 2008; Toth et al., 2011) while *D*. *dianthicola* is dominant in temperate climates (Laurila et al., 2008).

[1] Part of the literature review was accepted for publishing by the African Journal of Agricultural Research

Dickeya species occurrence differs from one continent to the other. *Dickeya chrysanthemi* has been reported in Spain (Palacio-Bielsa et al., 2006), *D. zea* in Israel (Tsror (Lahkim) et al., 2008) and *D. dadantii* in Zimbabwe (Ngadze et al., 2010). There is also a cold-tolerant strain, which is adapted to lower temperatures than other *Dickeya* species (Pitman et al., 2008; Czajkowski et al., 2011). Another strain, *D. solani,* virulent at higher temperatures compared to the other six *Dickeya* species has also been isolated from potato in many European countries such as France, Finland, Poland, Netherlands and Israel (Toth et al., 2011; Czajkowski et al., 2011).

2.3 Host range and disease expression

Pectobacterium and *Dickeya* species cause diseases in many identical hosts but their host ranges do not completely overlap (Ma et al., 2007). Of the 21 dicot and 16 monocot plant families reported as hosts of either *Pectobacterium* or *Dickeya*, 6 dicot and 4 monocot families have been reported as hosts for both genera. The lack of overlap has been noted at higher levels of classification, for example, *Pectobacterium* species have been recorded in avocado (magnoliids clade species) but there are no reports of *Dickeya* causing disease in magnoliids. Because of a few reports on some clades, this could have caused a lack of overlap in their host ranges (Ma et al., 2007).

Pectobacterium and *Dickeya* pathogens have similar blackleg and tuber soft rot symptoms, which are difficult to distinguish in the field (Diallo et al., 2009). However, symptom and disease expression by each pathogen depends on prevailing weather conditions as these determine their successful establishment or infection. Cool temperate climates favour wilting with low stem rot incidence, wet and humid conditions in the tropics promote extensive stem and tuber rotting with less desiccation while hot and dry conditions result in wilting, stunting and desiccation (Laurila et al., 2010; Tsror (Lahkim) et al., 2008; Palacio-Bielsa et al., 2006).

In cool temperate climates, *Pa* causes blackleg in the field and soft rot in storage (Pitman et al., 2008; Laurila et al., 2008). *Pectobacterium carotovorum* subsp. *carotovorum* is the main cause of tuber soft rot (Pérombelon, 2002) although it can cause typical blackleg symptoms in the field (de Haan et al., 2008). *Pectobacterium carotovorum* subsp. *brasiliensis* is a highly virulent soft rot pathogen that causes severe soft rot and blackleg in humid, subtropical climates at temperatures of around 17^{o}C to 20^{o}C (van der Merwe et al., 2009).

Genomic studies in *Pcc*, *Pcb* and *Pa* strains revealed that these taxa share 77 to 81% of their genes, with *Pa*, *Pcc* and *Pcb* accounting for 18%, 11% and 13% of these genomes respectively (Marquez-Villavicencio et al., 2011). These genes include those homologous to regulatory, cell wall degrading enzymes and toxin encoding genes. Differences in host range and virulence among these strains can be explained by differences in these genes.

Dickeya species have been known to cause soft rot and blackleg at higher temperatures or in the humid and sub-tropical climates (Pérombelon, 2002). However, *Pcc* and *Dickeya* species also cause similar symptoms at temperatures above 25^{o}C (Duarte et al., 2004). There are some cold tolerant strains that cause diseases in the temperate regions.

2.4 Pathogenesis of soft rot pathogens

A common feature of soft rot pathogens is that they cause pectin hydrolysis (Lionetti et al., 2007). Soft rot pathogens are able to macerate host tissue by secreting cell wall degrading enzymes, the main ones being pectin lyase (PnL), polygalacturonases (PG), pectin methyl esterase (PME) and several isoforms of pectate lyase (PL) (Flego et al., 1997; Pérombelon, 2002; Juge, 2006).

These enzymes have several isoforms encoded by different genes. Pectin lyase enzymes break down the pectin content of adjacent cell walls leading to the disintegration of cells. This creates an osmotic pressure within the detached cells causing an ex-osmosis of salts and sugars into the intercellular spaces. Bacteria latently present in the plant tissue will feed on these nutrients, furthering their growth and numbers. Once the cells reach a threshold value of 10^{7}cells g^{-1} of diseased tissue, rotting begins as the bacteria produce more enzymes which advance maceration (Bhat et al., 2010; Pérombelon, 2002).

2.5 The role of cell wall degrading enzymes

The cell wall is composed of methylated polygalacturonic acid chains linked to neutral sugars (Conway et al., 1992). To break down these linkages, the bacteria produce pectin methyl esterase (PME), which transforms pectin to pectic acid (Kotoujansky, 1987). Pectin methyl esterase catalyses the de-methylation of the pectin, followed by PG, which prefers the de-methylated pectin. These two enzymes catalyse the splitting of the polygalacturonic acid chains by either hydrolysis or elimination (Bhat et al., 2010).

Pectin lyase and pectate lyase are important in the hydrolysis of pectin and pectate respectively. Pectin lyase cleave pectin of the de-methylated polygacturonic chains and pectate lyase cuts the α1, 4 glycosidic bond at random within the polygalacturonic acid chains by β-elimination (Kotoujansky, 1987; Dorrel et al., 1996).

2.6 Identification and detection of soft rot pathogens

The characteristics common to all soft rot pathogens are that they are Gram negative, non-sporing, facultative anaerobes and produce large quantities of extracellular pectic enzymes, together with other cell degrading enzymes. They are non-pigmented straight rods mainly single with peritrichous flagellae (Bhat et al., 2010; Czajkowski et al., 2011). Soft rot bacteria generally grow well in most laboratory media, producing white colonies on nutrient agar and cloudy growth in nutrient broth.

Identification of specific species is based on standard biochemical tests are used. These tests permit the differentiation of all sub-species but can only be applied to pure cultures. Classical methods based on selective media have also proved to be efficient tools for the identification and enumeration of soft rot *Pectobacterium* but are laborious and time consuming. The other disadvantage of classical methods based on isolation is that they are inadequate for determining the persistence of low bacterial numbers in the environment (De Boer and Ward, 1995). Therefore, molecular methods with the required degree of specificity, sensitivity and user friendliness were developed for testing potato seed stocks for *Pa.*

Polymerase chain reaction based methods using specific primers appear to be the most satisfactory method of detection because they are the most rapid and allow detection regardless of serogroup. Polymerase chain reaction technology and gel electrophoresis have been used to detect specific bacteria (Yamamoto and Harayama, 1995).

2.7 The polymerase chain reaction technique

The polymerase chain reaction (PCR) makes use of polymerase enzymes, which copy and proofread genetic material and then correct the copies. It requires a template DNA molecule and two primer molecules to get the copying process started. The detection of the specific genes highly depends on the specificity of the primer sets used (Yamamoto and Harayama, 1995).

There are three basic steps in PCR. Firstly, the target genetic material is denatured and the double helix is unwound by heating. The mixture is then cooled and the primers added to the mixture. The primer defines the boundaries of the target section during annealing. DNA polymerase is added to the mixture and it adds the corresponding base pair to the strand, creating two identical copies of target DNA and this process is known as extension (Mullis et al., 1995; Pérombelon and van der Wolf, 2002). For high precision, *Pyrococcusfurioscus* (*Pfu*) polymerase is used as it incorporates a step known as proofreading, where it attempts to remove incorrectly bonded molecules and fix such mistakes. The process is repeated to get more DNA.

2.8 Control of soft rot pathogens

The basis of control of soft rot pathogens lies mainly in the avoidance of the pathogen or the disease, eradication of the pathogen and development of resistance (Elphinstone, 1988). It is difficult, however, to avoid contamination by the pathogen because latent infections are almost always unavoidable and symptoms only appear later in the season during the growing cycle (Snijder and van Tuyl, 2002).

Although several chemical and physical methods have been used against *Pectobacterium* and *Dickeya* species, effective control of blackleg and soft rot has not yet been achieved. The liquid disinfectants that are used to reduce infection outside tubers are not effective against bacteria inside the tuber. Treatment of potatoes in storage with bactericides with a systemic action is not effective because tubers in storage no longer possess an active translocation system (Elphinstone, 1988; Czajkowski et al., 2011). Although antibiotics are effective against these pathogens, the risk of introducing resistance to pathogens has led to their ban in crop protection (Czajkowski et al., 2011; Pasco et al., 2006). Tissue culture allows for the production of pathogen-free microplants and subsequently bacteria-free minitubers. Bulking of the disease-free seed stock is then done in the field. It is after two or three field generations that the seed becomes progressively more contaminated around the third year (Czajkowski et al., 2011). Another alternative control method is biological control, which uses antagonist plant pathogens to suppress other pathogen populations directly through production of various anti-bacterial metabolites, competition for nutrients or induction of systemic acquired resistance. *In-vitro* experiments have established potential candidates for bio-control of soft rot pathogens like *Pseudomonas* species and lactic acid bacteria. The major setback in the use of these bio-agents has been the difficulty in getting an antagonist

which satisfies several criteria. It has to reach its target and in this case the bacteria are located in the lenticels and vascular system which are not readily available. Another challenge has been the preparation of a stable formulation which can still remain effective when there are variations in field weather (Czajkowski et al., 2011).

Reduction of losses over the past years can be attributed to improved crop management systems. Soft rot pathogens are best managed using an integrated disease control approach, using cultural methods such as clean seed, good handling and sanitary practices and planting tolerant varieties (Snijder and van Tuyl, 2002; Pérombelon, 2002; Smid et al., 1995). Use of different physical, chemical or biological control strategies has led to a reduction, but never total elimination of the pathogens (Czajkowski et al., 2009). Genetic resistance presents the best method to control bacterial diseases that have no chemical control (Hélias et al., 2000).

2.9 Genetic resistance as a tool of disease control

Genetic resistance is a management strategy that can be easily adopted by farmers because the control is simply found within the seed of a resistant cultivar. There are two major types of genetic resistance in plants, namely qualitative (monogenic) and quantitative (polygenic) resistance (Gebhardt and Valkonen, 2001; Poland et al., 2008; Kröner et al., 2011). These two types of resistances co-exist in potato genotypes (Ewing et al., 2000). Qualitative resistance is based on the theory that for every avirulence (Avr) gene from the pathogen, the plant produces a corresponding resistance gene (R-gene) due to elicitor recognition. The R-genes will then induce defence mechanisms controlled by many other genes. This type of defence usually results in complete resistance which is evidenced by the absence of the disease (Yang et al., 2013).

The quantitative mode of resistance is controlled by multiple genes or polygenes of minor gene complexes (Gebhardt and Valkonen, 2001; Lindhout, 2002; Poland et al., 2008). Genes that control quantitative resistance are located in genomic regions called quantitative trait loci (QTL) (Lindhout, 2002; Poland et al., 2008). The QTLs may form clusters of structurally related R-genes that confer resistance to the same or different pathogens. The individual genes that control quantitative resistance have a small and inconsistent effect to the overall resistance, which also depends upon the interaction of these genes with the environment (Gebhardt and Valkonen, 2001; Poland et al., 2008). This resistance is partial and leads to a reduction in the severity of the disease, as opposed to its absence.

Although no potato cultivar has been reported to be immune to soft rot pathogens, partial resistance exists. Since partial resistance has been reported to exist (Bain and Pérombelon, 1988; De Maine et al., 1998), evaluation of such cultivars will provide a basis for the cultural control against these pathogens through the use of tolerant cultivars.

2.10 The nature of resistance in the *Solanaceae* family to soft rot bacteria

The two most important bacterial diseases of potato are tuber soft rot and stem blackleg (Zolobowska and Posieszny, 1999; Gebhardt and Valkonen, 2001). No source of qualitative resistance exists to soft rot pathogens. However, quantitative resistance has been observed in both wild and cultivated potato species against soft rot pathogens (Pasco et al., 2006). Quantitative trait loci for tuber and foliar resistance to *P. atroseptica* were obtained from crossing *S. tuberosum* with *S. chacoense* and *S. yungasense*. From the F_1 hybrid population, loci for genetic factors affecting resistance were found on all 12 potato chromosomes, with chromosome I having the most reproducible effect on tuber resistance (Gebhardt and Valkonen, 2001; Vreugdenhil et al., 2011).

2.11 Current status of resistance of potato cultivars to *Pectobacterium* and *Dickeya* species

Pasco et al., (2006) noted that cultivars with resistance do exist but the resistance is partial. Several cultivars have been screened in different countries and differences in resistance noted (Bain and Pérombelon, 1988) but there is no data showing resistance across cultivars (Degefu etal., 2013). There are some variations in susceptibility to *Pectobacterium* and *Dickeya* pathogens in S. *tuberosum* subsp. *tuberosum* cultivars (commercial cultivars) being grown, with very few of them being classified as highly resistant (De Maine et al., 1998). The level of resistance of these commercial cultivars to soft rot is relatively low (Lebecka and Zimnoch-Guzowska, 2004).

Very high levels of resistance to soft rot pathogens are found in the germplasm of wild non-*tuberosum* species such as *S. andigena, S. tarijense, S. canasense, S. stenomum* and *S. brevidens* though none of them has been commercialised (Huaman et al., 1988; De Maine et al., 1998; Czajkowski et al., 2011). Introgression of these wild species with commercial cultivars is difficult because of factors such as the complexity, incompatibility and the risk of carrying over undesired traits into progeny seed (Pasco et al., 2006; Czajkowski et al., 2011). Crosses between *S. phureja* and *S. tuberosum* produced hybrids that were resistant to soft rot and blackleg but produced low yields. Other examples are hybrids between *S. tuberosum* and *S. chacoense, S. sparsillum* and *S. multidissectum* which were all resistant to both *Pectobacterium* and *Dickeya* pathogens, but their

glycoalkalloid content was toxic to both humans and animals (Czajkowski et al., 2011). Understandably, no cultivar can be said to be completely resistant to *Pectobacterium* or *Dickeya* (Czajkowski et al., 2011).

2.12 Why screening for soft rot resistance across potato cultivars is difficult

The level of resistance among closely related potato species is not very distinct, making it difficult to distinguish between resistant and susceptible cultivars (Huaman et al., 1988). There are no clear cut categories or threshold values and data from most experiments exhibit a normal distribution, which characterises the polygenic nature of soft rot resistance (Lebecka and Zimnoch-Guzowska, 2004; Pasco et al., 2006). Therefore, while some varieties are considered resistant, intermediate or susceptible (Hélias et al., 2000), others changed ranking from experiment to experiment in different countries, depending on other factors such as the inoculation method, oxygen concentration, incubation temperatures and whether it is a field or greenhouse experiment and environmental conditions (Bain and Pérombelon, 1988; De Maine et al., 1998; Czajkowski et al., 2011).

Stem resistance can be used to rank blackleg resistance, (Bains et al., 1999), although the level of blackleg resistance depends on the initial latent infection of the mother tuber (Degefu et al., 2013) but this also differs with cultivars under different field conditions (Smid et al., 1995). However, there is a lack of correlation between soft rot and blackleg resistance which makes it difficult to rank the cultivars. Cultivars resistant to blackleg might not be necessarily resistant to soft rot (Pasco et al., 2006). Some inoculation methods, for example, the tuber slice test used to screen for soft rot resistance bypasses the periderm which would otherwise offer protection against pathogen entry, as opposed to stem inoculations used to screen cultivars for resistance against blackleg (Pasco et al., 2006). This lack of correlation could possibly mean that different factors, mechanisms and genes control resistance in different parts of the same plant (Bain and Pérombelon, 1988; Carputo et al. 1997; Bains et al., 1999). It is therefore very difficult to compare the ranking of different investigators and obtain successive rankings which could otherwise be used to classify cultivars (Lojkowska and Kelman, 1989).

2.13 How plants respond to infections

Plants, including potatoes, respond to infection by producing defence signalling molecules such as jasmonic acid, ethylene and salicylic acid, some of which are produced from the phenylpropanoid

pathway (Lyon et al., 1992; Montesano et al., 2005; Cao et al., 2006). In potato, during an attack of the pectin by the cell wall degrading enzymes produced by soft rot pathogens, some pectic fragments and oligossacharides from the cell wall may act as elicitors (Collmer and Keen, 1986; Baysal et al., 2003; Wegener and Olsen, 2004; Montesano et al., 2005). These molecules regulate plant gene defence expression and eventually lead to the production of defence molecules such as the phenylpropanoid pathogenesis-related (PR) proteins which accumulate in the intercellular spaces. Pathogenesis-related proteins include chitinases, β1,3glucanases and proteases, which have a direct effect on pathogens (Lyon et al., 1992; Baysal et al., 2003). Other responses include the biosynthesis of phytoalexins, proteinase inhibitors and phosphorylation of membrane proteins. Successful defence depends on factors such as the genetic and physiological characteristics of the host and the pathogen, in combination with the environment (Collmer and Keen, 1986; Montesano et al., 2003).

2.14 The role of the phenylpropanoid pathway in plant defence systems

Genes involved in defence reactions against a pathogen have a linkage with the QTL for resistance to that pathogen. In potato, for example, genes encoding phenylalanine ammonia-lyase (PAL) and 4-Coumarate-CoA ligase belong to the same cluster and both are key enzymes in the phenylpropanoid pathway that yields phytoalexins. The phenylpropanoid pathway (PPP) is a biochemical process that yields phenylpropanoid compounds which are important compounds in the plant's natural defence system. The process involves the deamination of L-phenylalanine catalysed by the enzyme phenylalanine ammonia lyase (PAL) (Dixon et al., 2002). Phenylpropanoids are pre-formed inducible compounds, chemical barriers and signal molecules. These compounds form the three classes of natural products that are important in plant defence, namely phytoalexins, phytoanticipins and signal molecules. The enzyme PAL provides precursors for lignin and other PPP secondary plant products involved in resistance. Examples of various soluble PPP derivatives found in potato are chlorogenic, salicylic, and hydroxynnamic acid (Dixon and Paiva, 1995; Sticher et al., 1997)

2.15 The important enzymes in the phenylpropanoid pathway

There are three principal enzymes that catalyse the various reactions of the phenylpropanoid pathway namely PAL, polyphenol oxidase and peroxidase. Significant increases in the levels of these enzymes have been recorded in plants inoculated with various pathogens. Examples are

cacao plants inoculated with the fungus *Phytophthora palmivora* (Okey et al., 1997); potato tubers inoculated with *Pectobacterium atroseptica*, *P. carorotovorum* subsp. *brasiliensis* and *Dickeya* spp (Ngadze et al., 2012); tomato seedlings inoculated with *Fusarium oxysporum* f. sp. *lycopersici* (Rai et al., 2011); banana inoculated with *F. oxysporum* f. sp. *cubense* (Sarwar et al., 2011) and cucumber seedlings damaged by *Bemisia tabaci* (Zhang et al., 2008).

Phenylalanine ammonia lyase is a key enzyme in the phenylpropanoid pathway which provides precursors of lignin and other phenylpropanoid-derived secondary compounds important in disease resistance, for example isoflavonoids, phytoalexins and salicylic acid (Sticher et al., 1997; Li and Steffens, 2002). It catalyses the transformation of phenylalanine to trans-cinnamic acid (Kröner et al., 2011; Ngadze et al., 2012), an important compound in the biosynthesis of phenol and lignin (Chandra et al., 2007; Vanitha et al., 2009). Inhibition of PAL increases disease susceptibility due to the absence of other phenylpropanoids.

Polyphenol oxidase is a nuclear encoded enzyme found in almost every plant. It is important in the early stages of infection where it catalyses the oxidation of phenols to quinones (Okey et al., 1997). Quinones are highly reactive compounds that covalently modify and cross link with other molecules such as carbohydrates, proteins and lipids (Wegener and Olsen, 2004; Melo et al., 2006) via a 1,4 addition mechanism resulting in condensation polymers (Li and Steffens, 2002). The condensation polymers provide additional polymerised phenolic barriers.

Some of the quinone oxidation products create an unfavourable environment for pathogen development because they possess an antibiotic and cytotoxic effect (Łojkowska and Kelman, 1989; Okey et al., 1997). They undergo reverse disproportionation to semi-quinones resulting in the production of reactive oxygen species (ROS) like hydrogen peroxide. Reactive oxygen species have a direct antimicrobial effect and they also activate other defence responses, for example, the hydrogen peroxide produced by PPO catalysed phenolic oxidation can drive other reactions through the action of peroxidases (Li and Steffens, 2002). Moreover, quinones further inhibit pathogen growth by limiting the bioavailability of certain proteins like histidine through alkylation with those amino acids (Melo et al., 2006).

Peroxidase is important in the last step of lignin biosynthesis (Brisset et al., 2000; Zhang et al., 2008). It catalyses the oxidative polymerisation of hydroxycinnamyl alcohols to yield lignin and the cross-linking of isoditryosine bridges in the cell wall (Ngadze et al., 2012).

2.16 Factors of resistance within potato cultivars

Since the mother tuber carries the source of infection latently, any factors of resistance lie mainly with the characteristics of the tuber, although environmental factors play a role in the disease cycle (Elphinstone, 1988). In potatoes, there is varietal resistance which is associated with certain characteristics within the tubers. Cultivars with high resistance to soft rot and blackleg have high starch content, low levels of reducing sugars (Zimnoch-Guzowska and Łojkowska, 1993; Agrios, 1997; Abo-Elyousr et al., 2010), low levels of electrolyte leakage (Conway et al., 1992), high levels of calcium in the medullary and periderm cells (McGuire and Kelman, 1984; Conway et al., 1992; Flego et al., 1997; Goodwin et al., 2009), high concentration of phenolic compounds and their products (Lyon et al. 1992; Beckman, 2000; Wegener and Olsen, 2004; Melo et al., 2006) and good cell wall structure (Lyon et al., 1992). All these physiological characteristics and cell constituents vary with cultivars (Huaman et al., 1988; Abo-Elyousr et al., 2010).

2.16.1 The cell wall structure

Potatoes have suberin and lignin which bind to the cell wall, enhancing its structural integrity which offers a physical barrier to the entry of the soft rot pathogens (Lyon et al., 1992). Cell wall fortification through lignification offers resistance in various ways. Strengthening of the cell wall by lignin makes it more resistant to degradation by pathogen enzymes, limiting the progression of bacteria as they multiply in the intercellular spaces (Brisset et al., 2000). Since soft rot pathogens do not have enzymes that can degrade pectin or suberin, this completely blocks further penetration by bacteria (Lyon, 1989; Wegener and Olsen, 2004). The barrier provided by lignin also prevents free water and nutrient movement through cells, starving the pathogen. If the invading pathogen is a fungus, lignin may bind to the pathogen leading to lignification of its cell structures. This makes it more rigid and impermeable thus hindering water and nutrient uptake (Sticher et al., 1997).

During blackleg development, bacteria move via the transpiration stream through the xylem vessels of the mother tuber to that of the stem. Variations in the susceptibility of stems of different cultivars may be due to differences in the extent of lignification at the junction between the mother tuber and the stem. Extensive lignifications at such a point would restrict translocation up the stem. In cultivars that are resistant to blackleg and stem rots, lignification tends to develop early and more extensively than in susceptible cultivars (Pérombelon, 2002). Successful defence depends on

how quickly the plant activates the defence system before it is overwhelmed by the pathogen. Resistant cultivars have been shown to have higher levels of defence enzymes (Kröner et al., 2011; Zhang et al., 2008).

2.16.2 Pectin methylation

The pectin in the cell wall complex has methyl and acetyl groups, making it highly esterified (Lionetti et al., 2012). The enzyme, pectin methyl esterase, which is produced by soft rot pathogens, causes the de-methylation of pectin, making pectin more susceptible to pathogen attack as this process collapses the integrity of the cell wall. The degree and pattern of methylation or acetylation therefore has a bearing on the susceptibility or resistance of a plant to degradation (Bateman and Millar, 1966). Resistant genotypes of *S. tuberosum* species inoculated with *Pa* were characterised by a higher content of highly methylated and branched water soluble pectin while susceptible species contained an acidic isoform of PME, which could be important in the demethylation of pectin (Marty et al., 1997).

2.17 Distinguishing between resistant and susceptible cultivars

The resistance of the plant depends on several factors. The combined genetic makeup of both the pathogen and the host, together with their interaction of the environment is important in the plant's defence system. In addition, elicitor recognition and the respective signal transduction pathways also determine the timeliness of defence responses. In general, resistant genotypes have a timely perception of pathogen elicitors which leads to the rapid accumulation of gene transcripts that generate higher levels of defence responses to prevent pathogen colonisation. On the contrary, susceptible cultivars exhibit a much slower response system that fails to restrict pathogen growth and spread, leading to a heavily diseased plant (Yang et al., 2013).

2.18 Resistance inducing chemicals as a tool for disease control

Another approach that has been indicated as promising for controlling post-harvest losses and reducing the severity of diseases caused by plant pathogens is the induction of resistance to infection (Baysal et al., 2003; Cao et al., 2006). The plant's natural defence system consists of pre-formed chemical and mechanical barriers as well as inducible defence systems to protect the plant against attack from various pathogens (Montesano et al., 2003, Petrov and Andonova, 2012). Induction of resistance is based on activating the plant's natural defence system using synthetic low weight molecules (Ghazanfar et al., 2011). Exogenous application of inducer chemicals or

inoculation of pathogens leads to the production of signal chemicals such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Buzi et al., 2004; Montesano et al., 2005, Sarwar et al., 2011). Defence signals then result in a coordinated induction of genes controlling diverse defence pathways such as lignin biosynthesis and expression of pathogenesis-related (PR) proteins (Brisset et al., 2000; Louws et al., 2001; Sarwar et al., 2011). Some of the PR proteins that show enhanced activity are the defence-related enzymes, for example, β1.3 glucanases, chitinases and peroxidases (Louws et al., 2001; Baysal et al., 2005a; Ghazanfar et al., 2011).

Different pathogens trigger different signal pathways that induce a specific plant defence response. Induced resistance may be local at the site of infection or systemic away from the site of infection (Oostendorp et al., 2001; Montesano et al., 2003; Ghazanfar et al., 2011). Local resistance is manifested as a hypersensitive response that is characterised by necrosis at the site of infection that limits the spread of the infection (Durner et al., 1997; Melo et al., 2006). Systemic acquired resistance (SAR) is a broad spectrum disease resistance that is activated systemically by a localised pathogen infection throughout the whole plant within hours or days after infection (Petrov and Andonova, 2012). It depends on the production of salicylic acid and systemic induction of genes responsive to SAR. This pathway provides protection against a broad range of pathogens, including bacteria, viruses and fungi (Brisset et al., 2000; Baysal et al., 2003). The activated disease resistance is against both the pathogen and other unrelated pathogens (Raskin, 1992; Oostendorp et al., 2001).

Synthetic chemicals have been developed which induce SAR in plants. This technology enhances the plant's resistance through the intense activation of defence responses without any alteration to the plant's genetic constitution (Ghazanfar et al., 2011). For a chemical to be considered as a SAR inducer, it should protect against a wide range of pathogens without specificity and activate host defence mechanisms in the same manner as a biological inducer without having a direct anti-microbial activity (Brisset et al., 2000; Louws et al., 2001; Baysal et al., 2005). Various chemicals that have been used as SAR inducers are acibenzolar-S-methyl (ASM), marketed as Bion® or Actigard™, salicylic acid (SA), benzothiadiazole (BTH) and polyacrylic acid (Bokshi et al., 2003; Baysal et al., 2005; Cao et al., 2006; Ghazanfar et al., 2011).

Bion is a functional analogue of SA as it activates the same set of genes as those activated by exogenous SA or biotic inducers (Buzi et al., 2004). It is fully systemic at very low rates and is

considered one of the best chemical elicitors to induce SAR in many crops against a broad spectrum of diseases, including fungi, viruses and bacteria. Its effect is very long lasting in monocots and less pronounced in dicots (Oostendorp et al., 2001; Ghazanfar et al., 2011; Sarwar et al., 2011).

Resistance to pathogens and the production of some PR proteins can also be induced by SA even in the absence of a pathogenic organism. In previous studies, SA reduced the number and size of lesions on tobacco leaves which had been inoculated with tobacco mosaic virus (Raskin, 1992). Salicylic acid can also induce SAR in potato tubers inoculated with *Pcc* causing an increase in chitinase and β1.3 glucanase (Bokshi et al., 2003). Salicylic acid treatment also enhances disease resistance of some growing plants or detached organs like the fruit, reducing both disease incidence and severity (Cao et al., 2005).

CHAPTER 3

Identification of soft rot pathogens causing tuber soft rot and blackleg symptoms in potatoes grown on farms in peri-urban Harare area

ABSTRACT

The blackleg/soft rot disease complex causes economic losses in potato production in Zimbabwe, estimated to be between 20 and 60%, depending on climatic and storage conditions. The aim of the study was to identify the pathogens which cause potato blackleg/soft rot disease complex in farms in peri-urban Harare areas. Infected samples, comprising of stems and tubers from potato plants with blackleg/soft rot disease complex symptoms (soft rot, wilting, internal and external darkening on stems), were collected from commercial farms where disease outbreaks occurred. The isolates from these plants and tubers yielded pectolytic bacteria on crystal violet pectate medium, and colonies were characterized after purification on King's B medium. All isolates were Gram negative rods producing soft rot symptoms on inoculated tubers. Identification was based on biochemical and phenotypic characteristics, PCR and sequences of *gyrB* and *recA* genes. *Dickeya dadantii* (*Dd*) was the dominant pathogen and was isolated from all samples collected. This confirms earlier findings which classified *D. dadantii* as the major cause of the blackleg and soft rot disease complex.

3.1 INTRODUCTION

Globalisation has allowed for the creation of free trade zones, which has accounted for the increased movement of goods and services across borders. Currently, Zimbabwe imports about 120 tonnes of potatoes from neighbouring South Africa monthly (Makoni et al., 2014). This has led to the spread of pests and diseases as countries trade in agricultural produce. The potato (*Solanum tuberosum* L.) is one crop that is of significant global importance and this has led to its increased production and movement across countries. Like any other crop, pests and diseases are some of the major constraints to its production. Among the bacterial pathogens of potato, soft rot pathogens stand out to be the most important because they are virulent under all climatic conditions (Pérombelon, 1988). They fall under the genera *Pectobacterium* and *Dickeya*, and cause bacterial tuber soft rot and stem rots (Pitman et al., 2008).

Successful control of bacterial diseases lies mainly in the accurate detection and identification of the causal pathogen since control depends on prophylactic control practices (Kang et al., 2003; Palacio-Bielsa, 2009). Though pathogen detection can be done through symptom assessment, this does not give an accurate diagnosis since two or more pathogens or environmental conditions, can lead to similar symptoms, for example, both *Pectobacterium* and *Dickeya* pathogens have similar blackleg and tuber soft rot symptoms that are difficult to distinguish in the field (Diallo et al., 2009).

This study describes the identification of soft rot pathogens presumed to be the cause of potato soft rot and blackleg affecting potatoes in commercial farms around the Harare area in Zimbabwe using standard biochemical tests and a polymerase chain reaction.

3.2 MATERIALS AND METHODS

3.2.1 Collection of samples

A farm survey was conducted from May-June 2012 in commercial farms within a 60 km radius of Harare. From thirteen farms visited, samples were obtained from eight of the farms. The GPS co-ordinates for the places where samples were collected are as follows (S17˚41,992; E031˚05,141), (S17˚43,695; E031˚03,923), (S17˚41,294; E030˚52,068), (S17˚40,771; E030˚51,358), (S17˚46,138; E031˚25,119), (S17˚41,760; E031˚22,469), (S17˚44,251; E030˚58,552) and (S17˚45,900; E031˚20,721) (Figure 1). A total of twenty-four samples were collected, three from

each farm. Samples that were collected were potato tubers and stems that showed typical soft rot or blackleg symptoms respectively.

3.2.2 Bacterial isolations

Isolations were done according to protocols described by Pérombelon and van der Wolf (2002). The plant material was sterilized in 2% sodium hypochlorite for 2 minutes and rinsed in sterile water. A loopful of tissue was removed from the rotting lesion and placed in 0.2 ml sterile water in a petri dish. This was left for 5 minutes to allow bacteria to diffuse out after which the suspension was then streaked onto a crystal violet pectate (CVP) plate. The plates were incubated for 48 h at 27°C. A well-spaced single colony was re-streaked onto a fresh CVP plate and then on nutrient agar (NA). The single colony technique was used to obtain a pure culture.

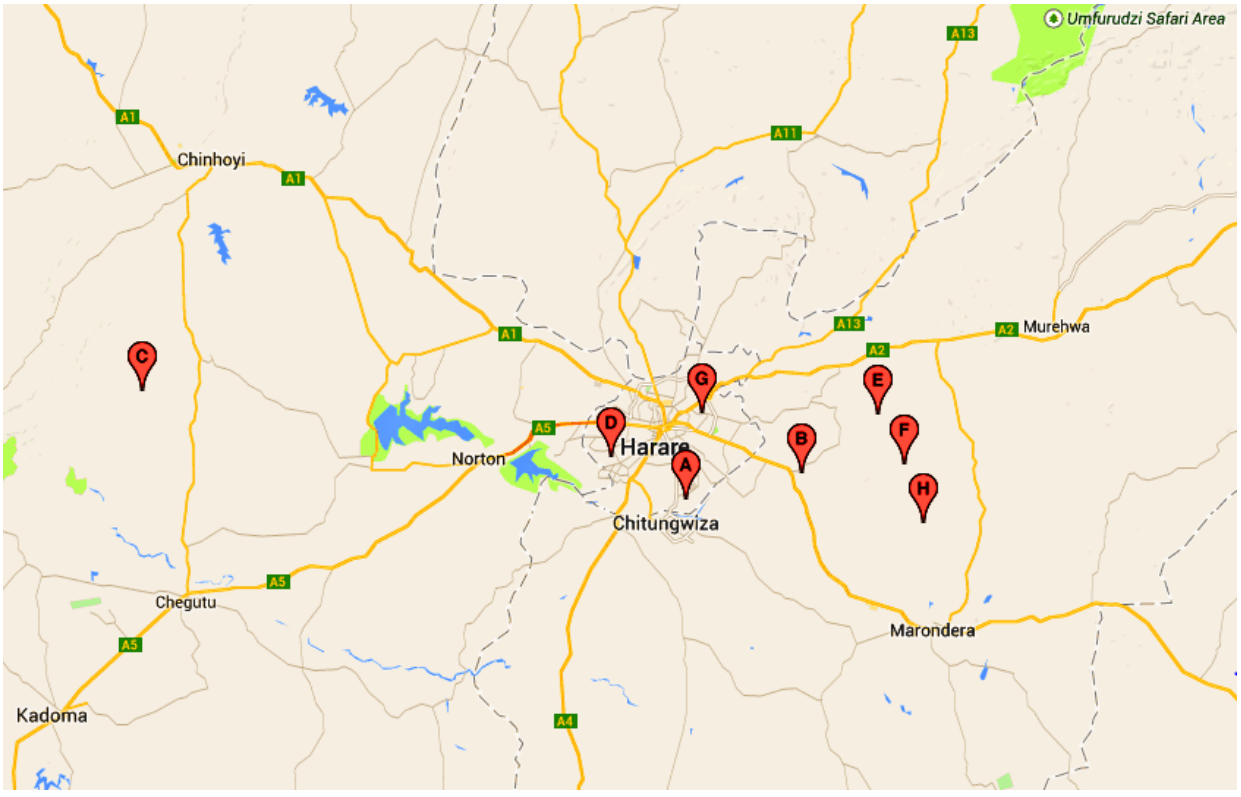

Figure 1: The location of farms where samples were collected

3.2.3 Biochemical identification and classification of strains

The determinative tests were conducted as described previously by Perombelon and van der Wolf (2002) and Schaad (1988). The following tests were conducted: potato soft rot, growth on NA at 37^{o}C, growth in 5% NaCl, gram staining, production of reducing substances from sucrose, acid production from lactose and maltose, gas from d-glucose, catalase, oxidase, oxidative-fermentative, utilization of organic substances and gelatin liquefaction.

3.2.4 Pathogenicity tests

Potato plants of cultivar BP1 were grown in 12 cm diameter pots in sterile red clay soils. Pure cultures of bacteria isolated from the eight representative samples were used in the test. At six weeks after crop emergence, plants were stem inoculated by injecting using a micropipette 10 µl of the 48 h old cultures adjusted to a concentration of 6 X 10^{8} cfu ml^{-1}. Control plants were inoculated with sterile water. Each bacterial strain was inoculated into ten plants and the wound covered by petroleum jelly. The plants were then watered and covered with clear polyethene bags to maintain a 100% relative humidity. Disease development and symptoms were assessed over a one week period. Isolations were done from the inoculated plants which developed typical blackleg symptoms and the potato soft rot test was performed on tubers as a confirmatory test.

3.2.5 Isolation of bacterial DNA for PCR identification

For the PCR reaction, bacterial genomic DNA was extracted from 48 h old bacterial suspensions in sterile distilled water. The DNA was extracted and purified using a Quick-gDNA™ MiniPrep kit as described by the manufacturer (Zymo Research Corp). Purified DNA was kept on ice. A total of 5µl of the DNA was used for the PCR.

3.2.6 PCR amplification

Primer sets used in standard PCR assays were obtained from Whitehead Scientific (South Africa) and the amplification was carried out using a GeneAmp® PCR System 9700. Detection of bacteria with primers ADE1 (5'-GAT CAG AAA GCC CGC AGC CAG AT-3') and ADE2 (5'-CTG TGG CCG ATC AGG ATG GTT TTG TCG TGC-3') (Table 1) specific for *Dickeya* species was performed in a 50µl PCR mixture containing 25µl of the PCR master mix (Thermo Scientific), 1µl forward primer, 1µl reverse primer, 5µl of the template DNA and 18µl of ultra-pure water. The PCR was run for 4 minutes with an initial temperature of 94^{o}C, followed by 40 cycles of 30 s at

94^{o}C, 60 s at 72^{o}C and a final extension at 72^{o}C for 10 minutes according to Palacio-Bielsa et al., (2006). The expected fragment length of the amplicons was 420 bp. Products from the PCR amplification from all samples were separated by electrophoresis in a 1.5% agarose gel in TAE buffer, stained with ethidium bromide and viewed under UV light.

3.2.7 Sequencing and phylogenetic analysis

Eight representative samples were selected and subjected to further analysis. Genomic DNA was extracted from overnight broth cultures using a DNeasy™ Blood and Tissue Kit (Qiagen, Southern Cross Biotechnology). For selected bacterial strains, the *recA* and *gyrB* genes were amplified using *rec*AF, *rec*AR and *gyr*B-01F, *gyr*B-02F respectively (Table 2). PCR amplification was performed with a GeneAmp® PCR System 9700 and products were purified using a QIAquick™ Purification Kit (Qiagen, Southern Cross Biotechnology). The *gyrB* and recA genes were sequenced in both directions using primers and conditions previously described (Young and Park, 2007; Brady et al., 2008). Sequencing products were cleaned using a sodium acetate precipitation step and sequenced on an ABI Prism DNA Automated Sequencer (Perkin Elmer). Incorrect basecalls were corrected using Chromas Lite v 2.01. The corrected sequences were edited and aligned with BioEdit Sequence Alignment v 7.0.0 and both ends were trimmed to the following final sizes *recA* 697 positions and *gyrB*, 740 positions. Searches were performed on each consensus sequence generated in BioEdit using the BLAST algorithm from GenBank.

Table 1: List of primers used in the study

Primer Code	Sequence	Source
ADE1	5'-GAT CAG AAA GCC CGC AGC CAG AT-3'	Palacio-Bielsa et al., 2006
ADE2	5'-CTG TGG CCG ATC AGG ATG GTT TTG TCG TGC-3'	Palacio-Bielsa et al., 2006
*gyr*B 01-F	TAA RTT YGA YGA YAA CTC YTA YAA AGT	Brady et al., 2008
*gyr*B 02-R	CMC CYT CCA CCA RGT AMA GTT	Brady et al., 2008
*gyr*B 07-F	GTV CGT TTC TGG CCV AG	Brady et al., 2008
*gyr*B 08-R	CTT TAC GRC GKG TCA TWT CAC	Brady et al., 2008

*rec*AF	GAR KCB TCN GGT AAA ACV AC	Young and Park, 2007
*rec*AR	TTC GCY TTR CCC TGR CCR ATC	Young and Park, 2007
*rec*AR2	RTT GAT RCC TTC GCC GTA SA	Young and Park, 2007

3.3 RESULTS

3.3.1 Results of biochemical tests

Seven isolates were homogenous in their physical, cultural and biochemical properties (Table 2). The isolates were gram negative, with cells appearing as single rods or in short chains. They tested negative for oxidase activity and positive in the catalase and oxidative/fermentative reaction, three tests that confirm their identity as soft-rot enterobacteria. The isolates also tested positive for gelatin liquefaction, growth at 37^{o}C, utilisation of organic substances lactose and maltose as well as growth in 5% NaCl. All isolates did not produce a blue pigment on YDC agar. There was a slight variation in the reducing sugars test, with two of the seven isolates testing positive while the other six were negative (Table 2). The eighth isolate that appeared distinctively different from the other seven tested negative for two of the three main tests which identify soft-rot enterobacteria, the oxidase and the oxidative/fermentative reaction although the other tests produced the same results.

From the results, the ability of the isolates to utilize glucose makes them more likely to be *Dickeya* species. Growth at 37^{o}C means that the isolates cannot be *Pectobacterium atrosepticum* but *Pectobacterium carotovorum* or *Dickeya* species. However, *Pectobacterium carotovorum* species are not able to utilize maltose. This leaves out the identity of the isolates most likely to be *Dickeya* species. Table 2 shows the results of the biochemical tests.

Table 2: Biochemical tests for the identification of soft rot pathogens isolated from farms around Harare (n 8)

Test	**Sample**										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	***Pcc***	***Pa***	***Dd***
Potato soft rot	+	+	+	+	+	+	+	+	+	+	+
Gram stain	-	-	-	-	-	-	-	-	-	-	-

Catalase	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	-	-	-	-	-	-	-	-	-	-
Gelatin liquefaction	+	+	+	+	+	+	+	+	+	+	+
Growth at 37˚C	-	+	+	+	+	+	+	+	+	-	+
Oxidative/fermentative	-/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Gas from d-glucose	+	+	+	+	+	+	+	+	-	-	+
Blue pigment on YDC agar	-	-	-	-	-	-	-	-	-	-	-
Reducing sugar	+	-	-	-	-	+	-	-	-	+	-
Growth in 5% NaCl	-	+	+	+	+	+	+	+	+	+	-
Acid production from: lactose	+	+	+	+	+	+	+	+	+	+	-
maltose	+	+	+	+	+	+	+	+	-	+	-

Soft rot bacteria isolated from farms - samples 1, 2, 3, 4, 5, 6, 7, 8; *Pcc*, *Pectobacterium carotovorum* subsp. *carotovorum* - strain LMG 2404^T; *Pa*, *Pectobacterium atrosepticum* - strain LMG 2386^T; *Dd*, *Dickeya dadantii* - strain 3937^T. (+) positive reaction, (-) negative reaction

3.3.2 Pathogenicity tests

Plants developed typical blackleg symptoms from all the eight isolates. A brown to black rotting was observed on most inoculated stems. The rotting lesions extended upwards and downwards from the point of inoculation. Depending on severity, the disease resulted in stem death on some plants whereas other stems became hollow. With the control plants, no disease symptoms were observed except a scar which did not proliferate beyond the point of inoculation.

Isolations of bacteria were done from the diseased plants and the pure cultures used in the potato soft rot tests where all the isolates tested positive.

3.3.3 PCR test results

There was amplification of the 420 bp fragment from all the eight isolates, indicative of *Dickeya* species (Figure 2).

M 1 2 3 4 C

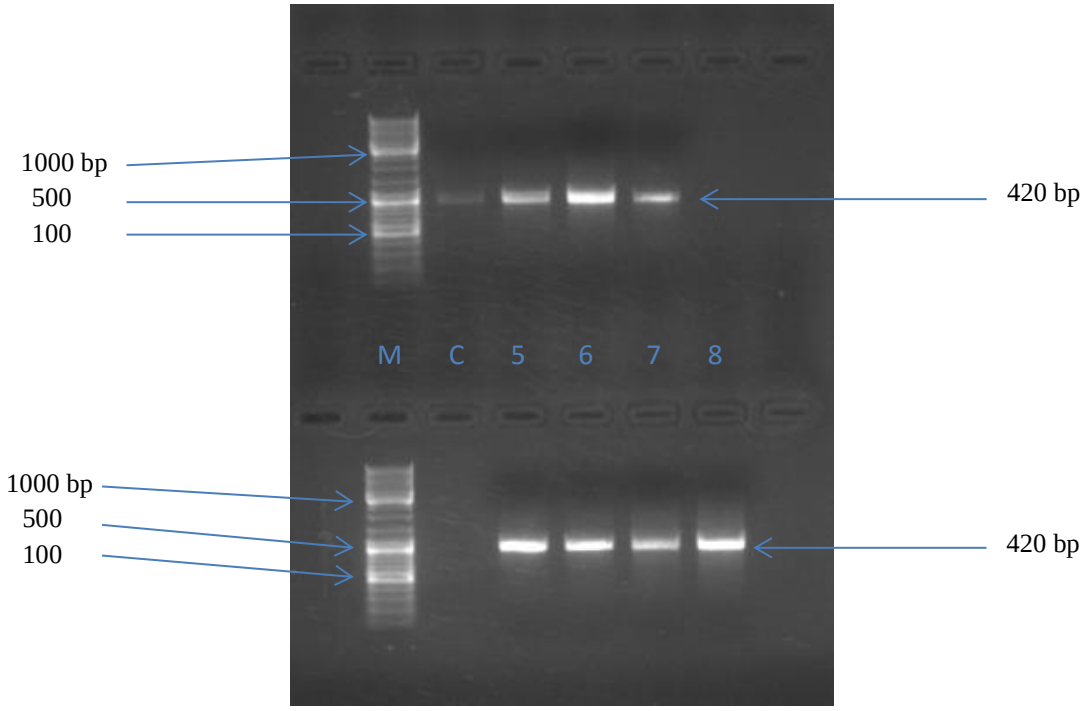

Figure 2: Characterisation of strains isolated on farms with PCR amplification using *Dickeya* spp specific primers ADE 1 and ADE 2. Lanes M, DNA molecular marker; C, negative control (water); 1-8, isolates.

3.3.4 Sequencing

The sequences of *recA* and *gyrB* were compared to those available in the Genbank database using BLAST N algorithm. All isolates tested were confirmed as *Dickeya dadantii* (Table 3).

Table 3: Blast search results for *gyrB* and *recA* gene sequences of isolates from potato

Symbol of strain	Closest match in NCBI Genebank database accession number	Genebank number	% similarity for 697 bp *recA* gene	% similarity for 750 bp *gyrB* gene
1	BCC 1035 ECH 586	(J311647) *gyrB* (NC_0135921) *recA*	95	99
2	BCC 1035 ECH 586	(J311647) *gyrB* (NC_0135921) *recA*	96	99
3	BCC 1035 ECH 586	(J311647) *gyrB* (NC_0135921) *recA*	98	99
4	BCC 1035 ECH 586	(J311647) *gyrB* (NC_0135921) *recA*	98	98

5	BCC 1035 ECH 586	(J311647) *gyrB* (NC_0135921) *recA*	98	98
6	BCC 1035 ECH 586	(J311647) *gyrB* (NC_0135921) *recA*	99	95
7	BCC 1036 ECH 586	(J311646) *gyrB* (NC_0135921) *recA*	99	99
8	BCC 1035 ECH 586	(J311647) *gyrB* (NC_0135921) *recA*	98	99

3.4 DISCUSSION

Results from the study using standard biochemical tests and the polymerase chain reaction confirmed that the pathogens isolated from tubers and plants showing soft rot and blackleg symptoms were from the *Dickeya* species. To identify the species, standard biochemical tests were used according to Pérombelon and van der Wolf (2002) and Schaad (1988). Bacteria belonging to the *Pectobacterium* and *Dickeya* genera are the causal agents of blackleg and tuber soft rot as confirmed by the results of the study (Czajkowski et al., 2011). The blackleg disease of potato is mainly caused by *Pectobacterium atroseptica* (*Pa*) in cool climates but under higher temperatures, *Dickeya* species and *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) cause similar symptoms (Duarte et al., 2004; van der Merwe et al., 2009). Since it is difficult to identify causal pathogens based on the field symptoms, the pure cultures used in the biochemical tests permitted the differentiation of sub-species (De Boer and Ward, 1995). Globally, *Dickeya* species are becoming more important as soft rot pathogens in the potato industry than *Pa* and *Pectobacterium carotovorum* subspecies (Degefu et al., 2013). This trend has been noted in many countries including Europe (Slawiak et al., 2009; Toth et al., 2011), Finland (Degefu et al., 2013), Israel (Tsror (Lahkim) et al., 2008) and Spain (Palacio-Bielsa et al., 2006) although species occurrence differs from one continent to the other.

Presently, *Dickeya dadantii* is one of the main causes of blackleg and soft rot in Zimbabwe. Of the four soft rot pathogens *Pcb*, *Pa*, *Pcc* and *Dickeya dadantii* isolated from infected potatoes in several regions during a recent study by Ngadze et al., (2012), *Dickeya dadantii* was found to be the most prevalent pathogen. This presents a shift on the blackleg and soft rot disease aetiology because the soft rot pathogens that used to be the predominant cause of soft rot and blackleg

diseases in Zimbabwe were *Pcc* and *Pa* (Manzira, 2010). Since seed tubers are considered to be the major source of infection and long distance dispersal of *Pectobacterium* and *Dickeya* species (Czajkowski et al., 2011; Pérombelon, 1992), the movement of potatoes regionally into the country through seed imports might have led to the introduction of *Dickeya* pathogens. In addition, *Dickeya* species are also known to have a wider host range, having been isolated in several crops such as banana, maize, chicory and other ornamental plants (Czajkowski et al., 2009; Toth et al., 2011). This could have contributed to their spread in several geographical areas as compared to *Pa*, which has always been known to be almost exclusively a potato pathogen (Smid et al., 1995). Furthermore, potato growing in the country used to be predominantly in Natural Region I, an area with the lowest average temperatures than any other region in the country. The temperatures in this region were optimum for *Pa*, whose optimum temperature is around 15°C (Pérombelon, 1988), which is also the reason why the *Pa* pathogen is found almost exclusively on potato in temperate regions (Duarte et al., 2004; Ma et al., 2007; Czajkowski et al., 2011). A recent increase in the number of farmers now growing seed potato in other parts of the country, especially in Natural Region II which has maximum temperature ranges of 21°C - 25°C has probably led to the increased presence of *Dickeya* species. These species are pathogenic in tropical, subtropical and warm climates (Tsror (Lahkim) et al., 2008; Laurila et al., 2010) with an optimum > 25°C, the reason why they are becoming a problem in warmer climates (Toth et al., 2011). Degefu et al., (2013) reported that the adaptability of *Dickeya* species to higher temperatures has given it a selective advantage over other *Pectobacterium* species especially in warmer environments as found in this case, in Natural Region II. Competition within rotting mother tubers is regulated by environmental conditions especially temperature which determines the pathogens that will prevail if more than one are present (Czajkowski et al., 2011). Higher temperatures result in *Dickeya* species being more aggressive than *P. carotovorum* and *P. atrosepticum* species (Henz et al., 2006). These conditions apply to the area under study, probably the reason why *Dickeya* species were found to be more prevalent in the samples collected from farms around Harare.

Dickeya chrysanthemi, formerly, *Erwinia chrysanthemi*, is the causal agent of tuber soft rot and blackleg-like symptoms in many potato growing areas of the world (Young et al., 1992). However, the study found *D. dadanti* to be the most predominant species in Zimbabwe in the area around Harare. This finding was not surprising because *D. dadantii* is a warm climate pathogen

(Pèrombelon, 2002) and Ngadze et al., (2010), also isolated and identified *D. dadantii* isolates from plants with typical wilting symptoms and rotting tubers in Zimbabwe. The strains isolated in this study shared biochemical and genetic features with the species *D. dadantii*. Phylogenetic analysis of the *rec*A and *gyr*B gene sequences demonstrated high genetic homogeneity between the potato isolates from Zimbabwe (Harare) and *D. dadantii* reference strains, with sequence similarity of 95% and above.

The information on the increasing presence and spread of *Dickeya* pathogens due to climate change is valuable for all stakeholders in the potato industry such as seed growers, suppliers and exporters. Results of this study will help to strengthen awareness among seed growers and seed companies about the potential threat of *Dickeya* species in the country. Knowing species occurrence is important in that it facilitates the monitoring of outbreaks and spread of the pathogen. This can help avoid cases of reputational damage that can occur if seed with latent *Dickeya* infections is exported to warmer regions often causing huge crop losses. This information can potentially be used to provide basic information for effective control. This can be facilitated through the adoption of plant health regulatory measures which encompass extra phytosanitary systems in the potato seed industry such as mandatory seed testing and the use of sensitive and specific diagnostic tools for the detection of the pathogens.

3.5 CONCLUSION AND RECOMMENDATION

In this study, the hypothesis that *Pectobacterium* and *Dickeya* species associated with soft rot and blackleg can be isolated from plants showing soft rot and blackleg symptoms was accepted. Physiological and molecular tests confirmed that the most common pathogen causing blackleg and soft rot in potatoes grown in areas around Harare is from the *Dickeya* genus. Since early diagnosis is an important part of effective control, identification may need to be carried out using the PCR and sequencing techniques which are fast, sensitive, specific and reliable methods used for the rapid detection of *Pectobacterium* and *Dickeya* species.

CHAPTER 4

The response of different potato cultivars to *Pectobacterium carotovorum* subspecies *brasiliensis* causal agent of blackleg

ABSTRACT

Two experiments were carried out with potted plants to evaluate five potato cultivars for their response to blackleg caused by *Pectobacterium carotovorum* subspecies *brasiliensis* in an outside environment. The experiments were arranged in a randomised complete block design with four blocks, with replication across blocks. Bacterial inoculation was carried out on six-week old plants by injecting 10 µl of the 6×10^8 cfu ml^{-1} bacterial suspension into the stem, 5 cm above the ground and covering the plant with a clear polyethene bag. Control plants were inoculated with sterile distilled water. Data collection was done one week after inoculation. Based on the $\log_{10}$ transformed disease incidence data, there were no significant differences in the response of the cultivars to *Pectobacterium carotovorum* subspecies *brasiliensis* ($P > 0.05$). All the cultivars inoculated with the pathogen produced typical blackleg symptoms. There was no disease expression in control plants. Results of the experiments suggest that under the given conditions, the locally grown potato cultivars Amethyst, BP1, Jasper, Montclaire and KY20 are all equally susceptible to *Pectobacterium carotovorum* subspecies *brasiliensis* which causes stem blackleg.

4.1 INTRODUCTION

Genetic resistance is a useful tool for controlling soft rot pathogens since no chemical control is available (Hélias et al., 2000; Pasco et al., 2006). Plant disease resistance and susceptibility depend on the combined genotypes of both the pathogen and the host (Yang et al., 2013). Under field conditions, cultivar resistance is a factor that determines symptom development, blackleg incidence and severity together with environmental conditions such as temperature and humidity (Bain and Pérombelon, 1990; Hélias et al., 2000). Field experiments are the only way to evaluate potato cultivars against soft rot and blackleg diseases (Czajkowski et al., 2011).

There are two types of disease resistance, quantitative and qualitative. Qualitative resistance results in complete resistance controlled by a single gene factor as opposed to quantitative resistance which is partial and is conditioned by multiple genes (Poland et al., 2008). The narrow range of genetic diversity in potatoes has made it difficult to find cultivars immune to soft rot pathogens. Potatoes exhibit a quantitative resistance to soft rot pathogens (Gebhardt and Valkonen, 2001). Though this resistance is partial, some cultivars show high levels of soft rot resistance. These cultivars can then be used in integrated crop management systems that involve prophylactic measures in soft rot control.

The objective of this study was to assess the variation in blackleg resistance among local potato cultivars currently available to farmers.

4.2 MATERIALS AND METHODS

A ppure culture of *Pectobacterium carotovora* subsp. *brasiliensis* (*Pcb*) obtained from the Plant Pathology Laboratory, University of Zimbabwe, was used as the inoculum. The pure cultures were maintained on nutrient agar (NA).

Bacterial suspensions in sterile distilled water were prepared from cultures grown on NA at 27^0C for 48 h. The concentration was calculated from optical density readings at 600nm on a UV-VIS spectrophotometer (Model ST-UV 752N) and adjusted to 6×10^8 cfu ml^{-1} (OD_{600}- 0.6) (Ngadze et al., 2012)

4.2.1 Pathogenicity tests

To obtain host plants for testing pathogenicity, certified, disease free seed potato of five cultivars obtained from the Potato Seed Co-op of Zimbabwe was used. The cultivars were selected on the

basis of their popularity among Zimbabwean farmers with Amethyst, BP1, Montclaire and Jasper being the top four cultivars respectively, and KY20 being a new cultivar yet to be registered.

Planting was done in 25 cm diameter pots with a depth of 20 cm and a volume of 5 589.2 cm^3 containing sterile red clay soils. One tuber per pot was planted and 41.5 g Compound S fertilizer (7N: 21P_2O_5: 7K_2O) was applied as a basal fertilizer. Ten plants per block were used for each variety. Plants were grown outside and inoculation was done on six-week old plants. Inoculation was done on the stem, 5 cm above the ground, by injecting 10µl of the inoculum using a micropipette. The stem was previously punctured below the inoculation hole to avoid back pressure (Lapwood and Read, 1986b). Control plants were inoculated in the same way with sterile distilled water. After the inoculation, the wound was covered with petroleum jelly. Plants were then watered and enclosed in polythene bags to maintain a 100% relative humidity (Palacio-Bielsa et al., 2006). Plants were then examined for symptom development for 7 days.

4.2.2 Experimental design and analysis

The experiment was a randomised complete block design with four blocks and replication was done across blocks. The experiment was performed twice. Disease incidence was expressed as the number of plants showing at least one stem with blackleg or blackleg associated symptoms (Hélias et al., 2000). Disease incidence data was transformed using the $\log_{10}$ transformation, assumptions of the analysis of variance checked and data subjected to ANOVA. Data analysis was done using Genstat 14th Edition statistical package. Fischer's Protected Least Significant Difference was calculated at $P < 0.05$.

4.3 RESULTS

4.3.1 Blackleg disease expression

Some stems that were inoculated with bacteria developed blackleg but uninoculated stems on the same plant remained healthy. The diseased stems of various cultivars showed a brown to black discolouration as a result of rotting, which is typical of blackleg. The infection spread upwards and downwards from the inoculation site, and in severe cases eventually led to stem collapse and death (Figure 3). Some stems also became hollow. In the control plants, a scar developed at the inoculation site which later dried up without proliferation.

4.3.2 Disease incidence across cultivars

There was no significant difference in disease incidence among the five cultivars namely Amethyst, BP1, Jasper, KY20 and Montclaire in both seasons ($P > 0.05$) (Appendix 4.1 and 4.2) although the trend observed showed that disease incidence in the first season was higher than in the second season (Table 4).

Figure 3: Blackleg symptoms on six-weeks old potato plants after artificial inoculation of stems with *P. carotovorum* subsp. *brasiliensis* in cv.Amethyst (a); cv. BP1 (b); cv. Jasper (c); cv. KY20 (d); cv. Montclaire (e).

Table 4: The susceptibility of five potato cultivars to blackleg measured based on disease incidence (log_{10} transformed data) caused by the inoculated soft rot pathogen *P. c.* subsp. *brasiliensis*

Cultivar	Disease incidence Experiment 1	Disease incidence Experiment 2
Amethyst	0.595 (4)	0.464 (3)
BP1	0.901 (8)	0.464 (3)
Jasper	0.690 (5.25)	0.520 (3.5)
KY20	0.714 (5.25)	0.651 (4.5)
Montclaire	0.702 (5.25)	0.476 (3.25)
P-value (0.05)	0.065	0.365
L.s.d	NS	NS

Note: Figures in brackets are the means analysed from the original data.

4.4 DISCUSSION

Pathogenicity tests carried out on the five cultivars based on disease incidence showed that Amethyst, BP1, Jasper, KY20 and Montclaire are all equally susceptible to blackleg caused by *Pcb*. Blackleg symptoms appeared as those described by other authors (Palacio-Bielsa et al., 2006; Tsror (Lahkim) et al., 2008; van der Merwe et al., 2010), showing the ability of *P.c.* subsp. *brasiliensis* to cause blackleg. *Pectobacterium carotovorum* subsp. *brasiliensis* is a member of the *P. carotovorum* species (Nabhan et al., 2012) which was first reported in Brazil by Duarte et al., (2004) as causing blackleg and being more aggressive than *P. atrosepticum*. Since then, it has been isolated in other countries including New Zealand (Panda et al., 2012) and South Africa (van der Merwe et al., 2010) where it caused severe losses in the potato industry as a result of blackleg. In Zimbabwe, studies conducted by Ngadze et al., (2012) established that in terms of prevalence, it comes second after *Dickeya dadantii,* hence the need to evaluate the response of locally grown cultivars to this pathogen.

Based on disease incidence, the experimental data exhibited a normal distribution across cultivars with no clear cut differences or categories. Therefore, cultivars in this study could not be grouped into different classes of susceptibility as has been done in other studies in which cultivars were classified into distinct groups as either susceptible, intermediate or resistant (Reeves et al., 1999; Hélias et al., 2000). Although a study by Ngadze et al., (2012) grouped Amethyst and BP1 as susceptible to soft rot and Montclaire as intermediate based on the rotting zone diameters caused by the inoculated bacteria correlated with the level of defence enzymes and phenolic compounds present in those cultivars, cultivars tend to change rankings depending on several factors. Environmental conditions and seasons (De Maine et al., 1998), method of inoculation and inoculation time (Bain and Pérombelon, 1988; Reeves et al., 1999) and inoculum concentration (Lapwood and Gans, 1984) are some of the important factors that affect results of resistance screening tests.

What the results of the experiments seem to consistently confirm though is the polygenic nature of the resistance of potatoes to soft rot pathogens (Gebhardt and Valkonen et al., 2001; Pasco et al., 2006). Polygenic or quantitative resistance is characterised by normally distributed data with no distinct or clear-cut categories (Lebecka and Zimnoch-Guzowska, 2004; Pasco et al., 2006). This continuous variation is a result of the simultaneous segregation of many genes together with other non-genetic factors (Geiger and Heun, 1989) which leads to narrow differences in the level of responses making it difficult to distinguish susceptible from resistant cultivars (Huaman et al., 1988; Reeves et al., 1999). Polygenic resistance is controlled by several genes which have a small and additive effect to the overall resistance. This type of resistance results in quantitative differences in the level of resistance or disease severity rather than its complete absence as observed with qualitative resistance (Geiger and Heun, 1989; Lindhout, 2002; Pasco et al., 2006). The susceptibility of all the five cultivars to blackleg as a result of their inherent ability to sustain pathogen establishment upon its inoculation clearly shows that the evaluated cultivars are susceptible to blackleg.

4.5 CONCLUSION AND RECOMMENDATION

In this study, the hypothesis that the cultivars would show different levels of resistance to blackleg was rejected. Results showed that the cultivars developed blackleg symptoms and disease incidence was the same across all the cultivars. However, results of these cultivars are limited to

factors such as the environment, the soft rot pathogen and method of inoculation. For more reliable screening tests, it is necessary to carry out assessments with several cultivars available in the country over a number of years at different sites. This information will then be added on to the country specific data-base

CHAPTER 5

Induction of polyphenol oxidase (PPO) and peroxidase (POD) enzymes in different potato cultivars (*Solanum tuberosum*) as a result of pathogen inoculation and mechanical damage

ABSTRACT

The response of plants to biotic and abiotic stress is often seen by the induction of plant defence enzymes such as polyphenol oxidase (PPO) and peroxidase (POD). The activity of PPO and POD was evaluated in the leaves of five potato cultivars. Six week old plants were subjected to two treatments, wounding and artificial inoculation with *Pectobacterium carotovorum* subsp. *brasiliensis.* The activity of PPO and POD were assayed at 0 h, 12 h, 24 h, 48 h, 84 h and one week after treatments. Results indicated that the treatments were not significantly different on their effect on both enzymes ($P > 0.05$). However, significant differences in PPO and POD activity were observed across cultivars ($P < 0.05$). The moderately resistant control cultivar, Montclaire, exhibited the highest enzyme activity at most time intervals compared to Amethyst and BP1 and Jasper were intermediate while KY20 exhibited the lowest enzyme activity. The interaction between cultivar and time was also significant ($P <0 .05$) with Montclaire showing the highest PPO and POD activity 12, 24 and 84 h. The activity of PPO in the cultivars was characterised by an early induction within 12 h followed by a sudden drop thereafter, while POD activity showed a gradual increase in activity throughout the experiment. These results suggest that both pathogen infection and mechanical damage can cause an induction of PPO and POD but the level of induction is also influenced by other factors such as cultivar and time after treatment.

5.1 INTRODUCTION

The phenylpropanoid pathway is a biochemical process that yields phenylpropanoid compounds which are important compounds in the plant's natural defence system. The phenylalanine ammonia lyase (PAL) is a key enzyme in the phenylpropanoid pathway that provides precursors for lignin and other metabolites involved in resistance (Chandra et al., 2007; Vanitha et al., 2009). An abiotic

or biotic stress causes elicitors such as oligosaccharides to be released and these trigger the decompartmentation of phenols. Phenols are synthesized during the plant's normal development and are compartmentalized in a reduced state in the vacuoles (Beckman, 2000). With the action of enzymes, phenols are oxidised to polymerise with other host constituents like carbohydrates to form lignin. They are important in conferring resistance against the pathogen (Im et al., 2008).

Products of phenol oxidation confer resistance by directly inhibiting pathogen multiplication, preventing plant cell wall degradation by pathogen enzymes or act as precursors in the formation of structural components such as lignin (Lyon et al., 1992). The three principal enzymes in the phenylpropanoid pathway are polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and peroxidase (POD). The enzyme PAL catalyses the transformation of phenylalanine to trans-cinnamic acid (Kröner et al., 2011; Ngadze et al., 2012). Cinnamic acid is an important compound in the biosynthesis of phenols and lignin. In the early stages of infection, PPO becomes the important enzyme because it catalyses the oxidation of phenols to quinones (Okey et al., 1997). Quinones are highly reactive compounds that can complex with other molecules such as carbohydrates, proteins and lipids to form polymers which offer additional phenolic barriers against pathogens (Wegener and Olsen, 2004; Melo et al., 2006). Peroxidase is important in the last step of lignin biosynthesis (Brisset et al., 2000; Zhang et al., 2008). It catalyses the oxidative polymerisation of hydroxycinnamyl alcohols to yield lignin and the cross-linking of isoditryosine bridges in the cell wall (Ngadze et al., 2012).

The objective of this experiment was to evaluate the defence mechanisms of five potato cultivars namely Amethyst, BP1, Jasper, Montclaire and KY20 in response to pathogen inoculation and mechanical damage by analysing the changes in the activities of peroxidase and polyphenol oxidase.

5.2 MATERIALS AND METHODS

5.2.1 Host plants

Potato plants were grown during the months of November 2012 and October 2013 at the University of Zimbabwe's Crop Science Department. Certified AA seed obtained from the Potato Seed Coop was used. AA seed is the purest form of potato seed released to farmers for commercial production. When grown for one season it is designated A1, and A2 when grown for the second season. This designation ends at A4 (fourth season) at which stage the resultant product is no longer certifiable

as seed. Five cultivars were used namely Amethyst, BP1, Jasper, Montclaire and KY20. Tubers were planted outside in 12 cm diameter pots, one tuber per pot, in red clay soils. Forty grammes per pot Compound S (7:28:7 NPK) was applied as a basal fertiliser. Watering was done as necessary to maintain the soils at field capacity. The experiment was arranged in a Completely Randomised Design with a 5*6 treatment structure replicated twice.

5.2.2 Bacterial culture

Bacterial suspensions of *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pcb*) in sterile distilled water was prepared as described in Section 4.2.

5.2.3 Treatments

Six week old plants were used in the experiment. The plants were inoculated by injecting 10 µl of the inoculum into the stems, about 5 cm from the ground. The wound was then covered with petroleum jelly to prevent infection by other airborne pathogens. Wounding was done by cutting off the plant's apical bud. Plants that were sampled at 0 h did not receive any treatment. Leaves were collected by destructive sampling on each occasion. Four leaves from the middle of the plant, with mid-ribs removed, were excised at 0, 12, 24, 48, 84 hours and 1 week after treatment and samples were frozen for later use in the enzyme assays. Five plants were used from each treatment group. A UV-VIS spectrophotometer (Model ST-UV 752N) was used for spectrophotometric analysis.

5.2.4 Enzyme assays

5.2.4.1 Polyphenoloxidase (PPO)

The assays were carried out as described by Zhang et al., **(**2008). Leaves were ground in liquid nitrogen. One gramme of the leaves was homogenized in a mortar and pestle and was mixed with 5 ml of 0.05 M phosphate buffer solution (pH 5.8) containing 5% (w/v) polyvinylpyrrolidone (PVP). The homogenate was then filtered through a muslin cloth and the filtrates centrifuged at 10 000 r/min for 5 minutes at room temperature. The final reaction mixture contained 3.9 ml of 0.05 M phosphate buffer solution (pH 5.5), 1.0 ml of 0.1 M catechol and 0.5 ml enzymatic extract. The same reaction mixture without the enzymatic extract was used as the blank. The reaction was monitored spectrophotometrically for 2 minutes at 20 second intervals at room temperature. Absorbance was read at 525 nm and results expressed as change in absorbance Δ_{525} min/g fresh mass.

5.2.4 Peroxidase (POD)

The leaf sample was ground in liquid nitrogen. One gramme of leaves was homogenized in a mortar and pestle in 0.05 M phosphate buffer solution (pH 5.8) containing 5% (w/v) PVP. The homogenate was then filtered through muslin cloth and the filtrates centrifuged at 10 000 r/min for 5 minutes at room temperature. The reaction mixture contained 2.9 ml 0.05 M phosphate buffer solution (pH 5.5), 1.0 ml 0.05 M guaicol, 1.0 ml 2% hydrogen peroxide (v/v) and 0.1 ml enzymatic extract.The same reaction mixture without the enzymatic extract was used as the blank. Oxidation of guaiacol was monitored spectrophotometrically for 2 minutes at 20 second intervals at room temperature. Absorbance was read at 470 nm and results expressed as Δ_{470} min/g fresh mass.

5.2.5 Statistical analysis

The experiment was performed twice and was arranged in a completely randomised design with a 5*6 factorial treatment structure replicated twice. Data obtained for the enzyme activity of different cultivars at different time intervals were subjected to ANOVA using Genstat 14th Edition. All significant means were separated using the Fischer's protected Least Significant Difference where ($P < 0.05$).

5.3 RESULTS

5.3.1 Effect of treatments on enzyme activity

There were no significant differences in polyphenol oxidase (Fig 4) and peroxidase activity (Fig 5) as a result of the two treatments in both seasons ($P > 0.05$) (Appendix 5.1- 5.4) in all the cultivars.

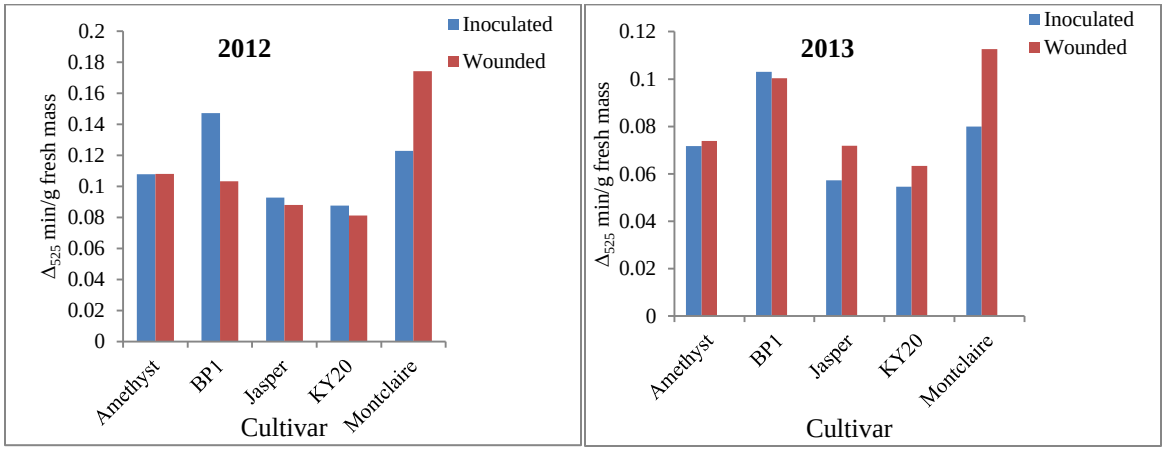

Figure 4: The effect of treatments on polyphenol oxidase (PPO) activity assayed in the leaves of wounded and inoculated potato cultivars

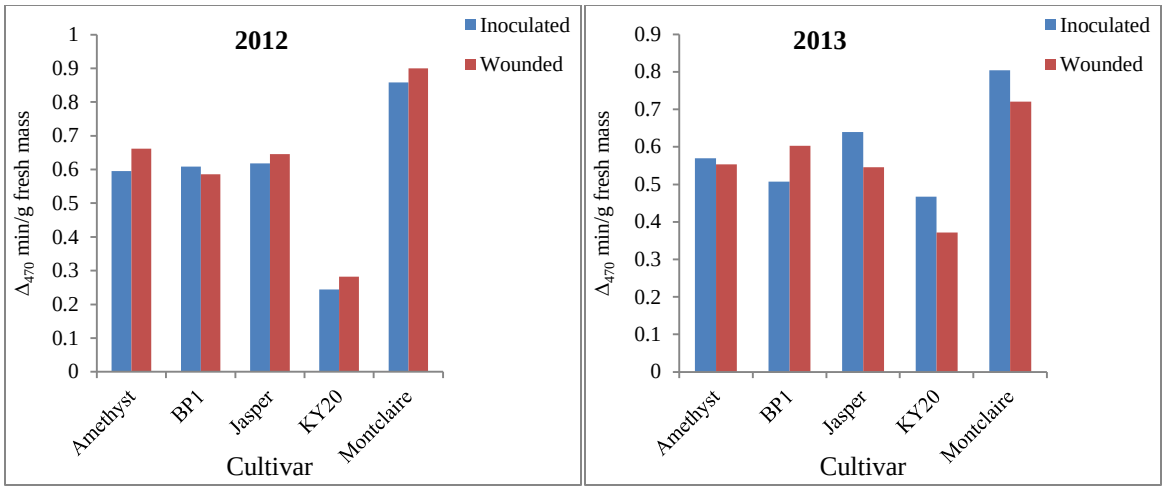

Figure 5: The effect of treatments on peroxidase (POD) activity assayed in the leaves of inoculated and wounded potato cultivars.

5.3.2 The effect of cultivar and time on enzyme activity

5.3.3 Peroxidase activity

The interaction between cultivar and time produced significant effects on peroxidase activity in both seasons ($P < 0.05$) (Appendix 5.3 and 5.4). During the 2012 season, Amethyst, BP1, Jasper and Montclaire started off with the same enzyme activity while KY20 had an initial lower POD activity whereas with the 2013 season, the initial POD activity in all the cultivars was not significantly different (Figure 6). During the 2012 season, significant differences in enzyme activity among cultivars were recorded throughout the experiment, during which the control cultivar Montclaire recorded the highest activity, KY20 exhibited the lowest POD activity and Amethyst, Jasper and BP1 were intermediate at 12, 24 and 84 hours. The same trend was observed in 2013 at 12, 24 and 84 hours. At the end of one week, KY20 exhibited a lower enzyme activity than the other four cultivars which recorded the same level of enzyme activity in both seasons. Generally, POD activity showed a continued gradual increase in activity during the whole seven day period (Figure 6).

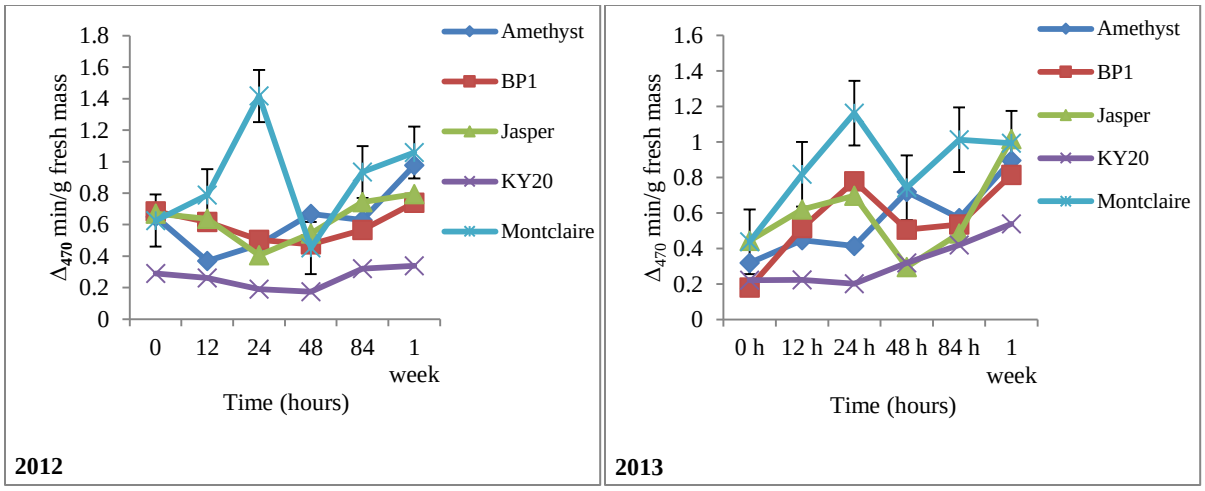

Figure 6: The interaction effect between time and cultivar on peroxidase activity assayed in the leaves of five potato cultivars harvested at different time intervals in 2012 and 2013.

5.3.4 Polyphenol oxidase

The interaction between time and cultivar was significant only during the 2012 season ($P < 0.05$) (Appendix 5.1), with the initial PPO activity across cultivars not significantly different. Significant differences in PPO activity among the cultivars were observed at 12, 24 and 84 hours after treatments. At 12 h, the highest enzyme activity was in the control cultivar Montclaire, followed by Amethyst, BP1 and Jasper which were intermediate with the least PPO activity recorded in KY20. At 24 and 84 hours, Montclaire had a higher PPO activity than the other four which were not significantly different. At the end of the experiment, enzyme activity in the five cultivars was the same. In the 2013 season, although cultivar and time individually had significant effect on enzyme activity, their interaction had no significant effect on PPO activity (Appendix 5.2). The interactions for both seasons however showed a similar trend of a sudden rise in enzyme activity at 12 h after induction in all the cultivars except KY20, followed by a rapid decrease in enzyme activity thereafter (Figure 7).

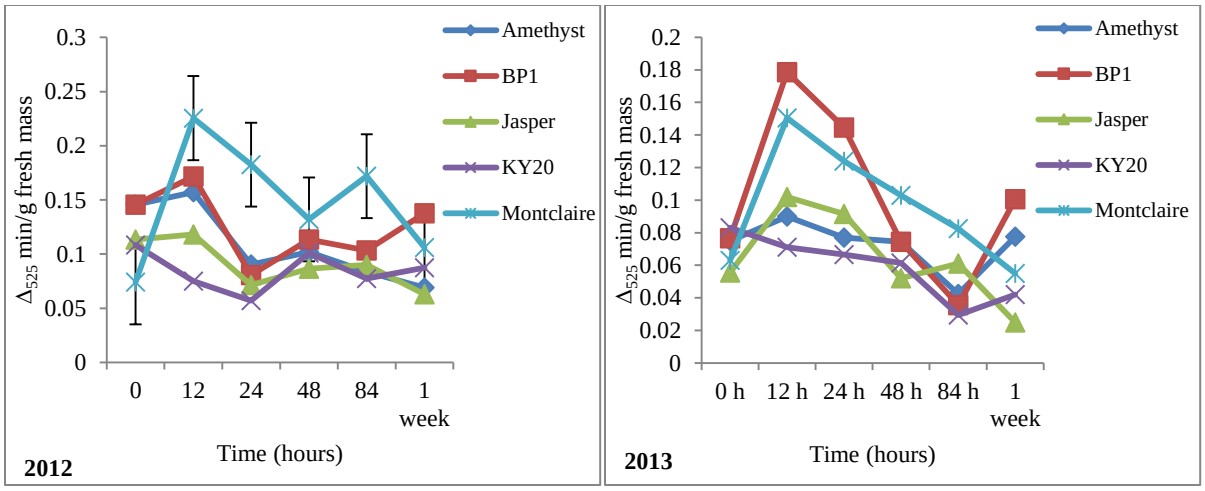

Figure 7: The interaction effect between time and cultivar on polyphenol oxidase activity assayed in the leaves of five potato cultivars harvested at different time intervals in 2012 and 2013.

5.4 DISCUSSION

The results show that the two treatments applied to the plants were not significantly different on their effects on PPO and POD activity. These results confirm findings that enhancement of PPO and POD activity due to infection by a pathogen is not different from that induced by mechanical damage as observed by Uritani (1971). This is because plants produce defence enzymes in response to both abiotic and biotic stresses (Raju et al., 2008). Similarly, in this experiment, an abiotic stress was created by wounding the plants and pathogen inoculation represented a biotic stress and both treatments were equally able to induce PPO and POD. When plants are exposed to environmental stresses, either biotic or abiotic, it leads to an induction of local and systemic defence responses (van Loon et al., 2006; Li and Steffens, 2002). The biotic or abiotic elicitors signal the activation of genes that coordinate different defence pathways such as the synthesis of defence enzymes like PPO and POD (Sarwar et al., 2011). Other researchers have also shown higher levels of PPO and POD in wounded and inoculated samples compared to the healthy ones (Okey et al., 1997; Ngadze et al., 2012). Other plants in which significant increases of defence enzymes have been recorded upon infection are tomato seedlings inoculated with *Fusarium oxysporum* f. sp. *lycopersici* (Rai et al., 2011); banana inoculated with *F. oxysporum* f. sp. *cubense* (Sarwar et al., 2011) and cucumber seedlings damaged by *Bemisia tabaci* (Zhang et al., 2008).

There was a significant increase in PPO activity in most cultivars immediately after treatments. During the experiment, there was rapid accumulation of PPO in Amethyst, BP1, Jasper and

Montclaire just 12 hours after induction followed by a rapid decrease thereafter. These results imply that enzymes involved in polyphenol synthesis are produced in response to infection or injury, further indicating the importance of PPO as an oxidative enzyme in the initial stages of plant defence (Melo et al., 2006; Rai et al., 2011). The disruption of the cell membrane as a result of wounding or pathogen inoculation triggered the release of a large amount of phenols, and it is the presence of phenols that in turn led to a rapid activation of latent PPO or its synthesis (Vitti et al., 2011). Since phenols are a substrate of PPO, there was a rise in the enzyme's activity (Lewis et al., 1998; Zheng et al., 2005). The oxidation of phenols to quinones as a first line of defence is important because quinones have an antibiotic and cytotoxic effect to pathogens (Mayer and Harel, 1979; Li and Steffens, 2002). The induction of PPO was most pronounced in the cultivars Montclaire and BP1, followed by Amethyst and Jasper which were intermediate and the least induction was in KY20. Polyphenol oxidase levels correlate with the capability of the plant's response to stress (Zheng et al., 2005). The implication of these results is these cultivars with higher PPO levels, in this case Montclaire and BP1 are likely to experience the least damage in the event of a stress, followed by Amethyst and Jasper while the cultivar KY20 will be the most susceptible.

There were significant differences in POD activity among cultivars. Enzyme activities recorded at 12, 24 and 84 hours showed that the cultivar Montclaire had the highest levels of POD activity; Amethyst, BP1, and Jasper recorded intermediate levels while KY20 exhibited the lowest activity. However, the general trend showed a continuous increase in POD activity in all cultivars throughout the experiment. Again, this shows that both infection and wounding stimulate the production of POD as it plays a role against various abiotic and biotic stresses and its formation continues for several days (Uritani, 1971; Goud and Kachole, 2011). Peroxidases catalyse several reactions such as suberisation, lignification, cross-linking isoditryosine bridges into cell walls (Okey et al., 1997), phytoalexin synthesis, auxin and reactive oxygen species metabolism (Almagro et al., 2009). Strengthening of the cell wall by lignin makes it more resistant to mechanical damage or degradation by pathogen enzymes (Brisset et al., 2000).

The two enzymes play a complementary role in plant defence and they occur together in injured or infected tissues (Uritani, 1971).While PPO provides a timely response against mechanical damage or pathogen attack by creating an environment that is unfavourable for pathogen survival,

POD further reinforces the line of defence by providing mechanical support. Resistant cultivars are the ones which tend to exhibit a higher concentration of defence enzymes and their rapid accumulation upon infection compared to susceptible ones (Rai et al., 2011; Vitti et al., 2011) as shown by the moderately resistant cultivar, Montclaire.

The different types of metabolic systems that occur during enzyme degradation explain the different trends exhibited by PPO and POD activity during the study. With some systems, there is an initial synthesis of an enzyme followed by its gradual decomposition. An example is phenylalanine ammonia lyase which is directly linked to polyphenol synthesis. The presence of polyphenols then leads to an induction of PPO. As the concentration of polyphenols continues to increase, it triggers the inactivation of PAL through a lyase-inactivating system and this leads to a decrease in PAL activity. A low PAL activity and their polymerization into lignin by POD resultantly cause a decrease in polyphenols (Uritani, 1971; Lagrimini, 1991; Lewis et al., 1998). It is this unavailability of the substrate that explains a gradual decrease in PPO activity after an initial quick induction at 12 hours. This same trend was observed by other researchers who reported an immediate induction of PPO followed by a marked drop in activity (Zheng et al., 2005; Goud and Kachole, 2011; Ngadze et al., 2012).

The other metabolic system involves the continuous synthesis of an enzyme for several days without its degradation, a trend observed for POD (Uritani, 1971). Peroxidase is not affected by the PAL inactivating enzyme and this explains the continued increase in POD activity observed in all the five cultivars during the study. There were similar findings by Zheng et al., (2005) who also recorded a continued increase in POD activity during a nine day experiment.

Based on the study, the level of PPO and POD induction differed with cultivar. The highest PPO and POD induction was in Montclaire, followed by Amethyst, BP1 and Jasper that were in an intermediate position while KY20 showed the weakest PPO and POD induction. Differences in the amount of initial latent PPO, POD or phenols within leaves of different cultivars account for genotypic differences among cultivars (Lewis et al., 1998). Furthermore, PPO and POD enzymes occur as different isozymes produced differentially depending on their distribution within cells, and this leads to the simultaneous differences observed across cultivars (Zacheo et al., 1987).

5.5 CONCLUSION AND RECOMMENDATION

The hypothesis that wounding and pathogen inoculation will increase the activities of PPO and POD in potato plants was accepted. The results indicate that both wounding and pathogen inoculation have the same effect on the activity of PPO and POD although the five cultivars exhibited differences in the activities of the enzymes. The control cultivar, Montclaire, exhibited the highest PPO and POD activity while KY20 had the lowest activity. The PPO and POD activity of Amethyst, BP1 and Jasper were intermediate between those of Montclaire and KY20. Understanding these plant pathogen systems is important in developing control strategies for pathogens with no chemical control such as soft rot pathogens. Since there was no disease expression in all the cultivars in the treatments inoculated with *Pcb*, no inference can be made about the role of PPO and POD as suitable markers o resistance of these cultivars to the pathogen.

CHAPTER 6

The use of salicylic acid and acibenzolar-s-methyl to induce resistance in different potato cultivars through the activation of polyphenol oxidase and peroxidase

ABSTRACT

Salicylic acid (SA) and its functional analogue acibenzolar-s-methyl (ASM) are both chemical inducers of systemic acquired resistance (SAR). Four week old plants of five different potato cultivars were sprayed with 1.5 mM SA, 100 mg active ingredient/litre (a.i/l) ASM and sterile distilled water (control) till run-off. Polyphenol oxidase (PPO) and peroxidase (POD) activity were assayed in leaves at different time intervals of 0 (before treatment), 3 and 6 days after spraying. Results showed that cultivars had a significantly different effect on the level of PPO and POD ($P < 0.05$). The interactions between time and treatment had a significant effect on PPO and POD activity ($P < 0.05$), although the control recorded PPO and POD levels higher or equal to the other treatments. These results suggest that 1.5 mM SA and 100 mg a.i/l ASM applied as foliar sprays were generally not effective in enhancing PPO and POD levels in potatoes.

6.1 INTRODUCTION

The use of resistance inducing chemicals offers another option of an environmentally friendly strategy to control diseases that have no chemical control like soft rot. Resistance inducing chemicals are able to induce resistance to a wide range of pathogens in several crops through the activation of defence genes (Prats et al., 2002). Products of defence genes are pathogenesis-related (PR) proteins such as chitinases, glucanases and enzymes such as phenylalanine ammonia lyase (PAL) which is important in phenol and phytoalexin synthesis, peroxidase (POD) and polyphenol oxidase (PPO) which play a great role in phenol oxidation and lignin biosynthesis (Raju et al., 2008). Therefore, these PR-proteins are appropriately used as markers for induced resistance.

Exogenous application of chemicals such as salicylic acid (SA), isonicotinic acid (INA), β-aminobutyric acid (BABA), acibenzolar-s-methyl (ASM,Bion®) and potassium phosphates can enhance disease resistance both at the site of infection (local) or in tissues away from the treated area (systemic acquired resistance, SAR) (Ishii et al., 1999; Prats et al., 2002; Ghazanfar et al., 2011). Salicylic acid is a phenolic metabolite that acts as a secondary signal compound in SAR reactions and is required for the expression of PR proteins. Other compounds such as INA and ASM are functional analogues of SA that directly induce SAR genes (Métraux, 2001). Salicylic

acid and ASM have been used in crops such as tomato (Louws et al., 2001; Petrov and Andonova, 2012), potato (Bokshi et al., 2003), cucumber (Ishii et al., 1999), sunflower (Prats et al., 2002) and fruit trees (Brisset et al., 2000; Cao et al., 2006). Following foliar sprays of resistance inducing compounds such as SA and ASM, these compounds bind to receptor sites which then activate systemic acquired resistance (SAR) genes (Huang et al., 2000). These genes code for the synthesis of pathogenesis-related (PR) proteins such as chitinases and glucanases, peroxidases and polyphenol oxidases (Brisset et al., 2000; Baysal et al., 2005; Chandra et al., 2007).

Induced resistance offers a new technology that has great potential for the control of diseases, giving an additional option for the farmer to complement genetic resistance. The objective of the study was to evaluate the potential of ASM and SA in locally grown potato cultivars by analysing the induction of two defence related enzymes, PPO and POD to assess a possible relationship between the activation of these enzymes and the use of ASM and SA.

6.2 MATERIALS AND METHOD

Potato plants of cultivars Amethyst, BP1, Jasper, KY20 and Montclaire were grown outside under a shade cloth in 12 cm diameter plastic pots. One plant was maintained in each pot in which red clay soil was used as the planting media and Compound S (7:21:7 NPK) was applied as the basal fertiliser at planting. No pesticide was used throughout the experiment. Aqueous solutions SA (1.5 mM) (Sigma) and ASM (100 mg a.i/l) (active ingredient, a.i) (Syngenta) were applied as resistance inducing chemicals. There were three treatment groups of SA, ASM and the control. At four weeks, three replications for each treatment were selected for treatment application. Plants were sprayed to run-off using a 2-litre hand held pressure sprayer. The control plants were sprayed with water. Leaf samples were collected by a destructive method on each occasion. Four middle leaves were excised from the treated and control plants at 0 (before treatment), 3 and 6 days after spraying. The leaf samples were then stored in a freezer for enzyme assays. A UV-VIS spectrophotometer (Model ST-UV 752N) was used for all spectrophotometric analyses.

6.2.1 Enzyme assays

6.2.1.1 Polyphenol oxidase

The enzyme assay was carried out as described in section 5.2.5.

6.2.1.2 Peroxidase

The enzyme assay was carried out as described in section 5.2.6.

6.2.2 Statistical analysis

The experiment was a Randomised Complete Block Design with three blocks and a 5*3*3 factorial treatment structure. Replication was done across blocks. Data obtained for enzyme activity for each treatment at different time intervals was subjected to ANOVA using Genstat 14th Edition. Significant means were separated using the Fischer's Protected Least Significant Difference at $P < 0.05$.

6.3 RESULTS

6.3.1 Enzyme activity in different cultivars

Cultivars had a significant effect on PPO and POD levels ($P<0.05$) (Appendix 6.1- 6.4). In both experiments, Montclaire exhibited highest POD activity, followed by Amethyst, Jasper and BP1 which were not significantly different and the least activity was in KY20. With regards to PPO activity, lowest levels were recorded in BP1, Jasper and Montclaire while KY20 had significantly the highest activity in the first experiment. In the second experiment, KY20 significantly maintained a higher PPO activity while BP1 and Jasper exhibited the lowest PPO activity (Table 5).

Table 5: The levels of peroxide (POD) and polyphenol oxidase (PPO) enzymes measured in the leaves of different potato cultivars after spraying with salicylic acid and acibenzolar-s-methyl

	POD activity (Δ_{470} min/g fresh mass)		**PPO activity (Δ_{525} min/g fresh mass)**	
Cultivar	**Experiment 1**	**Experiment 2**	**Experiment 1**	**Experiment 2**
Amethyst	0.4314 b	0.5352b	0.01078b	0.01289b
BP1	0.4557b	0.5123b	0.00944b	0.01098a
Jasper	0.4469b	0.4954b	0.00938a	0.01071a
KY20	0.2779a	0.3968a	0.01287c	0.01309b
Montclaire	0.6284c	0.8586c	0.00867a	0.01280b
P value	<0.001	<0.001	<0.001	0.011
L.s.d	0.0724	0.0951	0.00135	0.00174

Means followed by the same letter in each column are statistically not different at $P < 0.05$

6.3.2 Effect of time and treatments on enzyme activity

The interaction between time and treatment produced a significant effect on the activity of both enzymes in both seasons ($P<0.05$) (Appendix 6.1- 6.4). However, the two resistance inducing compounds, SA and ASM did not have the expected effect on the resistance markers (PPO and POD) that were being measured. When compared with the control, SA and ASM treatments did not enhance polyphenol oxidase activity since the values for the control treatments were equal or higher than those for SA and ASM treatments at 3 and 6 days after treatment (Figure 8).

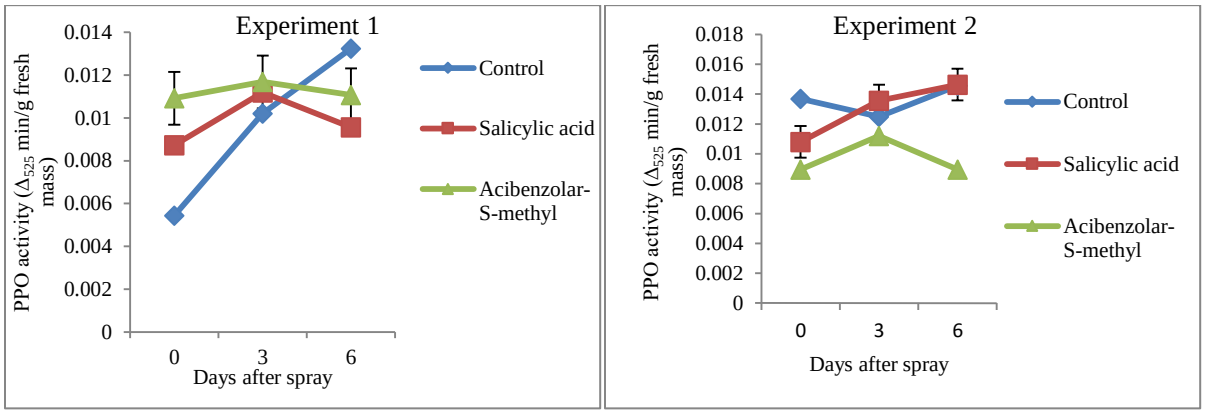

Figure 8: Time course of polyphenol oxidase in potato plants following treatments with water (control), salicylic acid (SA) 1.5 mM and acibenzolar-s-methyl (ASM) 100 mg a.i/l at 0, 3 and 6 days after foliar sprays

In the first experiment, ASM treated plants had a higher POD activity than SA and control treatments at 3 days but at 6 days the three treatments were not significantly different. In the second experiment, peroxidase activity increased over time in ASM and control treatments with ASM consistently mantaining a higher POD level than the SA and control treatments at 3 and 6 days (Figure 9). Acibenzolar-s-methyl seemed to effectively enhance the activity of POD in this experiment.

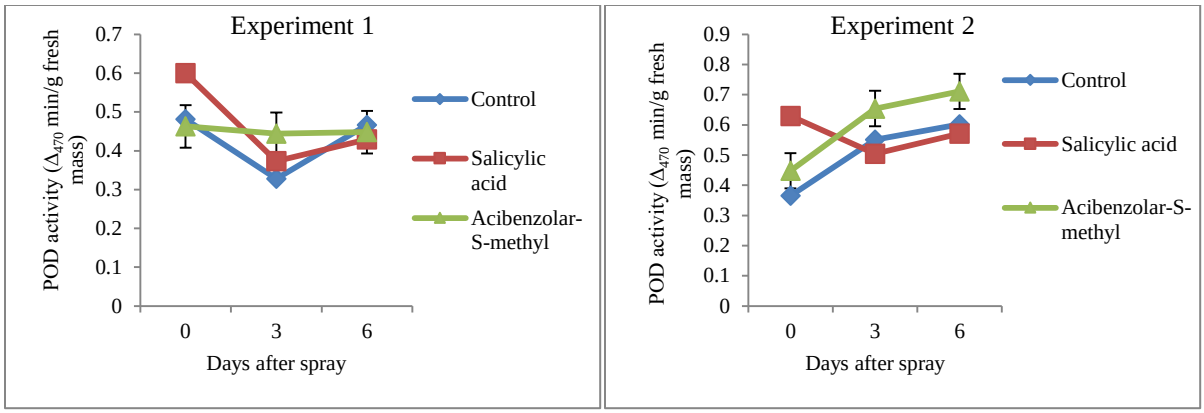

Figure 9: Time course of peroxidase activity in potato plants following treatments with water (control), salicylic acid (SA) 1.5 mM and acibenzolar-s-methyl (ASM) 100 mg a.i/l at 0, 3 and 6 days after foliar sprays.

6.4 DISCUSSION

Results from the present study showed that cultivar had a significant effect on the activity of peroxidase and polyphenol oxidase. The cultivars assayed showed some variations in their levels of PPO and POD. A notable feature is that of Montclaire, which expressed higher values of POD, had lower corresponding values of PPO. Similarly, KY20 which had the lowest POD values had its corresponding PPO values being the highest. These two cultivars clearly show that there is a time dependent activation of PPO and POD induction because of the different roles they play in plant defence. While PPO is important in the initial stages of plant defence when it oxidises phenols into the more reactive quinones which have a direct antibiotic and cytotoxic effect to pahogens (Li and Steffens, 2002), POD catalyses lignin biosynthesis by oxidising hydroxycinnamyl alcohols and their polymerisation into lignin to form physical barriers against pathogens at later stages (Okey et al., 1997; Zhang et al., 2008, Thakker et al., 2012). The corresponding activities of PPO and POD for the other three cultivars however remained in the middle range. Plant genotype has been shown to have an influence on the level of PPO and POD. Different initial latent level of phenols can account for the different levels of PPO and POD expressed in different cultivars (Lewis et al., 1998). Polyphenol oxidases and peroxidases also have several isoforms which are expressed differently depending on the substrate, signal stimulant (Melo et al., 2006) and their distribution within cells and this also leads to differences observed amongst cultivars (Zacheo et al., 1987). Expression of different PPO and POD isoforms has also been observed in four cowpea cultivars sprayed with salicylic acid (Chandra et al., 2007). A more desirable cultivar, however, would be one that shows consistently higher levels of both PPO and

POD as they are both equally important and play a complementary role as oxidative enzymes in plant defence mechanisms.

The use of salicylic acid and acibenzolar-s-methyl in this study did not produce the desired effect. In fact, SA at 1.5 mM and ASM at 100 mg a.i/l, did not provide significant activation of the two defence markers, PPO and POD. Ideally, the control treatments were expected to have the lowest enzyme activity compared to SA and ASM induced treatments. However, activities of enzymes during the different days of evaluation were fluctuating, with control plants sometimes expressing PPO and POD levels higher or equal to SA and ASM treated plants. This could have been due to the influence of environmental factors such as temperature since this was an outside experiment. There is a possibility that defence compounds could also have been produced in control plants in response to changes in temperatures (Bokshi et al., 2003; Sarwar et al., 2011), hence their elevated levels in the control plants. Nevertheless, conducting these experiments in a natural environment is still a more practical approach since it assimilates the conditions under which the crops will later be grown. Conclusions drawn from such experiments have a better practical application.

Another possibility for the limited efficacy of these compounds could have been due to the concentrations used that might have failed to effectively cause PPO and POD induction in potatoes even though the same concentrations have been used in other plant species with success. Salicylic acid at 1.5mM has been used in chickpea (Ghazanfar et al., 2011; War et al., 2011), cotton (Sarwar et al., 2011), even at lower concentrations of 1.4 mM in cowpea (Chandra et al., 2007). The same concentration of ASM used in the experiment has also been used with other researchers in potato (Bokshi et al., 2003), sunflower (Prats et al., 2002) and cucumber (Ishii et al., 1999) to induce resistance. In addition to the effect of the environment and the concentration of the chemicals, the time needed for SAR induction is variable depending on the type of plant and the stimulant (Sticher et al., 1997). According to Louws et al., (2001) SAR induction by ASM is within four days while other researchers give a range of between one and seven days after application (Baysal et al., 2005). An effort was made during the experiment to work within that time range. Salicylic acid has been shown to display very limited induction of PPO and POD in the absence of a pathogen or its elicitor (Buzi et al., 2004). Because of the several variables to be considered, several factors have to be taken into account before conclusive results can be drawn from this experiment.

6.5 CONCLUSION AND RECOMMENDATION

The experiment failed to fulfil the objective which sought to establish a possible relationship between the activation of PPO and POD with the use of SA and ASM. The use of SA and ASM did not produce the desired PPO and POD induction because the interaction of factors such as time, concentration of chemicals and the environment confounded the overall effect of SA and ASM. However, this experiment can serve as a preliminary investigation to identify factors that need to be considered in designing the subsequent experiments with appropriate modifications such as the concentration at which the chemicals will be applied when working with the potato plant in an outside environment, whether there is need for repeated routine sprays before the desired SAR induction can be achieved and then establish time at which resistance is at its maximum and possibly measure its persistence in this plant species.

CHAPTER 7: GENERAL DISCUSSION

Bacterial soft rot and blackleg caused by *Pectobacterium* or *Dickeya* pathogens are major diseases in potato production (Czajkowski et al., 2011). These diseases can be managed using cultural control methods but combining genetic resistance through the use of tolerant or resistant cultivars is a potential control strategy. In this view, the objectives of the study was to ascertain the presence of soft rot pathogens as the causal agents of blackleg and soft rot symptoms on potatoes in commercial farms around Harare and then to screen five locally grown cultivars for resistance against one soft rot pathogen. Enzyme assays were then carried out to evaluate the induction of defence enzymes in each of the five cultivars as one way to measure their inherent resistance. The last experiment involved the use of two resistance inducing compounds to induce defence enzyme as a way to complement the plant's genetic resistance against pathogens.

Biochemical and molecular tests confirmed the pathogens causing soft rot and blackleg symptoms as *Dickeya* species. Though this finding is not surprising since the presence of the pathogen has been reported before in other regions of Zimbabwe, what is of concern is its increasing geographical spread within the country. The pathogens that were known to be the causative agents of soft rot and blackleg in Zimbabwe were *Pcc* and *Pa*. The presence and dispersal of *Dickeya* specis has been attributed to several factors. There is movement of potatoes in the region, for example, potato imports from South Africa (Makoni et al., 2014); the pathogen has a wide range of hosts which facilitate its spread in a larger geographical area (Czajkowski et al., 2009) and the higher temperatures which are a result of climate change offer *Dickeya* pathogens a selective advantage over *Pa* and *Pcc* (Degefu et al., 2013).

With this clear knowledge of the presence of *Dickeya* species and the potential threat of soft rot and blackleg outbreaks, five cultivars namely Amethyst, BP1, Montclaire, Jasper and KY20 were screened for their resistance to *Pcb*, which is another highly virulent soft rot pathogen. Based on disease incidence data, the cultivars exhibited equal susceptibility to blackleg caused by *Pcb*. No cultivar is completely immune to soft rot pathogens and potatoes also exhibit a quantitative resistance against these pathogens which is partial. Cultivars can only be ranked as highly resistant, moderate or susceptible. This could have been possible if several cultivars had been used which would give clear-cut categories to group cultivars. However, the results are a clear indication of

the potential losses that can occur if an outbreak of *Pcb* was to occur with any one of these cultivars in the field.

A follow-up experiment was done with the same cultivars to analyse induction of PPO and POD in each of the cultivars after pathogen inoculation and mechanical damage. The essence of the experiment was to distinguish between genetically resistant and susceptible cultivars. Higher levels and rapid accumulation of defence enzymes is synonymous with resistant genotypes while susceptible genotypes have a slower response system. The moderately resistant control cultivar, Montclaire, exhibited highest enzyme activity; followed by Amethyst, BP1 and Jasper while KY20 exhibited the least enzyme activity. Ideally, observations made during this experiment were supposed to correlate with those of the screening resistance test or vice-versa. However, all cultivars proved equally susceptible despite the different levels of defence enzymes. Since the plant's defence system depends on the host, the pathogen and the environment, the response of cultivars to a disease might correspond accordingly with the defence enzyme levels if one factor changes, for example, the pathogen. The significance of differences in enzyme activity among cultivars might not have been apparent in the study but the results may be useful in a different setting.

The experiment on the use of SA and ASM to induce PPO and POD activity was done in order to evaluate their potential as resistance inducing compounds in potato. Enzyme activity in control treatments which were sprayed with water was sometimes higher or equal to SA and ASM treatment. There was no consistency as the values for enzyme activity in all treatments kept fluctuating. Conclusive results on the two compounds could not be drawn from the experiment. This experiment can serve as a preliminary investigation on the potential of SA and ASM as resistance inducing compounds in potatoes. A redesigned experiment which takes into account factors such as different levels of SA and ASM concentrations and more readings of shorter time intervals to measure the induction more precisely within a controlled environment might also give a better indication on the effectiveness of these two compounds to induce PPO and POD.

In conclusion, increasing *Dickeya* species are a cause for concern for all stakeholders because they are a potential threat to the potato industry. It is even more important to note that the most popular cultivars with farmers are all susceptible to soft rot and blackleg.

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APPENDICES

Appendix 4.1: Analysis of variance on the response of different cultivars to inoculation with *Pectobacterium carotovora* subsp. *brasiliensis* based on blackleg disease incidence (log_{10} transformed data) (Experiment 1).

Variate: Log_{10} incidence

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	3	0.01645	0.00548	0.33	
Block.*Units* stratum					
Cultivar	4	0.19910	0.04978	2.96	0.065
Residual	12	0.20196	0.01683		
Total	19	0.41751			

Appendix 4.2: Analysis of variance on the response of different cultivars to inoculation with *Pectobacterium carotovora* subsp. *brasiliensis* based on blackleg disease incidence (log_{10} transformed data) (Experiment 2).

Variate: Log_{10} incidence

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	3	0.06347	0.02116	1.00	
Block.*Units* stratum					
Cultivar	4	0.10024	0.02506	1.19	0.365
Residual	12	0.25324	0.02110		
Total	19	0.41695			

Appendix 5.1 Analysis of variance on the effect of pathogen inoculation and mechanical damage on polyphenol oxidase activity assayed in different potato cultivars at different time intervals (2012).

Variate: Enzyme activity

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment stratum	1	0.0000088	0.0000088	0.00	1.00
Treatment.Cultivar stratum					
Cultivar	4	0.0331616	0.0082904	2.39	0.210
Residual	4	0.0139014	0.0034754	3.77	
Treatment.Cultivar.*Units* stratum					
Time	5	0.0211500	0.0042300	4.58	0.004
Cultivar.Time	20	0.0408916	0.0020446	2.22	0.031
Residual	25	0.0230743	0.0009230		
Total	59	0.1321876			

Appendix 5.2: Analysis of variance on the effect of pathogen inoculation and mechanical damage on polyphenol oxidase activity assayed in different potato cultivars at different time intervals (2013).

Variate: Enzyme activity

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment stratum	1	0.0018593	0.0018593	3.30	0.081
Treatment.Cultivar stratum					
Cultivar	4	0.0176008	0.0044002	7.80	0.036
Residual	4	0.0022569	0.0005642	0.89	
Treatment.Cultivar.*Units* stratum					
Time	5	0.0331889	0.0066378	10.48	<.001
Cultivar.Time	20	0.0215946	0.0010797	1.70	0.103
Residual	25	0.0158318	0.0006333		
Total	59	0.0923323			

Appendix 5.3: Analysis of variance on the effect of pathogen inoculation and mechanical damage on peroxidase activity assayed in different potato cultivars at different time intervals (2012).

Variate: Enzyme activity

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment stratum	1	0.01390	0.01390	4.26	0.050
Treatment.Cultivar stratum					
Variety	4	2.32201	0.58050	178.06	<.001
Residual	4	0.01304	0.00326	0.11	
Treatment.Cultivar.*Units* stratum					
Time	5	0.57895	0.11579	3.80	0.011
Cultivar.Time	20	1.36005	0.06800	2.23	0.029
Residual	25	0.76120	0.03045		
Total	59	5.04915			

Appendix 5.4: Analysis of variance for peroxidase activity assayed in different potato cultivars at different time intervals following pathogen inoculation and mechanical damage (2013).

Variate: Enzyme activity

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment stratum	1	0.02230	0.02230	1.11	0.302
Treatment.Cultivar stratum					
Cultivar	4	0.72247	0.18062	9.02	0.028
Residual	4	0.08006	0.02001	0.59	
Treatment.Cultivar.*Units* stratum					
Time	5	1.54102	0.30820	9.10	<.001
Cultivar.Time	20	1.95722	0.09786	2.89	0.007
Residual	25	0.84712	0.03388		
Total	59	5.17020			

Appendix 6.1: Analysis of variance on the effect of salicylic acid and acibenzolar-S-methyl treatments on polyphenol oxidase assayed in different potato cultivars at different time intervals (Experiment 1).

Variate: Enzyme activity

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	2	2.315E-05	1.158E-05	0.71	
Block.Treatment stratum					
Treatment	2	6.840E-05	3.420E-05	2.09	0.240
Residual	4	6.560E-05	1.640E-05	2.65	
Block.Trtmnt.*Units* stratum					
Cultivar	4	2.981E-04	7.452E-05	12.05	<.001
Time	2	2.368E-04	1.184E-04	19.14	<.001
Treatment.Cultivar	8	1.712E-05	2.140E-06	0.35	0.945
Treatment.Time	4	2.794E-04	6.986E-05	11.29	<.001
Cultivar.Time	8	9.520E-05	1.190E-05	1.92	0.067
Treatment.Cultivar.Time	16	1.670E-04	1.043E-05	1.69	0.065
Residual	84	5.196E-04	6.186E-06		
Total	134	1.770E-03			

Appendix 6.2: Analysis of variance on the effect of salicylic acid and acibenzolar-S-methyl treatments on polyphenol oxidase assayed in different potato cultivars at different time intervals (Experiment 2).

Variate: Enzyme activity

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	2	0.00001682	0.00000841	1.71	
Block.Treatment stratum					
Treatment	2	0.00039189	0.00019594	39.79	0.002
Residual	4	0.00001970	0.00000492	0.48	
Block.Treatment.*Units* stratum					
Cultivar	4	0.00014252	0.00003563	3.46	0.011
Time	2	0.00006261	0.00003130	3.04	0.053
Treatment.Cultivar	8	0.00005502	0.00000688	0.67	0.718
Treatment.Time	4	0.00013792	0.00003448	3.35	0.014
Cultivar.Time	8	0.00018478	0.00002310	2.24	0.032
Treatment.Cultivar.Time	16	0.00009605	0.00000600	0.58	0.888
Residual	84	0.00086469	0.00001029		
Total	134	0.00197198			

Appendix 6.3: Analysis of variance on the effect of salicylic acid and acibenzolar-S-methyl treatments on peroxidase assayed in different potato cultivars at different time intervals (Experiment 1)

Variate: Enzyme activity

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	2	0.02755	0.01377	0.52	
Block.Treatment stratum					
Treatment	2	0.04120	0.02060	0.78	0.519
Residual	4	0.10617	0.02654	1.48	
Block.Treatment.*Units* stratum					
Cultivar	4	1.66914	0.41729	23.34	<.001
Time	2	0.39857	0.19928	11.14	<.001
Treatment.Cultivar	8	0.07881	0.00985	0.55	0.815
Treatment.Time	4	0.23759	0.05940	3.32	0.014
Cultivar.Time	8	0.19389	0.02424	1.36	0.228
Treatment.Cultivar.Time	16	0.19008	0.01188	0.66	0.820
Residual	84	1.50202	0.01788		
Total	134	4.44500			

Appendix 6.4: Analysis of variance on the effect of salicylic acid and acibenzolar-S-methyl treatments on peroxidase assayed in different potato cultivars at different time intervals (Experiment 2)

Variate: Enzyme activity

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	2	0.01207	0.00604	0.39	
Block.Treatment stratum					
Treatment	2	0.22275	0.11137	7.18	0.047
Residual	4	0.06207	0.01552	0.50	
Block.Treatment.*Units* stratum					
Cultivar	4	3.31769	0.82942	26.89	<.001
Time	2	0.49603	0.24802	8.04	<.001
Treatment.Cultivar	8	0.37851	0.04731	1.53	0.158
Treatment.Time	4	0.66145	0.16536	5.36	<.001
Cultivar.Time	8	0.39370	0.04921	1.60	0.138
Treatment.Cultivar.Time	16	0.42919	0.02682	0.87	0.605
Residual	84	2.59111	0.03085		
Total	134	8.56458			