EFFICACY OF PLANT EXTRACTS FOR MANAGEMENT OF POTATO BACTERIAL WILT (*Ralstonia solanacearum* Smith) IN RWANDA

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A Thesis Submitted to the Graduate School in Partial Fulfilment of the Requirements for the Doctor of Philosophy Degree in Horticulture of Egerton University

EGERTON UNIVERSITY

APRIL, 2021

DECLARATION AND RECOMMANDATION

Declaration

This thesis is my original work and has not been presented in this university or any other for the award of a degree.

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DEDICATION

This PhD research is dedicated to my lovely husband Eugène Hakuzimana and our children Jean Baptiste Mutuyimana, Josée Noella Dukuzimana, and Hervé Anova Hakuzwimana as well as anyone else who has been there for me as a source of my inspiration, courage, faith, and hope.

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ABSTRACT

Potato (*Solanum tuberosum* L.) is one of staple foods for food security program and income generation in Rwanda. However, potato yield in Rwanda is still below the potential yield. Bacterial wilt caused by *Ralstonia solanacearum* (Smith) is one of the major pathogens limiting potato production. The use of plant extracts with antibacterial activities, which are locally available and environmental friendly, could be an alternative in the management of the disease. Thus, the aim of this study was to: (i) To screen different plant extracts for their efficacy in the control of potato bacterial wilt; (ii) To evaluate the effective concentration of plant extracts in management of *R. solanacearum*; (iii) To determine the effective application frequency of plant extracts in management of *R. solanacearum,* potato growth, yield, tuber quality, post-harvest infection and post-harvest yield losses; and (iv) To identify the bioactive compounds in effective plant extracts. An *in vitro* screening of the antibacterial activity of methanol, water and chloroform extracts of ten local plant materials against the pathogen was performed. Higher antibacterial activity was found in tobacco, wild marigold and garlic extracts (19.6, 18.6, and 18.3 mm inhibition zones, respectively). An *in vivo* study was also conducted to determine the effective concentration and frequency of application of selected bioactive plant extracts over potato bacterial wilt, potato growth, tuber yield, quality, and post-harvest-infection and yield loss due to the pathogen under field conditions in two growing seasons (Season A and B). The field experiments, were laid out in a Randomized Complete Block Design (RCBD) with factorial arrangements. In field experiments, weekly and bi-weekly application of plant extracts showed higher biological control efficacy (BCE) against pathogen in potato plants (58.3 and 57.4 %, respectively) than monthly application (47.6 %) at p 0.05. Weekly and bi-weekly application of tobacco and wild marigold extracts at 50 mg mL^{-1} performed better all analysed parameters. Identification of bioactive compounds in methanol extract of tobacco was performed through high performance liquid chromatography (HPLC) technique with standards. It was detected that methanol extract of tobacco contains four bioactive compounds with activity against *R. solanacearum* (chlorogenic acid, rutin, unknown flavonoids, and 5-caffoylquinic acid). It is concluded and recommended that methanol extract from tobacco or wild marigold once applied weekly or bi-weekly at 50 mg mL^{-1} can be used in management of potato bacterial wilt and improvement of potato production and tuber quality in sustainable and organic horticulture.

TABLE OF CONTENT

DECLARATION AND RECOMMANDATION .... **ii**

COPYRIGHT .... **iii**

DEDICATION .... **iv**

ACKNOWLEDGMENTS .... **v**

ABSTRACT .... **vi**

LIST OF TABLES .... **xiii**

LIST OF FIGURES .... **xvii**

LIST OF ABBREVIATIONS AND ACRONYMS/SYMBOLS .... **xix**

CHAPTER ONE **1**

INTRODUCTION **1**

1.1 Background Information 1

1.2 Statement of the Problem 2

1.3 Objectives 3

1.3.1 Overall Objective 3

1.3.2 Specific Objectives 3

1.4 Hypotheses 3

1.5 Justification of the Study 4

1.6 Scope and Limitations 4

1.7 Definition of Terms 5

CHAPTER TWO **6**

LITERATURE REVIEW **6**

2.1 Origin and Description of Potato 6

2.2 Economic Importance of Potato 6

2.3 Constraints to Potato Production 7

2.4 Bacterial Wilt of Potato 9

2.4.1 Causal Agent 9

2.4.2 Races and Biovars of *Ralstonia solanacearum* and their Geographical Distribution 10

2.4.3 Pathogen Cycle, Survival and Dispersal 12
2.4.4 Mechanisms of Infection and Symptoms 14
2.4.5 Economic Importance of Bacterial Wilt 16
2.5 Methods of Controlling Potato Bacterial Wilt 16
2.5.1 Chemical Control Methods 16
2.5.2 Cultural Practices 18
2.5.3 Use of Transgenic Potato Plants 20
2.5.4 Biological Control Measures 22
2.6 Modes of Action of Plant Antimicrobial Compounds 32
2.7 Gaps in Knowledge 34

CHAPTER THREE 35
VIRULENCE AND CHARACTERIZATION OF ISOLATES OF POTATO BACTERIAL WILT CAUSED BY *Ralstonia solanacearum* (Smith) IN RWANDA 35
Abstract 35
3.1 Introduction 35
3.2 Materials and Methods 37
3.2.1 Origin of Bacterial Isolates 37
3.2.2 Isolation, Identification and Storage of Isolates 39
3.2.3 Pathogenicity Test 40
3.2.4 Biovar Identification 42
3.3 Results and Discussion 42
3.3.1 Bacterial Streaming Test 42
3.3.2 Cultural and Morphological Characteristics of Colonies on Growth Media . 43
3.3.3 Morphological Characteristics of Bacterial Cells through Gram Staining 45
3.3.4 Pathogenicity and Virulence of the Bacterial Isolates 45
3.3.5 Biovar Identification 49
3.4 Conclusion 51

CHAPTER FOUR 52
IN VITRO ANTIBACTERIAL ACTIVITY OF SELECTED PLANT

EXTRACTS AGAINST POTATO BACTERIAL WILT (*Ralstonia solanacearum* Smith) IN RWANDA **52**
Abstract 52
4.1 Introduction 52
4.2 Materials and Methods 54
4.2.1 Plant Extracts 54
4.2.2 Extraction of Bioactive Compounds 55
4.2.3 Preparation of Bacterial Inoculum 56
4.2.4 Screening for Antibacterial Activity of Plant Extracts 56
4.2.5 Determination of Minimal Inhibitory Concentration (MIC) 57
4.3 Results and Discussion 58
4.3.1 Screening of Plant Extracts against Ralstonia solanacearum 58
4.3.2 Minimal Inhibitory Concentration (MIC) of Plant Extracts 63
4.4 Conclusion 66

CHAPTER FIVE **67**
EFFECT OF FREQUENCY OF APPLICATION OF SELECTED PLANT EXTRACTS ON POTATO GROWTH, YIELD, QUALITY, POST-HARVEST INFECTION AND YIELD LOSS **67**
Abstract 67
5.1 Introduction 68
5.2 Materials and Methods 69
5.2.1 Description of Study Area 69
5.2.2 Preparation of Bacterial Inoculum 73
5.2.3 Preparation of Potato Seeds and Planting 73
5.2.4 Experimental Layout 74
5.2.5 Data Collection 76
5.2.6 Data Analysis 79
5.3 Results and Discussion 80
5.3.1 Effect of Plant Extracts on Potato Growth Parameter 80
5.3.2 Effect of Plant Extracts on Yield Parameters 105

5.3.3 Effect of Plant Extracts on Potato Tuber Quality Parameters 114
5.3.4 Effect of Plant Extracts on Control of DI and their BCE in Potato Plants and Tubers 131
5.3.5 Effect of Plant Extracts on Potato Tuber Post-harvest Infection (PHI) and Post-harvest Yield Losses (PHL) due to Ralstonia solanacearum 155
5.4 Conclusion 163

CHAPTER SIX 165
IDENTIFICATION OF BIOACTIVE COMPOUNDS AGAINST POTATO BACTERIAL WILT IN SELECTED PLANT EXTRACTS 165
Abstract 165
6.1 Introduction 166
6.2 Materials and Methods 168
6.2.1 Phytochemical Screening of Bio-compounds in Plant Extracts 169
6.2.2 Fractionation of Plant Extracts and Test of Antibacterial Activity of Fractions 172
6.2.3 Identification of Bioactive Compounds 174
6.3 Results and discussion 175
6.3.1 Phyotochemical Screening of Bio-compounds 175
6.3.2 Fractionation of Plant Extracts and their Antibacterial Activity 182
6.3.3 Identification of Bioactive Compounds in Tobacco Plant Extract against Ralstonia solanacearum 193
6.4 Conclusion 202

CHAPTER SEVEN 204
GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS 204
7.1 General Discussion 204
7.2 Conclusions 209
7.3 Recommendations **........ 210**
7.4 Areas for Further Studies 210

REFERENCES **212**
APPENDICES **229**
Appendix A: Published paper 1 229
Appendix B: Published paper 2 230
Appendix C: Collected plant species from Rwanda for in vitro antibacterial screening Against Ralstonia solanacearum 231
Appendix D. Raw data of effect of plant extracts (S), solvent extracts (E) and concentrations (C) on disease incidence (DI %) in greenhouse experiment 232
Appendix E. Raw data and ANOVA tables of effect of different concentrations of plant extracts on control of disease incidence (DI) under greenhouse conditions.. 234
Appendix F. Mean separation for effect of different concentrations of plant extracts on number of days to 50% sprouting and flowering in greenhouse experiment 238
Appendix G. Mean separation for effect of different concentrations of plant extracts on yield (t ha-1) in greenhouse experiment 239
Appendix H. Mean separation of effect of different concentrations of plant extracts, on tuber size (quality parameter) in greenhouse experiment 240
Appendix I. Mean separation of effect of different concentrations of plant extracts on potato tuber post-harvest infection (PHI) and yield loss (PHL) in greenhouse experiment 241
Appendix J. Raw data of effect of different application frequency of plant extracts on total yield (t ha-1) in season B 242
Appendix K. Data of interactions between analysed factors in days to 50 % sprouting and flowering in field experiment 243
Appendix L. Data of interactions between analysed factors in plant height (28, 56, and 70 AP) in field experiment 245
Appendix M. Data of interactions between analysed factors in number of stems per plant in field experiment 248
Appendix N. Data of interactions between analysed factors in yield parameters (number of tubers per plant and total yield t ha-1) in field experiment 251

Appendix O.	Data of interactions between analysed factors in quality parameters (tuber size, DM, SG, and RS content) in field experiment	253
Appendix P.	Data of interactions between analysed factors in disease incidence (DI) and biological control efficacy (BCE) in plants in field experiment	258
Appendix Q.	Data of interactions between analysed factors in disaese incidence (DI) and biological control efficacy (BCE) in tubers in field experiment	263
Appendix R.	Data of interactions between analysed factors in post-harvest infection and post-harvest yield loss in stored potato tubers from field experiment	265
Appendix S.	Comparison between chromatograms of different extracts from wild marigold through HPLC recorded at 230 nm	267
Appendix T.	Comparison between chromatograms of different extracts from garlic through HPLC recorded at 230 nm	268
Appendix U.	Greenhouse experiment	269
Appendix V.	Comparison between potatoes treated with plant extracts and potatoes treated with controls under field conditions	270
Appendix W.	Isolation of bioactive compounds from methanolic extract of tobacco through open column chromatography and Thin Layer Chromaatography (TLC)	271
Appendix X.	Research permit	272

LIST OF TABLES

Table 2.1. Biovars of Ralstonia solanacearum based on oxidation/utilization of alcohols or sugars 11
Table 2.2. Equivalence between biovars and races of R. solanacearum and their location 12
Table 2.3. Modes of action of botanicals against plant pathogens 33
Table 3.1. Disease incidence (DI) and disease severity index (DSI) over time caused by inoculation of potato seedlings with different isolates of Ralstonia solanacearum 48
Table 4.1. Collected plant materials for screening against Ralstonia solanacearum 55
Table 4.2. Growth inhibition zone (mm) of methanol, water and chloroform extracts from ten selected plants against Ralstonia solanacearum 56
Table 4.3. Growth inhibition zone (mm) of solvent extracts (methanol, water and chloroform) against *Ralstonia solanacearum* 59
Table 4.4 Minimum inhibitory concentration 64
Table 5.1. Environmental conditions in study area during field experiments 72
Table 5.2. Experimental layout of field experiments 75
Table 5.3. Effect of plant extracts, application frequency, and solvent extracts on number of days after planting (DAP) to 50% sprouting in season A and B 81
Table 5.4. Effect of plant extracts, application frequency, and solvent extracts on number of days after planting (DAP) to 50% flowering in season A and B 83
Table 5.5. Effect of growing seasons (A and B) on days to 50 % sprouting and flowering 85
Table 5.6. Effect of plant extracts on number of stems per plant in season A and B on various days after planting (DAP) 94
Table 5.7. Effect of application frequency of plant extracts on number of stems per plant in season A and B on various days after planting (DAP) 95
Table 5.8. Effect of solvent extracts on number of stems per plant in season A and B on various days after planting (DAP) 97
Table 5.9. Effect of plant extracts on plant height (cm) in season A and B on various days after planting (DAP) 98
Table 5.10. Effect of application frequency of plant extracts on plant height (cm) in season A and B on various days after planting (PA) 100

Table 5.11. Effect of solvent extracts on plant height (cm) in season A and B on various days after planting (DAP) 101
Table 5.12. Effect of growing seasons (A and B) on number of stems per plants and plant height.. 102
Table 5.13. Effect of plant extracts, application frequency and solvent extracts on number of tubers per plant in season A and B 106
Table 5.14. Effect of plant extracts, application frequency and solvent extracts on total yield (t ha-1) in season A and B.. 108
Table 5.15. Effect of growing seasons (A and B) on yield parameters 110
Table 5.16. Effect of plant extracts on tuber size in season A.. 115
Table 5.17. Effect of plant extracts on tuber size in season B 116
Table 5.18. Effect of application frequency of plant extracts on tuber size in season A..... 117
Table 5.19. Effect of application frequency of plant extracts on tuber size in season B 118
Table 5.20. Effect of solvent extracts on tuber size in season A 119
Table 5.21. Effect of solvent extracts on tuber size in season B.. 120
Table 5.22. Effect of growing seasons (A and B) on tuber size 121
Table 5.23. Effect of plant extracts, application frequency and solvent extracts on potato dry matter (DM %) content in season A and B.. 124
Table 5.24. Effect of plant extracts, application frequency and solvent extracts on specific gravity (SG) of potato in season A and B.. 126
Table 5.25. Effect of plant extracts, application frequency and solvent extracts on reducing sugars (RS) content of potato in season A and B.. 128
Table 5.26. Effect of growing seasons (A and B) on dry matter (DM), specific gravity (SG), and reducing sugar (RS) content of potato tuber 130
Table 5.27. Effect of plant extracts on disease incidence (DI %) in potato in season A on various days after incoluation (DAI).. 132
Table 5.28. BCE (%) of plant extracts against potato bacterial wilt in season A on various days after inoculation (DAI) 133
Table 5.29. Effect of application frequency of plant extracts on disease incidence (DI %) in potato in season A on various days after inoculation (DAI) 134

Table 5.30. BCE (%) of application frequency of plant extracts against potato bacterial wilt in season A on various days after inoculation (DAI)................. 135

Table 5.31. Effect of solvent extracts on disease incidence (DI %) in potato in season A on various days after inoculation (DAI) 136

Table 5.32. BCE (%) of solvent extracts against potato bacterial wilt in season A on various days after inoculation (DAI) 137

Table 5.33. Effect of plant extracts on disease incidence (DI %) in potato in season B on various days after inoculation 138

Table 5.34. BCE (%) of plant extracts against potato bacterial wilt in season B on various days after inoculation (DAI) 139

Table 5.35. Effect of application frequency of plant extracts on disease incidence (DI %) in potato in season B on various days after inoculation (DAI)............ 140

Table 5.36. BCE (%) of application frequency of plant extracts against potato bacterial wilt in season B on various days after inoculation (DAI)............................... 141

Table 5.37. Effect of solvent extracts on disease incidence (DI %) in potato in season B on various days after inoculation (DAI) 142

Table 5.38. BCE (%) of solvent extracts against potato bacterial wilt in season B on various days after inoculation (DAI) 143

Table 5.39. Effect of growing seasons (A and B) on disease incidence (DI %) in potato on various days after inoculation (DAI) 144

Table 5.40. Effect of growing season (A and B) on BCE of plant extracts against disease incidence (DI %) in potato on various days after inoculation (DAI) ... 145

Table 5.41. BCE (%) of plant extracts against DI (%) in potato tubers in season A and B 146

Table 5.42. BCE (%) of application frequency of plant extracts against DI (%) in potato tubers in season A and B... 147

Table 5.43. BCE (%) of solvent extracts against DI (%) in potato tubers in season A and B 148

Table 5.44. Effect of growing season (A and B) on BCE of plant extracts against disease incidence (DI %) in potato tubers.. 149

Table 5.45. Effect of plant extracts on potato tuber post-harvest infection (PHI) and post-harvest yield losses (PHL) in season A and B 150

Table 5.46. Effect of application frequency of plant extracts on potato tuber post-harvest infection (PHI) and post-harvest yield losses (PHL) in season A and B 157
Table 5.47. Effect of solvent extracts on potato tuber post-harvest infection (PHI) and post-harvest yield losses (PHL) in season A and B 158
Table 5.48. Effect of growing season (A and B) on potato tuber post-harvest infection (PHI) and post-harvest yield losses (PHL) due to *Ralstonia solanacearum* 159
Table 6.1. Method and conditions of HPLC used for fractions of plant extracts 173
Table 6.2. Isolated fractions in methanol fraction of tobacco extract by open column chromatography and TLC 174
Table 6.3. Phytochemical screening of the methanol and water extracts of tobacco, marigold, and garlic materials 176
Table 6.4. Growth inhibition zone (mm ± SD) of fractions of plant extracts from tobacco, wild marigold and garlic against *Ralstonia solanacearum* 189
Table 6.5. Growth inhibition zone (mm ± SD) of isolated fractions in methanol fraction of tobacco extract against *Ralstonia solanacearum* 194

LIST OF FIGURES

Figure 2.1. Ranking of potato production in African countries 7
Figure 2.2. Symptoms of bacterial wilt of potato 15
Figure 3.1. Symptoms of potato bacterial wilt (*Rasltonia solanacearum*) on potato plants and tubers collected from Kinigi site 38
Figure 3.2. Identification of isolated R. *solanacearum* by vascular flow test.................... 43
Figure 3.3. Morphological features of colonies of R. *solanacearum* on TTC and CPG culture media 44
Figure 3.4. Microscopic observation of Gram staining of RsKinigi, RsGikungu, and RsKirundo bacterial isolates (A, B, and C, respectively) 45
Figure 3.5. Wilting of potato seedlings at 5, 10, 15, and 20 days after inoculation (1st, 2nd, 3rd, and 4th row, respectively) with *Ralstonia solanacearum* isolate) 46
Figure 3.6. Biovar identification of three isolates of R. solanacearum from Rwanda 50
Figure 4.1. Characterization of R. solanacearum isolate RsGikungu by vascular flow test (A). Morphological features of the colonies on TTC and CPG culture (B, C). Microscopic observation of gram stained *R. solanacearum* (D) 56
Figure 4.2. Micro-dilution assay for Minimal inhibitory concentration (MIC) of methanol and water extracts from active plant species against R. *solanacearum* on 96-well micro titer plates 63
Figure 5.1. Map of location of study area (Busogo) in Rwanda 70
Figure 6.1. Flowchart of main activities in identification of bioactive compounds in plant extracts 168
Figure 6.2. Chromatograms of initial methanol (a) and water extract of tobacco (b) recorded at 230 nm 183
Figure 6.3. UV-spectra of the main peaks of initial methanol extract of tobacco recorded at 230 nm 184
Figure 6.4. Chromatograms of initial methanol (a), diethyl ether (b) and n-hexane (c) fractions of tobacco extract recorded at 230 nm 185
Figure 6.5. Chromatograms of initial methanol (a) and methanol fraction (b) of tobacco extract recorded at 230 nm 186

Figure 6.6. Chromatograms of methanol fraction of tobacco extract (a), a mixture of chlorogenic acid (tr= 27.558 min) and rutin (tr= 42.803 min) (b) and caffeic acid (tr= 27.564 min) (c) recorded at 230 nm.......................... 187

Figure 6.7. TLC of methanol extract from tobacco (T) compared to Rutin (R) and chlorogenic acid mixed with rutin (CHA+R) (left). Comparison of methanol extract of tobacco (T) with caffeic acid (CA) (middle). Overlaid UV-spectra of chlorogenic acid and caffeic acid (right).. 188

Figure 6.8. Antibacterial activity of initial methanol of wild marigold (20) and tobacco (21), Mmethanol fraction of wild marigold (22) and tobacco extract (23)... 189

Figure 6.9. In vitro growth inhibitory zone of five (14, 11, 12, 13, and 15) promising fractions of methanol fraction of tobacco extract against Ralstonia solanacearum 195

Figure 6.10. Chromatograms of fraction 14 (387-393) (a), standards, a mixture of chlorogenic acid (tr= 27.558 min) and rutin (tr = 42.803 min) (b), and caffeic acid (tr= 28.012 min) (c) recorded at 230 nm. Overlaid UV- spectra of rutin standard at tr= 42.803 min and of a compound in fraction 14 (tr= 42.687 min) (d) 196

Figure 6.11. Chromatogram of fraction 11 (286-325) (a) and fraction 12 (326-373) (b) recorded at 17.568 min. UV-Spectrum of a major bioactive compound isolated in 11 and 12 fractions at tr= 17.568 min (c)...................................... 197

Figure 6.12. Chromatograms of fraction 13 (374-386) (a), standards, a mixture of chlorogenic acid (tr=27.558 min) and rutin (tr =42.803 min) (b) and caffeic acid (tr=28.012 min) (c) recorded at 230 nm 197

Figure 6.13. Chromatograms of fraction 15 (394-404) (a), standards, a mixture of chlorogenic acid (tr=27.558 min) and rutin (tr =42.803 min) (b) and caffeic acid (tr=28.012 min) (c) recorded at 230 nm 198

LIST OF ABBREVIATIONS AND ACRONYMS/SYMBOLS

APPPC: Asia and Pacific Plant Protection Commission
ATP: Adenosine triphosphate
CABI: Centre for Agriculture and Biosciences International
CFU: Colony forming units
CIP: Centre International de la Pomme de terre (International Potato Center)
COSAVE: Comite de Sanidad Vegetal del Cono Sur
DAFF: Department of Agriculture, Forestry and Fisheries
DI: Disease incidence
DNA: Deoxyribonucleic acid
DS: Disease severity
EPPO: European and Mediterranean Plant Protection Organization
FAO: Food and Agriculture Organization of the United Nations
GIS: Geographic Information System
IAPSC: Inter-African Phytosanitary Council
INES: Institut Superieur d'Enseignement
IPDN: The International Plant Diagnostic Network
MINIRENA: Ministère des Resources Naturelles (Ministry of Natural Resources)
NAPPO: North American Plant Protection Organization
RAB: Rwanda Agriculture Board
REMA: Rwanda Environment Management Authority

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Potato (*Solanum tuberosum* L.) is the world's most widely grown tuber crop (Strange & Scott, 2005; Were *et al.*, 2013) and the fourth major food crop of the world after rice, wheat and maize (Mulugeta *et al.*, 2019; Wagura *et al.*, 2011). Potato is regarded as high-potential food security crop because of its ability to provide a higher yield per unit input with a shorter crop cycle (mostly less than 120 days) than major cereal crops like maize (Guchi, 2015). Potato is mainly grown in the cool highland areas (REMA, 2011; Were *et al.*, 2013). Its starchy tuber is used as staple food and vegetable (Mulugeta *et al.*, 2019; Strange & Scott, 2005). In Rwanda, potato is one of the main food crops, source of national food security and income generation especially in the Northern regions (Muhinyuza *et al.*, 2014; RAB, 2012; REMA, 2011). Annual consumption of potato is around 125 kg per person per year and it is the country's second most important source of energy after cassava (REMA, 2011). Rwanda is the sixth most important potato producer in Sub-Saharan African region after South Africa, Kenya, Tanzania, Nigeria, and Malawi followed by Angola, and Ethiopia (FAO, 2018).

Although potato is an important food and cash crop especially in developing countries, its actual yield is below the potential yield (Mulugeta *et al.*, 2019; Wagura *et al.*, 2011). In Rwanda, the average yield is estimated at 9 t ha^{-1} in farmers' fields compared to potential yield of 40 to 60 t ha^{-1} (Masengesho *et al.*, 2012; Muhinyuza *et al.*, 2014). Insect pests and diseases are among the major limiting factors in potato production worldwide (Muhinyuza *et al.*, 2007; Mulugeta *et al.*, 2019) which decrease the production of the crop by 36% (Yuliar *et al.*, 2015). Among plant diseases, soil-borne diseases are considered to be the most limiting than seed-borne or air-borne diseases. They cause annual losses estimated at 30 to 100% in potato crop (Ariena van Bruggen 2006; Mwaniki *et al.*, 2017; Rado *et al.*, 2015; Shili *et al.*, 2016).

In Rwanda, one of the most destructive soil-borne diseases in potato and other solanaceous plants like tomato, eggplant, pepper and tobacco is bacterial wilt which is also known as brown rot, a disease caused by *Ralstonia solanacearum* Yabuuchi *et al.*, 1995 formerly known as *Pseudomonas solanacearum* Smith (Muhinyuza *et al.*, 2007; REMA, 2011). *Ralstonia*

solanacearum threats the production of different crops mainly in warm ereas like tropical and subtropical but also in some warm and cool temperate regions of the world (Fock *et al.*, 2001; Rahman *et al.*, 2012). Bacterial wilt is the most economically serious pathogen of potato with a zero tolerance in different European countries (Mansfield *et al.*, 2012; OEPP/EPPO, 2004). Futhermore, *R. solanacearum* is one of the top ten plant pathogenic bacteria that lead to high yield loss throughout the world (Guchi, 2015; Yuliar *et al.*, 2015).

1.2 Statement of the Problem

Ralstonia solanacearum is currently one of the most serious problems, which threatens the production of potato in different parts of Rwanda and causing extensive yield losses. The losses caused by bacterial wilt of potato are currently estimated at between 30-90% in the field and 98% of tubers during storage. The disease is considered to be more problematic because its control with synthetic chemicals is not easy, effective chemicals are not readily available and costly. Furthermore, most used antibiotics including penicillin, streptomycin, ampicillin, and tetracycline are expensive and beyond the reach of small scale farmers and these pesticides were shown to be less effective against the pathogen. However, overuse of synthetic chemicals like streptomycin can result to pesticide resistance by the pest forcing the farmers to either increase dosage or frequency of application like streptomycin. The increased levels and repeated applications of these chemicals have impacted negatively on compliance with requirements concerning food safety, environmental safety, occupational health and safety and animal welfare. Currently, farmers tend to use mainly cultural control practices such as crop rotation, use of clean seeds, planting in non-infected soils and growing tolerant varieties. However, these techniques have different limitations. For example, crop rotation is not feasible because the pathogen can survive in the soil for long periods even in the absence of host plants and has a wide host range. Moreover, the disease has been observed even in first planting in newly cleared land. In addition, small farm size is also another challenge. Furthermore, it is not easy to find clean seeds because potato is mainly propagated by using tubers and this vegetative propagation method favors disease spread from mother tubers to next generation of propagules. The use of tolerant varieties is also limited because they are scarce, farmers do not appreciate some of them like Cruza, and they can harbor the latent infection in tubers (Fock *et al.*, 2001; Muthoni, 2014;

REMA, 2011). Therefore, there is need to seek for alternative methods which are environmental friendly and with minimal occupational health issues.

1.3 Objectives

1.3.1 Overall Objective

To contribute to improvement of potato production and quality through effective management of potato bacterial wilt by application of plant extracts.

1.3.2 Specific Objectives

i. To screen different plant extracts for their efficacy in the control of potato bacterial wilt.

ii. To evaluate the effect of different concentrations of plant extracts in management of *Ralstonia solanacearum*, potato growth, yield and tuber quality, post-harvest infection and post-harvest yield losses.

iii. To determine the effect of different application frequencies of plant extracts in management of *Ralstonia solanacearum,* potato growth, yield and tuber quality, post-harvest infection and post-harvest yield losses.

iv. To identify the types of bioactive compounds in effective plant extracts.

1.4 Hypotheses

i. Plant extracts have no effect on the control of potato bacterial wilt.

ii. The different concentrations of plant extracts have no effect in management of potato bacterial wilt, growth of potato, yield and tuber quality, post-harvest infection and post-harvest yield losses.

iii. There is no significant effect of different application frequencies of plant extracts in management of potato bacterial wilt, potato growth, yield and tuber quality, post-harvest infection and post-harvest yield losses.

iv. The types of effective bioactive compounds in the plant extracts are not different.

1.5 Justification of the Study

In Rwanda, potato production is hampered by *R. solanacearum*, a pathogen that cannot be effectively controlled by chemicals or agronomic and cultural practices like other main potato diseases such as late blight. Therefore, potato protection against *R. solanacearum* by using plant extracts with antimicrobial activities, which are locally available, easy to prepare, nontoxic to non-target organisms, cheaper, and environmental friendly is among promising control methods. However, effects of local plant materials on management of potato bacterial wilt, improvement of plant growth, yield, and tuber quality of potato grown in Rwanda is still missing. In addition, post-harvest infection and yield loss due to bacterial wilt in potato crop as well as the effect of plant extracts in controlling these damages in Rwanda has been not fully studied. Hence, in this study, screening of ten local plant extracts was done to determine their efficacy in the control of incidence and severity of bacterial wilt in potato grown in Rwanda as well as their effects on potato growth, yield, quality, post-harvest infection and post-harvest yield loss. Moreover, the present research contributed towards identification of bioactive compounds present in selected plant species against *R. solanacearum*. The findings from this study will provide an alternative management strategy for the potato bacterial wilt. This will also help in increasing yield and quality of potato produced in Rwanda. The increase of potato yield as well as seed quality will help to enhance the income generation and food security for small-scale farmers.

1.6 Scope and Limitations

Both *in vitro* and *in vivo* experiments were conducted using plant extracts from local plant and pathogen isolates from potato cultivars, which are growing in Rwanda. *In vitro* experiments covered the screening of plant species for their potential in the control of potato bacterial wilt, determination of minimum inhibitory concentration, and identification of bioactive compounds against *R. solanacearum*. *In vivo* studies were applied both under greenhouse (pilot experiment) and open field conditions. They were used to determine the virulence of bacterial wilt isolates, efficacy of screened plant extracts, application concentrations, and effective frequency of application in the control of bacterial wilt in potato crop and also their effects on potato growth, yield, quality of tubers, and post-harvest infection and post-harvest yield loss. It was assumed that plant extracts which inhibited bacterial growth in *in vitro* experiments were also able to control bacterial wilt under greenhouse and field conditions.Furthemore, the best promising plant extracts against the pathogen could also have the ability to improve potato growth, yield, and tuber quality and reduce post-harvest infection and yield loss caused by *R. solanacearum.*

1.7 Definition of Terms

Bioactive compound: A type of chemical in small amounts in plants and certain foods which are biologically active in promotion or destruction of good health in living organism, tissue, or cell.

Biovar: A group of microorganisms, usually bacteria that have identical genetic but different biochemical or physiological characters.

Botanical: A natural substance obtained or derived from plant such as a plant part or extract and used typically in medical or cosmetic products.

Disease incidence: Number of individuals that develop a specific disease or experience a specific health-related event in the population at a given time.

Disease severity: Term used to characterize the proportion of relevant host tissues or organ covered by symptom of lesion or damaged by the disease.

Pathogenicity: The potential disease-causing capacity of the pathogen i.e ability to harm the host and the overt damage in host-pathogen interactions.

Post-harvest infection: Infection that develops on or in harvested parts of plant

Soil-borne pathogen: Pathogen that lives within the soil and causing root disease.

Virulence: Degree of pathogenicity caused by the organism.

CHAPTER TWO

LITERATURE REVIEW

2.1 Origin and Description of Potato

Potato (*Solanum tuberosum* L.) is an autotetraploid (2n=4x=48) crop (Sequeira & Rowe, 1969) which belongs to the family of Solanaceae among which includes other crops such as tomato and eggplant (Guchi, 2015). Potato originated in the Andean regions of South America where it has been grown for over 2400 years (Muthoni, 2014). Currently, potato is grown in more than 150 countries worldwide from latitudes 65ºN to 50ºS and up to altitude of 4700 m a.s.l. Potato is a predominantly self-pollinated crop with flowering and fruit set mainly affected by genotype, day length, and temperature conditions (Muthoni *et al.*, 2013). It is classified as a short-day and cool-season crop (DAFF, 2013). It is one of the temperate crops mainly grown in the cool highland regions of the tropics (Were *et al.*, 2013) and other world areas with regular rainfall and temperatures ranging between 10 to 23ºC (Strange & Scott, 2005). Potato can adapt to different ecological conditions (Abalo *et al.*, 2001) but in tropics the optimum tuber development requires the temperature ranging about 15ºC and not above 27ºC (DAFF, 2013; REMA, 2011). This crop has a short growing cycle since it can be harvested in 3 to 4 months and this allows double or even triples cropping of the same land in a single year (Abalo *et al.*, 2001). In Rwanda, potato is mostly planted during the rainy seasons and thus, two times per year (Muhinyuza *et al.*, 2014).

2.2 Economic Importance of Potato

To date, potato is the world's most widely grown tuber crop (Guchi, 2015; Wagura *et al.*, 2011). More than a billion people worldwide consume potato and the Food and Agriculture Organization (FAO) recommend it as a food security crop (Uwamahoro *et al.*, 2018). At global scale, average potato yield is 17 t ha^{-1} and the direct consumption as human food is 31.3 kg per capita (Muthoni, 2014). Asia and Europe are the major potato producing continents with more than 80%, whereas Africa produces about 5% of world production. At the regional level, East and Central Africa regions account for over 45% of potato production (Muthoni, 2014). In Africa, Rwanda is the ninth producer of potato after Egypt, Algeria, South Africa, Kenya, Morocco, Tanzania, Nigeria, Malawi, and the sixth in sub-Saharan Africa countries (Figure 2.1; FAO, 2018).

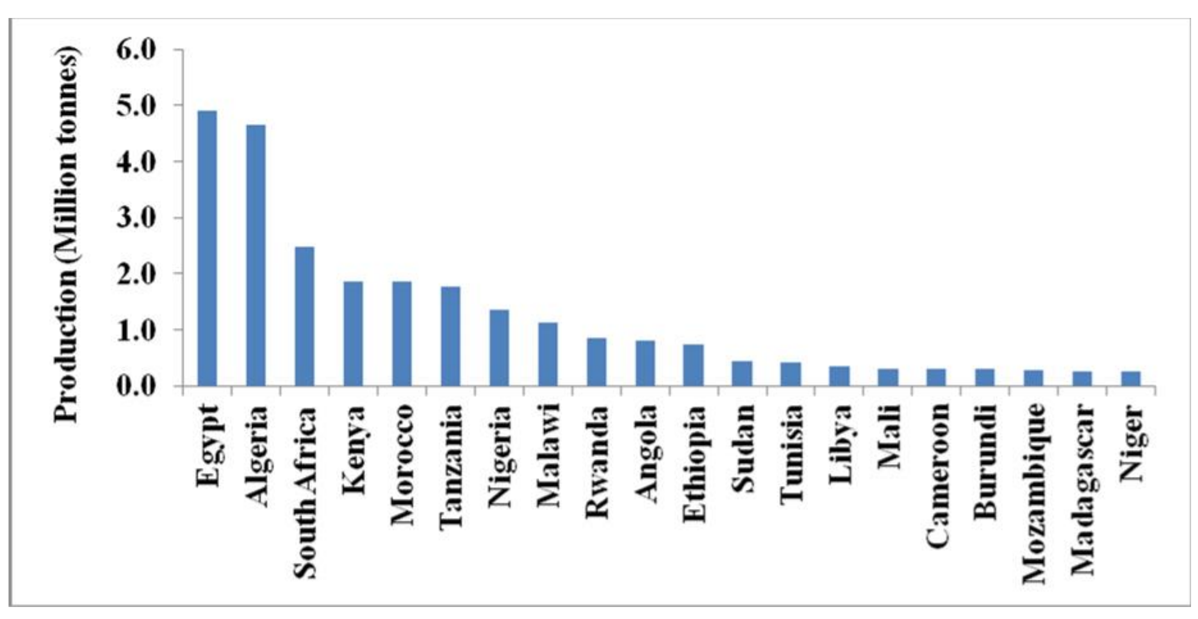

Figure 2.1. Ranking of potato production in African countries

Source: FAO (2018)

Potato is one of the most important crops in developing countries, its production is expanding more rapidly than that of most other crops, and it is becoming an important source of food for growing populations, rural employment and income generation (Guchi, 2015). Its starchy tuber is used as staple and vegetable (Were *et al.*, 2013). In Rwanda, potato is among six priority crops in the country, used as source of national food security and income generation (RAB, 2012; Uwamahoro *et al.*, 2018). Annual consumption of potato is around 125 kg per person per year and it is the country's second most important source of energy after cassava and the second food crop after banana (Muhinyuza *et al.*, 2014; REMA, 2011). Potato grows in different parts of Rwanda but the major producing areas are located in Northern regions where it is grown in rotation with maize (Muhinyuza *et al.*, 2007; RAB, 2012). In Rwanda, the average yield is around 9 t ha^{-1} which is far below the potential yield of 40 to 60 t ha^{-1} in developed countries (Masengesho *et al.*, 2012; Uwamahoro *et al.*, 2018).

2.3 Constraints to Potato Production

Although potato is an important food and cash crop especially in developing countries, its actual production is below the potential yield (Wagura *et al.*, 2011). Among limiting factors in potato production include shortage of good quality seed, pests and diseases, lack of disease resistant

varieties, lack of high yielding varieties, poor agronomic practices like fertilizer application, and post-harvest losses (Muhinyuza *et al.*, 2014; Wagura *et al*., 2011). Generally, plant diseases and pests decrease the production of crops worldwide by 36%, and diseases alone reduce crop yields by 14% (Yuliar *et al.*, 2015).

The most important diseases in potato production include fungal diseases like late blight (*Phytophthora infestan*s Mont De Bary), early blight (*Alternaria solani* Sorauer), Fusarium dry rot (*Fusarium solani* Mart. Sacc. and *F. oxysporum* Schlechtend), Verticillium wilt (*Verticillium alboatrum* Reinke and Berth and *V. dahlia* Kleb.), stem canker (*Rhizoctonia solani* Kuhn), and powdery scab (*Spongospora subterranea* Sacc.). In addition, bacterial diseases such as bacterial wilt (*Ralstonia solanacearum* Yabuuchi *et al.*, 1995), bacterial soft rot (*Erwinia carotovora* Jones Dye), common scab (*Streptomyces scabies* Waksman and Henrici), blackleg (*E. chrysanthemi* Burkholder), and viral diseases limit potato production in the world (CIP, 1996; DAFF, 2013; Monther & Kamaruzaman, 2010; Rahman *et al.*, 2012).

In Rwanda, the severe diseases causing yield loss include late blight (*P. infestans* Mont De Bary) followed by bacterial wilt (*R. solanacearum* Yabuuchi *et al.*, 1995) (Muhinyuza *et al.*, 2007; REMA, 2011). Although late blight is ranked at the first line, its negative impact on potato crop can be managed by applying fungicides like Dithane M45 or Ridomil in combination with the use of resistant varieties (REMA, 2011). However, bacterial wilt should be considered as more problematic than late blight because there are no known chemical control procedures once potato plant is infected (Masengesho *et al.*, 2012; Wagura *et al*., 2011). This is due to the fact that the pathogen has a wide host range (more than 200 crops species) and geographic distribution, long survival (more than 8 years in soil) even in absence of host crop, its lethality, its spread through seed, rainfall water, and its association with weeds (Mansfield *et al.*, 2012; Yuliar *et al.*, 2015). Beside the lack of effective pesticides, the effects of chemicals on consumer health, natural enemies as well as pesticide resistance restrict their use at global level (REMA, 2011). In a survey conducted by Uwamahoro *et al.* (2018), they also confirmed that potato bacterial wilt is the most severe pathogen in potato production in Rwanda among the others.

According to CIP (1996), bacterial wilt or brown rot caused by *Pseudomonas solanacearum* Smith or *R. solanacearum* (Yabuuchi *et al.*, 1995) is ranked first among three most important bacterial diseases that inhibit potato production at global scale. This is followed by bacterial soft rot and black leg caused by *E. carotovora* Jones Dye and *E. chrysanthemi* Burkholder and common scab caused by *S. scabies* Waksman and Henrici follow in that order of importance. In addition, *R. solanacearum* is one of the top ten bacterial species that have been listed based on their scientific and economic importance as plant pathogens (Guchi, 2015; Yuliar *et al.*, 2015). Bacterial wilt causes yield losses estimated from 30-100% and almost all tubers (98%) can be lost during storage according to the bacterial race and biovar, host, cultivar, climate, soil type, and cropping system (Guchi, 2015; Monther & Kamaruzaman, 2010; Rahman *et al.*, 2012; Uwamahoro *et al.*, 2018). This pathogen is also among the quarantine pathogens and this causes indirect losses when quarantine measures involve a restriction movement or destruction of plant products (Guchi, 2015).

2.4 Bacterial Wilt of Potato

2.4.1 Causal Agent

Bacterial wilt or brown rot is a plant disease caused by a bacterium *Pseudomonas solanacearum* Smith currently renamed *Ralstonia solanacearum* (Yabuuchi *et al.*, 1995) and which was described for the first time as *Bacillus solanacearum* by Smith in 1896 (Fock *et al.*, 2001; Muthoni *et al.*, 2012). The seed and soil-borne pathogenic bacterium affects more than 50 botanical families among which the crops belonging to Solanaceae family are the most susceptible (Denny, 2006; Mansfield *et al.*, 2012; Monther & Kamaruzaman, 2010). In addition to solanaceous crops, it causes serious yield losses of banana and groundnut (French, 1994; Huet, 2014; Mansfield *et al.*, 2012; Muthoni *et al.*, 2012; Priou *et al.*, 2001).

Based on taxonomic tree, *R. solanacearum* is classified under the domain of Bacteria, phylum Proteobacteria, class Betaproteobacteria, order Burkholderiales, family Ralstoniaceae, genus Ralstonia, species *Ralstonia solanacearum* (Denny, 2006; Mansfield *et al.*, 2012; Priou *et al.*, 2001). Different synonyms include *Bacterium solanacearum* Smith, *Burkholderia solanacearum* Yabuuchi *et al.*, 1992, *Pseudomonas solanacearum* Smith and its common names are brown rot (potato), southern bacterial wilt (tomato), Moko disease (banana), and Granville wilt (tobacco).

Ralstonia solanacearum is a non-spore forming, non-fluorescent species of the genus *Pseudomonas*, aerobic and Gram-negative bacterium with round-shaped cells, 05-1.5 μm in length, motile with a single, polar flagellum (EPPO/CABI, 1992; Rahman *et al.*, 2012).

2.4.2 Races and Biovars of Ralstonia solanacearum and their Geographical Distribution

Great variation has led to classification systems of *R. solanacearum* such as race and biovar systems. The determination of races or strains is based on the host range (Mansfield *et al.*, 2012; Monther & Kamaruzaman, 2010) and five races are recognized (Muthoni *et al.*, 2012). Race 1 affects a wide range of plant species including potato, tomato, eggplant, tobacco, chili, groundnut, olive, eucalyptus, geranium, and several weeds (CIP, 1996; Muthoni *et al.*, 2012). Race 2 affects plants of the Musaceae family such as banana and plantains and *Heliconia* species (CIP, 1996; Mansfield *et al.*, 2012). Race 3 affects mainly potato and tomato but it is weakly virulent to other solanaceous crops (Mansfield *et al.*, 2012; Muthoni *et al.*, 2012). This race has a narrow range of plant hosts in comparison to race 1 and it is mostly referred as potato race (Denny, 2006; Monther & Kamaruzaman, 2010). Race 3 is well adapted to cool-temperate areas (Rahman *et al.*, 2012). Race 4 affects particularly ginger but in Australia it has been also detected in tomato, eggplant, pepper and some native weeds (Muthoni *et al.*, 2012). Race 5 affects the mulberry trees and found only in China (CIP, 1996; Rahman *et al.*, 2012). Beside race classification, bacterial wilt strains can also be distinguished into different five biovars on the basis of their ability to acidify disaccharides (cellobiose, lactose, and maltose) and hexose alcohols (dulcitol, mannitol and sorbitol) (CIP, 1996; Rahman *et al.*, 2012) (Table 2.1).

Table 2.1. Biovars of *Ralstonia solanacearum* based on oxidation/utilization of alcohols or sugars

Biochemical Test		Biovars				
Oxidation of		1	2	3	4	5
	Mannitol	-	-	+	+	
	Sorbitol	-	-	+	+	-
	Dulcitol	-	-	+	+	-
Utilization of	Lactose	-	+	+	-	+
	Maltose	-	+	+	-	+
	Cellobiose	-	+	+	-	+

Source: Muthoni *et al.* (2012)

Bacterial wilt is mainly distributed in the tropical, subtropical, warm temperate regions and even in some cool temperate regions of the world (Fock *et al.*, 2001; Mansfield *et al.*, 2012; Rahman *et al.*, 2012). In tropical regions plants are mostly damaged by race 1 biovars 1, 3, and 4 of *R. solanacearum* whereas in tropical highlands and temperate areas potatoes and tomatoes are affected by race 3 biovar 2 strain, a race which is well adapted to cool temperatures (Muthoni *et al.*, 2012; Priou *et al.*, 2001; Strange & Scott, 2005) (Table 2.2). Biovars of race 1 are mainly soil-borne strains and have a higher genetic and phenotypic variation whereas biovar 2 of race 3 is mostly a seed-borne and genetically homogenous (Monther & Kamaruzaman, 2010; Rahman *et al.*, 2012). Bacterial wilt spread to potato producing countries like Australia, South-Eastern United States, Asia, and Europe. In Africa, it is the most serious potato disease throughout central and southern regions mainly in Uganda, Rwanda, Ethiopia, Kenya, Burundi, Nigeria, Madagascar, and Cameroon and its infection in tubers has restricted potato exports to European markets (Hammes, 2013; Priou *et al.*, 2001).

Table 2.2. Equivalence between biovars and races of *Ralstonia solanacearum* and their location

Race	Biovars	Hosts	Location
1	1, 3, 4	All solanaceous crops and other hosts	Lowland tropics
2	1, 3	Musaceae species	American and Asian tropics
3	2	Potato and tomato	Cool climate worldwide
4	3, 4	Ginger	Asia
5	5	Mulberry	China

Source: Muthoni *et al.* (2012)

2.4.3 Pathogen Cycle, Survival and Dispersal

Ralstonia solanacearum is usually closely associated with its living host plants mainly solanaceous crops and temporarily in infected host-plant debris or host weeds (Mansfield *et al.*, 2012; Monther & Kamaruzaman, 2010). Usually, once a plant host is infected it contains a very high population of *Ralstonia* cells. The inoculum survives in the dead plant tissues until the next infection of another host plant (Rahman *et al.*, 2012). This plant pathogenic bacterium also survives for a period of 5 to 8 years in soil or other environment where there are host plants or weeds like *Datura* and *Portulaca* depending on interrelating biotic or abiotic factors (Denny, 2006; Hammes, 2013). Various environmental factors such as temperature, moisture and rainfall, soil type, inoculum potential, and other soil biological factors such as nematode populations have been reported to correlate with development rate, survival and incidence of the pathogen (Hammes, 2013; Yuliar *et al.*, 2015).

In general, increase in ambient temperature between 24-35°C increases the incidence and rate of onset of bacterial wilt on hosts whereas temperature, which is above 40°C is fatal to the bacterium. In the regions with temperature below 10°C it is rarely found and this behavior varies among races and biovars (Hammes, 2013; Muthoni *et al.*, 2012; Muthoni, 2014). It has been observed that the virulence of biovar 3 and biovar 4 increase with the increase in ambient temperature whereas biovar 2 (race 3) gives significantly more wilt at lower temperatures but its incidence and severity decrease when temperature drops below 15°C and considerably below to 4°C (Guchi, 2015; Hayward, 1991). In addition, high soil moisture and periods of wet weather

are associated with high disease reproduction, survival and high incidence (Denny, 2006; Hammes, 2013; Hayward, 1991). However, excess soil moisture is harmful to the survival of the *Ralstonia* due to lack of oxygen (Mansfield *et al.*, 2012). Bacterium can survive for more than 40 years in water at ambient temperature ranging from 20 to 25°C. At 4°C temperature, extremes pH, and presence of contaminants and salts in water and soil decrease survival rate (Denny, 2006; Mansfield *et al.*, 2012). However, although cold storage (from 4 to 6°C) reduces the population level of bacteria in plant tissues, once plants are shifted from cold to warm conditions, the bacteria develop rapidly (Yuliar *et al.*, 2015).

In the soil, the pathogen may remain there almost indefinitely because it can survive saprophytically and/or it can parasitize a number of very common weeds (REMA, 2011). For short-term survival initial inoculum population and bacterial strain, soil moisture levels, type, depth and organic matter or nutrient content as well as host debris, latent infected tubers and soil microfauna and microflora are most important factors for its survival (Hammes 2013; Muthoni et al., 2012; Muthoni, 2014). In the absence of plant host, bacteria can colonize the rich soil near roots or asymptomatically then latently infect roots for long term survival. After multiplying, they return to the soil layers to repeat the cycle (Denny, 2006; Mansfield *et al.,* 2012).

Spread of *R. solanacearum* race 3 between countries mainly involves the vegetative propagating materials that carry latent infections when contaminated with the pathogen (Muthoni *et al.*, 2012; REMA, 2011; Yuliar *et al.*, 2015). For instance, a resistant cultivar such as Cruza 148 acts as a vehicle of latent infection and this type of tolerance is unsafe because it helps to spread the disease with apparently healthy material (Hayward, 1991; Yuliar *et al.*, 2015). Race 2, is mainly transmitted by infected seed and insect vectors and has a potential for rapid spread and also causes Moko disease of banana (Denny, 2006; Mansfield *et al.*, 2012). Race 1 and 3 spread in irrigation water when infected weeds grow with their roots and stem parts in water. Disease transmission by seed in groundnut was reported in Indonesia only (EPPO/CABI, 1992). In addition, transmission of *R. solanacearum* may occur through symptomless weeds, movement of soil and run-off water, farm implements as well as animal hooves (Hayward, 1991; Yuliar *et al.*, 2015). Mansfield *et al.* (2012) reported that *Ralstonia* naturally may live in the anti-virulent form to save energy and cellular resources for long survival in absence of host and once the host

becomes available, the pathogen reproduces, and once the cell mass is plenty, the extracellular virulence factors are produced.

2.4.4 Mechanisms of Infection and Symptoms

Ralstonia solanacearum enters plant through stomata or natural wounds or wounds made by farm tools during agricultural practices, or by nematodes and insects in the soil like xylem-feeding spittlebugs (CIP, 1996; Guchi, 2015). At high bacteria population, pathogen can also infect the plant roots at formation points of secondary roots. Usually, the incubation time differs and this depends on environmental conditions, host age and species, and resistance level of the host. After entrance, the next step in the pathogen life cycle is to colonize host tissues (Denny, 2006; Mansfield *et al.*, 2012). Once inside the plant, it moves towards the vascular bundles, and colonizes the xylem where the bacteria adhere by polar attraction to the vessel walls, or invade the lumen in process accelerated at high temperatures.

The presence of the bacteria inside the xylem is coupled with the production of exopolysaccharides which block the vascular vessels inducing a water shortage throughout the plant and a systemic infection that leads to plant wilting even in soil with high moisture content (CIP, 1996; Guchi, 2015; Muthoni *et al.*, 2012) (Figure 2.2B). Blocking of the vessels causes initial wilting of part of stems of a plant or one side of a leaf or stem while the remainder of plant appears healthy. This wilting occurs during the hot hours of the day when transpiration rate is high with recovery at night (Denny, 2006). As the disease develops, the plant fails to recover, vascular tissue rots, forming pits and die (Figure 2.2A: Mansfield *et al.*, 2012; Muthoni *et al.*, 2012; Priou *et al.*, 2001)**.** Sometimes the leaves turn yellow and then ultimately turn brown until they totally die (Denny, 2006; Mansfield *et al.*, 2012).

External symptoms on tubers may or may not be visible depending on the state of development of the disease. An infected potato tuber may show droplets of bacterial slime oozing from the eyes and clumps of soil sticking to this slime (Priou *et al.*, 2001; Muthoni *et al.*, 2012) (Figure 2.2C). Other pathogens inducing wilting in plants do not produce such kind of ooze. When tubers are cut, grayish white droplets of bacterial cream come out of them (Figure 2.2A: Priou *et al.*, 2001; Mansfield *et al.*, 2012). Infected tubers often shows a brownish discoloration of the

vascular ring (Figure 2.2D) and this is where brown rot name was derived from (Muthoni *et al.*, 2012; Pradhanang *et al.*, 2003; Priou *et al.*, 2001). This brown discoloration of the vascular tissue in tuber can be confused with ring rot caused by *Clavibacter michiganesis* subsp. *Sepidonicus* (Denny, 2006; Mansfield *et al.*, 2012).

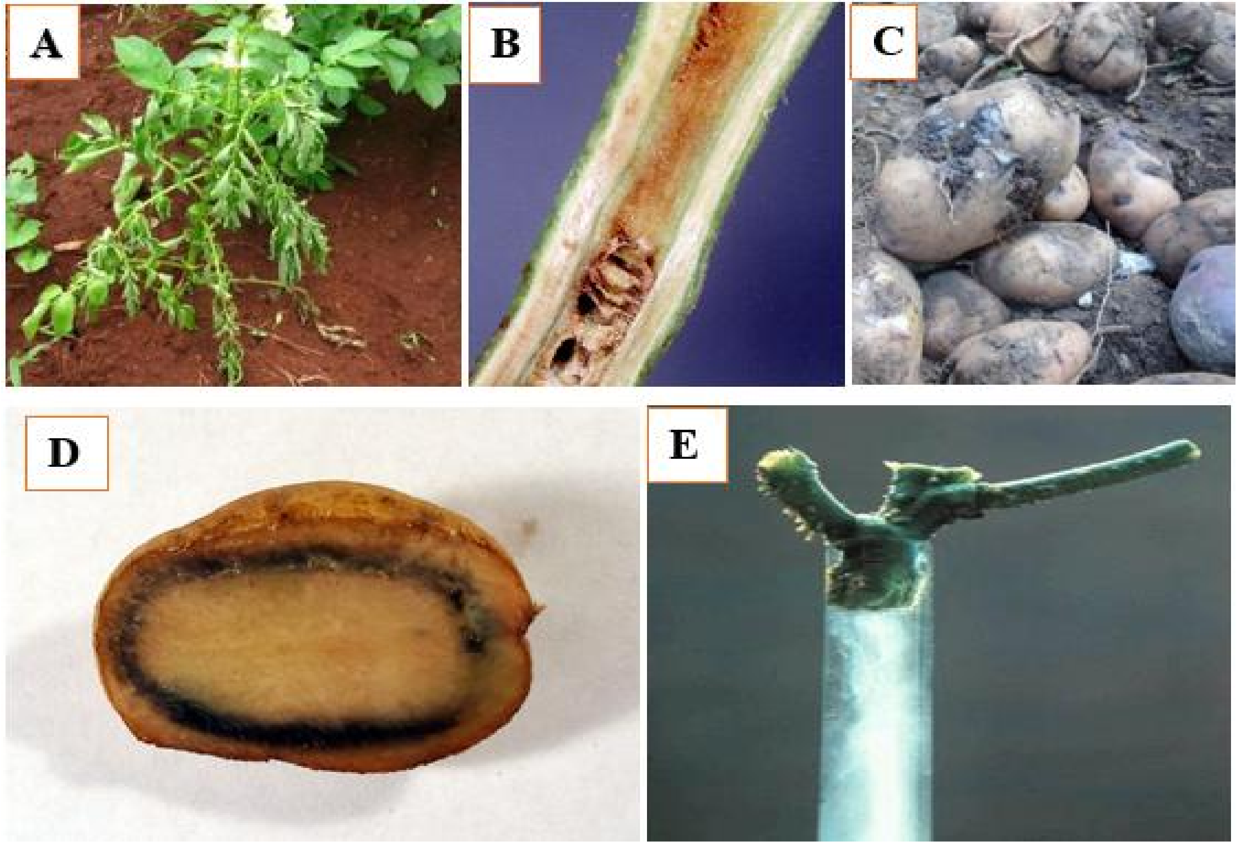

Figure 2.2. Symptoms of bacterial wilt of potato: stem wilting (A), vascular vessel with presence of exopolysaccharides produced by bacteria (B), soil adherence to potato eyes (C), brownish discoloration of the vascular ring (D), bacterial slime oozing from vascular bundles (E)

Source: Muthoni *et al.* (2012); Priou *et al.* (2001)

In addition, symptoms of bacterial wilt may be distinct from those of ring rot caused by *C. michiganensis* subsp. *sependonicus* Spieckermann & Kotthoff by the bacterial ooze that often emerges from cut stems, eyes or stem-end attachment of infected tubers (Figure 2.2E). Threads of ooze exude when a cut tissue is dipped in water and these threads are not formed by other

pathogens of potato. For ring rot, squeezed or pressed tubers produce yellowish vascular tissue and bacterial slime (Muthoni *et al.*, 2012; Priou *et al.*, 2001).

2.4.5 Economic Importance of Bacterial Wilt

European and Mediterranean Plant Protection Organization (EPPO), North American Plant Pro tection Organization (NAPPO), Inter-African Phytosanitary Council (IAPSC), Asia and Pacific Plant Protection Commission (APPPC), and Comite de Sanidad Vegetal del Cono Sur (COSAVE) have defined bacterial wilt as the most economically cruel pathogen of potato and a quarantine pathogen with a zero tolerance (CIP, 2017; EPPO/CABI, 1992; IPDN 2014; OEPP/EPPO, 2004). The economic losses caused by *Ralstonia* result from either direct pathogen infection in crops during growth or post-harvest loss, cost of control measures or quarantine and rejection of infected potatoes at old or new export markets (EPPO/CABI, 1992; Mansfield *et al.*, 2012).

Economic losses due to *R. solanacearum* relate to different factors namely climate, host plant, cultivar, soil properties, cultural practices as well as bacterial wilt strains. The pathogen is the most important disease in potatoes in warm temperate, tropical and subtropical regions. Bacterial wilt can also takes place in cool regions like in tropical highlands. It is more rigorous in tropical areas where it can lead to 100% of wilting incidence (Denny, 2006; Mansfield *et al.*, 2012). Some of *Ralstonia* strains are also more severe than others. A study conducted in India showed that bacterial wilt causes disease incidence estimated at 10 to 100% and results to yield losses, which can reach 91% in tomatoes. *Ralstonia solanacearum* causes important loss in potato crop, which is a staple food for millions of farmers throughout the world. This pathogen affects more than 1.5 million Ha of potato plantation in more than 80 countries with an annual global loss estimated at $ 950 million. In some countries like Bolivia, the pathogen induces yield loss ranging from 30-98% (Guchi, 2015).

2.5 Methods of Controlling Potato Bacterial Wilt

2.5.1 Chemical Control Methods

Plant pathogenic bacteria are extremely hard to control because they cause systemic infection in vascular bundle of plant that make it difficult to destroy them by spraying chemicals or removing

infected plant organs (Guchi, 2015; Yuliar *et al.*, 2015). Furthermore, phythopathogenic bacteria grow exponentially by the time and once the symptoms are visible, they are already well established in plant at high rate of severity to devastate the whole plant. They are spread either by rain, wind, or animals to the new sites (Yuliar *et al.*, 2015). In addition, management of bacterial wilt by chemicals seems to be nearly impossible to apply or more complicated since it has no known chemicals that control it effectively once potato plant is infected (Fock *et al.*, 2001; Guchi, 2015). This is due to its soil-borne nature, lethality, persistence, wide host range and broad geographic distribution (Monther & Kamaruzaman, 2010; Rahman *et al.*, 2012; Yuliar *et al.*, 2015).

Chemical control of plant pathogenic bacteria is more problematic in crop protection due to insufficient bactericides. In addition, several bacterial pathogens invade a high range of plant hosts and transmitted for long distance by infected planting materials, irrigation water or farming tools (Guchi, 2015). Apart from copper compounds and antibiotics, there are no other chemicals existing for controlling plant bacterial pathogens (Yuliar *et al.*, 2015). Moreover, even when the chemicals are available they are expensive and not affordable by most of potato growers (Masengesho *et al.*, 2012; Wagura *et al.*, 2011). Diverse chemicals were assessed for their effectiveness in the control of *Ralstonia* but their efficacy was limited (Yuliar *et al.*, 2015).

Chemicals which are being used to control this pathogen include streptomycin, ampicillin, tetracycline and penicillin. However, these antibiotics have shown hardly any effect or increased the resistance and incidence of bacterial wilt, for example streptomycin (EPPO/CABI, 1992; Yuliar *et al.*, 2015). In addition, chemical treatment can be achieved with, metam sodium, chloropicrin, and 1,3-dichloropropene for fumigation of contaminated soils while sodium hypochlorite can be applied at field level for general field sanitation (Yuliar *et al.*, 2015). Plant activators which generate systemic resistance namely validoxylamine and validamycin and other chemicals like algicide (3-[3-indolyl] butanoic acid were also used for tomato crop protection (Yuliar *et al.*, 2015). However, these are very expensive, pollutants or cannot be used on large areas and not practical in many parts of the world (Muthoni *et al.*, 2012). In general, excessive use of chemicals can lead to pesticide resistance and environmental pollution on a global basis that may result to the death of people, livestock; beneficial insects, birds, fish, and other wildlife

as well as desirable plants (Muthoni *et al.*, 2012). Some researchers have reported that the use of synthetic chemicals is the best way to control plant pathogens but it not all the time the case (Monther & Kamaruzaman, 2010). For instance, careless application of pesticides may result into the resistance or remaining of toxic residues in the environment, contamination of soil and/or groundwater, and toxic to farmers and animals (Yuliar *et al.*, 2015). For example, it is documented that use of different antibiotics leads to development of resistance in different bacterial strains and to horizontal transfer of virulence to other pathogenic bacteria or cross-resistance in human pathogens (Yuliar *et al.*, 2015). Most of these chemicals are banned in most of the countries.

Furthermore, copper compounds are more toxic and are harmful to the environment and this why their use is constrained in developed countries (Yuliar *et al.*, 2015). Therefore, it is mandatory that all such materials be carefully managed and used only if it is necessary (REMA, 2011). All the mentioned challenges and disadvantages in use of antiobitics and other chemicals in the control of bacterial diseases provide a stimulus to researchers and farmers in development of new control measures. For managing phyto-pathogenic bacteria, use of biological control methods including plant extracts are a promising alternative since they are environmental friendly especially in organic farming system (Yuliar *et al.*, 2015).

2.5.2 Cultural Practices

Once potato crop is already infected by *R. solanacearum* there are no curative methods which are effective against this pathogen. However, some treatments can be applied for containing disease incidence and severity and spread of inoculum in new areas or new hosts. Therefore, preventive measures are highly recommended to avoid any initial infection of bacterial wilt (Guchi, 2015). Today, farmers tend to use mainly cultural practices as single or integrated strategies to manage plant pathogenic bacteria. These practices involve a crop rotation program with non-host plants, intercropping of potato with other crops such as carrot, onion, maize, beans, wheat, or barley to reduce inoculum density and disease development. The use of clean seeds, planting in non-infected soils, growing tolerant varieties, control of nematodes, removal of infected or suspect plants and destroying them, and weeding are also recommended. Other agronomic practices include the use of clean water for irrigation and sanitation of farm implements (Guchi, 2015; Pal,

2006; Uwamahoro *et al.*, 2018). *Ralstonia solanacearum* is an EPPO A2, APPPC and IAPSC quarantine organism and the absence of the bacterium is an important consideration for countries exporting seed potatoes (EPPO/CABI, 1992). Therefore, application of quarantine measures is another practice that may reduce spread of bacterial wilt in new areas. Quarantine strategy include the avoidance of introduction of contaminated potato seeds or planting material in an area where the pathogen was not present (Pal, 2006; Strange & Scott, 2005; Yuliar *et al.*, 2015).

Cultural methods that may be applied to modify plant growth environment in order to ensure optimum plant growth involve selection of suitable growing sites for the crop; adequate fertilization mostly by organic manure; adequate tillage and proper cultivation to improve root growth and avoid plant injury. This method also implies other techniques such orientation of plantings to improve exposure to sun and air currents; water management through proper drainage for instance by raising planting beds to manage root and stem rots; irrigation or altering soil pH to ensure the vigor of plant and to increase soil microflora to the disadvantage of a soil borne pathogen. It may also involve changing date or depth of seeding, plant spacing or other practices that allow plants to escape infection or reduce severity of pest or disease (Muthoni *et al.*, 2012).

However, most of these techniques have individual practical, technological and economic limitations (Wagura *et al.*, 2011; Yuliar *et al.*, 2015). For example, crop rotation is not sufficient because the pathogen can survive in the soil for long periods in the absence of host plants. This requires a crop rotation program of five to eight years without susceptible crops. Moreover, the disease has been observed even in first planting in newly cleared land. In addition, small farm size is also another challenge in crop rotation plan (Guchi, 2015; Muthoni *et al.*, 2012). Furthermore, it is not easy to find clean seeds to be used for a long period because vegetative propagation in potato favors disease spread in different generations (REMA, 2011; Wagura *et al.*, 2011; Yuliar *et al.*, 2015). In addition, quarantine measures are either expensive or difficult to apply and may also limit production and commercialization of ware potatoes (Muthoni *et al.*, 2012).

The use of resistant varieties is among other means of managing bacterial wilt severity (Muhinyuza *et al.*, 2014; Muthoni *et al.*, 2012; REMA, 2011). However, there is no high level of resistance in potato to bacterial wilt (Champoiseau *et al.*, 2009; EPPO/CABI, 1992; REMA, 2011; Yuliar *et al.*, 2015). Although some potato cultivars with moderate tolerance such as Cruza 148 and Molinera have been cultivated in some countries like Rwanda, Burundi, Madagascar, Brazil, and Congo; these did not control the disease effectively (Champoiseau *et al.*, 2009; Fock *et al.*, 2001). Therefore, there is need to develop more resistant or tolerant cultivars (Fock *et al.*, 2001; Yuliar *et al.*, 2015). In addition, although some potato cultivars seem tolerant and do not show visible symptoms like wilting especially in cool regions, they harbor latent infection in tubers and can spread the disease via progeny tubers (DAFF, 2013; Muthoni, 2014). Furthermore, some cultivars have shown some defects such as high glycoalkaloid content and sensitivity to temperature conditions (Fock *et al.*, 2001). The use of tolerant varieties is also hindered by some farmers perception and preferences, for instance Cruza cultivar in Rwanda (REMA, 2011). All these aspects make effective management of bacterial wilt complicated (Champoiseau *et al.*, 2009).

2.5.3 Use of transgenic potato plants

Another technique of managing potato pathogens is the use of transgenic potato cultivars which are resistant against various biotic and abiotic stresses through plant genetic engineering. This technique consists of a direct modification of plant genetic material by using biotechnology. Genetic engineering is more advantageous than conventional breeding in potato protection since it allows not only the modification, silencing or removal of an undesired gene but also introduction and interchange of gene of interest across plants. This genome-editing technology enables the creation of new potato genotypes resistant against a specific pathogen even in crop species which are naturally multiplied through vegetative mean like potato. Therefore, genetic engineering allows the exploitation of the existing genes of the plant and also the transfer of foreign gene of interest. Genetic engineering is performed though plant transformation by inserting new DNA into plant genome at a chosen location, or editing of the existing plant DNA and RNA machineries by removing, inserting, or replacing the target DNA fragment to get desired plant genome with or without foreign genetic material (Dong & Ronald, 2019).

Genome-editing technology was recently used to enhance plant defence mechanisms against different fungal, viral, and bacterial diseases. For instance, plant micro RNA (miRNA) and RNA interference (RNAi) were exploited to get edited plant genome resistant against different viral diseases like cucumber vein yellowing virus, wheat dwarf virus, potato virus, tomato yellow leaf curl, and cassava brown steak virus (Schenke & Cai, 2020). In addition, genetic engineering also permitted the creation of plant varieties resistant against fungal pathogens such as *P. infestans in tomato* and *Magnaporthe oryzae* in rice, (Boschi *et al.*, 2017; Schenke & Cai, 2020). Furthermore, plant genome was also exploited to obtain transgenic plants resistant against bacterial diseases namely *Xanthomonas* sp., *Pseudomonas sp,* and *E. amylovora* in apple (Boschi *et al.*, 2017; Dong & Ronald, 2019).

In potato and tomato, transgenic varieties were also created to enhance the resistant of these crops against bacterial wilt. For instance, it has been reported that transgenic potatoes carrying *Arabidopsis thaliana* (At) pattern recognition receptor elongation factor-Tu (EF-Tu) receptor (EFR), which is a Pattern Recognition Receptor (PRR) are able to detect the bacterial pathogen-associated molecular pattern (PAMP). This recognitions of bacterial molecules released during the infection confer the ability of synthetizing antimicrobial compounds that inhibit cell wall degrading enzymes released by bacterium or neutralizing the toxins secreted by pathogen for host tissues colonization (Dong & Ronald, 2019). This positive effect of plant genetic engineering in management of *R. solanaceraum* was confirmed by Boschi *et al.* (2017) in a greenhouse experiment where they have found that potato varieties expressing AtEFR were much more resistant against *R. solanacearum* in both plants and tubers at the same level as wild type as compared to the control.

Although plant genetic engineering is environmentally friendly and effective in obtaining transgenic potato lines resistant against various plant pathogens, this technology is still facing various challenges. Most of results were obtained from greenhouse experiments and the assessment of the durability of this resistance under field conditions where plants are challenged to be infected by high bacterial inoculum in combination with different environmental factors is still needed. In some European countries, transgenic plants are still classified under genetically modified organisms (GMOs) and regulatory measures have to been applied. In those countries

also, GMOs are less acceptable by many consumers (Boschi *et al.*, 2017; Schenke & Cai, 2020). Moreover, it has been reported that the increasing of plant immunity against pathogens affects the original plant physiology and this results to negative side effects associated with plant growth like dwarfism, reduced plant resistance to other, accelerated senescence, and low yield (Boschi *et al.*, 2017; Dong & Ronald, 2019). Therefore, further studies should be carried out carefully to determine all negative effects of this technology and how far it can be exploited to produce resistant varieties with good agronomic traits. In addition, plant genome editing especially in a potato which is a polypoid crop with complex genome is an expensive, time consuming and difficult technique which requires highly skilled labour and enough equipment (Schenke & Cai, 2020).

2.5.4 Biological Control Measures

Biological control is a protection measure in crop protection comprising the use of non-chemicals. This implies the use of living and antagonistic organisms as well as natural products to control pests and pathogens (Yuliar *et al.*, 2015). Although these methods are novel, they have more potential in plant production and with simultaneous ability to protect environment and non-target organisms. In bio-control methods, plant pathogens are controlled by different mechanisms such as antagonism, root colonization, plant growth promotion or localized and systemic induced resistance (LIR and SIR). These mechanisms are not achievable through chemical control. For instance, rhizobacteria, bacteria that colonize plant root enhance biological control of pathogens through exudation of antibiotics or inducing plant resistance. They can also compete for nutrients and space against pathogens in rhizosphere. In addition, these microorganisms also act in promoting plant growth by supplying nutrients under harsh conditions or reducing the population of harmful organisms. Moreover, incompatible races of pathogens, avirulent strains of a pathogen, and natural products can trigger either localized or systemic induced resistance (LIR and SIR) in plant hosts (Guchi, 2015; Yuliar *et al.*, 2015).

Use of Biological Control Agents

Biological control can involve the reduction of disease population, its incidence and severity through antagonistic microorganisms or biological control agents (BCAs) (Alabouvette *et al.*, 2006). The critical criteria for selecting a BCA are short time for generation, mobility and

perseverance of the organism in the roots of host plants (Yuliar *et al.*, 2015). The BCAs suppress plant pathogens either by direct action when they interact with pathogen mainly during its saprophytic phase or by indirect effect by inducing resistance of the host plant. Direct action of these organisms is achieved through parasitism (causing the death of the target organism), antibiosis (producing toxic compounds) and competition for nutrients. Indirect action may be accomplished by production of defense compounds like phytoalexins, pathogenesis-related (PR) proteins and by reinforcement of cell walls which result to an induced systemic resistance (ISR) (Alabouvette *et al.*, 2006).

In bacterial wilt control, this technique is still in its infancy and has been accomplished through use of an antagonistic bacteria *B. polymyxa* and *Pseudomonas fluorescens* Migula. These microorganisms have been used and positively controlled potato bacterial wilt *in vitro* and in field experiments (Alabouvette *et al.*, 2006; Guchi, 2015). These bacteria produce siderophores that are actively liable in the control of numerous soil-borne pathogens. Siderephore compounds act in biological control by deprivation of nutrients especially iron to competitors/plant pathogens (Yuliar *et al.*, 2015). Furthermore, rhizobacteria can also produce different enzymes, antibiotics, volatiles and other substances that can suppress different plant pathogens or induce resistance mechanisms in plants (Guchi, 2015; Yuliar *et al.*, 2015). A study was conducted in biological control of tomato both in greenhouse and field experiments and it revealed that two strains of *Pseudomonas fluorescens* (VK58 and B16) and one strain of *B. subtilis* (B.16) were recognized as effective BCAs against *R. solanacearum* (Yuliar *et al.*, 2015).

Some bacteria that are able to produce bacteriocins have been identified. These bacteria have antibacterial compounds that have an inhibitory effect against a specific organism that is closely related to the producers (Alabouvette *et al.*, 2006). The advantage of using these BCAs in bio-control is that both bacteriocin producers and related strains live in the same environmental niche. Different organisms such as *E. carotovora* subsp. *carotovora*, *E. herbicola, P. solanacearum, P. syringae,* and *Agrobacterium radiobacter* are able to produce bacteriocins and can be used as BCAs in plant protection (Yuliar *et al.*, 2015). In addition, avirulent strains of *R. solanacearum*, *Streptomyces* sp., *Bacillus* sp, *Pseudomonas* sp, *Acinetobacter* sp., *Burkholderia*

sp., and *Paenibacillus* sp. have also been identified as BCAs against bacterial wilt (Alabouvette *et al.*, 2006; Guchi, 2015).

Numerous new BCAs were identified against *R. solanacearum*, such as *R. pickettii*, *Delftia acidovorans*, *Burkholderia* sp., *Acinetobacter* sp., *B. thuringiensis*, *B. amyloliquefaciens*, *Chryseobacterium* sp., *Clostridium* sp., *Flavobacterium johnsoniae*, *Chryseomonas luteola*, and *Myroides odoratimimus*. Others include *Enterobacter* sp., *Paenibacillus* sp., *Sphingomonas paucimobilis*, *Stenotrophomonas maltophilia*, *Pseudomonas brassicacearum*, *Serratia* sp., *Staphylococcus auricularis*, *Streptomyces* sp., *and Xenorhabdus nematophila*, *Pythium oligandrum, Scutellospora* sp., *Gigaspora margarita*, *Glomus* sp., and *Parmotrema tinctorum* (Yuliar *et al.*, 2015). However, some of these BCAs are not available commercially, and their efficacy is yet to be determined on a commercial scale (Muthoni, 2014; Yuliar *et al.*, 2015).

Biological Control by Natural Compounds

Natural products are substances or chemical compounds produced by a living organism (Hassan *et al.*, 2009). Plant natural compounds contain antimicrobial properties, which can be used in biological control (Momol *et al.*, 2003). Plant natural compounds perform in plant pathology either by their direct antimicrobial effect against pathogens or indirectly by inducing and enhancing plant host resistance against harmful organism (Hassan *et al.*, 2009; Yuliar *et al.*, 2015). Different plant species have been reported to contain secondary metabolites that are able to control plant pathogens (Malkhan *et al.*, 2012; Momol *et al.*, 2003). Those plants which contain bioactive compounds with antimicrobial activities include garlic, onion, citronella, mint, ginger, basil, lion's ear, turmeric, sweet potato, stinging nettle, wild fennel, marigold, cauliflower, neem, eucalyptus, tobacco, thyme, cumin, aloe, tobacco, caraway, lemongrass, rosemary, and coriander (Cowan, 1999; Körpe *et al.*, 2013). The main classes of botanicals that can be found in plants include phenols, phenolic acids, flavones, flavonoids, flavonols, tannins, coumarins, and different types of alkaloids (Hammer *et al.*, 1999; Oboh *et al.*, 2009; Stangarlin *et al.*, 2011). For *R. solanacearum* among different botanicals, phenols, alcohols, ketones, aldhydes, ether and esther control it at different levels (Oboo *et al.*, 2014; Zeller & Ullrich, 2006).

Plant taxonomic families such as Alliaceae, Urticaceae, Asteraceae, Poaceae, Lamiaceae, Euphorbiaceae, and Solanaceae are reported to contain plant species with antimicrobial properties. For instance onion and garlic plants belonging to Alliaceae family contain volatile antimicrobial substance allicin which is thought to be the basis of antimicrobial action of those species against a broad range of plant pathogenic fungi, bacteria, and oomycetes *in vitro* (Ankri & Mirelman, 1999). Allicin has been reported to have effective control of seed-borne *Alternaria* spp. in carrot, *Phytophthora* tuber blight of potato and leaf blight of tomato as well as *Magnaporthe* on rice and downy mildew of *Arabidopsis* (Amini *et al.*, 2016; Slusarenko *et al.*, 2008). Allium plants have a biofumigant property and are effective in the control of soil-borne pathogens such as *Pythium ultimum* Trow and *F. oxysporum* Schlechtend as well as nematodes (Mwitari *et al.*, 2013).

In previous studies, garlic extracts were reported to possess antibacterial properties against a variety of gram-negative and gram-positive bacteria and antifungal activities (Benkeblia, 2004). It contains the powerful sulfur and other numerous phenolic compounds that are the main antimicrobial components. Current reports states that the antibiotic activity of allicin are equated to that of penicillin, a synthetic chemical. Thus, *Allium* plants are used as a potential source to control bacterial and fungal diseases by their allicin, thiosulfonates and related compounds (Benkeblia, 2004). Moreover, *in vitro* and *in vivo* antimicrobial properties in *Allium* are also supported by a research conducted to investigate the biofumigant effects of onion and leek residues against soil-borne plant pathogens (Arnault *et al.*, 2013). The results showed that onion by-products have a high level of biofumigant activity. They also stimulate vegetative growth and ultimate productivity of strawberry and asparagus by 15 to 20%, when compared to those obtained from Brassica-based biofumigation. Therefore, it can be stated authoritatively, that onion has a practical potential for use as a new biofumigant and its by-products used as an alternative to methyl bromide. Also as pesticide, it has been used for soil fumigation to kill the soil-borne diseases and pests as well as to reduce the germination of weeds. Currently, the use of methyl bromide is prohibited in the European Union because it is highly poisonous as fumigant and has residual toxic effect on almost all living-organisms in the soil (Arnault *et al.*, 2013).

Furthermore, it was found that the extracts of other *Allium* plants contain the antimicrobial properties against bacterial wilt of tomato (*R. solanacearum*) either by *in vitro* growth inhibition or *in vivo* bacterial incidence reduction when it is used in pre-plant soil treatment. As a result, the researchers concluded that *Allium* extracts at concentration of 100 and 50% are able to manage bacterial wilt of tomato (Deberdt *et al.*, 2012). Other study was carried out to evaluate the *in vitro* bactericidal action of plant extracts from *Allium sativum* bulbs, *Bixa orellana* and *Caesalpinia coriaria* fruits, *Pterocarpus officinalis* sap as well as rhizhome of *Zingiber officinale* against *Xanthomonas axonopodis* pv. *allii.* The results showed growth inhibitory zone ranging between 90 and 100% in the first four extracts and 55% in the last one (Yuliar *et al.*, 2015). Antibacterial activity of *Allium* plants has also been confirmed by another *in vitro* and *in vivo* study of efficacy of garlic, Datura, and Nerium against *R. solanacearum* of tomato which was conducted by Abo-elyousr & Asran (2009). These researchers reported that water extracts from garlic had the strongest antibacterial activity against bacterial wilt both *in vitro* and *in vivo* followed by Datura and then Nerium respectively.

Urticaceae species (nettles*: Urtica* spp.) have secondary metabolites that are mainly phenolic compounds such as flavanols, anthocyanins and phenolic acids in all parts of plants and possess an ideal structural chemistry for free radical scavenging activity and antimicrobial properties (Kukri *et al.*, 2012). Stinging nettle extract was tested for antibacterial activity against various Gram-positive and Gram-negative bacteria namely *B. subtilis* Ehrenberg, *Lactobacillus plantarum* Orla-Jensen, *Pseudomonas aeruginosa* Schoter, and *Escherichia coli* Theodor Esherich) isolated from food and *Escherichia coli* isolated from urine samples (Kukri *et al.*, 2012). The results showed that stinging nettle extracts inhibit the growth of test microorganisms with a minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extract ranging from 9.05 to more than 149.93 mg mL^{-1}. Another study was carried out to evaluate antibacterial activities of methanol and aqueous leaf, root, stem, and seed extracts of stinging nettle *(Urtica dioica* L.) and Roman nettle (*Urtica pilulifera* L.) on both food-borne pathogens (*B. pumilus* Ehrenberg, *Shigella* sp. and *Enterococcus gallinarum* Thiercelin & Jouhaud) and plant-borne pathogens (*C. michiganensis* Smith, *Xanthomonas vesicatoria* Dowson, *E. amylovora* Winslow, *Pseudomonas* sp., and *Xanthomonas campestris* Dowson) (Körpe *et al.*, 2013). From the results, all methanol extracts (roots, seed, stems and leaves) from

both *U. dioica* L. and *U. pilulifera* L. were effective against all test strains with MIC in the range of 128 to 1024 µg mL^{-1} (Körpe *et al.*, 2013).

Asteraceae is another plant family in which belong some plant species like marigold (*Tagetes* spp.) with antimicrobial properties. It was revealed that flavonoids from leaves of *T. minuta* show activity against Gram-positive and Gram-negative bacteria, and *T. lucida* L. leaf extracts are reported to be active against Gram-positive bacteria. In addition, volatile oils of *T. minuta* have a suppressive biological activity against some insects and pathogens (Senatore *et al.*, 2003). Furthermore, *Tagetes* spp. were reported to contain compounds of therapeutic importance especially essential oils and flavonols (Irum & Mohammad, 2015). Flavonols are natural products containing the principal physiologically active constituents, which have been employed in curing many diseases in human since ages.

From a study of 13 essential oils of African plant species, including *T. minuta*, it was suggested that the most active oils were all rich in oxygenated compounds (Senatore *et al.*, 2003). The minimum inhibitory concentrations (MIC) were 6.25–25 µg mL^{-1} for test Gram-positive bacteria (*B. subtilis* Ehrenberg, *Staphylococcus aureus* Rosenbach) and 25–50 µg mL^{-1} for Gram-negative bacteria *Escherichia coli* Theodor Esherich, *Pseudomonas aeruginosa* Schoter, and *Salmonella typhi* Lignieres. The antimicrobial activity of *T. minuta* L. oil has previously been ascribed to ketone fractions, dihydrotagetones, tagetones and ocimenones (Hassan *et al.*, 2009; Senatore *et al.*, 2003). Some studies also reported that bacterial wilt is suppressed by plant residues of marigold. The residues control the pathogen through their antimicrobial activities, or by the indirect suppression of the pathogen through improved chemical, physical, and biological soil properties. The antibacterial activity of *T. patula* against *R. solanacearum* was confirmed in *in vitro* experiment (Momol *et al.*, 2003; Yuliar *et al.*, 2015).

Poaceae family also contains plant species namely lemongrass (*Cymbopogon citratus*) which can be used in the control of different pathogens. For instance, chloroform leaf and root extracts of *C. citratus* were assessed for their phytochemical properties and their antimicrobial potency against *Staphylococus aureus* Rosenbach, *Salmonella typhi* Lignieres, *Escherichia coli* Theodor Esherich and *Candida albicans* Berkhout. Phytochemical screening of *C. citratus* showed that

flavonoids, phenols, volatile oils, tannins, and carbohydrates were the main active ingredients present in both the root and leaf parts (Ewansiha *et al.*, 2012). Tannins and phenolic compounds have been found to inhibit bacterial and fungal growth and also to protect certain plants against infection. Furthermore, the leaf and root extracts of *C. citratus* exhibited an intermediate antimicrobial activity against the bacteria species although they did not inhibit the growth of *C. albicans* (Ewansiha *et al.*, 2012).

A study was performed to evaluate the effectiveness of lemongrass essential oils on population density of *R. solanacearum* in soil and on incidence of bacterial wilt of tomato in the greenhouse (Pradhanang *et al.*, 2003). The results showed that lemongrass used as soil fumigants has the potential to suppress *R. solanacearum* populations and bacterial wilt incidence of tomato grown in infested soil (Pradhanang *et al.*, 2003). The oils have also showed antifungal activity against *Botrytis cinerea* Pers. The study further revealed that these essential oils have fungicidal, nematicidal, and antibacterial activities. Therefore, they could be used in integrated management of soil-borne diseases in tomato and can be an alternative to the use of methyl bromide (Hassan *et al.*, 2009; Pradhanang *et al.*, 2003).

In Lamiaceae family, basil (*Ocimum* spp.); Lion's (*Leonotis nepetifolia* L.) and rosemary (*Rosemarinus officinalis* L.) have also been reported to have a potential to control different microorganisms. Phythochemical analysis showed that *O. gratissimun* contains essential oils (eugenol, linalol, and estragol), alkaloids, resins, flavonoids, phenolics, glycosides, saponin, and streroidal terpenes (Colpas *et al.,* 2009; Nascimento *et al.,* 2000; Sartoratto *et al.,* 2004). These compounds have antifungal, antibacterial and antioxidant activities in human and animal health. However, limited information exists for usage of basil as crop protectants (Ngoci *et al.,* 2013; Ochola *et al.,* 2015; Ogayo *et al.*, 2015). *Leonotis neptifolia* mainly contains alkaloid, phenolic, flavonoids, tannins, streroids, glycosides and saponins. These compounds were reported to be responsible for the antimicrobial properties against pests and diseases in plants (Benini *et al.,* 2010; Ngoci *et al.,* 2013; Ochola *et al.,* 2015).

In a recent study, the essential oils of *Ocimum suave* L. showed positive *in vitro* antibacterial effects against *R. solanacearum*. This finding demonstrated that essential oils of this plant

possess antibacterial activity that is effective in control of *R. solanacearum*. In *in vivo* tests (soil bio-fumigation) also showed that *Ocimum* reduced the disease by 38% but was not significantly different from untreated plots. A further study on application methods and the mode of action of these essential oils in the control of *R. solanacearum* has been suggested (Oboo *et al.*, 2014).

Rosemary (*Rosemarinus officinalis* L.) is another plant belonging to the family Lamiaceae and which contains secondary metabolites that can control different pathogens (Genena *et al.,* 2008; Rožman & Jeršek, 2009). Many active components in this plant include flavones, diterpenes, steroids, and triterpenes, flavonoids, phenolic acids (caffeic, chorogenic and rosmarinic acids) and essential oils (camphor and cineole). It has been suggested that two phenolic diterpenes (carnosic acid and carnosol) are responsible for antioxidant activity of rosemary extracts whereas the antimicrobial properties may be attributed to these phenolic diterpenes and -pinene, bornyl acetate, camphor and 1,8-cineole (Genena *et al.,* 2008; Khaleel, 2010). Methanolic extract of rosemary shows high inhibition activity against the bacterial strain resistant to several antibiotics including ampicillin and erythromycin (Mwitari *et al.,* 2013). Rosemary plants have important antimicrobial activity, but their antimicrobial activities have not been deeply characterized (Rožman & Jeršek, 2009).

In *in vitro* tests, crude extract of *R. officinalis* inhibited mycelial growth of *Colletotrichum graminicola* Politis, *Phytophthora* sp., *R. solani* Kuhn, and *Sclerotium rolfsii* Tulsane and *Alternaria alternata* Keissl (Gasparin *et al.,* 2010). Other *in vitro* and greenhouse experiments were conducted to assass the biocontrol efficiency of essential oils of *R. officinalis* on *Pectobacterium carotovorum* Waldee (causal agent of potato soft rot) and *R. solanacearum* Yabuuchi *et al.*, 1995 (causal agent of wilt in potato and tomato). The results showed moderate *in vitro* antibacterial effects of the essential oils from *R. officinalis* against both *P. carotovorum* and *R. solanacearum* (Alamshahi & Nezhad, 2015). Essential oils from *R. officinalis* leaf also inhibited the growth of *C. michiganensis* subsp. *michiganensis* Smith, *Salmonella aureus* Lignieres*, Escherichia coli* Theodor Esherich, and *Pectobacterium carotovorum* subsp. *carotovorum* Waldee (the causal agent of potato blackleg) in *in vitro* test (Khaleel, 2010). Therefore, these essential oils from *R. officinalis* are being considered as a new class of natural bactericides that can be used in the field of integrated management of plant disease in the near

future (Alamshahi *et al.,* 2015). Under field conditions, the aqueous extracts of rosemary showed inhibitory effect on *Rhizopus* sp. and *Colletotrichum fragariae* Brooks in strawberry cultivars (Franzener *et al.,* 2007).

Castor bean (*Ricinus communis* L.) is a plant species belonging to Euphorbiaceae family. The parts of this plant especially seeds contain a toxic compound named ricin which inhibits the growth of different bacteria. Another toxic compound ricinoleate is also present in seeds and castor oil has 90% of its composition (Irshad *et al.,* 2012). Seeds also contain alkaloids ricinine and toxalbumin ricin (Mwitari *et al.,* 2013). Castor oil plant also contains sterols, tannins, phenols, saponin glycosides and essential oils (Rahmati *et al.,* 2015). All these components attribute a biological activity of castor bean against different pathogens (Irshad *et al.,* 2012; Rahmati *et al.,* 2015).

Studies have shown that, methanol extracts of *R. communis* seeds inhibited the growth of both test Gram-negative and Gram-positive bacteria (*B. subtilis* Ehrenberg, *Staphylococcus aureus* Rosenbach, *Pseudomonas aeruginosa* Schoter, *Salmonella typhi* Lignieres and *Escherichia coli* Theodor Esherich) and yeast (*Candida albicans* Berkhout) (Rahmati *et al.,* 2015). In another study, ethanol and chloroform extracts of *R. communis* in different concentrations alone or combined with drugs were evaluated for their antibacterial activity against four bacterial strains (*Lactobacillus subtilis* Hansen & Mocquot, *B. thuringiensis* Berliner, and *Escherichia coli* Theodor Esherich) (Irshad *et al.,* 2012). They found that *R. communis* extracts showed higher antibacterial activity against *B. thuringiensis* and *E. coli* at 20 mg mL^{-1} even without the combination of drugs. This study suggested that the ethanol extracts of these medicinal plants contain compounds that can form the basis for the development of a novel broad spectrum antibacterial formulation (Irshad *et al.,* 2012). In addition, leaf extract of castor bean in different solvents (methanol, ethanol and water extracts) was used to investigate its antimicrobial properties against different bacteria. From the results, methanol leaf extracts were found to be more active against Gram-positive bacteria (*B. subtilis* Ehrenberg and *Staphylococcus aureus* Rosenbach) as well as Gram-negative bacteria (*Pseudomonas aeruginosa* Schoter and *Klebsiella pneumoniae* Trevisan) than ethanol and aqueous extracts (Naz & Bano, 2012).

A large number of species of family *Solanaceae* (among which belongs tobacco) which grow mainly in the tropical and temperate region are rich in phytochemicals of medicinal values. Some of these plants have great antibacterial activity against human pathogenic bacteria (Sharma *et al.,* 2016). Phytochemicals analysis of leaf aqueous and methanol extracts of tobacco (*Nicotiana tabacum* L.) showed that tobacco contains phenolic compounds, flavonoids, alkaloids, terpenoids, saponin, tannin, cardiac glycosides, steroids, and carbohydrates. Flavonoids contribute directly to antibacterial and antioxidant activity and nicotine (an alkaloid) compound has shown antibacterial activity against different strains of Gram-positive and Gram-negative bacterial strains (Sharma *et al.,* 2016; Singh *et al.,* 2010). The antibacterial activity of the extracts were evaluated on five human pathogenic bacteria namely *B. cereus* Frankland & Frankland, *B. fusiformis* Ahmed, *Salmonella typhimurium* Lignieres, *Staphylococcus aureus* Rosenbach and *Pseudomonas aeruginosa* Schroter. This study demonstrated that aqueous and methanol leaf extracts exhibited potential antibacterial activity against all the test bacterial strains (Singh *et al.,* 2010).

Tobacco extracts in different polar solvents (ethanol, ethyl acetate, n-Hexane, acetone, butanol and water) were also used to study their antibacterial activity against pathogenic bacteria (*B. cereus* Frankland and Frankland, *E. carotovora* Winslow, *Staphlococcus aureus* Rosenbach, *Agrobacterium tumefaciens* Smith & Townsend). The result of *in vitro* antibacterial screening showed that all extracts from *Nicotiana tabacum* revealed antibacterial activities against test bacteria at highest concentration (24 mg mL^{-1}) (Bakht *et al.,* 2012). The antibacterial activity of aqueous and alcoholic (ethanol, acetone and methanol) stem extracts of *N. tabacum* at 20% concentration also acted against Gram-positive bacteria (*B. amyloiquefaciens* Priest, *Staphylococcus aureus* Rosenbach) and Gram-negative bacteria *(E. coli*, *P. aeruginosa*). Furthermore, tobacco has a reputation for its antifungal activity against *F. solani* Link and the bioactive extracts serve as an active antibacterial agent against microbial diseases (Sharma *et al.,* 2016).

2.6 Modes of Action of Plant Antimicrobial Compounds

Mechanisms by which bioactive compounds can affect plant pathogenic organisms vary with types of components and also from cells, tissues up to molecular targets in the organs. Generally, these compounds cause degradation of the cell wall, disruption of the cytoplasmic membrane, leakage of cellular components, alteration of fatty acid and phospholipid constituents, changes in the synthesis of DNA and RNA and destruction of protein translocation (Lou *et al.*, 2011; Ribera & Zuñiga, 2012; Witkowska *et al.,* 2013). These modes by which the plant natural compounds can control phytopathogenic organisms involve binding to adhesins and inhibiting adhesion ability of pathogens to plant cells, interfering and intercalating into cell wall which increases cell permeability and influx and efflux of ions and membrane disruption which leads to the loss of membrane integrity (Malkhan *et al.*, 2012; Witkowska *et al.,* 2013). In addition, different bioactive compounds may bind to proteins or inhibit their biosynthesis or activate the synthesis of undesirable proteins, inactivate enzymes and inhibit their activities, substrate deprivation to prevent the nutrient uptake, interacting with DNA and forming disulfide bridges (Lou *et al.*, 2011; Malkhan *et al.*, 2012) (Table 2.3).

Table 2.3. Modes of action of botanicals against plant pathogenes

Class	Sub-class	Mechanism of action
Phenolics	Simple phenols	-Membrane disruption,
		-Substrate deprivation
Phenolic acids	Phenolic acids	-Bind to adhesins,
		-complex with cell wall
		-Inactivate enzymes
Terpenoids, essential oils		-Membrane disruption
Alkaloids		-Intercalate into cell wall
Tannins		-Bind to proteins
		-Enzyme inhibition
		- Substrate deprivation
Flavonoids		-Bind to adhesins,
		-complex with cell wall
		-Inactivate enzymes
Coumarins		-Interaction with eukaryotic DNA
Lectins and Polypeptides		-Form disulfide bridges

Source: Malkhan *et al.* (2012)

Currently, it has been found that the effectiveness of phenolic compounds against phytopathogenic bacteria results from their effects on structural and functional damage to plasma membranes and change of its permeability (Lou *et al.*, 2011; Witkowska *et al.,* 2013; Zeller & Ullrich, 2006). Research, has revealed that hydrophobic constituents of essential oils such as thymol and carvacrol are capable of gaining access to the periplasm of Gram-negative bacteria (inclusive of *R. solanacearum)* through the porin proteins of the outer membrane. On entry, they cause disruption of the cell membrane and increased cell permeability, dissipation of pH gradient (pH), and the electrical potential (   ) of pathogen that leads to the death of bacteria (Witkowska *et al.,* 2013). Furthermore, natural plant compounds can cause other damages related to nucleic acid synthesis, ion leakage (leakage of potassium and phosphate ions, nucleic acids and amino acids due to phenols), nutrient uptake and ATPase activity (impairs pH homeostasis

of cells or respiratory activity) and this will disrupt the pathogenicity of an organism (Lou *et al.*, 2011).

2.7 Gaps in Knowledge

Though the bio-control methods of plant pathogens has greater potential in reducing the use of synthetic pesticides, studies in this new approach and application of their findings are still limited or unexploited (Yuliar *et al.*, 2015). Most of medicinal plants have been tested against fungal diseases, and only few studies were conducted to evaluate their efficacy on bacterial diseases especially in plant pathology. In addition, most of the studies have been mainly limited to the assessment of the antibacterial activity *in vitro* in medical and clinical microbiology and the exploitation of active components in plant protection especially under greenhouse and field conditions is still in its infancy. Furthermore, the efficacy of different concentrations of plant extracts against growth of the pathogen is not yet well known. Moreover, effective application frequency of different plant extracts in management of *R. solanacearum* as well as their effect on potato growth, yield and tuber quality and post-harvest infections is also unknown. Hence, the attempts by current research to identify how far the bioactive compounds from different plant extracts can control this pathogen. Therefore, in-depth study on efficacy of selected plant extracts and the composition of their bioactive compounds to control potato bacterial wilt (*R. solanacearum)* is a step that could help in the management of this disease in potato production.

CHAPTER THREE

VIRULENCE AND CHARACTERIZATION OF ISOLATES OF POTATO BACTERIAL WILT CAUSED BY *Ralstonia solanacearum* (Smith) IN RWANDA

Abstract

Bacterial wilt caused by *Ralstonia solanacearum* is one of the major potato diseases in Rwanda. An *in vitro* study was carried out to identify and characterize the pathogen isolated from three potato cultivars (Kinigi, Kirundo and Gikungu) which are highly suceptibe to bacterial wilt in Rwanda. This was achieved by cultural and morphological tests on triphenyl tetrazolium chloride (TTC) and casamino peptone glucose (CPG) agar as well as biochemical tests through Gram staining and biovar test. An *in vivo* experiment was also performed to assess the pathogenicity and virulence of those isolates on potatoes. The isolates were named based on the bacterial species infected by *R. solanacearum* (*Rs*) and potato cultivar they were collected from. Thus, the three isolates were named'' *Rs*KIRUNDO, *Rs*KINIGI, and *Rs*GIKUNGU. All the three isolates showed typical morphological traits of virulent *R. solanacearum* on TTC and CPG media. The test isolates were Gram-negative bacteria. Biovar test confirmed that all the isolates belonged to race 1 biovar 3 of the pathogen. The highest disease severity index (DSI=100.00 %) and disease incidence (DI= 100.00 %) were observed in *Rs*GIKUNGU isolate followed by *Rs*KINIGI (DSI= 97.33 % and DI= 98.25 %) and *Rs*KIRUNDO (DSI= 94.67 % and DI= 92.61 %). From this study, all three isolates were typical *R. solanaceraum* belonging to race 1 biovar 3 and were all pathogenic to potato plants. *Rs*GIKUNGU isolate were highly virulent than *Rs*KINIGI and *Rs*KIRUNDO isolates. Therefore, *Rs*GIKUNGU isolate can be used for further studies in plant protection in management of the disease.

Key words: Biovar test, Gram-negative, Gram-positive, pathogenicity test.

3.1 Introduction

Potato is ones of the most important staple foods and vegetables in the world's (Guchi, 2015; Were *et al.*, 2013). In Rwanda, potato is one of the most important food and cash crops for small scale farmers especially in the Northern regions (RAB, 2012; REMA, 2011; Uwamahoro *et al.*, 2018). In Rwanda, the most grown potato cultivars are namely Kinigi, Kirundo, Mizero, Ngunda, Gikungu, Mabondo, Nderera, Sangema, Kigega, Victoria, Bineza, Nyirakabondo, IPO6, and Cruza. Some of these cultivars such as Kirundo, Ngunda, Sangema, Mizero, Mabondo, Nderera,

Kivu, were locally originated, whereas the others like Victoria, IPO6, and Cruza were distributed by CIP between 1980 to 1992 (Muhinyuza *et al.*, 2014; Rukundo *et al.*, 2019). The most desired potato cultivars are Gikungu, Mabondo, Kinigi, and Kirundo while other like Cruza are not appreaciated in the country due to their quantitative (total yield per unit area) and qualitative tubers characteristics (desirable shape and shallow eyes) (Muhinyuza *et al.*, 2007; Rukundo *et al.*, 2019). Annual consumption of potato is around 125 kg per person per year and it is the country's second most important source of energy after cassava (Muhinyuza *et al.*, 2014; REMA, 2011).

Although Rwanda is among the most important potato producer in sub-Saharan African (FAO, 2018), average yield is estimated at 9 t ha-1 which is below the potential yield of 40 to 60 t ha-1 (Masengesho *et al.*, 2012; Muhinyuza *et al.*, 2014). In Rwanda, brown rot or bacterial wilt caused by *R. solanacearum* (Smith) (Yabuuchi *et al.*, 1995) should be considered as more problematic pathogen threating potato production because it cannot be controlled by chemicals or agronomic practices like other main potato diseases (Huet, 2014; RAB, 2012; Uwamahoro *et al.*, 2018). This is due to the fact that there is no known chemical that can be used to control it once potato plant is infected because of its lethality, persistence, wide host range and broad geographic distribution (Guchi, 2015; Huet, 2014; Strange & Scott, 2005).

Ralstonia solanacearum is a Gram-negative bacterium with round-shaped cells (Hayward, 1991; Priou *et al.*, 2001; Stevenson *et al.*, 2001). The presence of the bacteria inside the plant xylem is coupled with the production of exopolysaccharides which block the vascular vessels inducing a water shortage throughout the plant (CIP, 1996; Guchi, 2015; Muthoni *et al.*, 2012). External disease symptoms of *R. solanacearum* in potato are mainly wilting of the plant and droplets of bacterial ooze from the eyes of the potato tubers. The internal symptoms are brownish discoloration of the vascular ring inside the tuber and grayish white droplets of bacterial cream which come out of them (IPDN, 2014; Muthoni *et al.*, 2012; Pradhanang *et al.*, 2003; Priou *et al.*, 2001; Strange & Scott, 2005). *Ralstonia solanacearum* isolates can be classified into five different races based on the host range and five biovars on the basis of their ability to utilize the disaccharides (cellobiose, lactose, and maltose) and to oxidize the hexose alcohols (dulcitol, mannitol and sorbitol) (IPDN, 2014; Muthoni *et al.*, 2012). Race 1 of *R. solanacearum* affects a

wide range of plant species in the Solanaceae family including potato, tomato and eggplant. It has been observed that *R. solanacearum* race 2 affects some plants of the Musaceae family such as banana and plantains. Race 3 affects mainly potato and tomato and to a less extent other solanaceous species. Race 4 affects particularly ginger whereas race 5 affects mulberry trees (CIP, 1996; OEPP/EPPO, 2004; Sikirou *et al.*, 2017). In addition, there is also a relationship between biovars and races of *R. solanacearum* and their location. This means that each race contains specific biovars and the races can also adapt to different regions due to the specific requirements in climatic conditions for their survival (Muthoni *et al.*, 2012; Sikirou *et al.*, 2017).

Although Rwanda is one of African countries where bacterial wilt is threatening potato production, the pathogen is poorly studied and its management is getting more difficult due to the challenges mentioned earlier. Identification of the biovars and characterization of the isolates are important for example in management of the disease because there is a correlation between the biovar type, race and ultimately the host range of *R. solanacearum* as well as the climatic conditions it may adapt to (Fock *et al.*, 2001; Popoola *et al.*, 2015; Strange & Scott, 2005). In addition, deep understanding on the virulence of different isolates on plant hosts can also provide the main key in application of adequate control measures of the pathogen such as development of potato resistant cultivars. The objectives of this study were, therefore, to identify the biovars of different *R. solanacearum* isolates and to evaluate their pathogenicity and virulence on potato plants in order to obtain an identy the most virulent bacterial isolate to be used in the futher experiments.

3.2 Materials and Methods

3.2.1 Origin of Bacterial Isolates

Three isolates of *R. solanacearum* were obtained from three infected potato cultivars grown in Rwanda namely Kinigi, Kirundo, and Gikungu with typical bacterial wilt symptoms as shown in Figure 3.1. These three cultivars were choosen because they are the most susceptible to bacterial wilt than other potato gynotypes grown in Rwanda (Ntizo from RAB-Musanze station, Personal information). Apart from their susceptibility to *R. solanacearum*, these cultivars have other traits that characterise them such as tuber shape, skin and flesh color, depth of tuber eyes as well as flower color. Thus, Gikungu has oblong tubers while Kinigi and Kirundo are round-shaped. In

terms of skin color, Kinigi and Gikungu are red whereas Kirundo is white. Tuber flesh of Kirundo and Kinigi is while Gikungu is yellow. Kinigi and Kirundo also have deep tuber eyes while Gikungu eyes are shallow. In addition, Kinigi and Gikungu have purple flowers while the ones of Kirundo are white (Muhinyuza *et al.*, 2014). Infected potato samples were collected from farmers' fields in Kinigi Sector, Musanze District, and Nothern Province of Rwanda in November 2017. Kinigi is located in the highland of volcanic soils at an altitude of 2,300 m above sea level (m.a.s.l.), with low temperature (average of 20°C) and high rainfall (1,400 mm to 1,800 mm and well distributed throughout the year) (Birasa *et al.*, 1990; Lepoint & Maraite, 2002; MINIRENA, 2013).

Figure 3.1. Symptoms of potato bacterial wilt (*Rasltonia solanacearum*) on potato plants and tubers collected from Kinigi site. Stem/Leaf wilting of Kinigi, Gikungu, and Kirundo cultivars (A, B, and C, respectively). Bacterial ooze and soil clumps on eyes of Kinigi Gikungu, and Kirundo cultivars (D, E, and F, respectively). Brown ring and ooze from tubers of Kinigi, Gikungu, and Kirundo cultivars (G, H, and I, respectively)

3.2.2 Isolation, Identification and Storage of Isolates

All activities for isolation, culturing and identification of bacterial isolates were done in the Plant Pathology Laboratory of Rwanda Agriculture Board (RAB)-Northern zone at Musanze. A vascular flow test and observation of natural slime drop formation after cutting tubers or plants are specific to *R. solanacearum* (Chaudhry & Rashid, 2011; IPDN, 2014; Priou *et al.*, 2001). In this study, diseased tubers from Kinigi, Kirundo and Gikungu potato cultivars collected from grower's fields were used. Tubers were first washed in tap water, then surface sterilized by soaking them in 70% ethanol solution for 5 min and well rinsed three times in sterile distilled water and left to dry. Then, tubers were cut into circular pieces which were suspended for 10 min in sterile distilled water in a glass container to detect the presence or absence of exudation from the tubers. Bacterial isolates were named based on R.solanacearum pathorgen and potato cultivars they were colleted from. Thus, there isolates were named *Rs*KINIGI, *Rs*GIKUNGU, and *Rs*KIRUNDO (bacterial isolate from Kinigi, Gikungu and Kirundo potato cultivars, respectively).

Kelman's Triphenyl Tetrazolium Chloride (TTC) and Casamino Peptone Glucose (CPG) agar were used as selective media for isolation, identification and biochemical characterization of *R. solanacearum* (IPDN, 2014; Kelman, 1954). Both TTC and CPG media were used to confirm the presence of *R. solanacearum* on these media because the traits of the bacterium are different from non-virulent colonies which are usually smaller, dry, and uniformly dark-red on TTC agar medium and smaller, regularly white round and dry on CPG agar (El-Habbaa *et al.*, 2016; IPDN, 2014). TTC medium was prepared by mixing 1 g of casamino acids with 10 g of Bacto-peptone, 2.5 g of dextrose, 20 g of Bacto-agar in 1 L of distilled water. The mixture was then sterilized by autoclaving at 121°C for 15 minutes. After sterilization, the medium was cooled at 50°C before 5mL of 2,3,5 triphenyl tetrazolium chloride (TTC) were added to the mixture. CPG contained similar reagents like TTC except TTC in glucose was used instead of dextrose. Culture of bacteria on both TTC and CPG was achieved by fivefold serial dilution in order to estimate the number of bacteria cells or colony forming units (CFU) in 1 mL of original inocula from three bacterial isolates. Initial bacterial inocula were obtained by collecting suspension of bacterial streaming in sterile distilled water through the vascular flow technique as described above. Serial dilution was done from 10^{-1} up to 10^{-5} dilution factor in five tubes each filled with sterile nutrient

broth containing peptone, sodium chloride, meat and yeast extracts as described by Marangoni *et al.* (2001). Each suspension of serially diluted cells were plated on TTC and CPG agar media and incubated at 28°C for 48 h, a period after which bacterial colonies were counted and calculation of CFU mL^{-1} was performed by the following formula of IPDN, (2014):

$$CFU = \frac{Number\ of\ colonies\ x\ Dilution\ factor}{Volume\ of\ culture\ plate}$$

Counting of the colonies was possible at dilution factor of 1:100,000 (plate labelled as 10^{-5}) on both TTC and CPG media. From the counted colonies, the CFU mL^{-1} in the original bacterial suspension ranged from 4.3×10^7 for Kirundo to 4.8×10^7 for both Gikungu and Kinigi bacterial isolates, respectively on TTC agar and from 4.8×10^7 for Kirundo and Kinigi to 4.9×10^7 for Gikungu isolate on CPG agar.

Purification of bacterial colonies was achieved through subculturing two times single colony of *R. solanacearum* isolated from TTC and CPG media on new TTC or CPG growth media. The colonies or cell mass were transferred into a sterile glycerol stock (80% of glycerol mixed with 20% of nutrient sucrose broth) in which a loop full of two days old colonies from TTC or CPG were transferred and kept at -20°C for subsequent uses (IPDN, 2014). A further diagnosis was performed to distinguish this Gram-negative bacterium from Gram-positive bacteria and this was achieved by simple Gram staining as described by Chaudhry & Rashid (2011). Cultural, morphological, and physiological characteristics of single colonies from the pure cultures were determined on the specific culture medium TTC and CPG. *Ralstonia solanacearum* colonies were identified through the shape, size and colour on TTC and CPG selective media. A further diagnosis was performed to distinguish this gram-negative bacterium from gram-positive bacteria by simple gram staining as described by Chaudhry & Rashid (2011).

3.2.3 Pathogenicity Test

Pathogenicity test for the three bacterial isolates (*Rs*KINIGI, *Rs*GIKUNGU and *Rs*Kirundo) was carried out by soil inoculation under greenhouse condiitons. Healthy potato seeds of Kirundo cultivar, which is the most susceptible potato cultivar to *R. solanacearum,* were obtained from RAB, Kinigi station. After washing and surface sterilizing the tubers, the later were planted in plastic pots filled with pasteurized mixture of soil, organic matter and sand (2:1:1) and grown

under greenhouse conditions (16.7 to 37.4°C temperature and 31 to 75% relative humidity) at RAB, Musanze. Bacterial inoculation was done when seedlings were 30 days old. Soil around plant roots was removed, and then half of the roots of each potato plant were slightly cut. Then 10 ml of bacterial suspension at concentration of 4.8×10^7 CFU mL^{-1} were inoculated around the roots of each pot. Seedlings inoculated with sterile water served as control.

This test was performed by using a randomized complete block design (RCBD) in which the three bacterial isolates (*Rs*KINIGI, *Rs*GIKUNGU, and *Rs*KIRUNDO) were defined as treatments. Each treatment was replicated three times (blocks). In each block, five potato seedlings were inoculated with the bacterial isolate or sterile water that served as the control. This means that 20 potato plants per block were used and a total of 60 plants for the greenhouse experiment were used. Disease incidence (DI %) and disease severity index (DSI %) in potato plants were evaluated starting from the appearance of symptoms (five days after inoculation) until all the plants inoculated with the most virulent bacterial isolate died (20 days after inoculation) with an interval of 5 days. The disease severity index (DSI %) in plants was evaluated using the scale of Kempe & Sequeira (1983), where 0 = no symptoms; 1 = 1 to 25% leaves wilted; 2 = 26 to 50% leaves wilted; 3 = 51 to 75% leaves wilted; 4 = more than 75% but less than 100% of leaves wilted; 5 = all leaves wilted and plant dead. The disease severity index and incidence was calculated using the following formula of Kempe and Sequeira (1983):

$$DSI\ \% = \left[\sum (ni\ x\ vi) \div (Vx\ N)\right]\ x\ 100$$

where DSI = Disease severity index; *ni* = number of plants with the respective disease rating; *vi* = disease rating; *V* = the highest disease rating; and *N* = the number of plants observed.

$$DI\ \% = \frac{n}{N}\ x\ 100$$

where DI = disease incidence; n = number of infected plants; and N = total number of plants assessed.

From the diseased potato plants, the bacteria were re-isolated and cultured on TZC media and CPG to confirm the presence or absence of the typical colonies of *R. solanacearum* to proof Koch's postulates as well as for further uses. The analysis of variance (ANOVA) was carried out using SAS software, to determine the difference in wilt incidence and wilt severity due to the

three isolates of the pathogen. The treatments means were separated using Tukey's honestly significant difference test at P 0.05.

3.2.4 Biovar Identification

Biovar determination of the three bacterial isolates was done on both the isolated bacteria strains from Kinigi site and the re-isolated ones from diseased plants during pathogenicity test which was performed under greenhouse conditions. The biovars were determined based on the ability of isolates to oxidize hexose alcohols namely dulcitol, mannitol and sorbitol or to utilize disaccharides namely cellobiose, maltose, and lactose. Basal medium as described by Sikirou *et al.* (2017) was composed of 1 g $NH_4H_2PO_4$, 0.2 g KCl, 0.2 g $MgSO_4.7H_2O$, 1 g of peptone, 0.03 g of bromothymol blue, and 3 g of agar per 1 L of distilled water. The medium was sterilized by autoclaving at 121°C for 15 min. After sterilization, 10% of sugar or alcohol solutions preliminary sterilized by boiling them in water bath for 20 min at 60°C for three successive days were amended to the basal medium. Then, this mixture was dispensed on 96 well plates by pouring 200 µl of medium in each well. Sterile distilled water served as control. Thereafter, 20 µl of bacterial suspension at concentration of 4.8 × 107 CFU mL^{-1} were added to each well. The plates were incubated at 28°C for seven days and observed for colour change.

Utilization of sugars and oxidation of alcohols were shown by a positive (+) reaction which leads to changing in colour from green (initial colour of medium) to yellow. Otherwise it remains green (-) because the bacterial strains do not utilize the test sugars or oxidize the alcohols (Muthoni *et al.*, 2012; Sikirou *et al.*, 2017). The results of colour change of the medium were visually observed from four to seven days. The experiment was set up in completely randomized design (CRD) with three treatments that corresponded to each bacterial isolate that was replicated four times.

3.3 Results and Discussion

3.3.1 Bacterial Streaming Test

In this study, infected potato tubers from Kinigi, Kirundo and Gikungu cultivars were used to identify and confirm the presence of *R. solanacearum* in the samples. A vascular flow test was performed to confirm whether the isolated pathogen from infected potato plant extracts was *R.*

solanacearum. Other pathogens can also cause wilting symptoms on potato plants such as *F. solani* (Mart. Sacc.), *V. alboatrum* (Reinke and Berth), *E. chrysanthemi* (Burkholder) and *C. michiganensis* subsp. *sepedonicus* (Spieckermann and Kotthoff) (El- Habbaa *et al.*, 2016; Priou *et al.*, 2001). Through this test, the presence of bacterial wilt in tubers was characterized by smoke-like milky stream that streamed downward from all the cut tubers (Figure 3.2). Typically, this streaming differentiates *R. solanacearum* from other bacteria which may lead to similar symptoms in potatoes in which these threads are not formed (Nadia *et al.*, 2013; Priou *et al.*, 2001). Since the results confirmed the presence of *R. solanacearum*, each isolate was named according to the cultivar that they were isolated from. Therefore, bacterial isolate from Kinigi, Gikungu, and Kirundo were named as *Rs*KINIGI, *Rs*GIKUNGU and *Rs*KIRUNDO, respectively.

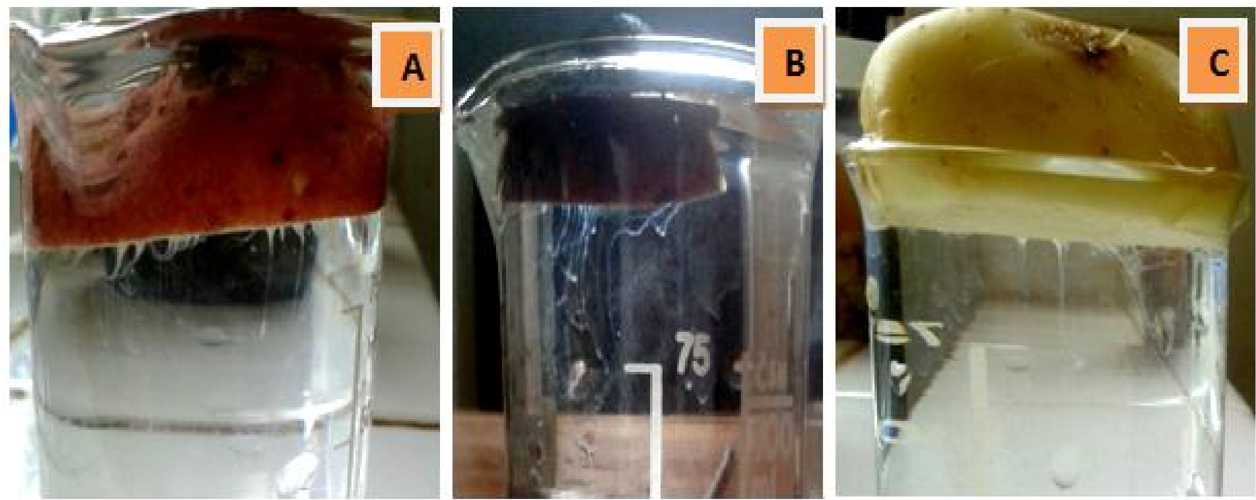

Figure 3.2. Identification of isolated *R. solanacearum* by vascular flow test: Bacterial streaming characterized by smoke-like milky exudates from infected tubers of Kinigi (A), Gikungu (B), and Kirundo (C) cultivars

3.3.2 Cultural and Morphological Characteristics of Colonies on Growth Media

Shape, size and color of colonies on TTC and CPG media are characteristics which are used to identify the pathogen and to distinguish the virulent and nonvilurent colonies of *R. solanacearum* (Kelman, 1954; Nadia *et al.*, 2013; Sikirou *et al.*, 2017). The colonies which developed on TTC growth medium were fluidal, big, irregularly shaped, and white with pink or red colored center whereas on CPG, they were also big with irregular shape and white color (Figure 3.3). These features of bacteria on both TTC and CPG agar media confirmed that all the test isolates had typical morphological and cultural characteristics of *R. solanacearum* and were able to infect

potato plants and to lead to plant wilting. Similar bacterial traits of *R. solanacearum* on culture media were confirmed by Priou *et al.* (2001) and Sikirou *et al.* (2017). These traits are different from non-virulent colonies which are usually smaller, dry, and uniformly dark-red on TTC agar medium and smaller, regularly round and dry on CPG agar (El-Habbaa *et al.*, 2016; Nadia *et al.*, 2013; Sikirou *et al.*, 2017).

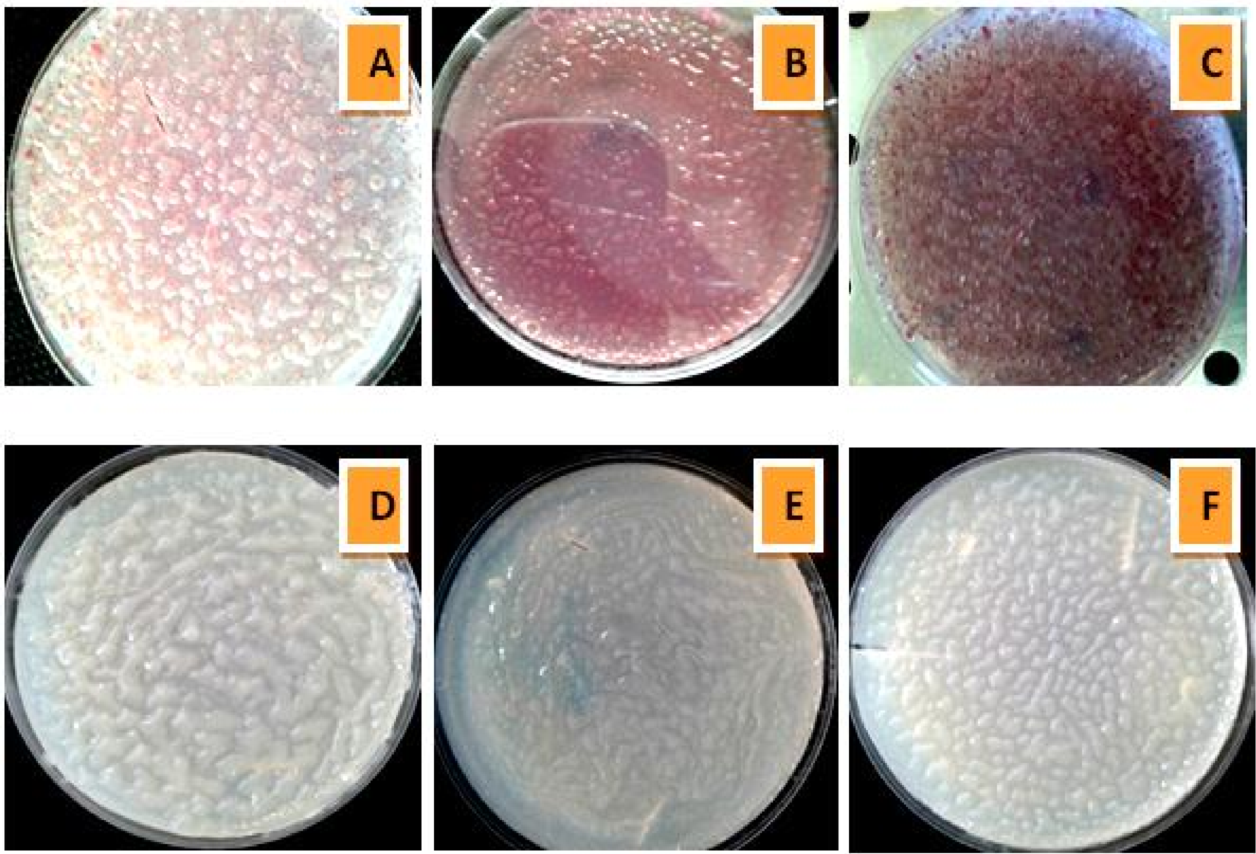

Figure 3.3. Morphological features of colonies of *R. solanacearum* on TTC and CPG culture media. (A, B, C) *Rs*KINIGI, *Rs*GIKUNGU, and *Rs*KIRUNDO isolates respectively on TTC agar at serial dilution 10^{-3}: colonies are mucoid, big with irregular shape, and white with pink colored center. (D, E, F) *Rs*KINIGI, *Rs*GIKUNGU, and *Rs*KIRUNDO isolates respectively on CPG agar at serial dilution 10^{-3}: fluidal, big and irregular white shaped colonies

3.3.3 Morphological Characteristics of Bacterial Cells through Gram Staining

Gram staining was performed to confirm the gram type of *Ralstonia* isolates namely *Rs*KINIGI, *Rs*KIRUNDO and *Rs*GIKUNGU. From the results of this test, the microscopic observation showed that bacterial cells stained reddish (Figure 3.4) and this confirmed that the isolated pathogen was a Gram-negative bacterium and distinguished *R. solanacearum* from Gram-positive bacteria which are usually stained purple by this test (Chaudhry & Rashid, 2011; Nadia *et al.*, 2013). In addition, isolated bacterial cells were rod shaped (Figure 3.4) and this observation also supported the fact that the isolated pathogen was *R. solanacearum* since its round shape was confirmed according to Hayward (1991), Popoola *et al.* (2015), and Stevenson *et al.* (2001).

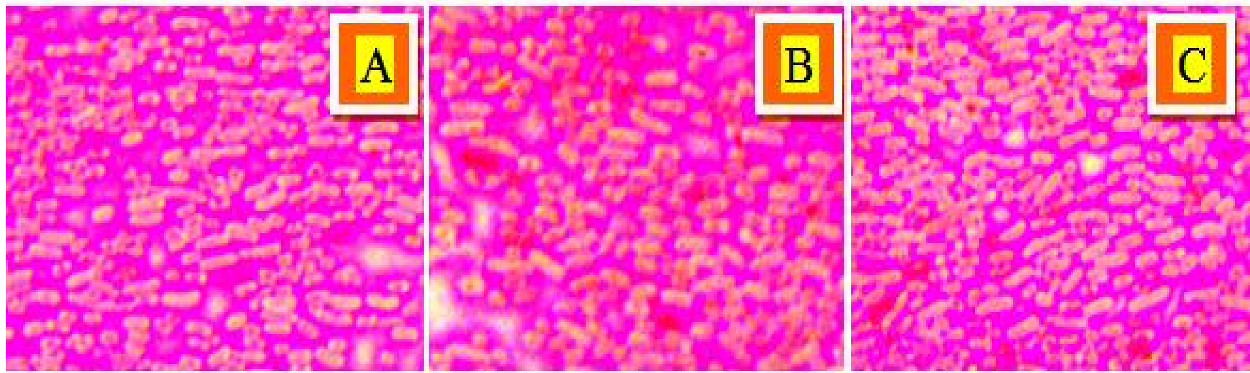

Figure 3.4. Microscopic observation of Gram staining of *Rs*KINIGI, *Rs*GIKUNGU, and *Rs*KIRUNDO bacterial isolates (A, B, and C, respectively)

3.3.4 Pathogenicity and Virulence of the Bacterial Isolates

Pathogenicity of *Rs*KINIGI, *Rs*GIKUNGU, and *Rs*KIRUNDO bacterial isolates was tested under greenhouse conditions from 5 to 20 days after inoculation (5DAI - 20DAI). The results showed that at 5DAI (Figure 3.5, 1st row), inoculated plants started to wilt in potatoes treated with Kinigi, Gikungu and Kirundo isolates whereas the control plants did not show any symptom of bacterial wilt. Wilting rate of potato plants increased over time in seedlings inoculated with all the three bacterial isolates especially from 10 DAI (Figure 3.5, 2nd row). Wilting was higher especially in potatoes inoculated with *Rs*KINIGI and *Rs*GIKUNGU bacterial isolates than in plants inoculated with *Rs*KIRUNDO isolate at 10, 15 and 20DAI (Figure 3.5, 2nd, 3rd, and 4th row respectively). At 20DAI all plants (100%) inoculated with *Rs*GIKUNGU isolate died and almost all plants (98.25%) inoculated with *Rs*KINIGI also died. At 20DAI plants inoculated with *Rs*KIRUNDO

showed disease wilting calculated at 92.6%. During the whole experimental period, there was no wilting in plants treated with sterile water which served as the control.

Figure 3.5. Wilting of potato seedlings at 5, 10, 15, and 20 days after inoculation (1st, 2nd, 3rd, and 4th row, respectively) with *Ralstonia solanacearum* isolates

Wilting of potatoes inoculated with bacterial isolates increased over time either within the isolate or between isolates. At 20DAI all plants inoculated with *Rs*GIKUNGU isolate died. In addition, based on the rates of the potato plant wilting, it was observed that DI as well as DSI caused by

the three bacterial isolates increased over time. Thus, from 5 to 10 DAI potatoes inoculated with *Rs*KINIGI, *Rs*KIRUNDO and *Rs*GIKUNGU bacterial isolates resulted to DI and DSI which increased over time but not significantly different between them at P 0.05 (Table 3.1). However, from 15 to 20DAI, there was a significant difference at P 0.05 in disease incidence and severity index caused by the three bacterial isolates. At 15DAI, *Rs*KINIGI and *Rs*GIKUNGU isolates caused higher disease incidence and severity index in comparison with *Rs*KIRUNDO isolate but with no significant difference between them. Furthermore, at 20DAI of potatoes with *Rs*GIKUNGU isolate caused significantly higher disease incidence and severity index in comparison with *Rs*KIRUNDO but not with *Rs*KINIGI. Morever, no significant difference in DI and DSI was observed between *Rs*KINIGI and *Rs*KIRUNDO at 20DAI. Sterile water inoculation (control) did not cause wilting of potatoes from 5 to 20DAI whereas all three test bacterial isolates were pathogenic to potato plants (Table 3.1).

Table 3.1. Disease incidence (DI) and disease severity index (DSI) over time caused by inoculation of potato seedlings with different isolates of *Ralstonia solanacearum*

Treatment	**DI (%)**				**DSI (%)**			
	5DAI	10DAI	15DAI	20DAI	5DAI	10DAI	15DAI	20DAI
*Rs*Kinigi	10.0±3.9^{a}	27.2±5.6^{a}	87.1±4.4^{a}	98.3±1.2^{ab}	30.0±10.0^{a}	60.0±17.3^{a}	84.0±4.0^{a}	97.3±2.5^{ab}
*Rs*Kirundo	9.5±5.6^{a}	16.7±6.3^{a}	69.1±8.7^{b}	92.6±4.0^{b}	23.3±2.9^{a}	44.7±0.6^{a}	70.7±13.4^{b}	94.7±4.7^{b}
*Rs*Gikungu	1.6±1.6^{ab}	23.0±6.3^{a}	88.5±4.1^{a}	100.0±0.0^{a}	20.0±10.0^{a}	58.3±2.9^{a}	84.0±6.1^{a}	100.0±0.0^{a}
Control	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{c}	0.0±0.0^{c}
P =0.05	0.0134	0.0022	<0.0001	<0.0001	0.0040	0.0004	<0.0001	<0.0001

DAI: Days after inoculation. Values within a column followed by the same letter are not significantly different at p 0.05 according Tukey's Honestly Significant Difference (HSD) test.

The *Rs*GIKUNGU isolate was more virulent to potatoes followed by *Rs*KINIGI and *Rs*KIRUNDO isolates. Usually, the difference in virulence may be a result of inoculum from different races and biovars of *R. solanacearum*, different plant hosts, or different plant cultivars due to their genetic base as well as isolate collected from different areas (El-Habbaa *et al.*, 2016; Sikirou *et al.*, 2017). In this study, the isolates were obtained from the same site (Kinigi sector) and the same plant host (potato) and were also inoculated in the same host and the same cultivar (Kirundo potato cultivar). In addition, all test isolates belonged to the same biovar (biovar 3 of race 1). These may explain why similar pathogenicity rate of these isolates on one potato cultivar during some periods of the study (5 to 10 DAI) were observed. On the other hand, the isolates were from three different potato cultivars (Kinigi, Kirundo and Gikungu). Thus, it is not surprising that the same pathogen isolated from different cultivars resulted to slight difference in the virulence level in the potatoes during this study especially at 20 DAI. In addition, it has been found that *Rs*GIKUNGU showed a higher number of bacterial cells (CFU $mL^{-1} = 4.9 \times 10^7$) than *Rs*KIRUNDO and *Rs*KIRUNDO (CFU $mL^{-1} = 4.8 \times 10^7$). Hammes (2013) and Yuliar *et al.* (2015) reported that inoculum potential is among the factors that affect the incidence of bacterial wilt in potatoes. This in agreement with the results of the present study where *Rs*GIKUNGU that contained higher number of bacterial cells led to higher DI and DS in potato plants than other bacterial isolates.

3.3.5 Biovar Identification

From the findings of biovar identification, the three bacterial isolates acidified all sugars (cellobiose (C), maltose (M), and lactose (L)) and alcohols (dulcitol (D), mannitol (M), and sorbitol (S)) since the medium color changed from green to yellow after four and seven days of plate incubation at 28°C (Figure 3.6). The color of initial medium remained unchanged in the control (C= Sterile distilled water). The ability to utilize both sugars and alcohols characterize biovar 3 of *R. solanacearum* (Lepoint & Maraite, 2002; Muthoni *et al.*, 2012; Sikirou *et al.*, 2017). Therefore, all the three Rwandan collected isolates causing wilting to potato seedlings belonged to *Ralstonia* biovar 3.

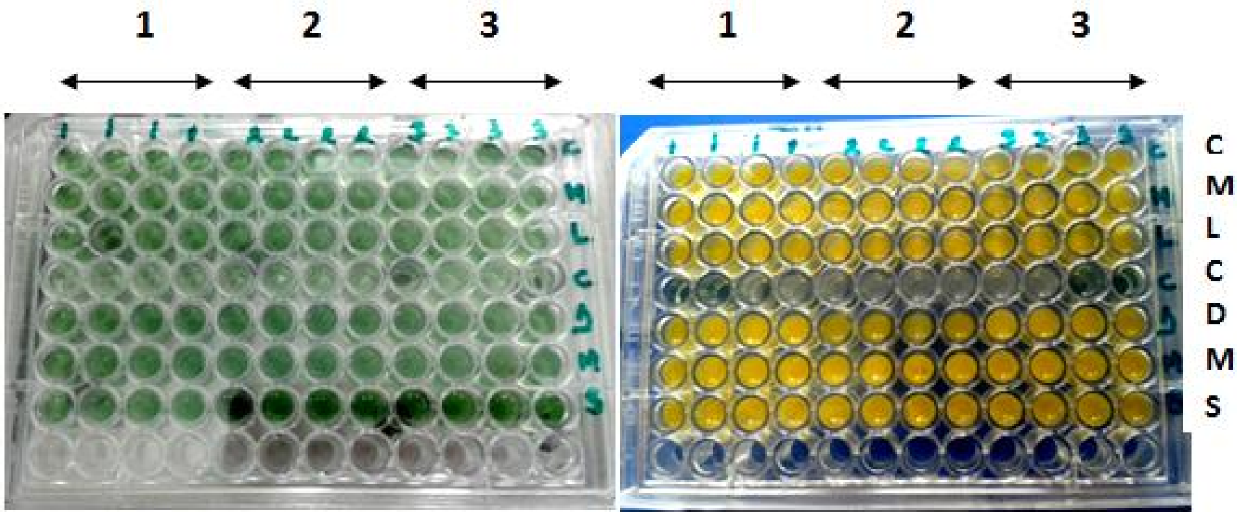

Figure 3.6. Biovar identification of three isolates of *R. solanacearum* from Rwanda. Green color (left) of initial basal medium changed to yellow color (right) at 4 and 7 days of incubation at 28ºC in the wells inoculated with *Rs*KINIGI (1), *Rs*KIRUNDO (2), and *Rs*GIKUNGU (3) isolates. C: Cellobiose; M: Maltose; L: Lactose; D: Dulcitol; M: Mannitol; S: Sorbitol, Sterile distilled water (C) in 4th row of plate served as control

It has been reported that there is a relationship between biovars and races of *R. solanacearum* and their location (Muthoni *et al.*, 2012; Priou *et al.*, 2001; Sikirou *et al.*, 2017). In this context, biovar 3 generally belongs to race 1 of *R. solanacearum,* a race which usually affects potato and other plant species such as tomato, eggplant, tobacco, chili, peanut, groundnut and several weeds in tropical lowland regions (Fock *et al.*, 2001; Sikirou *et al.*, 2017). However, all the three isolates were collected from Kinigi site in Musanze District, a region characterized by tropical highland conditions (Birasa *et al.*, 1990; MINIRENA, 2013). Under such conditions, potatoes are mainly affected by *R. solanacearum* biovar 2 race 3, a race which is well adapted to cool temperatures and which affect potatoes and tomatoes (Muthoni *et al.*, 2012; Popoola *et al.*, 2015; Priou *et al.*, 2001). However, the presence of biovar 3 in Kinigi site was previously confirmed by studies conducted by Butare (1987) and Lepoint and Maraite (2002). All these findings confirmed that in Rwanda *R. solanacearum* race 1 biovar 3 can also adapt to tropical highland areas and affect potatoes under these conditions. Therefore, it is concluded that all three isolates from Kinigi site (Rwanda) belonged to race 1 biovar 3 of *R. solanacearum*.

Race 1 biovar 3 is the most widely distributed type of *R. solanacearum* in the world because it has a wide host range with potatoes included among the others (Popoola *et al.*, 2015). In addition, potatoes are mainly propagated through vegetative planting materials, a method which favors the dissemination of the bacteria from mother tubers (Hayward, 1991). Therefore, it is not surprising to find race 1 biovar 3 of this pathogen in Musanze since long time ago it is one of the major potato growing areas and the main site of potato seed production in Rwanda (RAB, 2012; Uwamahoro *et al.*, 2018). Thus, the occurrence may be due to the introduction of this strain through latently infected potato seeds. To sustain potato production in this region, different potato genotypes should be introduced in order to identify cultivars which are high yielding and adaptable to this region with resistance to the different abiotic and biotic stresses, including *R. solanacearum* race 1 biovar 3.

3.4 Conclusion

The aim of this study was to evaluate pathogenicity of three bacterial wilt isolates on potato host plant in Rwanda. From *in vitro* experiment, all the three (*Rs*KINIGI, *Rs*KIRUNDO and *Rs*GIKUNGU) bacterial isolates from Rwanda are virulent *R. solanacearum* race 1 biovar 3. Furthermore, all isolates are pathogenic to potato plants. *Rs*GIKUNGU isolate is more virulent to potatoes and among others. Therefore, from this study, it is recommended to use *Rs*GIKUNGU bacterial wilt isolate in future tests that may be conducted on *R. solanacearum*.

CHAPTER FOUR

IN VITRO ANTIBACTERIAL ACTIVITY OF SELECTED PLANT EXTRACTS AGAINST POTATO BACTERIAL WILT (*Ralstonia solanacearum* Smith) IN RWANDA

Abstract

Bacterial wilt caused by *Ralstonia solanacearum* Smith is the most severe potato disease in Rwanda because there is no known pesticide for it and cultural control methods seem almost impossible to implement. Therefore, use of plant extracts with antibacterial activities which are locally available, economically affordable and environmental friendly could be an alternative in the management of the disease. This research focused on *in vitro* screening of the antibacterial activity of methanol, water and chloroform extracts of ten local plants against the pathogen. The results showed higher inhibition zone of methanol extracts (16.85 mm) against the bacteria followed by water (14.42 mm) and chloroform (14.19 mm) extracts. All ten plant extracts inhibited the growth of the pathogen at varying levels. Higher antibacterial activity was found in tobacco, wild marigold and garlic extracts (19.61, 18.56, and 18.3 mm inhibition zones respectively). From this screening, methanol and water extracts of three promising plant species, *i.e.* tobacco, wild marigold and garlic were also used for determination of minimal inhibitory concentration (MIC). The MIC of methanol extracts from tobacco and wild marigold was 6.25 mg mL^{-1} whereas, garlic methanol extract was 12.5 mg mL^{-1}. Furthermore, MIC of water extract was 12.5 mg/mL^{-1} in all the three plant species. The findings from this study confirmed that tobacco, garlic and wild marigold extracts are the best in controlling the growth of *R. solanacearum.* Moreover, methanol extracts were the most effective in inhibiting growth of the pathogen and would, therefore, be the most efficient in comparison to water and chloroform extracts.

Key words: Antibacterial activity, botanicals, growth inhibition zone, minimum inhibitory concentration, *Ralstonia solanacearum*.

4.1 Introduction

Bacterial wilt or brown rot disease caused by *R. solanacearum* (Smith) is the second most serious potato disease in sub-tropical and tropical regions and even in some cool temperate areas of the world (Muthoni *et al.*, 2013) after late blight caused by *P. infestan*s (Mont De Bary) (CIP, 2017). In Africa, it is the most serious potato disease throughout central and southern regions mainly in Uganda, Rwanda, Ethiopia, Kenya, Burundi, Nigeria, Madagascar, and Cameroon. Its

infection of tubers restricted potato exports to European markets (Hammes, 2013; Priou *et al.*, 2001). Bacterial wilt is the most problematic disease due to the fact that its management using chemicals seems to be nearly impossible or more complicated since it has no known chemicals that can control it effectively (Guchi, 2015; Masengesho *et al.*, 2012; Wagura *et al.*, 2011). Beside the lack of effective chemicals, pesticide resistances as well as negative effects of chemicals on consumer health and natural enemies limit their use at global level (REMA, 2011; Yuliar *et al.*, 2015).

Currently, farmers tend to use mainly cultural practices such as crop rotation, planting in non-infected soils, use of disease-free planting materials, growing tolerant varieties, removal of infected or suspect plants and destroying them, control of nematodes, use of clean water for irrigation, sanitation of farm implements, and application of quarantine measures (Pal, 2006; Yuliar *et al.*, 2015). However, all these methods have individual practical, technological or economic limitations (Wagura *et al.*, 2011). For example, crop rotation has some limitations because of long survival of the pathogen in the soil even in the absence of host plants (REMA, 2011; Yuliar *et al.*, 2015). In addition, small farm sizes pose challenge for crop rotation program (Guchi, 2015; Muthoni *et al.*, 2012). Transgenic potato cultivars which are resistant against the pathogen with good agronomic traits are not available to smallholder farmers (Schenke & Cai, 2020). Quarantine measures are also either costly or hard to apply and may limit production and commercialization of ware potatoes (Muthoni *et al.*, 2013).

Furthermore, it is not easy to find clean seeds because potato is mainly propagated vegetatively, and this method favours disease spread from mother tubers to next offspring (REMA, 2011; Wagura *et al.*, 2011). The use of resistant varieties is also limited because there is no high level of resistance in potato to bacterial wilt (Muthoni *et al.*, 2012; Priou *et al.*, 2001). In addition some tolerant potato varieties with no visible symptoms especially in cool regions, harbour latent infection in tubers and can spread the disease via progeny tubers (Muthoni *et al.*, 2013; Priou *et al.*, 2001). Furthermore, some of these varieties are not appreciated by farmers, for instance 'Cruza' in Rwanda (REMA, 2011) or show some defects such as high glycoalkaloid content and sensitivity to temperature conditions (Fock *et al.*, 2001).

It has been reported that some plants contain secondary metabolites with antimicrobial properties and can be used to control plant pathogens by either inducing systemic resistance or antibacterial activity (Körpe *et al.*, 2013; Wagura *et al.*, 2011). Therefore, the use of locally available, economically and environmentally friendly plant extracts with antimicrobial properties could be an alternative in the management of potato bacterial wilt (Körpe *et al.*, 2013). Plant antimicrobial metabolites may inhibit pathogens either by their natural bioactive compounds (phytoanticipins) or compounds synthesized *de novo* in response to pathogen attack or other stress conditions (phytoalexins) (Cowan, 1999; Ribera & Zuñiga, 2012).

Most of the studies done have been mainly limited to the assessment of the antifungal and antibacterial activities of plant extracts in medical and clinical microbiology but exploitation of their importance in plant bacteriology is still in its infancy (Yuliar *et al.*, 2015; Zeller & Ullrich, 2006). In addition, the efficacy of botanicals on bacterial wilt (*R. solanacearum*) has not been studied exhaustively (Deberdt *et al.*, 2012; Guchi, 2015; Pradhanang *et al.*, 2003). Moreover, it has been reported that the yield and composition of bioactive compounds of each species is affected by diverse factors such as geographical and environmental conditions (temperature, altitude, radiation, precipitation, humidity, soil fertility salinity and pH, and pests and pathogens), genetic factors (species, varieties), plant organs, stage of growth, extraction techniques and even the extraction solvents (Alamshahi & Nezhad, 2015; Arnault *et al.*, 2013; Senatore *et al.*, 2003). Therefore, this *in vitro* study was carried out to determine the inhibitory effect of methanol, chloroform and water extracts of ten selected plant extracts from Rwanda on *R. solanacearum*.

4.2 Materials and Methods

4.2.1 Plant Extracts

Ten plant species (Table 4.1) were collected randomly from Northern and Southern regions of Rwanda and identified by a botanist from the University of Rwanda, School of Forestry and Biodiversity Conservation, Department of Forest and Nature Conservation (FNC). The plants were selected based on their active compounds with antimicrobial properties (Amini *et al.*, 2016; Cowan, 1999; Körpe *et al.*, 2013), local availability, and low cost. For onion and garlic, the bulbs

were used, whereas for the other plants only leaves were collected for extraction of bioactive compounds.

Table 4.1. Collected plant materials for screening against *Ralstonia solanacearum*

English name	Scientific name	Family	Source	Organ used
Onion	*Allium cepa* L.	Alliaceae	Ankri & Mirelman (1999)	Bulbs
Garlic	*Allium sativum* L.			
Lemongrass	*Cymbopogon citratus* Stapf	Poaceae	Ewansiha *et al.* (2012)	Leaves
Castor bean	*Ricinus communis* L.	Euphorbiaceae	Irshad *et al.* (2012)	Leaves
Rosemary	*Rosmarinus officinalis* L.	Lamiaceae	Nascimento *et al.* (2000); Genena *et al.* (2008); Benini *et al.* (2010)	Leaves
Lion's ear	*Leonotis nepetifolia* R.Br			
African basil	*Ocimum gratissimum* L.			
Tobacco	*Nicotiana tabacum* L.	Solanaceae	Sharma *et al.* (2016)	Leaves
Wild marigold	*Tagetes minuta* L.	Asteraceae	Senatore *et al.* (2003)	Leaves
Stinging nettle	*Urtica massaica* Mildbr.	Urticaceae	Kukri *et al.* (2012)	Leaves

4.2.2 Extraction of Bioactive Compounds

Collected plant materials were shade-dried in a room at ambient temperature for four weeks and then placed in oven at 40ºC for two days for complete drying (Malkhan *et al.*, 2012; Mwitari *et al.*, 2013). Water and methanol solvents were chosen to extract polar metabolites, whereas chloroform was used for extraction of non-polar active compounds (Cowan, 1999; Ncube *et al.*, 2008; Sasidharan *et al.*, 2011). The extraction was performed by macerating 50 g of dried powdered plant material in 200 ml of water, methanol, and chloroform (ratio of 1: 4) separately and left to stand for three days on a rotary shaker (RO 20, Gerhardt, Bonn; 100 rpm at 27°C). Thereafter, the mixture was filtered using Whatman N°42 filter papers. The solvents were evaporated by RotarVapor at 40ºC and 280rpm until complete drying (Malkhan *et al.*, 2012). Subsequently, extracts were diluted to 50 mg mL^{-1} (w/v) concentration with 1 % dimethylsulfoxide (DMSO) and stored at 4ºC until use (Mwitari *et al.*, 2013).

4.2.3 Preparation of Bacterial Inoculum

A virulent isolate of *R. solanacearum* '*Rs*GIKUNGU' was used in this study. From infected materials, a vascular flow technique was used to get bacterial suspension of *R. solanacearum* (Figure 4.1A.). Cultural, morphological, and physiological diagnoses of bacterial colonies were performed on Kelman's Triphenyl Tetrazolium Chloride (TTC) and Casamino peptone glucose (CPG) agar media to distinguish *R. solanacearum* from other bacteria (Figure 4.1B and 4.1C) and to differentiate virulent colonies from non-virulent ones (Kelman, 1954; Narasimha & Srinivas, 2012). Gram staining was also performed to distinguish *R. solanacearum,* which is a Gram-negative bacterium, from Gram-positive strains (Figure 4.1D). Pure culture colonies were transferred into a sterile glycerol stock and kept at -20°C until use.

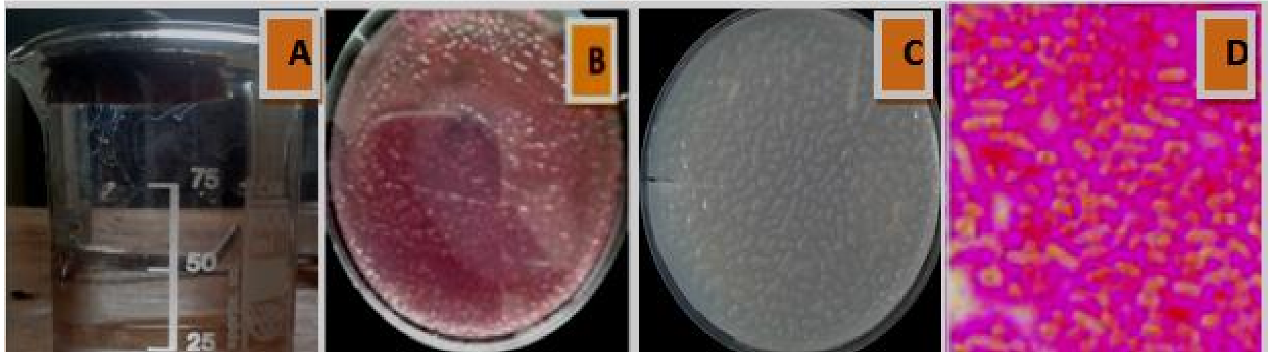

Figure 4.1.Characterization of *R. solanacearum* isolate *Rs*GIKUNGU by vascular flow test (A). Morphological features of the colonies on TTC and CPG culture (B, C). Microscopic observation of gram stained *R. solanacearum* (D)

4.2.4 Screening for Antibacterial Activity of Plant Extracts

Initial screening was achieved by agar disc diffusion method as described by Ncube *et al.* (2008). Some 50 µL bacterial suspension at 4.9 x 10^7 CFU mL^{-1} was inoculated on the surface of Mueller-Hinton agar plates. Afterward, filter paper discs of 5 mm diameter saturated with 20 µL (at the concentration of 50 mg mL^{-1}) of methanol, chloroform or water extracts from the ten plant materials were placed on the surface of each plate. Sterile water, absolute methanol, chloroform, and 1% DMSO served as negative controls while 1 % streptomycin was used as positive control. DMSO was used as a control because it was used for the dilution of plant extracts and it was better to check if it could have an affect on the antibacterial acitivity of the extracts. The plates were kept in the fridge at 4°C for 1 h to allow plant extract diffusion and then incubated at 37°C

for 24 h. After this period, growth inhibition zone was determined by measuring the total diameter of zone inhibition around each disc and substracting from the diameter of paper disc (5 mm) (Hassan *et al.*, 2009). A completely randomized design (CRD) with factorial arrangement was used and each treatment (methanol, water and chloroform extracts from each plant species) was replicated three times. Analysis of variance (ANOVA) was performed using SAS software, to determine the difference in growth inhibition zone due to the antibacterial activity of the plant extracts. The treatments means were separated using Tukey's honestly significant difference test at P 0.05.

4.2.5 Determination of Minimal Inhibitory Concentration (MIC)

The methanol and water extracts from three plant species (tobacco, wild marigold, and garlic) that showed the highest antibacterial activity during screening were tested to determine the minimal inhibitory concentration (MIC). This test was achieved by broth micro-dilution method, a potential technique for quantitative determination of MIC (Mounyr *et al.*, 2016). In this method, 96 wells micro titer plates were used and into each of the 12 wells in a row, 50 µL of nutrient broth was added. From the 1st to 10th well, 50 µL of plant extracts initially dissolved in 1% DMSO were added at two fold serial dilutions (50 to 0.098 mg mL^{-1}). In the 11th and 12th well, 50 µL of positive (streptomycin) and negative (methanol, sterile distilled water, or DMSO) controls as well as positive growth control (nutrient broth) were added. Then, 50 µL of bacterial suspension at concentration of 4.8 x 10^{-7} CFU mL^{-1} overnight grown in Mueller Hinton broth at 28ºC was added to each well.

The plates were incubated at 37ºC for 18 h, after which 50 µL of 0.01% of triphenyl tetrazolium chloride (TTC) was added to each well. The TTC was used as a dye reagent to indicate the viability or death of bacteria (Mounyr *et al.*, 2016). Thereafter, the plates were incubated at 37ºC for 1h, after which MIC was evaluated by visual observation of colour change of the medium from initial color of extract to red (Ncube *et al.*, 2008). Three plates were used for three plant extracts (tobacco, wild marigold and garlic). In each plate, three rows were used for ten serial diluted concentrations of methanol extracts and other three next rows for water extracts. Each concentration of methanol or water extracts from three plant species was replicated three times.

4.3 Results and Discussion

4.3.1 Screening of Plant Extracts against Ralstonia solanacearum

Methanol, water and chloroform extracts of the ten plant materials significantly inhibited the growth of *R. solanacearum* at different levels at p 0.05 compared to the negative controls (Table 4.2). It was also observed that all plant extracts inhibited the growth of the pathogen at the same level of streptomycin (positive control) except methanol, water, and chloroform extracts of rosemary, lion's ear, and stinging nettle (Table 4.2). Furthermore, methanol, water and chloroform extracts of the ten selected plant materials showed different levels of activity against the pathogen. For methanol extracts, the highest antibacterial activity was obtained with tobacco, wild marigold, garlic, and onion (21.00, 20.67, 20.33, and 18.33 mm inhibition zone, respectively). For water extracts, the highest antibacterial activity was also found in tobacco, wild marigold, and garlic plus lemongrass, African basil, and stinging nettle (20.33, 18.00, 17.67, 16.67, 16.00, and 15.00 mm of zone inhibitions, respectively). In the case of chloroform extracts, comparable strong inhibitory effect was also observed in tobacco (17.50 mm) followed by wild marigold (17.00 mm) then garlic (17.00 mm) plus castor bean (16.00 mm). Plant methanol extracts, which had slight inhibitory effect, were stinging nettle (17.73 mm) and lion's ear (17.67 mm). Similar effect was exhibited by rosemary (14.67 mm), castor bean (13.67 mm), and onion (13.33 mm) for water extracts as well as by African basil (14.67 mm) and onion (14.50 mm) for chloroform extracts. The lowest inhibitory effect was obtained with methanol extract of rosemary (14.67 mm), water extract of lion's ear (10.67 mm), and chloroform extract of stinging nettle (12.33 mm) (Table 4.2).

Although almost all the plant extracts exhibited almost similar antibacterial property as the streptomycin, they showed different levels of activity against the pathogen. The highest antibacterial activity was exhibited by tobacco, wild marigold, and garlic: 19.61, 18.56, and 18.33 mm, respectively. African basil, lemongrass and castor bean extracts displayed strong efficacy in controlling the pathogen with growth inhibition zone of 15.92, 15.89, and 15.61 mm, respectively. Additionally, onion, stinging nettle, and rosemary had a moderate inhibitory effect (15.39; 15.00; 14.45 mm, respectively) on the bacterium. However, the lowest inhibitory effect was obtained with lion's ear (14.22 mm) (Table 4.2).

Table 4.2. Growth inhibition zone (mm) of methanol, water and chloroform extracts from ten selected plants against *Ralstonia solanacearum*

Plant extract	Methanol extract	Water extract	Chloroform extract	Mean of Growth inhibition zone
Tobacco	21.00±2.00[a]	20.33±2.89[a]	17.50±1.32[a]	19.61±1.86[a]
wild marigold	20.67±1.15[ab]	18.00±3.46[ab]	17.00±1.00[ab]	18.56 ±1.90[ab]
Garlic	20.33±2.08[ab]	17.67±2.08[ab]	17.00±1.30[ab]	18.33±1.76[abc]
Onion	18.33±0.58[abc]	13.33±5.69[bc]	14.50±2.29[cd]	15.39±2.62[cde]
Stinging nettle	17.73±0.46[bc]	15.00±2.00[abc]	12.33±1.15[d]	15.00±2.67[de]
Lion's ear	17.67±2.08[bcd]	10.67±3.06 [c]	14.33±0.58[cd]	14.22±3.50[e]
Castor bean	17.17±0.29[cd]	13.67±5.13[bc]	16.00±1.00[abc]	15.61±1.78[bcde]
African basil	17.10±0.85[cd]	16.00±3.61[abc]	14.67±2.08[bcd]	15.92±1.22[bcde]
Lemongrass	16.67±3.51[cd]	16.67±1.53[ab]	14.33±0.58[cd]	15.89±1.35[bcde]
Rosemary	14.67±1.53[d]	14.67±2.89[bc]	14.00±1.73[cd]	14.45±0.39[de]
Controls (+; -)				
(+): Streptomycin	18.67±3.51[abc]	17.00±3.46[ab]	16.33±0.76[abc]	17.33±1.21[abcd]
(-): Methanol	1.33±0.12[e]	*	*	1.33±0.12[f]
(-): Water	*	0.00 ± 0.00[d]	*	0.00 ± 0.00[f]
(-): Chloroform	*	*	2.33±0.31[e]	2.33±0.31[f]
(-) DMSO	0.00 ± 0.00[e]	0.00 ± 0.00[d]	0.00 ± 0.00[e]	0.00 ± 0.00[f]
P =0.05	P<0.0001	P<0.0001	P<0.0001	P<0.0001

The values are an average of growth inhibitory zone (mm ± SD) from triplicates of methanol, water, or chloroform extracts from each of ten plant materials as well as the controls. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive and Negative controls. Water, Methanol, and chloroform were used as negative controls in water extract, methanol extract, and chloroform extracts, respectively. Streptomycin and DMSO were used as positive and negative controls for all extracts. (*: Not applicable). SD: Standard deviation.

Antibacterial activity of methanol, water and chloroform extracts against *R. solanacearum* was also determined. From an *in vitro* experiment, the results showed that all the methanol, water and chloroform extracts inhibited growth of *R. solanacearum* compared to positive and negative controls at p 0.05 (Table 4.3). In general, the average of antibacterial activity from solvent extracts was significantly higher in methanol (16.85±3.58 mm) than both water (14.42±1.97 mm) and chloroform (14.19±1.38 mm) extracts. Methanol extracts controlled potato bacterial wilt to the same extent as the positive control streptomycin, a synthetic antibiotic. In addition, methanol extracts had a significant difference in growth inhibition of the bacterium in comparison with water and chloroform extracts while water and chloroform extracts were similar (Table 4.3).

Table 4.3. Growth inhibition zone (mm) of solvent extracts (methanol, water and chloroform) against *Ralstonia solanacearum*

Solvent extract	**Growth inhibition zone (mm)**
Methanol extract	16.85 ± 3.58^{a}
Water extract	14.42 ± 1.97^{b}
Chloroform extract	14.19 ± 1.38^{b}
Controls (+; -)	
(+) Streptomycin	17.33 ± 1.21^{a}
(-): Methanol	1.33 ± 0.12^{c}
(-): Water	0.00 ± 0.00^{c}
(-): DMSO	0.00 ± 0.00^{c}
P =0.05	P<0.0001

The values are an average of growth inhibitory zone (mm ± SD) from triplicates of methanol, water, or chloroform extracts from ten plant materials as well as the controls. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (Streptomycin) and Negative controls (Water and DMSO). SD: Standard deviation.

The results from the present study are in agreement with different researchers who found that some plant species contain bioactive compounds with antimicrobial activities (Abo-elyousr &

Asran, 2009; Körpe *et al.*, 2013). A large number of species in the family *Solanaceae* (to which tobacco (*Nicotiana tabacum* L.) belongs) are rich in biochemicals of medicinal values (Sharma *et al.*, 2016). Tobacco extract was mainly reported to have insecticidal activities, but it also has antibacterial and antifungal properties against human diseases (Bakht *et al.*, 2012; Sharma *et al.*, 2016; Singh *et al.*, 2010). Phytochemical analysis of leaf aqueous and methanol extracts of tobacco revealed it contains flavonoids and alkaloids, which contribute directly to antibacterial activity against different strains of Gram-positive and Gram-negative bacterial strains (Sharma *et al.*, 2016; Singh *et al.*, 2010). This earlier findings of antibacterial activity of tobacco concur with the present where tobacco extract showed a stronger growth inhibitory effect against potato bacterial wilt.

Apart from tobacco, many authors also confirmed that wild marigold (*Tagetes minuta*) belonging in Asteraceae family has antimicrobial activities against plant pathogens (Gakuubi *et al.*, 2016; Irum & Mohammad, 2015). It was found that flavonoids, flavonols, and essential or volatile oils from *T. minuta* had a suppressive biological activity against pathogens and some insects (Gakuubi *et al.*, 2016; Senatore *et al.*, 2003). These bioactive compounds inhibited the growth of different Gram-negative bacteria and Gram-positive bacteria (Gakuubi *et al.*, 2016). For instance, antibacterial activity of *T. patula* against *R. solanacearum* was confirmed in *in vitro* experiment carried out by Yuliar *et al.* (2015). They concluded that the *Tagetes* residues controlled the pathogen through their possible mechanisms of action of antimicrobial activities and by the indirect suppression of the pathogen through improved chemical, physical, and biological soil properties. All these researches support the present study results that showed a stronger antibacterial activity of *Tagetes minuta* against *R. solanacearum.*

In the present study garlic also showed potential inhibitory effect against potato bacterial wilt and different previous studies are in agreement with it. It was reported that Alliaceae family (mainly garlic: *Allium sativum* L. and onion: *Allium cepa* L.) have antibacterial properties (Curtis *et al.*, 2005; Rahmati *et al.*, 2015). Allium plants contain volatile substance allicin which is the basis of antimicrobial action of those species against a broad range of plant pathogenic fungi, Gram-negative and Gram-positive bacteria, and Oomycetes (Borlinghaus *et al.*, 2014; Slusarenko *et al.*, 2008). For instance, it has been reported that *Allium* plants contain the

antimicrobial properties against bacterial wilt of tomato (*R. solanacearum*) and other soil-borne pathogens as well as nematodes through biofumigation and intercropping system (Abo-elyousr & Asran, 2009; Deberdt *et al.*, 2012; Rongquan *et al.*, 2011). *Allium* by-products can be used as an alternative to the phased out methyl bromide, a pesticide which was widely used for soil fumigation to kill the soil-borne diseases and pests (Arnault *et al.*, 2013; Mwitari *et al.*, 2013). In addition, Allicin has an effectiveness, which is equal to the other antibiotics namely kanamycin, penicillin, and ampicillin (Borlinghaus *et al.*, 2014; Curtis *et al.*, 2005). All these findings support the results from the present *in vitro* experiment in which tobacco, wild marigold and garlic extracts highly inhibited the growth of potato bacterial wilt.

In this research, methanol plant extracts inhibited the growth of *R. solanacearum* at the highest level among the other extracts (water and chloroform). They also inhibited the growth of bacteria to the same level as the synthetic antibiotic streptomycin which was used as a positive control. Similar effectiveness of methanol plant extracts from nettle compared to water extracts in the control of test Gram-positive and Gram-negative bacteria was confirmed by Körpe *et al.* (2013). In another *in vitro* experiment, methanol leaf extracts from castor bean were found to be more active against Gram-positive and Gram-negative bacteria than ethanol and water extracts (Naz & Bano, 2012). All these findings support observations in the present study that methanol extracts had a higher performance in the control of *R. solanacearum* than water extracts and chloroform extracts. It was reported that the extraction solvent is one of the factors that affect the yield and composition of natural compounds (Arnault *et al.*, 2013; Kukri *et al.*, 2012). Usually, methanol and water are organic solvents which are mainly used to extract polar compounds, whereas chloroform is used for non-polar metabolites (Cowan, 1999; Mwitari *et al.*, 2013; Ncube *et al.*, 2008). Methanol has been revealed to be the best solvent to extract a high range of polar metabolites (Arnault *et al.*, 2013; Cowan, 1999). Apart from polar compounds, chloroform was used for extracting non-polar compounds (Cowan, 1999; Rahman et al., 2012). In general, the results from this study showed lower antibacterial performance in chloroform than methanol extracts. This suggests that all or most of the ten selected plant extracts may contain much more polar antibacterial metabolites than non-polar compounds.

4.3.2 Minimal Inhibitory Concentration (MIC) of Plant Extracts

From *in vitro* experiment, it was found that the MIC of methanol extracts from tobacco and wild marigold which completely restrained the growth of *R. solanacearum* was 6.25 mg mL^{-1} whereas methanol extract from garlic inhibited the growth of bacterium at 12.5 mg mL^{-1}. All water extracts tested killed the target bacterium at 12.5 mg mL^{-1} (Figure 4.2, Table 4.4). These findings showed that both methanol extracts from tobacco and wild marigold killed the pathogen at the low concentration (6.25 mg mL^{-1}) while their water extracts inhibited the growth of *R. solanacearum* at high concentration (12.5 mg mL^{-1}). Both methanol and water extracts from garlic controlled the bacterium at high concentration of 12.5 mg mL^{-1}.

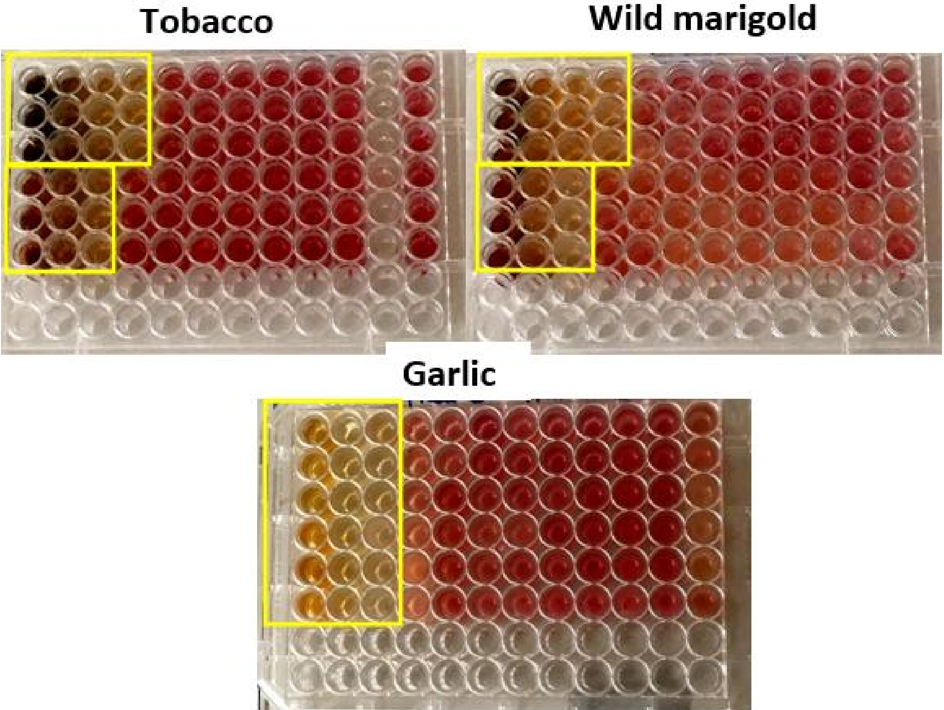

Figure 4.2. Micro-dilution assay for Minimal inhibitory concentration (MIC) of methanol and water extracts from active plant species against R. solanacearum on 96-well micro titer plates

Tobacco, wild marigold, and garlic extract (1st, 2nd, and 3rd plate, respectively). In each plate the first three rows were used for methanol extracts and the next three rows for water extracts. The first to tenth wells of each row were used for serial diluted concentration (from 50 to 0.098 mg mL^{-1}). The eleventh and twelfth wells were used for the controls (streptomycin and nutrient

broth in plate 1; sterile water and DMSO in plate 2; sterile water and methanol in plate 3). The presence of viable bacterial cells was indicated by the reduction of TTC into a red colour, otherwise it retains the same colour of initial medium (wells encircled by yellow line).

Table 4.4. Minimal inhibitory concentration (MIC) (mg mL^{-1}) of three plant extracts against *Ralstonia solanacearum*

Plant material	Solvent extract	MIC (mg mL^{-1})
Tobacco	Methanol	6.25
	Water	12.5
Wild marigold	Methanol	6.25
	Water	12.5
Garlic	Methanol	12.5
	Water	12.5

From this experiment, the MIC of methanol was lower than the one of water extracts especially in tobacco and wild marigold extracts. This means that these methanol extracts had higher antibacterial effect than water extracts against *R. solanacearum*. Similar results were obtained in the initial screening carried out in this study to evaluate the efficacy of three selected solvent extracts (methanol, chloroform, and water) in management of the pathogen in Rwanda. In addition, two out of three test plant extracts (tobacco and wild marigold) had the same MICs in methanol extracts. All three plant extracts inhibited growth of the target bacterium at the same MIC in water extracts.

In previous studies, it was found that the efficacy of some plant extracts against different pathogens is dose-dependent (Curtis *et al.*, 2005; Gakuubi *et al.*, 2016; Sharma *et al.*, 2016). This supports the findings from the present study in which *R. solanacearum* was killed at higher concentrations and remains viable at the low concentrations both in methanol and water extracts from all three tested plant extracts. In *in vitro* experiment to determine the effectiveness of tobacco extracts at different concentrations (6, 12, 18 and 24 mg mL^{-1}) against different pathogenic bacteria was observed that all extracts had antibacterial activities against the test bacterium at the highest concentration (24 mg mL^{-1}) (Bakht *et al.*, 2012). This was higher than the MICs that inhibited *R. solanacearum* in the present study in both methanol and water extracts

from tobacco. Various researchers listed different factors that affect the yield and composition of antimicrobial compounds for each species such as climatic conditions under which the plant has grown, organ used, stage of growth, extraction techniques and even the extraction solvents (Alamshahi & Nezhad, 2015; Kukri *et al.*, 2012; Senatore *et al.*, 2003). The difference in MICs may be due to one or combination of some of these factors.

Similar concentration-dependent effect of plant active metabolites was also found in garlic and onion against bacteria and fungi. In *in vitro* study, plant extracts were assessed at different concentrations and all of them controlled the pathogens at the highest concentration and garlic had higher antimicrobial activity than onion extracts (Borlinghaus *et al.*, 2014; Curtis *et al.*, 2005). All these results demonstrate that all the three plant extracts which were tested in the present study have strong antibacterial activity, which is also dose-dependent in the control of potato bacterial wilt. In general, methanol extracts inhibit the growth of this pathogen at lower concetration compared to water extracts.

In addition, Gakuubi *et al.* (2016) reported that Gram-negative bacteria are usually more resistant to bioactive compounds than Gram-positive ones. For instance, it has been found that MIC of *T. minuta* was 6.25 to 25 µg mL^{-1} for test Gram-positive bacteria and 25 to 50 µg mL^{-1} for Gram-negative bacteria (Senatore *et al.*, 2003). From another study, it was also observed that antibacterial activity of *T. minuta* increased with increasing concentration levels and Gram-positive bacteria are less resistant to volatile oils than Gram-negative bacteria (Gakuubi *et al.*, 2016). The researchers reported that the MIC of the essential oils from *T. minuta* on Gram-negative bacteria was 16.5 mg mL^{-1}, whereas the one against Gram-positive ones was 6.7 mg mL^{-1}. The *T. minuta* affects strongly the multiplication and growth of Gram-positive bacteria than Gram-negative ones (Gakuubi *et al.*, 2016). The results confirmed that natural compounds led to higher sensitivity on Gram-positive than on Gram-negative bacteria (Gakuubi *et al.*, 2016; Senatore *et al.*, 2003). *Ralstonia solanacearum* is a Gram-negative bacterium (Priou *et al.*, 2001; Uwamahoro *et al.*, 2018) and this could explain why the MIC of all tested extracts in the control of this pathogen may be higher than the one reported to inhibit the growth of other bacterial strains.

4.4 Conclusion

From this study, it is concluded that all the ten tested plant extracts are effective in growth inhibition of *R. solanacearum* of potato under *in vitro* conditions compared to controls. Among the ten tested plants, the best performing against growth of *R. solanacearum* were tobacco, wild marigold and garlic. Furthermore, methanol extracts have higher potential against bacterial wilt of potatoes followed by water and chloroform extracts. Moreover, minimum inhibitory concentration (MICs) is lower in methanol extracts than in water extracts. Thus, methanol extract has stronger antimicrobial properties than water extracts and is highly recommended in management of *R. solanacearum*. From this study, methanol extracts from tobacco, wild marigold and garlic are recommended to be used for extraction of natural compounds against bacterial wilt with non-toxicity to the environment.

CHAPTER FIVE

EFFECT OF FREQUENCY OF APPLICATION OF SELECTED PLANT EXTRACTS ON POTATO GROWTH, YIELD, QUALITY, POST-HARVEST INFECTION AND YIELD LOSS

Abstract

Bacterial wilt (*Ralstonia solanacearum* (Smith) is a major limiting factor for potato production in Rwanda. Plant extracts of tobacco (*Nicotiana tabacum* L.), wild marigold (*Tagetes minuta* L.) and garlic (*Allium sativum* L.) were identified to contain antibacterial activity that can control the disease. Therefore, the current study was conducted to evaluate the effective frequency of application (weekly, bi-weekly and monthly) and extractiom method of selected plant extracts on potato bacterial wilt under field conditions (Season A and B). In the same experiment, effects of weekly, bi-weekly and monthly application of plant extracts on potato growth, yield, quality, post-harvest infection (PHI) and yield loss (PHL) were also investigated. Methanol and water extracts of tobacco (*Nicotiana tabacum* L.), wild marigold (*Tagetes minuta* L.) and garlic (*Allium sativum* L.) at 50 mg mL^{-1} (concentration selected from a greenhouse pilot experiment) were tested. The experiment was designed as a Randomized Complete Block Design (RCBD) with factorial arrangements. From the results, weekly, bi-weekly application showed higher biological control efficacy (BCE) (58.28 and 57.40 %) against the pathogen in potato plants than monthly application (47.62 %) at p 0.05. In potato tubers, weekly, bi-weekly application also had higher BCE (75.92 and 67.39 %) than monthly application (52.49 %) at p 0.05. Weekly, bi-weekly and monthly application gave higher tuber yield (27.72, 24.13, and 23.19 t ha^{-1} respectively) than positive and negative controls (14.22 t ha^{-1} and 5.29 t ha^{-1}). Weekly application reduced PHI (18.33 %) compared to bi-weekly (30.97 %) and monthly application (36.83 %). From the experiment, it is concluded that Weekly or bi-weekly application of methanolic extract of tobacco and wild marigold at 50 mg mL^{-1} is an effective approach in management of potato bacterial wilt in field or during storage and also significantly improved potato growth, yield, and quality in sustainable horticultural production.

Key words: Biological control efficacy, growth, post-harvest infection, quality, yield.

5.1 Introduction

Potato is used for income generation mainly in Northern and Western regions of Rwanda where are located the major potato growing areas like Musanze, Burera, Nyabihu, and Gicumbi districts of the country (Manishimwe *et al.*, 2019; Uwamahoro *et al.*, 2018). However, potato yield in Rwanda (9 t ha^{-1}) is still below the potential yield, which is estimated at 40 t ha^{-1} and supply is also lower compared to the demand (Manishimwe *et al.*, 2019). Bacterial diseases are among the major limiting factors in potato production worldwide with bacterial wilt or brown rot caused by *R. solanacearum* (Smith) at the first line especially in tropical, subtropical and warm temperate regions (Giri *et al.*, 2020; Li *et al.*, 2016; Rado *et al.*, 2015; Shili *et al.*, 2016).

Ralstonia solanacearum is also the most problematic pathogen of potato in Rwanda because there are no known effective chemicals used against it and cultural practices fail to manage it (Uwamahoro *et al.*, 2018). Agrochemicals such as antibiotics or copper derived compounds have been used to control bacterial wilt but they also fail to controls it effectively (Derid *et al.*, 2013). This is due to the fact that this pathogen has a wide range of plant hosts (mainly solanaceous and Musaceaa crops), biological diversity (races and biovars), long survival in the soil even in the absence of host, easy transmission by seeds, soil, rain or irrigation water or farm tools (Derid *et al.*, 2013; Giri *et al.*, 2020; Rado *et al.,* 2015; Shili *et al.,* 2016). In addition, overuse of these conventional chemicals is associated with environmental pollution and endangering the consumers and wild animals' welfare as well as development of new strains of bacterium which are more resistant to the bactericides (Li *et al.*, 2016; Mitali *et al.*, 2012; Mulugeta *et al.*, 2019; Vu *et al.*, 2017).

Therefore, identification of new alternatives that can control this pathogen in sustainable agriculture for food security, safety and which is environmental friendly can be helpful to overcome the damage caused by plant pathogen (Hosam, 2016; Mulugeta *et al.*, 2019; Vu *et al.*, 2017). Plant species belonging to different families are reported to contain renewable secondary metabolites ''botanicals'' with antimicrobial effects which can substitute for synthetic pesticides (Hosam, 2016; Li *et al.*, 2016). These materials have inhibitory properties against a wide range of fungal and bacterial diseases in human, animals and plants. They contain compounds which are not toxic to environment, have rapid decomposition, low cost and easily renewable (Hosam,

2016; Mulugeta *et al.*, 2019; Shili *et al.*, 2016). Earlier studies carried out showed that biological control by natural plant products can be used as a promising strategy to manage potato bacterial wilt in an integrated disease management (IDM) (Giri *et al.*, 2020; Li *et al.*, 2016; Mulugeta *et al.*, 2019). Most of these studies have focused on *in vitro* antibacterial activity of plant extracts but the studies on these active compounds against phythopathogenic bacteria under field conditions are crucial but still limited (Mulugeta *et al.*, 2019; Vu *et al.*, 2017).

An *in vitro* screening of methanol, chloroform and water extracts of ten selected plant extracts against *R solanacearum* was carried out in Rwanda in this present study as described in Chapter Four. The results showed that methanol and water extracts of tobacco, wild marigold and garlic are the most promising in management of potato bacterial wilt. In addition, highest, moderate and minimal inhibitory concentration (MIC) of these extracts were also determined in the previous study. Thus, the objective of this *in vivo* study was to evaluate the efficacy of different frequencies of application of methanolic and water extracts of tobacco, wild marigold and garlic materials on the control of potato bacterial wilt, potato growth, tuber yield and quality as well as post-harvest infection and yield loss during storage. Before the field experiments, a pilot greenhouse experiment with these extracts at three different concentrations (50, 25, 12.5 or 6.25 mg mL^{-1}) was conducted to determine the effective application concentration for the field experiment. From all analysed parameters, application of plant extracts at 50 mg mL^{-1} was recommended.

5.2 Materials and Methods

5.2.1 Description of Study Area

Field experiments for both growing season A (September, 2018 to January, 2019) and B (February to June, 2019) were performed at experimental farm of College of Agriculture, Animal sciences and Veterinary medicine (CAVM), Busogo Campus located in Musanze District of Rwanda (Figure 5.1). Musanze District is located in Northern Province at 1°30 27.47 S latitude and 29°36 23.83 E longitude (Birasa *et al.,* 1990; MINIRENA, 2013).

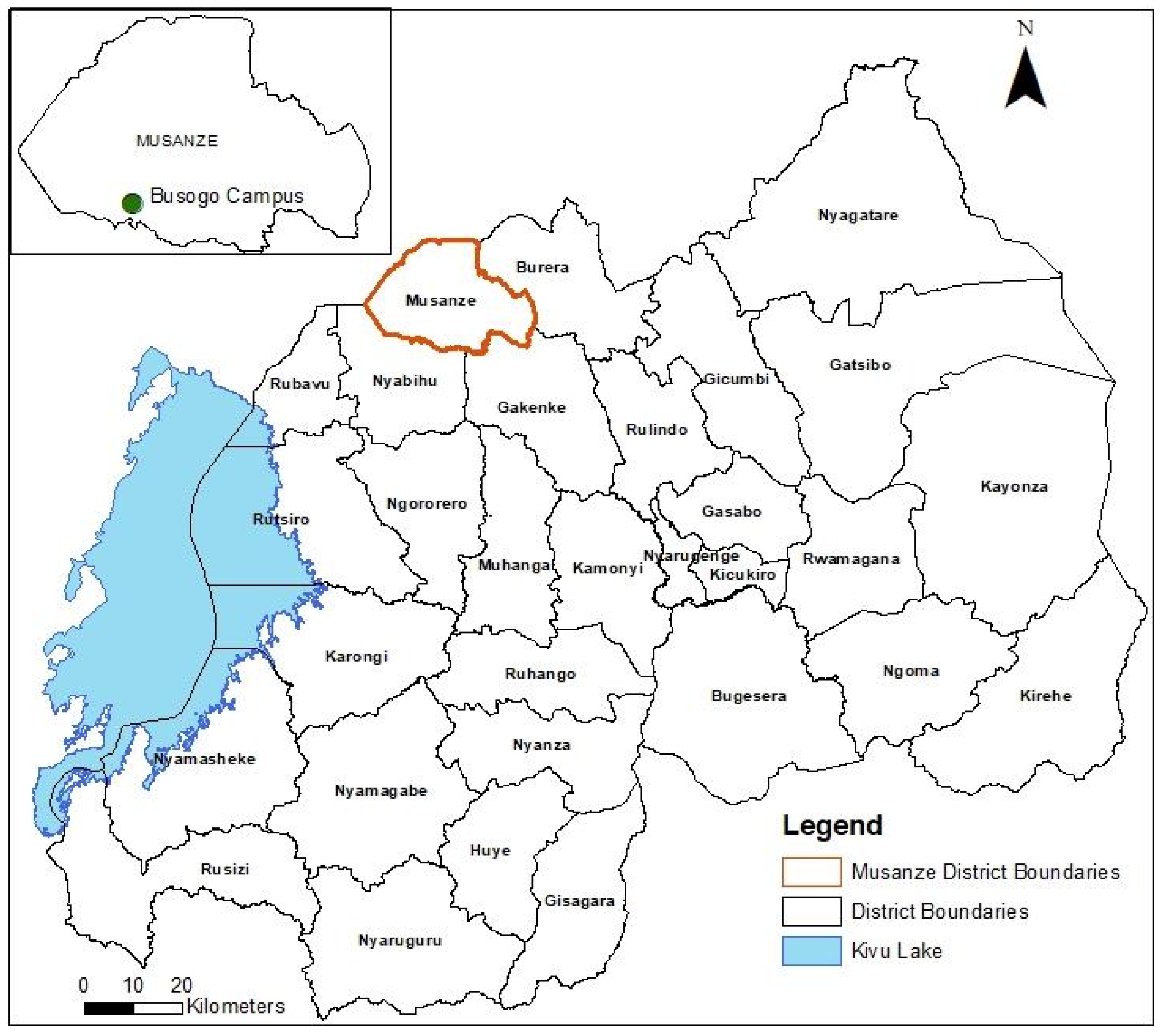

Figure 5.1. Map of location of study area (Busogo) in Rwanda

Source: GIS, University of Rwanda

This district is characterized by tropical highland conditions with high altitude (average of 2000m a.s.l), low temperature (average temperature of 20ºC) and high rainfall (1,400 mm to 1,800 mm well distributed throughout the year). This is the most mountainous part of Rwanda and is mainly characterized by hills with steep slopes. The soil is predominantly volcanic type of andisols (Birasa *et al.,* 1990; MINIRENA, 2013). The district has an average population density of 695 inhabitants /km^2 and the mean size of land cultivated per household is less than 0.3 ha. This region is among the major potato producing areas in Rwanda (RAB, 2012). Climatic and edaphic conditions in study area during field experiment season A and B were collected by

Meteo-Rwanda. Season A was a short rain season (average of rainfall= 708.7 mm) while season B was a long rain season (average of rainfall= 816 mm). During season A and B, the average of minum ambient temperature ranged from 11.2 to 10.8°C respecivelty whereas the maximum was 22.2 and 22.0°C. Soil temperature was 20.4 and 20.3 °C in Season A and B respectively. Ambient relative humidity was 75.2 and 75.5% % in season A and B respectively whereas soil moisture content was 26.3% in season A and 26.8% in season B (Table 5.1).

Table 5.1. Climatic and edaphic conditions in study area during field experiments

Parameter	Season A (2018-2019)						Season B (2019)					
	S	**O**	**N**	**D**	**J**	**Mean**	**F**	**M**	**A**	**Ma**	**Jn**	**Mean**
Ambiant temperature (°C)												
Maximum	23.2	22.8	23.0	21.9	24.3	**23.0**	22.9	22.7	22.4	21.5	21.3	**22.2**
Minimum	10.9	12.2	11.0	10.1	9.9	**11.2**	9.9	9.7	11.0	13.3	12.2	**10.8**
Ambiant relative humidity (%)	71.3	76.0	78.5	80.3	69.8	**75.2**	70.6	74.3	85.3	79.4	68.1	**75.5**
Rainfall (mm)	147	126.2	115.7	186.0	133.8	**708.7**	97.8	111.9	271.8	200.1	175.1	**856.7**
Soil temperature (°C)	20.8	20.6	20.1	20.0	20.4	**20.4**	20.7	20.2	20.5	20.6	20.0	**20.3**
Soil moisture (%) at 50 cm depth	24.8	25.6	27.0	26.8	26.0	**26.3**	26.5	26.3	28.0	27.8	23.2	**26.8**

S= September, O= October, N= November, D= December, J= January, F=February, M= March, A= April, Ma= May, Jn= June

5.2.2 Preparation of Bacterial Inoculum

During these *in vivo* experiments (Season A and B), laboratory preparation of bacterial inoculum was done in the Plant Pathology Laboratory of RAB-Northern zone at Musanze and Gikungu isolate of *R. solanacearum* was used in this experiment. Gikungu isolate was chosen among the others based on the virulence test which was carried out earlier in chapter three. After isolation of the bacterium from Gikungu potato cultivar, it was identified through a vascular flow technique, cultural and morphological characteristics on Kelman's Triphenyl Tetrazolium Chloride (TTC) and Casamino peptone glucose (CPG) culture media as well as biovar and Gram staining tests to distinguish this Gram-negative bacterium. The bacterium was purified and stored in a sterile glycerol stock at -20°C until use.

5.2.3 Preparation of Potato Seeds and Planting

The efficacy of plant extracts for the control of potato bacterial wilt, potato growth, yield and quality parameters, as well post-harvest infections and yield losses was evaluated on susceptible potato cultivar (Kirundo) under field conditions in two successive growing seasons (Season A and B). Certified and healthy potato seeds of Kirundo cv. were obtained from RAB-Kinigi station. Uniform tubers in size and with five eyes were selected for these experiments. Tubers were washed in running tap water, surface sterilized by using 70% ethanol solution for 5 min and after rinsing in sterile distilled water they were left to dry for one hour under room temperature conditions. Then, the tubers were soaked overnight in methanolic and water extracts of tobacco, wild marigold, and garlic previously diluted at 50 mg mL^{-1} with 1% DMSO. The following day, the tubers were left standing under shade for a day to allow sticking of active compounds. The tubers were then inoculated with 50 µL of bacterial suspension at concentration of 10^8 CFU mL^{-1} and incubated for 24 hours at room temperature to enhance sticking of the bacteria prior to planting. After that, they were grown under field conditions for four months. The haulm was again injected with 50 µL of 50 mg mL^{-1} methanol and water extracts of tobacco, marigold, and garlic when the plants were 30 days old. The treatment was repeated once per week (F1), once bi weekly (F2), and once per month (F3) for 50 days. Copper oxychloride (2%) and DMSO (1%) spray were applied as positive and negative controls, respectively. Methanol and water extracts were chosen because they inhibited the growth of bacteria at the higher level than chloroform extracts in *in vitro* screening experiment (Chapter 4). Tobacco, wild marigold and garlic were

also revealed to be the most promising plant extracts in the control of *R. solanacearum* among ten test plant extracts in the same experiment. The concentration of 50 mg mL^{-1} was selected from pilot greenhouse experiment.

5.2.4 Experimental Layout

The field experiments, were laid out in a Randomized Complete Block Design (RCBD) with factorial arrangements. The four factors used were plant extracts (S), solvent extracts (E), and application frequency (F), and growing seasons (G). During two growing seasons (A and B), two solvent extracts (methanol and water) from three plants (tobacco, wild marigold, and garlic) were applied weekly, bi-weekly or monthly (F1, F2, and F3, respectively) at concentration of 50 mg mL^{-1}. Positive control (2% copper oxychloride) was applied bi-weekly whereas negative control (1% DMSO) was applied weekly, bi-weekly or monthly, the same as plant extracts. Therefore, 22 treatments (18 treatments of plant extracts + 4 treatments of controls) were evaluated during each growing season. The following were 22 treatments used in field experiments (Table 5.2): In tobacco, wild marigold, or garlic plot (S1, S2, or S3 respectively), six treatments in each (i.e 18 treatments in total) were used namely both methanolic (E1) and water (E2) extracts from tobacco, wild marigold, or garlic applied weekly (F1), bi-weekly (F2), or monthly (F3) during each growing season. In addition, in control plot (C); four treatmennts such as negative control (C1= DMSO) applied weekly, bi-weekly or monthly and positive control (C2= Copper oxychloride) applied only bi-weekly were used and each, treatment was replicated three times in the whole experiment.

Table 5.2. Experimental layout of field experiments

S1E1F1 S1E1F2 S1E1F3 S1E2F1 S1E2F2 S1E2F3	S2 E1F1 S2E1F2 S2E1F3 S2E2F1 S2E2F2 S2E2F3	S3 E1F1 S3E1F2 S3E1F3 S3E2F1 S3E2F2 S3E2F3	C1F1 C1F2 C1F3 C2F2	B1
C1F1 C1F2 C1F3 C2F2	S3 E1F1 S3E1F2 S3E1F3 S3E2F1 S3E2F2 S3E2F3	S2 E1F1 S2E1F2 S2E1F3 S2E2F1 S2E2F2 S2E2F3	S1E1F1 S1E1F2 S1E1F3 S1E2F1 S1E2F2 S1E2F3	B2
S1E1F1 S1E1F2 S1E1F3 S1E2F1 S1E2F2 S1E2F3	S2 E1F1 S2E1F2 S2E1F3 S2E2F1 S2E2F2 S2E2F3	S3 E1F1 S3E1F2 S3E1F3 S3E2F1 S3E2F2 S3E2F3	C1F1 C1F2 C1F3 C2F2	B3

S= Plant extract (S1, S2, and S3= Tobacco, wild marigold, and garlic, respectively); E= Solvent extract (E1 and E2= Methanol and water extracts, respectively); F= Application frequency of plant extracts (F1, F2, and F3= weekly, bi-weeks, and monthly application, respectively); **C**= Controls (C1: Negative control= 1% DMSO), C2: Positive control= 2% Copper oxychloride).

Each plot of 8 m x 3 m was double dug to a depth of 20 cm, lined with polyethylene sheet and filled with the top soil mixed with NPK 17:17:17 (300 kg ha^{-1}). In both experiments, preceding crop for rotation program (two previous seasons) were beans and maize. No specific soil nutrient analysis or bacterial wilt inoculum were done prior to planting. In each treatment, four potato

plants were grown at spacing of 75 cm (inter-row) and 30 cm (intra-row). Therefore, 24 plants per plot were used. The statistical model is shown below:

$$Y_{ijklm} = \mu + {}_{i} + S_j + E_k + F_l + G_m + ES_{jk} + FS_{jl} + SG_{jm} + EF_{kl} + EG_{km} + SEF_{jkl} + SEFG_{jklm} + \mathcal{E}_{ijklm}$$

Where; Y_{ijkl}: observations made; μ: Overall mean; ${}_{i}$: Effect due to the ith blocks; S_j: Effect due to plant extracts; E_k: Effect due to the kth solvent extracts; F_l: Effect due to the jth application frequencies; G_m: Effect due to mth growing season; SE_{jk}: Effect due to interaction between between plant extracts and solvent extracts; SF_{jl}: Effect due to interaction between plant extracts and application frequency; SG_{jm}: Effect due to interaction between plant extracts and growing season; EF_{kl}: Effect due to interaction between solvent extracts and application frequencies; EG_{km}: Effect due to interaction between solvent extracts and growing season; SEF_{jkl}: Effect due to interaction between plant extracts, solvent extracts and application frequency; $SEFG_{jklm}$: Effect due to interaction between plant extracts, solvent extracts, application frequency and growing season; $\mathcal{E}_{ijklm}$: Random Error.

5.2.5 Data Collection

Evaluation of Growth Parameters

Simultaneous evaluation of the disease incidence on potato cultivar under field experiments and growth parameters such as days to 50% sprouting, plant height, number of stems per plant, and days to 50% flowering were conducted to determine the efficacy of plant extracts on crop growth. To evaluate the days required to 50% sprouting, the number of sprouted plants per treatment were counted and the mean of days to 50% sprouting in each treatment was calculated. Plant height was measured at 28, 56, and 70 days after planting (DAP) under field conditions by using a tape measure on plants from each treatment. The number of main stems per plant for the sampled plants was also counted three times at 28, 35 and 42 DAP in all experimentss. In addition, the effect of extracts on days to 50% flowering was evaluated by a daily counting of number of flowering plants from each treatment from 40th day after planting and this was stopped when a half of plants per treatment were flowered and this was considered as 50% flowering. In these field experiments (Season A and B), sampling on growth parameters was done on 12 plants per row of each treatment.

Evaluation of Yield Parameters

To evaluate the effect of plant extracts on yield of potato tubers harvested from field experiments (Season A and B), the following parameters were determined: Number of tubers per plant and total yield in each treatment. The potatoes were harvested at 90 DAP and the number of tubers per plant from each treatment was counted. Total yield of potato was determined by weighing all the harvested tubers per treatment (a plot with known area) and then this was converted to yield in t ha^{-1}. In these field experiments (Season A and B), sampling on yield parameters was done on 12 plants per row of each treatment.

Evaluation of Tuber Quality Parameters

To evaluate the effect of plant extracts on quality of potato, the following parameters were considered: Tuber size, dry matter (DM) content, specific gravity and reducing sugar content. Tuber size was evaluated by weighing six tubers picked randomly from each treatment from each block and then classified under three categories of size (small, medium and large). Small, medium, and large tubers were the ones with weight <39; 39-75, and >75 g (Biruk-Masrie *et al.*, 2015). In addition, both medium and large tubers (39-75, and >75 g) were considered as marketable tubers while small tubers (<39g) were taken as non-marketable tubers. Proportion of small, medium, large and marketable tubers was also calculated from total tuber samples over the number of tubers classified into small, medium, large, and marketable categories (Biruk-Masrie *et al.*, 2015). The DM content which is mostly important for culinary purposes was determined by taking 100g fresh weight of six tubers from each treatment from each block, oven dried at 80ºC for three days and then weighed using an electronic balance and DM content in percentage calculated according to the formula of Abong *et al.* (2010):

$$DM\ \% = \frac{Weight\ of\ sample\ after\ drying\ (g)}{Initial\ weight\ of\ sample\ (g)}\ x\ 100$$

The specific gravity is another measurement of tuber quality and is one of the vital indicators of tuber quality for processing purposes. This was measured by weighing in air (Wa) sampled 6 tubers from each treatment from each block and the same samples were again weighed in tap water (Ww), according to the method and formula by Abong *et al.* (2010):

$$SG = \frac{Wa}{(Wa - Ww)}$$

Where SG=Specific gravity; Wa= Weight in air, Ww= Weight in water.

Reducing sugars content which influences the colour of fried products was evaluated through Fehling's test or titration method. By this method, two Fehling's solutions (A and B) were used. Fehling's solution A was prepared by mixing 7 g of $CuSO_4.5H_2O$ with distilled water containing 2 drops of dilute sulfuric acid whereas solution B was obtained by adding 35 g of potassium tartrate and 12 g of NaOH in 100 ml of distilled water. Then 15 ml of solution A and B were thoroughly mixed. Thereafter, 10 g of potato tuber sample (from four oven-dried and gounded tubers of each treatments) were diluted with 100 ml of distilled water, mixed viguorously with orbital shaker for 30 minutes. The mixture was then filtered with Whatman N°42 filter paper in a 250 ml volumetric flask. The 10 ml of diluted 0.5 M HCl was added to the filtrate and the mixture was boiled at 100 C for 5 minutes. After cooling, the solution was neutralized with 10% NaOH. The solution was made up to 250 ml in a volumetric flask using distilled water. Three drops of phenolphthalein (indicator) were added. The solution was titrated by mixture of Fehling's solutions until the appearance of red brick color. The volume (mL) of Fehling's solution used for titration was recorded and the calculation of reducing sugar content (RS %) was done using the formula below according to Ooko and Kabira (2011) :

$$RS\ \% = \frac{4.95\ (Factor) x\ 250\ (Dilution)\ x\ 100}{Weight\ of\ the\ sample\ x\ Title\ x\ 1000}$$

Disease Incidence and Biological Control Efficacy of Plant Extracts

Bacterial wilt incidence (DI %) on potato plants was evaluated and recorded from 10 to 50 days after inoculation of bacterial suspension. In these field experiments (Season A and B), sampling on disease incidence was done on the 12 plants per row of each treatment. Plant was considered as diseased when it showed the usual symptoms of bacterial wilt. In addition, disease incidence (DI %) on tubers were evaluated at harvesting time (Priou *et al.,* 2001).

Disease incidence on plant or tuber was evaluated by the following formula:

$$DI\,\% = \frac{n}{N}\ x\ 100$$

Where; n is the number of infected stems/tubers and N is the total number of plants or tubers inspected per block.

From this experiment, biological control efficacy (BCE %) of plant extracts was also calculated according to Guo *et al.* (2004) :

$$BCE\,\% = \frac{(DI\ of\ control - DI\ of\ treatment\ group)}{DI\ of\ control}\ x\ 100$$

Evaluation of Post-harvest Infection and Total Yield Loss due to *Ralstonia solanacearum*

Post-harvest infections and the efficacy of extracts in the protection of potato in storage against bacterial wilt were determined from ten healthy harvested tubers from field which were picked from each treatment. The tubers were submerged in bacterial suspension at concentration of 10^8 CFU mL^{-1} for 30 minutes and air-dried at room ambient temperature. Then, treated tubers were also submerged in water and methanol extracts of tobacco, marigold, and garlic once per week, once per two weeks or once per month in stock for 30 minutes and air-dried again at room temperature. DMSO at 1% and copper oxychloride at 2 % were used as negative and positive controls, respectively. The experiment was carried out in a Randomized Complete Block Design (RCBD) with factorial arrangements with 22 treatments, each replicated three times. The tubers were stored in polythene bags for 30 days, after which the observation for tuber infection and total yield loss caused by *R. solanacearum* was evaluated. The number and weight of infected tubers and total number and fresh weight of stored tubers were used to calculate the percentage of post-harvest infection and percentage of total loss as described by Rahman *et al.* (2012).

5.2.6 Data Analysis

The analysis of variance (ANOVA) was carried out using SAS software version 9.00 (TS M0), to determine the difference in growth, yield, and quality parameters and post-harvest infections and yield due to application of the methanol and water extracts from tobacco, wild marigold, and garlic species applied every week, every two weeks and every month in the control of Gikungu

isolate of the pathogen. The treatments means were separated using Tukey's honestly significant difference test at P 0.05.

5.3 Results and Discussion

5.3.1 Effect of Plant Extracts on Potato Growth Parameters

Effect of Plant Extracts on Days to 50 % Sprouting and Flowering

Effect of solvent extracts (methanol and water) of three plants (tobacco, wild marigold, and garlic) applied either weekly, bi-weekly or monthly on number of days required to 50% sprouting was studied under field conditions (Season A and B). From the results, no interaction between these factors was observed on number of days to 50% flowering at p 0.05. However, one interaction between season and application frequency was observed on days to 50 % sprouting. (Appendix K). In both seasons A and B, tobacco, wild marigold and garlic extracts showed reduced number of days required for sprouting of potato (Season A= 19.17, 17.00, and 16.67 DAP respectively; Season B= 20.33, 18.89, and 18.61 DAP respectively) compared to copper oxychloride (Season A=29.00 DAP; Season B= 27.00 DAP), at p 0.05. In addition, application of tobacco extract resulted to a delay of sprouting as compared to negative control (DMSO) at p 0.05. Thus, in season A; tobacco extracts required much more days to 50% sprouting (season A =19.17 DAP; season B=20.33 DAP) than negative control (Season A=16.47 DAP; Season B= 16.00 DAP) though marigold and garlic did not at p 0.05 (Table 5.3). In addition, tobacco extract delayed the sprouting more than garlic but not than marigold in season A while in season B, there was no significant difference in number of days to 50% sprouting between the test plant extracts at p 0.05 (Table 5.3).

Table 5.3. Effect of plant extracts, application frequency, and solvent extracts on number of days after planting (DAP) to 50% sprouting in season A and B

Plant extracts			**Frequency**			**Solvent extracts**		
	Season A	**Season B**		**Season A**	**Season B**		**Season A**	**Season B**
Tobacco	19.17 ± 0.72^{b}	20.33 ± 0.50^{b}	**Weekly**	20.06 ± 0.95^{b}	22.00 ± 0.44^{b}	**Methanol**	18.33 ± 1.81^{b}	19.63 ± 1.03^{b}
Wild marigold	17.00 ± 1.00^{bc}	18.89 ± 0.86^{bc}	**Bi-weekly**	19.00 ± 0.58^{bc}	17.94 ± 0.75^{c}	**Water**	16.89 ± 1.87^{b}	18.93 ± 0.50^{b}
Garlic	16.67 ± 1.06^{c}	18.61 ± 0.63^{bc}	**Monthly**	18.72 ± 0.38^{bc}	18.04 ± 0.28^{c}			
Controls (+; -)								
Copper (+)	29.00 ± 1.00^{a}	27.00a$\pm4.36^{a}$	**Copper**	29.00 ± 1.00^{a}	27.00 ± 4.36^{a}	**Copper**	29.00 ± 1.00^{a}	27.00 ± 4.36^{a}
DMSO (-)	16.47 ± 0.58^{c}	16.00 ± 0.88^{c}	**DMSO**	16.67 ± 0.58^{d}	16.00 ± 0.88^{c}	**DMSO**	16.67 ± 0.58^{b}	16.00 ± 0.88^{b}
P =0.05	<0.0001	0.0003	**P =0.05**	<0.0001	0.0002	**P =0.05**	<0.0001	0.0011

The values are means of days required after planting (DAP ± SD) for 50% tuber sprouting. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2 % copper oxychloride) and negative (1% DMSO) controls. SD: Standard deviation.

The effect of three different application frequencies (weekly, bi-weekly, and monthly) of plant extracts on number of days to 50% sprouting was also evaluated under two successive seasons (A and B) (Table 5.3). From the findings, in both seasons A and B; application of extracts weekly, bi-weekly and monthly shortened the days to sprouting than synthetic chemical at p 0.05. However, in season A, all three test frequencies (weekly, bi-weekly and monthly) of extract application showed a delay of sprouting (20.06, 19.00, 18.72 DAP, respectively) more than negative control (16.67 DAP); while in season B, only the weekly application significantly delayed sprouting (22.00 DAP) more than negative control (16.00 DAP) but bi-weekly and monthly (17.94 and 18.04 DAP, respectively) did not. Moreover, no significant difference in the number of days to 50% sprouting was observed between frequencies in season A; but in season B only a weekly application of extract significantly (p 0.05) delayed sprouting (Table 5.3).

The efficacy of the two solvent (methanol and water) extracts was verified on number of required days to 50 % sprouting in potato under field conditions. In both seasons A and B, methanol and water extracts significantly (p 0.05) hastened the number of days to sprouting (A=18.33, 16.89, and 16.67 DAP; B=19.63, 18.93, and 16.00 DAP) compared to positive control (A= 29.00 and B= 27.00 DAP). However, there was no significant difference ((p 0.05) in days to 50 % sprouting between extracts and negative control in both seasons (Table 5.3).

In terms of days to 50% flowering an interaction between all test factors (plant extracts, solvent extracts, and frequency of application) was observed in season A whereas no interaction was observed in season B. On the other hand in season A; plant extract of tobacco, marigold and garlic extracts required significantly (p 0.05) shorter time to 50% flowering (43.00, 41.28, 41.11 DAP respectively) as compared to positive control (68.00 DAP). In season B, only tobacco extract delayed flowering (56.44 DAP) to a closer extent as a positive control (62.11 DAP) whereas marigold and garlic (53.59 and 53.72 DAP) shortened it (Table 5.4). In addition, no difference in number of days to 50% flowering was observed among the plant extracts or between them and negative control in both seasons A and B (Table 5.4).

Table 5.4. Effect of plant extracts, application frequency, and solvent extracts on number of days after planting (DAP) to 50% flowering in season A and B

Plant extracts			Frequency			Solvent extracts		
	Season A	**Season B**		**Season A**	**Season B**		**Season A**	**Season B**
Tobacco	43.00±1.73^{b}	56.44±4.26^{ab}	**Weekly**	44.17±2.20^{c}	59.44±1.84^{a}	**Methanol**	42.19±1.81^{b}	55.26±4.58^{a}
Wild marigold	41.28±2.22^{b}	53.59±3.16^{b}	**Bi-weekly**	40.83± 1.80^{c}	51.28±2.04^{b}	**Water**	41.41±1.87^{b}	55.04±2.18^{a}
Garlic	41.11±0.84^{b}	53.72±1.42^{b}	**Monthly**	40.39±1.54^{c}	53.72±2.47^{b}			
Controls (+; -)								
Copper (+)	68.00± 1.73^{a}	62.11±2.59^{a}	**Copper**	68.00± 1.73^{a}	62.11±2.59^{a}	**Copper**	68.00± 1.73^{a}	62.11±2.59^{a}
DMSO (-)	41.00± 1.00^{b}	54.44±1.54^{b}	**DMSO**	41.00± 1.00^{c}	54.44±1.54^{b}	**DMSO**	41.00± 1.00^{b}	54.44±1.54^{a}
P =0.05	<0.0001	0.0133	**P =0.05**	<0.0001	0.0033	**P =0.05**	<0.0001	0.0962

The values are means of days required after planting (DAP ± SD) for 50% plant flowering. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2 % copper oxychloride) and negative (1% DMSO) controls. SD: Standard deviation.

Furthermore, frequency of application also influenced the number of days required to 50% flowering. In season A, all test frequencies of plant extract application required fewer days to flowering (44.17, 40.83, 40.39 DAP in weekly, bi-weekly, and monthly, respectively) compared to positive control (68.00 DAP). However, no difference in days for 50% flowering was found between application frequencies or between them and negative control in season A. In season B, both weekly application of extracts and positive control were observed to delay flowering (59.44 and 62.11 DAP respectively) of potato at the same level. However, bi-weekly or monthly application did not significantly (p 0.05) delayed flowering (51.28 and 53.72 DAP, respectively) comparing to negative control in season B (Table 5.4). The weekly application also resulted to delayed flowering than negative control (54.44 DAP) and than both bi-weeks and monthly application (51.28 and 53.72 DAP, respectively) in season B (Table 5.4).

For solvent extracts, no significant difference in days to 50% flowering was observed between methanol and water extracts or the two solvents and negative control in both season A and B. Plants treated with methanol and water extracts required shorter time (42.19 and 41.41 DAP, respectively) to reach 50% flowering than positive control (68.00 DAP) in season A but no difference in number of days to reach 50% flowering was recorded between solvent extracts and copper in season B (Table 5.4).

The effect of growing seasons (A and B) on number of days required to 50 % sprouting and flowering in potato under field conditions was also assessed. The results showed that there was no significant effect of growing season on number of days required to 50 % sprouting (p 0.05). However, both seasons A and B shortened the number of days to sprouting (A= 18.7 DAP; B= 19.3 DAP) compared to positive control (28.0 DAP). However, there was no significant difference ((p 0.05) in days to 50 % sprouting between seasons and negative control (Table 5.5). The growing season, A showed reduced number of days required for flowering of potato (41.8 DAP) comparing to season B (54.8 DAP) at p 0.05. In addition, seasons B resulted to a delay of flowering as compared to negative control (DMSO), eventhough season A did not. Both seasons A and B resulted to reduced number of days required to 50 % flowering than positive control (Table 5.5).

Table 5.5. Effect of growing seasons (A and B) on days to 50 % sprouting and flowering

Season	Days to 50% Sprouting	Days to 50% Flowering
A	18.7±2.0^{b}	41.8±1.01^{c}
B	19.3±1.3^{b}	54.8±1.5^{b}
Controls (+; -)		
Copper (+)	28.0±1.0^{a}	65.1±1.7^{a}
DMSO (-)	16.2±0.5^{b}	43.0±1.0^{c}
P =0.05	0.0043	<0.0001

The values are means of days required after planting (DAP ± SD) for 50% tuber sprouting and flowering. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2 % copper oxychloride) and negative (1% DMSO) controls. SD: Standard deviation.

In summary, all the results from season A and B showed that methanol and water extracts of tobacco, wild marigold and garlic at three different application frequencies had positive effect on potato growth compared to controls. In general, all plant extracts shortened days to 50% sprouting by ten and seven days respectively in season A and B as compared to positive control. However, they also delayed 50% sprouting by three days in season A and four days in season B than negative control especially methanolic and water extracts from tobacco applied every week. No significant effect of growing seasons was observed in days required to 50 % potato sprouting. In addition, there was an interaction between season and application frequency for days to 50 % sprouting. For days required for 50% flowering, there was no interaction between test factors. Methanol or water extracts of tobacco, wild marigold or garlic whether applied weekly, bi-weekly or monthly, initiated flowering earlier than positive control and negative control. However, tobacco extracts showed a delay in flowering similar to synthetic pesticide in season B. In the same season, weekly application of plant extracts prolonged time to 50% flowering comparable to positive control. Season B resulted to a delayed flowering (13 days) than season A. Therefore, under field conditions; methanolic and water extracts of tobacco applied weekly delayed 50% sprouting and flowering than negative control and other treatments except pesticide treatment used as positive control especially in season B.

Similar delay of tuber sprouting and flowering in potato treated with plant extracts were reported by different researchers. For instance, a greenhouse experiment was carried out in Ethiopia to find out the effect of potato seed treatment with eucalyptus, dill weed, black cumin and spearmint essential oils on growth and yield parameters of potato by Biruk-Masrie *et al.* (2015). Analysed parameters were sprouting and flowering rate, plant height, number of leaves, number of main stems per plant, physiological maturity, number of tubers per plant, tuber weight, and tube size. The researchers found that treatment of potato seeds with plant extracts resulted into delay of sprouting, flowering and tuber maturity compared to untreated tubers or negative control (Biruk-Masrie *et al.*, 2015; Ulfa *et al.*, 2013). In addition, the difference in number of days required for sprouting and flowering was also associated with the type of plant extracts applied. Futhermore, plant extracts from dill weed and eucalyptus showed greater delay of sprouting and flowering than the others (Biruk-Masrie *et al.*, 2015).

Another study was conducted to evaluate the influence of seeds treatment with different plant extracts on potato sprouting rate. Plant extracts used were *Azadirachta indica, Cymbopogon, Ultica dioica, Mentha, and Acoros calamus* (Giri *et al.*, 2020). From this study, it was concluded that application of plant extracts resulted to delay of potato sprouting in comparison to control (untreated tubers). Inhibitory effect also varied between the types of applied plant extracts. Thus, at 60 days after planting; the highest rate of sprouting was found in the control (untreated seeds) followed by *Ultica* and *Azadirachta* whereas *Mentha, Cymbopogon* and *Acoros* extracts completely inhibited potato sprouting. Sprouting rate changed over time and at 75 days, the lowest and the highest sprouting was recorded in *Cymbopogon* and control respectively. Among plant extracts, *Mentha* recorded the highest sprouting rate at 75 days. It was then concluded that *Cymbopogon* extract is the most effective anti-sprouting agent in potato and can be used in storage. All these experiments confirmed that application of plant extracts can suppress or delay the emergence of potato (Biruk-Masrie *et al.*, 2015; Giri *et al.*, 2020). From the results, it was concluded that treatment of potato seeds with plant essential oils results to the delay or inhibition of emergence as well as delay to 50% flowering (Biruk-Masrie *et al.*, 2015). The results of the above studies are in agreement with the present study where; methanolic and water extracts of tobacco applied every week significantly delayed days to 50% sprouting and flowering.

Song *et al.* (2009) also confirmed the potential of plant extracts in potato sprouting inhibition. They detected inhibition of potato sprouting for 6 months in all potato cultivars treated with essential oils (MOE) from mint ((*Mentha spicata* L.) especially applied at high concentration (4.5 and 5 µL L^{-1}). The same extract also protected potato tuber against post-harvest losses and microbial infection during that storage period. Interestingly, low concentration of MOE induced early potato sprouting. In addition, the effect of high concentration MOE against tuber sprouting in the first stage decreased over time and some weeks later during experiment all treated tubers started to emerge. These researchers recorded R-carvone as the main sprouting inhibitor from MOE. They reported that this organic compound was converted into degraded compounds with less or non-biological activity in dormancy maintenance later during storage. The researchers reported that R-carvone from mint inhibits potato sprouting by causing local necrosis of tuber apical meristem without skin damage, later the axillary buds develop in the same sprouting eyes. This new induction of axillary buds from a damaged meristem together with degradation of R-carvone were suggested to be the reason for delayed sprouting of tubers treated with MOE at high concentration. In the same experiment it was shown that low concentration initiated earlier formation of axillary buds and thus triggered early emergence. It was concluded that some plant natural compounds like R-corvone from MOE can be used to induce early potato sprouting (at low concentration) or to prevent sprouting (at high concentration or repeated/high frequency of application) in organic farming (Song *et al.*, 2009).

In earlier studies, natural compounds from tobacco extract were also reported to influence seed germination in different plants (Bhalla *et al.*, 1974; Heena & Swati, 2018). For instance, Bhalla *et al.* (1974) conducted a study to investigate the effect of tobacco smoke condensate (TSC) at different concentrations (by condensating tobacco cigarette in water) on germination of onion and tomato seeds. In general, all treatments inhibited seed germination of both tomato and onion seeds. In addition, the highest TSC concentration (0.25%) highly inhibited the germination of tomato seeds and completely prevented the germination of onion seeds. In addition, onion was reported to be more sensitive to TSC than tomato and this shows that seeds react differently to tobacco extract in terms of germination. Greatest inhibitory effect on seed germination from TSC treatment at highest concentration was attributed to higher concentration of nicotine (germination inhibitory agent) in this tobacco extract (Bhalla *et al.*, 1974; Yu *et al.*, 2014).

A recent study was also carried out by Heena and Swati (2018) to evaluate the effect of pure tobacco extract and nicotine (an alkaloid from tobacco) on seed germination and plant height of *Trigonellafoenum* plant. From the results, the researchers reported that pure tobacco extract at high concentration (3%) decreased significantly the germination rate compared to other treatments including normal/untreated seed. The highest plant height was recorded in nicotine treatment followed by pure tobacco extract. The researchers concluded that pure tobacco extract can be used as seed germination retardant while both nicotine and pure tobacco extract can be used as growth stimulant since they may have positive effect on plant height. In addition, it was reported that tobacco has the ability to stimulate production of some phytohormones in plant like abscisic acid (ABA) and cytokinin. Therefore, its inhibitory effect against germination may be attributed to ABA effect whereas the positive effect on root and shoot length may be associated to cytokinin (Heena & Swati, 2018).

In the present study, it was observed that potato tubers treated with tobacco plant extracts took longer to reach 50 % sprouting and flowering than negative control when they were applied every week. Delay of sprouting and flowering due to high frequency of tobacco extract application may be associated with the high concentration of ABA in plant extracts which may cause the delay to potato emergence. Similar inhibitory effect of plant extracts against plant sprouting and flowering in dose-dependent manner was also confirmed in earlier studies. Biruk-Masrie *et al.* (2015) confirmed that the suppression or delay of sprouting by plant extracts was high when the extracts were applied at higher dose (135 mg kg^{-1}) and this was due to both type and concentration of natural compounds present in a plant extract.

In addition, in a study conducted by Giri *et al.* (2020), it was surprising to find out that *Mentha* which inhibited completely potato sprouting at 60 days also gave the highest sprouting rate at 75 days. This also showed that a single application is similar to low concentration of active compounds in plant which is not enough for total suppression of sprouting. It also supports the idea that suppression of seed germination by plant extracts at low concentration is always reversible and may resume after some time (Biruk-Masrie *et al.*, 2015). Thus, a repeated application of plant extracts is recommended to suppress sprouting or maintain seed dormancy (Giri *et al.*, 2020). In the present study, the highest application frequency (every week) was not

concentrated enough to suppress the total sprouting of potato tubers but was sufficient for delaying it and to allow it to resume later.

In the present study, tobacco extract was reported to delay tuber sprouting and flowering than other treatments. This delay may be related to the effect of different bioactive compounds which have been recorded in tobacco by earlier studies. For instance, many studies reported that tobacco contained nitrogen-containing components such as alkaloids and related volatile bases, proteins and amino acids; saponin, tannin, flavonoides, cardiac glycosides, phenolic compounds (phenols, phenolic acids, quinones), carbohydrates steroids, terpenes, sulfur-containing compounds; hydrocarbons, alkenes, alkanes, alkynes, polycyclic and monocyclic aromatics (Amzad & Salehuddin, 2013; Perfetti, 2013; Sharma *et al.,* 2016; Singh *et al.,* 2010). Usually, these natural compounds were reported to play role in plant defense against biotic and abiotic stresses but Mulugeta *et al.* (2019) and Teper-Bamnolker *et al.* (2010) confirmed that plant natural compounds are not only able to protect plants against those stresses but can also induce plant growth. Thus, therefore provides explaination as to why tuber sprouting was delayed in treatments with tobacco extract.

Other researchers also confirmed that inhibitory effect of plant extracts on sprouting is associated to the active role of natural compounds present in a given extract in maintaining plant dormancy (Mani & Hannachi, 2015; Song *et al.*, 2009; Ulfa *et al.*, 2013). For instance, Biruk-Masrie *et al.* (2015); Song *et al.* (2009); and Teper-Bamnolker *et al.* (2010) attributed the delay of tuber spouting from plant extracts to active compounds like monoterpene S (+)-carvone and citronellol in dill, mint and eucalyptus respectively. The higher concentration of S (+)-carvone and citronellol in dill and eucalyptus respectively may be the cause of total inhibition of sprouting which was resumed later during growth. The early emergence of seeds treated with plant extracts at low concentration is attributed to its lower content in bio-active compounds required for delaying emergence (Biruk-Masrie *et al.*, 2015; Ulfa *et al.*, 2013). Biruk-Masrie *et al.* (2015) and Giri *et al.* (2020) also reported that suppression of seed emergence by plant extracts is a reversible state because metabolites from plant are volatile and that the sprouting can resume later, leading to a delay of emergence especially for extracts applied at low concentration. Therefore, plant extracts can be used for suppressing or delaying sprouting depending on the

types and concentrations applied. The above also helps to explains what was observed in the present study.

Song *et al.* (2009) gave complimentary details on mechanism behind delay of sprouting in tubers treated with plant natural compounds. They explained that active compounds delay potato sprouting by delaying and disrupting metabolic changes required in tuber to induce sprouting (transaction of source assimilate to sink for developing bud). The active compounds also cause physical damage and stress to bud and apical meristem which delays sprouting. In potato tuber, axillary buds develop from apical meristem and usually, anything that suppresses apical dominance triggers outgrowth of axillary buds and vice versa (Song *et al.*, 2009; Teper-Bamnolker *et al.*, 2010). This involves internal hormonal balance, by which auxin induces apical dominance while cytokinin inhibits it. This provides an explanation of development of sprouts from axillary buds after loss of apical meristem in tuber treated with plant extracts. This also confirmed that a treatment of tubers with natural active compounds alters auxin: cytokinin ratio in tuber (Ulfa *et al.*, 2013). In addition, high application of extracts may also prevent the hormone production, thus suppression of axillary bud development from damaged apical bud (Song *et al.*, 2009; Ulfa *et al.*, 2013). These reports may explain why in the current study frequent application of plant extracts delayed tuber sprouting.

Gómez-Castillo *et al.* (2013) conducted a study to investigate inhibitory effect of peppermint (*Mentha piperita* L.), caraway (*Carum carvi* L.), eucalyptus (*Eucalyptus globules* Labill.), and coriander (*Coriandrum sativum* L.) essential oils against potato sprouting. Results confirmed greater sprouting suppression in peppermint and coriander treatments with inhibiton effect evaluated at 65 to 95% comparing to control. This inhibitory effect was attributed to germination inhibitory agents namely citral, caravan (terpenoid), and menthol (monoterpene) in *Cymbopogon, Mentha,* and *Acoros* respectively, natural compounds with the ability to suppress seed emergence in storage (Gómez-Castillo *et al.*, 2013).

In other studies, it was also reported that the inhibitory agents against seed germination are abscisic acid (ABA), ethylene, lactone, different types of flavonoid and alkaloid, benzoic acid, and cinnamic acid, S-carvone, cineole, neomenthol, menthone, fenchone, cyclodextrin, -

aldehydes, and -ketones (Bhalla *et al.*, 1974, Yu *et al.*, 2014). These studies revealed that apart from nicotine, tobacco also contain two organic acids (benzoic and cinnamic acids) with the major germination inbihitory effect on different crops. In addition, it was also confirmed that tobacco contain most of those compounds like flavonoids, alkaloids (mainly nicotine), terpenes, benzoic and cinnamic acid (Heena & Swati, 2018; Yu *et al.*, 2014) but with also other organic compounds in high concentration like phenolic acids (Perfetti, 2013; Sharma *et al.,* 2016) as well as some plant growth regulators like solanacol (a strigolactone) and ABA- derivatives (Pandey *et al.*, 2016; Zwanenburg & Blanco-Ania, 2018). Therefore, it is evident that a plant extract like tobacco which was used in the present study, which contains a wide range of bioactive compounds with germination inhibitory potential significantly influenced potato sprouting.

Moreover, in the current study, methanolic and water extracts from tobacco applied every week delayed potato plant flowering in the present study. Similar effects of plant extracts on potato flowering were also recorded in previous studies. Biruk-Masrie *et al.* (2015) reported that treatment of potato tubers with plant extracts resulted to a delayed flowering compared to untreated tubers. The delay was also recorded at different levels among treatments and this may be linked to the various contents of bioactive compounds in different extracts used. The same treatments which delayed sprouting also led to delayed flowering of potato. It was therefore, concluded that late sprouting may go together with a delay of the next vegetative growth and ultimately a late flowering and maturity (Biruk-Masrie *et al.*, 2015). The same observation was also made in the present study, where treatments with delayed 50% sprouting had also late flowering. This was common with treatment of methanolic and water extracts from tobacco when applied every week.

During long rain season B compared to short rain season A. These results are in agreement with above-cited literatures which reported a delayed flowering when precipitation increased and temperatures decreased. Growing seasons did not affect sprouting and this may due to the fact that the variation in rainfall and/or temperatures was not enough to trigger In the present study, growing season B resulted to delayed potato flowering comparing to season A. In previous literature, it has been reported that potato is a short-day and cool-season crop (DAFF, 2013) which is mainly grown in the cool highland regions of the tropics (REMA, 2011; Were *et al.*,

2013). Among the environemental requirements for adequate potato growth and production a combined regular rainfall of 850-1500 mm and temperature ranging between 10 to 23ºC during the growing season is crucial (REMA, 2011; Rymuza *et al.*, 2015). Below or above these growing conditions, potato faces to different biotic and abiotic stresses and fails to grow well which leads to low tuber set and bulking as well as low yield and tuber quality (Escuredo *et al.*, 2020; Rymuza *et al.*, 2015). It was also reveald that increase in temperatures combined with decreased precipitation leads to short potato life cycle, short plants, low tuber bulking and low yield. On the other hand, lower temperature with more rainfall result to long potato cycle, tall plants, delayed flowering and high yield (Escuredo *et al.*, 2020). In the current study, meteorological data showed that growing season B which is usually a long rain season was characterised by more rainfall and low temperatures compared to season A (short rain season) (Table 5.1). These meteo conditions alone or combined with other analysed parameters especially bacterial wilt incidence and non-analysed factors (soil nutrient and pH status) during these two growing seasons, could have contributed to the delayed flowering, production of tall potato plants, high yield and tuber quality remarkable physiological changes required for accelerating or delaying potato sprouting.

In the experiments where, copper oxchloride, a synthetic pesticide was used as positive control, also resulted to a delayed sprouting and flowering and also inhibited subsequent potato growth. Copper treatments were revealed to have some positive response against plant pathogens but it was also reported to be toxic due to its ability to induce reactive oxygen species (ROS) production either in rhizosphere or in plant (Ferreira *et al.*, 2014). ROS are known to be toxic compounds in various metabolic processes of living organisms like proteins and nucleic acids synthesis, lipid, and carbohydrates production. Thus, copper-based treatment leads to oxidative stresses in plants. Therefore, any substance that can induce ROS production may harm plant growth, development and ultimate yield parameters (Ferreira *et al.*, 2014). Therefore, delayed sprouting and flowering observed in positive controls in the present study could be attributed to copper toxicity.

Effect of Plant Extracts on Number of Stems per Plant and Plant Height

Number of stems per plant were counted at 28, 35, and 42 days after planting**.** For number of stems per plant no interaction was found between plant extracts, solvent extracts, and frequency of application in both growing season A and B (Appendix M). However, these three factors affected number of stems per plant separately and at different level during the two growing seasons. In seasons A and B, no difference between plant extracts was recorded interms of the number of stems per plant throughout the experiment. However, at 28 and 35 DAP, tobacco, marigold, and garlic extracts resulted to higher number of stems per plant (at 28 DAP= 2.54, 2.53, and 2.30, respectively; at 35 DAP= 2.65, 2.66, and 2.58, respectively) than positive control (at 28 DAP= 1.00 and at 35 DAP= 1.25) at p 0.05 (Table 5.6). At 28 DAP tobacco, marigold, and garlic extracts also resulted to higher number of stems per plant than negative control (1.16) but at 35DAP, only tobacco and marigold had higher number of stems than negative control (1.50) while garlic did not (Table 5.6). In addition, at 42DAP in season A only tobacco extracts resulted to higher number of stems per plant (3.79) than negative control (1.59) while marigold and garlic (2.83 and 2.76, respectively) did not. In addition, there was no significant difference (p 0.05) in number of stems between plant extracts and positive control at 42 DAP (Table 5.6).

In season B, no difference between plant extracts was recorded in term of stems per plant throughout the experiment. In addition, no difference number of stems per plant was found between plant extracts and positive control at 28 and 35 DAP. At 28 and 35 DAP, tobacco alone showed higher number of stems per plant (2.86 and 3.10) than negative control (1.56 and 1.92) but marigold and garlic did not. At 42 DAP, tobacco extract also showed higher number of stems (3.39) per plant compared to positive control (2.25) but not marigold and garlic (2.83 and 2.81) did not at p 0.05 (Table 5.6). Moreover, at 42 DAP; all plant extracts gave higher number of stems per plant (3.39, 2.83, and 2.81 for tobacco, marigold, and garlic respectively) than negative control (2.06). Overall, treatment of plant extracts resulted to higher number of stems per plant comparing to negative control in the two field experiments. In addition, tobacco extracts led to higher number of stems per plant than positive control followed by marigold and garlic extracts in some of the observation (Table 5.6).

Table 5.6. Effect of plant extracts on number of stems per plant in season A and B on various days after planting (DAP)

Plant extracts	Number of stems per plant Season A			Number of stems per plant Season B		
	28DAP	35DAP	42DAP	28DAP	35DAP	42DAP
Tobacco	2.54±0.41^{a}	2.65±0.49^{a}	3.79±0.71^{a}	2.86±0.45^{a}	3.10±0.33^{a}	3.39±0.25^{a}
Marigold	2.53±0.13^{a}	2.66±0.10^{a}	2.83±0.15^{ab}	2.30±0.20^{ab}	2.68±0.42^{ab}	2.83±0.52^{ab}
Garlic	2.30±0.95^{a}	2.58±0.60^{ab}	2.76±0.74^{ab}	2.40±0.48^{ab}	2.65±0.42^{ab}	2.81±0.36^{ab}
Controls (+; -)						
Copper	1.00±0.01^{b}	1.25±0.43^{c}	2.69±0.19^{ab}	1.97±0.43^{ab}	2.08±0.52^{ab}	2.25±0.50^{bc}
DMSO	1.16±0.10^{b}	1.50±0.06^{bc}	1.59±0.14^{b}	1.56±0.25^{b}	1.92±0.17^{b}	2.06±0.21^{c}
P =0.05	0.0445	0.0027	0.0039	0.0204	0.0302	0.0222

The values are average of stems (± SD) from plants of each treatment at 28, 35, and 42 days after planting. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2 % Copper oxychloride) and negative (1% DMSO) controls. SD: Standard deviation.

Effect of frequency of application of plant extracts on stems per plant was also examined. At 28 and 35 DAP season A, all frequencies of application showed higher number of stems than negative and positive control. At 42 DAP season A, all frequency gave higher number of stems (3.80, 2.93, and 2.65 for weekly, bi-weekly, and monthly respectively) compared to negative control (1.59) but not more than positive control (2.69). No significant difference (p 0.05) in number of stems per plant was found between three tested application frequencies at 28 and 35 DAP. At 42 DAP, the weekly application gave higher number of stems than monthly application but not than bi-weeks application of plant extract (Table 5.7).

The effect of application frequencies was also found in season B where from 28 to 35 DAP only weekly application led to higher number of stems (2.79 and 3.03) than positive (1.97 and 2.08) and negative (1.56 and 1.92) controls at p 0.05. Application of plant extracts bi-weekly weeks or monthly did not result to a significant difference of stems per plant comparing to negative or positive controls at 28 and 35 DAP. At 42 DAP, all frequencies resulted to higher stems (3.38,

2.71, ad 2.65 in weekly, bi-weekly, and monthly respectively) than negative control (2.06). The weekly and bi-weekly application of extracts gave higher number of stems per plant than positive control (2.25) but a monthly application did not (Table 5.7). In season A and B, frequency of application did not influence the number of stems per plant in early stages of potato growth (from 28 to 35 DAP). However, at 42 DAP in season A, application of plant extracts weekly resulted to higher number of stems per plant (3.80) than monthly application (2.65). In season B, weekly application gave higher stems number per plant (3.38) than both bi-weekly and monthly application (2.71 and 2.65) at p 0.05. Overall, application of plant extracts at all studied frequencies resulted to higher number of stems per plant than negative control at the end of both season A and B. In addition, in season A all frequencies also gave higher number of stems especially early in growing season than positive control. However, in season B only a weekly application of extracts (early in the season) and also both weekly and bi-weeks application (at the end of season) resulted to higher number of stems per plant than positive control (Table 5.7).

Table 5.7. Effect of application frequency of plant extracts on number of stems per plant in season A and B on various days after planting (DAP)

	Number of stems per plant Season A			Number of stems per plant Season B		
Frequency	**28DAP**	**35DAP**	**42DAP**	**28DAP**	**35DAP**	**42DAP**
Weekly	2.63±0.16[a]	2.83±0.33[a]	3.80±0.80[a]	2.79±0.49[a]	3.03±0.50[a]	3.38±0.36[a]
Bi-weekly	2.39±0.25[a]	2.52±0.23[a]	2.93±0.40[ab]	2.26±0.25[ab]	2.47±0.24[ab]	2.71±0.23[b]
Monthly	2.35±0.30[a]	2.56±0.26[a]	2.65±0.40 [b]	2.15±0.31[ab]	2.63±0.27[ab]	2.65±0.11[bc]
Controls (+; -)						
Copper (+)	1.00±0.00[b]	1.25±0.43[b]	2.69±0.19[ab]	1.97±0.43[b]	2.08±0.52[b]	2.25±0.50[cd]
DMSO (-)	1.16±0.10[b]	1.50±0.06[b]	1.59±0.14[c]	1.56±0.25[b]	1.92±0.17[b]	2.06±0.21[d]
P =0.05	<0.0001	0.0001	0.0239	0.0143	0.0089	0.0002

The values are average of stems (± SD) from plants of each treatment at 28, 35, and 42 days after planting. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2 % Copper) and Negative (1% DMSO) controls. SD: Standard deviation.

Solvent (methanol and water) extracts did not differ in term of stems number per plant in both season A and B along the whole period of observation (from 28 to 42 DAP). Nevertheless, in season A from 28-35 DAP both methanol and water extracts resulted to higher number of stems (at 28 DAP= 2.39 and 2.52; at 35 DAP=2.69 and 2.58) than both negative (1.16 and 1.50) and positive (1.00 and 1.25) controls. At 42 DAP; only methanol extract gave higher stems number (3.48) than negative control (1.59). At this time, no difference in stems number was revealed between solvent extracts and positive control at p 0.05 (Table 5.8). In season B, no difference in stems (p 0.05) was found between solvent extracts and both negative and positive controls at 28 DAP. However, at 35 DAP; methanol extracts gave a higher number of stems per plant (2.69) than negative control (1.92) while water extract (2.64) did not. At 35 DAP, no significant difference (p 0.05) in stems was reported between solvent extracts and positive control. At 42 DAP, both methanol and water extracts resulted to higher number of stems (3.00 and 2.83) than both positive (2.25) and negative (2.06) controls (Table 5.8).

Throughout the experiments A and B no difference in number of stems per plant was observed between methanol and water extracts. However, treatment of potato seeds with these solvent extracts recorded higher number of stems per plant than negative and positive controls early in the season A. Later, only methanol extract gave higher number of stems per plant than negative control. This was the opposite in season B where the difference in stems number between solvent extracts and negative was only observed later during seedling growth from 35 to 42 DAP. Furthermore, methanol extracts gave higher number of stems compared to positive control. Thus, treatment of potato with methanol and water extracts produce higher number of stems per plant than negative control. In addition, methanol extracts also gave many stems than copper oxychloride, a standard pesticide used as positive control (Table 5.8).

Table 5.8. Effect of solvent extracts on number of stems per plant in season A and B on various days after planting (DAP)

Solvent extracts	Number of stems per plant Season A			Number of stems per plant Season B		
	28DAP	**35DAP**	**42DAP**	**28DAP**	**35DAP**	**42DAP**
Methanol	2.39 ± 0.17^{a}	2.69 ± 0.14^{a}	3.48 ± 0.28^{a}	2.35 ± 0.45^{a}	2.69 ± 0.28^{a}	3.00 ± 0.19^{a}
Water	2.52 ± 0.33^{a}	2.58 ± 0.60^{a}	2.87 ± 0.32^{ab}	2.41 ± 0.23^{a}	2.64 ± 0.37^{ab}	2.83 ± 0.30^{a}
Controls (+; -)						
Copper	1.00 ± 0.00^{b}	1.25 ± 0.43^{c}	2.69 ± 0.19^{ab}	1.97 ± 0.43^{a}	2.08 ± 0.52^{ab}	2.25 ± 0.50^{bc}
DMSO	1.16 ± 0.10^{b}	1.50 ± 0.06^{bc}	1.59 ± 0.14^{b}	1.56 ± 0.25^{a}	1.92 ± 0.17^{b}	2.06 ± 0.21^{c}
P =0.05	<0.0001	0.0014	0.0255	0.0650	0.0230	0.0018

The values are average of main stems (± SD) from plants of each treatment at 28, 35, and 42 days after planting. Values within a column followed by the same letter are not significantly different at p 0.05 according to Honestly Significant Difference (HSD) test. (+, -): Positive (2 % Copper oxychloride) and negative (1% DMSO) controls. SD: Standard deviation.

Plant height in season A and B was measured at 28, 56, and 70 DAP. An interaction between seasons and plant extracts, solvent extracts and application frequency was observed at 28 DAP. In addition, an interaction between plant extracts, application frequency, and solvent extracts was confirmed at 56 and 70 DAP (Appendix I). Apart from interaction, effect of those separate factors on plant height of potato seedlings was also evaluated. Consequently, in season A at 28 DAP; tobacco, marigold and garlic extracts produced significantly (p 0.05) taller seedlings (9.65, 9.19, 6.91 cm, respectively) than positive control (3.10 cm) (Table 5.9). However, only tobacco and marigold extracts gave taller plants than negative control at 28 DAP. At the same time, both tobacco and wild marigold showed taller plants than garlic extract at 28 DAP. At 56 DAP, all plant extracts gave higher plant height (32.88, 35.11, and 29.46 cm, respectively) than both negative and positive controls (19.78 and 9.417 cm, respectively). At the same time, marigold extract produced taller plants than garlic but tobacco did not. No significant difference (p 0.05) in plant height was observed between marigold and tobacco extracts at 56 DAP. At 70 DAP, no significant difference was recorded between plant extracts themselves at p 0.05. However, tobacco, marigold and garlic extracts produced taller potato seedlings (60.75, 56.32,

and 54.11 cm, respectively) than both positive (26.5 cm) and negative (41.53 cm) controls (Table 5.9).

In season B, plant extracts had no significant effect on plant height throughout the experiment (from 28 to 70 DAP) at p 0.05. In addition at 28 DAP, no significant difference in plant height was found between plant extracts and positive or negative controls. At 56 DAP, tobacco and garlic extracts gave taller plants (44.64 and 42.56 cm) than positive control (34.62 cm) whereas marigold (40.31 cm) did not. In the same experiment, only tobacco extract resulted to higher plant height than negative control (35.93 cm). At 70 DAP, tobacco and garlic also produced taller seedlings (65.72 and 63.43 cm) than negative control (55.06 cm) but marigold (61.84 cm) did not. Furthermore, tobacco extract alone resulted to higher plant height than positive control (56.17 cm) (Table 5.9). In summary, treatment of potato seedlings with plant extracts resulted to taller plants compared to negative and positive controls. In addition tobacco and marigold extracts gave taller plants than garlic in season A while in season B tobacco and garlic performed better than marigold extract (Table 5.9).

Table 5.9. Effect of plant extracts on plant height (cm) in season A and B on various days after planting (DAP)

Plant extracts	Plant height (cm) Season A			Plant height (cm) Season B		
	28DAP	**56DAP**	**70DAP**	**28DAP**	**56DAP**	**70DAP**
Tobacco	9.65±0.84^{a}	32.88±1.02^{ab}	60.75±1.16^{a}	19.88±2.20^{a}	44.64±1.79^{a}	65.72±3.21^{a}
Marigold	9.19±0.36^{a}	35.11±2.04^{a}	56.32±1.71^{a}	15.44±1.53^{a}	40.31±2.46^{abc}	61.84±2.53^{abc}
Garlic	6.91±0.87^{b}	29.46±1.79^{b}	54.11±3.36^{a}	16.81±2.15^{a}	42.56±3.05^{ab}	63.43±3.78^{ab}
Controls (+; -)						
Copper	3.10±1.06^{c}	9.417±4.63^{d}	26.5±3.90^{c}	14.04±3.98^{a}	34.62±4.96^{c}	56.17±7.97^{bcd}
DMSO	6.86± 0.1^{b}	19.78±1.37^{c}	41.53±4.82^{b}	19.00±4.46^{a}	35.93±3.79^{bc}	55.06±6.18^{dc}
P =0.05	<0.0001	<0.0001	<0.0001	0.2552	0.0391	0.0263

The values are average of plant height (± SD) from plants of each treatment at 28, 56, and 70 days after planting. Values within a column followed by the same letter are not significantly

different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2 % Copper) and Negative (1% DMSO) controls. SD: Standard deviation.

The effect of application frequency on plant height in season A and B was also evaluated. In season A from 28 to 70 DAP, all frequencies resulted to higher plant height than both negative and positive controls at p 0.05. Thus at 28 DAP, weekly, bi-weekly and monthly application gave taller plants (9.56, 8.10, and 8.09 cm, respectively) than both negative (6.86 cm) and positive (3.10 cm) controls. At 56 DAP, also weekly, bi-weekly and monthly application gave taller plants (35.22, 31.17, and 31.05 cm, respectively) than both negative (19.78 cm) and positive (9.417 cm) controls. Finally, at 70 DAP; taller seedlings were also produced by weekly, bi-weekly and monthly application (60.14, 56.40, 54.64 cm, respectively) than negative (41.53 cm) and positive (26.5 cm) controls. The difference in plant height between frequencies was only observed early in the experiment in season A (28 to 56 DAP). Thus, at 28 and 56 DAP; weekly application of extracts (F1) produced taller plants (9.56 and 35.22 cm) than both bi-weekly and monthly application (at 28DAP= 8.10 and 8.09 cm; at 56 DAP= 31.17 and 31.05 cm) (Table 5.10).

In season B, no difference in plant height was observed between frequencies of application at 28 and 56 DAP. However, at 70 DAP; weekly application led to taller plants (68.32 cm) than monthly application (60.26 cm) but there was no significant difference (p 0.05) between weekly and bi-weekly application (Table 5.10). Moreover, frequencies of application did not affect plant height compared to controls at 28 DAP. However, at 56 DAP all application frequencies resulted to taller plants (43.99, 42.65, and 40.87 cm in weekly, bi-weekly and monthly respectively) than positive control (34.62 cm). In addition, at 56 DAP, weekly and bi-weekly led to taller potato plants than negative control (35.93 ± 3.79 cm) but monthly application did not. At 70 DAP, only weekly application of extracts recorded taller plants (68.32 cm) than both positive (56.17 cm) and negative (55.06 cm) controls while bi-weekly and monthly (62.40 and 60.26 cm) did not (Table 5.9). These results confirmed that application of plant extracts weekly, bi-weekly or monthly have a positive response in potato growth in term of plant height. In general, application of extracts weekly gave taller plants among other treatments.

Table 5.10. Effect of application frequency of plant extracts on plant height (cm) in season A and B on various days after planting (DAP)

Frequency	Plant height (cm) Season A			Plant height (cm) Season B		
	28DAP	56DAP	70DAP	28DAP	56DAP	70DAP
Weekly	9.56±0.97^{a}	35.22±1.68^{a}	60.14±0.77^{a}	19.45±2.31^{a}	43.99±1.45^{a}	68.32±4.95^{a}
Bi-weekly	8.10±0.66^{b}	31.17±2.38^{b}	56.40±1.97^{a}	16.76±1.12^{a}	42.65±2.52^{a}	62.40±1.85^{ab}
Monthly	8.09±0.80^{b}	31.05±2.05^{b}	54.64±2.43^{a}	15.93±2.29^{a}	40.87±1.60^{ab}	60.26±3.19^{bc}
Controls (+; -)						
Copper (+)	3.10±1.06^{d}	9.417±4.63^{d}	26.5±3.90^{c}	14.04±3.98^{a}	34.62±4.96^{c}	56.17±7.97^{bc}
DMSO (-)	6.86±0.1^{b}	19.78±1.37^{c}	41.53±4.82^{b}	19.00±4.46^{a}	35.93±3.79^{bc}	55.06±6.18^{bc}
P =0.05	<0.0001	<0.0001	<0.0001	0.3284	0.0173	0.0088

The values are average of plant height (± SD) from plants of each treatment at 28, 56, and 70 days after planting. Values within a column followed by the same letter are not significantly different at p 0.05 according to Honestly Significant Difference (HSD) test. (+, -): Positive (2% Copper) and Negative (1% DMSO) controls. SD: Standard deviation.

Methanol and water extracts did not show any significant difference between them in plant height at p 0.05 either in season A or B. However, treatment of potato tuber with these solvent extracts resulted to taller plants than the controls. At 28DAP in season A, both methanol and water extracts gave taller seedlings (9.33 and 7.83 cm, respectively) than positive control (3.10 cm) at p 0.05 (Table 5.11). In addition, methanol extract resulted to taller plants than negative control (6.86 cm) but water extract did not. From 56 to 70 DAP in season A treatment of potatoes with methanol and water extracts resulted to taller plants (at 56 DAP= 33.19 and 31.78 cm; at 70 DAP= 59.61 and 54.51 cm) than positive (9.417 and 26.5 cm) and negative (19.78 and 41.53 cm) controls (Table 5.11).

In season B, significant difference (p 0.05) in plant height due to solvent extracts was observed from 56 DAP. At that time, both methanol and water extracts gave taller plants (44.16 and 40.84 cm, respectively) than positive control (34.62 cm) and only methanol extract gave taller plants than negative control (35.93 cm). At 70 DAP, only methanol extracts influenced positively the

plant height (65.89 cm) than both positive (56.17 cm) and negative (55.06 cm) controls while water extracts (61.44 cm) did not (Table 5.11). All these results show that methanol and water extracts have a positive effect on plant height of potatoes compared to positive control although there is no significant difference (p 0.05) between them. Overall, methanol extract resulted to taller plants than both negative and positive controls.

Table 5.11. Effect of solvent extracts on plant height (cm) in season A and B on various days after planting (DAP)

Solvent extracts	Plant height (cm) Season A			Plant height (cm) Season B		
	28DAP	**56DAP**	**70DAP**	**28DAP**	**56DAP**	**70DAP**
Methanol	9.33±0.94^{a}	33.19±1.46^{a}	59.61±1.62^{a}	18.48±2.09^{a}	44.16±1.14^{a}	65.89±4.51^{a}
Water	7.83±0.87^{ab}	31.78±1.34^{a}	54.51±0.87^{a}	16.28±1.70^{a}	40.84±0.25^{ab}	61.44±2.24^{ab}
Controls (+; -)						
Copper	3.10± 1.06^{d}	9.42±4.63^{c}	26.5±3.90^{c}	14.04±3.98^{a}	34.62±4.96^{c}	56.17±7.97^{bc}
DMSO	6.86± 0.10^{bc}	19.78±1.37^{b}	41.53±4.82^{b}	19.00±4.46^{a}	35.93±3.79^{bc}	55.06±6.18^{bc}
P =0.05	0.0001	<0.0001	0.0002	0.4076	0.0203	0.0399

The values are average of plant height (± SD) from plants of each treatment at 28, 56, and 70 days after planting. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2% Copper oxychloride) and negative (1% DMSO) controls. SD: Standard deviation.

For field experiments no difference in number of stems per plant was observed between two growing seasons (A and B). However, growing seasons recorded higher number of stems per plant than negative and positive controls during earlier observation (28-35 DAP). At 42 DAP, seasons gave higher number of stems per plant than only negative control (Table 5.11). Plant height was also affected by growing seasons. Thus, throughout the experiments, season B led to taller potato seedlings (17.4, 42.5, and 63.7 cm at 28, 56, and 70 DAP respectivey) than season A (8.6, 32.5, and 57.1 cm at 28, 56, and 70 DAP respectivey). In addition, growing seasons resulted to taller plants than both negative and positive controls at 56 to 70 DAP. At 28 DAP, only season B gave taller plants than positive control (Table 5.12).

Table 5.12. Effect of growing seasons (A and B) on number of stems per plants and plant height

Season	Number of stem per plants			Plant height (cm)		
	28 DAP	35 DAP	42 DAP	28 DAP	56 DAP	70 DAP
A	2.4±0.5[a]	2.6±0.1[a]	2.7±0.3[a]	8.6±4.6[b]	32.5±1.3[b]	57.1±7.9[b]
B	2.4±0.4[a]	2.7±0.4[a]	2.9±0.5[a]	17.4±1.4[a]	42.5±4.8[a]	63.7±4.5[a]
Controls (+; -)						
Copper (+)	1.5±0.1[b]	1.7±0.2[b]	2.5±0.5[ab]	8.6±0.8[b]	22.0±3.9[c]	41.3±0.2[c]
DMSO	1.4±0.4[b]	1.7±0.5[b]	1.8±0.1[b]	12.9±0.9[ab]	27.9±1.3c	48.3±1.1[c]
P =0.05	0.0467	0.0028	0.0341	0.0001	0.0003	0.0209

The values are average of main stems (± SD) from plants of each treatment at 28, 35, and 42 days after planting. Values within a column followed by the same letter are not significantly different at p 0.05 according to Honestly Significant Difference (HSD) test. (+, -): Positive (2 % Copper oxychloride) and negative (1% DMSO) controls. SD: Standard deviation.

In these experiments application of plant extracts gave higher number of stems per plant and taller seedlings than controls. These effects increased with increase of application frequency of of plant extracts. Methanolic and water extract from tobacco applied every week and every two weeks gave higher number of stems per plant than both negative and positive controls. In term of plant height, only methanolic extract from tobacco applied every week led to taller seedlings than other treatments. Methanolic extracts of tobacco applied weekly performed better in terms of plant growth parameters compared to the other treatments. This was followed by methanol extract of wild marigold whereas garlic showed low efficacy among other factors. Field experiments revealed that treatment of potato with plant extracts not only control bacterial wilt but also enhances number of main stems per plant and plant height in comparison to controls. No interaction was observed between test factors in number of stems per plants during field experiment. However, an interaction between season and plant extracts, solvent extracts, application frequency, and season was observed at 28 DAP in plant height. In addition, an

interaction between plant extracts, application frequency, and solvent extracts was confirmed at 56 and 70 DAP. Methanolic extracts of tobacco applied weekly performed better in terms of plant growth parameters compared to the other treatments especially in season B.

The findings of the current study are in agreement with different results from earlier studies. For instance, in a greenhouse experiment carried out to analyse the effect of potato seed treatment with eucalyptus, dill weed, black cumin and spearmint essential oils on growth and yield parameters of potato, it was found that plant extracts resulted to higher number of stems and leaves per plant and taller seedlings in potato compared to negative control (untreated seeds) (Biruk-Masrie *et al.*, 2015). It has also been observed that the delay of emergence and flowering positively affects the general growth of seedlings since plant extracts which caused delayed sprouting and flowering also resulted into higher number of stems and taller plants. In addition, Biruk-Masrie *et al.* (2015) also reported that application of plant extracts with active compounds on growth may suppress the apical dominance and thus leading to production of many sprouts or main stems from a mother tuber. In the present study it was also observed a high number of main stems developed from potato tuber treated with methanolic or water extracts from tobacco when applied frequently. This is in agreement with results of Biruk-Masrie *et al.* (2015).

Song *et al.* (2009) also confirmed that active compounds in a given plant extract cause physical damage to bud apical meristem in tuber, which delays sprouting but triggers the development of axillary buds from the same damaged apical meristem. This delay of sprouting due to active substance may later induce axillary bud outgrowth (Song *et al.*, 2009). This may be an explanation of development of sprouts from axillary buds after loss of apical meristem in tuber treated with plant extracts (Song *et al.*, 2009). In addition, suppression of apical dominance was revealed to be important physiological process used to activate shoot branching or development of many sprouts from tuber (Celis-Gamboa *et al.*, 2003; Song *et al.*, 2009). These studies goes further to support the results of the present studies, potato tubers treated with plant extracts especially tobacco significantly produced many stems as compared to both controls in the current experiment.

In addition, a study carried out by Heena and Swati (2018) on effect of tobacco extracts on germination and plant height of *Trigonella foenum-graecum* also showed that the application of pure tobacco reduced germination rate but germinated seedlings grow taller than untreated ones (normal plant). Tobacco was reported to be a suppressant of seed germination and stimulant of plant growth (Heena & Swati, 2018). In another study it has been shown that application of tobacco extracts at higher concentration delayed seed germination and accelerated plant growth (root and shoot length) of Bengal gram (*Cicer arietinum* L.) compared to control (distilled water) (Mondal *et al.*, 2014). It has also reported that tobacco photochemical is able to trigger the abnormal secretion of Kinetic and indole acetic acid (IAA) which are growth regulator hormones (Mondal *et al.*, 2014).

In the present study, the results are in agreement with (Heena & Swati, 2018; Mondal *et al.*, 2014). The tobacco phytochemicals triggered the abnormal secretion of Kinetic and indole acetic acid (IAA) growth regulator hormones which accelerated plant growth hence taller plants. Positive effect of plant extracts treatment on potato growth was also observed to be frequency-dependent process, the higher the frequency of application, the higher the number of stems and the taller the plant in height as was the case in the present study. Although few studies were reported on frequency of application of plant extracts, similar results were confirmed. A typical example is a field experiment conducted by Istifadah *et al.* (2019) to examine the effect of concentration and frequency of application of mixture of microbial consortium (*B. subtillis, Trichoderma harzianum,* and *Pseudomonas* sp.) with animal manure or compost against soil-borne and air-borne pathogens of potato namely bacterial wilt and late blight. The results revealed that effectiveness of biological control by using manure-microbe mixture is dose-dependent. Thus, high suppressive effect (75%) against the pathogens was found in a weekly application of microbial consortium + manure at 50 g per plant after eight weeks of planting. It was also reported that application of a mixture of animal manure with *B. subtilis* or *Trichoderma harzianum* reduced the rate of the usual dose of NPK in potato production by 50% (Istifadah *et al.*, 2019). Even if the main objective of the study focused on the control of those important diseases of potato, they also evaluated the efficacy of different treatments on plant height. It was observed that two weeks after planting, a weekly application of microbial consortium + manure at 50g per plant (the same control measure that controlled bacterial wilt at the highest level)

affected positively plant height (56.6 cm) compared to the control (43.3 cm). It was concluded that a weekly application of tested control measures improved plant growth than every two weeks application or the control (Istifadah *et al.*, 2019). Mondal *et al.* (2014) also confirmed that bio-active response on *Trigonella foenum-graecum* growth due to tobacco extract reduced gradually as concentration of extract decreased. This supports the results from the present study where the performance of plant extracts on potato growth was high when they were applied frequently.

Furthermore, in the present study, it was also concluded that methanolic extract performed the best in potato growth than water extract. Studies on efficacy of solvent extracts on growth parameters are limited. But, some literatures attribute this difference to the effectiveness of solvent used during bioactive-products' extraction (Alamshahi &Nezhad, 2015; Kukri *et al.*, 2012). Some studies have stated that extraction solvent is one of the major factors that affect the yield and composition of bioactive compounds for each species (Alamshahi & Nezhad, 2015; Arnault *et al.,* 2013; Kukri *et al.*, 2012). Both methanol and water are organic solvents, which are used to extract polar compounds (Cowan, 1999; Mwitari *et al.*, 2013; Ncube *et al.*, 2008). In addition, methanol solvent was reported to be the most powerful in extraction of high range of polar bioactive components from plants (Arnault *et al.*, 2013; Kukri *et al.*, 2012; Malkhan *et al.*, 2012). Therefore, it is evident that methanolic extract contains some different bioactive compounds than water extract. This may explains the best performance of methanolic extracts in plant growth over water extracts in the present study.

5.3.2 Effect of Plant Extracts on Yield Parameters

Effect of plant extracts (tobacco, wild marigold, and garlic), solvent extracts (methanolic and water), and frequency of application (weekly; bi-weekly, and monthly) on yield parameters of potato was evaluated in field season A and B. Examined yield parameters included the number of tubers per plant and total yield per treatment. In both season A and B, no interaction between those three factors was observed in number of tubers per plant. However, an interaction between plant extracts, application frequency, and solvent extracts was observed (Appendix N). Apart from interaction, effect of tested factors or variables on potato yield was evaluated separately.

Thus, in both season A and B no significant difference (p 0.05) in number of tubers per plant was observed between plant extracts or between them and controls (Table 5.13).

Table 5.13. Effect of plant extracts, application frequency, and solvent extracts on number of tubers per plant in season A & B

Plant extracts			Frequency			Solvent extracts		
	Season A	**Season B**		**Season A**	**Season B**		**Season A**	**Season B**
Tobacco	8.89±0.67^{a}	12.53±0.37^{a}	**Weekly**	9.53±0.38^{a}	13.59±0.69^{a}	**Methanol**	8.65±0.06^{a}	12.58±0.22^{a}
Wild marigold	8.47±0.47^{a}	11.00 ±0.43^{a}	**Bi-weekly**	8.63±0.08^{ab}	11.26±0.75^{a}	**Water**	8.35±0.13^{a}	11.18±0.16^{a}
Garlic	8.14±3.02^{a}	12.12 ±1.21^{a}	**Monthly**	7.74±0.44^{abc}	10.79±0.32^{a}			
Controls (+; -)								
Copper (+)	5.83±1.75^{a}	11.58 ±4.35a	**Copper**	5.83±1.75^{c}	11.58 ±4.35^{a}	**Copper**	5.83±1.75^{a}	11.58 ±4.35^{a}
DMSO (-)	6.97±0.47^{a}	11.33 ±3.69a	**DMSO**	6.97±0.47^{bc}	11.33 ±3.69^{a}	**DMSO**	6.97±0.47^{a}	11.33 ±3.69^{a}
P =0.05	0.1755	0.8557	**P =0.05**	0.0435	0.5552	**P =0.05**	0.1880	0.7842

The values are average of number of tubers (± SD) from plant in each treatment. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2% Copper oxychloride) and Negative (1% DMSO) controls. SD: Standard deviation.

Influence of frequency of application of plant extracts on potato yield was also analysed. It was oberved that contrary to season B where it did not affect yield at significant level, in season A, frequency of application increased the number of tubers per plant. Thus, although there was no significant difference (p 0.05) between frequencies of application themselves, they resulted to higher number of tubers than controls. Therefore, a weekly and bi-weekly application of plant extracts led to higher tubers per plant (9.53 and 8.63) than positive control (5.83) at p 0.05. In addition, a weekly application of plant extracts also resulted to higher number of tubers per plant than negative control (6.97) while other frequencies did not (Table 5.13). There was no

significant difference (p 0.05) between solvents in the number of tubers per plant in season A and B. Additionally, no significant difference was observed between solvent extracts and negative or positive control (Table 5.13).

Plant extracts, solvent extracts, and frequency of application were also tested against potato tuber yield in both season A and B. In season A, solvent extracts interacted with frequency of application in total yield of potato whereas no interaction was observed between factors in season B. Although there was no significant effect of plant extracts on potato yield, they positively affected yield of potato than controls in both season A and B. Therefore, in season A all tested plant extracts resulted to higher yield (20.39, 19.64, and 20.34 t ha^{-1} in tobacco, wild marigold, and garlic, respectively) than negative control (6.75 t ha^{-1}) at p 0.05. However, no significant (p 0.05) difference in total yield was observed between plant extracts and positive control in season A (Table 5.14). Furthermore, tobacco, marigold, and garlic extracts gave higher potato yield (27.69, 24.13, and 23.22 t ha^{-1}, respectively) than negative control (5.29 t ha^{-1}) in season B at p 0.05. In addition, both tobacco and wild marigold extracts led to higher yield than positive control (14.22 t ha^{-1}) (Table 5.14).

Table 5.14. Effect of plant extracts, application frequency and solvent extracts on total yield (t ha^{-1}) in season A and B

Plant extracts			**Frequency**			**Solvent extracts**		
	Season A	**Season B**		**Season A**	**Season B**		**Season A**	**Season B**
Tobacco	20.39 ±5.99^{a}	27.69 ±3.39^{a}	**Weekly**	25.32 ±4.67^{a}	27.72 ± 4.67^{a}	**Methanol**	21.26 ± 2.99^{a}	26.33 ±2.58^{a}
Wild marigold	19.64± 3.92^{a}	24.13 ±0.62^{a}	**Bi-weekly**	19.89 ± 2.99^{ab}	24.13 ± 2.79^{a}	**Water**	19.06± 4.22^{ab}	23.70 ± 0.65^{a}
Garlic	20.34± 2.94^{a}	23.22 ± 1.98^{ab}	**Monthly**	15.15 ± 4.36^{bc}	23.19 ± 4.37^{a}			
Controls (+; -)								
Copper (+)	14.03± 7.09^{ab}	14.22 ±1.42^{bc}	**Copper**	14.03± 7.09^{bc}	14.22 ±1.42^{b}	**DMSO**	14.03± 7.09^{bc}	14.22 ±1.42^{b}
DMSO (-)	6.75± 2.60^{c}	5.29 ± 2.00^{c}	**DMSO**	6.75± 2.60^{d}	5.29 ± 2.00^{c}	**P =0.05**	6.75± 2.60^{d}	5.29 ± 2.00^{c}
P =0.05	0.0046	<0.0001	**P =0.05**	0.0008	0.0006	**P =0.05**	0.0037	0.0004

The values are average of yield (t h^{-1}± SD) from plants of each treatment (conversion of weight of tubers harvested from each treatment at t h^{-1}). Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2% Copper oxychloride) and Negative (1% DMSO) controls. SD: Standard deviation.

Effect of frequency of application of plant extracts on potato yield was also determined. In season A, a weekly application of extracts resulted significantly (p 0.05) to higher potato yield (25.32 t ha^{-1}) than monthly application (15.15 t ha^{-1}) but was not significantly different to bi-weekly application (19.89 t ha^{-1}). In addition, all tested frequencies led to higher yield compared to negative control (6.75 t ha^{-1}) while only weekly application gave significantly higher total yield than positive control (14.03 t ha^{-1}) (Table 5.14). In season B, there was no significant difference in yield between all tested frequencies at p 0.05. However, all frequencies significantly (p 0.05) gave higher yield (27.72, 24.13, and 23.19 t ha^{-1} in weekly, bi-weekly, and monthly application, respectively) than both positive (14.22 t ha^{-1}) and negative (5.29 t ha^{-1}) controls (Table 5.14).

For solvent extracts, no significant difference (p 0.05) was observed in yield of potato between methaolic or water extracts in both seasons A or B, although, the extracts gave higher yield than controls in both seasons. In season A both methanol and water extracts led to higher potato yields (21.26 and 19.06 t ha^{-1}, respectively) than negative control (6.75 t ha^{-1}) (Table 5.14). Moreover, methanol extract resulted to higher yield than copper oxychloride used as positive control (14.03 t ha^{-1}). In season B, both methanol and water extracts gave higher yield (26.33 and 23.70 t ha^{-1}) than negative (5.29 t ha^{-1}) and positive (14.22 t ha^{-1}) controls at p 0.05 (Table 5.14).

Effect of growing seasons on yield parameters was also evaluated. During field experiments, no significant difference (p 0.05) was observed in number of tubers per plant between growing seasons A and B. However, season B gave significantly higher total yield (25.0 t ha^{-1}) than season A (20.1 t ha^{-1}) (Table 5.15). Season B also led to higher number of tubers per plant than copper oxychloride used as positive control. Morever, season A and B resulted to higher yield than positive control (14.1 t ha^{-1}) and negative control (6.0 t ha^{-1}) (Table 5.15).

Table 5.15. Effect of growing seasons (A and B) on yield parameters

Season	Number of tubers per plant	Total yield (t ha^{-1})
A	8.4±0.5^{b}	20.1±6.0^{b}
B	11.8±0.6^{a}	25.0±4.7^{a}
Controls (+; -)		
Copper (+)	8.7±0.1^{b}	14.1±1.4^{c}
DMSO (-)	9.2±0.4^{ab}	6.0± 2.0^{d}
P =0.05	0.0437	<0.0001

The values are average of number of tubers (± SD) from plant in each treatment. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2% Copper oxychloride) and Negative (1% DMSO) controls. SD: Standard deviation.

In the present study under field conditions, methanolic and water extracts from tobacco, wild marigold, and garlic, when applied weekly and bi-weekly gave higher number of tubers per plant than both positive and negative controls. In terms of total yield of potato, a weekly application of methanolic extract from tobacco and wild marigold resulted to higher potato yield than negative and positive controls especially in season B. Comparable positive effect of plant extracts on potato yield was reported in different research literatures. Biruk-Masrie *et al.* (2015) conducted a greenhouse experiment to investigate efficacy of tuber treatment with eucalyptus, dill weed, black cumin, and spearmint extracts at different concentrations (45, 90, and 135 mg kg^{-1}) on potato growth and yield. In terms of yield parameters, the results showed that tested plant extracts gave higher tuber number and tuber weight per plant than untreated tubers. In addition, positive effect of extracts on potato yield was dose-dependent since the greatest potential was observed when plant extracts were applied at the highest concentration of 135 mg kg^{-1} (Biruk-Masrie *et al.*, 2015).

In the present study beside tobacco extract, wild marigold (*Tagetes minuta* L.) also showed great potential in potato yield parameters. The same positive effect of *Tagetes minuta* are in agreement with the studies conducted in tomato crops where extract of *Tagetes* minuta and *Tagetes patula*

highly increased number of tomato fruits, enhanced growth and reduced bacterial pathogens than the control under field conditions (Mitali *et al.*, 2012). It was suggested that plant extracts from marigold do not only contain antimicrobial properties but also contain hormones that are required for plant growth and development and can increase the nutrient uptake (Gayatri & Rajani, 2016; Mitali *et al.*, 2012).

Garlic plant extracts in the present study also significantly increased the number of tubers per plant. Similar positive effect of garlic extract on potato productivity was reported in other studies conducted by Dahshan *et al.* (2018). The studies tested the efficacy of plant growth regulators indole-3-butyric acid (IBA) and gibberellic acid (GA3) and garlic, green tea and yeast extracts on potato total yield and tuber quality/chemical composition. For the total yield alone; garlic extract and IBA treatments showed greatest potential. Garlic extracts also significantly increased N and K uptake as well as protein content in potato tubers. The effectiveness of garlic plant extracts in all the investigated parameters could have been due to the bioactive compounds, flavonoids and organo-sulfur compounds found in the plant extract, which are scavengers of plant reactive oxygen species (ROS) and effective compounds in plant defense system (Dahshan *et al.*, 2018).

Biruk-Masrie *et al.* (2015) concluded that application of plant extracts has a positive impact on tuber physiology, which leads to enhanced potato productivity. All these findings concur with the results of the current study, which showed greatest effect of plant extracts on potato yield (number of tubers and total yield) especially in methanolic extract from tobacco and wild marigold applied at high frequency (every week). Although garlic had shown the highest positive response on potato yield than other treatments (Dahshan *et al.*, 2018), however, in the present study it gave the tuber number not significantly different from tobacco and wild marigold. The lower yield produced in garlic treatment than tobacco and wild marigold could be attributed to the high proportion of small tubers recorded in the current study.

Different studies showed a correlation between growth and yield parameters in potato production. It was revealed that plant growth parameters such as shoot branching, plant height, leaf number and structure, as well as flowering time are the key determinants for plant yield

(Gayatri & Rajani, 2016; Jyotirmaya *et al.*, 2016). Plant growth parameters are major components for subsequent plant yield because they determine photosynthetic efficiency and transport of assimilates as well as a source and sink interaction. Consequently, optimization of plant growth may result to enhanced crop yield (Jyotirmaya *et al.*, 2016; Mani & Hannachi, 2015). Celis-Gamboa *et al.* (2003) indicated that the rate of number of stems is the determinant of leaf area, and duration of potato plant cycle, as well as the total number of tubers. Therefore, higher number of stems correlates to production of more tubers (Biruk-Masrie *et al.*, 2015; Celis-Gamboa *et al.*, 2003; Jyotirmaya *et al.*, 2016). Stem number is also associated positively to the tuber number, thus the higher the stem number, the higher the tuber number and potato yield (Celis-Gamboa *et al.*, 2003). The results of the present study are in agreement with Biruk-Masrie *et al.* (2015), Celis-Gamboa *et al.* (2003), Jyotirmaya *et al.* (2016), and Mani and Hannachi (2015). The plant extracts from tobacco and marigold which produced many stems also had increased number of tubers per plant developed from them, increased proportion of bigger sized tubers and finally gave higher yields. The study also showed that the higher production of the number of tubers and yield from treatment with plant extracts especially in season B may be attributed to positive effect of extracts on plant physiology during that period as is reported in the current study.

Furthermore, treatment of crop with plant extracts suppresses apical dominance. Suppression of apical dominance via application of plant extracts induces outgrowth of lateral buds from which arise many and uniform stems (Biruk-Masrie *at al.*, 2015; Mani & Hannachi, 2015). Physiologically, it was reported that tubers with multiple sprouts induce production of strong plant and subsequently high yield. In potato, number of developed tubers correlates to the number of main stems per plant. Thus, higher potato tubers and yields are produced when a plant has many main stems (Biruk-Masrie *et al.*, 2015). The above studies elucidates why significantly increased yields were reported in treatments with plant extracts as compared to the control in the present study (Biruk-Masrie *at al.*, 2015; Mani & Hannachi, 2015).

It was also recorded that tuber weight is highly associated to plant height. Usually, there is plant height required for a given plant to be mature for flower initiation and production of reproductive or storage organs (Biruk-Masrie *et al.*, 2015; Celis-Gamboa *et al.*, 2003). In addition, plant

height was also found to be correlated with number of stems and leaves as well as number of tubers and tuber weight (Biruk-Masrie *et al.*, 2015). Thus, taller plant produces more leaves than dwarf one. Usually, dwarf plant initiate flower buds early which may limit vegetative growth required for development of stems from wich leaves are produced (Biruk-Masrie *et al.*, 2015). This also is a limiting factor for carbohydrates production during photosynthesis which in turns results to low plant productivity.

A correlation between flowering time and subsequent plant productivity was recorded by many authors. For instance, Biruk-Masrie *et al.* (2015) and Celis-Gamboa *et al.* (2003) confirmed that under normal growth conditions, flowering time in potato is associated with tuber initiation. It was also reported that expansion of vegetative growth allows plant to produce more leaves and ultimate high growth and yield rate (Celis-Gamboa *et al.*, 2003). A prolonged vegetative growth and delayed flowering contributes to the accumulation of primary metabolites for potato production (Biruk-Masrie *et al.*, 2015; Celis-Gamboa *et al.*, 2003). This explains why plant extracts which delayed potato flowering also had in turn a significant increase in yields in the present study. Furthermore, any factor which supports production of vigorous sprout and prolonged vegetative growth i.e delay of tuber initiation also has positive effect on number of tubers initiated and tuber bulking (Biruk-Masrie *et al.*, 2015; Celis-Gamboa *et al.*, 2003). The earlier the tuber initiation, the smaller are the plants with low leaf area, earlier senescence and lower potato yield. In contrast, late tuber initiation resulted into larger and vigorous plant, which gives higher yield as was observed in the present study.

It was also confirmed that application of plant extracts delay potato flowering without negative effect on subsequent growth and yield of potato crop (Biruk-Masrie *et al.*, 2015). Therefore, the same treatments that delayed potato flowering also resulted to higher number of tubers and yield per plant in our present study. From all these findings, it was reported that plant extracts had positive response on physiology of potato growth which in turns also affect positively tuber bulking for higher yield. The results of the present studies support the previous findings of other experiments (Biruk-Masrie *et al.*, 2015; Celis-Gamboa *et al.*, 2003; Mani & Hannachi, 2015) whereby plant extracts resulted to higher tuber number and yield than negative and positive

controls especially methanol extracts of tobacco and wild marigold when applied weekly during season B.

5.3.3 Effect of Plant Extracts on Potato Tuber Quality Parameters

Effect of Plant Extracts on Potato Tuber Size

Efficacy of methanolic and water extracts (solvent extracts) from tobacco, wild marigold, and garlic (plant extracts) applied either weekly, bi-weekly or monthly (frequency of application) on tuber quality was evaluated in both season A and B. In both season A and B, no interaction was observed between these factors for tuber size (Appendix O). In season A, no significant effect due to plant extracts between themselves for tuber size (small, medium, large and marketable) at p 0.05 (Table 5.16). However, these plant extracts showed a positive effect on tuber size as compared to control. That is why tobacco, wild marigold, and garlic extracts resulted to reduced proportion of small-sized tubers (9.26, 11.11, and 12.96 %) than both positive (66.67 %) and negative (61.37 %) controls at p 0.05 (Table 5.15). Although there was no significant difference (p 0.05) in medium-sized tubers between plant extracts and control, there was a higher proportion of large and marketable tubers in plant extract treatments than in control. The plant extracts tobacco, marigold, and garlic (58.33, 52.78, and 48.15 % respectively) significantly (p 0.05) had increased proportion of large sized tubers than negative (11.11 %) control (Table 5.16). Furthermore, both tobacco and marigold extracts gave increased proportion of large–sized tubers than the positive (22.22 %) control. In addition, all plant extracts resulted to significantly (p 0.05) increased proportion of marketable tubers (90.74, 89.82, and 87.04 % in tobacco, marigold, and garlic, respectively) than both negative (38.63 %) and positive (33.33 %) controls (Table 5.16).

Table 5.16. Effect of plant extracts on tuber size in season A

Plant extracts	Small (%)	Medium (%)	Large (%)	Marketable (%)
Tobacco	9.26± 9.76^{c}	40.74±5.63^{a}	58.33±16.90^{a}	90.74± 9.76^{a}
Wild marigold	11.11± 12.11^{c}	38.89±9.62^{a}	52.78±10.02^{ab}	89.82±10.52^{a}
Garlic	12.96± 3.21^{c}	36.11±12.11^{a}	48.15±13.70^{abc}	87.04±3.21^{a}
Controls (+; -)				
Copper	66.67± 16.67^{a}	11.11±9.62^{a}	22.22±14.70^{cd}	33.33±16.67^{b}
DMSO	61.37± 9.85^{ab}	27.52±9.75^{a}	11.11 ±5.56^{d}	38.63±5.69^{b}
P =0.05	<0.0001	0.0776	0.0151	<0.0001

The values are proportion (% ± SD) of small, medium, and large (35, >35-75 , >75 g respectively) of total sampled tubers from each treatment. Marketable tuber is average of proportion of medium + large of sampled tubers. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2% Copper oxychloride) and negative (1% DMSO) controls. SD: Standard deviation.

Effect of plant extracts on tuber size in season B was also determined. All plant extracts gave reduced proportion of small-sized tubers (0.00, 1.39, and 2.78 % in tobacco, marigold, and garlic, respectively) compared to both positive (41.67 %) and negative controls (55.56 %) at p 0.05 (Table 5.17). However, no significant difference (p 0.05) in the proportion of medium tubers was detected between plant extracts themselves or between them and controls. Conversely, the proportion of large and marketable tubers, were increased in tobacco, wild marigold and garlic extracts (large= 41.67, 50.00, and 41.67 %; marketable= 100, 98.61, and 97.22 %, respectively) than both positive (large= 16.67 %; marketable= 58.33 %) and negative controls (large= 11; marketable= 44.44 %) at p 0.05 (Table 5.17).

Table 5.17. Effect of plant extracts on tuber size in season B

Plant extracts	Small (%)	Medium (%)	Large (%)	Marketable (%)
Tobacco	0.00±0.00^{b}	58.33±9.62^{a}	41.67±16.67^{a}	100.00±0.00^{a}
Wild marigold	1.39±1.39^{b}	48.61± 8.45^{a}	50.00±7.22^{a}	98.61±1.39^{a}
Garlic	2.78±4.81^{b}	55.56±17.35^{a}	41.67±14.43^{a}	97.22±2.78^{a}
Controls (+; -)				
Copper	41.67±15.28^{a}	41.67±10.41^{a}	16.67±14.43b	58.33±15.28^{b}
DMSO	55.56±33.68^{a}	33.33±22.05^{a}	11.11±19.25b	44.44±19.44^{b}
P =0.05	0.0095	0.3989	0.0256	0.0050

The values are proportion (% ± SD) of small, medium, and large (35, >35-75 , >75 g respectively) of total sampled tubers from each treatment. Marketable tuber is average of proportion of medium + large of sampled tubers. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2% Copper oxychloride) and negative (1% DMSO) controls. SD: Standard deviation.

Frequency of application of plant extracts also affected potato tuber size in season A. For small-sized tubers, the higher proportion was reported in both negative (61.37 %) and positive controls (66.67 %) than in weekly, bi-weekly, and monthly application (2.78, 9.26, and 20.37 %, respectively) at p 0.05 (Table 5.18). No significant difference in proportion of small tubers was found between frequencies themselves at p 0.05. Likewise, no significant difference (p 0.05) was observed between all treatments in the proportion of medium-sized tubers. However, frequency of application of plant extracts influenced the proportion of large and marketable tubers. Thus, weekly and bi-weekly application resulted to higher proportion of large and marketable tubers (large= 67.59 and 53.70 %; marketable= 97.22 and 91.67 % in weekly and bi-weekly application, respectively) than positive (large= 22.22 %; marketable= 33.33 %) and negative control (large= 11.11; marketable= 38.63 %) and positive control at p 0.05 (Table 5.17). No significant difference in proportion of large tubers was assessed between monthly application of extracts and controls. In addition, weekly application of plant extracts led to higher proportion of large and marketable tubers than monthly application at p 005. However, no

significant difference (p 0.05) was found between weekly and bi-weekly or bi-weekly, and monthly application in large or marketable tubers (Table 5.18).

Table 5.18. Effect of application frequency of plant extracts on tuber size in season A

Frequency	**Small (%)**	**Medium (%)**	**Large (%)**	**Marketable (%)**
Weekly	2.78 ± 2.78^{d}	29.63 ± 16.28^{a}	67.59 ± 19.71^{a}	97.22 ± 2.78^{a}
Bi-weekly	9.26 ± 7.91^{cd}	37.04 ± 6.42^{a}	53.70 ± 7.23^{ab}	91.67 ± 6.99^{ab}
Monthly	20.37 ± 5.8^{cd}	40.74 ± 6.99^{a}	38.89 ± 7.35^{bc}	79.63 ± 5.78^{bc}
Controls (+; -)				
Copper	66.67 ± 16.67^{a}	11.11 ± 9.62^{a}	22.22 ± 14.70^{c}	33.33 ± 16.67^{d}
DMSO	61.37 ± 9.85^{ab}	27.52 ± 9.75^{a}	11.11 ± 5.56^{c}	38.63 ± 5.69^{d}
P =0.05	<0.0001	0.0706	0.0111	<0.0001

The values are proportion (% ± SD) of small, medium, and large (35, >35-75 , >75 g respectively) of total sampled tubers from each treatment. Marketable tuber is average of proportion of medium + large of sampled tubers. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2% Copper oxychloride) and negative (1% DMSO) controls. SD: Standard deviation.

Frequency of application of plant extracts also affected potato tuber size in season B. For small-sized tubers, the higher proportion was reported in both negative (55.56 %) and positive controls (41.67 %) than in weekly, bi-weekly, and monthly application (0.00, 0.00, and 4.17 % respectively) at p 0.05 (Table 5.19). No significant difference (p 0.05) in proportion of small tubers between frequencies themselves and between all treatments in term of proportion of medium-sized tubers were observed at p 0.05. However, frequency of application of plant extracts influenced the proportion of large and marketable tubers. Thus, weekly and bi-weekly application resulted to higher proportion of large tubers (63.89 and 40.28 %) than negative control (11.11 %) but monthly application did not (29.17 %) at p 0.05 (Table 5.19). In addition, weekly application of plant extracts led to higher proportion of large tubers than positive control (16.67 %) but bi-weekly or monthly application did not. Furthermore, weekly application resulted to higher proportion of large tubers than monthly application but not than bi-weekly

application of extracts. In terms of marketable tubers, no significant difference (p 0.05) was observed between frequencies. However, all frequencies of application resulted to higher proportion of marketable tubers (100, 100, and 95.83 % in weekly, bi-weekly, and monthly application, respectively) than both negative (44.44 %) and positive controls (58.33 %) at p 0.05 (Table 5.19).

Table 5.19. Effect of application frequency of plant extracts on tuber size in season B

Frequency	Small (%)	Medium (%)	Large (%)	Marketable (%)
Weekly	0.00±0.00^{c}	36.11±13.39^{a}	63.89±7.73^{a}	100.00±0.00^{a}
Bi-weekly	0.00±0.00^{c}	59.72±20.55^{a}	40.28±11.85^{ab}	100.00±0.00^{a}
Monthly	4.17±7.22^{c}	66.67±16.67^{a}	29.17±11.02^{bc}	95.83±7.22^{ab}
Controls (+;)				
Copper	41.67±15.28^{ab}	41.67±10.41^{a}	16.67±14.43^{bc}	58.33±15.28^{c}
DMSO	55.56±33.68^{a}	33.33±22.05^{a}	11.11±19.25^{c}	44.44±19.44^{c}
P =0.05	0.0099	0.1445	0.0096	0.0052

The values are proportion (% ± SD) of small, medium, and large (35, >35-75 , >75 g respectively) of total sampled tubers from each treatment. Marketable tuber is average of proportion of medium + large of sampled tubers. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2% Copper oxychloride) and negative (1% DMSO) controls. SD: Standard deviation.

Solvent extracts (both methanolic and water) were also evaluated to test their effect on potato tuber size in season A (Table 5.20). In this season, no significant difference (p 0.05) in small, medium, large, or marketable tubers was observed between methanolic and water extracts. However, methanol and water extracts resulted significantly to lower proportion of small tubers (11.11 and 11.73 % respectively) than both positive and negative control (66.67 and 61.37 % respectively). No significant different was observed between two solvent extracts and controls in proportion of medium tubers. Nevertheless, methanolic and water extracts led to higher proportion of large tubers (59.26 and 47.53 %) than negative control (11.11 %) at p 0.05. In addition, methanolic extract gave higher proportion of large tubers than positive control (22.22

%). For marketable tubers, both methanolic and water extracts led to high proportion of these tubers (89.506 and 88.272 %) than both negative (38.63 %) and positive (33.33 %) controls (Table 5.20).

Table 5.20. Effect of solvent extracts on tuber size in season A

Solvent extracts	**Small (%)**	**Medium (%)**	**Large (%)**	**Marketable (%)**
Methanol	11.11± 8.49^{b}	29.63±6.68^{a}	59.26±13.35^{a}	89.506± 7.48^{a}
Water	11.73±2.14^{b}	40.74±7.48^{a}	47.53±12.96^{ab}	88.272±2.14^{a}
Controls (+; -)				
Copper	66.67± 16.67^{a}	11.11±9.62^{a}	22.22±14.70^{bc}	33.33±16.67^{b}
DMSO	61.37± 9.85^{a}	27.52±9.75^{a}	11.11±5.56^{c}	38.63±5.69^{b}
P =0.05	0.0001	0.0661	0.0326	<0.0001

The values are proportion (% ± SD) of small, medium, and large (35, >35-75 , >75 g respectively) of total sampled tubers from each treatment. Marketable tuber is average of proportion of medium + large of sampled tubers. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2% Copper oxychloride) and negative (1% DMSO) controls. SD: Standard deviation.

In season B, no significant difference in small, medium, large, or marketable tubers was observed between methanolic and water extracts at p 0.05 (Table 5.21). Furthermore, there was no difference reported between these two solvent extracts and controls in the proportion of medium-sized tubers. A significant difference was found between methanol and water extracts and both negative and positive controls in proportion of small tubers. On the other hand, methanolic and water extracts led to higher proportion of large tubers (50.93 and 37.96 %) than negative control (11.11 %) at p 0.05. In addition, methanolic extract gave higher proportion of large tubers than positive control (16.67 %) while water extract did not. Both methanolic and water extracts led to higher proportion of these marketable tubers (97.22 and 100 %) than negative (44.44 %) and positive (58.33 %) controls (Table 5.21).

Table 5.21. Effect of solvent extracts on tuber size in season B

Solvent extract	Small (%)	Medium (%)	Large (%)	Marketable (%)
Methanol	2.78±4.81^{b}	46.30±12.86^{a}	50.93±10.68^{a}	97.22±2.78^{a}
Water	0.00±0.00^{b}	62.04±9.76^{a}	37.96±5.63^{ab}	100.00±0.00^{a}
Controls (+; -)				
Copper	41.67±15.28^{a}	41.67±10.41^{a}	16.67±14.43^{bc}	58.33±15.28^{b}
DMSO	55.56±33.68^{a}	33.33±22.05^{a}	11.11±19.25^{c}	44.44±19.44^{b}
P =0.05	0.0280	0.3742	0.0388	0.0174

The values are proportion (% ± SD) of small, medium, and large (35, >35-75 , >75 g respectively) of total sampled tubers from each treatment. Marketable tuber is average of proportion of medium + large of sampled tubers. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2% Copper oxychloride) and negative (1% DMSO) controls. SD: Standard deviation.

Effect of growing seasons (A and B) on tuber size was also analysed. Season B resulted to significicantly lower proportion of small-sized tubers (1.4 %) than season A (10.8 %) at p 0.05 (Table 5.22). Furthermore, season B gave higher propotion of medium and marktable tubers (medium= 54.2 %, marketable= 98.6 %) than season A (medium= 35.8 %, marketable= 89.5 %). On the other hand, season A and B led to lower proportion of small tubers than negative control and positive controls at p 0.05. In addition, season A and B gave higher proportion of large and marketable tubers than positive control and negative controls (Table 5.22).

Table 5.22. Effect of growing seasons (A and B) on tuber size

Season	Small (%)	Medium (%)	Large (%)	Marketable (%)
A	10.8±19.3^{b}	35.8±5.6^{b}	53.7±13.4^{a}	89.5± 8.4^{b}
B	1.4±4.8^{c}	54.2±10.7^{a}	44.4±12.9^{b}	98.6±7.4^{a}
Conrtols (+; -)				
Copper (+)	54.2±10.6^{a}	26.4±9.8^{c}	19.5±6.7^{c}	45.8±7.5^{c}
DMSO (-)	58.5±15.3^{a}	30.4±6.7^{b}	11.1±5.6^{c}	41.5±5.7^{c}
P =0.05	0.0002	<.0001	0.0054	0.0002

The values are proportion (% ± SD) of small, medium, and large (35, >35-75 , >75 g respectively) of total sampled tubers from each treatment. Marketable tuber is average of proportion of medium + large of sampled tubers. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2% Copper oxychloride) and negative (1% DMSO) controls. SD: Standard deviation.

In brief, for both seasons A and B, the three plant extracts (tobacco, wild marigold, and garlic) showed significantly reduced proportion of small tubers than both negative and positive controls. Season B gave lower proportion of small tubers than seasonA. In addition, all treatments gave increased proportion of marketable tubers compared to both negative and positive controls. Season B also gave higher proportion of marketable tubers than season A. Furthermore, a weekly application of plant extracts led to higher proportion of marketable tubers than both the negative and positive controls. Therefore, methanol and water extracts from tobacco, wild marigold, and garlic significantly increased the production of higher proportion of marketable tubers when applied weekly especially in season B.

Comparable positive effect of plant extracts application on potato tuber size was previously observed in other studies. For instance, Biruk-Masrie *et al.* (2015) reported that proportion of potato tuber size was affected by treating potatoes with different plant extracts. Black cumin extract resulted to a higher number of small tubers whereas dill weed extracts and negative control gave the lowest proportion of small tubers. The results of the above study are in

agreement with the present study where treatments with the three extracts gave the lowest proportion of small potato tubers and a positive correlation between number of tubers per plant and tuber size was recorded. Furthermore it was observed that increased number of tubers may be associated with increased number of small tubers. This could be explained by the principle of source: sink interaction whereby higher number of tubers per plant compete for photosynthetic assimilates and therefore some do not develop properly (Celis-Gamboa *et al.*, 2003; Jyotirmaya *et al.*, 2016).

However, this was not always the case because in the same experiment, the same dill weed extract which produced higher number of tubers per plant and higher potato yield also showed the lowest proportion of small tubers (Biruk-Masrie *et al.,* 2015). These observations were similar to those of the current study where a weekly application of plant extracts resulted to higher number of tubers and also high proportion of marketable tubers at the expense of small tubers. In this case, plant extracts that resulted to numerous stems also gave lower proportion of small tubers, and ultimately higher proportion of medium and large-sized (marketable) tubers. However, in the previous study application of some plant extracts like cumin resulted to higher proportion of small tubers than control (Biruk-Masrie *et al.,* 2015). These results are contrary to the current study where higher proportion of small tubers was found in potatoes treated with DMSO (negative control) than in all tested plant extracts' treatments. In the present study, plant extracts that resulted to more tuber number per plant also gave lower proportion of small tubers, and ultimately higher proportion of medium and large-sized (marketable) tubers. This may be because beside the effect of plant extracts on growth and production, this current study was also dealing with the control of potato bacterial wilt which was not the case for Biruk-Masrie *et al.* (2015). Therefore, it would not be surprising if the plant extract, solvent extract, frequency application, and growing season which were able to manage potato bacterial wilt, are also the best performing in increasing number of stems and tubers, and tuber size as well as yield and quality of potato crop. Production of higher proportion of marketable (medium plus large) tubers in response to potato treatment with plant extracts may be due to optimization of early growth stages like number of stems, plant height and delayed flowering which allowed maximization of photosynthates production from proper tuber bulking in combination with resistance against potato bacterial wilt.

Effect of Plant Extracts on Dry Matter (DM), Specific Sravity (SG), and Reducing Sugar (RS) content of Potato Tuber

In addition to tuber size, dry matter (DM) content was another parameter, which was analysed to evaluate whether it was affected by treatment of potatoes with methanol or water extracts of tobacco, wild marigold or garlic and applied either weekly, bi-weekly or monthly. In both season A and B, there was no interaction between these factors on dry matter content of potato (Appendix O). Separate analysis of the factors for their effect on DM content of potato tubers, revealed that there was no significant difference (p 0.05) in DM content between tobacco, marigold, and garlic extracts in season A. However, in season B, tobacco extracts resulted into higher DM content (23.65 %) than garlic (21.78 %) but not wild marigold (22.55 %) at p 0.05 (Table 5.23). In both seasons A and B the plant extracts also gave higher DM in tubers in comparison to controls. Therefore, in season A, tobacco and wild marigold resulted into higher DM content in tubers (22.59 and 22.07 %) than negative control (19.05 %) even though garlic extract (21.35 %) did not. Moreover in season B, all tested plant extracts led to higher DM (23.65, 22.55, and 21.78 % in tobacco, marigold, and garlic, respectively) than both negative and positive controls (18.77 and 18.98 %) (Table 5.23).

Table 5.23. Effect of plant extracts, application frequency and solvent extracts on potato dry matter (DM %) content in season A and B

Plant extracts			Frequency			Solvent extract		
	Season A	**Season B**		**Season A**	**Season B**		**Season A**	**Season B**
Tobacco	22.59 ± 1.45^{a}	23.65 ± 1.09^{a}	**Weekly**	22.95 ± 2.24^{a}	24.61 ± 0.78^{a}	**Methanol**	23.27 ± 0.46^{a}	23.89 ± 0.47^{a}
Wild marigold	22.07 ± 0.55^{a}	22.55 ± 0.12^{ab}	**Bi-weekly**	22.25 ± 1.01^{ab}	21.71 ± 0.70^{b}	**Water**	20.74 ± 1.51^{b}	21.43 ± 0.03^{b}
Garlic	21.35 ± 0.48^{ab}	21.78 ± 1.18^{b}	**Monthly**	20.82 ± 0.89^{ab}	21.66 ± 0.34^{b}	**-**	**-**	**-**
Controls (+; -)								
Copper	20.79 ± 1.67^{ab}	18.98 ± 1.44^{c}	**Copper**	20.79 ± 1.67^{ab}	18.98 ± 1.44^{c}	**Copper**	20.79 ± 1.67^{b}	18.98 ± 1.44^{c}
DMSO	19.05 ± 0.61^{b}	18.77 ± 0.61^{c}	**DMSO**	19.05 ± 0.61^{b}	18.77 ± 0.61^{c}	**DMSO**	19.05 ± 0.61^{b}	18.77 ± 0.61^{c}
P =0.05	0.0193	0.0003	**P =0.05**	0.0441	0.0001	**P =0.05**	0.0214	0.0005

The values are average of DM (% ± SD) of total sampled tubers from each treatment. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2 % Copper oxychloride) and Negative (1% DMSO) controls. SD: Standard deviation.

Effect of frequency of application of plant extracts on DM content was also evaluated in potato tubers grown in season A and B. In season A, there was no significant difference (p 0.050) in DM content in tubers treated with plant extracts weekly, bi-weekly and monthly at p 0.05 (Table 5.23). However, in season B, weekly application led to higher DM content (24.61 %) in tubers than both bi-weekly and monthly application (21.71 and 21.66 %). In addition, all three tested frequencies of application gave higher DM content than (24.61, 21.71, and 21.66 % in weekly, bi-weekly and monthly application respectively) than both negative and positive controls (18.77 and 18.98 %). In season A, only weekly application of plant extracts showed significantly (p 0.05) higher DM content than negative control (Table 5.23).

Dry matter (DM) content in tuber due to treatment of potato with methanolic or water extracts was also evaluated for season A and B. In both seasons A and B, methanolic extract resulted to higher DM content (season A= 23.27 and season B= 23.89 %) in tubers than water extract (season A= 20.74 and season B= 21.43 %) at p 0.05 (Table 5.23). Additionally, only methanolic extract gave higher DM content than both positive (20.79 %) and negative controls (19.05 %) in season A while in season B both solvent extracts gave higher DM than controls (negative= 18.98 % and positive= 18.77 %) at p 0.05 (Table 5.23).

Apart from DM content, another quality parameter, which was evaluated from season A and B was specific gravity (SG). An interaction between the plant extracts and seasons as well as between plant extracts and application frequency on SG was assessed. Besides interaction, effect of independent variables on SG were also tested separately in both season A and B. In season A, tobacco extract resulted to higher SG (1.123) followed by both marigold and garlic (both at 1.103) at p 0.05 (Table 5.24). Moreover, both tobacco and marigold extracts gave higher SG than garlic extract in season B. In addition, all tested plant extracts led to higher SG (season A= 1.123, 1.103, and 1.103; season B=1.139, 1.137, and 1.113 in tobacco, marigold and garlic, respectively) than both negative (A= 1.048; B= 1.060) and positive controls (A=1 .081 and B= 1.064) at p 0.05 in both season A and B (Table 5.24).

Table 5.24. Effect of plant extracts, application frequency and solvent extracts on specific gravity (SG) of potato in season A and B

Plant extracts			Frequency			Solvent extracts		
	Season A	**Season B**		**Season A**	**Season B**		**Season A**	**Season B**
Tobacco	1.123±0.02^{a}	1.139±0.01^{a}	**Weekly**	1.122±0.03^{a}	1.142±0.03^{a}	**Methanol**	1.113±0.013^{a}	1.139±0.02^{a}
Wild marigold	1.103±0.01^{b}	1.137±0.01^{a}	**Bi-weekly**	1.104±0.01^{b}	1.130±0.01^{b}	**Water**	1.105±0.011^{a}	1.121±0.01^{b}
Garlic	1.103±0.01^{b}	1.113±0.02^{b}	**Monthly**	1.102±0.02^{b}	1.118±0.01^{c}	**-**	**-**	**-**
Controls (+; -)								
Copper	1.081±0.02^{c}	1.064±0.03^{c}	**Copper**	1.081±0.02^{c}	1.064±0.03^{d}	**Copper**	1.081±0.02^{b}	1.064±0.03^{c}
DMSO	1.048±0.01^{d}	1.060±0.01^{c}	**DMSO**	1.048±0.01^{d}	1.060±0.01^{d}	**DMSO**	1.048±0.01^{c}	1.060±0.01^{c}
P =0.05	<0.0001	<0.0001	**P =0.05**	<0.0001	<0.0001	**P =0.05**	<0.0001	<0.0001

The values are average of SG ± SD of total sampled tubers from each treatment. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2 % Copper oxychloride) and negative (1% DMSO) controls. SD: Standard deviation.

Effect of frequency of application of plant extracts on specific gravity in potato tubers was also determined. In season A, a weekly application resulted to higher SG followed by both bi-weekly and monthly application of plant extracts at p 0.05. In season B, weekly application gave higher SG followed by bi-weekly whereas monthly application resulted to the lowest SG. In addition, weekly, bi-weekly and monthly application of plant extracts showed higher SG in season A (1.122, 1.104, and 1.102, respectively) than both negative (1.048) and positive (1.081) controls. The results were similar in season B, where weekly, bi-weekly and monthly applications also gave higher SG (1.142, 1.130, and 1.118, respectively) than both negative (1.060) and positive controls (1.064) at p 0.05 (Table 5.24).

Solvent (methanolic and water) extracts were also assessed to examine their effect on SG in potato tubers. In season A, there was no significant difference (p 0.05) in SG between mathanolic and water extracts. However, mathanolic extract gave higher SG (1.139) than water extract (1.121) in season B. Moreover, both methanol and water extracts showed higher SG (A= 1.113 and 1.105; B= 1.139 and 1.121) compared to both negative (A= 1.048 and B= 1.060) and positive controls (A= 1.081 and B= 1.064) at p 0.05 in both season A and B (Table 5.24).

Reducing sugars (RS) content in potatato tubers was also another quality parameter which was evaluated in season A and B. In both seasons A and B, no interaction was detected between plant extracts, solvent extracts and frequencies of application. Thus, the factors were evaluated separately for their effectiveness on reducing sugars' content in potato tubers. In both seasons A and B, there was no significant difference (p 0.05) in RS between three tested plant extracts at p 0.05. However, in season A tobacco, wild marigold and garlic extracts resulted to lower RS (0.209, 0.247, and 0.314 %, respectively) than negative control (0.525 %) at p 0.05 (Table 5.25). In addition, only tobacco extract gave lower RS content (0.209 %) than positive control (0.403 %). In season B, both tobacco and wild marigold led to lower RS (0.133 and 0.147 %) than both negative (0.321 %) and positive (0.347 %) controls while garlic (0.159 %) did not (Table 5.25).

Table 5.25. Effect of plant extracts, application frequency and solvent extracts on reducing sugars (RS %) content of potato in season A and B

Plant extracts			Frequency			Solvent extracts		
	Season A	Season B		Season A	Season B		Season A	Season B
Tobacco	0.209±0.08^{c}	0.133±0.02^{b}	**Weekly**	0.205±0.04^{d}	0.129±0.03^{b}	**Methanol**	0.235±0.05^{b}	0.129±0.01^{c}
Wild marigold	0.247±0.02^{bc}	0.147±0.02^{b}	**Bi-weekly**	0.255±0.03^{cd}	0.159±0.03^{b}	**Water**	0.250±0.01^{b}	0.165±0.02^{bc}
Garlic	0.314±0.08^{bc}	0.159±0.03^{ab}	**Monthly**	0.269±0.03^{bcd}	0.151±0.02^{b}			
Controls (+; -)								
Copper (+)	0.403±0.07^{ab}	0.347±0.04^{a}	**Copper**	0.403±0.07^{ab}	0.347±0.04^{a}	**Copper**	0.403±0.07^{ab}	0.346±0.04^{a}
DMSO (-)	0.525±0.13^{a}	0.321±0.10^{a}	**DMSO**	0.525±0.13^{a}	0.321±0.10^{a}	**DMSO**	0.525±0.13^{a}	0.321±0.10^{a}
P =0.05	0.0289	0.0083	**P =0.05**	0.0039	0.0088	**P =0.05**	0.0129	0.0202

The values are average of RS ± SD of total sampled tubers from each treatment. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2 % Copper oxychloride) and negative (1% DMSO) controls. SD: Standard deviation.

For the three tested frequencies of application, there was no significant difference (p 0.05) between them in RS content in potato in seasons A and B. However, frequency of application affected positively RS content in potato tubers in both seasons A and B. Thus in season A, all tested frequencies resulted to lower RS (0.205, 0.205, and 0.269 % in weekly, bi-weekly, and monthly application respectively) content than negative control (0.525 %) at p 0.05. In the same experiment, weekly and bi-weekly application of extracts led to RS even lower than positive control (0.403 %) whereas monthly application did not. In season B, all frequencies of application resulted to lower RS (0.129, 0.159, and 0.151 % in weekly, bi-weekly, and monthly application respectively) than both negative (0.321 %) and positive (0.347 %) controls (Table 5.25).

Reducing sugars' content was also analysed in potato tubers treated with methanolic or water extracts in both season A and B. In seasons A and B, there was no significant difference (p 0.05) in RS between solvent extracts. However, solvent extracts affected RS content in tubers in comparison to controls. This was observed in season A where both methanolic and water extracts resulted to lower RS (0.235 and 0.250 %) than negative control (0.525 %) at p 0.05. In season B, methanolic and water extracts gave lower RS (0.129 and 0.165 %) than negative (0.321 %) and positive controls (0.346 %) at p 0.05 (Table 5.25).

Effect of growing season on tuber quality parameters was also evaluated. From the results, no significant effect of seasons was observed in tuber DM content while both seasons resulted to higher tuber DM content (A= 22.01 %; B= 22.66 %) than both positive (19.89 %) and negative (18.91 %) controls at p 0.05 (Table 5.26). Futhermore, growing season B resulted to higher tuber SG content (1.13) than season A (1.11). Similar effect was also observed in tuber RS content where season B also gave tubers containing lower RS (0.15 %) than season A (0.24 %). In addition, both seasons A and B resulted to higher SG and lower RS than both positive and negative controls at p 0.05 (Table 5.26).

Table 5.26. Effect of growing seasons (A and B) on dry matter (DM), specific gravity (SG), and reducing sugar (RS) content of potato tuber

Season	DM (%)	SG	RS (%)
A	22.01±0.55[a]	1.11±0.02[b]	0.24±0.03[c]
B	22.66±1.45[a]	1.13±0.01[a]	0.15±0.02[d]
Controls (+;-)			
Copper (+)	19.89±0.61[b]	1.07±0.03[c]	0.38±0.04[b]
DMSO (-)	18.91±1.44[b]	1.05±0.01[c]	0.42±0.07[a]
P =0.05	0.0427	<.0001	<.0001

The values are average of DM, SG, and RS (± SD) of total sampled tubers from each treatment. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2 % Copper oxychloride) and Negative (1% DMSO) controls. SD: Standard deviation.

In this present study, a weekly application of methanolic extract from tobacco and wild marigold gave potato tuber with high dry matter (DM) and specific gravity (SG) contents especially in season B. In addition, application of methanolic and water extracts from tobacco weekly and bi-weekly resulted to lower reducing sugar content specially in season B. In summary, a weekly application of methanolic extract from tobacco at 50 mg mL^{-1} led to tubers of high quality among the tested treatments particularly in season B. Studies by different researchers (Abong *et al.*, 2010; Giri *et al.*, 2020; Kabira & Lemaga, 2003; Ooko & Kabira, 2011) have confirmed that beside tuber size, other potato qualities which are considered for tuber marketability and acceptance for processing industries are tuber dry matter (DM), specific gravity (SG), and reducing sugars (RS) content. In the current study, a weekly application of methanolic extract from tobacco at 50 mg mL^{-1} exclusively in season B led to tubers with high DM and SG content together with low RS content, these quality standards required for potato by processing industries. Furthermore, the standards required for potato processing reported by different authors are high dry matter, specific gravity and low reducing sugars (DM 20% SG 1.080 and RS 0.25) respectively for crisps and French fries production (Abong *et al.*, 2010; Kabira & Lemaga, 2003; Muhammad *et al.*, 2012; Ooko & Kabira, 2011). It has been revealed that potato

tubers with high dry matter and specific gravity suck up a smaller amount of oil and provide high yield of final products from processing (Ooko & Kabira, 2011). Higher RS content results to dark brown color while low level of RS leads to a desirable golden brown color (Abong *et al.*, 2010; Ooko & Kabira, 2011). The results of the present study are in full agreement with the standards recommended above for potato processing. The DM content, SG, and RS from the treatment with plant extracts resulted in potato with high quality attributes for processing and other uses in Rwanda. High tuber quality in potato treated by plant extracts in season B in the present study could be attributed to the positive effects on plant growth parameters during field experiments.

5.3.4 Effect of Plant Extracts on Control of DI and their BCE in Potato Plants and Tubers

Efficacy of two solvent extracts of three plant materials at the three different frequencies of application on the control of disease incidence (DI) of bacterial wilt in potato plants grown under field conditions (Season A and B) was evaluated at various days after inoculation (DAI). During the whole period of observation, there was no interactions observed between these factors on disease incidence in potato plants (Appendix P). Therefore, the effect of these independent variables on DI were tested separately. In season A, DI was evaluated from 10 (when the first symptoms of the disease appeared) to 50 DAI (when almost all plant in controls wilted and died). From 10 to 30 DAI, there was no significant difference (p 0.05) in DI between plant extracts but this changed from 40 to 50 DAI (Table 5.27). At 40 DAI; both tobacco and wild marigold reduced DI by (37.65 and 39.53 %) of potato bacterial by a higher percentage than garlic extract (48.63 %) at p 0.05. At 50 DAI, tobacco extract gave reduced DI (39.45 %) than garlic extract (51.13 %) but not than wild marigold (45.12 %). Garlic and wild marigold extracts did not differ between them in DI in potato at 50 DAI (Table 5.27). Plant extracts also had higher activity against the pathogen than controls during season B. At 10 DAI, extracts from all selected plant extracts reduced DI by (1.24, 1.88, and 2.60 % in tobacco, marigold, and garlic respectively) in potatoes than negative control (8.43 %) even if it was not the same with positive control at p 0.05. At 20 DAI, all the extracts reduced DI by (12.82, 16.56, and 20.18 %, respectively) more than both negative and positive controls (44.55 and 36.75 %). From 30 to 50 DAI, plant extracts also reduced DI more than negative control and positive controls at p 0.05 (Table 5.27).

Table 5.27. Effect of plant extracts on disease incidence (DI %) in potato in season A on various days after inoculation (DAI)

Plant extracts	10DAI	20DAI	30DAI	40DAI	50DAI
Tobacco	1.24 ± 0.27^{b}	12.82 ± 3.21^{b}	28.29 ± 9.56^{b}	37.65 ± 9.24^{c}	39.45 ± 4.77^{c}
Marigold	1.88 ± 0.75^{b}	16.56 ± 4.29^{b}	35.15 ± 4.83^{b}	39.53 ± 4.17^{c}	45.12 ± 4.40^{bc}
Garlic	2.60 ± 0.44^{b}	20.18 ± 3.32^{b}	36.15 ± 3.84^{b}	48.63 ± 2.67^{b}	51.13 ± 4.95^{b}
Controls (+; -)					
Copper	3.79 ± 1.57^{ab}	36.75 ± 3.55^{a}	61.06 ± 10.43^{a}	90.11 ± 3.12^{a}	95.71 ± 2.710^{a}
DMSO	8.43 ± 3.32^{a}	44.55 ± 9.20^{a}	83.97 ± 10.71^{a}	95.71 ± 3.86^{a}	99.54 ± 0.32^{a}
P =0.05	0.0081	<0.0001	0.0002	<0.0001	<0.0001

The values of DI (% ± SD) are a percentage of diseased plants over total sampled plants from each treatment. Values within a column followed by the same letter are not significantly different at p 0.05 according to Turkey's Honestly Significant Difference (HSD) test. (+, -): Positive (2 % Copper oxychloride) and negative (1% DMSO) controls. SD: Standard deviation.

Biological Control Efficacy (BCE) of solvents (methanol and water) from three plant extracts (tobacco, wild marigold, and garlic), applied at three different frequencies (weekly, bi-weekly and monthly application) was determined in season A. At 10 and 30 DAI, plant extracts did not show significant difference (p 0.05) in BCE between them (Table 5.28). However, along the season, all solvent extracts reduced significantly DI as compared to negative control. In addition, plant extracts also reduced DI at significant level of BCE higher than positive control throughout the season except at 10 DAI. Apart from these potential BCE of plant extracts as compared to controls; BCE of plant extracts also differed between themselves at 20; 40; and 50 DAI. Therefore, at 20 DAI; tobacco extract reduced DI at higher BCE (71.33 and 44.73 % as compared to negative and positive controls) than garlic (56.86 and 30.26 % as compared to negative and positive controls) but not more than marigold (62.96 and 36.36 % as compared to negative and positive controls) at p 0.05. At 40 DAI both tobacco and marigold had higher BCE (60.77 and 57.66 % as compared to negative control and 55.06 and 51.95 % as compared to negative and positive controls) than garlic extract (BCE= 49.21 and 43.50 % as compared to negative and positive controls). Finally, at 50 DAI tobacco extract also reduced DI with the

highest BCE (60.34 and 56.49 % as compared to negative and positive controls) than garlic (48.62 and 44.77 % as compared to negative and positive controls) but not more than wild marigold (54.66 and 50.81 % as compared to negative and positive controls) at p 0.05 (Table 5.28).

Table 5.28. BCE (%) of plant extracts against potato bacterial wilt in season A on various days after inoculation (DAI)

Plant extracts	10DAI	20DAI	30DAI	40DAI	50DAI
Tobacco	84.40±5.79^{a}	71.33±4.50^{a}	66.37±3.93^{a}	60.77±3.91^{a}	60.34±3.33^{a}
Marigold	74.67±8.33^{a}	62.96±4.22^{ab}	58.67±3.38^{a}	57.66±3.41^{a}	54.66±3.24^{ab}
Garlic	65.54±7.32^{a}	56.86±2.99^{b}	54.34±4.92^{ab}	49.21±1.82^{b}	48.62±3.56^{b}
Controls (+; -)					
Copper	44.37±23.61^{ab}	26.60±7.99^{c}	16.16±5.97^{c}	5.71±3.53^{c}	3.85±2.69^{c}
DMSO	0.00±0.00^{b}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{c}	0.00±0.00^{c}
P =0.05	0.0016	<0.0001	<0.0001	<0.0001	<0.0001

BCE= [(DI in the control-DI in treatment)/ DI in the control]*100. Positive control (2% copper oxychloride) and negative (1% DMSO) controls. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. SD: Standard deviation.

Frequency of application of plant extracts also affected the DI in potato plants in season A from 10 to 50 DAI. Although therefore was no significant difference (p 0.05) in DI between tested frequencies of application at 10 and 30 DAI, but the difference between frequencies was clear at 20, 40 and 50 DAI. At 20 DAI, weekly application resulted to lower DI (13.62 %) than monthly application (20.19 %) although it did not differ from bi-weekly application (15.76 %). At 40 DAI, both weekly and bi-weekly application reduced DI (39.02 and 36.71 %) more than monthly application (50.09 %). At the end of the observation, weekly application resulted once again to more reduced DI (41.52 %) than monthly application (51.83 %) even though it did not have significant difference with bi-weekly application (42.40 %) at p 0.05 (Table 5.29).

Tested frequencies of application of plant extracts also reduced DI in potato compared to the controls in season A from 10 to 50 DAI as showed by reduced DI in weekly, bi-weekly, and monthly application (1.21, 1.97, and 2.55 %, respectively) compared to negative control (8.43 %) at 10 DAI. In addition, at 20 DAI weekly, bi-weekly, and monthly application led to significantly reduced DI (13.62, 15.76, and 20.19 %, respectively) more than both negative and positive controls (44.55 and 36.75 %) at p 0.05. Similar result was observed at 30, 40 and 50 DAI where weekly, bi-weekly, and monthly application of extracts reduced the incidence of bacterial wilt at significant rate than both negative and positive controls (Table 5.29).

Table 5.29. Effect of application frequency of plant extracts on disease incidence (DI %) in potato in season A on various days after inoculation (DAI)

Frequency	10DAI	20DAI	30DAI	40DAI	50DAI
Weekly	1.21±0.66^{b}	13.62±3.04^{d}	29.52±9.18^{c}	39.02±6.68^{c}	41.52±5.11^{c}
Bi-weekly	1.97±1.08^{b}	15.76±4.47^{cd}	31.51±4.58^{c}	36.71±5.04^{c}	42.40±3.66^{bc}
Monthly	2.55±0.21^{b}	20.19±4.13^{c}	38.56±5.94^{c}	50.09±3.86^{b}	51.83±5.44^{b}
Controls (+; -)					
Copper	3.79±1.57^{ab}	36.75±3.55^{b}	61.06±10.43^{b}	90.11±3.12^{a}	95.71±2.71^{a}
DMSO	8.43±3.32^{a}	44.55±9.20^{a}	83.97±10.71^{a}	95.71±3.86^{a}	99.54±0.32^{a}
P =0.05	0.0097	<0.0001	0.0002	<0.0001	<0.0001

The values of DI (% ± SD) are a percentage of diseased plants over total sampled plants from each treatment. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2% copper oxychloride) and negative (1% DMSO) controls. SD: Standard deviation.

BCE of frequencies of application of plant extracts against DI in potato plants was tested in season A from 10 to 50 DAI. Early in the experiment (10 to 20 DAI), there was no significant difference (p 0.05) in BCE between frequencies of application. However, these frequencies reduced DI in potato plants at BCE of 86.25, 72.48, and 65.88 % in weekly, bi-weekly, and monthly application, respectively as compared to negative control and BCE of 41.88, 28.11, and 21.1 %, respectively as compared to positive control at 10 DAI. At 20 DAI, weekly, bi-weekly, and monthly application were able to protect potato plant against *R. solanacearum* at BCE of

69.42, 65.00, and 54.21 %, respectively as compared to negative control and BCE of 42.82, 38.40, and 27.61 %, respectively as compared to positive control (Table 5.30). Additionally, the difference in BCE between frequencies was also expressed from 30 to 50 DAI in season A. At 30 DAI, weekly application controlled bacterial wilting at BCE of 65.00 and 48.84 % as compared to negative and positive controls and higher than monthly application (53.36 and 37.20 %% as compared to negative and positive controls) although it was not higher than bi-weekly application (62.53 and 46.37 % as compared to negative and positive controls). At 40 and 50 DAI, both weekly and bi-weekly resulted to significantly higher BCE than monthly application at p 0.05 (Table 5.30).

Table 5.30. BCE (%) of application frequency of plant extracts against potato bacterial wilt in season A on various days after inoculation (DAI)

Frequency	**10DAI**	**20DAI**	**30DAI**	**40DAI**	**50DAI**
Weekly	86.25±3.55$_{a}$	69.42±4.33^{a}	65.00±3.31^{a}	59.29±3.48^{a}	58.28±3.37^{a}
Bi-weekly	72.48±7.38^{a}	65.00±4.73^{a}	62.53±3.15^{ab}	61.75±2.88^{a}	57.40±2.76^{a}
Monthly	65.88±9.34^{a}	54.21±4.75^{a}	53.36±3.73^{b}	47.93±3.94^{b}	47.62±2.72^{b}
Controls (+; -)					
Copper	44.37±23.61^{ab}	26.60±7.99^{c}	16.16±5.97^{c}	5.71±3.53^{c}	3.85±2.69^{c}
DMSO	0.00±0.00^{b}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{c}	0.00±0.00^{c}
P =0.05	0.0026	<0.0001	0.0001	<0.0001	<0.0001

BCE= [(DI in the control-DI in treatment)/ DI in the control]*100. Positive control (2% copper oxychloride) and negative (1% DMSO) controls. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. SD: Standard deviation.

Solvent extracts also affected the level of DI in potato plants. Although there was no significant difference in DI between methanolic and water extracts in season A, they induced a reduction of DI compared to controls at p 0.05. A 10 DAI both methanolic and water plant extracts showed reduced DI (1.97 and 1.84 %) than negative control (8.43 %) but not the positive control. However, at 20 DAI, 30, 40, and 50 DAI, methanolic and water extracts significantly (p 0.05) reduced DI more than both negative and positive controls (Table 5.31).

Table 5.31. Effect of solvent extracts on disease incidence (DI %) in potato in season A on various days after inoculation (DAI)

Solvent extract	10DAI	20DAI	30DAI	40DAI	50DAI
Methanol	1.97±0.15^{b}	16.57±3.98^{b}	30.32±5.20^{c}	41.10±3.26^{b}	41.52±5.11^{b}
Water	1.84±0.57^{b}	16.47±3.25^{b}	36.08±6.76^{c}	42.40±3.66^{b}	42.77±7.43^{b}
Controls (+; -)					
Copper	3.79±1.57^{b}	36.75±3.55^{a}	61.06±10.43^{b}	90.11±3.12^{a}	95.71±2.710^{a}
DMSO	8.43±3.32^{a}	44.55±9.20^{a}	83.97±10.71^{a}	95.71±3.86^{a}	99.54±0.32^{a}
P =0.05	0.0235	<0.0001	0.0009	<0.0001	<0.0001

The values of DI (% ± SD) are a percentage of diseased plants over total sampled plants from each treatment. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2 % Copper oxychloride) and negative (1% DMSO) controls. SD: Standard deviation).

The BCE of solvent extracts compared to negative control and between themselves against bacterial wilt was also analysed in season A from 10 to 50 DAI. No significant difference (p 0.05) in BCE was reported between solvent extracts except at 30 DAI where methanolic extract expressed higher BCE (62.91 %) than water plant extract (56.62 %). Although there was no significant difference (p 0.05) in BCE observed between solvent extracts at 10, 20, 40, and 50 DAI, the solvent extracts controlled DI in plants at higher level of BCE as compared to both negative and positive controls during those periods (Table 5.32).

Table 5.32. BCE (%) of solvent extracts against potato bacterial wilt in season A on various days after inoculation (DAI)

Solvent extract	**10DAI**	**20DAI**	**30DAI**	**40DAI**	**50DAI**
Methanol	73.86±5.63[a]	63.97±2.87[a]	62.91±3.91[a]	57.17±2.72[a]	56.59±2.48[a]
Water	75.88±6.40[a]	62.84±3.95[a]	56.62±2.80[b]	55.27±2.75[a]	52.48±3.20[a]
Controls (+; -)					
Copper	44.37±23.61[ab]	26.60±7.99[b]	16.16±5.97[c]	5.71±3.53[b]	3.85±2.69[b]
DMSO	0.00±0.00[b]	0.00±0.00[c]	0.00±0.00[d]	0.00±0.00[b]	0.00±0.00[b]
P =0.05	0.0067	<0.0001	0.0005	<0.0001	<0.0001

BCE= [(DI in the control-DI in treatment)/ DI in the control]*100. Positive control (2% copper oxychloride). Negative control (1% DMSO). Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. SD: Standard deviation.

Efficacy of plant extracts of selected plant extracts applied at different frequencies against *R. solanacearum* in potato plants was also determined in season B. In season B, the DI was evaluated from 20 DAI when the first symptoms of wilting appeared in plants) to 50 DAI (when almost all plants in the controls wilted and died). From 20 to 40 DAI, there was no significant (p 0.05) difference between plant extracts in DI in potato plants. However, at 50 DAI, both tobacco and wild marigold extracts resulted in significantly (p 0.05) reduced DI (28.55 and 33.16 %) than garlic extract (42.54 %). In addition, treatment of potato plants with tobacco, wild marigold and garlic extracts had a positive effect in the control of DI of bacterial wilt compared to the controls (Table 5.33).

Consequently, at 20 DAI; all three tested plant extracts led to reduced DI (0.32, 1.04, and 0.74 % in tobacco, marigold, and garlic, respectively) than negative control (17.48 %) but not positive control (9.05 %). From 30 to 50 DAI, all three tested plant extracts resulted to reduced DI than the controls. Thus, at 30 DAI, tobacco, wild marigold, and garlic led to reduced DI (3.67, 6.31, and 8.76 %, respectively) than both negative (34.68 %) and positive (28.09 %) controls at p 0.05. Similar results were observed at 40 DAI where tobacco, wild marigold, and garlic led to

reduced DI (12.34, 18.72, and 24.72 %, respectively) than both negative (71.76 %) and positive (66.40 %) controls. Finally, at 50 DAI tobacco, marigold, and garlic extracts also led to reduced DI (28.55, 33.16, 42.54 %, respectively) than both negative (98.25 %) and positive controls (98.80 %) at p 0.05 (Table 5.33).

Table 5.33. Effect of plant extracts on disease incidence (DI %) in potato in season B on various days after inoculation (DAI)

Plant extracts	20DAI	30DAI	40DAI	50DAI
Tobacco	0.32±0.56^{b}	3.67±1.72^{b}	12.34±3.65^{b}	28.55±7.88^{c}
Marigold	1.04±0.90^{b}	6.31±3.46^{b}	18.72±2.26^{b}	33.16±2.15^{c}
Garlic	0.74±1.27^{b}	8.76±3.77^{b}	24.72±3.29^{b}	42.54±5.03^{b}
Controls				
Copper	9.05±9.49^{ab}	28.09±19.79^{a}	66.40±11.67^{a}	98.80±0.94^{a}
DMSO	17.48±6.58^{a}	34.68±2.38^{a}	71.76±5.5 ^{a}	98.25±1.20^{a}
P =0.05	0.0008	0.0058	0.0001	<0.0001

The values of DI (% ± SD) are average in percentage of diseased plants over total plants from each treatment. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2% Copper oxychloride) and Negative (1% DMSO) controls. SD: Standard deviation.

Biological control efficacy (BCE) of methanolic and water extracts of tobacco, wild marigold, and garlic materials at weekly, bi-weekly, and monthly application was evaluated to examine the effectiveness of the plant extracts against *R. solanacearum* in season B from 20 to 50 DAI. At 20 DAI there was no significant difference (p 0.05) in BCE between plant extracts. However, tobacco, wild marigold, and garlic extracts reduced bacterial incidence (BCE= 97.73, 94.23, and 97.07 %, respectively) as compared to negative control (BCE=0.0%) and with BCE of 49.50, 46.00, and 48.84 %, respectively as compared to positive control (Table 5.34).

From 30 to 50 DAI selected plant extracts also controlled potato against bacterial wilt at different level between themselves in addition to their higher efficacy as compared to controls. At 30DAI tobacco controlled bacterial wilt in potato plants with the highest percantage (BCE= 89.63 and 70.63 % as compared to negative and positive controls) than garlic extract (75.09 and 55.09 % as

compared to negative and positive controls) but not more than wild marigold (81.93 and 62.93 % as compared to negative and positive controls) at p 0.05. In addition, at 40 DAI, tobacco extract also reduced DI at the highest rate (BCE= 83.00 and 75.53 % as compared to negative and positive control) followed by wild marigold (73.91 and 66.44 %) while garlic gave the lowest BCE of 65.66 and 58.19 % as compared to negative and positive controls. At the end of observation, both tobacco and marigold extracts reduced DI in plants at higher BCE (70.97 and 66.20 % as compared to negative control) and 70.41 and 65.64 % as compared to positive control) than garlic extract (BCE= 56.70 and 56.14 % as compared to negative and positive controls) (Table 5.34).

Table 5.34. BCE (%) of plant extracts against potato bacterial wilt in season B on various days after inoculation (DAI)

Plant extracts	20DAI	30DAI	40DAI	50DAI
Tobacco	97.73±2.27^{a}	89.63±8.08^{a}	83.00±9.58^{a}	70.97±12.13^{a}
Marigold	94.23±3.91^{a}	81.93±17.23^{ab}	73.91±13.16^{b}	66.20±9.65^{a}
Garlic	97.07±2.93^{a}	75.09±19.34^{b}	65.66±10.27^{c}	56.70±9.95^{b}
Controls (+; -)				
Copper	48.23±1.06^{b}	19.00±1.80^{c}	7.47±1.43^{d}	0.56±0.04^{c}
DMSO	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{e}	0.00±0.00^{c}
P =0.05	<0.0001	<0.0001	<0.0001	<0.0001

BCE= [(DI in the control - DI in treatment)/ DI in the control]*100. Positive control (2% copper oxychloride) and negative control (1% DMSO) controls. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. SD: Standard deviation.

Effective frequency of application of plant extracts against bacterial wilt was also evaluated in both season B. It was revealed that from 20 to 40 DAI there was no significant difference (p 0.05) in the control of DI between the tested frequencies of application. However, at 50 DAI; both weekly and bi-weekly application of plant extracts showed higher potential (29.94 and 33.96 % in the control of bacterial incidence than monthly application (40.37 %) at p 0.05 (Table 5.35). Beside the comparison between frequencies of application, there were also comparison with the controls and it was noted that from 20 to 50 DAI, plant extracts at different

frequencies of application reduced DI in season A. At 20 DAI, weekly, bi-weekly, and monthly application resulted to lower DI (0.41, 0.05, and 1.64 %, respectively) than negative control (17.48 %) but not the positive one (9.05 %). From 30 to 50 DAI, all tested frequencies of application reduced effectively the DI than both negative and positive controls. Thus at 30 DAI, the DI in weekly, bi-weekly, and monthly application was recorded at 4.81, 5.08, and 8.85 %, respectively while the DI in negative and positive controls reached 34.68 and 28.09 % . Similar results were found at 40 DAI where weekly, bi-weekly, and monthly application led to lower DI (15.49, 17.38, and 22.91 %) than negative and positive controls (71.76 and 66.40 %). The same findings were reported at 50 DAI where weekly, bi-weekly, and monthly application also led to lower DI (29.94, 33.96, and 40.37 %, respectively) than both negative and positive controls (98.25 and 98.80 %) (Table 5.35).

Table 5.35. Effect of application frequency of plant extracts on disease incidence (DI %) in potato in season B on various days after inoculation (DAI)

Frequency	**20DAI**	**30DAI**	**40DAI**	**50DAI**
Weekly	0.41 ± 0.50^{b}	4.81 ± 0.92^{b}	15.49 ± 1.46^{b}	29.94 ± 2.57^{c}
Bi-weekly	0.05 ± 0.09^{b}	5.08 ± 1.97^{b}	17.38 ± 4.20^{b}	33.96 ± 1.88^{c}
Monthly	1.64 ± 1.67^{b}	8.85 ± 5.48^{b}	22.91 ± 5.33^{b}	40.37 ± 5.08^{b}
Controls				
Copper	9.05 ± 9.49^{ab}	28.09 ± 19.79^{a}	66.40 ± 11.67^{a}	98.80 ± 0.94^{a}
DMSO	17.48 ± 6.58^{a}	34.68 ± 2.38^{a}	71.76 ± 5.53^{a}	98.25 ± 1.20^{a}
P =0.05	0.0008	0.0062	0.0001	<0.0001

The values of DI (% ± SD) are a percentage of diseased plants over total sampled plants from each treatment. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2 % Copper oxychloride) and negative (1% DMSO) controls. SD: Standard deviation.

BCE of frequency of application was compared to negative control as from 20 to 50DAI in season B. At 20 DAI there was no significant difference (p 0.05) in BCE between frequencies of application of plant extracts. However, all frequencies of application resulted to potent BCE (99.79, 97.41, and 91.83 % for weekly, bi-weekly, and monthly application, respectively) against potato bacterial wilt incidence as compared to negative control and BCE of 51.56, 49.18, and

43.18 % for weekly, bi-weekly, and monthly application, respectively as compared to positive control. In addition to their efficacy against bacterial wilt as compared to the controls, from 30 to 50 DAI all tested frequencies of application also controlled bacterial wilt at different level between them (Table 5.36). Thus, at 30 DAI both weekly and bi-weekly application of extracts controlled bacterial wilt at the highest level (BCE= 86.20 and 85.56 % as compared to negative control and BCE= 67.2 and 66.56 % as compared to positive control) than monthly application (BCE= 74.89 and 55.89% as compared to negative and positive controls) at p 0.05. In addition, at 40 and 50 DAI, weekly application also reduced DI at the highest rate (BCE at 40 DAI= 78.38 and 70.91 % as compared to negative and positive control; BCE at 50 DAI= 69.50 and 68.94 %) as compared to negative and positive controls) than monthly application (BCE at 40 DAI= 68.20 and 60.73 % as compared to negative and positive control; BCE at 50 DAI= 58.94 and 58.38 % as compared to negative and positive control). Weekly application BCE was not significantly different (p 0.05) from bi-weekly application. Likewise, BCE of the latter did not differ from monthly application at 40 and 50 DAI (Table 5.36).

Table 5.36. BCE (%) of application frequency of plant extracts against potato bacterial wilt in season B on various days after inoculation (DAI)

Frequency	20DAI	30DAI	40DAI	50DAI
Weekly	99.79±0.90^{a}	86.20±9.52^{a}	78.38±11.37^{a}	69.50±9.71^{a}
Bi-weekly	97.41±9.65^{a}	85.56±10.23^{a}	75.99±11.87^{ab}	65.44±13.05^{ab}
Monthly	91.83±8.02^{a}	74.89±23.82^{b}	68.20±14.13^{b}	58.94±11.24^{b}
Controls (+; -)				
Copper	48.23±1.06^{b}	19.00±1.80^{c}	7.47±1.43^{c}	0.56±0.04^{c}
DMSO	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{c}	0.00±0.00^{c}
P =0.05	<0.0001	<0.0001	<0.0001	<0.0001

BCE= [(DI in the control-DI in treatment)/ DI in the control]*100. Positive control (2% copper oxychloride) and negative control (1% DMSO) controls. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. SD: Standard deviation.

Effectiveness of mathanolic and water extracts against bacterial wilt incidence in potato plants was also analysed in field experiment season B. It was found that from 20 to 40 DAI, there was no significant difference (p 0.05) between solvent extracts in the control of bacterial wilt incidence at p 0.05. However, at 50 DAI methanolic extract significantly reduced DI (32.87 %) than water extract (36.65 %) at p 0.05. Furthermore, the solvent extracts significantly reduced DI of bacterial wilt more than controls from the first to the last day of observation. At 20, 30, 40, and 50 DAI, both methanolic and water extracts gave lower DI than negative and positive controls (Table 5.37).

Table 5.37. Effect of solvent extracts on disease incidence (DI %) in potato in season B on various days after inoculation (DAI)

Solvent extract	20DAI	30DAI	40DAI	50DAI
Methanol	0.39±0.58[c]	4.91±0.72[b]	16.20±1.75[b]	32.87±1.48[c]
Water	1.01±1.24[c]	7.59±4.73[b]	20.98±4.11[b]	36.65±1.10[b]
Controls				
Copper	9.05±9.49[ab]	28.09±19.79[a]	66.40±11.67[a]	98.80±0.94[a]
DMSO	17.48±6.58[a]	34.68±2.38[a]	71.76±5.53[a]	98.25±1.20[a]
P =0.05	0.0025	0.0201	0.0011	<0.0001

The values of DI (% ± SD) are a percentage of diseased plants over total sampled plants from each treatment. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2 % Copper oxychloride) and negative (1% DMSO) controls. SD: Standard deviation.

BCE of solvent extracts as compared to negative control against bacterial wilting in potato plants was also analysed from 20 to 50 DAI in season B. Along the experiment, there was no significant difference (p 0.05) in BCE of the two tested solvent extracts except at 40 DAI where methanolic extract reduced DI at 77.46 and 69.99 % as compared to negative and positive controls more than water extract (BCE= 70.92 and 63.45 % as compared to negative and positive control) (Table 5.38). Although there was no significant difference in BCE between solvent extracts at some times during the experiment, they were active against bacterial wilt as compared to controls. It was observed that at 20 DAI, methanolic and water extracts reduced DI in potato

plants with BCE of 97.38 and 95.30 % as compared to negative control and BCE of 49.15 and 47.07 % as compared to positive control. At 30 DAI, methanolic and water extracts reduced DI with BCE of 85.89 and 85.89 % as compared to negative control and BCE of 66.89 and 59.55 % as compared to positive control. At 50 DAI, BCE of methanolic and water extracts was calculated at 66.56 and 62.70 % as compared to negative control and 66.00 and 62.14 % as compared to positive control (Table 5.38).

Table 5.38. BCE (%) of solvent extracts against potato bacterial wilt in season B on various days after inoculation (DAI)

Solvent extract	20DAI	30DAI	40DAI	50DAI
Methanol	97.38±12.87^{a}	85.89±10.67^{a}	77.46±12.19^{a}	66.56±12.40^{a}
Water	95.30±13.36^{a}	78.55±20.32^{a}	70.92±13.25^{b}	62.70±11.56^{a}
Controls (+; -)				
Copper	48.23±1.06^{b}	19.00±1.80^{b}	7.47±1.43^{c}	0.56±0.04^{c}
DMSO	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{c}
P =0.05	<0.0001	<0.0001	<0.0001	<0.0001

BCE= [(DI in the control-DI in treatment)/ DI in the control]*100. Positive (2% copper oxychloride) and negative (1% DMSO) controls. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. SD: Standard deviation.

Effect of growing seasons (A and B) on effectiveness of plant extracts against bacterial wilt incidence in potato plants was also analysed in field experiments. It was found that throughout the field experiment from 20 to 50 DAI, there was significant difference (p 0.05) between growing seasons in the control of bacterial wilt incidence at p 0.05 (Table 5.39). Therefore, at 20 DAI season B significantly reduced DI (0.70%) than season A (16.52 %) at p 0.05. Similar effect was found at 30 DAI where season B resulted to reduced DI (6.25 %) in potato comparing to season A (33.20). Furthermore, at both 40 and 50 DAI season B reduced DI of bacterial wilt (18.59 and 34.76 % respectively) more than season A (41.94 and 45.25 % respectively). On the other hand, both seasons significantly reduced DI more than positive and negative controls throughout the periods of observation (Table 5.39).

Table 5.39. Effect of growing seasons (A and B) on disease incidence (DI %) in potato on various days after inoculation (DAI)

Season	20DAI	30DAI	40DAI	50DAI
A	16.52 ± 3.21^{c}	$33.20\ \pm4.83^{c}$	41.94 ± 4.40^{c}	45.25 ± 9.20^{b}
B	0.70 ± 1.24^{d}	6.25 ± 4.73^{d}	18.59 ± 1.75^{d}	34.76 ± 4.83^{c}
Controls (+; -)				
Copper (+)	22.9 ± 3.32^{b}	44.58 ± 2.67^{b}	78.26 ± 9.24^{b}	97.26 ± 2.71^{a}
DMSO (-)	31.02 ± 3.55^{a}	59.33 ± 10.43^{a}	83.74 ± 10.71^{a}	98.90 ± 0.32^{a}
P =0.05	<.0001	<.0001	<.0001	0.0006

The values of DI (% ± SD) are a percentage of diseased plants over total sampled plants from each treatment. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2 % Copper oxychloride) and negative (1% DMSO) controls. SD: Standard deviation.

The effect of growing seasons on BCE of plant extracts compared to negative control and between themselves against bacterial wilt was also analysed in field experiments from 20 to 50 DAI. Along the experiments, there was a significant difference between effect of growing season A and B at p 0.05. Thus, at 20 DAI, higher effect on BCE was reported in season B (BCE= 96.34 %) comparing to season A (BCE= 62.89 %). Similar effect was observed at 30 DAI where season B resulted to higher BCE (82.22 %) of plant extracts than season A (BCE= 60. 30 %). Later during potato growth, plant extracts controlled DI in plants at higher level of BCE in season B (BCE= 74.19 and 64.63 % at 40 and 50 DAI respectively) as compared to season A during those periods (56.22 and 54.54 % respectively). Furthermore, growing seasons controlled DI in potato plants more than positive and negative controls throughout the observation (Table 5.40).

Table 5.40. Effect of growing season (A and B) on BCE of plant extracts against disease incidence (DI %) in potato on various days after inoculation (DAI)

Season	20DAI	30DAI	40DAI	50DAI
A	62.89±11.56^{b}	60.30±11.56^{b}	56.22±3.41^{b}	54.54±3.24^{b}
B	96.34±12.87^{a}	82.22±10.67^{a}	74.19±8.33^{a}	64.63±3.91^{a}
Controls (+; -)				
Copper (+)	37.42±1.06^{c}	17.58±1.80^{c}	6.59±1.43^{c}	2.21±2.69^{c}
DMSO (-)	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{c}	0.00±0.00^{c}
P =0.05	<.0001	<.0001	<.0001	<.0001

BCE= [(DI in the control-DI in treatment)/ DI in the control]*100. Positive control (2% copper oxychloride). Negative control (1% DMSO). Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. SD: Standard deviation.

Biological control efficacy (BCE) of methanolic and water extracts (solvent extracts) from tobacco, wild marigold, and garlic (plant materials) applied weekly, bi-weekly or monthly (frequency of application) in the control of DI of bacterial wilt was also tested in potato tubers harvested from season A and B. No interaction was found in DI and BCE between these factors during field experiments A and B (Appendix Q). Apart for interaction, these three factors were also analysed separately to determine their efficacy (BCE) on the control of potato bacterial wilt incidence (DI) in tubers.

In season A, there was no significant difference (p 0.05) in DI between plant extracts and this in turn resulted into no clear difference in BCE between themselves against bacterial incidence in tubers. However, treatment of potatoes with all tested plant extracts reduced DI in tubers (30.37, 28.22, and 42.65 % in tobacco, wild marigold, and garlic, respectively) than both positive (80.49 %) and negative (91.75 %) controls at p 0.05. This also led to higher BCE (66.80, 69.29, and 53.48 % in tobacco, wild marigold, and garlic, respectively) as compared to negative control and BCE of 54.51, 57.00, and 41.19 %, respectively as compared to positive control (Table 5.41). Similar results were reported in tubers from season B where plant extracts did not show any significant difference (p 0.05) in DI between them and consequently no difference in their BCE

between them at p 0.05. However, tobacco, wild marigold, and garlic extracts led to lower DI compared to both negative and positive control and as result, they reduced disease in tubers at very higher rate (BCE= 68.75, 65.22, and 61.82 %, respectively) over negative control and BCE of 63.62, 60.09, and 56.69 %, respectively as compared to positive control (Table 5.41).

Table 5.41. BCE (%) of plant extracts against DI (%) in potato tubers in season A and B

Plant extracts	**DI (%) Season A**	**BCE (%) Season A**	**DI (%) Season B**	**BCE (%) Season B**
Tobacco	32.92±14.34[b]	66.80±7.41[a]	29.10±8.72[b]	68.75±7.24[a]
Marigold	28.22±8.06[b]	69.29± 6.52[a]	32.13±4.70[b]	65.22±2.01[a]
Garlic	42.65±5.91[b]	53.48± 5.20[a]	35.32±7.53[b]	61.82±5.58[a]
Controls (+; -)				
Copper	80.49±0.86[a]	12.29±1.39[b]	86.92±3.46[a]	5.13±7.76[b]
DMSO	91.75±0.75[a]	0.00±0.00[b]	92.05±4.96[a]	0.00±0.00[b]
P =0.05	0.0005	0.0001	<0.0001	<0.0001

The values of DI (% ± SD) are a percentage of diseased tubers over total sampled tubers from each treatment. BCE= [(DI in the control-DI in treatment)/ DI in the control]*100. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2% Copper oxychloride) and negative (1% DMSO) controls. SD: Standard deviation.

Beside plant materials, BCE of application frequency of these plant extracts against *R. solanacearum* comparing to controls was determined in tubers from both season A and B. In season A, a weekly application of extracts resulted to reduced DI (25.50 %) in tubers than monthly application (47.50 %) at p 0.05. No significant difference (p 0.05) was found in DI between weekly and bi-weekly application or between the latter and monthly application of extracts. Subsequently, both weekly and bi-weekly application showed higher BCE (72.23 and 69.20 % over negative control and 59.94 and 56.91 % over positive control) than monthly application (48.13 and 35.84 % over negative and positive controls) in season A at p 0.05 (Table 5.42). Similar findings were also observed in season B where both weekly and bi-weekly application showed reduced DI (22.33 and 29.97 %) than monthly application (48.13 %) and thus also led to higher BCE (75.92 and 67.39 % as compared to negative control and 70.79 and

62.26 % as compared to positive control) than monthly application (52.49 and 47.36 % as compared to negative and positive controls) at p 0.05 (Table 5.42).

Table 5.42. BCE (%) of application frequency of plant extracts against DI (%) in potato tubers in season A and B

Frequency	**DI (%) Season A**	**BCE (%) Season A**	**DI (%) Season B**	**BCE (%) Season B**
Weekly	25.50±8.63^{c}	72.23±6.38^{a}	22.33±4.61^{c}	75.92±2.97^{a}
Bi-weekly	28.22±9.60^{bc}	69.20± 4.53^{a}	29.97±4.44b^{c}	67.39± 4.17^{a}
Monthly	47.50±9.13^{b}	48.13± 7.17^{b}	44.24±12.66^{b}	52.49± 5.72^{b}
Controls (+; -)				
Copper	80.49±0.86^{a}	12.29±1.39^{c}	86.92±3.46^{a}	5.13±7.76^{c}
DMSO	91.75±0.75^{a}	0.00±0.00^{c}	92.05±4.96^{a}	0.00±0.00^{c}
P =0.05	0.0002	<0.0001	<0.0001	<0.0001

The values of DI (% ± SD) are a percentage of diseased tubers over total sampled tubers from each treatment. BCE= [(DI in the control-DI in treatment)/ DI in the control]*100. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2% Copper oxychloride) and negative (1% DMSO) controls. SD: Standard deviation.

BCE of solvent extracts against *R. solanacearum* was also evaluated in tubers from both season A and B. In season A, there was no significant difference (p 0.05) in BCE between methanol and water extracts against DI in potato tubers. However solvent extracts controlled DI in tubers at significantly different level with BCE of 64.73 and 62.07 % in methanol and water extract as compared to negative control and BCE of 52.44 and 49.78 % as compared to positive control at p 0.05 (Table 5.43). In season B, methanolic extract showed reduced DI (26.54%) than water extract (37.82%) and thus also led to higher BCE (71.26 and 66.13 % as compared to negative and positive controls) than water extract (59.27 and 54.14 % as compared to negative and positive controls) at p 0.05 (Table 5.43).

Table 5.43. BCE (%) of solvent extracts against DI (%) in potato tubers in season A and B

Solvent extract	DI (%) Season A	BCE (%) Season A	DI (%) Season B	BCE (%) Season B
Methanol	34.43 ± 6.23^{b}	64.73 ± 8.03^{a}	26.54 ± 3.50^{c}	71.26 ± 5.10^{a}
Water	34.77 ± 4.62^{b}	62.07 ± 8.97^{a}	37.82 ± 5.63^{b}	59.27 ± 7.70^{b}
Controls (+; -)				
Copper	80.49 ± 0.86^{a}	12.29 ± 1.39^{b}	86.92 ± 2.00^{a}	5.13 ± 7.76^{c}
DMSO	91.75 ± 0.75^{a}	0.00 ± 0.00^{b}	92.05 ± 4.96^{a}	0.00 ± 0.00^{c}
P =0.05	0.0002	<0.0001	<0.0001	<0.0001

The values of DI (% ± SD) are a percentage of diseased tubers over total sampled tubers from each treatment. BCE= [(DI in the control-DI in treatment)/ DI in the control]*100. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2% Copper oxychloride) Negative (1% DMSO) controls. SD: Standard deviation.

BCE of plants extracts against bacterial wilt incidence (DI) was also compared in tubers from both season A and B. From the results, there was no significant difference (p 0.05) in DI between tubers from season A or B. Furthermore, no significant difference in BCE of plant extrcats against DI in potato tubers was observed between both seasons (Table 5.44). However, growing seasons affected positively the effectiveness of plant extracts against DI in tubers at p 0.05 more than positive and negative controls. Thus, both season A and B resulted into higher BCE (63.19 and 65.26 % in A and B respectively) than positive (8.71 %) and negative (0.00 %) controls due to their higher potential in the control of DI in potato tubers (Table 5.44).

Table 5.44. Effect of growing season (A and B) on BCE of plant extracts against disease incidence (DI %) in potato tubers

Season	DI (%) in tubers	BCE (%) in tubers
A	33.74±14.34[c]	63.19±3.33[a]
B	32.17±12.06[c]	65.265±4.22[a]
Controls (+; -)		
Copper (+)	83.71±3.46[b]	8.71±3.53[b]
DMSO (-)	91.90±7.76[a]	0.00±0.00[b]
P =0.05	0.0487	0.0023

The values of DI (% ± SD) are a percentage of diseased tubers over total sampled tubers from each treatment. BCE= [(DI in the control-DI in treatment)/ DI in the control]*100. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2% Copper oxychloride) Negative (1% DMSO) controls. SD: Standard deviation.

Briefly, weekly and bi-weekly application of potato with methanolic extract from tobacco and wild marigold at 50 mg mL^{-1} significantly reduced disease incidence (DI) in potato plants and tubers at higher level than other treatments under field conditions especially in season B. In summary, all tested plant extracts performed better in reduction of bacterial wilt incidence in both potato plants and tubers than positive and negative controls especially in season B. In both season A and B, it was also concluded that both weekly and every two weeks application of methanolic extract from both tobacco and marigold at 50 mg mL^{-1} are able to reduce bacterial wilt incidence in plants and tubers more than the other treatments. Similar efficacy of tobacco, and wild marigold extracts against plant pathogens was reported in different earlier studies.

Plant species of family *Solanaceae* including tobacco (*Nicotiana tabacum* L.) were reported to have great antibacterial activity against pathogenic bacteria (Sharma *et al.,* 2016; Singh *et al.,* 2010). Phytochemicals that were found in methanol and aqueous extracts of tobacco have shown antibacterial and antioxidant activity against different strains of Gram-positive and Gram-negative bacteria (Sharma *et al.,* 2016; Singh *et al.,* 2010). In the present study, excellent performance of plant extracts against potato bacterial wilt was observed when they were applied

repeatedly i.e the performance was frequency-dependent. Comparable dose-dependent effect of tobacco extract in the control of pathogens was confirmed by different researchers. Singh *et al.* (2010) reported that tobacco extract in a higher concentration showed potential antibacterial activity against *Salmonella typhimurium, B.cereus, B. fusiformis, Staphylococcus aureus* and *Pseudomonas aeruginosa* in human medicine. Another *in vitro* inhibitory activity of ethanol, ethyl acetate, n-Hexane, acetone, butanol and water extract of tobacco extracts at different concentrations (6, 12, 18 and 24 mg mL^{-1}) was also done on pathogenic bacteria such as *Agrobacterium* t*umefaciens, B. cereus*, *E. carotovora,* and *Staphlococcus aureus*. The results proved that all solvent extracts from tobacco had antibacterial activity against test bacteria especially at highest concentration (24 mg mL^{-1}) (Singh *et al.,* 2010).

Similarly, a study done by Ekefan *et al.* (2018) to evaluate the *in vitro* and *in vivo* growth inhibitory capacity of plant extracts (including *Nicotiana tabacum)* applied at different concentrations against *F. solani,* showed that all test plant extracts including tobacco controlled effectively the growth of the pathogen *in vitro.* It was also reported that the level of inhibitory activity depended on the concentration of extract. The higher the concentration of a plant extract, the higher the growth inhibition percentage. This confirmed that the content of bioactive compounds in an extract increase with increase concentration. It has been found that the higher inhibition zone of *F. solani* resulting from application of *Piper guineense* and *Nicotiana tabacum* at high concentration was attributed to higher content in phytochemicals piperine and nicotine respectively (Ekefan *et al.*, 2018).

In another study, tobacco extract also inhibited the growth of tested pathogens with dose-dependent activity. Tobacco extracts were effective over Gram-negative and Gram-positive bacteria (*Klebsiella pneumoniae*, *Escherichia coli*, *Streptococcus faecalis*, *Mycobacterium phlei*, *B. subtilis*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium*) as well as the fungi like *Candida albicans* and *Cryptococcus neoformans* (Bakht *et al.*, 2012). The antibacterial activity of aqueous, ethanol, acetone and methanol extracts of tobacco at 20% concentration was also observed against Gram-positive bacteria (*B. amyloiquefaciens*, *Staphylococcus aureus*) and Gram- negative bacteria *(Escherichia coli* and *Pseudomonas aeruginosa*). Tobacco is also known for its antifungal activity against *F. solani* (Bakht *et al.,* 2012). From the above

researches authors concluded that bioactive extracts of *N. tabacum* can be utilized as an active antibacterial agent against pathogenic diseases and all the above studies support the positive effect of tobacco extract against bacterial wilt in potato plants and tubers as was observed in the present study.

In the present study, wild marigold (*T. minuta* L.) extract also demonstrated good performance in the reduction of potato bacterial wilt incidence and high biological control efficacy against *R. solanacearum* after tobacco extracts. Similar efficacy of wild marigold extract against pathogens was also detected in previous studies (Gayatri & Rajani 2016; Irum & Mohammad, 2015; Senatore *et al.,* 2003; Yuliar *et al.,* 2015). *Tagetes* which belongs to Asteraceae family has been reported to contain compounds with active role against different pathogens and physiological disorders (Gayatri & Rajani, 2016). For example, Gayatri and Rajani (2016) confirmed that *Tagetes* extracts inhibited the growth of *Fusarium* wilt, canker, early blight, fruit spot, and blossom end rot and sunscald in tomato compared to control. Anti-pathogenic effect of this marigold extracts also led to higher growth and fruit yield in tomato in comparison to untreated plants. From the findings, the authors concluded that marigold contains growth stimulant compounds like flavonoids and their derivatives which have antimicrobial and antioxidant properties and its extract can be used to control tomato diseases and to stimulate growth, yield and improve the quality of tomato fruits (Gayatri & Rajani, 2016).

It was also revealed that *T. minuta* is active against some insects, Gram-positive, Gram-negative bacteria and other pathogens (Irum & Mohammad, 2015; Senatore *et al.,* 2003). *Tagetes* extracts were found out to be efficient against different bacteria like *Escherichia coli* Theodor Esherich, *B. subtilis* Ehrenberg, *Staphylococcus aureus* Rosenbach, *Pseudomonas aeruginosa* Schoter, and *Salmonella typhi* Lignieres (Irum & Mohammad, 2015; Senatore *et al.,* 2003). Some studies also reported that bacterial wilt was suppressed by plant residues derived from marigold (Yuliar *et al.,* 2015). The residues control the pathogen through their possible mechanisms of action being antimicrobial activities and the indirect suppression of the pathogen through improved chemical, physical, and biological soil properties (Yuliar *et al.,* 2015). All these findings concur with the results from the current study where wild marigold showed antibacterial activity against *R. solanacearum* in potato plants and tubers.

In vitro and *in vivo* studies were also carried out to investigate the antibacterial effect of seven aqueous plant extracts including marigold and garlic in different concentrations against *R. solanacearum* in tomato (Naseerud *et al*., 2016). These experiments also confirmed the effectiveness of marigold and garlic, marigold (*T. patula*) exhibited a stronger inhibitory effect in *in vitro* condition which was 60% more than streptomycin over the pathogen (Naseerud *et al*., 2016). In present study, biological control efficacy of wild marigold against *R. solanacearum* also increased with increasing frequency of application i.e increased concentration of extract. Dose-dependent effect of *Tagetes* was also confirmed by earlier studies. The higher dose (40 g kg^{-1} of soil) of marigold extracts reduced disease severity of *R. solanacearum* at higher level and enhanced tomato growth and yield than standard antibiotic (Naseerud *et al*., 2016). In addition, they did not notice any residues or toxic compounds from these plant extracts on tomato plants or fruits (Naseerud *et al*., 2016). From this result, it was concluded that marigold extract at high concentration is effective in management of *R. solanacearum* in tomato (Naseerud *et al*., 2016). All these results support the findings from the present study where methanolic extract of wild marigold at high frequency of application also performed exceptionally well in the control of potato bacterial wilt in season A and B.

In the current study, garlic extract also performed in the management of bacterial wilt over the controls although it showed low efficacy than tobacco and marigold. Earlier studies support these findings about antimicrobial activity of garlic extract. In the same study carried out by Naseerud *et al.* (2016), garlic showed less effect against *R. solanacearum* in tomato compared to marigold but higher than the ones that did not show any antimicrobial capacity. Vu *et al.* (2017) also reported that *Allium* species including garlic contain natural compounds with antibacterial properties. Some literatures showed *in planta* potential ability of garlic in the control of *R. solanacearum* in different crops. For instance, a study conducted on methanolic extract of *Allium fistulosum* against bacterial wilt in tomato showed higher potential of this extract in reduction of bacterial wilt incidence (Vu *et al*., 2017). Moreover, *in vitro* and *in vivo* antimicrobial properties in Allium against soil-borne pathogens have also been supported by Arnault *et al.* (2013). The latter researchers proved that onion by-products have biofumigant activity against soil-borne pathogens and stimulate vegetative growth and ultimate productivity of strawberry and asparagus by 15 to 20%, when compared to those obtained using Brassica-based biofumigation. They also

concluded that A*llium* can be used as a new biofumigant and can be used as a substitute of methyl bromide to kill the soil-borne pathogens and pests or to lessen the germination of different weeds.

In current study, garlic extract also performed better in the control of potato bacterial wilt under field conditions at high frequency of application. Similar increased suppression of the bacterial wilt with increased concentration was also reported by other researchers. For instance, Vu *et al.* (2017) reported that methanolic extract of *Allium fistulosum* applied at high concentration (1000 and 2000 µg $mL^{-1)}$ reduced the disease incidence at the rate of 63 and 83% respectively. *Allium* plants also contain the antimicrobial properties against *R. solanacearum* in tomato either by *in vitro* growth inhibition or *in vivo* bacterial incidence reduction when it is used in pre-plant soil treatment (Deberdt *et al.,* 2012). Different researchers confirmed that garlic and other *Allium* extracts at high concentrations can manage tomato bacterial wilt either *in vitro* or *in vivo* conditions (Abo-elyousr & Asran, 2009; Deberdt *et al.,* 2012). This explains why garlic extracts controlled the incidence of potato bacterial under field conditions. Although this extract led to lower BCE than tobacco and wild marigold extract, but it performed well in the management of disease incidence in potato plants and tubers compared to controls.

In the current study, tested plant extracts greatly inhibited bacterial wilt incidence at the highest level in methanolic extract compared to water extract. Less effect of water extract was similarly reported by Sangoyomi *et al.* (2011). The latter conducted a study to evaluate the antibacterial efficacy of water extract from ten different plant extracts (including *Allium sativum* L.) at different concentrations (0.5%, 1% and 10%) against tomato bacterial wilt. From the results, it was found that none of the tested extracts showed growth-inhibitory property against the pathogen even at the highest level (Sangoyomi *et al.*, 2011). However, Vu *et al.* (2017) have confirmed that methanolic extract of *Allium* was able to reduce the bacterial wilt incidence in tomato. Other researchers like Abo-elyousr and Asran (2009), Deberdt *et al.* (2012) have also confirmed that garlic and other *Allium* extracts can manage tomato bacterial wilt either *in vitro* or *in vivo* conditions. It was concluded that the type of organic solvent used for extraction of active botanicals from plant materials may be the reason of lack of activity of all tested plant extracts and that other solvents than water must be used to check whether they can perform well

(Sangoyomi *et al.*, 2011). Apart from garlic, in the current study tobacco and wild marigold extracts also performed better in reducing bacterial wilt incidence in potato especially when methanolic extract was used over water extract. All these studies are in agreement that the type of solvent used for extraction of active botanicals from plant materials is one of the factors that affect yield and composition of extract, which in turn influence antimicrobial activity of a given plant extract (Mwitari *et al.*, 2013; Ncube *et al.*, 2008; Sangoyomi *et al.*, 2011). The same was observed in the current study and further explains why in the present study, methanolic extract was more effective in the control of potato bacterial than water extract.

In the present study, disease incidence was lower and biological control efficacy of plant extracts was higher in growing season B compared to season A. Different factors were reported to affect the incidence of bacterial wilt in potatoes including temperature, moisture and rainfall, soil type, inoculum potential, and other soil biological factors such as nematode populations (Hammes, 2013; Yuliar *et al.*, 2015). The report of thse previous studies are in agreement with the current study that disease incidence increases with increased air temperature especially for biovar 3 (used in the present study, Chapter three) while bacterial wilt is rarely found in regions with temperature below 10°C (Guchi, 2015). In addition, high soil moisture and periods of wet weather are associated with high disease reproduction, survival and high incidence (Hammes, 2013). However, excess rainfall and soil moisture is harmful to the survival of the *Ralstonia* due to lack of oxygen. Extreme soil pH, and presence of contaminants and salts in soil decrease survival rate. Morever, high population of nematodes in soil also increase the development of bacterial wilt (Mansfield *et al.*, 2012). In the present study, ambient temperature and precipitation varied in both seasons and rainfall was much more in season B compared to season A while the temperature conditions were opposite (Meteo data, Table 5.1). In addition, soil type, existing inoculum or other microorganisms in soil were not analysed prior to planting. Therefore, this could have affected the disease incidence in seasons. The high rainfall combined with low temperature or combination of rainfall and those other factors may have contributed to the results observed in season A. The reduced bacterial wilt incidence during season B may also explain why potatoes grown in this season perfomed the better in term of growth, yield, and quality parameters as well as post-harvest infection and yield loss due to *R. solanacearum*.

5.3.5 *Effect of Plant Extracts on Potato Tuber Post-harvest Infection (PHI) and Post-harvest Yield Losses (PHL)* **due to** ***Ralstonia solanacearum***

Plant extracts, solvent extracts and application frequency of these plant extracts were tested against post-harvest infection (PHI) and post-harvest yield losses (PHL) due to infection of bacterial wilt during storage. No interaction was found in PHI and PHL between these factors during field experiments A and B (Appendix R). In both season A and B, there was no significant difference (p 0.05) in PHI between potato tubers treated with different plant extracts (tobacco, wild marigold and garlic). However, all tested plant extracts reduced PHI at significantly higher rate compared to controls at p 0.05. Therefore, in both season A and B tobacco, wild marigold, and garlic extracts resulted to lower infection rate in tubers than both negative and positive controls. Effect of plant extracts against yield losses during storage of tubers was also assessed for season A and B. For both seasons A and B, there was no significant difference (p 0.05) in post-harvest yield losses (PHL) between plants extracts (Table 5.45). However, in both seasons A and B all tested plant extracts significantly reduced PHL compared to controls. In season A, both tobacco and wild marigold reduced PHL (34.69 and 35.61 %) than both negative and positive control (83.09 and 70.26 %) although garlic did not. It was also shown that in season B tobacco, wild marigold, and garlic resulted to reduced PHL (31.16, 34.06, and 38.48 %, respectively) than both negative and positive controls (Table 5.45).

Table 5.45. Effect of plant extracts on potato tuber post-harvest infection (PHI) and post-harvest yield losses (PHL) due to *Ralstonia solanacearum* in season A and B

	Season A		**Season B**	
Plant extracts	**PHI (%)**	**PHL (%)**	**PHI (%)**	**PHL (%)**
Tobacco	26.48±19.89^{b}	34.69±19.38^{c}	21.67±11.67^{b}	31.16±7.33^{b}
Marigold	26.85±4.10^{b}	35.61±13.56^{c}	27.94±9.28^{b}	34.06±10.90^{b}
Garlic	35.56±9.77^{b}	46.81±14.41^{bc}	36.53±6.01^{b}	38.48±4.44^{b}
Controls (+; -)				
Copper	70.00±10.00^{a}	70.26±10.90^{ab}	73.33±5.77^{a}	70.27±2.66^{a}
DMSO	76.40±3.37^{a}	83.09±4.82^{a}	77.78±1.92^{a}	78.70±1.92^{a}
P =0.05	<0.0001	0.0038	<0.0001	<0.0001

The values are average of number or weight of diseased tubers over total number or weight of stored tubers (% ± SD) from each treatment. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2 % Copper) and negative (1% DMSO) controls. SD: Standard deviation.

Frequency (weekly, bi-weekly, and monthly) of applications of plant extracts were evaluated against (PHI) and (PHL) due to infection of bacterial wilt during storage. In season A there was no significant difference (p 0.05) in PHI between frequencies of application. However, all tested frequencies of application reduced PHI to significantly lower levels compared to controls at p 0.05. Therefore, weekly, bi-weekly, and monthly application resulted to lower PHI (20.19, 29.44, and 39.26 %, respectively) in tubers than both negative (76.40 %) and positive (70.00 %) controls (Table 5.46). In season B, it was observed that weekly application led to lower PHI (18.33 %) in tubers than monthly application (36.83 %) and bi-weekly application (30.97 %) at p 0.05. In addition, all tested frequencies of application significantly reduced PHI than both negative (77.78 %) and positive (73.33 %) controls (Table 5.46).

For post-harvest yield losses, the results showed that there was no significant difference (p 0.05) between all tested frequencies for season A. However, from season B both weekly and bi-weekly application reduced PHL significantly (p 0.05) (PHL= 26.45 and 33.51 %) compared to monthly application (43.58 %). Furthermore, it was observed that all tested frequencies of application both for season A and B resulted to reduced PHL than both negative (A= 83.09 %; B= 78.70 %) and positive controls (A= 70.26 %; B= 70.27 %) (Table 5.46).

Table 5.46. Effect of application frequency of plant extracts on potato tuber post-harvest infection (PHI) and post-harvest yield losses (PHL) due to *Ralstonia solanacearum* in season A and B

	Season A		**Season B**	
Frequency	**PHI (%)**	**PHL (%)**	**PHI (%)**	**PHL (%)**
Weekly	20.19±1.40^{b}	29.61±13.38^{b}	18.33±2.89 ^{c}	26.45±6.73^{c}
Bi-weekly	29.44±14.94^{b}	39.83±12.10^{b}	30.97±13.18 ^{bc}	33.51±3.26^{c}
Monthly	39.26±15.72^{b}	47.69±15.36^{b}	36.83±13.14 ^{b}	43.58±4.52^{b}
Controls (+; -)				
Copper	70.00±10.00^{a}	70.26±10.90^{a}	73.33±5.77^{a}	70.27±2.66^{a}
DMSO	76.40±3.31^{a}	83.09±4.82^{a}	77.78±1.92^{a}	78.70±1.92^{a}
P =0.05	<0.0001	0.0008	<0.0001	<0.0001

The values are average of number or weight of diseased tubers over total number or weight of stored tubers (% ± SD) from each treatment. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2 % Copper) and negative (1% DMSO) controls. SD: Standard deviation.

Effectiveness of solvent extracts against post-harvest infection and yield losses due to *R. solanacearum* was analysed at 30 days of storage of potato tubers harvested from season A and B. From both seasons, there was no significant difference (p 0.05) in PHI between the two solvent extracts. However, from season A the methanolic and water extracts significanlty reduced the PHI in tubers (A=24.69 and 34.57; B=26.77 and 30.65 %) than both negative (A= 76. 40 %; B= 77.78 %) and positive (A= 70.00 %; B= 73.33 %) controls (Table 5.47). For post-harvest loss, there was no significant difference (p 0.05) in PHL between solvent extracts from season A. However, from season B methanolic extracts reduced post-harvest yield loss by (PHL= 28.72 %) compared to water extract (PHL= 40.42 %) at p 0.05. In addition, from both season A and B; methanolic and water extracts decreased PHL more significantly than both negative and positive controls at p 0.05 (Table 5.47).

Table 5.47. Effect of solvent extracts on potato tuber post-harvest infection (PHI) and post-harvest yield losses (PHL) due to *Ralstonia solanacearum* in season A and B

	Season A		Season B	
Solvent extracts	**PHI (%)**	**PHL (%)**	**PHI (%)**	**PHL (%)**
Methanol	24.69±5.50^{b}	33.57±9.02^{b}	26.77±8.24^{b}	28.72±1.57^{d}
Water	34.57±15.42^{b}	44.51±15.29^{b}	30.65±8.61^{b}	40.42±1.19^{c}
Controls (+;-)				
Copper	70.00±10.00^{a}	70.26±10.90^{a}	73.33±5.77^{a}	70.27±2.66^{b}
DMSO	76.40±3.37^{a}	83.09±4.82^{a}	77.78±1.92^{a}	78.70±1.92^{a}
P =0.05	0.0001	0.0020	<0.0001	<0.0001

The values are average of number or weight of diseased tubers over total number or weight of stored tubers (% ± SD) from each treatment. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2 % Copper) and Negative (1% DMSO) controls. SD: Standard deviation.

Effectiveness of growing seasons against post-harvest infection and yield losses due to *R. solanacearum* was analysed at 30 days of storage of potato tubers harvested from season A and B. From the results, there was significant difference (p 0.05) in PHI and PHL between the two growing seasons. Therefore, season B significanlty reduced the PHI and PHL and in tubers (PHI= 20.71 %; PHL= 31.57 %) than season A (PHI= 29.63 %; PHL= 39.04 %). In addition, both season A and B decreased PHI and PHL more significantly than both negative and positive controls at p 0.05 (Table 5.48).

Table 5.48. Effect of growing season (A and B) on potato tuber post-harvest infection (PHI) and post-harvest yield losses (PHL) due to *Ralstonia solanacearum*

Season	PHI (%)	PHL (%)
A	29.63±4.10^{b}	39.04±11.67^{c}
B	20.71±9.28^{c}	31.57±13.56^{d}
Controls (+; -)		
Copper (+)	70.67±10.90^{a}	74.27±9.28^{ab}
DMSO (-)	77.09±3.37^{a}	80.90±4.82^{a}
P =0.05	0.0002	0.0030

The values are average of number or weight of diseased tubers over total number or weight of stored tubers (% ± SD) from each treatment. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Honestly Significant Difference (HSD) test. (+, -): Positive (2 % Copper) and Negative (1% DMSO) controls. SD: Standard deviation.

Effect of plant extracts, solvent extracts, application frequency, and growing season on post-harvest infection (PHI) and post- harvest yield loss (PHL) due to *R. solanacearum* were evaluated for a period of 30 days on potato grown under field conditions. From results, all tested treatments showed higher efficacy in reduction of PHI over both negative and positive controls. In addition, both tobacco and wild marigold extracts significantly reduced PHL than positive control although garlic did not. These findings showed that both tobacco and wild marigold extracts are able to reduce PHI and PHL more than the other treatments during potato storage. Furthermore, all tested treatments reduced PHI and PHL in a frequency-dependent manner i.e dose-dependent. All tested plant extracts performed better when they were applied weekly and bi-weekly. This indicates that the higher the concentration of bioactive compounds in potato, the more effective was the applied plant extracts. Moreover, analysis of effectiveness of solvent extracts showed that methanolic extracts reduced PHI and PHL more than water extract. In addition, PHI and PHL was reduced in tubers harvested from season B than in season A. From all these findings, it can be concluded that methanolic extract from tobacco and wild marigold extract at 50 mg mL^{-1} when applied weekly and bi-weekly are the best treatments to control both

PHI and PHL caused by *R. solanacearum* in stored potato. Therefore, it is confirmed that these plant extracts are able to manage *R. solanacearum* during potato storage.

Similarly, it was previously reported that plant natural metabolites are potent to control plant diseases during crop growth and in turn increases yield and post-harvest quality of products (Giri *et al.*, 2020; Mulugeta *et al.*, 2019; Ndivo *et al.*, 2018). A study was conducted to evaluate the influence of tubers treatment with different plant extracts on management of post-harvest losses in potato using plant extracts from *Azadirachta indica, Cymbopogon, Ultica dioica, Mentha,* and *Acoros calamus* (Giri *et al.*, 2020). The results showed that a lesser damage and weight losses were observed in treated tubers with plant extracts as compared to control. These results are also in a harmony with the findings of the present study where plant extracts from tobacco and marigold significantly reduced the PHI and PHL during the storage duration. The biological control efficacy of plant extracts is attributed to the content of bioactive compounds against plant pathogens (Ndivo *et al.*, 2018). These plant extracts were also observed to have antimicrobial activity against post-harvest pathogens in different crops and they can be used as an alternative to suppress pathogens causing yield losses during storage (Giri *et al.*, 2020).

Another study was conducted to investigate the capacity of aqueous extracts from 13 plant species in management of potato soft rot (*E. carotovora*), one of the major pathogens that cause yield losses during storage of potato tubers. *Datura stramonium* and *Ficuscarica* showed higher inhibitory effect on disease severity of this bacterium after 60 days of tuber storage (Viswanath *at al.*, 2018). Essential oils from tobacco were reported to control some post-harvest pests in different crops (Armachius & Zikankuba, 2017). In addition, tobacco extract was reported to contain a high range of natural metabolites with antibacterial activity like rutin and other flavonoids, chlorogenic acid and other phenolic compounds, alkaloids (nicotine and volatile bases), tannins, coumarins, and terpenes (Adamczak *et al.*, 2019; Chen *et al.*, 2007; Docheva & Gagnon, 2015; G rniak *et al.*, 2019; Roberto *et al.*, 2017; Rodrigues *et al.*, 2011; Taleb-Contini *et al.*, 2003). It was noticed that Gram-negative bacteria (including *R. solanacearum*) are more resistant to different antibiotics than Gram-positive bacteria but it was reported that chlorogenic acid is able to control them at high rate (Bouarab-Chibane *et al.*, 2019; Lou *et al.*, 2011). These studies are in agreement with the results of the current study where natural metabolites with

antibacterial activity like rutin and other flavonoids, chlorogenic acid and other phenolic compounds, alkaloids (nicotine and volatile bases), tannins, coumarins, and terpenes presence were confirmed in tobacco extract with potential to contro post-harvest infection caused by *R. solanacearum* which in turn resulted to reduced post-harvest losses in the current studies.

Wild marigold contains various natural compounds with antimicrobial activity like flavonoids and phenolic compounds, saponins, tannins, terpenoids, and alkaloids and tobacco extract contain a high range of natural metabolites with antibacterial activity like rutin and other flavonoids, chlorogenic acid and other phenolic compounds, alkaloids (nicotine and volatile bases), tannins, coumarins, and terpenes. Some of these compounds have been reported to control both the Gram-negative and Gram-positive bacteria in plants (Bouarab-Chibane *et al.*, 2019; Chkhikvishvili *et al.*, 2016; G rniak *et al.*, 2019). In addition, it was also reported from *in vitro* and *in vivo* experiments that wild marigold has a strong antibacterial activity against *R. solanacearum* of tomato especially at high concentration (Li *et al.*, 2007). Naseerud *et al.* (2016) and Yuliar *et al.* (2015) concluded that *Tagetes* extracts can be used in integrated management against *R. solanacaerum* and possibly other plant pathogenic bacteria. These results are in agreement with those of the current study where apart from tobacco, wild marigold extract also controlled post-harvest infection and losses caused by *R. solanacearum* in stored potatoes.

In the current study, garlic also showed antibacterial activity against bacterial wilt in potato during storage over controls although it was less effective than tobacco and wild marigold extracts. Similar antibacterial activity of garlic was reported in control of potato soft rot in storage in a study conducted by Ndivo *et al.* (2018). These researches declared that among three tested extracts, garlic showed the highest antibacterial activity against soft rot followed by neem whereas aloe recorded the lowest effect. Similar positive antibacterial effect of garlic against bacterial wilt in plants either *in vitro* or *in vivo* was reported by different researchers (Abo-elyousr & Asran, 2009; Naseerud *et al.*, 2016). Effectiveness of garlic against plant pathogens was reported to be associated to its content in sulphur-containing compounds, saponins and terpenoids (Deberdt *et al.,* 2012; Vu *et al.*, 2017). This active role of garlic extract in controlling post-harvest pathogens in potato is in agreement with the results of the current study where garlic

extract reduced post-harvest infection and yield loss due to *R. solanacearum* in stored tubers than controls.

The difference in activity of bioactive compounds from plant species has been attributed to genotypic variability in species or cultivars (Mulugeta *et al.*, 2019) and this explains why different plant species have different types and/or concentrations of natural compounds (G rniak *et al.*, 2019; Mulugeta *et al.*, 2019). This difference in type and concentration of bioactive compounds influence the efficacy of plant extracts against pathogens (G rniak *et al.*, 2019; Mulugeta *et al.*, 2019). Therefore, tobacco and wild marigold extracts, which are reported to have high range of bioactive compounds against bacterial pathogens (Adamczak *et al.*, 2019; G rniak *et al.*, 2019; Taleb-Contini *et al.*, 2003) were the most effective against *R. solanacearum* than garlic extract in the present study. Song *et al.* (2009) also reported that some plant natural compounds can be applied to control post-harvest infection and reduce post-harvest weight loss in potato by maintaining tuber firmness during long-term storage (Song *et al.*, 2009). This may explain why in the current study, treatment of potato tubers with tobacco wild marigold and garlic extracts controlled post-harvest infection of *R. solanacearum* as well reducing post-harvest yield loss more than controls.

Effect of plant extracts in a dose-dependent manner was reported by different authors and it was confirmed that plant extracts inhibited effectively different pathogens at high concentrations (Bouarab-Chibane *et al.*, 2019; Lou *et al.*, 2011; Vu *et al.*, 2017). These studies supported results from of the present study in which effectiveness of plant extracts in the control of post-harvest infection and loss due to *R. solanacearum* was higher at weekly and every two weeks application over monthly application. It was also reported that the type of solvent used during extraction influence the composition and yield of extracted bioactive compounds (G rniak *et al.*, 2019; Mulugeta *et al.*, 2019). This means that the higher performance of methanolic extract over water extract in the current study was due to its high content (both types and concentration) of antibacterial compounds. In the present study, it was also reported that PHI and PHL due to bacterial wilt was reduced in potato tubers harvested from season B than season A. This high post-harvest quality of tubers harvested from season B may be attributed to the best perfomanance of preceding growth parameters as well as bacterial wilt tolerance of potatoes

grown in this season. The good potato growth traits in this season resulted from combination of environmental conditions which guaranteed the best development of plants and tubers and tolerance against *R. solanacaerum*.

In the present study, it was found that weekly and bi-weekly application of tobacco and wild marigold at 50 mg mL^{-1} showed higher efficacy in the control of bacterial wilt and yield loss caused by bacterial wilt more than copper oxychloride especially in season B. It is known that the main purpose of post-harvest technology is to maintain both quantity and quality of harvested products (Giri *et al.*, 2020). Therefore, these selected plant extracts can be used in proper post-harvest strategies to minimize potato infection and yield loss due to *R. solanacearum* to guarantee high quality of products for market and processing industries. Further, adverse effects and toxicity of chemicals which are linked to the use of conventional pesticides cannot be neglected. It is known that these chemicals are harmful to environment, non-target organisms; toxicity in the foods chains and health related problems to human and animals as well as drug resistance in human pathogens which result to health problems like cancers (Armachius & Zikankuba, 2017; Ndivo *et al.*, 2018; Viswanath *et al.*, 2018). This is in agreement to negative effect reported in copper oxychloride treatment (used as positive control) in the current study against potato growth, yield, quality, and post-harvest infection and yield loss. Different literatures showed that copper-based treatments can be used against plant pathogens but they still have toxic effect on rhizosphere and plants due to its induction of reactive oxygen species (ROS) that cause oxidative stresses in crops (Ferreira *et al.*, 2014).

5.4 Conclusion

The aim of this study was to evaluate the efficacy of application frequency of plant extracts on disease incidence in potato plants and tubers as well post-harvest infection and yield loss due to *R. solanaceraum* in potato grown in Rwanda. From the results, it is concluded that a weekly or bi-weekly application of both methanolic and water extracts of tobacco and marigold at 50 mg L^{-1} has higher biological control efficacy (BCE) in reducing bacterial disease incidence (DI) in potato plants than garlic extract especially in season B. Futhermore, weekly or bi-weekly application of methanolic extract of tobacco, marigold, and garlic at 50 mg L^{-1} have higher BCE against potato bacterial wilting in tubers than other treatments. All tested plant extracts are able

to reduce post-harvest infection than copper oxychloride. From all the tested plant extracts, weekly and bi-weekly application of methanolic extract of tobacco and wild marigold has potential efficacy to limit post-harvest losses in potato. In summary, weekly or bi-weekly application of methanolic extract of tobacco or wild marigold at 50 mg mL^{-1} are the most effective treatments recommended for adequate potato growth, production of potato with higher yield and tuber quality. They are also the best in protection of potato plants and tubers against disease incidence from *R. solanacearum* during potato growth under field conditions especially in season B. Under storage conditions, these plant extracts are also able to reduce post-harvest infection and yield losses caused by *R. solanacearum* due to their potential biological control efficacy against the pathogen.

CHAPTER SIX

IDENTIFICATION OF BIOACTIVE COMPOUNDS AGAINST POTATO BACTERIAL WILT IN SELECTED PLANT EXTRACTS

Abstract

Potato bacterial wilt caused by *R. solanacearum* is a threatening pathogen in potato production in Rwanda. One of the promising alternative management against it is the use of plant extracts with antibacterial activity. Previously, it was reported that tobacco collected from Rwanda is effective against potato bacterial wilt. However, there was a knowledge gap in bioactive compounds in these plant extracts against *R. solanacearum*. Thus, the objective of this research focused on exploration of phytochemicals present in tobacco extract, and identification of the bioactive compounds against potato bacterial wilt which are present in it. Examination of bio-compounds in tobacco extract was performed by colorimetric and thin layer chromatography (TLC) techniques. Identification of bioactive compounds of isolated fractions from methanolic extract of tobacco that was reported to be effective against the pathogen was done through high performance liquid chromatography (HPLC) with standards. The results of phytochemical screening using colorimetric and TLC methods showed that methanolic and water extracts from tobacco contained flavonoids, alkaloids, terpenes, saponins, anthraquinones, tannins, phenolic compounds, coumarins, glycosides, and sulfur-containing compounds. Through HPLC analysis, the most active molecules in tobacco against *R. solanacearum* were flavonoids (rutin and unknown flavonoid) and phenolic acids (chlorogenic acid and 5-caffeoylquinic acid). From these results, it is concluded that methanolic extract from tobacco contains three bioactive compounds with stronger activity against *R. solanacearum,* namely chlorogenic acid, rutin, and unknown flavonoid and one more compound with minor activity against the pathogen which is 5-caffeoylquinic acid. Therefore, in sustainable and environmental friendly protection of potato crop methanolic extract from tobacco or its derived-molecules are highly recommended for application in disease management against *R. solanacearum.*

Key words: Bioactive compounds, phytochemical screening, *R. solanacearum*, thin layer chromatography (TLC), tobacco extract.

6.1 Introduction

Plant-derived botanicals have been reported to have an ability of managing different plant pathogens including bacterial wilt in environmental friendly way (Mulugeta *et al.*, 2019; Vu *et al.*, 2017). In addition, some botanicals act as plant protectors by stimulating plant defense systems and metabolic processes and can be used to reduce occurrence of plant pathogen. It has been also revealed that plant extracts with antimicrobial activities are sources of substances that may be used to control pathogens with high resistance against different drugs (Mulugeta *et al.*, 2019). This is due to the fact that different plants contain a mixture of bioactive ingredients which work synergically in the control of pathogens and with varying mechanisms of action against them (Vu *et al.*, 2017). Nowadays, the use of plant extracts in management of plant pathogens is getting high attention, since these bio-based compounds can be used as an alternative to replace pesticides for environmental safety (Mulugeta *et al.*, 2019; Naseerud *et al.*, 2016). Globally, the use of bio-pesticides is annually increasing especially in developed countries where there is a growing demand for organic crop products (Vu *et al.*, 2017).

In previous study which focused on screening of methanol, water and chloroform extracts from ten local plant species (Chapter Four), it has been found that methanol extracts followed by water extracts of tobacco, wild marigold, and garlic are able to inhibit the growth of *R. solanacearum* under *in vitro* conditions. In addition, the study of effective concentration (greenhouse pilot experiment) and application frequency (Chapter Five) of those three performing plant extracts against the pathogen revealed that weekly or bi-weekly application of methanolic extract of tobacco or wild marigold at 50 mg mL^{-1} are the most effective treatments recommended for protecting potato plants and tubers against disease incidence from *R. solanacearum* during potato growth under field conditions. The same treatments are also the best for adequate potato growth, production of potato with higher yield and tuber quality. Under storage conditions, these plant extracts are also able to reduce post-harvest infection and yield losses caused by *R. solanacearum* due to their potential biological control efficacy against the pathogen.

Different classes of natural secondary metabolites with antimicrobial activity have been found in different plant species. These are mainly alkaloids, carotenoids, carbohydrates, flavonoids, phenols, terpenes, tannins, glycosides, steroids, saponins, and sulphur-containing compounds

(Adamczak *et al.*, 2019; Cushnie & Cushnie, 2014; Kumar *et al.*, 2009; Li *et al.*, 2007; Taleb-Contini *et al.*, 2003). However, deep study on ingredients from local plant species which are active against *R.solanaceraum* is still limited. In addition, plant natural compounds vary in types and concentrations due to plant species, geographical (altitude) and environmental factors (air and soil temperature and humidity, light duration, soil fertility and pH), organs, age, and solvent used for extraction (Adamczak *et al.*, 2019; Li *et al.*, 2007).

The aim of this research was, therefore, to: i) identify bio-compounds present in water and methanol extracts of tobacco, wild marigold, and garlic materials collected from Rwanda ii) Fractionate tobacco, wild marigold, and garlic extracts iii) explore antibacterial potential of different fractions of extracts iv) examine efficacy of isolated compounds in methanol fraction extract of tobacco in the growth inhibition of potato bacterial wilt v) identify bioactive compounds in selected fractions against *R. solanacearum* (Figure 6.1).

6.2 Materials and Methods

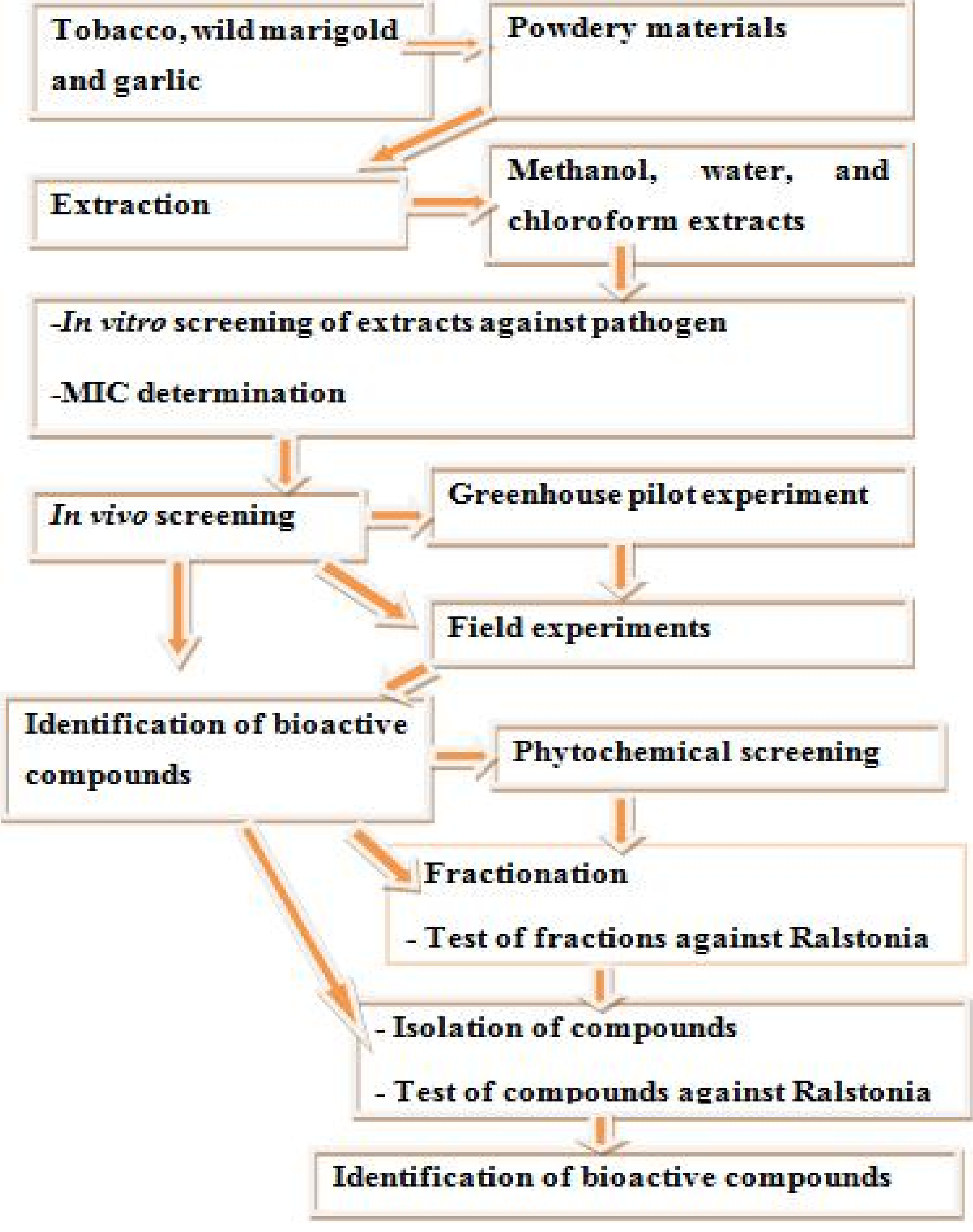

Figure 6.1. Flowchart of main activities in identification of bioactive compounds in plant extracts

6.2.1 Phytochemical Screening of Bio-compounds in Plant Extracts

Photochemical screening was performed mainly to identify classes of bio-compounds, which are present in selected plant extracts. This was achieved for methanol and water extracts of tobacco, wild marigold, and garlic species. Qualitative tests of bio-chemicals were conducted using two different techniques namely colorimetric and thin layer chromatography (TLC) methods. Colorimetric tests were done to detect the presence or absence of alkaloids, flavonoids, tannins, anthraquinones, diterpenes, steroids, saponins, coumarins, glycosides, carbohydrates, phenolic compounds and sulphur-containing compounds in plant extracts. Thin layer chromatography (TLC) technique as described by Wagner (1996) was also used to confirm the results obtained from colorimetric method on alkaloids, flavonoids, anthraquinones, terpenes, and coumarins in plant extracts. Colorimetric tests were carried out in Molecular laboratory at Egerton University, Kenya (August-October, 2019). TLC and further analyses related to identification of bioactive compounds were carried out in Laboratory of Pharmacology of University of Rwanda located in Southern Province of Rwanda, Huye District (November, 2019-February, 2020).

Qualitative Analysis through Colorimetric Tests

To check the presence of alkaloids in plant extracts, Wagner's (1996) test was used. By this method 0.5 g of powdery extract was dissolved in 5 mL of 2N hydrochloric acid and then spinned in a centrifuge for 5 minutes at 500 rpm. Thereafter, 1 mL of the supernatant was mixed with 1 mL of Wagner's reagent. Presence of alkaloids was shown by the appearance of brown color as described by Dash *et al.* (2017) and Nagy (2014). Flavonoids were detected by lead acetate test by which in 1 mL of plant extract three drops of lead acetate solution was added and appearance of yellow precipitate in test tube indicated the presence of flavonoids in the samples (Dash *et al.*, 2017; Nagy, 2014). The presence of tannins was determined by ferric chloride test by addition of 50 mL of hot water in 1 g of plant extract and keeping the solution boiling for 30 minutes. Then, after cooling and filtration, 10 mL of filtrate were mixed with equal volume of water and shaken vigorously. This step was followed by addition of 2 drops of 1 % ferric chloride in 2 mL taken from the test solution. The appearance of dark green colour indicated the presence of tannin in the samples (Gul *et al.*, 2017; Wagner; 1996). Phenolic compounds were also detected by ferric chloride test by adding 2 mL of distilled water in 200 μL of test solution.

Five drops of ferric chloride were added on the side of test tube. Appearance of dark green color was an indicator of the presence of phenolic compounds in the samples (Gul *et al.*, 2017).

Diterpenes were determined by modified Salkowiski's test by adding 1 mL of chloroform in 200µL of plant extract followed by addition of 3 drops of concentrated sulfuric acid. A grey color formation showed the presence of diterpenes in extracts (Gul *et al.*, 2017). Steroids were also checked with Salkowiski's test by which in 2 mL of plant extract was added 20 mL of chloroform and concentrated H_2SO_4. The appearance of red color in chloroform layer revealed the presence of steroids in the samples (Gul *et al.*, 2017). Anthraquinones were determined by ammonia test by which 2 mL of extract were mixed with 5 mL of chloroform followed by a vigorous shaking of solution for 5 minutes, filtration and addition of equal volume of 10 % ammonia solution to the filtrate. Then, formation of red or pink violet color in ammoniacal layer was an indicator of anthraquinones in extracts (Gul *et al.*, 2017; Nagy, 2014). Liebermann's test was used to determine glycosides in plant extracts, whereby 2 mL of acetic acid and chloroform were added to 0.2 g of plant extract. The mixture was cooled for a while and then 2 mL of concentrated H_2SO_4 were added. Appearance of green color showed the presence of glycosides (Dash *et al.*, 2017; Gul *et al.*, 2017; Nagy, 2014).

Sulfur-containing compounds were checked by cystein test. By this method, 1 mL of plant extract was mixed with 1 mL of 40 % sodium hydroxide. Then, 2 drops of lead acetate solution were added to the solution followed by heating for 3 minutes and color observation. Black precipitate indicated the presence of sulfur compounds. Cysteine at 1 % was used as a control (Altemimi *et al.*, 2017, Dash *et al.*, 2017). For saponins, froth test was used by which 5 mL of extract was mixed with equal volume of water in a test tube, then shaken vigorously for 15 minutes and let stand to observe stable froth formation as an indicator of the presence of saponins (Dash *et al.*, 2017; Nagy, 2014). Coumarin determination was performed through opened loop-closed loop response test (Nagy, 2014). Two drops of 1 % sodium hydroxide solution were added to 200 µL of test solution. The mixture was heated in boiling water for 3 minutes to get a clear solution. Then, four drops of 2 % hydrochloric acid were added. Change of clear solution into cloudy confirmed the presence of coumarin (Nagy, 2014). Carbohydrates were detected by Molish's test. By this method, 0.5 ml of test solution was mixed with five drops of

Molish's reagent. Then, 0.5 mL of concentrated H_2SO_4 was added. The appearance of purple color at the interface of the two layers indicated the presence on carbohydrates (Nagy, 2014).

Qualitative Tests with Thin Layer Chromatography

For alkaloids, 1 g of powdery extract was macerated in 1 mL of 10 % ammonia solution and 5 mL of methanol were added. The mixture was shaken for 30 minutes and filtrated. Filtrate of 20 and 40 µL was applied to TLC Silica-gel plate (Aluminium sheets 20 x 20 cm, DC Kieselgel 60 F_{254}). Quinine at 5 mg mL^{-1} was used as a reference solution. TLC separation was done in chromatographic chamber saturated with dichloromethane: methanol: 25 % ammonia (16:4:1) as a mobile phase. After a migration of 10 cm, the plate was dried at room temperature. Then, Draggendorff's reagent was sprayed to the plate followed by direct detection with a UV light at 254 and 366 nm. Appearance of yellow-brown or yellow-orange bands confirmed the presence of alkaloids (Perfetti, 2013; Wagner, 1996).

For flavonoids, 1 g of extract was mixed with 5 mL of methanol and shaken for 10 minutes and filtrated. Then 20 and 40 µL of filtrate was applied to TLC plate. Rutin at 5 mg mL^{-1} was used as standard. The mobile phase was composed of ethyl acetate, formic acid, glacial acetic acid, and distilled water (20:2.2:2.2:5.2). The detection after spraying of natural products-polyethylene glycol (NP/PEG) reagent was done at UV 254 and 366 nm (Perfetti, 2013; Wagner, 1996). The presence of flavonoids was indicated by yellow, blue or green fluorescence. The same solution prepared for flavonoids test was used to check the presence of anthraquinones in plant extract. 20 and 40 µl of solution was applied to TLC plate. Aloïn was used as a reference solution. Ethyl acetate: methanol: water (50: 6.75:5) was used as the mobile phase. After spraying the plate with 10 % ethanolic KOH, observation under UV light at 254 and 366 nm was conducted. With this test, the appearance of red fluorescence indicated the presence of anthraquinone while the aloïn was colored in yellow (Perfetti, 2013; Wagner, 1996).

Terpenes were determined by macerating 1 g of plant extract in 5 mL of dichloromethane and boiled in a water bath for 15 minutes. After cooling the solution and filtrating, the filtrate was evaporated to dryness. This was followed by a dilution of residues in 0.5 mL toluene. Then 20 and 40 µL of solution or thymol (5mg mL^{-1}, used as standard) were applied to TLC plate. Mobile

phase was a mixture of toluene and ethyl acetate (31:2.33). The plate was then sprayed with vanillin in sulfuric acid and heated at 100°C for 10 minutes. This was followed by an observation under UV lamp at 254 and 366 nm. The development of colors different from the reagent color (grey) indicated the presence of terpenes (Perfetti, 2013; Wagner, 1996). The same solution prepared for terpenes test was used to check the presence of coumarins. However, toluene and ether (15:15) saturated with 20 mL of 10% acetic acid was used as mobile phase while scopoletin at 5mg mL^{-1} was used as standard solution. After spraying with 10 % KOH, the plate was observed under UV 254 and 366 nm and coumarins were indicated as blue fluorescence (Perfetti, 2013).

6.2.2 Fractionation of Plant Extracts and Test of Antibacterial Activity of Fractions

Prior to fractionation, methanol and water extracts from tobacco, wild marigold and garlic were analysed by HPLC to determine the major compounds they contain. Preparation of samples for HPLC was done by mixing 1 g dry extract of sample with 10 mL HPLC methanol or water and sonicating the test solution for 1 hour, then filtrating through filter membrane (0.45µm). A volume of 500 µL of filtrate was later transferred to HPLC vial and injected into HPLC microcolumn (Hypersil ODS: 250 mm x 4.6 mm) at an injection rate of 1 mL/ min. The mobile phase of HPLC was composed by addition of 0.05 % trifluoroacetic acid (TFA) and acetonitrile (ACN). The table 6.1 summarizes the HPLC method and conditions used. From the obtained HPLC chromatograms, methanol extract was selected as starting material of fractionation because it showed stronger antibacterial activity against *R.solanacearum* than other treatments. This fractionation helped to discard some non-active compounds and to get the picture of the major compounds present in active fractions. Thereafter, initial methanol extract of tobacco, wild marigold and garlic were washed with n-hexane, diethyl ether, and methanol respectively.

Initial methanol extract was obtained by macerating 100 g of powdery plant materials with 400 mL of absolute methanol (1:4) and left to stand in ultrasonic water bath at 40°C for 1 hour. This was followed by filtration and evaporation of solvent to dryness as described in Chapter Four. For all solvents 400 mL were used under sonication for 1 hour. From this process, four sub-fractions namely initial methanol fraction, n-hexane fraction, diethyl ether fraction and methanol fraction (the residues of methanol extract re-dissolved in methanol) were obtained. In addition to

fractionation, those four fractions of plant extracts were also injected in HPLC used as an advanced technique to detect the main constituents of four fractions of plant extracts but also to get the fingerprint of these plant extracts. Furthermore, chromatographic peaks and the spectra of these fractions were compared to the spectra of rutin, chlorogenic acid, and caffeic acids which were used as standards.

Table 6.1. Method and conditions of HPLC used for fractions of plant extracts

Time (min)	% CAN	% 0.05 TFA
0	0	100
1	3	97
45	40	60
55	40	60
56	60	40
66	60	40
67	0	100
82	0	100
Temperature	25°C	
Flow rate	1 mL/ min	
Injection volume	10µl	
Column	Hypersil ODS(250 mm x 4.6 mm; 5µm particle size, Thermo)	

ACN= Acetonitrile, TFA=Trifluoroacetic acid, ODS=Octadecyl-silica

After fractionation, a new *in vitro* screening technology was carried out to evaluate the antibacterial activity of initial methanol, n-hexane, diethyl ether and methanol fractions of tobacco, wild marigold and garlic plant materials against *R. solanacearum*. Dilution of plant extracts as well as *in vitro* screening method and controls were the same as the ones described in Chapter Four. This experiment was performed to select the fraction of plant extract from a plant material to be used for isolation and identification of its bioactive compounds.

6.2.3 Identification of Bioactive Compounds

Identification of bioactive compounds was performed through three different steps: i) Isolation of bioactive compounds ii) evaluation of antibacterial activity of isolated fractions iii) HPLC analysis of promising isolated fractions with standards (rutin, chlorogenic acid, and caffeic acid). Isolation of bioactive compounds was only done for methanol fraction of tobacco material that showed higher potential in inhibiting *R. Solanacearum* growth among the others during screening and previous studies (Chapter Four and Five). Isolation and separation of bioactive compounds was performed by open column chromatography packed with silica gel under step gradient elution mode. By this mode, the composition as well as the ratio of mobile phase constituents changed from ethyl acetate and methanol (80:20) to 100% methanol (0:100) along the isolation process. TLC analysis helped in changing of a mobile phase composition and to classify isolated compounds into different fractions for further analyses. Analysis by open column chromatography and TCL resulted into 19 fractions isolated from methanol fraction of tobacco (Table 6.2).

Table 6.2. Isolated fractions in methanol fraction of tobacco extract by open column chromatography and TLC

No	Fraction	No	Fraction	No	Fraction	No	Fraction	No	Fraction
1	41-50	**5**	157-160	**9**	251-266	**13**	374-386	**17**	411-435
2	73-86	**6**	161-178	**10**	267-285	**14**	387-393	**18**	436-457
3	87-102	**7**	179-188	**11**	286-325	**15**	394-404	**19**	458-485
4	103-105	**8**	189-250	**12**	326-373	**16**	405-410		

Then, these 19 fractions were tested for their antibacterial activity against *R. Solanacearum*. Preparation of the fractions for antibacterial activity test was carried out by mixing each dry isolated fraction with methanol at 50 mg mL^{-1} concentration. Streptomycin and methanol were used as positive and negative controls respectively. This *in vitro* experiment was done as described in Chapter Four. From the *in vitro* screening of antibacterial activity, isolated fractions with higher growth inhibitory effect against the pathogen were injected in HPLC with different standards namely rutin, chlorogenic acid, and caffeic acid to identify their bioactive compounds. To confirm the identity of isolated compounds, retention time (tr) of chromatographic peaks of the samples were compared to the ones of rutin, chlorogenic acid and caffeic acid standards.

Identification of bioactive molecules was performed for five isolated fractions (11 to 15) from methanol fraction extract of tobacco.

6.3 Results and Discussion

6.3.1 Phyotochemical Screening of Bio-compounds

Phythochemical screening was performed to determine the classes of natural compounds in methanol and water extract from tobacco (T), wild marigold (M), and garlic (G) materials in comparison to standard (S). These qualitative tests were performed by TLC (only methanol extracts) and colorimetric methods (both methanol and water extracts). The bio-compounds with antimicrobial activity which were evaluated in plant extracts were flavonoids, alkaloids, tannins, coumarins, terpenes, carbohydrates, steroids, phenolic compounds, sulphur-containing compounds, anthraquinones, saponins, and glycosides. Saponins, tannins, glycosides, steroids, carbohydrates, phenolic compounds, and sulphur-containing compounds were only tested by colorimetric method while the remaining phytochemicals were checked by both colorimetric and TLC methods.

All the tested plant extracts contained saponins. Only methanol and water extracts of tobacco and wild marigold contained tannins and garlic extract lacked them. Methanol extract of tobacco and water extract of both tobacco and wild marigold showed positive results in term of glycosides while all garlic extracts and methanol extract of wild marigold lacked glycosides. In general, all tobacco extracts plus water extract of marigold contained glycosides while all garlic extracts lacked them (Table 6.3).

Table 6.3. Phytochemical screening of the methanol and water extracts of tobacco, marigold, and garlic materials

Compounds	Methanol extract			Water extract			Photos from TLC under 254 UV or colorimetric tests
	T	**M**	**G**	**T**	**M**	**G**	
Alkaloids	+	-	+	+	+	+	
Flavonoids	+	+	+	+	+	-	
Tannins	+	+	-	+	+	-	
Terpenes	+	+	-	+	+	-	

Anthraquinones	+	-	-	+	+	-	
Saponins	+	+	+	+	+	+	
Coumarins	+	-	-	+	-	-	
Steroid	-	-	+	-	-	+	

Glycosides	+	-	-	+	+	-	
Sulfur-containing compounds	+	-	+	+	+	+	
Phenolic compounds	+	+	-	+	+	-	
Carbohydrates	-	-	+	-	-	+	

T=Tobacco (1-2), M=Wild marigold (3-4), G=garlic (5-6); S=standards, C=control

Steroids and carbohydrates were only present in garlic extract (both methanolic and aqueous) whereas tobacco and marigold extracts lacked them. Phenolic compounds were detected in all tobacco and wild marigold (methanolic and water) while garlic extracts lacked them. By cystein test, all tested extracts showed positive results in Sulfur-containing compounds except methanol extract from wild marigold (Table 6.3). For alkaloids, colorimetric method by Wagner's test

showed appearance of brown color in water extract from all three tested plant materials and all methanol extracts except methanol extract of wild marigold. TLC also confirmed positive result in methanol extract of tobacco and garlic by appearance of yellow-brown or yellow-orange bands under UV light at 254 nm (the same color of quinine used as standard) after spraying of TLC plate with Draggendorff‘s reagent. Wild marigolds extract showed green bands which indicated that they do not contain alkaloids (Table 6.3). Therefore, both TLC and colorimetric results confirmed that methanol extract of tobacco and garlic contained alkaloids whereas wild marigold lacked them. However, water extract from wild marigold also contained alkaloids.

Lead acetate test showed presence of flavonoids in only water and methanol extracts from wild marigold and tobacco whereas garlic did not have. However, TLC method revealed that all test plant extracts (methanol extracts from tobacco, wild marigold, and garlic) contained flavonoids. This was confirmed by development of yellow or blue fluorescence bands in all plant extracts under UV light at 254 nm. The same color was found in rutin used as a reference or standard (S) (Table 6.3). Therefore, methanol extracts of tobacco, wild marigold and garlic, as well as water extract from wild marigold and tobacco contained flavonoids (Table 6.3).

Anthraquinones in plant extracts were detected through ammonia test for colorimetric method. The results revealed the formation of red or pink violet color in ammoniacal layer of methanol extract of tobacco and water extracts of both tobacco and wild marigold. All garlic extract and methanol extract of marigold lacked anthraquinones. In the TLC test, under UV 254 and 366 nm, appearance of red fluorescence was an indicator of anthraquinone while the aloïn used as standard was yellow colored. The TLC confirmed the presence of anthraquinones in methanol extracts of tobacco but not in wild marigold and garlic. These findings indicate that only tobacco extract (both methanolic and aqueous) and water extract from wild marigold contained anthraquinones while all garlic extracts and methanol extract of marigold lacked them (Table 6.3).

Coumarins determination was performed through opened loop-closed loop response test. From this test, positive results in coumarins were only found in methanol and water extracts from tobacco whereas methanol and water extracts of both marigold and garlic gave negative results.

TLC also confirmed positive result only in tobacco extract by observation of blue fluorescence bands under UV 254 nm. The same color was developed by scopoletin used as standard (Table 6.3). Diterpenes were determined by modified Salkowiski's test for colorimetric method. From the results, methanol and water extracts from tobacco and marigold contained diterpenes while garlic extracts did not have. The TLC also confirmed the presence of terpenes in only tobacco and marigold extracts with development of bands with red, green, and pink colors, whereas garlic extract did not express any bands and the zone remained grey under UV 254 and 366 nm. Development of red color (different from sprayed reagent) was also found in thymol (used as standard (Table 6.3).

In the present study twelve compounds were examined in tobacco, wild marigold, and garlic such as flavonoids, alkaloids, tannins, coumarins, terpenes, carbohydrates, steroids, phenolic compounds, sulphur-containing compounds, anthraquinones, saponins, and glycosides. Both TLC and colorimetric phytochemical screening showed that methanol extracts from tobacco contained almost all tested bio active compounds (except steroids and carbohydrates). Methanol extract of wild marigold contained all tested bioactive compounds except alkaloids, coumarins, anthraquinones, steroids, carbohydrates, sulfur-compounds, and glycosides. Some of these compounds were detected in water extract of marigold like glycosides, anthraquinones, and alkaloids. Both water and methanol extracts of garlic contained sulphur-containing compounds, saponins, steroids, and carbohydrates. In addition, methanol extract of garlic contained flavonoids and alkaloids. This means that garlic extracts lacked tannins, phenolic compounds, glycosides, terpenes, anthraquinones, and coumarins. Therefore, the highest range of botanicals was present in tobacco, followed by wild marigold while garlic contained fewer compounds.

Reports by different researchers concur with the findings of botanicals detected in tobacco, marigold and garlic extracts from the present study. For instance, different studies reported that tobacco contained alkaloids, saponins, tannins, flavonoids, cardiac glycosides, phenolic compounds, steroids, terpenoids and coumarins (Bakht *et al.,* 2012; Sharma *et al.,* 2016; Singh *et al.,* 2010). In addition, Perfetti (2013) also confirmed that tobacco species contains four major classes of natural compounds. The major class is composed of oxygen-containing components which are in the highest amount estimated at 75.7 %, which include phenolic compounds

(phenols, phenolic acids, quinones). This is followed by nitrogen-containing components (alkaloids and related volatile bases, proteins and amino acids), miscellaneous and other components (sulfur-containing compounds), and hydrocarbons (alkenes, alkanes, alkynes, polycyclic and monocyclic aromatics). Generally, tobacco extracts contained all botanicals in conformity to the previous studies reported in different literatures (Bakht *et al.,* 2012; Perfetti, 2013; Sharma *et al.,* 2016; Singh *et al.,* 2010).

On the other hand, it was reported that marigold (*Tagetes* spp), which include wild marigold (*Tagetes minuta* L.: Asteraceae family) contained alkaloids, flavonoids, ketone fractions, terpenes and volatile oils (tagetones, dihydrotagetones, and ocimenones), lutein esters, phenolic components, tannins and saponins (Irum & Mohammad, 2015; Li *et al.*, 2007; Naseerud *et al.*, 2016; Senatore *et al.,* 2003; Yuliar *et al.,* 2015). The results of these previous studies mentioned to some extent concur with the findings of present study that wild marigold contains flavonoids, terpenes, tannins, saponins and phenolic components which were in both methanolic and water extracts. However, in addition the results in the present study shows more compounds in water extract of *T. minuta* like sulphur-containing compounds and anthraquinones, which were missing in previous literatures. This disagreement with the previous studies stated above may be attributed to one or many factors that may influence the type and composition of plant extracts, namely genotypic traits (different cultivars in the same plant species), age of plant, organ used, solvent and method used in extraction, geographical location (climate and soils conditions under which a given plant species is grown), and agricultural practices (G rniak *et al.*, 2019; Malkhan *et al.*, 2012; Mwitari *et al.*, 2013).

In the present screening, garlic (*Allium sativum* L.: Alliaceae family) extract contained sulpfur-containing compounds, steroids, carbohydrates, alkaloids, flavonoids, and saponins. Different studies recorded the same results whereas in others, there were some missing or additional secondary metabolites. In most of literatures it was reported that garlic is mainly composed of sulphur-containing amino acid alliin which is converted into organosulfur compounds such as allicin, and ajoene by alliinase enzyme (Mulugeta *et al.*, 2019; Naseerud *et al.*, 2016). It has also been reported that garlic also contains other biochemicals like terpenes (geraniol, citral, and linalool), alkaloids, saponins (erubosides and sativosides), proteins and amino acids, lipids and

prostaglandins, and organo-selenium compounds (Chkhikvishvili *et al.*, 2016; Naseerud *et al.*, 2016). Furthermore, Roberto *et al.* (2017) revealed that garlic aqueous extract contained tannins, saponins and terpenoids, polyphenols, and flavonoids. However, in the present study, both colorimetric and TLC tests confirmed that garlic did not contain diterpenes or terpenes, tannins, phenolic compounds, coumarins, glycosides and anthraquinones. For instance, Naseerud *et al.* (2016) found that garlic aqueous extracts did not contain alkaloids, flavonoids and tannins whereas they were detected in ethyl acetate extract of the same material. In addition, Roberto *et al.* (2017) confirmed that garlic aqueous extract contained tannins, saponins terpenoids, polyphenols, and flavonoids and phenolic compounds. These differences in composition between the present study and other studies stated above is attributed to different solvents used for bioactive compounds extraction and other factors that affect the types of botanicals in plant species like geographical and environmental conditions, genetic profile, age of plant, organ used or extraction method. Furthermore organic solvents also influence the type as well as quantity of botanicals extracted (Kukri *et al.*, 2012; Ncube *et al.*, 2008).

In summary, most of reported bioactive chemicals by different researchers were also found in three plants extract materials used in the current in study. The few difference or disagreement with earlier studies are attributed to factors that have impact on composition and concentration of bio-compounds from plant species as were reported in various literatures (Arnault *et al.*, 2013; Kukri *et al.*, 2012; Mwitari *et al.*, 2013; Ncube *et al.*, 2008). In addition, most of the bio-compounds were extracted by methanol in tested plant extracts except in wild marigold where water extracted a high range of botanicals over methanol. Both methanol and water are polar solvents and the small difference in isolated compounds which was observed between these two solvent extracts may be due to the chemical nature of the compound which renders it less or more soluble to a given organic solvent (Cowan, 1999; G rniak *et al.*, 2019; Ncube *et al.*, 2008).

6.3.2 Fractionation of Plant Extracts and their Antibacterial Activity

Prior to fractionation, initial methanol extract was compared to water extracts to see if they have similarities of major compounds using HPLC technique. From chromatogramic peaks of methanol and water extracts only results for tobacco (which had the highest antibacterial activity) are presented here while the others are in appendices. Through HPLC, it was found that

methanol and water extract both contained almost the same major peaks. All of them contained six main peaks (from 1 to 6. Among the six main peaks, initial methanol (Figure 6.2a) and water (Figure 6.2b) extracts shared five (1-5), while the 6th peak in initial methanol extract was absent in water extract and the 6th in water was in both but at very low concentration in initial methanol extract.

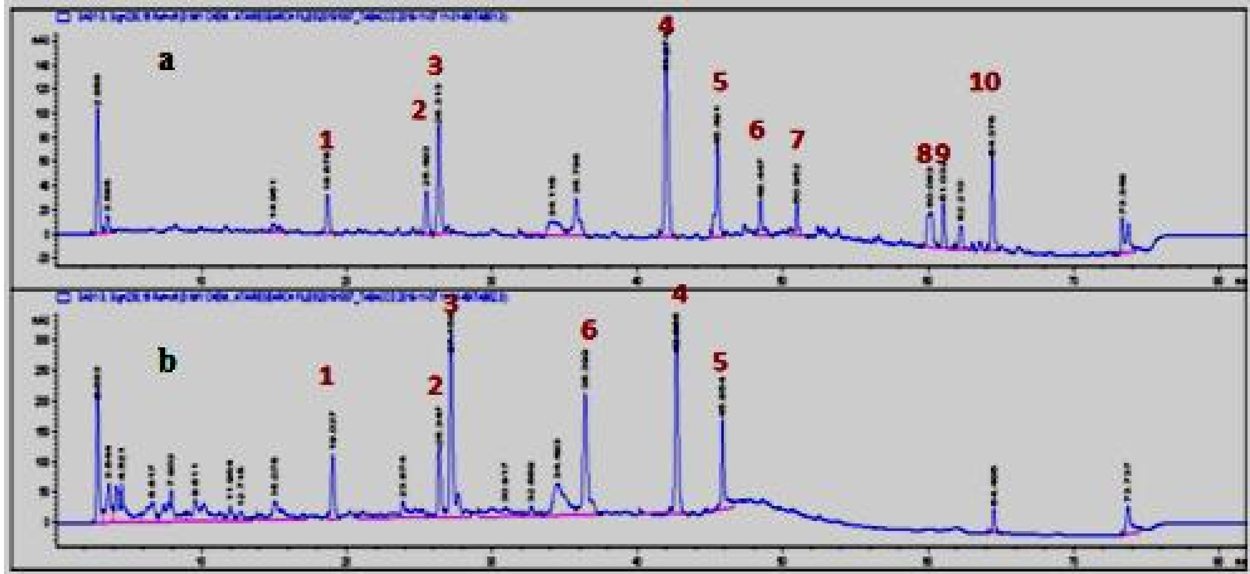

Figure 6.2. Chromatograms of initial methanol (a) and water extract of tobacco (b) recorded at 230 nm

In general both methanol and water extracts of tobacco contained five major peaks recorded at tr= 18.572 min (1), 25.505 min (2), 26.425 min (3), 42.025 min (4), 45.412 min (5). In addition, methanol extract contained extra main peak recorded at tr= 48.492 min whereas the sixth major peak of water extract was detected at tr= 36.300 min (Figure 6.2 and 6.3). Compared to chlorogenic acid, rutin, and caffeic acid standards, it may be stated that the main peaks in methanol extract of tobacco were an unknown flavonoid, (peak 1), isomer of chlorogenic acid (peak 2), chlorogenic acid (peak 3), rutin (peak 4), and two unknown compounds but very close to rutin and probably flavonoids (peak 5 and 6). From the UV-spectra, initial methanol of tobacco extract had the spectrum characteristic of phenolic compounds (both flavonoids and phenolic acids) (Figure 6.3). Beside the major peaks, methanol or water extract showed extra minor peaks which were present in one and absent in another. For instance, the 7th to 9th peaks in

metanolic extract (Figure 6.1a) were missing in water extract (Figure 6.1b). The first chromatogram detected at tr= 2.808 min was considered as injection peak and 10th peak was an impurity because they were detected in all tested samples.

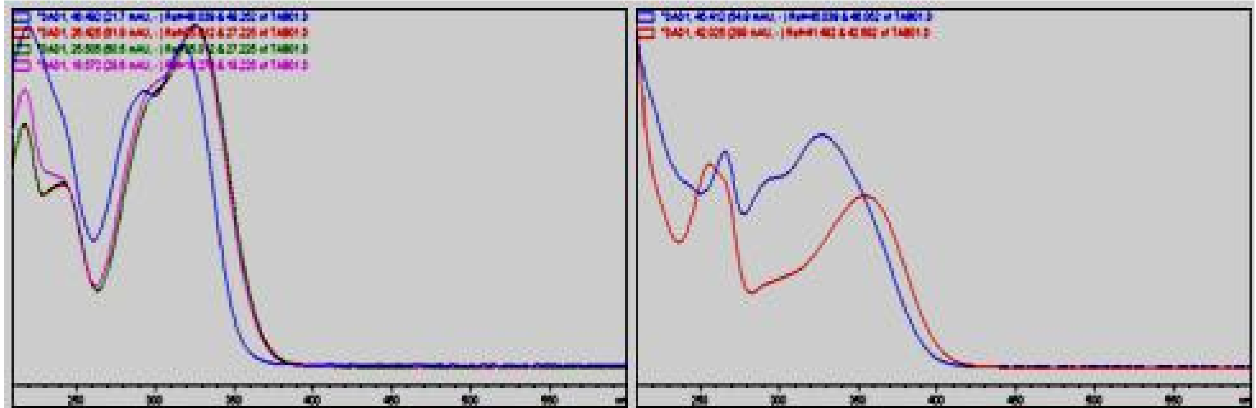

Figure 6.3. UV-spectra of the main peaks of initial methanol extract of tobacco recorded at 230 nm (tr= 18.572, 25.505, 26.425, 42.025, 45.412, and 48.492 min)

After comparing initial methanol and water extract, fractionation was done by washing initial methanol extract of tobacco, wild marigold, and garlic with n-hexane, diethyl ether, and methanol solvents. The main purposes of fractionation of plant extracts was to get finger prints of different fractions and to compare their chromatograms and standards as well as to separate fractions with active compounds from inactive ones against *R. solanacearum*. The first comparisons were conducted between initial methanol, a polar fraction (Figure 6.4a) and nonpolar fractions (diethyl ether and n-hexane extracts) (Figure 6.4b and 6.4c, respectively). From the results of chromatograms recorded at 230 nm, the main peaks of diethyl ether (Figure 6.4b) ether and n-hexane fraction (Figure 6.4c) were found in nonpolar zone (8 and 9) and there were no peaks polar zone (left part of the chromatogram = peak 1 to 7). This shows that treating initial methanol extract of tobacco with diethyl ether or n-hexane solvents led to the extraction of nonpolar compounds. Thus, diethyl ether and n-hexane extracts contained especially nonpolar constituents.

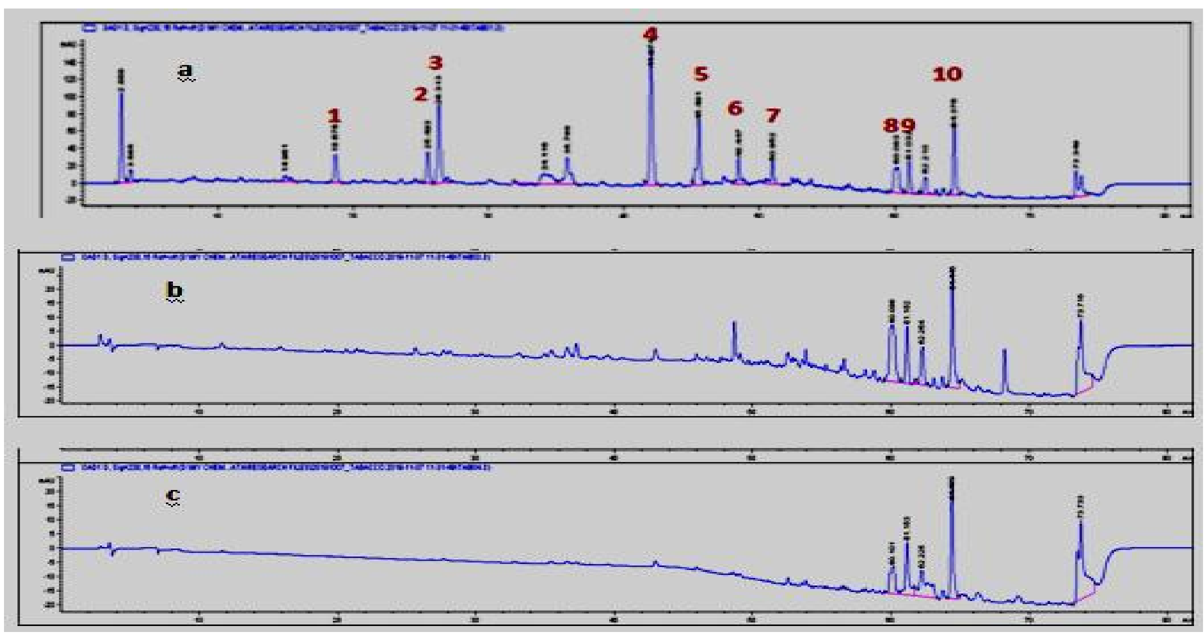

Figure 6.4. Chromatograms of initial methanol (a), diethyl ether (b) and n-hexane (c) fractions of tobacco extract recorded at 230 nm

Thereafter, initial methanol fraction (Figure 6.5a) was also compared to methanol fraction (Figure 6.5b). HPLC results showed that methanol fraction lacked four peaks (from 6 to 9) compared to initial methanol fraction. This shows that even if initial methanol fraction was mainly composed of polar compounds, it also contained few nonpolar compounds, which were washed by nonpolar solvents (diethyl and n-hexane) during fractionation. Thus, methanol fraction only contained five major polar biocompounds (from 1 to 5).

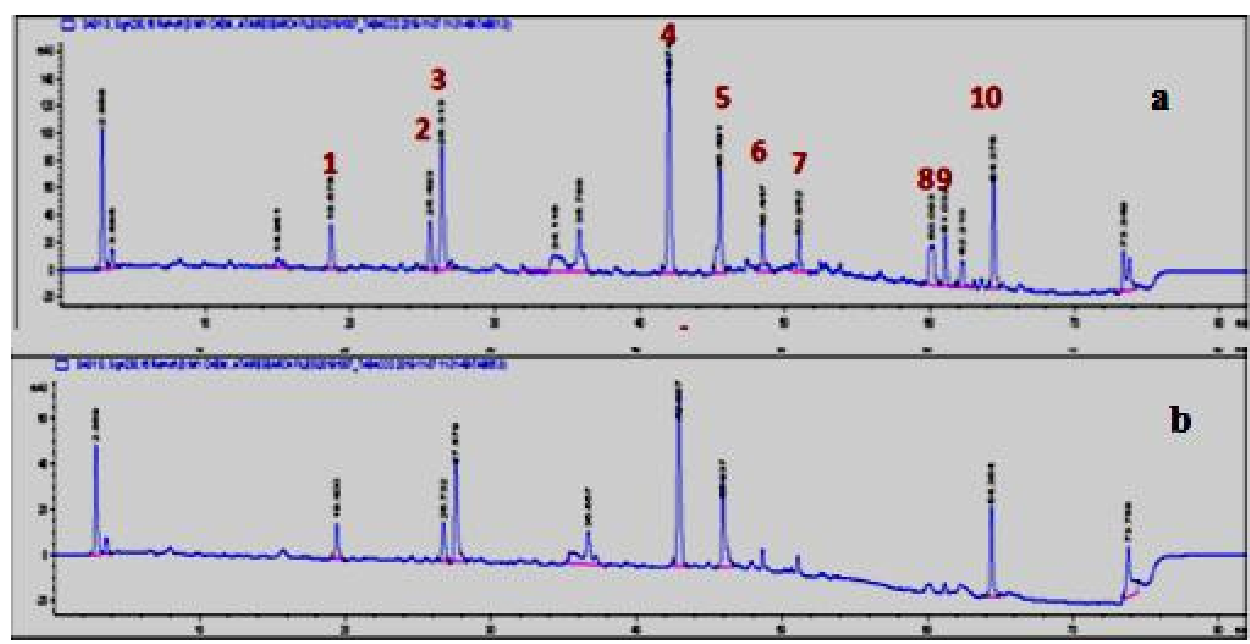

Figure 6.5. Chromatograms of initial methanol (a) and methanol fraction (b) of tobacco extract recorded at 230 nm

In the next step, methanol fraction of tobacco extract was compared to different standards namely rutin, caffeic acid and chlorogenic acid to get an overview of natural compounds present in tobacco extract. From earlier results, we have found that methanol fraction of tobacco extracts contained five main peaks of compounds detected at tr=18.572 min (1), 25.505 min (2), 26.425 min (3), 42.025 min (4), and 45.412 min (5). Chromatograms of methanol (Figure 6.6a) were compared with the ones of a mixture of chlorogenic acid and rutin recorded at 27.558 and 42.803 min, respectively (Figure 6.6b), as well as caffeic acid recorded at 27.564 min (Figure 6.6c). These results showed that the peak detected in tobacco extract which was recorded at 26.425 min (peak 3) was very close to chlorogenic acid or caffeic acid recorded at 27.558 and 27.564 min, respectively. Moreover, a compound recorded at 42.025 min in tobacco extract was also similar or close to rutin recorded at 42.803 min. All these findings showed that methanol extract of tobacco probably contained chlorogenic acid and/or caffeic acid as well as rutin molecules.

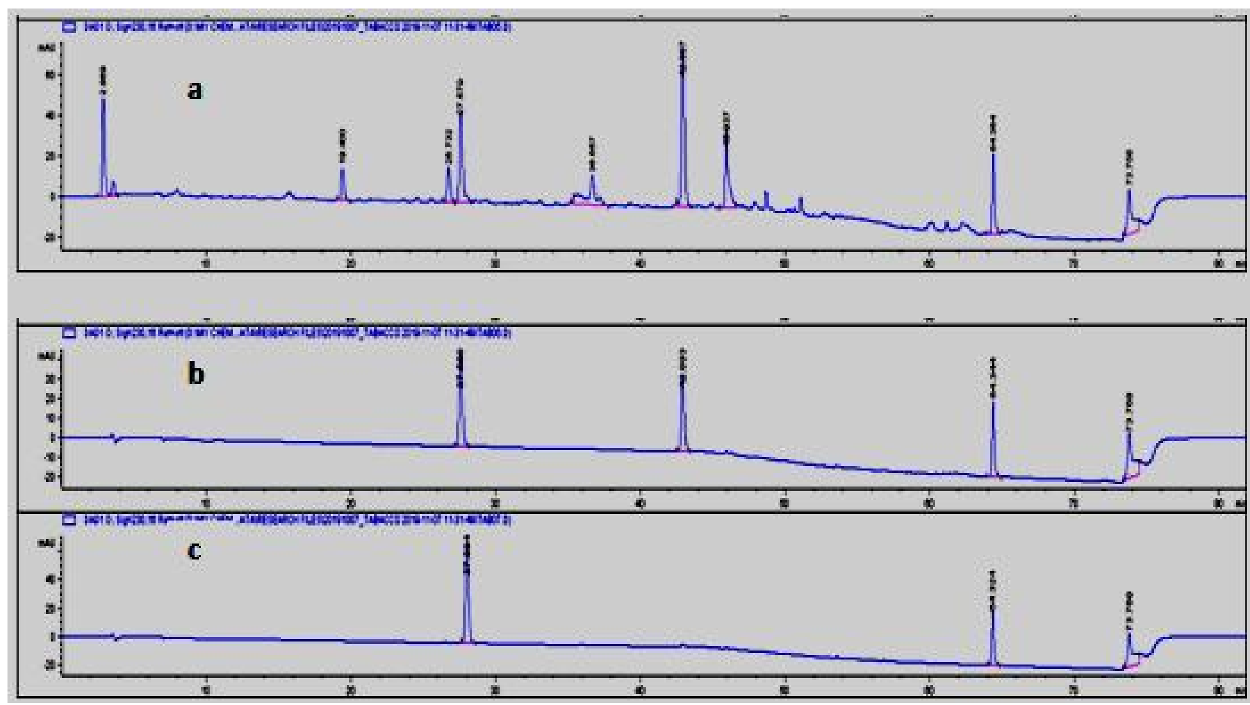

Figure 6.6. Chromatograms of methanol fraction of tobacco extract (a), a mixture of chlorogenic acid (tr= 27.558 min) and rutin (tr= 42.803 min) (b) and caffeic acid (tr= 27.564 min) (c) recorded at 230 nm

In addition to HPLC, the comparison of methanolic extract of tobacco with standards was also performed by TLC test. TLC showed that tobacco extract contained rutin (R) (yellow colored bands on Figure 6.7 left) and chlorogenic acid (CHA+R) (blue colored bands in Figure 6.7 left). This showed that methanol fraction of tobacco extract contained rutin and chlorogenic acid. Blue- colored bands in tobacco extract was also detected when it was compared with caffeic acid standard (CA) (Figure 6.7 middle). This shows that tobacco extract may also contain caffeic acid. Furthermore, it was found that UV-spectrum of chlorogenic acid overlaid the one of caffeic acid (Figure 6.7 right) but they were detected at reduced retention time. Chlorogenic and caffeic acids were detected at 27.714 min and tr= 28.012 min respectively. In Figure 6.6a, one main peak was shown to match with chlorogenic acid (Figure 6.6b) and or caffeic acid (Figure 6.6c). However, the higher probability is that the peak recorded at 26.425 min in tobacco is much closer to chlorogenic acid than caffeic acid. This shows that tobacco extract contained chlorogenic acid instead of caffeic acid.

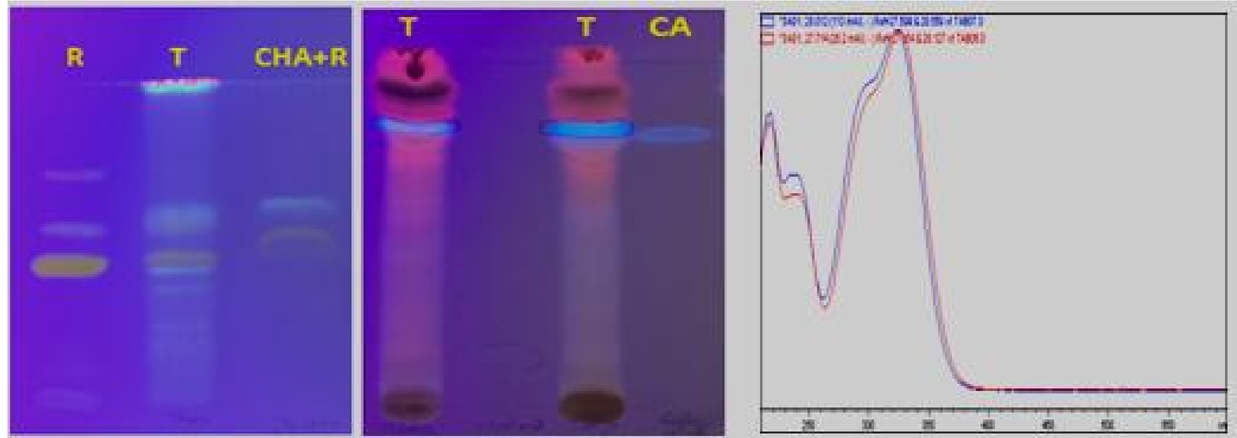

Figure 6.7. TLC of initial methanol extract from tobacco (T) compared to Rutin (R) and chlorogenic acid mixed with rutin (CHA+R) (left). Comparison of initial methanol extract of tobacco (T) with caffeic acid (CA) (middle). Overlaid UV- spectra of chlorogenic acid and caffeic acid (right)

Four fractions (initial methanol, diethyl ether, n-hexane, and methanol fraction) from tobacco, wild marigold and garlic extracts were used to study their *in vitro* antibacterial activity against *R. solanacearum*. From this experiment, it was revealed that initial methanol extracts of tobacco and wild marigold materials had the highest antibacterial activity (23.67 and, 20.67 mm of growth inhibition zone, respectively) than other fractions (Table 6.4 and Figure 6.8). The same potential ability to inhibit bacterial growth was also recorded in methanol fraction of tobacco extract (20.33 mm) (Table 6.4 and Figure 6.8). These extracts inhibited bacterial growth at the same level as streptomycin (23.00 mm), a standard antibiotic used as positive control (Table 6.4). Slightly high antibacterial activity was recorded in initial methanol extract of garlic and methanol fraction of wild marigold with growth inhibition zone against bacterium at 18.67 and 18.33 mm respectively (Table 6.4). The two latter fractions inhibited bacterial growth at the same extent as initial methanol extracts of tobacco and marigold as well as methanol fraction of tobacco but significantly lower than standard antibiotic at p 0.05 (Table 6.4 and Figure 6.8). Moderate efficacy against *R. solanacearum* was reported in methanol fraction from garlic extract (13.00 mm).

Lower growth inhibitory effect against the pathogen was reported in diethyl ether extracts from all three tested plant extracts (7.33 8.00, and 6.00 mm in tobacco, marigold, and garlic, respectively). The n-hexane extracts resulted to the lowest antibacterial activity (2.00, 1.33,and 0.00 mm in tobacco, marigold, and garlic respectively) than all the others and which was significantly equal to the negative control (1 % DMSO= 0.00 + 0.00 mm) at p 0.05 (Table 6.4). Therefore, higher growth inhibition property was reported in initial methanol extracts of tobacco and marigold as well as methanol fraction of tobacco. All these findings confirmed that both initial methanol and methanol fraction extracts from tobacco have high positive effect in the control of potato bacterial wilt with comparable efficacy as standard antibiotic.

Table 6.4. Growth inhibition zone (mm ± SD) of fractions of plant extracts from tobacco, wild marigold and garlic against *Ralstonia solanacearum*

Fraction extracts	**Growth inhibition zone (mm)**		
	Tobacco	**Marigold**	**Garlic**
Initial methanol	23.67±1.53^{ab}	20.67±0.58^{ab}	18.67±0.76^{b}
Methanol fraction	20.33±0.58 ^{ab}	18.33±1.53^{b}	13.00±1.00^{c}
Diethyl ether extract	7.33±1.53^{d}	8.00±2.00^{d}	6.00±2.65^{d}
N-hexane	2.00±1.00^{e}	1.33±0.58^{e}	0.00±0.00 ^{e}
Controls (+; -)			
Streptomycin (+)	23.00±1.00^{a}		
DMSO (-)	0.00±0.00 ^{e}		
P =0.05	< 0.0001		

Figure 6.8. Antibacterial activity of initial methanol of wild marigold (20) and tobacco (21), methanol fraction of wild marigold (22) and tobacco extract (23)

Prior to this fractionation, we already had methanol and water extracts of tobacco, wild marigold, and garlic in previous *in vitro* and *in planta* experiments. Chromatograms of methanol and water extracts from each of these three plant materials were developed and evaluated through HPLC technique. HPLC results showed that both methanol and water extracts of tobacco (Figure 6.2), wild marigold (Appendix S) and garlic (Appendix T) contained the same main peaks of compounds. Thus, tobacco extracts contained six major compounds (from 1 to 6). However, methanol extract of tobacco contained some other four minor compounds (from 7 to 9) which were not extracted by water (Figure 6.2). Peaks 10 and others ahead were considered as impurities because they were found in all fractions of all plant.

In vitro test on antibacterial activity of different four fractions of extracts from each plant extract against bacterial wilt showed that initial methanol of both tobacco and wild marigold, as well as methanol fraction extracts of tobacco had the highest antibacterial activity against the pathogen than other fractions. This indicated that both initial methanol and methanol fractions showed higher potential efficacy against *R. solanacearum* while diethyl ether and n-hexane extracts had the lowest antibacterial activity. Among plant materials, tobacco performed the best followed by wild marigold while garlic showed the lowest antibacterial activity.

Various plant species belonging to different plant families are reported to contain secondary metabolites that are able to control plant pathogens (Malkhan *et al.*, 2012; Yuliar *et al.,* 2015). The main classes of botanicals recorded in plants and having antimicrobial activity include phenols, phenolic acids, flavonoids, tannins, coumarins, and alkaloids (Cowan, 1999; Oboh *et al.,* 2009; Rahman *et al.,* 2012; Stangarlin *et al.,* 2011). Tobacco, marigold, and garlic are among plant species found containing these botanicals and which are able to control different pathogenic microorganisms (Wagura *et al.,* 2011; Yuliar *et al.,* 2015). Therefore, it was not unusual to find that tobacco extracts performed better in the control of potato bacterial wilt in the present study. Tobacco's high antibacterial activity may be attributed to its high content in various natural compounds which are already known to have antimicrobial properties (Amzad & Salehuddin, 2013; Sharma *et al.,* 2016). Although tobacco is mainly reported to have insecticidal and anti-oxidative effects, some few studies also confirm its antibacterial efficacy against both Gram-positive and Gram-negative bacteria. For instance, it was reported that methanol and

aqueous extracts of tobacco (*Nicotiana tabacum* L.) contain flavonoids and alkaloids which exhibited direct antibacterial potential against different strains of gram-positive and gram-negative bacterial strains in human medicine namely *B. cereus* Frankland & Frankland, *B. fusiformis* Ahmed, *Salmonella typhimurium* Lignieres, *Staphylococcus aureus* Rosenbach and *Pseudomonas aeruginosa* Schroter (Sharma *et al.,* 2016; Singh *et al.,* 2010).

Furthermore, ethanol, ethyl acetate, n-Hexane, acetone, butanol and water extracts from tobacco at highest concentration (24 mg mL^{-1}) were reported to control *E. carotovora* Winslow, *Agrobacterium* t*umefaciens* Smith and Townsend, *Staphlococcus aureus* Rosenbach, and *B. cereus* Frankland and Frankland (Bakht *et al.,* 2012). The antibacterial activity of aqueous and alcoholic (ethanol, acetone and methanol) stem extracts of *N. tabacum* at 20% concentration was also observed against Gram-positive bacteria (*B. amyloiquefaciens* Priest, *Staphylococcus aureus* Rosenbach) and Gram-negative bacteria *(E. coli*, *P. aeruginosa*). Some studies concluded that bioactive extracts of *N. tabacum* can be utilized as active antibacterial agent against microbial diseases (Sharma *et al.,* 2016). The results of the present study are in agreement that among the plants evaluated, tobacco performed the best against *R. solanacearum* than the others.

Beside tobacco, it was also reported that methanol extracts from wild marigold (*Tagetes minuta* L.) had strong antibacterial potential against *R. solanacearum* in potato. *Tagetes* genus was recorded to contain many bio-chemicals which can control different microorganisms. For instance, Chkhikvishvili *et al.* (2016) and G rniak *et al.* (2019) revealed that *T. patula* contains various flavonoids and phenolic compounds, saponins, tannins, terpenoids, and alkaloids which were traditionally used for curing a variety of infectious diseases in human medicine. In *in vitro* experiment, aqueous extract of *T. patula* at all tested concentrations (undiluted, diluted at 75, 50, and 25%) showed a strong antibacterial activity against *R. solanacearum* of tomato (Naseerud *et al.*, 2016). Li *et al.* (2007) also confirmed an *in vitro* antibacterial efficacy of *T. patula* against bacterial wilt. In *in vivo* experiment, the same material at high concentration (40 g kg^{-1} of soil) controlled bacterial severity and increased tomato yield by 60% more than control (Naseerud *et al.*, 2016).

The same researchers reported that *Tagetes* compounds may control *Ralsotonia solanacearum* pathogen either by stimulating competitive and antagonistic organisms in the soil when plant material is used as green manure or biofumigant or activating plant defense system through the supplying of natural elicitors or inducers (Naseerud *et al.*, 2016). They concluded that plant species belonging to *Tagetes* genus can be used in integrated management against *R. solanacaerum* and possibly other plant pathogenic bacteria. The antibacterial activity of *T. patula* against *R. solanacearum* was also confirmed in *in vitro* experiment (Yuliar *et al.,* 2015). In another experiment, it was reported reported that ethanol is the best oganic solvent for extraction of flavonoids and phenols from Tagaetes while *n*-hexane is the last to be used (Li *et al.*, 2007). In other study, it was revealed that flavonoids and volatile oils from *T. minuta* show activity against Gram-positive and Gram-negative bacteria and have a suppressive biological activity against some insects and pathogens (Irum & Mohammad, 2015; Senatore *et al.,* 2003). All these findings support the results of the present study in which wild marigold followed tobacco in controlling potato bacterial wilt. Furthermore, aqueous extract of garlic (*A. sativum* = Alliaceae family) showed antibacterial activity against different pathogens but only at the highest concentration (undiluted extract) but did not significantly affected growth and yield in tomato compared to the control (Naseerud *et al.*, 2016). They reported that aqueous *T. patula* contained flavonoids, alkaloids, saponins, terpenoids, and tannins whereas garlic contained only sulphur-containing compounds, saponins, terpenes among the tested compounds. In addition, no phytotoxicity was reported in plants treated with marigold or garlic extracts even at the highest concentration (Naseerud *et al.*, 2016). Moreover, other researches also confirmed that garlic is one of the two plant materials (beside *Datura*) that were tested in Africa against *R. solanacearum* of tobacco and potato and it showed antibacterial activity against it (Abo-elyousr & Asran, 2009; Mulugeta *et al.*, 2019).

It has been reported that the extracts of other *Allium* plants contain the antimicrobial properties against bacterial wilt of tomato (*R. solanacearum*) either by *in vitro* growth inhibition or *in vivo* bacterial incidence reduction when it is used in pre-plant soil treatment (Deberdt *et al.,* 2012). For instance, *Allium fistulosum* extract also significantly reduced bacterial wilt in tomato plants in a dose-dependent manner. At concentrations of 1000 and 2000 µg/mL, the methanol extract of this species controlled bacterial wilting at 63 and 83% after 14 days of inoculation, respectively

(Vu *et al.*, 2017). In addition, allicin, flavonoids, polyphenols, tannins, quercitin, saponin, kaempferol, and scopolamine from garlic controlled different Gram-negative and Gram-positive bacteria like *C. michiganensis* subsp. *Michiganensis, Xanthomonas vesicatoria,* and *Pseudomonas syringae* (Chkhikvishvili *et al.*, 2016; Roberto *et al.*, 2017). Garlic extract was also effective against fungal diseases *A. solani, P. infestans, Pythium ultimum* Trow and *F. oxysporum* Schlechtend as well as nematodes and oomycetes (Amini *et al.*, 2016; Mwitari *et al.*, 2013; Slusarenko *et al.*, 2008). In this recent study, garlic also showed antibacterial efficacy against potato bacterial wilt. The antibacterial effect of garlic in present study is in agreement to what has been reported in all the literatures referred above. Although garlic was among plants that showed inhibitory effect against *R. solanacearum* of potato (especially methanolic extract), it showed the lowest antibacterial activity than tobacco and wild marigold extracts. This is not surprising because from the phytochemical analysis, garlic extract contained few bioactive metabolites in comparison to tobacco and wild marigold extracts.

In the present study, different extracts (methanol or water) of the same species also contained different natural compounds. This difference in bio-compounds also affected the potential of plant extracts against bacterial wilt. This may be due to the fact that organic solvents used to isolate bio-compounds from plant materials do not only affect the types of compounds isolated but also their concentration in extracts (Naseerud *et al.*, 2016). This must influence the effectiveness of extract against pathogens (Naseerud *et al.*, 2016). This difference in both bioactive compounds as well as concentration may also be the reason why different extracts fractions (initial methanol, diethyl ether, n-hexane and methanol fraction) showed different antibacterial potential against *R. solanacearum* of potato in the current study.

6.3.3 Identification of Bioactive Compounds in Tobacco Plant Extract against Ralstonia solanacearum

From the results of HPLC and antibacterial activity of different fractions, it was found that methanol fraction of tobacco extract had higher ability to control *R. solanacearum* among other treatments. Thus, methanol fraction of tobacco extract was selected for isolation and identification of compounds which are active against the pathogen. Thus, 19 fractions were isolated from methanol fraction of tobacco extract (Table 6.5) and were evaluated for their

antibacterial activity against *R. Solanacearum*. The results showed that five fractions (14, 11, 12, 13, and 15) were the most effective in the control of potato bacterial wilt with growth inhibitory zone recorded at 23.33, 21.67, 21.67, 21.33, and 21.000 mm respectively (Table 6.5 and Figure 6.9). All these five fractions of compounds inhibited the pathogen at the same level as positive control at P 0.05 (Table 6.5).

Table 6.5. Growth inhibition zone (mm ± SD) of isolated fractions in methanol fraction of tobacco extract against *Ralstonia solanacearum*

Extract fraction	**Growth inhibition zone (mm)**	**Extract fraction**	**Growth inhibition zone (mm)**
14 (387-393)	23.33 ± 2.89^{a}	2 (73-86)	7.00 ± 1.00^{cd}
11 (286-325)	21.67 ± 1.20^{a}	3 (87-102)	4.67 ± 0.58^{def}
12 (326-373)	21.67 ± 2.08^{a}	5 (157-160)	4.00 ± 1.00^{ef}
13 (374-386)	21.33 ± 0.58^{a}	7 (179-188)	3.67 ± 1.15^{ef}
15 (394-404)	21.00 ± 1.00^{a}	6 (161-178)	3.00 ± 2.00^{ef}
4 (103-105)	14.00 ± 3.06^{b}	1 (41-50)	0.00 ± 0.00^{f}
18 (436-457)	13.67 ± 3.51^{bc}	10 (267-285)	11.67 ± 0.58^{bc}
8 (189-250)	13.67 ± 2.03^{bc}	17 (411-435)	11.33 ± 0.58^{bcd}
9 (251-266)	13.33 ± 2.85^{bc}	16 (405-410)	11.00 ± 1.00^{bcd}
19 (458-485)	12.00 ± 0.88^{bc}	2 (73-86)	7.00 ± 1.00^{cd}
10 (267-285)	11.67 ± 0.58^{bc}	3 (87-102)	4.67 ± 0.58^{def}
17 (411-435)	11.33 ± 0.58^{bcd}	5 (157-160)	4.00 ± 1.00^{ef}
16 (405-410)	11.00 ± 1.00^{bcd}	7 (179-188)	3.67 ± 1.15^{ef}
Controls (+; -)			
(+):Streptomycin	22.67 ± 1.15^{a}		
(-): Methanol	0.00 ± 0.00^{f}		
P =0.05	0.0001		

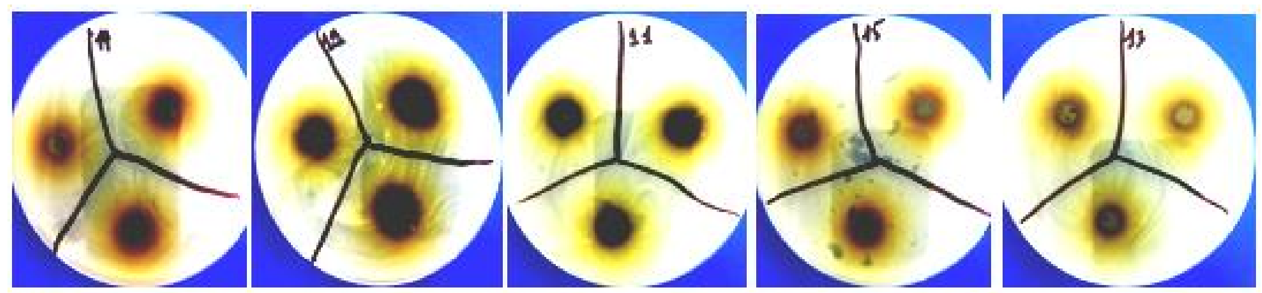

Figure 6.9. ***In vitro*** **growth inhibitory zone of five (14, 11, 12, 13, and 15) promising fractions of methanol fraction of tobacco extract against** ***Ralstonia solanacearum***

After antibacterial screening of isolated fractions, HPLC was also carried out for those which, showed higher antibacterial activity against the pathogen. These fractions (in decreasing order) were namely 14 (387-393), 11 (286-325), 12 (326-373), 13 (374-386), and 15 (394-404). The HPLC results showed that fraction 14 contained one major chromatographic peak of natural compounds recorded at tr= 27.117 min and two minor peaks at tr= 42.687 min and 26.288 min (Figure 6.10a). Chromatograms of standards chlorogenic acid, rutin, and caffeic acid were detected at tr= 27.558 min, tr= 42.803 min, and tr=28.012 min respectively (Figure 6.10b and 6.10c). This comparison confirmed that the major compound of fraction 14 of methanolic tobacco extract was chlorogenic acid while other minor compounds were rutin and the third one was unknown but probably related or close to chlorogenic acid. In addition, an overlaid UV-spectra of rutin standard and a compound detected in fraction 14 (Figure 6.10d) confirmed the presence of rutin in fraction 14. No caffeic acid was detected in this fraction. In general, fraction 14 contained mainly chlorogenic acid, rutin, and an unknown compound which may be an isomer or derivative of chlorogenic acid.

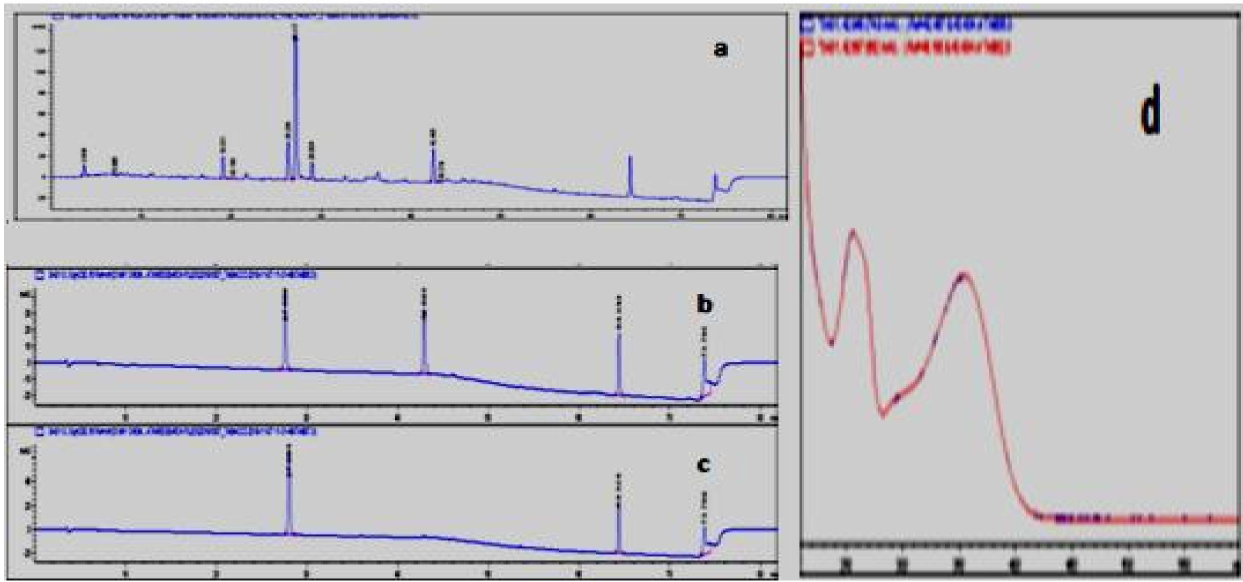

Figure 6.10. Chromatograms of fraction 14 (387-393) (a), standards, a mixture of chlorogenic acid (tr= 27.558 min) and rutin (tr = 42.803 min) (b), and caffeic acid (tr= 28.012 min) (c) recorded at 230 nm. Overlaid UV- spectra of rutin standard at tr= 42.803 min and of a compound in fraction 14 (tr= 42.687 min) (d)

HPLC results also showed that both fractions 11 and 12 contained one major peak of compound recorded at tr= 17.568 min (Figure 6.11a and 6.11b). This unknown bioactive molecule had a spectrum of a flavonoid molecule similar to rutin spectrum (comparison of Figure 6.11c and Figure 6.10d). However, this molecule and rutin were detected at very different retention times. This unknown molecule which is probably a flavonoid was released at tr= 17.568 min whereas rutin was at tr= 42.940 min. The UV-spectrum of this unknown flavonoid was also similar to the one detected in methanol fraction and recorded at tr= 18.572 min (Figure 6.3) which is yet to be identified.

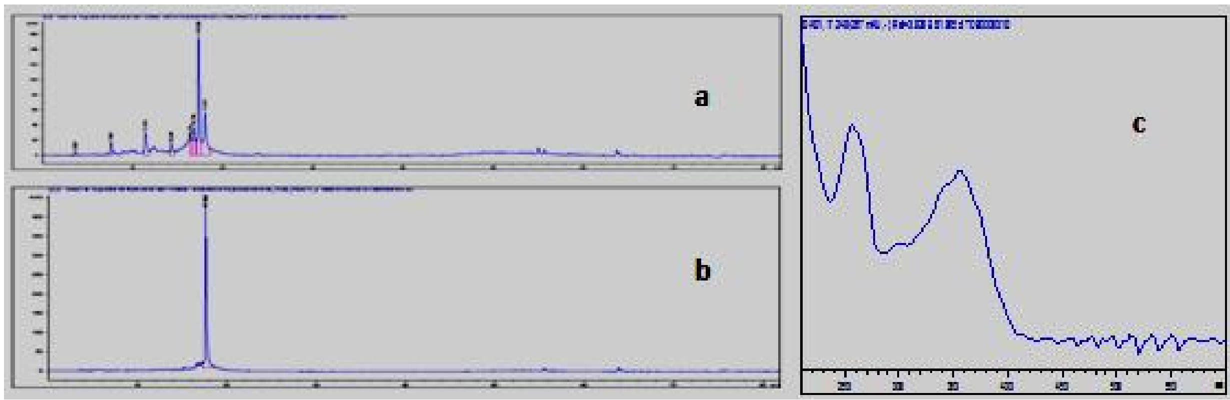

Figure 6.11. Chromatogram of fraction 11 (286-325) (a) and fraction 12 (326-373) (b) recorded at 17.568 min. UV-Spectrum of a major bioactive compound isolated in 11 and 12 fractions at tr= 17.568 min (c)

Moreover, HPLC results of fraction 13 contained mainly a chromatogram of compound detected at tr= 42.501 min. The minor molecule was also detected at tr= 27.186 min (Figure 6.12a). Compared to the tested standards (Figure 6.12b and 6.12c), the major bioactive compound in this fraction was rutin while the minor one was chlorogenic acid.

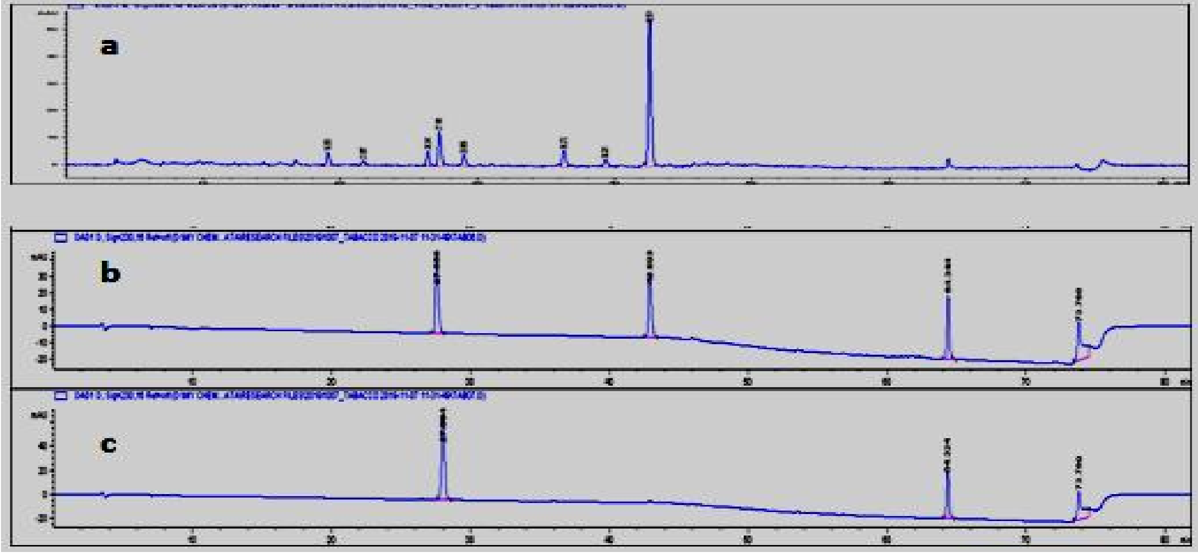

Figure 6.12. Chromatograms of fraction 13 (374-386) (a), standards, a mixture of chlorogenic acid (tr=27.558 min) and rutin (tr =42.803 min) (b) and caffeic acid (tr=28.012 min) (c) recorded at 230 nm

Finally, HPLC showed that fraction 15 contained a major bioactive compound detected at tr= 27.260 min while the minor constituents were recorded at tr= 42.590 and tr= 26.406 min (Figure 6.13a). Compared to the chromatographic peaks as well as the retention time of standards (Figure 6.13b and 6.13c), the major metabolite of fraction 15th was identified to be chlorogenic acid while the minor one was rutin. The third compound was similar to the one detected in fraction 14 at tr= 26.288 min which may be an isomer of chlorogenic acid.

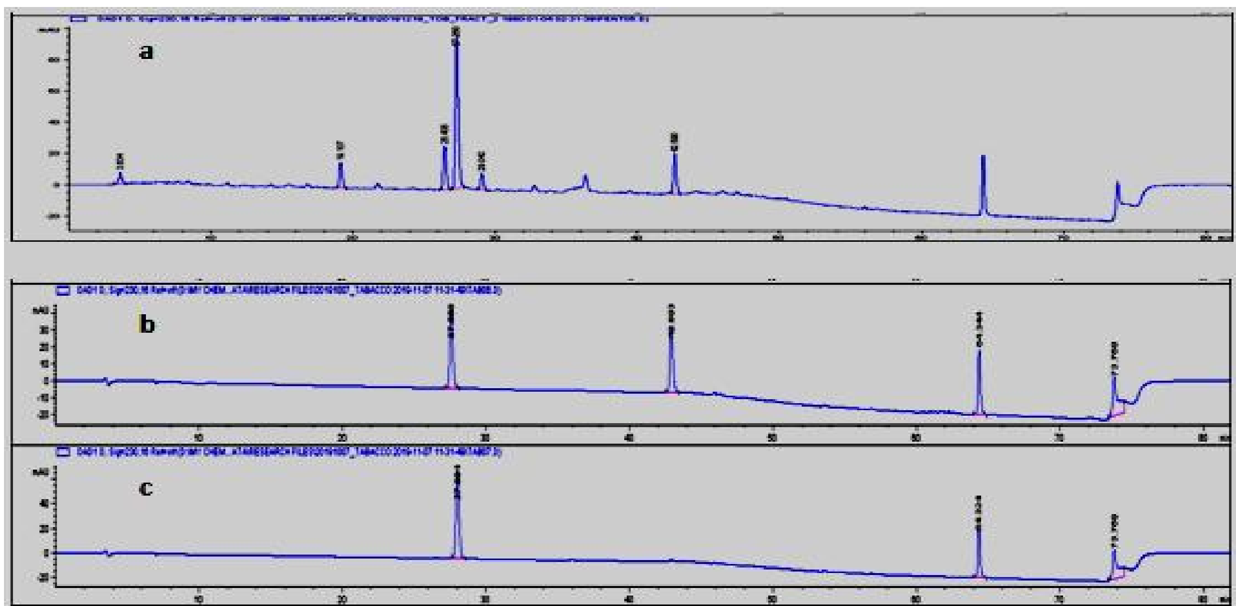

Figure 6.13. Chromatograms of fraction 15 (394-404) (a), standards, a mixture of chlorogenic acid (tr=27.558 min) and rutin (tr =42.803 min) (b) and caffeic acid (tr=28.012 min) (c) recorded at 230 nm

In general, the comparison of chromatographic peaks, UV-spectra and retention times between standards (rutin, chlorogenic acid and caffeic acid) and five active fractions showed that tobacco extract contains four bioactive compounds against *R. solanacearum.* Among them, three have chlorogenic acid as the major active ingredient, rutin and an unknown flavonoid whereas the fourth one has a minor effect against bacterial wilt and probably it is an isomer of chlorogenic acid.

From the previous *in vitro* experiment, it was established that both initial methanol and methanol fraction (the one washed with diethyl ether and n-hexane solvents) of tobacco extract had higher ability to control *R. solanacearum* than the other treatments. In addition, qualitative tests performed to determine the phytochemicals in these extracts through HPLC chromatograms confirmed that methanol and water extracts from tobacco contained almost the same main bioactive compounds. Thus, methanol fraction extract from tobacco was selected for isolation and identification of the bioactive compounds against *R. solanacearum* of potato. The 19 fractions (with fluorescent bands at UV 256 nm) were isolated from methanol fraction extract of tobacco. Isolation was followed by a test of their antibacterial efficacy against *R. solanacearum*. The results showed that five fractions (from 11 to 15) in decreasing order of 14 (387-393), 11 (286-325), 12 (326-373), 13 (374-386), and 15 (394-404) had the highest performance in the control of potato bacterial comparable to streptomycin. From these five fractions, HPLC was carried out and chromatographic peaks and their retention time and spectra of test samples were compared to the ones of rutin (flavonoid), chlorogenic, and caffeic acid (phenolic acids) standards. In comparison of HPLC results with the standards, it was confirmed that fraction 14 contained one major chromatographic peak of natural compounds recorded at tr 27.117 min and two minor peaks at tr 42.687 min at 26.288 min. Compared to the standards, chlorogenic acid was detected at tr=27.558 min, rutin recorded at tr =42.803 min whereas caffeic acid was tr=28.012 min. This comparison confirmed that the main compound of fraction 14 of tobacco was chlorogenic acid while the other compound was rutin. No caffeic acid was detected in this fraction.

The results of the present study are in agreement with those of Li *et al.* (2003) and Chen *et al.* (2007) who also proved through HPLC results that tobacco mainly contains chlorogenic acid (phenolic acid) and rutin (flavonoid) in very high concentration. In a quantitative test of natural compounds in tobacco leaves, it was also found that chlorogenic acid and rutin were in the highest concentration which was at 75–95% among the polyphenolic compounds (Chen *et al.*, 2007). Furthermore, the same authors also detected three other peaks before and two after chlorogenic acid and they concluded that tobacco contain other significant polyphenols apart from chlorogenic acid. The major polyphenols in tobacco are flavonoids (rutin and kaempferol-3

rutinoside) and phenolic acids (chlorogenic acid, its isomer 4-O-Caffeoylquinic acid, and neochlorogenic acid) (Chen *et al.*, 2007; Docheva & Gagnon, 2015).

Additional literatures also revealed that tobacco contains rutin, chlorogenic acid, scopolin, scopoletin, and caffeic acid in a respective decreasing order of concentration (Li *et al.*, 2003). Beside rutin, it was reported that tobacco also contains another flavones kaempferol-3-rutinoside homolog to rutin (Li *et al.*, 2003). The same authors confirmed that chlorogenic acid (3-caffeoylquinic acid or 5-O-caffeoylquinic) has its isomers which are present in tobacco like 5-caffeoylquinic acid, and 4-caffeoylquinic acid. Through HPLC, 5-caffoylquinic acid comes before chlorogenic acid peak whereas 4-caffeoylquinic acid comes after (Chen *et al.*, 2007). From these reports, it may also be proposed that the third compound which was found in fraction 14 and detected at 26.288 min (before chlorogenic acid detected at 27.117 min) in the current study may be 5-caffeoylquinic acid which is an isomer of chlorogenic acid. Furthermore, by another comparison of chromatographic peaks of standards and tobacco sample; it was also revealed that chlorogenic acid is in high amount, and tobacco was found to contain other minor polyphenolic substances (Chen *et al.*, 2007).

HPLC results in the present study also showed that both fractions 11 and 12 contained one major unknown bioactive compound recorded at tr= 17.568 min. This unknown bioactive molecule had a spectrum of a flavonoid molecule similar to rutin spectrum. However, this molecule and rutin were detected at very different retention times. This needs to be clear and requires further studies to reveal its true identity. HPLC results also showed that fraction 13 contained mainly a compound detected at tr 42.501 min and a minor molecule detected at 27.186 min. Compared to the standards, the major bioactive compound in this fraction was rutin while the minor one was chlorogenic acid. HPLC showed that fraction 15 contained a major bioactive compound which was chlorogenic acid while the minor one was rutin and an isomer of chlorogenic acid. The results of the present study are in concurrence with the previous reports by Li *et al.* (2003) and Chen *et al.* (2007) that tobacco contains rutin, chlorogenic acid and isomers of chlorogenic acid.
The comparison of chromatogramic peaks, UV-spectra and retention times of standards (rutin, chlorogenic acid and caffeic acid) and tests of active fractions showed that four compounds were active against *R. solanacearum*. Three main bioactive compounds against the pathogen were

cholorogenic acid, rutin and one unknown flavonoid. The fourth compound with a minor activity against the pathogen was reported to be an isomer of chlorogenic acid (5-caffeoylquinic acid). These findings from the current study confirmed that the main antibacterial activity against *R. solanacearum* of potato was in flavonoids (rutin and another unknown flavonoid) and phenolic acids (chlorogenic acid and its isomers). Both flavonoids and phenolic acids were reported to have potent antibacterial activity and it is associated to their various mechanisms of action against both Gram-positive and Gram-negative bacteria (Adamczak *et al.*, 2019; Roberto *et al.*, 2017). Therefore, the results from the current study are in agreement with above findings from previous studies, and this confirmed why flavonoids and phenolic acids from tobacco extract were active against potato bacterial wilt.

High antibacterial activity of flavonoids was confirmed against both Gram-negative and Gram-positive bacteria (Adamczak *et al.*, 2019). In recent years, rutin is among the flavonoids which were found to be the strongest antimicrobial agents (G rniak *et al.*, 2019). For instance, rutin, quercetin, hesperidin, morin, apigenin, naringin, kaempferol, and galangin isolated from *Castanea sativa* were tested against gram-negative and gram-positive bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and *Staphylococcus aureus*). The result showed that all test compounds inhibited the growth of all bacteria but with the highest antibacterial activity in rutin, quercetin, and apigenin (Adamczak *et al.*, 2019; Taleb-Contini *et al.*, 2003). In addition, rutin, quercetin, and apigenin were more active against gram-negative than gram-positive bacteria (Taleb-Contini *et al.*, 2003). Thus, the confirmation in the present study of a higher inhibitory effect of rutin from tobacco extract against *R. solanacearum* of potato, which is a Gram-negative bacterium.

Modes of action by which flavonoids (including rutin) and phenolic acids (including chlorogenic acid) can affect plant pathogenic organisms vary with types of components and also from cells, tissues up to molecular targets in the organs (Docheva & Gagnon, 2015; Malkhan *et al.*, 2012; Ribera & Zuñiga, 2012; Witkowska *et al.*, 2013). From different literatures, it has been reported that rutin is able to disrupt lipid monolayer structure and decrease bilayer thickness of bacterial cell wall which in turn leads to a disruption of membrane integrity and increase of membrane permeability (Bouarab-Chibane *et al.*, 2019; G rniak *et al.*, 2019). Rutin was also reported to be

able to enhance or promote antibacterial activity of other flavonoids when they are combined in inhibiting bacterial DNA synthesis (Arima *et al.*, 2002). This synergistic interaction of rutin and other flavonoids is an advantage in the control of pathogens which are less susceptible to antibiotics (Arima *et al.*, 2002). Therefore, flavonoids are promising resistance-modifying agents in plant (Adamczak *et al.*, 2019; G rniak *et al.*, 2019). Chlorogenic acid mainly kills bacteria by inducing irreversible cell membrane permeability and disruption of membrane integrity and leakage of cytoplasmic ions and nucleotides (Lou *et al.*, 2011). Chlorogenic acid due to its negative surface charge binds to membrane of Gram-negative bacteria, which are usually more resistant to phenolic compounds due to the rigidity of their outer membrane and removes the cations like magnesium which leads to the loss of barriers ability against antibacterial agent (Bouarab-Chibane *et al.*, 2019; Maddox *et al.*, 2010). All these previous findings support the results of the present study, in which flavonoids (rutin and unknown flavonoid) as well as phenolic acids (chlorogenic acid and its isomer) from tobacco extract are the most promising active compounds against *R. solanacearum* of potato.

6.4 Conclusion

The aim of this study was to identify the bioactive compounds against *R. solanacearum* in selected plant extracts. Among the tested plant materials, tobacco had a stronger antibacterial activity against *R. solanacearum* of potato followed by wild marigold while garlic exhibited low efficacy against the pathogen, therefore, the bioactive compounds were analysed. From the phytochemical screening results, it is concluded that tobacco extract contains almost all tested bio-compounds followed by wild marigold while garlic contains very few bioactive compounds. Both methanol and water extracts of tobacco contain flavonoids, phenolic compounds, alkaloids, tannins, coumarins, terpenes, sulphur-containing compounds, anthraquinones, saponins, and glycosides. The initial methanol and methanol fraction of plant extracts performed better in the control of *R. solanacearum* of potato followed by diethyl ether while n-hexane was the last. Methanol fraction extract of tobacco contains three bioactive compounds with stronger activity against *R. solanacearum* namely chlorogenic acid, rutin, unknown flavonoid and one more compound with minor active response against the pathogen which is 5-caffeoylquinic acid, an isomer of chlorogenic acid. From the conclusion, methanolic extract of tobacco or its isolated

compounds are recommended as botanical pesticides in the disease management program against potato bacterial wilt.

CHAPTER SEVEN

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7.1 General Discussion

In vitro antibacterial activity of methanolic, water and chloroform extracts (solvent extracts) of ten collected plant species (plant materials) was assessed against the growth of *R. solanacearum.* In addition, effective concentration of the most promising plant extracts against bacterial growth was identified. The result showed that methanol plant extracts inhibit the growth of the pathogen at the highest level among the other solvent extracts and inhibit the growth of bacteria to the same level as the synthetic antibiotic streptomycin. This was followed by water extract whereas chloroform extract showed the lowest antibacterial activity. Similar effectiveness of methanol over water plant extracts from plant species in the control of different Gram-positive and Gram-negative bacteria was reported by Körpe *et al.* (2013) and Naz and Bano (2012). Methanol and water are organic solvents which are mainly used to extract polar compounds, whereas chloroform is used for non-polar metabolites (Cowan, 1999; Mwitari *et al.*, 2013; Ncube *et al.*, 2008). Methanol has been revealed to be the best solvent to extract a high range of polar metabolites (Arnault *et al.*, 2013; Cowan, 1999; Kukri *et al.*, 2012; Malkhan *et al.*, 2012). In general, the results from this study showed lower antibacterial performance in chloroform (non-polar solvent) than methanol (polar solvent) extracts. This suggests that the ten selected plant materials may contain much more polar antibacterial metabolites than non-polar compounds.

In the present study, the highest antibacterial activity was exhibited by tobacco, wild marigold, and garlic among ten tested plant materials. It has been reported that a large number of species in the family *Solanaceae* (to which tobacco (*Nicotiana tabacum* L.) belongs) are rich in biochemicals of medicinal values (Sharma *et al.*, 2016). Tobacco extract was mainly reported to have insecticidal activities, but it also has antibacterial and antifungal properties against human diseases (Bakht *et al.*, 2012; Sharma *et al.*, 2016; Singh *et al.*, 2010). Phytochemical analysis of leaf aqueous and methanol extracts of tobacco showed that tobacco contains flavonoids and alkaloids, which contribute directly to antibacterial activity against different strains of Gram-positive and Gram-negative bacterial strains (Sharma *et al.*, 2016; Singh *et al.*, 2010). Apart from tobacco, many authors also confirmed that wild marigold (*Tagetes minuta*) belonging in Asteraceae family has antimicrobial activities against plant pathogens (Irum & Mohammad, 2015; Zeller & Ullrich, 2006). It was found that flavonoids, flavonols, and essential or volatile

oils from *T. minuta* had a suppressive biological activity against Gram-negative bacteria and Gram-positive bacteria (Gakuubi *et al.*, 2016; Senatore *et al.*, 2003). For instance, antibacterial activity of *T. patula* against *R. solanacearum* was confirmed in *in vitro* experiment carried out by Yuliar *et al.* (2015).

Alliaceae family also contains different species (mainly garlic: *Allium sativum* L. and onion: *Allium cepa* L.) which are reported to have antibacterial properties (Abo-elyousr & Asran, 2009; Malkhan *et al.*, 2012; Rahmati *et al.*, 2015). For instance, it has been reported that *Allium* plants contain the antimicrobial properties against bacterial wilt of tomato (*R. solanacearum*) and other soil-borne pathogens as well as nematodes through biofumigation and intercropping system (Deberdt *et al.*, 2012; Rongquan *et al.*, 2011). In addition, Allicin has an effectiveness, which is equal to the other antibiotics namely kanamycin, penicillin, and ampicillin (Borlinghaus *et al.*, 2014; Curtis *et al.*, 2005). All these findings support the results from the present *in vitro* experiment in which tobacco, wild marigold and garlic extracts highly inhibited the growth of potato bacterial wilt.

In the present study, a pilot greenhouse experiment with methanolic and water extracts of tobacco, wild marigold, and garlic at three different concentrations (50, 25, 12.5 or 6.25 mg mL^{-1}) was conducted, with the main purpose of determining the effective concentration of these plant extracts against bacterial wilt. From the results, application of methanolic and water extracts from tobacco, wild marigold, and garlic at 50 mg mL^{-1} was recommended for controlling of disease incidence in potato plants, improving plant growth, high yield and tubers quality, as well as reduction of post-harvest infection and yield loss during storage. Thus, the efficacy of selected plant extracts was dose-dependent. The higher the concentration, the higher the effectiveness of plant extracts in all analysed parameters.

These results are in agreement with previous studies, which also found that the efficacy of plant extracts against different pathogens is dose-dependent (Curtis *et al.*, 2005; Gakuubi *et al.*, 2016; Sharma *et al.*, 2016). A study was conducted by Bakht *et al.* (2012) to determine the effectiveness of tobacco extracts at different concentrations (6, 12, 18 and 24 mg mL^{-1}) against different pathogenic bacteria showed that the extracts had antibacterial activities against the test

bacteria at the highest concentration (24 mg mL^{-1}). Another *in vitro* study evaluated inhibitory activity of ethanol, ethyl acetate, n-Hexane, acetone, butanol and water extracts of tobacco at different concentrations (6, 12, 18 and 24 mg mL^{-1}) on pathogenic bacteria such as *Agrobacterium* t*umefaciens, B. cereus*, *E. carotovora,* and *Staphlococcus aureus*. The results proved that tobacco had antibacterial activity against test bacteria at highest concentration (24 mg mL^{-1}) (Singh *et al.*, 2010).

It was also observed in previous studies that antibacterial activity of wild marigold (*T. Minuta*) against different bacterial pathogens increased with increasing concentration levels (Gakuubi *et al.*, 2016; Naseerud *et al.*, 2016; Senatore *et al.*, 2003). Similar concentration-dependent effect of plant active metabolites was also found in garlic and onion against bacteria and fungi (Borlinghaus *et al.*, 2014; Curtis *et al.*, 2005). This supports the results of the present study in which the higher the concentration of methanolic extract from tobacco, wild marigold and garlic, the higher was the BCE of extract against bacterial incidence in both plants and tubers especially when applied every weeks or every two weeks. The positive effect of plant extracts against bacterial wilt in turn affected positively potato growth, yield, and tuber quality parameters as well as reduced post-harvest infection and losses from the pathogen.

In the current study, the effect of three application frequencies of plant extracts (every week, every two weeks, and every month) on *R. solanacearum*, plant growth, tuber yield, and quality as well as post-harvest infection and yield loss was determined under field conditions. Weekly or every two weeks application of methanolic extract of tobacco or wild marigold at 50 mg mL^{-1} were the most effective to manage the incidence of *R. solanacearum* in potato plants and tubers. Besides evaluating types and concentrations of plant extracts, few studies were also conducted to evaluate the effect of frequency of application of plant extracts on plant growth in term of plant height and control of *R. solanaceraum* in potato. A typical example is a field experiment conducted by Istifadah *et al.* (2019) to examine the effect of concentration and frequency of application of mixture of microbial consortium (*B. subtilis, Trichoderma harzianum* and *Pseudomonas* sp.) with animal manure or compost against soil-borne (bacterial wilt) and air-borne (late blight) pathogens of potato. The concentrations used were 25, 50 and 100 g per plant applied weekly or every two weeks (from planting date). The results from previous study

revealed that effectiveness of biological control by using manure-microbe mixture is dose-dependent. They also concluded that biological control suppressed the diseases at higher rate when they were applied at higher concentration and repeated (weekly) (Istifadah *et al.*, 2019).

Previous researches revealed that plant natural compounds are not only able to protect plants against pathogens and abiotic stresses but can also induce plant growth (Mulugeta *et al.*, 2019). Plant extracts able to control potato pathogens also have in turn a positive effect on potato growth (Biruk-Masrie *et al.*, 2015; Gayatri & Rajani, 2016). It is already known that *R. solanacearum* is the main threat of potato growth and production and it causes remarkable yield and quality losses in potato (Vu *et al.*, 2017). Therefore, it is not surprising if a plant material, solvent extract and concentration or frequency of application which is able to manage bacterial wilt, would also be can best performing in potato growth. This may explain why, a weekly or every two weeks treatment of potato with methanolic extract of tobacco or wild marigold at 50 mg mL^{-1} which were found to perform the best in management of *R. solanacearum,* also showed potential in improving potato growth, tuber yield and quality. It is also apparent that good quality tubers are able to resist to post-harvest infection and yield losses during storage.

The phytochemical screening revealed that methanolic tobacco extract collected from Rwanda contains a wide range of bio-compounds namely flavonoids, alkaloids, tannins, coumarins, terpenes, carbohydrates, phenolic compounds, sulphur-containing compounds, anthraquinones, and saponins. Tobacco extract was followed by wild marigold whereas garlic contained very few phytochemicals. Most of those compounds were previously reported to have antimicrobial properties like phenols, phenolic acids, flavonoids, tannins, coumarins, alkaloids, terpenes, steroids, glycocides, and sulphur-containing compounds (Cowan, 1999; Hammer *et al.*, 1999; Malkhan *et al.*, 2012; Oboh *et al.*, 2009; Rahman *et al.*, 2012). Moreover, tobacco, marigold, and garlic materials are among plant species reported to have botanicals which are able to control different pathogenic microorganisms (Cowan, 1999; Rahman *et al.*, 2012; Sharma *et al.*, 2016; Wagura *et al.*, 2011; Yuliar *et al.*, 2015). Therefore, it is not surprising to find that tobacco followed by wild marigold extracts performed the best in the control of potato bacterial wilt in the present study. Their high antibacterial activity is attributed to their high content in various natural compounds which are already known to have antimicrobial properties. In the present

study, it was also found that methanolic extracts contained high range of major botanicals during phytochemical screening (chapter six). This may be the reason why methanolic extracts had more activity against *R. solanacearum* than the other tested plant extracts.

Fractionation of the methanolic extract yielded to 19 fractions which were further tested for their antibacterial efficacy against *R. solanacearum*. The results showed that five fractions (from 11 to 15) in decreasing order of 14, 11, 12, 13, and 15 exhibited potential inhibitory effect against *R. solanacearum* growth comparable to the antibiotic streptomycin. Briefly, HPLC results and comparison of chromatogramic peaks, UV-spectra and retention times of standards (rutin, chlorogenic acid and caffeic acid) and test active fractions showed that in the present study four compounds were active against *R. solanacearum*. Three main bioactive compounds against bacterial wilt were chlorogenic acid, rutin and one unknown flavonoid. The minor compounds were a molecule which may be 5-caffeoylquinic acid, an isomer of chlorogenic acid. Different researchers proved by HPLC results that tobacco mainly contains chlorogenic acid (phenolic acid) and rutin (flavonoids) in very high concentration estimated at 75–95% among the polyphenolic compounds (Chen *et al.*, 2007; Li *et al.*, 2003). The major polyphenols in tobacco are flavonoids (rutin and kaempferol-3 rutinoside) and phenolic acids (chlorogenic acid, its isomers 4-O-Caffeoylquinic acid, neochlorogenic acid and 5-caffeolyquininc acid) (Chen *et al.*, 2007; Docheva & Gagnon, 2015).

The findings from isolation and identification of bioactive compounds against *R. solanacreaum* of potato confirmed that the main antibacterial activity was in flavonoids (rutin and another unkown flavonid) and phenolic acids (chlorogenic acid and its isomers). Both flavonoids and phenolic acids are reported to have potent antibacterial activity, which is associated with their various mechanisms of action against both Gram-positive and Gram-negative bacteria (Adamczak *et al.*, 2019; Roberto *et al.*, 2017). High antibacterial activity of flavonoids was confirmed against both Gram-negative and Gram-positive bacteria (G rniak *et al.*, 2019; Taleb-Contini *et al.*, 2003). This high antibacterial activity of flavonoids against both Gram-positive and Gram-negative bacteria was related to their ability to inhibit cytoplasmic membrane function by increasing membrane permeability and ions leakage (G rniak *et al.*, 2019). For instance, rutin (quercetin-3-O-rhamnoglucoside) was reported to be able to disrupt lipid monolayer structure

and decrease bilayer thickness of bacterial cell wall which in turn leads to a disruption of membrane integrity and increase of membrane permeability (G rniak *et al.*, 2019). For phenolic acids, it was shown that chlorogenic acid is able to bind to membrane and rend it more permeable. Chlorogenic acid mainly kills bacteria by inducing irreversible cell membrane permeability and disruption of membrane integrity and leakage of cytoplasmic ions and nucleotides (Lou *et al.*, 2011). Therefore, all these previous results explain why in our study, flavonoids and phenolic acids in methanol fraction of tobacco extract were the main active molecules against *R.solanacearum* of potato among the others.

7. 2 Conclusions

The present study aimed at evaluation of efficacy of selected plant extracts for management of potato bacterial wilt (*Ralstonia solanacearum* Smith) and improvement of potato production and tuber quality in Rwanda. From the results, the following conclusions are made:

i) Tobacco, wild marigold, and garlic extracts have the highest antibacterial activity against *R. solanacearum* among the ten plants tested. In addition, methanolic extract of tobacco, wild marigold, and garlic had the best performance in the control of bacterial wilt of potatoes.

ii) The highest concentration (50 mg mL^{-1}) of methanolic and water extracts from tobacco, wild margold and garlic extracts exhibited the best antibacterial activity and improvement of potato growth, yield, and quality parameters as well as control against post-harvest infection and yield losses during storage.

iii) Weekly or bi-weekly application of methanolic extract of tobacco or wild marigold at 50 mg mL^{-1} were the most effective treatments for optimum potato growth, production, yield, and tuber quality. The applications are also the best in protection of potato plants and tubers against *R. solanacearum* during potato growth under field conditions and during storage.

iv) Methanolic extract of tobacco contains three bioactive compounds with stronger antibacterial activity against *R. solanacearum* namely chlorogenic acid, rutin, and unknown flavonoid, and one more compound with minor activity against the pathogen which may be 5-caffeoylquinic acid, an isomer of chlorogenic acid.

7.3 Recommendations

i) Methanolic extract from tobacco, wild marigold, and garlic are recommended in management of *R. solanacearum* than water or chloroform extracts.

ii) A concentration of 50 mg mL^{-1} of methanolic and water extracts from tobacco, wild margold and garlic extracts is recommended for the control of bacterial wilt and improvement of plant growth, yield, and tuber quality under greenhouse and field conditions. It also recommended for reduction of post-harvest infection and yield losses of potato due to *R. solanacearum*.

iii) Weekly or bi-weekly plication of methanolic extract from tobacco and wild marigold at 50 mg mL^{-1} is recommended as the best approach in management of potato bacterial wilt and improvement of potato growth, yield and tuber quality. It is aslo recommended for reduction of post-harvest infection, and yield losses due to *R. solanacearum* in potato production.

iv) Rutin, chlorogenic acid, unknown flavonoid, and 5-caffeoylquinic acid are the bioactive compounds in methanolic extract of tobacco, which are recommended for formulation as botanical pesticides for management of potato bacterial wilt in disease management program to reduce environmental pollution and other side effects associated with synthetic pesticides.

7.4 Areas for Further Studies

i) Further studies on biovar and pathogenicity of *R. solanacearum* should be done using a high number of bacterial wilt isolates from different agro-ecological zones. Similar studies should be carried out by using different hosts to determine whether there are other biovars and races of the pathogen that affect potato plants in Rwanda or more virulent isolates than *Rs*Gikungu, *Rs*Kinigi and *Rs*Kirundo isolates used in this study.

ii) Proper formulations of rutin, chlorogenic acid, unknown flavonoid, and 5-caffoylquinic acid, is necessary to convert these active botanicals into commercially usable products at affordable price for smallholder farmers to improve potato yield and quality and with acceptable levels of toxicity at both local and international markets.

iii) Further studies should be carried out to identify the unknown flavonoid, which is active against the pathogen.

iv) Further studies should be done to identify the bioactive compounds in methanol extract from wild marigold as well as their mode of action against the pathogen.

iv) Studies should also be perfomed to determine the long-term positive or negative effects in environmental welfare which may be associated with the treatment of potatoes with methanolic extracts of tobacco and wild marigold or their derivative compounds. The production and maiantenance cost of production should also analysed.

iv) Advanced studies should also be conducted to determine the mode of action of the bioactive compounds (Rutin, chlorogenic acid, unkown flavonoids, and 5-caffoylquinic acid) in tobacco plant against *R. solanacearum* of potato.

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APPENDICES

Appendix A. Published paper 1

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Full Length Research Paper

Virulence and characterization of isolates of potato bacterial wilt caused by *Ralstonia solanacearum* (Smith) in Rwanda

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Bacterial wilt (*Ralstonia solanacearum*) is one of the major potato diseases in Rwanda. An *in vitro* study was carried out to identify and characterize the pathogen isolated from three potato cultivars (Kinigi, Kirundo and Gikungu) in Rwanda. This was achieved by cultural and morphological tests on triphenyl tetrazolium chloride (TTC) and casamino peptone glucose (CPG) agar as well as biochemical tests through Gram staining and biovar test. An *in vivo* experiment was also performed to assess the pathogenicity of those isolates on potatoes. All isolates showed typical morphological traits of virulent *R. solanacearum* on TTC and CPG media. The test isolates were Gram-negative bacteria. Biovar test confirmed that all the isolates belonged to race 1 biovar 3 of the pathogen. Furthermore, the highest disease severity (DS=100%) and disease incidence (DI=100%) were observed in Gikungu isolate followed by Kinigi (DS=97.33% and DI=98.25) and Kirundo (DS=94.67% and DI=92.61%). From this study, all three isolates were typical *R. solanaceraum* belonging to race 1 biovar 3 and were all pathogenic to potato plants. Gikungu and Kinigi isolates were highly virulent than Kirundo isolate. Therefore, Gikungu or Kinigi isolates can be used for further studies in plant protection in management of the disease.

Key words: Biovar test, Gram-negative, Gram-positive, pathogenicity test.

Appendix B. Published paper 2

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In vitro antibacterial activity of selected plant extracts against potato bacterial wilt (*Ralstonia solanacearum* Smith) in Rwanda

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Abstract

Bacterial wilt caused by *Ralstonia solanacearum* Smith is the most severe potato disease in Rwanda because there is no known pesticide for it and cultural control methods seem almost impossible to implement. Therefore, use of plant extracts with antibacterial activities which are locally available, economically affordable and environmental friendly could be an alternative in the management of the disease. This research focused on *in vitro* screening of the antibacterial activity of methanol, water and chloroform extracts of ten local plant materials against the pathogen. From this screening, methanol and water extracts of three promising plant species, *i.e.*, tobacco, wild marigold and garlic were also used for determination of minimal inhibitory concentration (MIC). The results showed higher inhibition zone of methanol extracts (16.85 mm) against bacteria followed by water (14.42 mm) and chloroform (14.19 mm) extracts. All ten plant extracts inhibited the growth of the pathogen. Higher antibacterial activity was found in tobacco, wild marigold and garlic extracts (19.61, 18.56, and 18.3 mm inhibition zones respectively). Minimal inhibitory concentration (MIC) of methanol extracts from tobacco and wild marigold was 6.25 mg mL^{-1} whereas, garlic methanol extract was 12.5 mg mL^{-1}. Furthermore, MIC of water extract was 12.5 mg/mL^{-1} in all three plant species. The findings from the test of plants confirmed that tobacco, garlic and wild marigold extracts are the best in the control of potato bacterial wilt. Moreover, methanol extracts are the most efficient in management of potato bacterial wilt in comparison to water and chloroform extracts.

Key words: Antibacterial activity, botanicals, growth inhibition zone, minimum inhibitory concentration, potato, *Ralstonia solanacearum*.

Appendix C: Collected plant species from Rwanda for *in vitro* antibacterial screening against *Ralstonia solanacearum* (Objective 1). (A) African basil (*Ocimum gratissimum* L.), (B) Lion's ear (*Leonotis nepetifolia* R.Br.), (C) Castor bean (*Ricinus communis* L.), (D) Wild marigold (*Tagetes minuta* L.), (E) Onion (*Allium cepa* L.), (F) Garlic (*Allium sativum* L.), (G) Rosemary (*Rosmarinus officinalis* L.), (H) Stinging nettle (*Urtica massaica* Mildbr.), (I) Lemongrass (*Cymbopogon citratus* Stapf), and (J) Tobacco (*Nicotiana tabacum* L.).

Appendix D. Raw data of effect of plant extracts (S), solvent extracts (E) and concentrations (C) on disease incidence (DI %) at 10, 15, 20, 25, and 30 days after inoculation (DAI) in greenhouse experiment (Objective 2). B= Block, C= Concentrations (1, 2, and 3= 50, 25, 12.5 or 6.25 mg mL^{-1}, respectively), S=Plant extracts (1, 2, and 3=Tobacco, wild marigold, and garlic, respectively), E= Solvent extracts (1= Methanol, 2= Water)

B	C	S	E	5DAI	10DAI	15DAI	20DAI	25DAI	30DAI
1	1	1	1	0	1.99	3.38	4.35	6.43	14.46
1	1	1	2	0	3.66	6.07	8.78	13.02	26.51
1	1	2	1	0	1.69	3.33	6.23	11.50	19.08
1	1	2	2	0	4.57	10.07	16.19	19.74	29.33
1	1	3	1	0	2.37	5.03	7.99	14.74	25.43
1	1	3	2	0	6.99	16.53	22.46	25.50	37.64
1	2	1	1	0	2.00	5.30	14.92	16.53	24.03
1	2	1	2	0	6.63	7.11	16.01	20.60	32.34
1	2	2	1	0	5.67	10.82	18.70	19.59	31.11
1	2	2	2	0	1.79	8.31	10.67	17.37	27.32
1	2	3	1	0	5.42	15.10	16.69	24.57	38.24
1	2	3	2	0	7.74	18.39	22.93	27.98	40.81
1	3	1	1	0	2.49	7.22	11.23	14.62	24.95
1	3	1	2	0	3.71	7.99	11.65	16.12	24.47
1	3	2	1	0	2.04	6.27	11.70	15.58	22.75
1	3	2	2	0	5.53	16.40	18.36	22.58	32.38
1	3	3	1	0	1.82	6.58	9.78	16.23	23.11
1	3	3	2	0	8.10	19.51	23.52	27.48	41.82
2	1	1	1	0	1.44	3.09	5.01	6.58	11.49
2	1	1	2	0	2.13	4.55	9.39	19.97	24.21
2	1	2	1	0	2.80	6.19	10.08	18.15	33.95
2	1	2	2	0	4.58	8.75	11.41	16.53	23.77
2	1	3	1	0	2.30	10.51	12.11	16.61	38.85
2	1	3	2	0	8.98	16.40	20.63	23.95	44.60
2	2	1	1	0	2.04	6.84	13.55	15.59	29.00
2	2	1	2	0	5.87	6.68	9.94	13.38	20.44
2	2	2	1	0	2.23	9.18	12.27	19.74	31.87
2	2	2	2	0	6.92	19.78	22.61	26.55	35.99
2	2	3	1	0	3.82	7.97	12.68	18.76	40.25
2	2	3	2	0	6.17	16.83	19.23	20.60	46.03
2	3	1	1	0	3.33	6.48	15.74	17.63	25.49
2	3	1	2	0	2.27	6.96	7.70	16.36	25.98
2	3	2	1	0	3.28	6.99	8.56	13.00	18.96
2	3	2	2	0	2.14	19.17	22.97	28.44	37.74

2	3	3	1	0	1.95	4.19	8.04	18.30	43.65
2	3	3	2	0	8.33	15.784	18.89	24.58	49.91
3	1	1	1	0	3.04	6.47	6.46	8.46	17.38
3	1	1	2	0	1.83	4.01	5.614	14.03	21.22
3	1	2	1	0	2.09	7.95	8.21	14.06	23.43
3	1	2	2	0	4.77	15.70	17.56	23.41	34.70
3	1	3	1	0	2.78	14.04	11.49	21.47	33.09
3	1	3	2	0	12.23	15.65	17.52	19.69	26.17
3	2	1	1	0	4.38	11.81	15.56	20.92	32.47
3	2	1	2	0	1.94	10.76	14.94	17.71	24.73
3	2	2	1	0	2.18	7.87	12.56	16.51	23.76
3	2	2	2	0	1.90	12.77	18.54	22.60	32.05
3	2	3	1	0	3.07	7.70	12.93	19.61	36.84
3	2	3	2	0	15.02	21.84	23.48	30.77	44.68
3	3	1	1	0	3.50	15.81	20.23	23.43	35.28
3	3	1	2	0	1.93	9.66	14.61	16.63	23.23
3	3	2	1	0	9.80	15.97	19.16	21.78	32.93
3	3	2	2	0	6.84	16.13	20.48	26.96	39.28
3	3	3	1	0	6.31	15.65	21.71	29.462	40.14
3	3	3	2	0	16.36	21.97	25.82	30.97	45.42

Appendix E. Raw data and ANOVA tables of effect of different concentrations of plant extracts on control of disease incidence (DI) under greenhouse conditions on various days after inoculation (DAI) (Objective 2). Treatment (1, 2, and 3 = concentrations of plant extracts at 50, 25, 12.5 or 6.25 mg mL^{-1}, respectivley) (4 and 5= Streptomycin and DMSO used as postivie and negative controls respectively). Days after inoculation= 5, 10, 15, 20, 25, and 30 DAI. * = significantly different

Obs	Block	Treatment	5DAI	10DAI	15DAI	20DAI	25DAI	30DAI
1	1	1	0.0000	2.6000	6.2300	7.4300	9.4500	21.320
2	1	2	0.0000	3.9000	9.2000	10.7900	12.6700	32.270
3	1	3	0.0000	6.5700	13.3600	17.1000	19.7500	38.710
4	1	4	1.0030	12.8457	28.6712	41.8458	56.0010	67.967
5	1	5	1.1905	29.1864	34.6993	60.7320	75.1709	82.171
6	2	1	0.0000	4.0000	8.0000	15.4300	17.4000	23.320
7	2	2	0.0000	4.1100	12.0000	16.8900	20.6700	26.670
8	2	3	0.0000	5.6700	12.4670	15.1100	23.9500	29.410
9	2	4	1.2379	14.9009	30.6243	51.4339	73.9100	35.215
10	2	5	5.0423	25.4283	53.4974	68.0725	78.5677	89.568
11	3	1	0.0000	2.4300	7.4600	11.4300	19.5000	28.320
12	3	2	0.0000	3.7800	12.4000	16.6900	25.6700	29.470
13	3	3	0.0000	7.7700	15.7840	19.1200	24.8500	48.010
14	3	4	1.5000	0.0000	24.0087	38.0064	59.3970	62.820
15	3	5	10.8314	20.3896	45.6614	64.8942	73.4738	79.474

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Dependent Variable: DI5DAI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	231.7576466	33.1082352	5.64	0.0075
Error	10	58.6782786	5.8678279		

Corrected Total	17	290.4359252			

R-Square	Coeff Var	Root MSE	5DAI Mean
0.797965	23.89547	2.422360	2.579847

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	30.8291142	15.4145571	2.63	0.1211
Treatment	5	200.9285323	40.1857065	6.85	0.0051*

....

Dependent Variable: 10DAI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	3123.304650	446.186379	36.71	<.0001
Error	10	121.545514	12.154551		
Corrected Total	17	3244.850164			

R-Square	Coeff Var	Root MSE	DI2 Mean
0.962542	24.19657	3.486338	14.40840

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	53.730864	26.865432	2.21	0.1604
Treatment	5	3069.573786	613.914757	50.51	<.0001*

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Dependent Variable: 15DAI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	6067.158687	866.736955	25.44	<.0001
Error	10	340.714211	34.071421		
Corrected Total	17	6407.872898			

R-Square	Coeff Var	Root MSE	15DAI Mean
0.946829	21.82520	5.837073	26.74464

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	211.706988	105.853494	3.11	0.0892
Treatment	5	5855.451699	1171.090340	34.37	<.0001*

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Dependent Variable: DI20DAI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	11808.84342	1686.97763	151.85	<.0001*
Error	10	111.09719	11.10972		
Corrected Total	17	11919.94061			

R-Square	Coeff Var	Root MSE	DI20DAI Mean
0.990680	8.763877	3.333125	38.03253

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	93.49234	46.74617	4.21	0.0472
Treatment	5	11715.35107	2343.07021	210.90	<.0001*

………..

Dependent Variable: DI25DAI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	15466.45889	2209.49413	110.61	<.0001*
Error	10	199.75562	19.97556		
Corrected Total	17	15666.21451			

R-Square	Coeff Var	Root MSE	DI25DAI Mean
0.987249	9.409879	4.469403	47.49692

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	2	153.57835	76.78917	3.84	0.0578
Treatment	5	15312.88054	3062.57611	153.32	<.0001*

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Dependent Variable: DI30DAI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	14170.25576	2024.32225	28.65	<.0001
Error	10	706.63659	70.66366		
Corrected Total	17	14876.89235			

R-Square	Coeff Var	Root MSE	DI30DAI Mean
0.952501	15.23581	8.406168	55.17375

Source	DF	Type III SS	Mean Square	F Value	Pr > F
B	2	184.73150	92.36575	1.31	0.3131
Treatment	5	13985.52426	2797.10485	39.58	<.0001*

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Appendix F. Mean separation for effect of different concentrations of plant extracts on number of days to 50% sprouting and flowering in greenhouse experiment (Objective 2)

Plant extracts	**DAP 50% Sprouting**	**DAP 50% flowering**
Tobacco	18.72± 2.34 a	65.00± 1.00 a
Wild marigold	16.45 ± 2.19 ab	59.67±2.08 ab
Garlic	13.00± 1.23 bc	54.56±2.50 b
Controls (+; -)		
Streptomycin (+)	14.67 ± 3.51 b	56.33±6.33 b
DMSO (-)	10.44± 0.57c	46.00±3.46 c
P =0.05	0.013	0.0090
Solvent extracts	**DAP 50% Sprouting**	**DAP 50% flowering**
Methanol	14.000± 1.15 a	60.30± 2.44a
Water	12.333± 1.73 a	59.19±1.74 a
Controls (+; -)		
Streptomycin (+)	14.67 ± 3.51 a	56.33±6.33 ab
DMSO (-)	10.44± 0.57a	46.00±3.46 b
P =0.05	0.3889	0.0192

Concentrations (mg mL^{-1})	**DAP 50% Sprouting**	**DAP 50% flowering**
50	18.00± 2.21a	62.50± 2.78 a
25	16.33 ± 2.89 ab	60.56±2.61 ab
12.5/6.5	13.39 ± 2.06 bc	56.17±1.94 ab
Controls (+; -)		
Streptomycin	14.67 ± 3.51 abc	56.33±6.33 ab
DMSO	10.44 ± 1.15 c	46.00±3.46 c
P =0.05	0.04	0.0193

The values are average of days to 50 % sprouting or flowering (± SD) in each treatment. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Studentized Range (HSD) test. (+, -): Positive (1 % Streptomycin) and negative (1% DMSO) controls. SD: Standard deviation. DAP= Days after planting.

Appendix G. Mean separation for effect of different concentrations of plant extracts on yield (t ha^{-1}) in greenhouse experiment (Objective 2)

Plant extracts	#Tubers per plant	Yield (g) per plant
Tobacco	8.00± 1.1 ab	191.85± 11.88a
Wild marigold	9.33±1.15 a	163.61±11.12ab
Garlic	8.33± 0.58 ab	133.89±2.09 bc
Controls (+; -)		
Streptomycin	7.333±1.53ab	123.33± 39.3 bc
DMSO	6.67±2.08 b	52.78± 19.32 d
P =0.05	0.0487	0.0004
Concentrations (mg mL^{-1})	**#Tubers per plant**	**Yield (g) per plant**
50	9.03±1.73a	181.57±18.9a
25	8.000±1a	154.81±11.97b
12.5/6.25	9.00±2.65a	152.96±15.31b
Controls (+; -)		
Streptomycin	7.333±1.53a	123.33± 39.3bc
DMSO	6.667±2.08 b	52.78± 19.32d
P =0.05	0.0391	0.0014
Solvent extracts	**#Tubers per plant**	**Yield (g) per plant**
Methanol	8.67±0.58 a	175.37± 8.81a
Water	8.42±1.15 ab	150.86± 15.33 ab
Controls (+; -)		
Streptomycin	7.33 ±1.53 ab	123.33± 39.3 bc
Water	6.667±2.08 b	52.78± 19.32 d
P =0.05	0.0496	0.0025

The values are average of days to 50 % sprouting or flowering (± SD) in each treatment. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Studentized Range (HSD) test. (+, -): Positive (1 % Streptomycin) and negative (1% DMSO) controls. SD: Standard deviation.

Appendix H. Mean separation of effect of different concentrations of plant extracts, on tuber size (quality parameter) in greenhouse experiment (Objective 2)

Plant extracts	Small (%)	Medium (%)	Large (%)	Marketable (%)
Tobacco	20.65±6.98b	69.17± 6.64 a	9.295± 4.24a	78.43± 6.18a
Marigold	37.96±3.70b	52.78±1.15 ab	9.259±6.42 a	62.04± 3.70a
Garlic	43.52± 6.48b	52.39±5.15 ab	4.093± 1.46a	56.48± 6.48 a
Controls (+; -)				
Streptomycin	33.33±19.25b	55.56±29.4 ab	11.11± 11.11a	66.67±19.25 a
DMSO	98.02±1.89 a	1.98±1.09 b	0.000±0.00 a	1.98±1.09 b
P =0.05	0.0020	0.0438	0.5948	0.0027
Concentration (mg mL^{-1})	**Small (%)**	**Medium (%)**	**Large (%)**	**Marketable (%)**
50	20.83±8.45b	69.91± 2.12 a	9.259± 3.70a	79.17±4.88a
25	34.26±1.60 b	63.89±2.78 ab	1.852±3.21a	65.74±1.60a
12.5/6.25	45.74±4.32 b	48.31±3.92ab	5.944± 0.63a	54.26± 4.32a
Controls				
Streptomycin	33.33± 19.25 b	55.56±29.4 ab	11.11± 11.11 a	66.67±19.25a
DMSO	98.02±1.89 a	1.98±1.09 b	0.000±0.00 a	1.98±1.09b
P =0.05	0.003	0.0443	0.6080	0.0025
Solvent extract	**Small (%)**	**Medium (%)**	**Large (%)**	**Marketable (%)**
Methanol	27.78±4.90b	62.96± 2.83a	8.642± 3.27a	71.6±8.75a
Water	40.12±5.49b	53.70±3.21a	5.556±1.85a	59.26±8.49a
Controls				
Streptomycin	33.33± 19.25b	55.56±29.4a	11.11± 11.11a	66.67±19.25a
DMSO	98.02±1.89 a	1.98±1.09b	0.000±0.00 a	1.98 ±1.09b
P =0.05	0.0088	0.0356	0.6505	0.0085

Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Studentized Range (HSD) test. (+, -): Positive (1 % Streptomycin) and negative (1% DMSO) controls. SD: Standard deviation.

Appendix I. Mean separation of effect of different concentrations of plant extracts on potato tuber post-harvest infection (PHI) and yield loss (PHL) in greenhouse experiment (Objective 2)

Plant material	**PHI (%)**	**Concentration (mg mL^{-1})**	**PHI (%)**	**Solvent extract**	**PHI (%)**
Tobacco	25.32±1.63 d	50	27.90± 1.52 d	Methanol	28.64±0.77c
Marigold	29.23±1.69 d	25	30.77±2.36 cd	Water	31.18±1.70 c
Garlic	35.19±2.33 c	12.5/6.25	31.77±1.53 c		
Controls (+; -)					
Streptomycin	68.42±1.7 b	Streptomycin	68.42±1.7 b	Streptomycin	68.42±1.70 b
DMSO	75.27±3.14a	DMSO	75.27± 3.14 a	DMSO	75.27± 3.14 a
P =0.05	<0.0001	P =0.05	<0.0001	P =0.05	<0.0001

Plant material	**PHL (%)**	**Concentration (mg mL^{-1})**	**PHL (%)**	**Solvent extract**	**PHL (%)**
Tobacco	37.38±1.99 d	50	41.80±1.51c	Methanol	42.56±1.45 c
Marigold	43.88±2.10cd	25	43.46±2.98c	Water	44.25±1.62 c
Garlic	48.97±1.69c	12.5/6.25	44.77±1.43c		
Controls (+; -)					
Streptomycin	61.80±2.80b	Streptomycin	61.80±2.80b	Streptomycin	61.80±2.80 b
DMSO	89.78± 3.57 a	DMSO	89.78± 3.57 a	DMSO	89.78± 3.57 a
P =0.05	<0.0001	**P =0.05**	<0.0001	**P =0.05**	<0.0001

The values are average of days to 50 % sprouting or flowering (± SD) in each treatment. Values within a column followed by the same letter are not significantly different at p 0.05 according to Tukey's Studentized Range (HSD) test. (+, -): Positive (1 % Streptomycin) and negative (1% DMSO) controls. SD: Standard deviation.

Appendix J. Raw data of effect of different application frequency of plant extracts on total yield (t ha-1) in season B (Objective 3). S= Plant extracts (S1, S2, and S3= Tobacco, wild marigold and galic, respectively), E= Solvent extracts (E1= methanol, E2= Water), F= application frequency (F1, F2, F3= Weekly, every two weeks and montly application of plant extracts, respectively). B = Block, C1= Negative control (DMSO), C2= Positive control (Copper oxychloride)

		B1		B2		B3	
	F	Yield (g)	Yield t ha-1	Yield (g)	Yield t ha-1	Yield (g)	Yield t ha-1
S1 E1	F1	2873.18	21.55	4446.83	33.35	2572.97	19.30
	F2	5537.08	41.53	1748.66	13.12	4209.39	31.57
	F3	1301.31	9.76	2746.07	20.60	3840.84	28.81
S1 E2	F1	3111.87	23.34	3402.27	25.52	3772.18	28.29
	F2	2446.47	18.35	4691.79	35.19	1509.7	11.32
	F3	4183.15	31.37	2666.45	20.00	2843.53	21.33
S2 E1	F1	3221.55	24.16	5384.48	40.38	1963.42	14.73
	F2	2700.18	20.25	3541.36	26.56	4360.18	32.70
	F3	1389.78	10.42	3837.66	28.78	4145.17	31.09
S2 E2	F1	2894.87	21.71	2641.87	19.81	3422.7	25.67
	F2	2821.55	21.16	901.23	6.76	3381.84	25.36
	F3	3864.39	28.98	2500.74	18.76	2754.51	20.66
S3 E1	F1	4738.8	35.54	6730.26	50.48	3940.21	29.55
	F2	6665.98	50.00	3657.72	27.43	2357.18	17.68
	F3	1722.79	12.92	3047.8	22.86	2092.13	15.69
S3 E2	F1	4276.08	32.07	3720.61	27.91	3407.08	25.55
	F2	1709.36	12.82	3582.07	26.87	2084.53	15.63
	F3	2313.06	17.35	4416.25	33.12	5998.51	44.99
C1	F1	544.27	4.08	4686.15	35.15	2091.34	15.69
	F2	567.39	4.26	4300.65	32.26	1427.52	10.71
	F3	738.31	5.54	3160.87	23.71	1626.52	12.20
C2	F2	1724.79	12.94	2631.77	19.74	3199.23	23.99

Appendix K. Data of interactions between analysed factors in days to 50 % sprouting and flowering in field experiment (Objective 3). S= Plant extracts, E= Solvent extracts, F= application frequency, NS= Not significantly different, *= significantly different

Days to 50% sprouting

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	0.0370370	0.0370370	0.01	0.9275 NS
BLOCK	2	9.4629630	4.7314815	1.06	0.3505 NS
S	2	25.3518519	12.6759259	2.85	0.0445 *
E	1	7.2592593	7.2592593	1.63	0.2055 NS
F	2	163.0185185	81.5092593	18.33	<.0001 *
SEASON*E	1	11.7962963	5.8981481	1.33	0.2719 NS
SEASON*S	2	0.9259259	0.9259259	0.21	0.6495 NS
SEASON*F	2	53.6851852	26.8425926	6.04	0.0038 *
S*E	2	0.6851852	0.3425926	0.08	0.9259 NS
S*F	4	27.9814815	6.9953704	1.57	0.1910 NS
S*E*F	4	45.8888889	7.6481481	1.72	0.1290 NS
SEASON*S*F	4	13.3148148	3.3287037	0.75	0.5622 NS
SEASON*S*E*F	10	23.9074074	2.9884259	0.67	0.7142 NS

Days to 50% flowering

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	4576.009259	4576.009259	138.50	<.0001 *
BLOCK	2	46.500000	23.250000	0.70	0.4982 NS
S	2	127.166667	63.583333	1.92	0.1536 NS
E	1	18.750000	18.750000	0.57	0.4538 NS
F	2	679.500000	339.750000	10.28	0.0001 *
SEASON*S	2	5.796296	2.898148	0.09	0.9161 NS
SEASON*E	1	0.083333	0.083333	0.08	0.9601 NS
SEASON*F	2	106.462963	53.231481	1.61	0.2070 NS
S*E	2	123.388889	61.694444	1.87	0.1622 NS
S*F	4	302.000000	75.500000	2.29	0.0687 NS
S*E*F	4	194.611111	32.435185	0.98	0.4443 NS
SEASON*S*F	4	53.481481	13.370370	0.40	0.8047 NS
SEASON*S*E*F	10	218.333333	27.291667	0.83	0.5825 NS

Appendix L. Data of interactions between analysed factors in plant height (28, 56, and 70 DAP) in field experiment (Objective 3). DAP= Days after planting. S= Plant extracts, E= Solvent extracts, F= application frequency, NS= Not significantly different, *= significantly different.

Plant height (cm) 28 DAP

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	2088.827171	2088.827171	316.23	<.0001*
BLOCK	2	121.510194	60.755097	9.20	0.0003*
S	2	175.904099	87.952049	13.32	<.0001*
E	1	92.253398	92.253398	13.97	0.0004*
F	2	128.399419	64.199709	9.72	0.0002*
SEASON*S	2	88.008651	44.004325	6.66	0.0022*
SEASON*E	1	3.366091	3.366091	0.51	0.4777 NS
SEASON*F	2	19.114697	9.557348	1.45	0.2422 NS
S*E	2	56.259322	28.129661	4.26	0.0180*
S*F	4	63.084378	15.771094	2.39	0.0592 NS
S*E*F	4	96.534074	16.089012	2.44	0.0339*
SEASON*S*E	2	7.057856	3.528928	0.53	0.5885 NS
SEASON*S*E*F	10	146.075859	14.607586	2.21	0.0267*

Plant height (cm) 56 DAP

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	2712.848583	2712.848583	98.98	<.0001 *
BLOCK	2	104.943836	52.471918	1.91	0.1550 NS
S	2	138.150433	69.075217	2.52	0.0477 NS
E	1	150.954015	150.954015	5.51	0.0218 *
F	2	253.704774	126.852387	4.63	0.0129 *
SEASON*S	2	321.297501	160.648751	5.86	0.0044 *
SEASON*E	1	24.573768	24.573768	0.90	0.3469 NS
SEASON*F	2	37.329851	18.664925	0.68	0.5094 NS
S*E	2	49.573551	24.786775	0.90	0.4095 NS
S*F	4	91.110057	22.777514	0.83	0.5099 NS
S*E*F	4	446.169784	74.361631	2.71	0.0199 *
SEASON*S*E	2	5.297316	2.648658	0.10	0.9080 NS
SEASON*S*E*F	10	271.687402	27.168740	0.99	0.4593 NS

Plant height (cm) 70 DAP

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	1176.780093	1176.780093	26.12	<.0001 *
BLOCK	2	428.019306	214.009653	4.75	0.0116 *
S	2	447.192917	223.596458	4.96	0.0097 *
E	1	615.378148	615.378148	13.66	0.0004 *
F	2	876.482639	438.241319	9.73	0.0002 *
SEASON*S	2	101.076713	50.538356	1.12	0.3315 NS
SEASON*E	1	2.900833	2.900833	0.06	0.8004 NS
SEASON*F	2	34.278935	17.139468	0.38	0.6850 NS
S*E	2	5.695324	2.847662	0.06	0.9388 NS
S*F	4	44.961528	11.240382	0.25	0.9091 NS
S*E*F	4	741.238611	123.539769	2.74	0.0189 *
SEASON*S*E	2	1.997639	0.998819	0.02	0.9781 NS
SEASON*S*E*F	10	371.339954	37.133995	0.82	0.6067 NS

Appendix M. Data of interactions between analysed factors in number of stems per plant in field experiment (Objective 3). S= Plant extracts, E= Solvent extracts, F= Application frequency NS= Not significantly different, *= Significantly different

Number of stems per plant 28 DAP

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	0.10808899	0.10808899	0.32	0.5706 NS
BLOCK	2	0.88310185	0.44155093	1.33	0.2719 NS
S	2	0.05671296	0.02835648	0.09	0.9184 NS
E	1	0.18061986	0.18061986	0.54	0.4638 NS
F	2	3.28279321	1.64139660	4.93	0.0099 *
SEASON*S	2	0.70846193	0.35423097	1.06	0.3505 NS
SEASON*E	1	0.01446759	0.01446759	0.04	0.8354 NS
SEASON*F	2	0.57343107	0.28671554	0.86	0.4270 NS
S*E	2	0.28176440	0.14088220	0.42	0.6565 NS
S*F	4	0.29938272	0.07484568	0.22	0.9236 NS
S*E*F	6	1.18171296	0.19695216	0.59	0.7358 NS
SEASON*S*E	2	0.17013889	0.08506944	0.26	0.7752 NS
SEASON*S*E*F	10	3.64416152	0.36441615	1.09	0.3782 NS

Number of stems per plant 35 DAP

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	0.09284979	0.09284979	0.22	0.6370 NS
BLOCK	2	1.41293724	0.70646862	1.71	0.1884 NS
S	2	0.10120885	0.05060442	0.12	0.8849 NS
E	1	0.01260288	0.01260288	0.03	0.8619 NS
F	2	2.31648663	1.15824331	2.80	0.0675 NS
SEASON*S	2	0.06262860	0.03131430	0.08	0.9271 NS
SEASON*E	1	0.12448560	0.12448560	0.30	0.5849 NS
SEASON*F	2	0.21540638	0.10770319	0.26	0.7713 NS
S*E	2	0.37281379	0.18640689	0.45	0.6388 NS
S*F	4	0.41808128	0.10452032	0.25	0.9070 NS
S*E*F	6	0.99305556	0.16550926	0.40	0.8763 NS
SEASON*S*E	2	0.15213477	0.07606739	0.18	0.8323 NS
SEASON*S*E*F	10	5.67425412	0.56742541	1.37	0.2109 NS

Number of stems per plant 42 DAP

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	0.86523230	0.86523230	2.71	0.1042 NS
BLOCK	2	1.17952078	0.58976039	1.85	0.1653 NS
S	2	0.29720041	0.14860021	0.47	0.6298 NS
E	1	0.17386564	0.17386564	0.54	0.4630 NS
F	2	4.94188282	2.47094141	7.74	0.0009 *
SEASON*S	2	0.71386337	0.35693169	1.12	0.3327 NS
SEASON*E	1	0.20164897	0.20164897	0.63	0.4295 NS
SEASON*F	2	1.59464300	0.79732150	2.50	0.0896 NS
S*E	2	1.46464208	0.73232104	2.29	0.1084 NS
S*F	4	1.16769928	0.29192482	0.91	0.4605 NS
S*E*F	6	3.56868765	0.59478128	1.86	0.0995 NS
SEASON*S*E	2	0.96463467	0.48231734	1.51	0.2279 NS
SEASON*S*E*F	10	5.91689897	0.59168990	1.85	0.0670 NS

Appendix N. Data of interactions between analysed factors in yield parameters (number of tubers per plant and total yield t ha^{-1}) in field experiment (Objective 3). S= Plant extracts, E= Solvent extracts, F= application frequency NS= Not significantly different, *= Significantly different.

Number of tubers per plants

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	308.6729038	308.6729038	57.57	<.0001*
BLOCK	2	5.9129372	2.9564686	0.55	0.5786 NS
S	2	16.6806842	8.3403421	1.56	0.2183 NS
E	1	0.1672454	0.1672454	0.03	0.8603 NS
F	2	6.3172582	3.1586291	0.59	0.5576 NS
S*E	2	5.5428241	2.7714120	0.52	0.5986 NS
S*F	4	15.6275720	3.9068930	0.73	0.5754 NS
S*E*F	6	19.4317130	3.2386188	0.60	0.7262 NS
SEASON*S	2	10.3573817	5.1786908	0.97	0.3857 NS
SEASON*E	1	9.0422454	9.0422454	1.69	0.1983 NS
SEASON*F	2	23.2069187	11.6034594	2.16	0.1225 NS
SEASON*S*E*F	12	58.1314300	4.8442858	0.90	0.5479 NS

Total yield t ha^{-1}

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	646.492850	646.492850	9.02	0.0037 *
BLOCK	2	90.596538	45.298269	0.63	0.5344 NS
S	2	117.475311	58.737656	0.82	0.4447 NS
E	1	152.187091	152.187091	2.12	0.1494 NS
F	2	988.960145	494.480072	6.90	0.0018 *
SEASON*S	2	90.054049	45.027024	0.63	0.5364 NS
SEASON*E	1	1.712820	1.712820	0.02	0.8776 NS
SEASON*F	2	149.570717	74.785358	1.04	0.3575 NS
S*E	2	62.110889	31.055444	0.43	0.6500 NS
S*F	4	187.161261	46.790315	0.65	0.6266 NS
S*E*F	6	1216.874627	202.812438	2.83	0.0159 *
SEASON*S*F	4	202.143959	50.535990	0.71	0.5909 NS
SEASON*S*E*F	8	387.407749	48.425969	0.68	0.7110 NS

Appendix O. Data of interactions between analysed factors in quality parameters (tuber size, DM, SG, and RS content) in field experiment (Objective 3). DM= Dry matter, SG= Specific gravity, and RS= Reducing sugars. S= Plant extracts, E= Solvent extracts, F= application frequency NS= Not significantly different, *= Significantly different.

Tuber size (% small tubers)

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	2392.618313	2392.618313	13.37	0.0005 *
BLOCK	2	294.495885	147.247942	0.82	0.4435 NS
S	2	194.187243	97.093621	0.54	0.5838 NS
E	1	77.803498	77.803498	0.43	0.5119 NS
F	2	2246.656379	1123.328189	6.28	0.0031 *
SEASON*S	2	9.002058	4.501029	0.03	0.9752 NS
SEASON*E	1	31.507202	31.507202	0.18	0.6761 NS
SEASON*F	2	811.471193	405.735597	2.27	0.1112 NS
S*E	2	178.755144	89.377572	0.50	0.6091 NS
S*F	4	565.843621	141.460905	0.79	0.5354 NS
S*E*F	6	374.228395	62.371399	0.35	0.9085 NS
SEASON*S*F	4	334.362140	83.590535	0.47	0.7597 NS
SEASON*S*E*F	8	460.390946	57.548868	0.32	0.9553 NS

Tuber size (% medium tubers)

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	9105.581276	9105.581276	22.85	<.0001 *
BLOCK	2	9468.878600	4734.439300	11.88	<.0001*
S	2	433.384774	216.692387	0.54	0.5830 NS
E	1	5324.717078	5324.717078	13.36	0.0005 *
F	2	8434.927983	4217.463992	10.58	<.0001*
SEASON*S	2	850.051441	425.025720	1.07	0.3497 NS
SEASON*E	1	77.803498	77.803498	0.20	0.6599 NS
SEASON*F	2	1953.446502	976.723251	2.45	0.0936 NS
S*E	2	32.150206	16.075103	0.04	0.9605 NS
S*F	4	943.930041	235.982510	0.59	0.6694 NS
S*E*F	6	1662.808642	277.134774	0.70	0.6540 NS
SEASON*S*F	4	342.078189	85.519547	0.21	0.9295 NS
SEASON*S*E*F	8	2775.205761	346.900720	0.87	0.5454 NS

Tuber size (% large tubers)

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	2314.81482	2314.81482	7.18	0.0092 *
BLOCK	2	12388.11728	6194.05864	19.21	<.0001 *
S	2	1033.95062	516.97531	1.60	0.2085 NS
E	1	4323.55967	4323.55967	13.41	0.0005 *
F	2	18244.59877	9122.29938	28.30	<.0001 *
SEASON*S	2	756.17284	378.08642	1.17	0.3155 NS
SEASON*E	1	2.57202	2.57202	0.01	0.9291 NS
SEASON*F	2	513.11728	256.55864	0.80	0.4552 NS
S*E	2	252.05761	126.02881	0.39	0.6779 NS
S*F	4	825.61728	206.40432	0.64	0.6355 NS
S*E*F	6	945.21605	157.53601	0.49	0.8147 NS
SEASON*S*F	4	223.76543	55.94136	0.17	0.9513 NS
SEASON*S*E*F	8	2184.92798	273.11600	0.85	0.5648 NS

Tuber size (% marketable tubers)

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	2238.297325	2238.297325	13.42	0.0005 *
BLOCK	2	225.051440	112.525720	0.67	0.5125 NS
S	2	209.619341	104.809671	0.63	0.5364 NS
E	1	52.083333	52.083333	0.31	0.5780 NS
F	2	2300.668724	1150.334362	6.90	0.0018 *
SEASON*S	2	24.434156	12.217078	0.07	0.9294 NS
SEASON*E	1	52.083333	52.083333	0.31	0.5780 NS
SEASON*F	2	819.187243	409.593621	2.46	0.0931 NS
S*E	2	142.746914	71.373457	0.43	0.6535 NS
S*F	4	488.683127	122.170782	0.73	0.5728 NS
S*E*F	6	412.808642	68.801440	0.41	0.8683 NS
SEASON*S*F	4	257.201646	64.300412	0.39	0.8182 NS
SEASON*S*E*F	8	416.666667	52.083333	0.31	0.9589 NS

Dry matter content

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	11.5509481	11.5509481	1.85	0.1787 NS
BLOCK	2	13.8857185	6.9428593	1.11	0.3356 NS
S	2	43.7985241	21.8992620	3.50	0.0356 *
E	1	167.9010704	167.9010704	26.82	<.0001 *
F	2	123.3867574	61.6933787	9.86	0.0402 *
SEASON*S	2	2.2320019	1.1160009	0.18	0.8371 NS
SEASON*E	1	0.0363000	0.0363000	0.01	0.9395 NS
SEASON*F	2	22.1643574	11.0821787	1.77	0.1778 NS
S*E	2	13.1290241	6.5645120	1.05	0.3558 NS
S*F	4	51.2360926	12.8090231	2.05	0.0973 NS
S*E*F	6	88.3790056	14.7298343	2.35	0.0697 NS
SEASON*S*F	4	19.1744926	4.7936231	0.77	0.5511 NS
SEASON*S*E*F	8	30.1694333	3.7711792	0.60	0.7727 NS

Specific gravity

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	0.01105170	0.01105170	57.77	<.0001 *
BLOCK	2	0.00062868	0.00031434	1.64	0.2007 NS
S	2	0.00982583	0.00491292	25.68	<.0001 *
E	1	0.00434793	0.00434793	22.73	<.0001 *
F	2	0.00908336	0.00454168	23.74	<.0001 *
SEASON*S	2	0.00266888	0.00133444	6.98	0.0017 *
SEASON*E	1	0.00065149	0.00065149	3.41	0.0692 NS
SEASON*F	2	0.00034509	0.00017254	0.90	0.4104 NS
S*E	2	0.00028733	0.00014366	0.75	0.4757 NS
S*F	4	0.00715896	0.00178974	9.36	<.0001 *
S*E*F	6	0.00211004	0.00035167	1.84	0.1041 NS
SEASON*S*F	4	0.00124666	0.00031167	1.63	0.1766 NS
SEASON*S*E*F	8	0.00064640	0.00008080	0.42	0.9039 NS

Reducing sugars

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	0.25001324	0.25001324	59.70	<.0001 *
BLOCK	2	0.02063398	0.01031699	2.46	0.0925 NS
S	2	0.03717436	0.01858718	4.44	0.1053 NS
E	1	0.00294064	0.00294064	0.70	0.4049 NS
F	2	0.04160774	0.02080387	4.97	0.0596 NS
SEASON*S	2	0.00616642	0.00308321	0.74	0.4826 NS
SEASON*E	1	0.01830534	0.01830534	4.37	0.0702 NS
SEASON*F	2	0.00814484	0.00407242	0.97	0.3832 NS
S*E	2	0.00614327	0.00307163	0.73	0.4839 NS
S*F	4	0.00881204	0.00220301	0.53	0.7169 NS
S*E*F	6	0.02213179	0.00368863	0.88	0.5137 NS
SEASON*S*E	4	0.00339656	0.00169828	0.41	0.6682 NS
SEASON*S*E*F	8	0.05883433	0.00588343	1.40	0.1963 NS

Appendix P. Data of interactions between analysed factors in disaese incidence (DI) and biological control efficacy (BCE) in plants (20, 30, 40, and 50 DAI) in field experiment (Objective 3). DAI= Days after inoculation. S= Plant extracts, E= Solvent extracts, F= application frequency NS= Not significantly different, *= Significantly different

Disease incidence (DI) 20 DAI

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	6756.631388	6756.631388	151.86	<.0001 *
BLOCK	2	301.938374	150.969187	3.39	0.0392 *
S	2	273.220038	136.610019	3.07	0.0527 NS
E	1	1.903006	1.903006	0.04	0.8368 NS
F	2	301.219690	150.609845	3.39	0.0795 NS
SEASON*S	2	217.895487	108.947743	2.45	0.0938 NS
SEASON*E	1	3.482501	3.482501	0.08	0.7805 NS
SEASON*F	2	128.004010	64.002005	1.44	0.2442 NS
S*E	2	51.034532	25.517266	0.57	0.5662 NS
S*F	4	94.623478	23.655870	0.53	0.7128 NS
S*E*F	6	40.320081	6.720013	0.15	0.9883 NS
SEASON*S*E	2	70.300988	35.150494	0.79	0.4578 NS
SEASON*S*E*F	10	75.431003	7.543100	0.17	0.9978 NS

Disease incidence (DI) 30 DAI

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	41074.52309	41074.52309	325.49	<.0001 *
BLOCK	2	510.21067	255.10533	2.02	0.1401 NS
S	2	1258.68359	629.34179	4.99	0.0904 NS
E	1	309.41506	309.41506	2.45	0.1219 NS
F	2	1146.27148	573.13574	4.54	0.0140 *
SEASON*S	2	192.98208	96.49104	0.76	0.4694 NS
SEASON*E	1	13.46362	13.46362	0.11	0.7449 NS
SEASON*F	2	215.29071	107.64536	0.85	0.4305 NS
S*E	2	46.05471	23.02736	0.18	0.8336 NS
S*F	4	169.99366	42.49841	0.34	0.8523 NS
S*E*F	6	440.59838	73.43306	0.58	0.7435 NS
SEASON*S*E	2	61.01732	30.50866	0.24	0.7859 NS
SEASON*S*E*F	10	298.06851	29.80685	0.24	0.9915 NS

Disease incidence (DI) 40 DAI

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	5760.088339	5760.088339	59.33	<.0001 *
BLOCK	2	969.953555	484.976777	5.00	0.0094 *
S	2	1900.107853	950.053927	9.79	0.0002 *
E	1	749.258880	749.258880	7.72	0.0070 *
F	2	1331.563070	665.781535	6.86	0.0019 *
SEASON*S	2	137.338165	68.669083	0.71	0.4965 NS
SEASON*E	1	6.330727	6.330727	0.07	0.7992 NS
SEASON*F	2	14.881264	7.440632	0.08	0.9263 NS
S*E	2	517.997682	258.998841	2.67	0.0765 NS
S*F	4	204.722906	51.180727	0.53	0.7161 NS
S*E*F	6	167.825573	27.970929	0.29	0.9407 NS
SEASON*S*E	2	76.784232	38.392116	0.40	0.6749 NS
SEASON*S*E*F	10	275.753599	27.575360	0.28	0.9828 NS

Disease incidence (DI) 50 DAI

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	1390.824731	1390.824731	12.40	0.0008 *
BLOCK	2	516.152452	258.076226	2.30	0.1077 NS
S	2	3018.588239	1509.294120	13.46	<.0001 *
E	1	200.815857	200.815857	1.79	0.1852 NS
F	2	2570.798392	1285.399196	11.46	<.0001 *
SEASON*S	2	50.025113	25.012557	0.22	0.8007 NS
SEASON*E	1	30.209400	30.209400	0.27	0.6054 NS
SEASON*F	2	267.154803	133.577401	1.19	0.3100 NS
S*E	2	51.835901	25.917951	0.23	0.7943 NS
S*F	4	525.988132	131.497033	1.17	0.3305 NS
S*E*F	6	979.052026	163.175338	1.45	0.2065 NS
SEASON*S*E	2	102.240387	51.120194	0.46	0.6358 NS
SEASON*S*E*F	10	1166.067342	116.606734	1.04	0.4203 NS

Biological control efficacy (BCE) 20 DAI

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	30236.51656	30236.51656	96.87	<.0001 *
BLOCK	2	58.12482	29.06241	0.09	0.9112 NS
S	2	1458.51470	729.25735	2.34	0.1042 NS
E	1	31.08139	31.08139	0.10	0.7533 NS
F	2	2362.19019	1181.09509	3.78	0.0275 *
SEASON*S	2	1263.86624	631.93312	2.02	0.1397 NS
SEASON*E	1	27.17700	27.17700	0.09	0.7688 NS
SEASON*F	2	441.42034	220.71017	0.71	0.4966 NS
S*E	2	92.49032	46.24516	0.15	0.8626 NS
S*F	4	790.37197	197.59299	0.63	0.6406 NS
S*E*F	6	676.62616	112.77103	0.36	0.9009 NS
SEASON*S*E	2	600.01736	300.00868	0.96	0.3874 NS
SEASON*S*E*F	10	1020.98406	102.09841	0.33	0.9711 NS

Biological control efficacy (BCE) 30 DAI

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	12970.99006	12970.99006	59.75	<.0001 *
BLOCK	2	1695.51821	847.75910	3.91	0.0247 *
S	2	2718.49146	1359.24573	6.26	0.0031 *
E	1	1456.70652	1456.70652	6.71	0.0117 *
F	2	2791.26103	1395.63051	6.43	0.0027 *
SEASON*S	2	188.61275	94.30637	0.43	0.6494 NS
SEASON*E	1	0.00054	0.00054	0.00	0.9988 NS
SEASON*F	2	17.17845	8.58923	0.04	0.9612 NS
S*E	2	325.99244	162.99622	0.75	0.4757 NS
S*F	4	443.41670	110.85417	0.51	0.7281 NS
S*E*F	6	392.06478	65.34413	0.30	0.9344 NS
SEASON*S*E	2	406.54087	203.27043	0.94	0.3969 NS
SEASON*S*E*F	10	799.36027	79.93603	0.37	0.9563 NS

Biological control efficacy (BCE) 40 DAI

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	8722.028249	8722.028249	70.23	<.0001 *
BLOCK	2	597.702659	298.851329	2.41	0.0976 NS
S	2	3824.238863	1912.119431	15.40	<.0001 *
E	1	480.788951	480.788951	3.87	0.0431 *
F	2	2875.322632	1437.661316	11.58	<.0001 *
SEASON*S	2	251.147066	125.573533	1.01	0.3690 NS
SEASON*E	1	145.473881	145.473881	1.17	0.2828 NS
SEASON*F	2	197.797957	98.898979	0.80	0.4550 NS
S*E	2	174.563529	87.281764	0.70	0.4986 NS
S*F	4	175.900395	43.975099	0.35	0.8403 NS
S*E*F	6	743.200583	123.866764	1.00	0.4340 NS
SEASON*S*E	2	90.980595	45.490298	0.37	0.6946 NS
SEASON*S*E*F	10	1303.711089	130.371109	1.05	0.4123 NS

Biological control efficacy (BCE) 50 DAI

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	2747.673777	2747.673777	17.06	<.0001 *
BLOCK	2	192.741334	96.370667	0.60	0.5526 NS
S	2	3076.538776	1538.269388	9.55	0.0002 *
E	1	429.250362	429.250362	2.66	0.1071 NS
F	2	2150.551183	1075.275592	6.67	0.0022 *
SEASON*S	2	58.285756	29.142878	0.18	0.8349 NS
SEASON*E	1	0.422166	0.422166	0.00	0.9593 NS
SEASON*F	2	56.817071	28.408535	0.18	0.8387 NS
S*E	2	193.051781	96.525890	0.60	0.5521 NS
S*F	4	293.033093	73.258273	0.45	0.7686 NS
S*E*F	6	632.939955	105.489992	0.65	0.6861 NS
SEASON*S*E	2	56.935536	28.467768	0.18	0.8384 NS
SEASON*S*E*F	10	989.792491	98.979249	0.61	0.7965 NS

Appendix Q. Data of interactions between analysed factors in disaese incidence (DI) and biological control efficacy (BCE) in tubers in field experiment (Objective 3). S= Plant extracts, E= Solvent extracts, F= application frequency NS= Not significantly different, *= Significantly different

Disease incidence in tubers

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	66.714094	66.714094	0.18	0.6769 NS
BLOCK	2	2542.276049	1271.138024	3.34	0.0413 NS
S	2	1966.647984	983.323992	2.58	0.0828 NS
E	1	582.210193	582.210193	1.53	0.2205 NS
F	2	9491.097843	4745.548922	12.46	<.0001 *
SEASON*S	2	568.884464	284.442232	0.75	0.4776 NS
SEASON*E	1	1200.078794	1200.078794	3.15	0.0802 NS
SEASON*F	2	149.652459	74.826230	0.20	0.8221 NS
S*E	2	1151.546254	575.773127	1.51	0.2277 NS
S*F	4	1886.199207	471.549802	1.24	0.3028 NS
S*E*F	6	3383.755724	563.959287	1.48	0.1975 NS
SEASON*S*E	2	1282.180355	641.090178	1.68	0.1932 NS
SEASON*S*E*F	10	599.281331	59.928133	0.16	0.9984 NS

Biological control efficacy (BCE) in tubers

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	116.49257	116.49257	0.26	0.6093
BLOCK	2	2133.89437	1066.94719	2.41	0.0968
S	2	2341.58088	1170.79044	2.65	0.0478
E	1	641.74610	641.74610	1.45	0.2322
F	2	11056.90835	5528.45418	12.51	<.0001
SEASON*S	2	693.22543	346.61271	0.78	0.4604
SEASON*E	1	1365.36980	1365.36980	3.09	0.0832
SEASON*F	2	206.81751	103.40876	0.23	0.7920
S*E	2	1470.10407	735.05204	1.66	0.1969
S*F	4	2240.19446	560.04862	1.27	0.2911
S*E*F	6	3712.00579	618.66763	1.40	0.2269
SEASON*S*E	2	1431.90857	715.95428	1.62	0.2052
SEASON*S*E*F	10	673.30092	67.33009	0.15	0.9986

Appendix R. Data of interactions between analysed factors in post-harvest infection (PHI) and post-harvest yield loss (PHL) due to *Ralstonia solanacearum* in stored potato tubers from field experiment (Objective 3). S= Plant extracts, E= Solvent extracts, F= application frequency. NS= Not significantly different, *= Significantly different

Post-harvest infection (PHI)

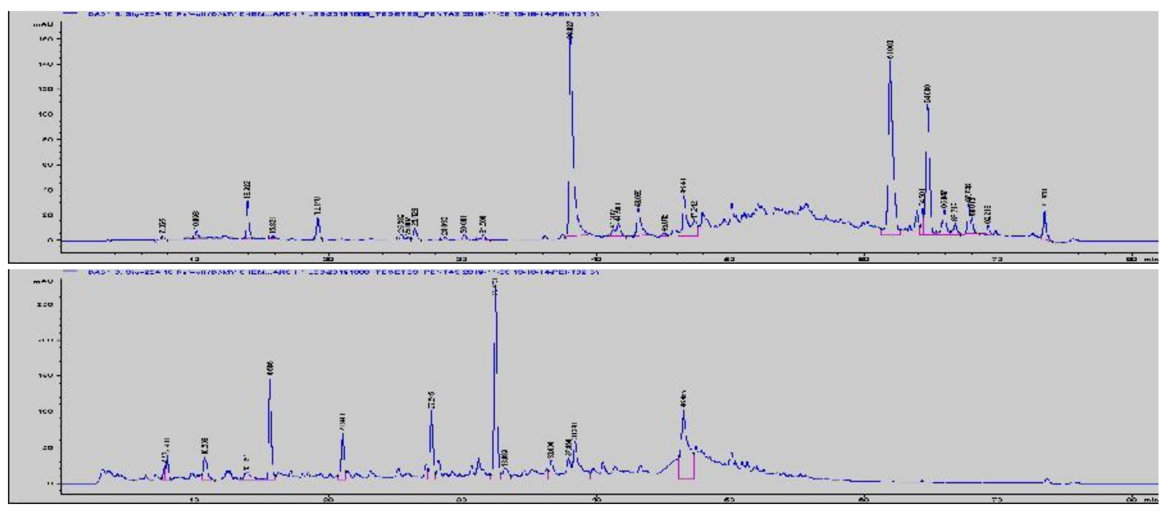
Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	22.818641	22.818641	0.05	0.0233 *
BLOCK	2	2203.860387	1101.930193	2.43	0.0958 NS
S	2	2748.304831	1374.152416	3.03	0.0549 NS
E	1	1276.581134	1276.581134	2.81	0.0981 NS
F	2	6408.688429	3204.344215	7.06	0.0016 *
SEASON*S	2	204.918588	102.459294	0.23	0.7986 NS
SEASON*E	1	243.071434	243.071434	0.54	0.4669 NS
SEASON*F	2	82.365678	41.182839	0.09	0.9134 NS
S*E	2	3.933873	1.966936	0.00	0.9957 NS
S*F	4	3259.153754	814.788439	1.79	0.1398 NS
S*E*F	6	2277.498268	379.583045	0.84	0.5465 NS
SEASON*S*E	2	750.406536	375.203268	0.83	0.4420 NS
SEASON*S*E*F	10	1529.112339	152.911234	0.34	0.9680 NS

Post-harvest yield loss (PHL)

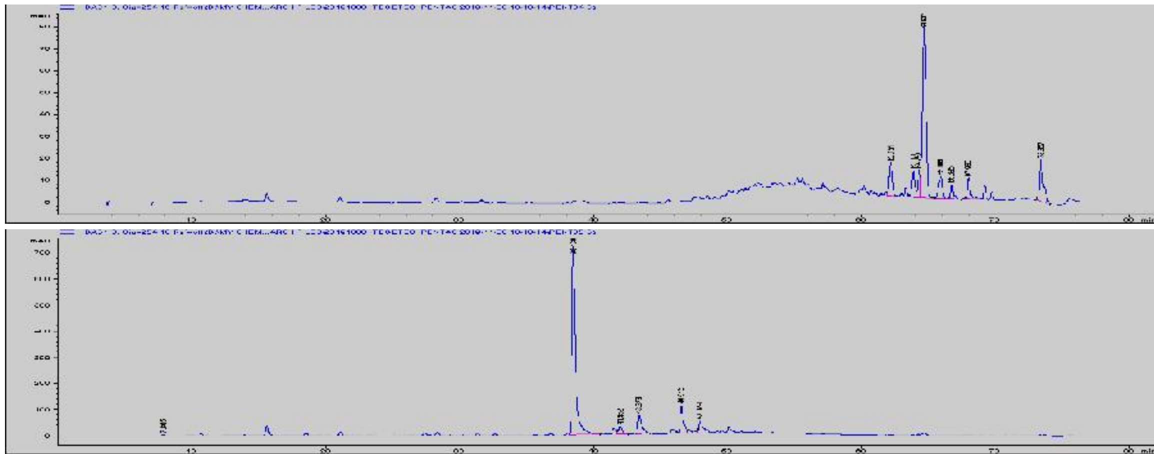
Source	DF	Type III SS	Mean Square	F Value	Pr > F
SEASON	1	539.655298	539.655298	1.47	0.0096 *
BLOCK	2	2444.991241	1222.495621	3.33	0.0417 NS
S	2	1909.163268	954.581634	2.60	0.0816 NS
E	1	3456.401381	3456.401381	9.41	0.0031 *
F	2	5580.259451	2790.129725	7.59	0.0010 *
SEASON*S	2	218.365260	109.182630	0.30	0.7439 NS
SEASON*E	1	3.925798	3.925798	0.01	0.9180 NS
SEASON*F	2	41.817407	20.908703	0.06	0.9447 NS
S*E	2	911.171105	455.585553	1.24	0.2957 NS
S*F	4	2409.189933	602.297483	1.64	0.1741 NS
S*E*F	6	991.254826	165.209138	0.45	0.8429 NS
SEASON*S*E	2	396.430928	198.215464	0.54	0.5855 NS
SEASON*S*E*F	10	903.938762	90.393876	0.25	0.9901 NS

Appendix S. Comparison between chromatograms of different extracts from wild marigold through HPLC recorded at 230 nm (Objective 4)

Chromatograms of Initial methanol (up) and water extracts (down)

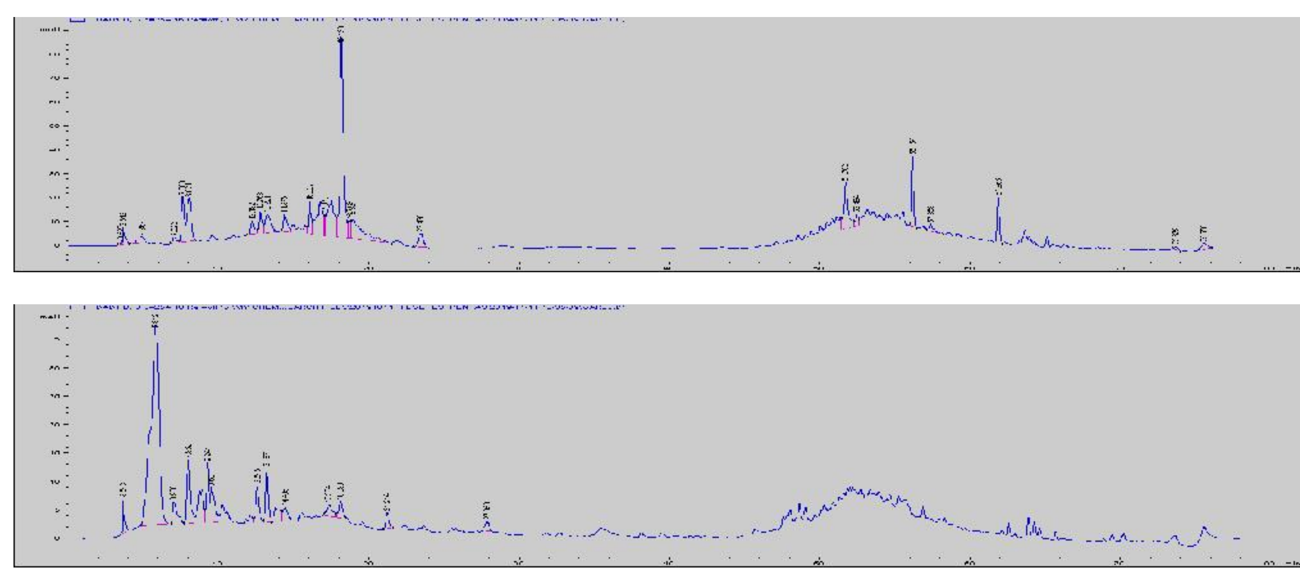


Chromatograms of Diethyl ether (up) and n-hexane (down) fractions

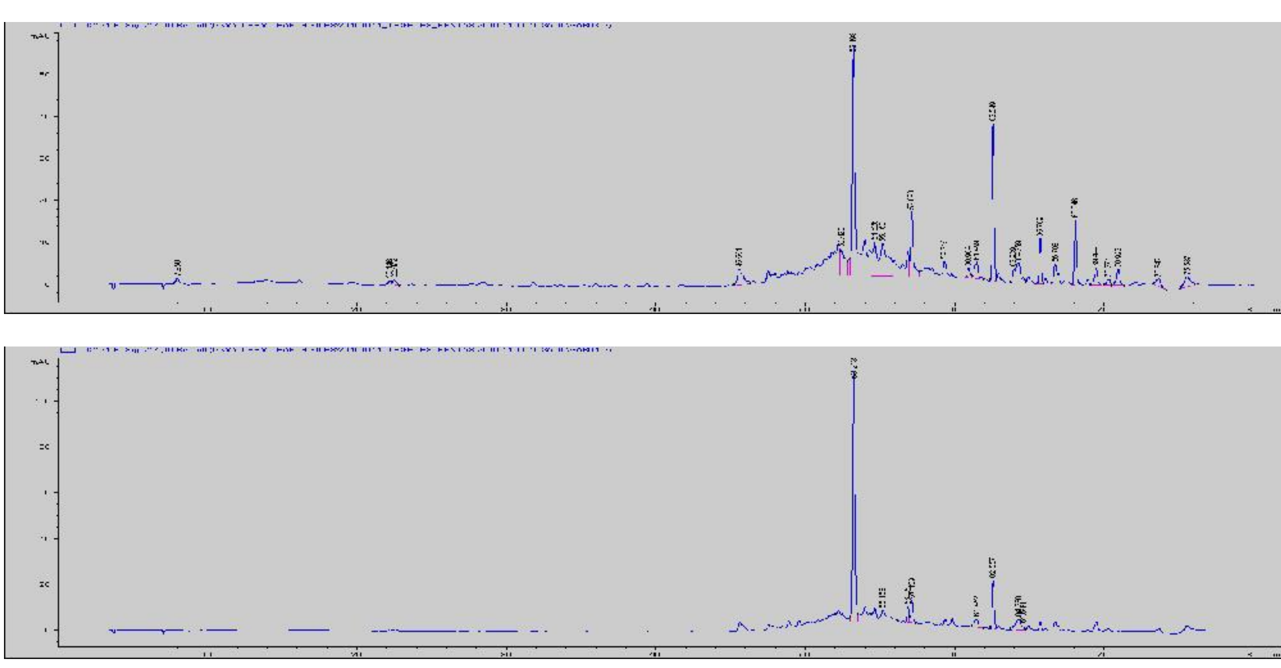


Appendix T. Comparison between chromatograms of different extracts from garlic through HPLC recorded at 230 nm (Objective 4)

Chromatograms of initial methanol and water extracts



Chromatograms of Diethyl ether (up) and n-hexane fractions (down)

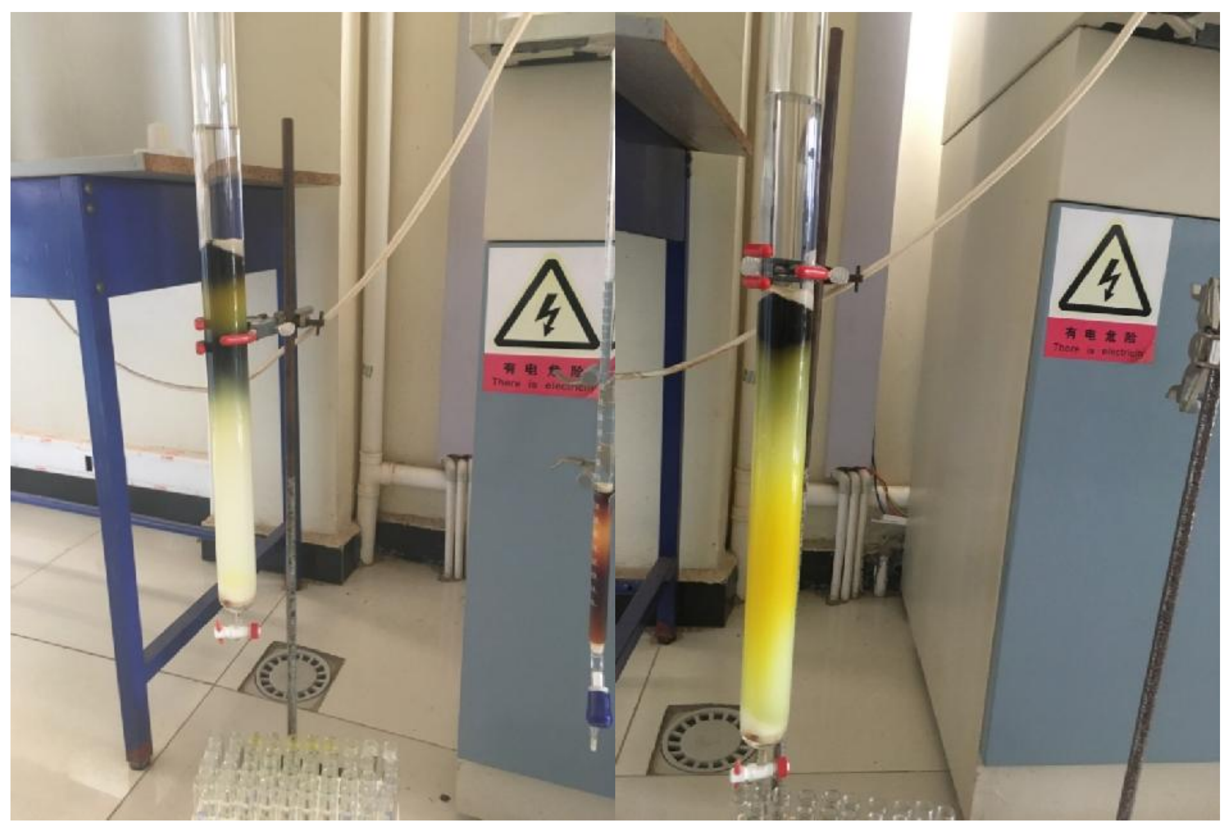


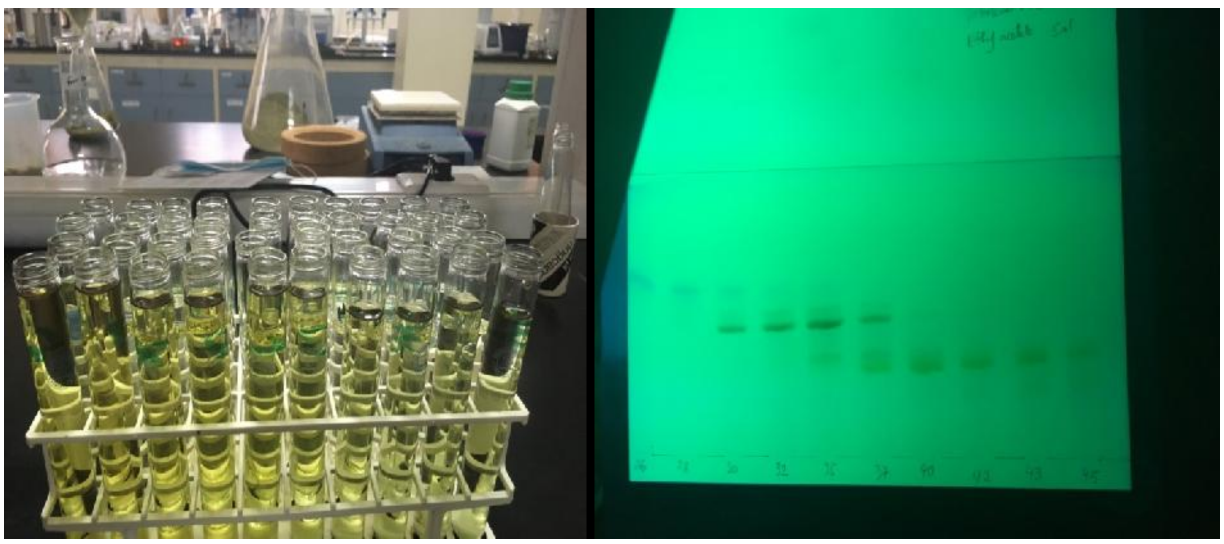
Appendix U. Greenhouse experiment (Objective 2).

Comparison of bacterial wilt incidence between potato treated with methanolic extract of tobacco at 50 mg mL^{-1} (left) and negative control (1% DMSO) (right) at 10 days after inoclutaion (DAI)

Appendix V. Comparison between potatoes treated with plant extracts and potatoes treated with controls (Copper oxychloride and DMSO) under field conditions (Objective 3)

Appendix W. Isolation of bioactive compounds from methanolic extract of tobacco through open column chromatography and Thin Layer Chromaatography (TLC) (Objective 4)





Appendix X. Research permit

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY & INNOVATION

Ref No: 621931

Date of Issue: 19/August/2020

RESEARCH LICENSE

This is to Certify that Ms.. Marie Chantal Mutimawurugo of Egerton University, has been licensed to conduct research in Nakuru on the topic: EVALUATION OF EFFICACY OF SELECTED PLANT EXTRACTS FOR MANAGEMENT OF POTATO BACTERIAL WILT (Ralstonia solanacearum) IN RWANDA for the period ending : 19/August/2021.

License No: NACOSTI/P/20/6223

621931

Applicant Identification Number

Director General

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