Growth and physiological and biochemical responses of enset *(Ensete ventricosum* (Welw.) Cheesman) clones infected with bacterial wilt: Induction of resistance using medicinal plant leaf extract

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June, 2016

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A Dissertation Submitted to the Department of Plant Biology and Biodiversity Management Presented in Fulfillment of the Requirements for the Degree of Doctor of Philosophy (Biology: Botanical Sciences)

Addis Ababa University

Addis Ababa, Ethiopia

June, 2016

ADDIS ABABA UNIVERSITY
GRADUATE PROGRAMMES

This is to certify that the Dissertation prepared by Getahun Yemata Lule, entitled: Growth and physiological and biochemical responses of enset *(Ensete ventricosum* (Welw.) Cheesman) clones infected with bacterial wilt and submitted in fulfillment of the requirements for the degree of Doctor of Philosophy (Biology: Botanical Sciences) complies with the regulations of the University and meets the accepted standards with respect to originality and quality

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Abstract

Enset *(Ensete ventricosum* (Welw.) Cheesman) is a perennial staple food crop widely cultivated in the south and southwestern Ethiopia. It supports the lives of approximately 20 million people. However, its cultivation is impeded by enset bacterial wilt (EBW). Therefore, the aim of the study was to evaluate the antibacterial activity of medicinal plant extracts and antibiotics against *Xanthomonas campestris pv. musacearum* (Xcm); to study induction of resistance in enset by leaf extract and evaluate its contribution in controlling enset bacterial wilt disease and to investigate growth and metabolic responses of enset clones under different treatments.The antibacterial activities of methanol leaf extracts of *Achyranthes aspera*, *Agarista salicifolia*, *Datura stramonium*, *Melia azedarach*, *Pycnostachys abyssinica* and *Vernonia amygdalina* and some antibiotics; amoxicillin, cephalexin, chloramphenicol, streptomycin sulphate and tetracycline were evaluated *in vitro*. Qualitative analysis of leaf extracts was carried out using standard methods. Total phenolics content was estimated with Folin Ciocalteau method. The antibacterial activity of methanol leaf extracts was evaluated by disc diffusion method. Minimum inhibitory concentration was determined using agar dilution method. The potential role of *A. salicifolia* leaf extract to trigger induced resistance, metabolic changes in resistant and susceptible enset clones and the subsequent suppression of EBW was evaluated. Growth,

photosynthetic and biochemical responses of the two enset clones, Viz., resistant (*Genticha*) and susceptible (*Midasho*) were evaluated 120, 270 and 360 days after treatment application. The phytochemical screening results revealed the existence of alkaloids, phenols, terpenoids, saponnins, tannins and glycosides in extract of *A. salicifolia* in abundant amount. Similar result was observed in *P.abyssinica* extract. However, glycosides were absent. Moreover, *A. salicifolia* leaf extract had the highest total phenolics content. It showed a 100, 85.7, 100, 61.2 and 12.3 % increment from *A. aspera, A. salicifolia, D. stramonium, M. azedarach, P. abyssinica* and *V. amygdalina.* The widest inhibition zone diameter was recorded in *A. salicifolia* extract followed by *P. abyssinica.* A statistically significant difference in inhibition zone diameter was seen between species and among serial concentrations within a species extract. The two species extracts also showed the lowest minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC). Similarly, antibiotics showed antibacterial activity against Xcm. However, the inhibition zone diameters of antibiotics were significantly higher fivefold times than leaf extracts. The highest anti-Xcm activity was shown by streptomycin sulphate (50.3 mm at 50 mg/mL) followed by amoxicillin (49.7 mm at 50 mg/mL). However, the lowest MIC (0.02 mg/mL) and MBC (0.049 mg/mL) values of antibiotics were recorded by tetracycline. In addition, extract treated and Xcm inoculated plants of both clones had an increased activity of phenylalanine ammonia lyase (PAL), peroxidase, polyphenol oxidase and greater amount of total phenolics content (TPC) and total soluble sugars (TSS). Induced plants of the resistant clone showed an 8, 4.6, 3, 13 and 4.6 fold increments in PAL activity compared to controls at 24, 48, 72, 96 and 120 h post treatment, respectively. Infected control plants on the other hand demonstrated a 7, 3.6, 2.3, 10.6 and 2.4 fold increments compared to controls. Similarly, induced plants of the susceptible clone exhibited 1.5, 1.7, 1.4, 4.2 and 4.4 fold increments in PAL activity

compared to controls at 24, 48, 72, 96 and 120 h post treatment, respectively. While infected control plants showed a 0, 1.3, 1.4, 3 and 2 fold increments in PAL activity compared to controls at similar sampling time. The activity of tested enzymes and amounts of TPC and TSS were significantly increased in plants of the resistant clone treated with leaf extract and Xcm as compared to plants of the susceptible clone. The results of the study suggested that the difference between the two clones was partially caused by variations in biochemical responses. Extract induced resistance brought a 33.33 % and 3.1% disease incidence reduction in plants of the susceptible and resistant clones, respectively, compared to infected controls. Plants of the two enset clones also showed variations in growth and physiological responses among themselves and between treatments at different measuring time periods. Nine months after treatment application, induced plants of the resistant clone had significantly lower pseudostem (22.2%), total leaf area (6.8%) and leaf area index (32%) than control plants. Similarly, infected control plants of the susceptible clone showed lower pseudostem girth (29.4%), total leaf area (37.3%) and leaf area index (37.3%) as compared to controls. Generally, the results suggest that extracts of *A. salicifolia* and *P. abyssinica* have high potency and the potential for further formulation development as biocides with broad spectrum activity. Tetracycline with the lowest MIC and MBC values is also a potential alternative in controlling EBW. Moreover, the results also suggest the feasibility of induced resistance to control EBW. However, further research should be conducted to formulate the extracts, identify the active compounds responsible for the antibacterial activity of extracts, and evaluate the performance of other application methods under field conditions.

Keywords: *Agarista salicifolia*, Antibiotics, Assimilation rate, Inhibition zone, *Ensete ventricosum*, Induced resistance, methanol extracts, phenylalanine ammonia lyase, Total phenolic compounds. *Xanthomonas campestris pv. musacearum*

ACKNOWLEDGEMENTS

The completion of this Doctoral Dissertation was possible with the support of several people. I would like to express my sincere gratitude to all of them. First of all, I am extremely grateful to my supervisor, Prof. Masresha Fetene for his valuable guidance, understanding, support, scholarly inputs, consistent encouragement, and most importantly his gracious treatment throughout the research work. The successful accomplishment of this work was possible only with his unreserved and consistent support. His infectious enthusiasm in science and hardworking have been the major driving forces throughout my graduate career. He taught me the sense of hardworking and shaped me to grow in that personality. For everything you have done for me, Prof. Masresha, I thank you a lot.

My field study was made easier and attractive to visit because of the presence of a few individuals; Fekadu Assefa, Dr. Solomon Zewdie and Dr. Tefera Mengistu. I am grateful to ato Fekadu Assefa for his generous support in facilitating all communications related to the research work in and around Wondo Genet College of Forestry and Natural Resources. Dr. Solomon Zewdie and Dr. Tefera Mengistu are also acknowledged for their contribution in getting the study plot in the College. My thank go out to Wondo Genet College of Forestry and Natural Resources for allowing me to laid down the study plot in the campus.

I would like to extend my gratitude to Addis Ababa University, Department of Plant Biology and Biodiversity Management for accepting my application for the PhD program, financially supporting the research work and facilitating administrative cases as the need arises. I thank Dr. Kassahun Tesfaye for providing some chemicals that were not available in the market. I am also

very much indebted to my beloved family, my wife Aynadis Anagaw and daughter Hanna for their patience and understanding at times I forced down their interests and for their support in every possible way to see the completion of this work.

I appreciate Bahir Dar University for permitting the study leave and financially supporting the research work. My heartfelt gratitude goes to Yewulsew Kebede, a friend at Bahir Dar University, who closely follows up and performs my personal affairs that could otherwise compete the time devoted to the research work. Thanks a lot Yewulsew. I am also grateful to my friends, Destaw Damtie, Getu Alemayehu, Awol Assefa and Bruktawit Desta for their moral support and encouragement

Above all, I am indebted all to the Almighty God for conceding me the wisdom, health and strength to undertake this research and enabling me to its completion.

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List of Acronyms

A = Net assimilation rate

AFLP = Amplified fragment length polymorphism

Ag = Agreleen

ASA = Acetyl salicylic acid

ASM = Acibenzolar acid

BBTV = Banana bunchy top virus

BYMV = Bean yellow mosaic virus

CCA = Celliobiose-cephalexin agar

CFU = Colony forming unit

Chl. a = Chlorophyll a

Chl. a+b = Chlorophyll a and b

Chl. b = Chlorophyll b

C_i = intercellular CO_2 concentration

CSA = Central Statistical Authority

DI = Disease incidence

DNA = Deoxy ribonucleic acid

DW = Dry weight

EBW = enset bacterial wilt

EUCAST = European Committee on Antimicrobial Susceptibility Testing

FW = Fresh weight

GLN = Green leaf number

g_s = Stomatal conductance

GTA = Gentisic acid

HR = Hypersensitive response

JA = Jasmonic acid

LAI = leaf area index

MAT = months after treatment

MBC = Minimum bactericidal concentration

MIC = Minimum inhibitory concentration

NCCLS = National Committee for Clinical Laboratory Standards

OD = Optical density

PAL = Phenylalanine ammonia lyase

PAMP = Pathogen associated molecular pattern

PH = Plant height

POX = Peroxidase

PPO = Polyphenol oxidase

PR = Pathogenesis related proteins

PRR = Pattern recognition receptors

PSG = Pseudostem girth

RAPD = Random amplified polymorphic DNA

RPM = Rotation per minute

RWC = Relative water content

SA = Salicylic acid

SAR = Systemic acquired resistance

SARI = South Agricultural Research Institute

SNNPS = South Nations Nationalities and Peoples State

T = Transpiration rate

TC = Total carotenoids

TLA = Total leaf area

TLL = Third leaf length

TLW = Third leaf width

TMV = Tomato mosaic virus

TN = Total nitrogen

TP = Total protein

TPC = Total phenolics content

TSS = Total soluble sugar

TW = Turgid weight

WUE = water use efficiency

Xcm = *Xanthomonas campestris pv. musacearum*

Xoo = *Xanthomonas oryzae pv. oryzae*

YPSA = Yeast extract peptone sucrose agar

YTSA-CC = Yeast extract tryptone sucrose agar cellobiose cephalexin

CHAPTER ONE

1. INTRODUCTION

1.1. Background

Some crop species which have been cultivated for millennia and contributing to the livelihoods of local communities are rarely known outside the regions of their cultivation and use. Crops with such localized cultivation and uses are mainly grown in subsistence farming systems in many developing countries. They substantially contribute to food security of small-holder farmers (Dansi *et al*., 2012; Temesgen Magule *et al*., 2014). One among such crops in Ethiopia is *Enseteventricosum* (Welw.) Cheesman), a crop commonly known by its vernacular name enset. It is a monocarpic perennial crop widely cultivated in the south and southwestern Ethiopia. Enset is a traditional crop that makes up the Enset-based farming system (Desalegn Rahmato, 1996). It serves as a staple/co-staple food nourishing approximately 20 million people in the country (Temesgen Addis, 2005; Temesgen Magule *et al*., 2014).

Enset is a multi-purpose crop cultivated for thousands of years only in Ethiopia, where it was domesticated. As a food crop, enset offers several merits. First, the plant can be harvested at any time during the year and the corm can be harvested at any stage over a period of several years. Second, enset foods can be stored for long periods (Dereje Fekadu, 2009; Abay Ayalew and Mikias Yeshitila, 2011). Third, compared to cereals, it gives a high food yield per unit area. Enset growing regions of Ethiopia are well known for their high population density which may not be supported by any other type of land use and crop (Admasu Tsegaye and Struik, 2001). Because of this, the land holding of each farmer is very small and yet the population rarely faces food shortage, because of the high productivity of enset. Fourth, enset grows in a wide range of

environments from about 1200 to 3100 meters above sea level (Taye Bezuneh and Asrat Feleke, 1966; Temesgen Addis, 2005). This allows farmers to grow the plant in all parts of the country including areas not suitable for cereal cultivation. Fifth, enset is considered tolerant to drought, heavy rains, flooding and other stress factors (Dereje Fekadu, 2009). Moreover, research results suggest that "those populations depending on enset have never suffered from famine, even during Ethiopia's tragic drought and famine prone decades of the 1970s and 1980s" (Shigeta, 1991; Brandt *et al*., 1997). This shows the potential role of enset to ensure national food security (Admasu Tsegaye and Struik, 2002; Almaz Negash and Niehof, 2004).

In spite of its merits, enset agriculture has received surprisingly little extension, development, or research attention. This perhaps is because: 1) the majority of enset farmers live in one of the least developed regions of Ethiopia, making access and logistics difficult in the past; 2) the system is unique when compared to cereal farming; 3) production processes are complex; and 4) there is the perception that it is eminently successful, sustainable, and trouble-free (Brandt *et al*., 1997; MacEntee *et al*., 2013). It was only in 1997 that the Ethiopian government declared enset as a national crop implying the start of research attention to the crop (Brandt *et al*., 1997). Nowadays, enset cultivation is threatened by various abiotic and biotic environmental factors such as diseases, insect pests, vertebrate pests, drought, declining soil fertility and an increasing human population (Admasu Tsegaye and Struik, 2002; Temesgen Addis, 2005; Temesgen Addis *et al*., 2008).

Of all the aforementioned environmental factors, enset bacterial wilt, also called *Xanthomonas* wilt is the major constraint to enset cultivation followed by drought (Solomon Zewdie, 2007). Enset bacterial wilt is caused by the Gram negative phytopathogenic bacterium *Xanthomonas campestris* pv *musacearum* (Dagnachew Yirgou and Bradbury, 1968; Dereje Ashagari, 1985;

Temesgen Addis, 2005). The disease is affecting the lives of millions of people in Ethiopia (Gizachew Welde-Michael *et al*., 2008). In addition to enset, the disease also affects other *Musa* species such as banana which is the main staple food source in east Africa region thus, posing a serious threat in the entire region (Mwangi *et al*., 2007). Unlike other diseases, enset bacterial wilt is both extreme and rapid causing gradually increasing losses over years. The economic impact of bacterial wilt is due to death of the mother plant that would otherwise contribute to the continuation of enset production cycles. Fields infested with *Xanthomonas campestris* pv *musacearum* cannot be replanted for at least 6 months due to carryover of soil borne inoculum (Tripathi *et al*., 2009).

The major transmission means of the disease across or within fields are insects, contaminated tools and infected plant materials (Mwangi *et al*., 2007). Therefore, the spread of the disease can be prevented by implementing cultural disease management practices such as burying infected plants, restricting movement of infected plant materials, sterilizing tools and raising awareness about the disease (Mwangi *et al*., 2007; Tripathi *et al*., 2009; Temesgen Addis *et al*., 2010). However, these methods are not effective as farmers are inconsistent and reluctant to employ labor-intensive disease controlling measures (Tripathi *et al*., 2009). Moreover, the disease is systemic and hence surface application of chemicals has little or no use to control the disease (Smith *et al*., 2008). Thus, investigations for alternative disease controlling strategies that are effective, eco-friendly, long-lasting, low-cost, and easy to prepare have prime importance.

In recent years, much interest has been developed in the antimicrobial effects of medicinal plants for plant disease control. Plant extracts are important sources of new agrochemicals for the control of plant diseases. They are used to control both pre and postharvest pests of plants. There are several research results in this area. The results showed that crude extracts of plants are

effective in inhibiting the growth of pathogens both *in vitro* and *in vivo* and the consequent development of disease in plants. For instance, the leaf extracts of *Ocimum basilicum, Azadirachta indica*, *Eucalyptus chamadulonsis*, *Datura stramonium*, *Nerium oleander* and *Allium sativum* showed high antimicrobial activity against *Alternaria solani* at different concentrations both *in vitro* and *in vivo* (Nashwa and Abo-Elyousr, 2012). Related to this, leaf extracts of *D. stramonium* and *A. sativum* at 5 % concentration (W/V) increased the fruit yield of tomato by 76.2 % and 66.7 %, respectively, compared to the infected control (Nashwa and Abo-Elyousr, 2012). Similarly, crude extracts of Neem, Chili and Pong-pong showed high inhibition against the mycelial growth of *Penicillium digitatum* on the surface of fruits in storage conditions at 25±2 °C (Al-Samarrai *et al*., 2012). Furthermore, biocides of plant origin are non-phytotoxic, systemic and easily biodegradable (Kagale *et al.* 2004; Guleria and Tiku, 2009). It is now well known that various plant extracts can reduce populations of foliar pathogens and control disease development. Thus, they have the potential as environmentally safe alternatives and components in integrated disease management programs (Bowers and Locke, 2004). In addition, plant extracts are rich sources of elicitors; compounds that activate chemical defense in plants. They act as signal compounds at low concentrations, providing information for the plant to trigger defense (Thakur and Sohal, 2013).

The ability of plants to express induced resistance to pathogens has been known for over a century and the issue was first reviewed by Chester, (1933). These early observations were referring to the ability of a plant to become resistant to subsequent inoculations after the occurrence of an initial infection. Induced resistance was named as systemic acquired resistance by Ross, (1961) who showed that inoculation of one lower leaf of tobacco with tobacco mosaic virus (TMV) increased the resistance of the upper leaves on the same plant to subsequent

infection with TMV. Nowadays, resistance induction by biotic and abiotic agents in plants has attracted the attention of researchers worldwide as a possible strategy for integrated plant disease management (Ghazanfar *et al*., 2011). Biotic inducers (elicitors) include infection by necrotizing pathogens and plant growth promoting rhizobacteria, and treatment with nonpathogens or cell wall fragments. Abiotic inducers include chemicals which act at various points in the signaling pathways involved in disease resistance (Walters *et al*., 2005). Resistance induced by these agents (resistance elicitors) is broad spectrum and long lasting, but rarely provides complete control of infection, with many resistance elicitors providing between 20 and 85% disease control (Walters *et al*., 2005). Results of several studies revealed that treating plants with extracts induces systemic resistance. Cucumber plants sprayed with aqueous extracts of *Ocimum gratissimum* leaves developed systemic resistance to *Colletotrichum lagenarium.*This is reflected by reduction in disease incidence and an increase in the activity defense enzymes (Colpas *et al*., 2009). Likewise, incidence of bacterial leaf blight disease of rice was reduced when seeds are pretreated with leaf extracts of *Adathoda vasica* (Govindappa et *al*., 2011). Physiological observation of *A. vasica* treated plants indicates that control of disease development in plant tissue was correlated with the marked increase in peroxidase, phenylalanine ammonia lyase, β-1, 3-glucanase, polyphenol oxidase and phenol activity after challenge inoculation with the target pathogen (Govindappa *et al*., 2011). Evidences show that induction of systemic resistance can lead to the direct activation of defense-related genes and priming of cells, resulting in stronger elicitation of defense responses (Conrath *et al*., 2001; Hammerschmidt, 2014).

Moreover, antibiotics have been used to control certain bacterial diseases of high-value fruits, vegetables and ornamental plants since 1950s in some developed countries (McManus *et al*., 2002). Several antibiotics such as neomycin (Toa *et al*., 2011), streptomycin, tetracycline,

chloramphenicol and gentamycin (Pawar, 2015) have been used to control different species of phytopathogenic *Xanthomonas*. Therefore, the aim of the present study was to evaluate the antibacterial activity of medicinal plant extracts and their potential to induce resistance in enset and investigate growth, physiological and biochemical responses of enset clones grown in the field and subjected to various treatments.

1.2. Research questions, hypotheses and objectives

1.2.1. Research questions

- Do the *in vitro* test of medicinal plant extracts and antibiotics show antibacterial activity against Xcm? Do medicinal plant extracts and antibiotics have the potential to be used as alternative strategies to control enset bacterial wilt?
- Do pretreating enset plants with medicinal plant leaf extract induce systemic resistance and reduce disease incidence in the two enset clones? Is there a significant difference in disease incidence between the two enset clones?
- Are there clear and significant differences between treatments in plant height, leaf number, leaf area, leaf area index, leaf relative water content and photosynthetic performance among the studied enset clones?
- Which clone and treatment exhibit remarkable variation in biochemical concentration such as total carbohydrate, phenols, total nitrogen and crude protein as compared to the control?
- Which measured parameters are recorded high and can be taken as indicators of resistance under infection and induction? Is it possible to identify mechanisms of resistance/tolerance in the resistant enset clone?

1.2.2. Hypotheses

- Methanolic leaf extracts of the different species show variations in their antibacterial activity.
- Treating plants of the two enset clones with medicinal plant leaf extracts induces disease resistance/tolerance and improves crop performance.
- Growth, physiological and biochemical responses vary between resistant and susceptible enset clones under the different treatments

- Maximum activity of induced enzymes such as phenylalanine ammonia lyase, peroxidase and polyphenol oxidase after induction with leaf extract and inoculation with the pathogen indicates the degree of tolerance and susceptibility.
- Enset bacterial wilt affects the growth, leaf relative water content, physiology and other metabolic activities of enset clones
- Inducing enset's natural immunity with leaf extract reduces disease incidence in the susceptible than the resistant clone

1.3. Objectives

General objectives

- To evaluate the antibacterial activity of some medicinal plant leaf extracts and commercially available antibiotics against Xcm.
- To study induction of resistance in enset by leaf extract and evaluate its contribution in controlling enset bacterial wilt disease
- To investigate growth, leaf relative water content and metabolic responses of enset clones treated with different treatments under field condition

Specific objectives

In this study, the under mentioned specific objectives were achieved.

- To assess the chemical composition of crude leaf extracts, and evaluate the antibacterial activity of these extracts and commercially available antibiotics *in vitro*.

- To examine the activity of induced enzymes and metabolic changes in enset clones under different treatments
- To evaluate the contribution of induced resistance in reducing disease incidence and compare the contribution between resistant and susceptible enset clones
- To study the photosynthetic and leaf relative water content responses of the two enset clones under the different treatments
- To study metabolic changes in control, induced and infected control plants of the two enset clones under field condition.
- To evaluate growth responses of the two enset clones and compare the differences between treatments

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Origin and distribution

Enset is an old genus first described by Paul Fedorowitsch Horaninow in 1862 (Cheesman, 1947). According to Deckers *et al*. (2001), it originated in south-east Asia. Contrary to this, Peregrine (1992) claimed that the centre of origin of enset is in south west Uganda. The wild representatives of the genus enset are found throughout Africa and southern Asia (Lye and Sue Edwards, 1997; Kress *et al*., 2001). In Africa, it occurs widely across the continent in the western, central, eastern and southern parts (Peregrine, 1992; Brandt, 1996; Jones, 2000). In Asia, some species of the genus are present in north eastern India, Burma, Maynamar, Thailand, Philippines, Papua New Guinea, Indonesia and China (Deckers *et al*., 2001). The greatest genetic diversity occurs in the Assam (northeast India)-Burma region. From there it spread into Africa (Peregrine, 1992; Brandt, 1996; Deckers *et al*., 2001; Jones, 2000). Although the genus *Ensete* has a wide distribution in Africa and Asia, most evidences show that it was domesticated only in Ethiopia. Even within Ethiopia, *enset* is confined to a relatively small region of the southwest, in areas inhabited by speakers of Semitic, Cushitic and Omotic languages (Brandt, 1996; Blench, 2007).

Nevertheless, evidences about where the first domestication of the plant took place are controversial. Few scholars argued that *Ensete ventricosum* was first domesticated in ancient Egypt while the overwhelming consensus is that the species was domesticated in Ethiopia in prehistoric times (Brandt, 1996). In addition, Pankhurst (1996) suggested that enset cultivation in Ethiopia may have started around the 15^{th} century, south of Ghibe river. However, historical

evidence regarding the origin and domestication of crops is seldom completely dependable (Pankhurst, 1996).

2.2. Biology of Enset

Ensete ventricosum is a perennial, monocarpic, herbaceous and monocotyledonous crop (Lye and Sue Edwards, 1997; Kress *et al*., 2001). It has a diploid chromosome number with n = 9 (Cheesman, 1947; Simmonds, 1962; Deckers *et al*., 2001). The crop has a genome size of approximately 547 megabases, similar to the 523-megabase genome of the closely related banana (*Musa acuminata)*. Additionally, enset contains genes that are absent in banana. These include reverse transcriptases, virus-like sequences and a homolog of the RPP8-like resistance gene (Harrison *et al*., 2014). This indicates the large gene pool in the species that could be utilized for improvement of the crop (Genet Birmeta, 2004).

Research results suggest high enset clone diversity. However, there is greater disparity in research reports in terms of number and type of clones. In a baseline survey, SARI, (2014) disclosed a total of 440 cultivar names in eight enset growing administrative zones in Ethiopia. The number of cultivars in each zone was 75 (Dawro), 26 (Gedeo), 63 (Gurage), 51 (Hadiya), 66 (Kembata-Tembaro), 62 (Sidama), 69 (Silte) and 28 (Wolaita). However, this number was reduced to 312 after known synonyms were replaced with distinct names. Zerihun Yemataw *et al*. (2014) reported a total of 278 clones with distinct names from seven enset growing zones. Hadiya is the richest zone with a total of 59 clones followed by Kembata (43), Dawro (42), Wolaita (39), Gamo Gofa (34), Gurage (31) and Sidama (30). In other studies, Admasu Tsegaye, (2002) described 146 different enset clones from Sidama, Wolaita and Hadiya zones. Similarly, Almaz Negash, (2001) recorded 146 different enset clones from four zones (65 from Kefa-Sheka, 30 clones from Sidama, 45 from Hadiya and 6 from Wolaita). Moreover, Genet Birmeta,

(2004) studied 111 enset clones from nine enset growing areas of Ethiopia. In all the studies, duplication of names is reported. The same enset clone is given different names in different areas and different enset clones are given the same name in different localities.

Based on genetical studies, Genet Birmeta, (2004) and Genet Birmeta *et al*. (2004) suggest that the current cultivated enset clones have been introduced to domestication from a limited number of wild progenitors. However, subsequent gene flow between wild and cultivated enset may have been inhibited by differences in modes of propagation and harvesting time. In cultivated enset, genetic diversity within populations is high (Dagmawit Chombe and Endashaw Bekele, 2011). Despite the large variation in agroecological conditions among enset growing areas, amplified fragment length polymorphism (AFLPs) studies reveal that only 4.8 % of the total genetic variation is found between regions and 95.2 % within regions or populations (Almaz Negash *et al*., 2002). This may be explained by regular long distance exchange of clones and the existence of substantial levels of phenotypic plasticity in enset due to changing weather and soil conditions (Almaz Negash *et al*., 2002; Zippel, 2005). Random Amplified Polymorphic DNA (RAPD) results show that the genetic diversity in cultivated enset in a particular area appears to be related to the extent of enset cultivation, the culture and distribution pattern of the different ethnic groups than geographical distance (Genet Birmeta, 2004).

Enset is a tall plant reaching up to 13 meters. The leaves are large, basal, spiral with long sheathing leaf bases overlapping and forming a pseudo-stem. They are 4-6 m long and 60-90 cm wide with prominent midrib. Unlike the genus *Musa*, a petiole is lacking in enset (Simpson, 2005).The stem has three parts. The upper most part, the pseudostem is made of a system of tightly clasping leaf bases or leaf sheaths (Figure 2.2.1). It is shorter and thicker than the pseudostem of banana. The pseudostem may be 2-3 m tall and contains an edible pulp and

quality fiber. The corm is the second part found underground. It is the enlarged lower portion of the stem. It may be up to 0.7 meters in length and diameter. A short section of stem near the soil line, between the pseudostem and corm, is the true botanical stem. Leaves and the single flower head start from the true stem at its center, grow up through the middle of the pseudostem, and emerge at the whorl in the middle of the leaf bases (Brandt *et al*., 1997).

Enset has a fibrous root system that grows out from the corm. It is a shallow root system that extends 2-3 m around the plant (Peregrine, 1992; Deckers *et al*., 2001). As compared to banana, enset has a larger root system with thicker main roots and lower shoot-root ratio. It is also true that the root density in enset sharply decreases with increasing soil depth (Blomme *et al*., 2008). Furthermore, more than 70 % of the fine root mass is found in the above 0-20 cm depth and live fine root mass shows a seasonal variation (Solomon Zewdie *et al*., 2008).

The wild relatives of *E. ventricosum* grow in Ethiopia in close proximity with cultivated forms and are morphologically discrete. Enset farmers can make a distinction between the two forms. Wild forms reproduce sexually and have a thick pseudostem base but unpalatable corm while the cultivated enset plants reproduce vegetatively and have a smaller pseudostem base. They also have a more palatable corm and a prominent epicuticular wax on abaxial leaf surfaces (Blench, 2007; Hildebrand, 2007).

As a monocarpic crop enset has a single flowering period throughout its life time. The flowers are hermaphrodite with a single petal protected by a large dark purple bract that does not drop after withering. Five stamens produce sticky pollen. The flowers form a massive and hanging spike inflorescence (Figure 2.2.1) 2-3 m in length (Peregrine, 1992; Deckers *et al*., 2001). The flowers form leathery fruits that are about 9 cm long and look like rounded banana. They have a

thin layer of orange colored pulp containing 3-12 brownish black hard seeds of about 2 cm in diameter. The fruits are inedible due to the hard seeds and little pulp (Deckers *et al*., 2001; Hildebrand, 2007). During the time of development of a flower and fruits, most of the plant's carbohydrate is translocated to these parts, used and then the plant dies altogether. Thus, harvesting, either for food and fiber or propagation is done before flowering (Taye Bezuneh and Asrat Feleke, 1966; Brandt *et al*., 1997).

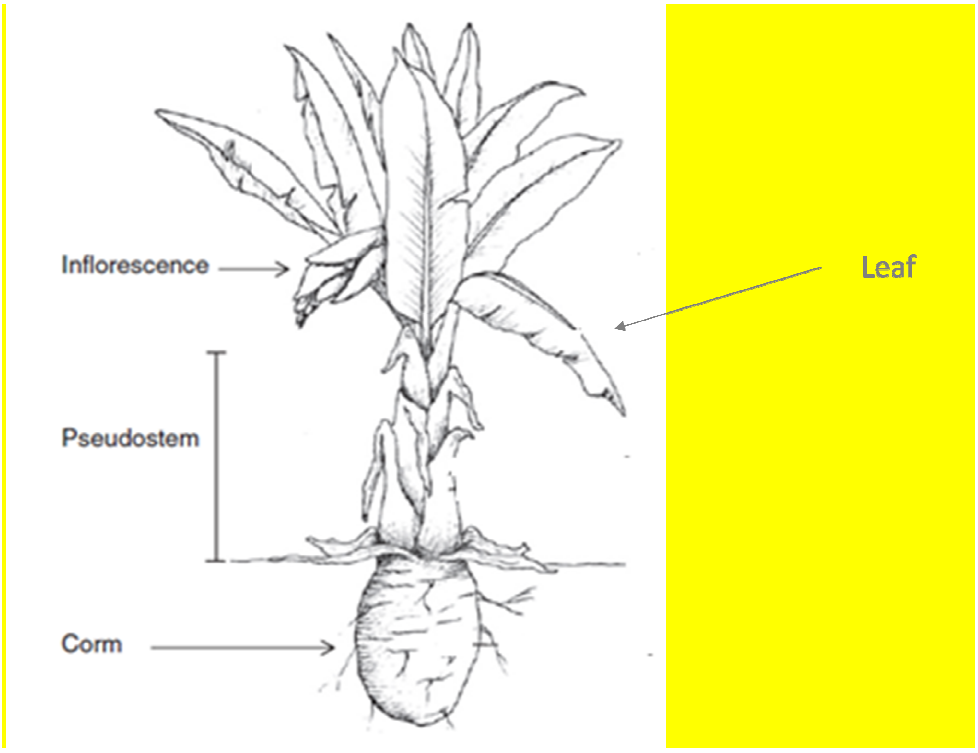


Figure 2.2.1: Morphology of cuultivated enset (Source: Sirawdink Fikreyesus *et al*., 2013)

2.3. Taxonomy of Enset

Ensete ventricosum (Welw.) Cheesman) belongs to order Zingiberales, family *Musaceae* and Genus *Ensete* (Lye and Sue Edwards, 1997; Kress *et al*., 2001; Simpson, 2005). *Musaceae* is a paleotropical family, forming the basal lineages of order Zingiberales. The family comprises of three genera, namely *Musa*, *Ensete* and *Musella* (Simpson, 2005; De Langhe *et al*., 2009). The third monotypic genus consists of *Musella lasiocarpa* (Golden Lotus Banana), a species endemic to southwest China. The genus *Ensete* was first described by Paul Fedorowitsch Horaninow in his *Prodomus Monographiae Scitaminarum* in 1862 in which he included a single species, *Ensete edule*. However, the genus was not recognized until its revival in the first series of papers in the Kew Bulletin in 1947 by Cheesman on the classification of bananas, with a total of 25 species (Cheesman, 1947).

According to the most recent revision of the genus by Simmonds (1962), *Ensete* comprises of six species. The species have wide distribution and notable species are African members. These are *Ensete ventricosum* (Ethiopia), *E. homblei* (south east Zaire to north Zambia), *E. gilletti* (west Tropical Africa to Malawi), *E. glaucum* (east central Nepal to Papuasia) and *E. superbum* (west India, Assam to north Thailand). Endemic species are *E. perrieri* (Madagascar) and *E. wilsonii* (Yunnan, China). Most of the *Ensete* species except *E. ventricosum* are well known for ornanmental use. *E. ventricosum* grows wild in Eritrea, Ethiopia and Sudan in the north, in Angola, South Africa and Mozambique in the south (Cheesman, 1947).

2.4. Ethnobotanical and ecological uses of enset

Characteristics of *E. ventricosum* that affected prehistoric strategies for hunting and gathering and set the foundation for human-plant interaction before food production include size, timing of

availability, environmental requirements and the ability to survive and reproduce around intensive use by humans (Blench, 2007; Hildebrand, 2007). Enset is large in size as compared to other plants and available throughout the year. It would therefore be more likely to be eaten by hunter-gatherers in seasons when other resources are not available. Moreover, the plant is an important year round source of fibre and shelter materials to hunter-gatherers. It is also visible from a distance, which could make it an easy target for use by people new to an area (Hildebrand, 2007). Such long time interaction shows the level of cultural entrenchment between enset and a particular people. An Ari (ethnic group in southern Ethiopia) ritual specialist explained the cultural entrenchment as: "We and enset have a long history of relatedness. We rely on it and it relies on us for survival. This means we cannot live without it and it cannot live without us either; we are created to support each other" (Gebre Yintiso, 1996).

The type and number of enset clones cultivated in southwestern Ethiopia vary from place to place. A large number of clones are cultivated for multiple purposes, different requirements of site and climate. In each area, different clones are grown for different purposes (Zippel, 2005). Therefore, the different livelihood requirements of a farmer are partially or wholly covered by enset products. As such the crop has several roles which can be categorized as nutritional, material, ecological, aesthetic, and medicinal values (Brandt *et al*., 1997).

In areas where enset is an indigenous crop, it is the major source of human food supplemented with legumes and animal products. The main food items prepared from enset are *Kocho, Bulla* and *Amicho. Kocho* is the fermented product obtained from the corm and pseudostem. *Bulla* is made by dehydrating the juice collected during the decortication of the pseudostem and grating of corm while, *Amicho* is the stripped corm of younger plants of enset, boiled and consumed (Liyuwork Zewdie, 1996; Sirawdink Fikreyesus *et al*., 2013).

Enset is processed annually from November to March and processing enset is an entirely women's task. The methods used in enset processing are more or less similar among the various enset growing areas (Liyuwork Zewdie, 1996). The starch deposits in the corm and pseudostem are extracted through fermentation. At harvest, enset plants are uprooted, the pseudostem and other edible parts are cut, sliced and torn. Before placing into the fermentation pit, the processed parts are squeezed by trampling and the liquid that oozes out, known as *Bulla* and it is collected in a small side pit lined with enset leaves. The pit is usually shallow but the depth can be increased by constructing above ground, a well-like structure with stick supports and enset leaves (Kelbessa Urga *et al*., 1996). The content is left to ferment for a period of time ranging from a few months to several years. The product is considered ready for consumption after 90 days from the initial processing day, but can also be kept for one or more years (Kelbessa Urga *et al*., 1996; Deckers *et al*., 2001). The fermented product is cooked into pancake-like flat bread and locally called *Kocho*. It is eaten with cabbage leaves and minced meat in some areas. Corms with few fibres are especially valued as they yield *Kocho* of high quality. Corm chunks of certain enset clones are not fermented but eaten as a boiled form called *Amicho* (Liyuwork Zewdie, 1996; Deckers *et al*., 2001).

The quality of enset products depends on the age, type of clone and method of processing. The best quality *Kocho* is white in color and less fibrous produced when no *Bulla* is extracted from enset plants of four to eight years old. The lowest grade *Kocho* is darker and more fibrous and is the end product of *Bulla* extraction (Liyuwork Zewdie, 1996). Likewise, *Bulla* is white in color and relatively small in production quantity as compared to *Kocho*, but it still fetches a much higher price than *Kocho* (Liyuwork Zewdie, 1996).

Enset is a strategic food reserve as it can be harvested at any time of the year and ideally suited to bridge food shortage that is common in Ethiopia (Brandt *et al*., 1997; Deckers *et al*., 2001).Outside Ethiopia, the inflorescence is eaten as an enjoyment in Malawi and the flower bud is consumed as a boiled vegetable in many parts of south-east Asia (Deckers *et al*., 2001). Furthermore, most part of enset is a good fodder for livestock. Enset leaves and pseudostem are cut down and used for cattle feeding especially in the dry season when grass is scarce. It is the best fodder during the dry season due to the fact that the plant contains much water in the thick leaves and pseudostem (Shigeta, 1990; Deckers *et al*., 2001*).*

The nutritive value of enset products is evaluated in terms of its chemical composition. The chemical composition of enset dry matter as whole plant is 90.87 % organic matter, 9.13 % ash of which 5.98 % is crude protein, 0.84 % is crude fat, 9.48 % is crude fibre, 74.57 % is soluble carbohydrates and 60.62 % is starch (Mohammed *et al*., 2013). This shows that enset is a rich source of carbohydrate (Solomon Zewdie *et al*., 2008). The unprocessed corm of enset is also rich in calcium (Ca), magnesium (Mg), potassium (K), zinc (Zn), and iron (Fe) (Ayalew Debebe *et al*., 2012; Sirawdink Fikreyesus *et al*., 2013). Processed forms such as *Kocho* and *Bulla* are rich in calcium (Ca) and zinc (Zn) compared to other similar food stuffs and contain comparable concentration of copper (Cu), iron (Fe) and magnanese (Mn). These products are free of heavy metal contaminants such as cadmium (Cd) and lead (Pb) (Minaleshewa Atlabachew, 2007). In addition, *Amicho* (boiled corm) has higher total phenolics content next to teff and corn (Sirawdink Fikreyesus *et al*., 2013).

Industrially, enset starch is used for various applications. Several studies reveal that enset starch has amylose content whereby granule size, X-ray diffraction pattern and gelatinization temperature comparable to potato starch, which is commonly used in pharmaceutical

applications (Abraham Wondimu *et al*., 2014). Research results of Tsige Gebre-Mariam and Nikolayev (1993) illustrate that enset starch can be used both as a tablet binder and disintegrant possessing a better binding ability and less disintegrating power than potato starch. It is also used as gelling agent. The cross-linked and acetylated form of enset starch shows the potential use as a novel drug delivery system (Abrham Wondimu *et al*., 2014). Furthermore, the squeezed and dehydrated product of enset (*Bulla*) is used as a gelling agent substituting agar in *in vitro* propagation. *Bulla* as a gelling agent is capable of producing an equivalent number of shoots, roots, leaves, shoot height and associated fresh weight of the plantlets in pineapple (Biruk Ayenew *et al*., 2012).

The ecological role of enset emanates from its vegetative organs morphology. Enset with deep roots and leaf canopies of long duration improve the hydrological dynamics of an area, increasing infiltration and decreasing surface runoff that results in more water in the soil and aquifers (Tadesse Kippie, 2001). Moreover, the very large leaf area and perennial canopy of the leaf provide protection to the soil from eroding effect of rain drops. Its extensive root system and leaf litter mulched beneath the plant protect soil from runoff (Brandt *et al*., 1997; Asnaketch Welde-Tensaye *et al*., 1998; Deckers *et al.*, 2001). Enset also serves the same role as trees, providing people, other plants such as coffee that need only moderate sunshine, and animals, protection from wind and sunshine (Shigeta, 1990). Aesthetically, enset beautifies the Ethiopian landscape by its thick, dark green foliage (Brandt *et al*., 1997).

Enset plays an important role in local medicine. Particular clones and parts of enset are used medicinally for both humans and livestock to cure bone fractures, broken bones, childbirth problems (i.e., assisting to discharge the placenta), diarrhea, and birth control (Peregrine, 1992; Brandt *et al*., 1997). For instance, *Amicho* and *Bulla* from certain enset clones such as *Astara*,

Gariyena, and *Sabara* are considered to have medicinal value in speeding up healing of bone fractures (Liyuwork Zewdie, 1996). In Uganda, the plant sap is also said to encourage the flow of milk in nursing mothers. Moreover, the Chaga tribe in the Kilimanjaro region uses the floury endosperm of the seeds as an aid to divination and augury (Deckers *et al*., 2001).

Furthermore, enset has numerous material uses. Cut leaves of enset are indispensable for wrapping *Kocho* and *Bulla* during fermentation, storage and transport to market, temporary roof covering, umbrella, baking bread, as a lunch-box, for serving food (Liyuwork Zewdie, 1996), thatching (Deckers *et al.,* 2001*),* sheeting to sit, making containers, dressing as women's skirts and personal ornaments for funeral and wedding cermonies (Shigeta, 1990). In rural areas, the pseudostem and leaf fibers are economically important to make sacks, bags, ropes, cordage, mats, construction materials such as tying materials in place of nails and sieves (Brandt *et al*., 1997; Deckers *et al.,* 2001*).* Additionally, the seeds are often made into attractive necklaces and as a component of various rituals (Peregrine, 1992).

2.5. Ecology and cultivation of enset

The genus enset is found throughout Africa and southern Asia (Lye and Sue Edwards, 1997; Kress *et al*., 2001). Enset flourishes in the cool tropics within a temperature range of 18 to 28°C. It requires a relative humidity of 60 to 80 % and an annual rainfall of 1100 to 1500 mm. In Ethiopia, these conditions are met in the enset belt at altitudes between 1700 to 2450 m above sea level (Deckers *et al*., 2001). The crop is mostly grown on soils of volcanic origin such as the deep, well drained red soils (Nitisols, Luvisols) or black soils (Phaeozems) with pH values between 5.6 and 7.3. Many enset soils are cultivated and enriched with organic matter to a depth of 60 cm (Deckers *et al*., 2001). Unlike banana, enset is more tolerant to wet conditions. This is because enset survives in the swampy areas of Uganda and northern Tanazania where bananas

die. It also competes well with grasses and other weeds to a degree that is exceptional in the *Musaceae* (Deckers *et al*., 2001). Enset can also survive short periods of frost and withstand prolonged drought periods (Deckers *et al*., 2001). In spite of its extensive distribution in the wild, enset has been domesticated only in Ethiopia. Wild enset propagates naturally by seed, and is restricted to altitudes of approximately 1200 to 1600 meters above sea level. However, farmers almost always propagate domesticated enset vegetatively and expanded its cultivation area from 1100 to 3000 m above sea level (Shigeta, 1991; Zippel, 1995; Brandt *et al*., 1997).

Wild enset grows in a number of countries in central and eastern Africa including Congo, Mozambique, Uganda, Tanzania and Zambia. In Ethiopia, the distribution of wild enset is restricted. It grows mainly around the city of Bonga and in a smaller area around the Omo river, inhabiting areas ranging from dense forests to open shrub land, or along riverbanks (Genet Birmeta *et al*., 2004). There are usually about 10-200 wild enset plants in a population and the areas where these enset plants grow are often not suitable for human settlements. On the riverbank side, wild enset plants are sometimes cut down by the Ari people when sorghum and other crops are cultivated since monkeys like the fruits of the wild enset and damage nearby crops (Shigeta, 1990). In contrast, cultivated enset grows in a wider area comprising the central, south and southwestern parts of Ethiopia, but mainly at higher altitudes ranging from 1500 to 3100 m (Genet Birmeta *et al*., 2004).

Enset farming is believed to be indigenous to Ethiopia and is a common feature of the farming systems in the south and southwestern parts of the country. It constitutes what is often termed as the ‘Enset system’ (Desalegn Rahmato, 1996). Agricultural land dedicated to enset has been increasing through the years and recent reports indicated that 263 890 ha of land are covered with enset (CSA, 2009). Moreover, according to Shank and Cherenet Eritro (1996), enset

accounts for 65 % of the total crop production in South Nations Nationalities Peoples State (SNNPS) and 9 % of the total in Oromiya. In the Enset-based agricultural system, enset growing areas are categorized into four sub-systems based on the extent to which people depend on enset as a staple crop; i) Sidama and Gurage: in this sub-system, enset is grown as a staple food and main crop. It is a sub-system where enset is grown in dense plantations and highly dependent on cattle manure for fertilizing enset fields. The main enset product in this sub-system is *kocho.* ii) The Gamo, Hadiya, Wolaita, and Ari people who use enset as a co-staple crop with cereals and tuber crops; iii) Oromo farmers of southwestern Ethiopia who rely upon cereals as the most important crops and grow enset and root crops as secondary crops. Enset is grown largely for security reasons and eaten in the form of *Kocho* and *Amicho;* iv) Sheko in southwestern Ethiopia growing root and cereal crops as primary and secondary dietary importance, respectively. Enset has a minor importance (Brandt *et al*., 1997). The permanent enset-based farming system is closely linked to livestock. Dung is collected in the enclosures where the animals are kept at night and applied to enset plantations to enrich the soil in organic matter (Deckers *et al*., 2001).

Naturally, enset can be multiplied by seed. Nonetheless, farmers multiply enset exclusively by vegetative propagation. One reason is scarcity of seeds since enset is harvested prior to seed production. Other reason is to maintain the specific characteristics of each landrace. However, changing weather and soil causes phenotypic variability. Both increasing as well as decreasing number of landraces have been observed over the years in the different regions due to changing food preferences, climate, pests and diseases, cultivation systems, and infrastructure. Thus, farmers tend to increase the number of cultivated landraces to broaden its use, and to respond to annual climate fluctuations (Zippel, 2005). Enset growing areas are characterized by diverse ethnic groups with distinct culture and cultivation practices. Thus, vegetative propagation of nset

varies from place to place. Generally, three approaches have been identified: i) uprooting of parent corm and use of half corm planting; a common approach in the Wolaita, the Gamo, the Gofa, and the Sidama areas; ii) sucker initiation at original place of parent corm and uprooting followed by transplanting after suckers emergence (Jemjem, Gedeo, Sidama, and some areas of Gofa); iii) uprooting and whole corm planting commonly used in Kambata-Hadiya and Chebo-Gurage (Mulugeta Diro and Endale Tabogie, 1992 cited in Mulgeta Diro *et al*., 1996). According to these authors, half corm planting is more productive than whole and quarter corm planting. In this approach, corm of 3-years old enset with some portion of pseudostem is cut off and vertically split into two and used as a starting material for vegetative propagation.

Enset farms are established from vegetatively propagated suckers and these are usually produced from 2-4 years old corm (10 to 20 cm in diameter) and the true stem. To initiate suckering, a mother plant is cut out and its pseudostem cut off at about 25 cm above the corm. The apical meristem and the roots are removed. The removal of the apical meristem eliminates the apical dominance and allows the development of side-shoots or suckers on the corm (Teketel Makiso, 1996; Brandt *et al*., 1997; Deckers *et al*., 2001). By using one of the propagation approaches mentioned above, the corms are buried in dung 20 cm below the soil surface. Suckers start emerging after 4 to 6 weeks and 40 to 200 suckers are produced per plant within a couple of months. When approximately 75 cm tall, at about 5 weeks after emergence, the suckers are separated from the corm and transplanted into a nursery where they remain for one year. They are then transplanted at close spacing (25 x 25 cm) in a weed free plot. As they grow taller, they are transplanted a few more times to optimally exploit the limited land area per farmer. The final spacing is 3 x 3 m, which seems adequate for small varieties but too close for larger ones. After the last transplanting, enset is intercropped with food crops (vegetables, maize, and beans) and

cash crops (chat, coffee) for two years (Teketel Makiso, 1996; Brandt *et al*., 1997; Deckers *et al*., 2001). Thereafter, the heavy enset canopy prevents further intercropping. Maintenance is simple and only consists of removing old leaves from the plant and mulching the field (Deckers *et al*., 2001).

2.6. Enset diseases

Diseases are collectively the most severe biological problems facing enset. They affect enset at any growth stage such as suckers, seedlings, young plants and mature plants. It is particularly a serious loss when diseases kill an enset plant late in its life cycle. This is because farmers have already invested several years of land, labor, and resources into the plant's production. In some Enset-growing areas, such situations have caused farmers to abandon their enset farming and replace it with annual crops (Brandt *et al*., 1997; Deckers *et al*., 2001). The lack of knowledge about implementation of preventive strategies contributes to the severity of enset plant diseases.

Enset diseases are caused by bacteria, nematodes, fungi, and viruses. Enset foliar diseases caused by fungi are numerous and widespread. Leaf spot diseases that commonly affect enset suckers, seedlings, and young plants are caused by *Phyllosticta Sp*., *Piricularia sp*. and *Drechslera sp*. Leaf spots due to *Cladosporium sp.* and to some extent *Deightoniella sp*. are frequently encountered in older plants (Quimio and Mesfin Tessera, 1996). Little is known about the fungal diseases affecting enset roots, corm and pseudostem. However, severe cases of *Sclerotium* wilt and root rot caused by *Sclerotium rolfi* on young seedlings and transplants are reported (Quimio and Mesfin Tessera, 1996).

Enset roots can also be affected by nematodes; the root lesion nematode, *Pratylenchus goodeyi*, the root knot nematode, *Meloidogyne sp*. These are commonly found and widely distributed in

enset farms. *P. goodeyi* is often found in large numbers associated with bacterial wilt affected plants and is, therefore, suspected to play a role in bacterial wilt development and severity. *P. goodeyi* induces extensive lesions on roots which normally unite and give way to other microorganisms to form a complex root rot (Quimio and Mesfin Tessera, 1996; Mesfin Bogale *et al*., 2004). Root knot galls due to *Meloidogyne sp*. usually develop at tips or near the tips of small rootlets. Galled rootlets are severely stunted, die and turn black presumably due to invasion by other microorganisms (Quimio and Mesfin Tessera, 1996). Moreover, insect pests such as enset root mealybugs is nonspecific and resulted in slow plant growth, lack of vigor, dried lateral leaves and subsequent death especially under stressful conditions (Temesgen Addis, 2005). With respect to viral diseases, mosaic and chlorotic streak diseases of enset are known. Mosaic is more destructive than chlorotic streak as it causes severe stunting of affected plants. According to Quimio and Mesfin Tessera, (1996) mosaic streak disease is the potential threat to enset farming.

The most devastating enset disease is caused by the bacterium *Xanthomonas campestris pv. musacearum*. Enset bacterial wilt was first reported in Ethiopia in 1968 and described as *Xanthomonas musacearum sp. n.* by Dagnachew Yirgou and Bradbury, (1968). Later on, the bacterium was renamed using accepted nomenclature as *Xanthomonas campestris pv. musacearum* (Young *et al*., 1991). The bacterium also infects banana under natural conditions and a natural epidemic of the disease emerged on banana in 1974 (Dagnachew Yirgou and Bradbury, 1974). In Uganda, the disease was first reported on bananas in the Mukono and Kayunga districts in 2001, from where it spread rapidly and gained economic importance (Tushemereirwe *et al*., 2003). The disease subsequently spread to Rwanda, Democratic Republic of Congo, Tanzania, Kenya, and Burundi (Ndungo *et al*., 2006). The disease is commonly known as *Xanthomonas* wilt or enset bacterial wilt (EBW). It damages enset at any growth stage. The

disease starts from the root and damages the whole plant. Infected plants exhibit symptoms first on the heartleaf or on one of the inner leaves where their tips become limp and hang down. The inner fold of a dropping heartleaf has grayish brown, rotting patches covered with a slimy secretion. In a more advanced stage of the disease, chlorosis develops and the leaf breaks at the petiole. In cross section, yellowish mass of bacteria oozes out from the vascular bundles of the leaf. Finally, all the leaves wilt, bend over and wither. Internal symptoms in cross-sections include yellow-orange discoloration of vascular tissue (vascular streaking) with some bacterial ooze in the psuedostems and grayish or yellowish bacterial exudates, brownish to bluish-black buckets in the corm. Eventually, the plant dies and rots to the ground (Dagnachew Yirgou and Bradbury, 1968; 1974; Dereje Ashagari, 1985; Temesgen Addis, 2005; Biruma, *et al*., 2007).

2.6.1. Disease control strategies

Disease controlling strategies go with the cause of the disease. In view of that, the traditional methods used to control fungal diseases of enset include establishing nursery in an open space, planting corms in well tilled and manured soil with proper spacing, thinning, removing and properly disposing old leaves, carryout regular weeding of nursery and avoid intercropping with tall plants (Quimio and Mesfin Tessera, 1996). On the other hand, root nematode diseases of enset could be controlled; a) using nematode free planting materials. This may be accomplished by i) dipping the corm in nematocides; ii) packing corms in mud mixed with nematocides; and iii) water treatment; b) reducing or eliminating nematode inocula in the soil before planting; and c) maintaining the plant in good health during cultivation (Quimio and Mesfin Tessera, 1996; Mesfin Bogale *et al*., 2004; Temesgen Addis, 2005).

Enset bacterial wilt is a systemic disease in nature. The major transmission means of the disease are infected planting materials and farming tools that are used to manage infested farms

(Gizachew Wolde-Michael *et al*., 2008; Temesgen Addis *et al*., 2010). There are no known commercial bactericides to control the disease. Therefore, the only controlling option is to take sanitary measures that would prevent, reduce or eliminate the spread of Xcm in the field. These include; a) flaming the enset cutting tools after using them in infected plants; b) deep tillage and turning over the soil to expose it under the sun during the dry period prior to planting; c) spot rotations of infested sites in the field with non-host plants of Xcm; and d) replacing wilt susceptible enset and banana with resistant ones. The cultivation of different enset clones with varying levels of resistance in a plot also prevents the rapid spread of bacterial wilt (Quimio and Mesfin Tessera, 1996).

2.6.2. Epidemology of enset bacterial wilt

2.6.2.1. *Xanthomonas campestris pv musacearum*

The pathogen, *Xanthomonas campestris pv Musacearum* (Xcm) belongs to a group of bacteria that are found only in association with plants or plant materials called *Xanthomonadaceae*. Xcm is a soil borne pathogen. It is straight rod in shape about 0.4 - 0.7 x 0.7 - 1.6 μm, motile by means of a single polar flagellum. The bacterium is chemo-organotrophic and obligately aerobic (Biruma *et al*., 2007). Metabolically, the bacterium is characterized as Gram negative, KOH and catalase positive; negative for nitrate reduction, do not denitrify or reduce nitrate; produce small amounts of acid from various carbohydrates and other carbon sources, but not from rhamnose, adonitol, sorbitol, dulcitol, meso-inositol, inulin or salicin. Gluconate is not immediately metabolized and asparagine is not used as a sole source of carbon and nitrogen simultaneously. Moreover, most isolates are insensitive to 2 % NaCl solution (Befekadu Haile *et al*., 2014). As a typical member of *Xanthomonadaceae*, the bacterium produces yellow, circular, mucoid, slimy colonies on nutrient agar and semi-selective medium YTSA-CC (Tripath *et al*., 2007).

Phylogenetic relationship studies of 20 Xcm isolates using different techniques reveal limited (< 2 %) nucleotide divergence among the isolates and thus the isolates are found to be homogenous The isolates are collected from Ethiopia, Uganda, Democratic Republic of Congo, Tanzania, and Rwanda for a period of four decades (1968-2005) (Biruma *et al*., 2007; Aritua *et al*., 2008).

Xcm grows slowly on synthetic media and can be overgrown easily by contaminants during culturing. Thus, to precisely diagnose the pathogen, two semi-selective media have been developed. These are cellobiose-cephalexin agar; CCA by Mwangi *et al*. (2007) and yeast extract, tryptone, sucrose, agar- cellobiose cephalexin; YTSA-CC by Tripath *et al*. (2007). On these media, Xcm is easily identified as yellowish, mucoid, circular colonies, and the bacterium can be easily isolated from infected plant tissues and infested soil. The yellow colored colonies of the pathogen are due to the abundant production of extracellular polysaccharide, called xanthan gum which contributes to significant blockage of vessels in infected plant tissues (Dagnatchew Yirgou and Bradbury, 1968; 1974; Biruma *et al*., 2007). Xcm is known to systemically invade all tissues of the hosts after infection. When infection starts from the lower parts of the hosts, the bacteria moves upward through the vascular tissues and the reverse occurs if infection occurs in the upper parts of the plants (Ssekiwoko *et al*., 2006; Blomme *et al*., 2008).

2.6.2.1.1. Hosts of *X. campestris pv. musacearum*

Natural hosts of Xcm are cultivated enset and banana (Thwaites *et al*., 2000; Dagnachew Yirgou and Bradbury, 1968; 1974). Although there are no studies on the sensitivity response of wild *Ensete ventricosum* to the pathogen, it is presumed to be susceptible and potential host (Jones, 2000). Artificial inoculation studies have shown that the ornamental *Canna orchoides* (Dejene Ashagari, 1985) and maize are found to be hosts (Aritua *et al*., 2007a). Moreover, artificial inoculation of wild relatives of banana (*Musa zebrina and M. ornate*) and *Canna indica*, an

ornamental/wild weed in Uganda with Xcm causes infection and produces symptoms (Ssekiwoko *et al*., 2006). However, there are inconsistencies on the host status of maize in research results. Dagnachew Yirgou and Bradbury, (1974) inoculated aubergine, barley, bean, broadbean, castor oil, lettuce, lucerne, maize, peanut, pelargonium, potato, sorghum, sunflower, sweet pepper, tobacco, tomato, and wheat with Xcm and confirms them as non-hosts. Likewise, Dereje Ashagari (1985) inoculated plants commonly found in enset growing areas such as *Chenopodium album*, *Colocasia antiquorium*, *Commelina* sp., *Guizotia scabra*, *Kalanchoe quartinia*, *Snowdenia polystachya*, *Solanum nigrum,* and *Tagetes minuta* and found to be unaffected after 10 weeks.

2.6.2. Mode of transmission

Enset *Xanthomonas* wilt is a transmissible plant disease. The major sources of Xcm inoculum are infected host plant parts, infested soil and traded parts of the hosts such as suckers, *Kocho*, *Bulla* and *Amicho* in enset, and seedlings, fruits, leaves and fibers in banana. The mode of disease spread varies depending on the agroecology, type of cultivation and cultivar. Garden tools, infected plant materials, infested soil, wind driven rain and insects are the means by which the pathogen spreads within and across fields (Gizachew Wolde-Michael *et al*., 2008; Tripathi *et al*., 2009; Temesgen Addis *et al*., 2010).

According to Gizachew Wolde-Michael *et al*. (2008) and Temesgen Addis *et al*. (2010) cutting of healthy green leaves, pseudostem and broken leaves with contaminated knife caused infection at various levels. In this experiment, cutting of green leaves and broken leaves results in high level of disese incidence both in enset and banana. The other means of Xcm transmission is through infected plant debris. In enset leaf sheath, Xcm can survive for at least 3 months in infected samples kept in the laboratory, 2 months in the samples used as soil surface mulch and a

month in the samples buried 15 cm deep in the soil. Similarly, viable cells of Xcm in infected banana samples are stable in the laboratory for three months. Unlike enset, Xcm survives in banana debris for 21 days in the field (Eden-Green, 2006; Mwebaze *et al*., 2006; Gizachew Wolde-Michael *et al*., 2008).

Moreover, the survival of Xcm in the soil varies with the amount of soil moisture and soil treatment. As soil moisture increases, the survival rate of Xcm increases. Also, soil treatment increases the survival rate of Xcm. In untreated soil, Xcm isolates can survive up to 9 days. Thenafter, the population of the bacteria reduces to a level that cannot initiate infection (Gizachew Wolde-Michael *et al*., 2008). In sterile soil, the pathogen can live for 90 and 45 days under high and low soil moisture, respectively (Mwebaze *et al*., 2006). Therefore, to be safe fields infested with Xcm should not be replanted for at least 6 months due to carryover of soil borne inoculum (Gizachew Wolde-Michael *et al*., 2008: Tripathi *et al*., 2009). Xcm exists in *Kocho* processed from bacterial wilt infected enset. *Kocho* samples examined up to 105 days from the time the pulverized pseudostem and corm added to the fermentation pit shows Xcm colonies at varying levels. This shows that *Kocho* is also another means of pathogen transmission (Fikre Handoro, 2014).

In enset, plants are harvested before flowering and thus transmission of the pathogen by insects is not a problem. However, Xcm has been isolated from the nectar and ooze exuding from the fresh opening made by fallen bracts of male and female flowers of banana (Tinzaara *et al*., 2006). Insects visit the banana inflorescence and pick up Xcm and then transmit it to healthy plants. The most common insects visiting banana inflorescence and potentially transmit the pathogen include stingless bees (Family *Apidae*), fruit flies (Family *Drosophilidae*) and grass flies (Family *Chloropidae*). This has been confirmed by isolating the pathogen from stingless

bees, honeybees, fruit flies and grass flies collected from male flowers of symptomatic and asymptomatic plants (Tinzaara *et al*., 2006; Gold and Bandyopadhyay, 2005). Moreover, animals that feed on leaves and pseudostems of infected enset are found to be potential means of disease transmission (Thwaites *et al*., 2000).

2.7. Plant reactions to pathogens

In the course of their development, plants face a wide range of potential pathogens, including bacteria, viruses, fungi, nematodes, and insects. Pathogens use different strategies to invade a plant, as well as to feed on and reproduce in the plant. Biotrophic pathogens propagate in living plant tissue and generally do not cause necrosis as a result of infection. They use various means such as haustoria production to extract nutrients from host (Agrois, 2005). Opposite to this, necrotrophic pathogens actively induce necrosis in infected tissues often through the production of toxins and obtain nutrients from the dead host tissue. Others called hemibiotrophic pathogens that incorporate aspects of both biotrophic and necrotrophic infection strategies. *Xanthomonas campestris pv. musacearum* is hemibiotrophic (Endah *et al*., 2010). This strategy often involves an initial biotrophic infection phase during which the pathogen spreads in host tissue followed by a necrotrophic phase during which host cell death is induced (Thomma *et al*., 2001; Dodds and Rathjen, 2010).

Plants are resistant to the majority of micro-organisms based on preformed and induced defense mechanisms (Berger *et al*., 2007). Preformed physical and chemical barriers such as cuticles, cell walls and constitutively produced antimicrobial compounds that protect the plant against most attempted invasions (non-host interactions). However, when a putative pathogen is able to pass over these barriers (host interaction) and recognized by the plant, a rapid induction of defense responses occur by the resistant plant and can prevent the bioagressor from developing

and causing infection. Disease spreads only in susceptible plants (compatible interaction) which are unable to recognize the pathogen or respond too slowly (Garcia-Brugger *et al*., 2006).

The recognition of the pathogen is the first step for the initiation of defense responses. Firstly, basal resistance response which is induced early during the invasion process and mediated by pathogen associated molecular patterns (PAMP). These PAMPs can arise from either non pathogenic or pathogenic organisms (Berger *et al*., 2007). They include elicitors such as glycoproteins from oomycetes, lipopolysaccharides, peptodoglucans, bacterial flagellin, chitins, which are essential for the survival of the pathogen (Jones and Dangl, 2006; Dodds and Rathjen, 2010). Plants in turn have evolved pattern recognition receptors (PRR) to sense and prevent the establishment of the pathogens. PRRs are mostly membrane localized proteins that counteract the effects of PAMPs. Secondly, pathogens that have developed ways of bypassing the plant's innate immunity (basal resistance) provoke the plant to mount an additional response known as hypersensitive response (HR). It is a form of voluntary suicide of plant cells at the site of invasion limiting access to nutrients and minerals for the pathogen and progression to other cells (Jones and Dangl, 2006). Thirdly, HR is not always effective in providing ample resistance and blocking pathogens from penetrating the interior of cells (Berger *et al*., 2007). However, it triggers an intensified state of readiness in which plant resources are assembled in preparation for future attack resulting in systemic acquired resistance (SAR), which leads to the activation of a chain of defense components in distal uninfected parts of the plant (Dodds and Rathjen, 2010).

In general, pathogen or elicitor-induced signal transduction pathways lead to; i) the reinforcement of cell walls; ii) the production of antimicrobial metabolites (phytoalexins), production of pathogenesis-related (PR) proteins and enzymes of oxidative stress protection; iii) lignification; and often, iv) the hypersensitive response (HR), a form of programmed host cell

death at the infection site associated with limiting pathogen development (Lorrain *et al*., 2003; Garcia-Brugger *et al*., 2006). This shows that, in the course of resistance development, plants undergo tremendous changes in secondary metabolism due to induction of defense responses and alterations in primary metabolism which, in turn affect growth and development of the plant. Therefore, pathogen attack causes crop yield losses even in interactions which do not end up with disease or death of the plant.

The contact with pathogens alters plant primary metabolism. Because; i) the induction of defense is cost-intensive causing an increased demand for assimilates in the plant; ii) the pathogen tries to manipulate plant carbohydrate metabolism for its own need and withdrawal of nutrients that will further increase the demand for assimilates; iii) pathogen infection often leads to the development of chlorotic and necrotic areas that consequently decrease photosynthetic assimilate production (Guoa *et al*., 2005; Berger *et al*., 2007; Berova *et al*., 2007; Hooks *et al*., 2008). These changes result in decline in photosynthesis and an increase in respiration, photorespiration, and cell wall invertases activity. Invertases are extracellular enzymes which cleave sucrose in the apoplast where pathogens are available into glucose and fructose, increasing the amount of soluble carbohydrates (Lobato *et al*., 2009). Sugars are not only nutrients needed for growth, respiration, and storage compounds but also signals which can regulate gene expression, suppressing photosynthetic genes (Berger *et al*., 2007). This down-regulation of photosynthesis and the simultaneous increased demand for assimilates very often leads to a transition of source tissue into sink tissue during plant-pathogen interactions (Roitsch *et al*., 2003).

2.8. Botanicals as crop disease controlling agents

Plants, as long-lived stationary organisms interact with environmental variables over their lifetime and overcome attackers by producing secondary metabolites. This shows that metabolites play an important role in defense mechanisms and chemical communication. The secondary metabolites of plants are a vast storehouse of compounds with a wide range of biological activities. Secondary compounds from plants are virtually guaranteed to have biological activity and that activity is likely to function in protecting the producing plant from a pathogen, herbivore, or competitor (Dubey *et al*., 2011). These natural plant products can be extracted through different techniques and the extracted chemicals are referred to as botanicals or phytochemicals (Guleria and Tiku, 2009).

Botanicals have wide applications in the field of agriculture and health. They have been used as curative drugs for both human and animal ailments from time immemorial (Dubey *et al*., 2011). Botanicals namely, nicotine and alkaloids, rotenone and rotenoids, pyrethrum and pyrethrins, and vegetable oils were used in plant protection from the end of the 19th century to the beginning of Second World War. After this war, botanicals were replaced by synthetic organic pesticides. Researches on biopesticides of plant origin were actively pursued again throughout the second half of the 20th century (Dubey *et al*., 2011; Ntalli and Menkissoglu, 2011). The development of botanicals as biopesticides resulted from two parallel methods: i) the observation of the traditional uses of plants and extracts for cattle and crop protection, followed by checking the efficiency of these practices and identification of the active molecules; ii) the systematic screening of botanical families followed by biological tests in order to discover the active molecules (Ntalli and Menkissoglu, 2011).

Pest controlling strategies using botanicals are safer to the user and the environment because they break down into harmless compounds within hours or days in the presence of sunlight and have no any residual effect. Due to their close chemical resemblance to those plants from which they are derived, a variety of microbes common in most soils can easily decompose botanicals (Kagale *et al.* 2004; Guleria and Tiku, 2009; Dubey *et al*., 2011). Also, the use of botanicals maintains the biological diversity of predators. Botanical pesticides tend to have broad-spectrum activity, stimulatory effect to the host immunity and avoid the emergence of resistant races of pest species (Guleria and Tiku, 2009; Isman, 2006). The occurrence of several bioactive constituents in botanicals creates difficulty in standardizing the product and is an asset as it avoids the development of resistance among the pests (Guleria and Tiku, 2009). Moreover, many of the botanicals are environmentally friendly, pose less risk to humans and animals and have a selective mode of action (Isman, 2006). This is an indicator that botanicals effectively meet criteria of sustainable agriculture and have enormous potential to influence modern agrochemical research in the future (Guleria and Tiku, 2009).

In the developing world, the uses of botanicals have solved enormous problems of farmers caused by an array of pests that reduce yield and make farming a harder process. They are used as alternatives to synthetic pesticides (Isman, 2006; Isman, 2008; Ofuya, 2009) and attributes of botanicals as alternative pest control strategies include easy to use and formulate, availability and accessibility of raw materials at all times of the year particularly in tropical regions, minimum demand for application equipment, nontoxic to other organisms, less costly and environmentally friendly. These attributes contribute to the production of cheap and safe food. Therefore, botanicals are assets to developing countries and efforts should be maximized for their large-scale usage (Isman, 2006; Isman, 2008; Guleria and Tiku, 2009; Dubey *et al*., 2011; Olufolaji,

2011). As a matter of this fact, the future of botanicals is bright and most of the researches on pest control in both developed and developing countries are now geared towards the use of botanicals as alternatives to synthetic pesticides (Guleria and Tiku, 2009; Ofuya, 2009). Thus, the use of botanicals is now emerging as one of the prime means to protect crops and their products, and the environment from pesticide pollution (Bowers and Locke, 2004; Guleria and Tiku, 2009).

2.9. Induction of resistance in plants

In addition to pre-existing defense barriers to pathogens, plants possess an inducible immune system that controls the activation of defence mechanisms after recognition of a pathogen. One of the strategies to overcome crop production problems caused by plant diseases is through the targeted use of inducible defense mechanism that represents plant's inherent and own resistance. Induced resistance is a physiological "state of enhanced defensive capacity'' elicited by specific environmental stimuli, whereby the plant's innate defenses are potentiated against subsequent biotic challenges (Edreva, 2004; Vallad and Goodman, 2004). Induced resistance is a novel technology in the management of plant diseases and in practicing it, plant defenses are preconditioned by prior treatment that results in resistance or tolerance against subsequent challenge by a pathogen (Vallad and Goodman, 2004). Induced resistance offers the prospect of durable, broad-spectrum, eco-friendly and cost-effective disease controlling approach using the plant's own resistance (Abd El-Rahman *et al*., 2012; Jadesha *et al*., 2012; Walters *et al*., 2013; Walters and Fountaine, 2009). It also increases the ability of susceptible plants to withstand pathogens in a non-genetic way (Ma and Cui, 2002).

Nowadays, resistance induction in plants through biotic and abiotic agents has attracted the attention of researchers worldwide as a possible strategy for integrated plant disease management. This in addition, minimizes the environmental impact of chemical pesticides (Ghazanfar *et al*., 2011). Plants can be induced locally and systemically and become more resistant to diseases. To this end, various chemicals have been discovered that seem to act at various points in this defense, activating networks and mimic all or parts of the biological activation of resistance. For instance, acibenzolar-5-methyl at low rate activates resistance in many crops against a broad spectrum of diseases, including fungi, bacteria and viruses. Similarly, extracts from plants and microorganisms have also been described as resistance inducers (Oostendorp *et al.*, 2001; Zeller, 2006). Plant extracts are widely used all over the world and found to be effective and feasible means of plant disease management (Govindappa *et al*., 2011; Negashi *et al*., 2011).

Plants undergo both biochemical and enzymatic changes when infected or induced with abiotic agents (Misra *et al*., 2008). These physiological/biochemical changes are associated with induced resistance as the basis of resistance in plants to pathogens. Moreover, most researches on resistance mechanisms have shown that plants use defenses that are activated after infection to stop pathogen development. Many biochemical changes occur in plants after infection and some of these have been associated with the expression of defense as they have activity against pathogens *in vitro* (Hammerschmidt, 1999).

The activity of various defense related enzymes increases during plant-pathogen interaction or when treated with elicitor chemicals. Some of these enzymes are pathogenesis related (PR) proteins. These are plant protiens produced in response to infection by pathogens. They are also found in infected plants showing induced resistance to a second infection and are produced in

response to chemical elicitor application (Edreva, 2005). Peroxidases are among the PR proteins. They are heme-containing glycoprotiens that catalyze the oxidation by H_2O_2 of a wide range of organic and inorganic substances (Chittoor *et al*., 1999). Moreover, peroxidase production is also correlated to systemic acquired resistance (Chittoor *et al*., 1999). The function of peroxidase during infection and induction is the strengthening of plant cell walls by catalyzing lignin deposition and generating monolignol radicals for polymerization (Edreva, 2005). Polyphenol oxidases (PPO) are other types of defense related enzymes. They are enzymes catalyzing the oxygen dependent oxidation of phenols to quinones (Li and Steeffens, 2002). By doing this, the anti-pathogen effects of polyphenol oxidase include; i) general toxicity of PPO produced quinones to pathogens and plant cells resulting in accelerated cell death; ii) alkylation and low bioavailability of cellular proteins to pathogens; iii) cross-linking of quinones with protein or other phenolics and iv) the conversion of the endogenous phenolic pool to quinones further triggers an intricate series of non-enzymatic secondary reactions that might directly or indirectly affect the ability of plants to resist diseases (Li and Steffens, 2002).

Furthermore, phenylalanine ammonia lyase (PAL) is a key enzyme of phenylpropanoid metabolism. Phenylpropanoid pathway is one of the most important plant secondary metabolism pathways involved in the synthesis of a wide variety of phenolic compounds such as flavonoids, lignins, coumarins, and stilbenes (Osakabe *et al*., 2007; Lu *et al*., 2012). The transcriptional activation of PAL genes is controlled during plant growth and development and by abiotic and biotic stresses. PAL catalyses the first step in the conversion of primary metabolism to secondary metabolism (Osakabe *et al.*, 2007). It diverts the central flux of carbon from primary metabolism to the synthesis of countless phenolics (Zhang and Liu, 2015). Phenolics are aromatic benzene

ring compounds with one or more hydroxyl groups produced by plants mainly for protection against stress. Plants need phenolic compounds for pigmentation, growth, reproduction, resistance to pathogens and for many other functions. Importantly, phenolic phytoalexins secreted by wounded or otherwise perturbed plants repel or kill many microorganisms. Therefore, they represent adaptive characters that have been subjected to natural selection during evolution (Lattanzio *et al*., 2006; Bhattacharya *et al*., 2010). Overall, inducible defense related enzymes possess antimicrobial activities through hydrolytic activities on pathogen cell wall, contact toxicity of their products, cell wall lignification and involvement in defense signaling (van Loop *et al*., 2006).

The changes in carbohydrate metabolism during plant-pathogen interactions are also relevant because; first, the carbohydrate status affects the defense as well as general metabolism of the plant. Second**,** sugars are not only nutrients and signals for the plant partner but also for the microbial partner. Therefore, changes in assimilate levels may influence the spreading of the pathogen and might regulate gene expression of the pathogen. Third, certain pathogens possess extracellular sucrolytic enzymes such as invertases, fructoexohydrolases, and levansucrases. With the expression of these enzymes, the pathogen would be able to alter the hexose and sucrose levels in the apoplast (Berger *et al*., 2007; Lobato *et al*., 2009). The cumulative changes undergone in the primary and secondary metabolism of the plant consequently results in a significant reduction in growth parameters such as leaf area, leaf number, psuedostem diameter, plant height and others. It may be plausible to assume that the low productivity of infected plants is partly attributed to physiological stress associated with low photosynthetic rate of chlorotic leaves (Hooks *et al*., 2008).

Besides, the use of resistant clones is another alternative and a cost-effective method of managing diseases. In enset, all clones show disease symptoms at various intensity levels during the first 45 days after inoculation. However, some enset clones revive from infection and become healthy after 4 - 6 months depending on locations. The susceptible enset clones die altogether (Fikre Handoro and Gizachew Wolde-Michael, 2007; Biruma, *et al*., 2007; Gizachew Welde-Michael *et al*., 2008). These reports show differential responses of enset clones to the pathogen both in the field and greenhouse conditions. However, the mechanisms leading to these differential responses of enset clones, the role of plant extracts and induction of resistance as possible enset bacterial wilt management tools are not yet known and need to be investigated.

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Evaluation of the Antibacterial Activity of Some Medicinal Plant Leaf Extracts and Antibiotics against *Xanthomonas campestris pv. musacearum*

3.1.1. Medicinal plant material collection

Fresh leaves of medicinal plants Viz. *Achyranthes aspera L. (Amaranthaceae*), *Agarista salicifolia* (*Com.ex Lamb.) Don (Ericaceae), Datura stramonium L. (Solanaceae), Melia azedarach L. (Meliaceae), Pycnostachys abyssinica (Fresen) (Lamiaceae)* and *Vernonia amygdalina Del. (Asteraceae)* were collected from different areas (Fig. 3.1.1). The study species were selected on the basis of follow-up of antimicrobial activity reports and ethnomedical or traditional uses against diseases (Fabricant and Farnsworth, 2001). Specimens of each species were freshly pressed, mounted and identified using experts and authenticated specimens in the National Herbarium, Department of Plant Biology and Biodiversity Management, Addis Ababa University. A voucher specimen of each species has been made available in the National Herbarium.

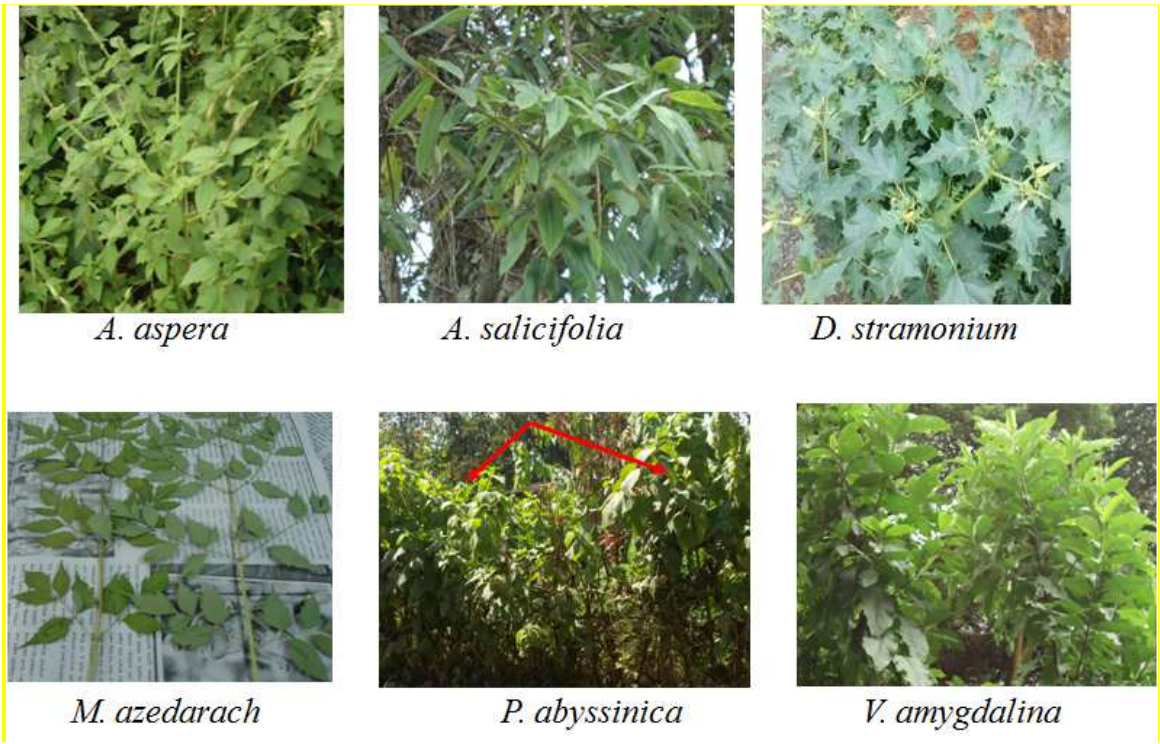


Figure 3.1.1: Medicinal plant species used in the present study

The leaves were washed with running tap water to remove dust and other debris, and air dried under shade at room temperature to constant weight. The dried leaves were ground to fine powder using mechanical grinder. The powder was then sieved through 0.6 mm wide mesh, homogenized and stored in an air tight container under room temperature until extraction.

3.1.2. Extraction

Extraction of crude leaf extracts were performed using maceration method. Leaf powder and the solvent (methanol) were added into conical flasks for each species separately in the ratio of 1:10 (W/V). Methanol was used because it is an all purpose solvent that dissolves most of the secondary metabolites and enhances the release of these chemicals from cellular matrix (Visht and Chaturvedi, 2012). The flasks were tightly closed and the mixture was shaken for 72 h using

orbital shaker at a speed of 250 rpm under room temperature. After 72 h, the extract was filtered first by four layer of cheesecloth and cotton followed by Whatman's No 1 filter paper. The extracts were dried and concentrated by evaporating methanol using rotary evaporator. The concentrated extracts were stored at 4°C until used for antibacterial testing.

Extraction yield

The percentage yield of the extract was determined gravimetrically using the dry weight of extract and the initial weight of the leaf powder as follows:

$$\% \text{ extract yield} = \frac{\text{dry weight of extract}}{\text{initial powder weight}} * 100$$

3.1.3. Infected enset material collection and isolation of *Xanthomonas campestris pv. musacearum*

Pseudostems and midribs of newly infected enset plants were collected from a homegarden in Wondo Genet College of Forestry and Natural Resource. The samples were cut into small pieces approximately 2-5 mm^2 in size using sterilized knife. Each piece was placed in separate plastic bags and kept as cool as possible in an ice box to prevent drying, microbial degradation and avoid tissue decomposition. The specimens were then brought to the laboratory to isolate Xcm; causal agent of enset bacterial wilt.

Isolation

In the laboratory, the infected pseudostem and midrib specimens were further cut into smaller pieces using sterile scalpel. The pieces were surface disinfected by dipping in 5 % sodium hypochlorite solution for one minute and immediately immersed in distilled water three times to

remove the disinfectant. Then after, the cut pieces were placed in a test tube containing 5 mL of distilled water and allowed to stand for 5 minutes until the bacterial population diffuses out of the cut tissue into the distilled water.

Loopful of bacterial suspension was streaked to sterilized semi-selective growth medium composed of yeast extract (10 g L^{-1}), peptone (10 g L^{-1}), sucrose (10 g L^{-1}), agar (15 g L^{-1}), cephalexin (50 mg L^{-1}) and amphotericin (150 mg L^{-1}) (Tripathi *et al*., 2007). Cephalexin and amphotericin were used to inhibit the growth of most saprophytes and fungal contaminants, respectively. The streaked Petri dishes were incubated in an inverted position at 28°C for 72 h. Isolated colonies were selected and streaked on a newly prepared growth medium of the same type. The subculturing of the bacterium was carried out using streak plate method. In this method, loopful of bacteria was directly taken from growth plates that contain uniform colonies of Xcm using wire loop. Subculturing was done several times until uniform yellow colored colonies were produced. The pure culture was stored at 4°C (Fig.3.1.3.1) and every time activated at 28°C before usage. Identification was made based on colony characteristics. Accordingly, yellowish, mucoid and circular colonies were identified as Xcm. The identity of the pathogen was confirmed by pathogenicity test.



Figure 3.1.3.1: Pure culture of Xcm

3.1.4. Pathogenicity test

Nine suckers of a susceptible enset clone were brought to Addis Ababa University, College of Natural Science and transplanted in plastic buckets in the glasshouse. During transportation, all the leaves were removed and the root part was covered by dried enset pseudostem. Plastic buckets were 33 cm high and 30 cm wide. The buckets were filled with soil, sand and manure in the ratio of 2:1:1. After establishment, individual enset plants were inoculated with 10 ml of Xcm suspension adjusted to $1.5x10^8$ CFU/mL (0.5 McFarland standard) at the base of midrib in three replications. The negative control was inoculated with the same amount of distilled water using syringes with metal needle. Three individual suckers were not treated for comparison purpose. A week after inoculation, symptom development was monitored for every other day. Yellowing of the inoculated leaf was seen after three weeks (Fig. 3.1.4.1). Pseudostem (leaf sheath) of the infected plants was taken and the bacterium was re-isolated using the previous isolation procedure.



Figure 3.1.4.1: Pathogenicity test showing inoculation of enset with Xcm (a) and yellowing of leaves of the inoculated enset plant (b) after three weeks

3.1.5. Hypersensitivity testing

Seeds of tobacco (*Nicotiana tabacum L.*) were sown in the same type of bucket with similar soil composition as used for pathogenicity test. Several seeds were sown and after germination, one seedling was left for the experiment and others were removed. The experiment was carried out in three replications. The ability of plant pathogenic bacteria to induce hypersensitive reaction in a non-host plant provides a useful test for pathogenicity. Bacterial suspension (10 mL) containing 1.5 x 10^8 CFU/mL was infiltrated into the intercellular spaces of tobacco leaf using syringes with metal needle. Tobacco plants were used due to the easy of infiltrating the fleshy leaves and the convenience of growing and maintaining the plants under greenhouse condition. Death of cells around the infiltrating area of the leaf, typical characteristics of hypersensitive response was observed three days after inoculation during monitoring period that was checked for every other day.

3.1.6. Antibacterial susceptibility testing of leaf extracts and antibiotics

The antibacterial potential of crude leaf extracts and antibiotics against Xcm was evaluated by disc diffusion method (EUCAST, 2012). This method was chosen due to the fact that it is suitable for testing the majority of bacterial pathogens and requires no special equipment. Petri dishes, paper discs, cotton swabs, forceps, test tubes and other materials used during the test were autoclaved every time before usage. All the tests were performed in three or more replications repeated for at least three times.

Extract preparation

Extracts of each species were dissolved in methanol and serial test concentrations were prepared. Approximately 1.2, 0.9, 0.6, 0.3, 0.15, 0.075, 0.04 and 0.02 g of each species extract was separately dissolved in 3 mL of methanol producing 400, 200, 100, 50, 25, 12.5, 6.25 and 3.12 mg/mL serial concentrations. Similarly, test concentrations of antibiotics that range from 50 – 0.02 mg/mL of distilled water were also prepared. Discs with a size of 5 mm in diameter were prepared from Whatman's No 1 filter paper using paper borer. The discs were sterilized and impregnated in each serial concentration of each species and antibiotics for 12 h. Subsequently, the discs were taken out and dried before application.

Inoculum preparation and inoculation

The inoculum was prepared from 72 h old bacteria grown in YPSA medium. The upper surfaces of several pure colonies were swabbed with cotton swab and dissolved in distilled water in a test tube. The content of the test tube was thoroughly shaken in a vortex until a homogenous suspension was formed. The absorbance of the bacterial suspension was measured with a spectrophotometer (NV202, Sunny) at 600 nm and adjusted to 0.132, which is equivalent to the

density of 0.5 McFarland standards (Sutton, 2011). The bacterial population at 0.5 McFarland standards is approximately equal to 1.5 x 10^8 CFU/mL.

Cotton swab was used for inoculation. The sterilized swab was dipped into the bacterial suspension and the excess fluid was removed by turning the swab against the inside of the test tube. This avoids over inoculation of the Petri dishes. The inoculum was spread evenly over the entire surface of the Petri dishes by swabbing in three directions. The dried discs were applied to the inoculated Petri dishes within 15 minutes of inoculation. During application, the discs were pressed downward and the Petri dishes were kept in a normal position until the discs get wet. Discs rinsed in methanol were used as negative controls. Then, all the Petri dishes were inverted and incubated at 28°C for 72 h. Inhibition zone was measured after 72 h of incubation. Zones of complete inhibition were measured using transparent ruler at the widest possible diameter including the disc (NCCLS, 1997).

3.1.7. Minimum inhibitory concentration

The minimum inhibitory concentrations (MICs) of leaf extracts and antibiotics were determined using agar dilution method as described in (EUCAST, 2000). One mL of each serial concentration of each species leaf extract and antibiotic was thoroughly mixed with 19 mL of molten growth medium and poured into Petri dishes. The medium was then allowed to solidify at room temperature. Bacterial suspension adjusted to 0.5 McFarland standards (0.3µl) was inoculated at three spots in each Petri dish (0.1µl of bacterial suspension at each spot). The inoculated Petri dishes were incubated at 28°C for 72 h. Parallel to this, control Petri dishes without extract and antibiotic were used and the result was compared against these controls

3.1.8. Minimum bactericidal concentration (MBC)

Minimum bactericidal concentrations (MBC) of leaf extracts and antibiotics were determined as described in Njinga *et al*. (2014). Bacterial growth medium composed of yeast extract (1%), peptone (1%), sucrose (1%) and agar (1.5%) (YPSA) was prepared and autoclaved at 121°C for 15 minutes. The medium was poured into sterile Petri-dishes and allowed to cool and solidify. The contents of the MIC Petri-dishes that did not show growth were sub-cultured onto the prepared Petri-dishes. The Petri-dishes were then incubated at 28°C for 72 h. Then after, the Petri-dishes were observed for growth. The Petri-dishes without growth represent the minimum bactericidal concentration (MBC). After 72 h, the results were recorded and taken as MBC.

3.1.9. Preliminary phytochemical screening of crude leaf extracts

Qualitative analysis was performed to confirm the presence or absence of major secondary metabolites in the methanol crude leaf extract of each species. The screening was carried out following standard chemical methods as described in Harborne, (1998), Tiwari *et al*. (2011), Jones and Kinghorn, (2006), Wadood *et al*. (2013), Kumar *et al*. (2013) and Rai *et al*. (2013).

Test for alkaloids

Wagner's test: Extract of each species (20 mg) was dissolved separately in 2 % HCl and filtered. The filtrate was used for the detection of alkaloids. Wagner's reagent was prepared by dissolving 1.27 g of iodine and 2 g of potassium iodide (PI) in 100 mL of distilled water. Wagner's reagent (1 mL) was added to 2 mL of filtrate. The formation of redish or brown solution was an indication for the presence of alkaloids.

Test for flavonoids

Sample from each plant extract (0.5 g) was taken in separate test tubes and 10 mL of distilled water was added, shaken and filtered. Five mL of diluted ammonia solution (10 %) was added to a portion of the aqueous filtrate (3 mL) of each plant extract followed by addition of 1 mL concentrated sulphuric acid. Yellow color formation was taken as a positive test for flavonoids.

Test for phenolic compounds

Ferric Chloride ($FeCl_3$): To 2 mL of the above filtrate, 3-4 drops of 5 % Ferric Chloride solution were added. Bluish black color production indicated the presence of phenols.

Test for terpenoids

Leaf powder (0.5 g) of each species and 10 mL of methanol were added in separate test tubes. The content was shaken well, centrifuged and filtered. An aliquot of extract or filtrate (5 mL) was taken and mixed with 2 mL of chloroform. Into the solution, 3 mL of sulphuric acid was added. Reddish brown layer production between chloroform and sulphuric acid was used as an indicator for the presence of terpenoids.

Test for saponins

Froth test: One gram of each extract was boiled with 5 mL of distilled water and filtered. To 1 mL of the filtrate, 3 mL of distilled water was added and shaken vigorously for about 5 minutes. Frothing that persisted for 15 minutes was taken as an evidence for the presence of saponins.

Test for tannins

Crude leaf extracts were taken separately in test tubes and 5 mL of distilled water was added, warmed and filtered. To 1 mL of the filtrate, 2 mL of 5 % (W/V) Ferric chloride solution prepared in distilled water was added. The appearance of deep blue color was used to confirm the presence of tannins.

Test for glycosides

Approximately 20 mg of extract was dissolved in 2 % HCl and filtered. The filtrate (1 mL) was treated with five drops of glacial acetic acid and two drops of 5 % Ferric Chloride solution and thoroughly mixed. Subsequently, 2 mL of concentrated sulfuric acid was added and two layers were formed; the lower white layer and upper acetic layer which turned bluish green indicated a positive test for glycosides

3.1.10. Estimation of total phenolics content of extracts

Extract of each species (0.5 g) and 10 mL of distilled water were added in separate test tubes, shaken and centrifuged. An aliquot of (0.1 mL) of the supernatant was taken and diluted to 3 mL with distilled water. Consecutively, 0.25 mL of Folin Ciocalteau reagent was added. After 3 minutes, 1 mL of 20 % (w/v) sodium carbonate was added and thoroughly mixed. The tubes were placed in boiling water for 1 min and cooled. The absorbance of the resulting solution was measured at 650 nm against a reagent blank using a spectrophotometer (NV202 Spectrophotometer, Sunny). The blank was composed of 3 mL of distilled water, 0.25 mL of Folin Ciocalteau and 1 mL of 20 % sodium carbonate. The absorbance of the blank was subtracted from each reading. Catechol was used to prepare the standard calibration curve from which the amount of total phenols in the sample was calculated. The amount of total phenols was expressed in mg catechol equivalent of phenol/g of sample (Zieslin and Ben-Zaken, 1993).

A calibration curve was prepared by dissolving 0.5 g of dry catechol in 100 mL of distilled water as a stock solution (Fig. 3.1.10.1). Zero (0), 1, 2, 3, 5, and 10 mL of the catechol stock solution were added into 100 mL separate volumetric flasks, and then diluted to volume with distilled water. These solutions had 0, 0.05, 0.1, 0.15, 0.25 and 0.5 mg/mL of catechol. From each calibration solution, sample, or blank, 0.2 µL was taken into separate test tubes, and to each 2.8

mL of distilled water and 0.25 µL Folin-Ciocalteu reagent were added, and mixed thoroughly. After 3 minutes, 1mL of 20 % sodium carbonate solution was added, well mixed and warmed in a boiling water for one minute, cooled and absorbance was determined against the blank at 650 nm.

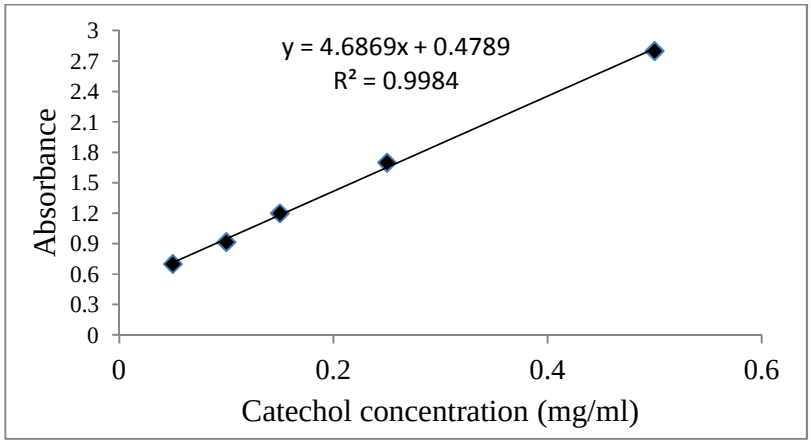


Figure 3.1.10.1: Calibration curve of total phenolics content using catechol

3.2. Induction of Systemic Defense Responses in enset (*Ensete ventricosum (Welw.) Cheesman*) Clones by the Leaf Extract of *Agarista Salicifolia* against *Xanthomonas campestris pv. Musacearum*

3.2.1. Description of the field study area

The field study was conducted in Wondo Genet College of Forestry and Natural Resources, 263 kms far from the capital, Addis Ababa (Fig. 3.2.1.1). It is located in the SNNPS at 7° 06' N to 7° 11' N and 38° 5' E to 38° 07' E), southeast of Shashemene town. The altitude of the place ranges between 1700 - 2100 m above sea level (a.s.l). The mean annual temperature varies between 17°C and 19°C and it receives a mean annual precipitation of about 1200 mm on a bimodal distribution pattern. The main rainy season occurs between June and September and the short rainy season that contributes about one third of the annual rainfall occurs between March and April. The soil is well drained loam and sandy loam, shallow at steep convex slopes but deep at low altitude (Eriksson and Stern, 1987).

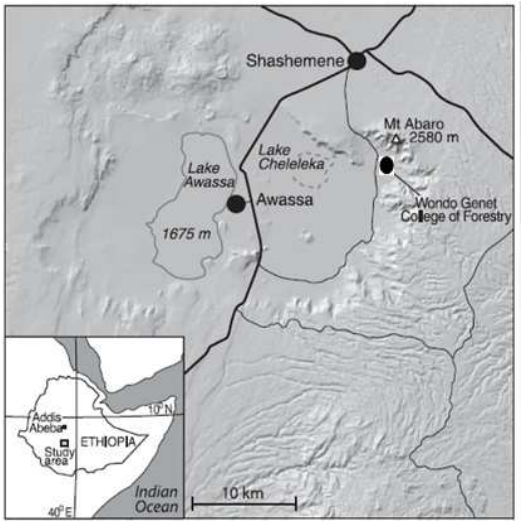


Figure 3.2.1.1: Map of the study area (Source: Gessesse Dessie and Kleman, 2007)

3.2.2. Enset clones

The study enset clones were selected based on research reports about their sensitivity to Xcm and the preference of enset growing farmers in terms of quantity and quality of products such as *Bulla, Kocho* and *Amicho*. Accordingly, *Genticha* is known as resistant/tolerant and *Midasho* as susceptible clones (Gizachew Wolde-Michael *et al*., 2008). In addition, the two enset clones and Xcm isolate were collected from the farmers' field in Sidama zone, Wondo Genet district where the study was conducted. This zone was selected as a source of enset clones and Xcm isolate since research reports of Fikre Handoro and Gizachew Wolde-Michael, (2007) revealed that natural epidemic of enset bacterial wilt is high in the zone and Xcm isolates from infected enset clones in this zone are more virulent.

3.2.3. Growing conditions

The study area was ploughed using tractor. The soil harrowed into small particles and the land was irrigated before plot preparation. The field experiment was conducted on an area of 1224 m^2; partitioned into three blocks, each consisting of six, 6 m X 6 m (36 m^2) plots (Fig. 3.2.3.2). There were 2 m and 3 m spacing between blocks and plots, respectively. In each plot, nine 33 cm deep and wide holes with 2 m spacing were prepared for enset sucker plantation. Each hole was 0.5 m far from the edge of the plot to avoid edge effect. Before transplanting the enset suckers, the perimeter of the site was tightly fenced around using wooden logs. One year old and approximately equal sized enset suckers of the study clones (*Ganticha* and *Midasho*) were bought from farmers who had suckers developed from one corm for each clone. Transplanting was carried out at the beginning of the rainy season, June, 2013. Each enset clone was randomly assigned to each plot in a completely randomized design in three replications (Fig. 3.2.3.2). Nine individual enset suckers were transplanted in each plot. After two weeks, equal amount of

undecayed cattle manure was added around each enset sucker. Enset management techniques such as tilling and weeding commonly practiced by the enset farmers were carried out as needed.

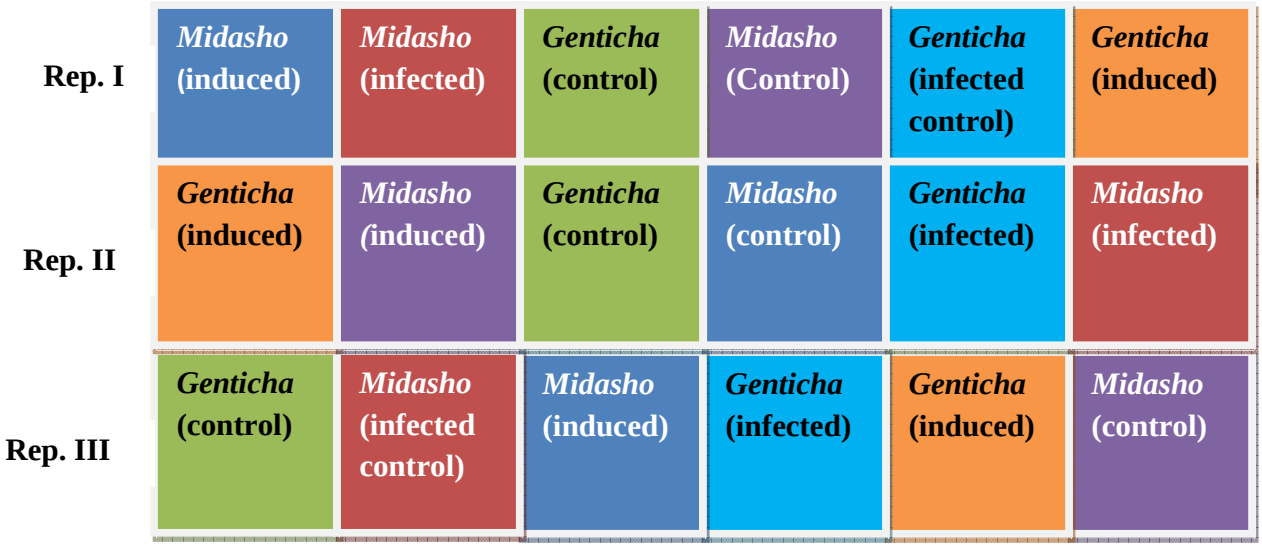


Figure 3.2.3.1: Layout of the field experimental plot showing the random distribution of treatments

3.2.4. *Agarista salicifolia*

Fresh leaves of *Agarista salicifolia* (*Com.ex Lamb.) G. Don (Ericaceae)* was collected from Amaro district, southern Ethiopia. The species was selected on the basis of follow-up of antimicrobial activity reports and traditional use of the plant against diseases (Fabricant and Farnsworth, 2001). It is a tree that grows on steep, rocky slopes in *Juniperus-Erica* scrub, open grassland with *Combretum, Protea, Erica* and *Helichrysum*, bamboo forest, evergreen forest relict in valleys at altitude ranging 2050 -2900 m above sea level. It is found widespread in Africa (Hedberg and Hedberg, 2003). The leaf samples were collected from a tree grown at 2131 m above sea level. The leaf of this species is traditionally used to treat gastrointestinal diseases such as diarrhea in humans (Shangal *et al*., 2008). The bark and the root are used to treat sores

(external boil) and toothache, respectively (Shangal *et al*., 2008). The shoot is also used to treat cattle disease such as Babesiosis (Engedasew Andarge *et al.*, 2015). Specimen of the species was freshly pressed, mounted and identified by experts in the National Herbarium, Department of Plant Biology and Biodiversity Management, Addis Ababa University. A voucher specimen of the species has been made available in the National Herbarium.

3.2.5. Application of treatments

The treatments were applied after one year from transplanting. In each block, there were six plots; three for each enset clone. The treatments were assigned randomly in plots. All plants grown in a plot received the respective treatments. The three treatments were; 1) enset plants inoculated with 10 mL of Xcm suspension adjusted to 1.5 x 10^8 CFU/mL (Fig. 3.2.5.1A). Xcm inoculated plants were taken as infected controls (positive controls); 2) enset plants treated with 10 mL of 12.5 mg/mL *A. salicifolia* crude leaf extract dissolved in methanol (Fig. 3.2.5.1B). The leaf extract was applied using mid vein injection method. The value 12.5 mg/mL was the minimum inhibitory concentration of the leaf extract. Plants pretreated with the leaf extract were inoculated with Xcm suspension after 30 days when symptoms of infected control plants fully develop. Both inoculants were thoroughly shaken before inoculation; 3) enset plants treated with 10 mL of methanol, the solvent of the leaf extract, were taken as the controls (negative controls) of the experiment. Separate 5 mL capacity hypodermic syringes with metal needles were used for application of the three treatments and applications were made at the base of the midrib of a newly expanding youngest leaf (Fig. 3.2.5.1A and B). To protect transmission of the pathogen, separate management tools were used for each treatment after inoculation.



Figure 3.2.5.1: Inoculation of enset with Xcm suspension (A) and treating with A. salicifolia leaf extract (B) one year after transplanting

3.2.6. Leaf sample collection

Leaf samples were collected soon after treatment application. Leaf discs approximately equal to 10 cm x 10 cm in size were collected from the middle of the 3rd leaf from the top excluding the midrib at 0, 24, 48, 48, 72, 96 and 120 hrs after treatment application. The leaf discs were taken from three to five randomly selected plants in each plot. The leaf samples were stored in liquid nitrogen and transported to Addis Ababa University. The leaf discs of each clone were sorted by blocks and treatments, and further cut into small pieces. The leaf pieces of each treatment were homogenized block wise and used for biochemical analysis separately. All assays were performed independently a minimum of two times for each enset clone, replication (block), treatment and sample collection time. Similar trends were observed between replicate samples under trail times during each bioassay.

3.2.7. Bioassay

3.2.7.1. Assay for the detection of phenylalanine ammonia lyase (PAL)

The activity of PAL was estimated according to Dickerson *et al*. (1984) as described in Eisa *et al.* (2013). One gram (1 g) of leaf sample from each treatment was crushed and homogenized in 10 mL of borate buffer (pH 8.8) using pre-chilled mortar and pestle. This was carried out in the presence of 0.4 mL of 5 mM mercaptoethanol L^{-1}. The homogenate was centrifuged at 12, 000 rpm at 4°C for 10 minutes and the supernatant was collected and used as an enzyme extract.

An aliquot (0.2 mL) of enzyme extract was transferred into a separate tube containing 2.5 mL of borate buffer and 1 mL of 0.1 mM L-phenylalanine (pH 8.8). This was done for each treatment and clone for every sampling time. The mixture was incubated for 30 minutes at 32±2°C. Enzyme reaction was stopped by the addition of 0.5 mL of 2N HCl. Enzyme extract (0.2 mL) along with 0.5 mL of borate buffer, 1.3 mL of distilled water, 0.5 mL of 2N HCl and 1 mL of L-phenylalanine were added into a test tube in sequence and used as a blank. The PAL activity was determined from the production of cinnamate (Qin and Tian, 2005) and absorbance was measured at 290 nm with a spectrophotometer (NV202 Spectrophotometer, Sunny). The amount of cinnamic acid produced was determined from cinnamic acid calibration curve and PAL activity was expressed as µmoles of cinnamic acid produced $min^{-1}g^{-1}$ of FW.

Dry cinnamic acid (0.5 g) was dissolved in ethanol (absolute) and made to 100 ml by adding ethanol. This was used as a stock solution. Due to the lower absorbance readings at low concentrations, higher volumes were taken from the stock (5mg/ml) solution as compared to other calibration curves in the thesis. Serial dilutions of 1, 4, 10, and 20 mL were taken from the stock solution and added into separate 100 mL volumetric flasks, each diluted to volume with

distilled water producing serial concentrations of 0.05, 0.2, 0.5 and 1 mg/ml, respectively. The absorbance of the stock solution (5mg/ml) was also measured. Absorbance was measured at 290 nm using a spectrophotometer and the standard curve was constructed as concentration of cinnamic acid versus absorbance (Fig. 3.2.7.1.1).

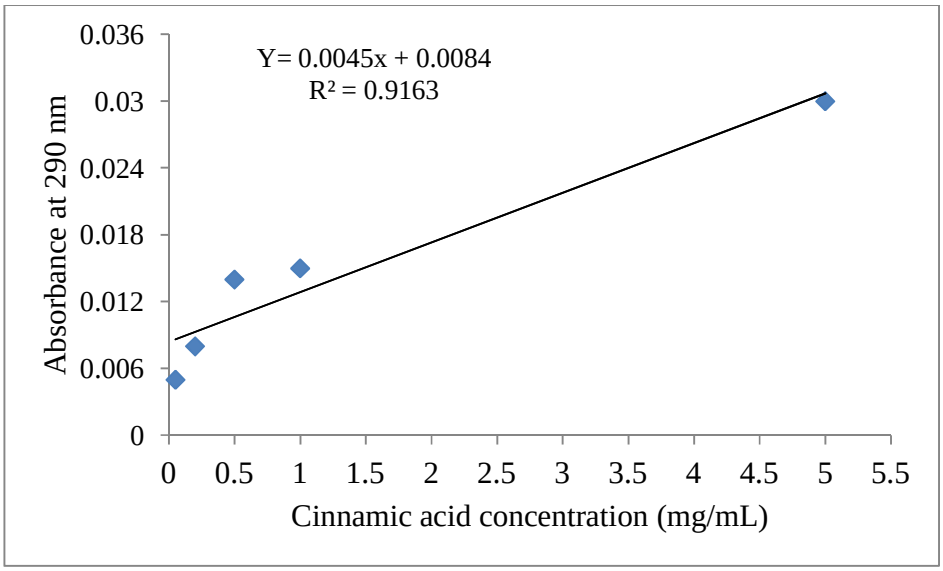


Figure 3.2.7.1.1: Calibration curve for phenylalanine ammonia lyase activity

3.2.7.2. Peroxidase assay

In the presence of hydrogen donor, pyrogallol peroxidase converts H_2O_2 to H_2O and O_2. The oxidation of pyrogallol to a coloured product called purpurogalli can be followed spectrophotometrically at 470 nm. One gram of enset leaf sample was weighed and crushed in pre-chilled (cooled) mortar and pestle in the presence of 0.1M sodium phosphate buffer (pH 7) in the ratio of 1:4 (W/V). The extract was allowed to pass through a funnel to exclude fibers and the filtrate was centrifuged at 4000 rpm for 20 minutes. The supernatant was used for estimation of peroxidase activity.

Two sets of tests were run representing the experimental test and the control. To all the test tubes of the two sets, 2.5 mL of sodium phosphate buffer (pH 7) and 0.2 mL of the enzyme extract were added. To the experimental set, 0.2 mL of 0.05M pyrogallol solution was added and mixed. Then 0.1 mL of 1% H_2O_2 was added to all the test tubes. Contents were mixed thoroughly and left at room temperature for 20 minutes. A solution consisted of 0.2 mL of distilled water, 2.5 mL of phosphate buffer (pH 7) and 0.1 mL of 1% H_2O_2 was used as a blank.

Absorbance of the experimental and control tests was measured at 470 nm using a spectrophotometer (NV202, Sunny). Each measurement was replicated six times to ensure maximum conformity of results. Optical density (OD) of all the tests was taken against the blank. Peroxidase activity was estimated according to Anjum *et al.* (2012) using the formula as follows:

$$\text{Units mg-1 fresh wt.} = \frac{\text{O.D of test} - \text{O.D of control}}{\text{O.D of control*mg of plant sample}}$$

3.2.7.3. Polyphenol oxidase assay

Polyphenol oxidase activity was determined according to the method described by Esterbauer *et al.* (1977). Using a pre-chilled mortar and pestle, 0.5 gram of leaf sample was homogenized in 2 mL of the extracting solvent containing 50 mM Tris-HCl (pH 7.2), 0.4 M sorbitol and 10 mM NaCl in the ratio of 3:1:1. The homogenate was centrifuged at 2000 rpm for 10 minutes at 4°C and the supernatant was used for the assay.

An aliquot of 2.5 mL of phosphate buffer, 0.3 mL of catechol solution and 0.2 mL of enzyme extract were added in a cuvette and the absorbance of the mixture was measured at 495 nm using a spectrophotometer (NV202, Sunny). Change in absorbance was recorded every 30 seconds

interval for 3 minutes. However, absorbance was nearly stable approximately after 2 minutes. One unit of polyphenol oxidase is defined as the amount of enzyme that transforms one μmole of dihydrophenol to one μmole of quinone per minute per FW. The activity of polyphenol oxidase was calculated using the formula:

$$\text{Enzyme unit in the sample} = K \times (\Delta A/\text{min})$$

Where, K for catechol oxidase (polyphenol oxidase) = 0.272

3.2.7.4. Estimation of total phenolics content

Enset leaf sample (1 g) from each treatment was homogenized with mortar and pestle in 10 mL of 80 % methanol (w/v). The homogenate was filtered in a funnel and centrifuged at 10,000 rpm for 10 min. The supernatant was used for total phenolics content determination. An aliquot of (0.2 mL) of the supernatant was taken and diluted to 3 mL with distilled water. Consecutively, 0.25 mL of Folin Ciocalteau reagent was added. After 3 minutes, 1 mL of 20 % (w/v) sodium carbonate was added and thoroughly mixed. The test tubes were placed in boiling water for 1 min and cooled.

A blue color was developed in each test tube because the phenols undergo a complex redox reaction with phosphomolibdic acid in Folin Ciocalteau reagent in alkaline medium. This blue colored complex was molybdenum blue. The absorbance was measured at 650 nm against a reagent blank using a spectrophotometer (NV202 Spectrophotometer, Sunny).The blank was composed of 3 mL of distilled water, 0.25 mL of Folin Ciocalteau and 1 mL of 20 % sodium carbonate. The absorbance of the blank was subtracted from each reading. Catechol was used to prepare the standard calibration curve (Fig. 3.1.10.1) from which the amount of total phenols in

the samples was calculated. The amount of total phenols was expressed in mg catechol equivalent of phenol/g FW (Zieslin and Ben-Zaken, 1993).

3.2.7.5. Estimation of total soluble sugars

The amount of total soluble sugars was estimated using phenol–sulfuric acid method as mentioned in Nielsen, (2010). Freeze dried enset leaf sample (0.5 g) was homogenized with 10 mL of 80 % ethanol as described in Salt *et al.* (1988). The solution was poured in a funnel and the filtrate was centrifuged at 2000 rpm for 20 minutes. The supernatant was collected separately for each clone, treatment and sampling time. In a test tube, 0.1 mL alcoholic leaf extract and 1 mL of 2 % phenol solution were added. This concentration of phenol solution was chosen due to the smallest difference in the absorbance readings among the three sugars (glucose, fructose and galactose). Moreover, measuring absorbance at 490 nm with the use of 2 % phenol in the sugar assay gives the closest match to the absorption coefficients of the three basic monomers without compromising the sensitivity of the assay (Chow and Landhausser, 2004). To the extract-phenol solution, 5 mL of concentrated sulphuric acid was added. Each tube was gently agitated during the addition of the acid and allowed to stand in a water bath adjusted to 30°C for 20 minutes.

The absorbance of the yellow orange color thus developed was measured at 490 nm in a spectrophotometer (NV202 Spectrophotometer, Sunny). The blank was composed of all the constituents of the test without the enzyme extract. The amount of total soluble sugars was determined from a standard curve prepared by using known concentration of glucose followed by the addition of equal amounts of phenol solution and sulfuric acid. The amount of sugar was expressed as mg/g fresh weight of leaf tissue.

A calibration curve was prepared by dissolving 0.5 g of dry D-glucose in 100 mL of distilled water as a stock solution (Fig. 3.2.8.5.1). Serial dilutions of 1, 2, 3, 5, and 10 mL of the stock solution were added into separate 100 mL volumetric flasks, and then diluted to volume with distilled water. These solutions had 0.05, 0.1, 0.15, 0.25 and 0.5 mg/mL of glucose, respectively. From each calibration solution 0.2 µL was taken into separate test tubes, and to each 1 mL of 2 % phenol solution and 5 mL concentrated sulfuric acid were added and mixed thoroughly. A solution composed 0.2 µL of distilled water, 1 mL of 2 % phenol solution and 5 mL of concentrated sulfuric acid was used as a blank. The absorbance of the resulting yellow orange colored solution was measured at 490 nm using a spectrophotometer (NV202 Spectrophotometer, Sunny)

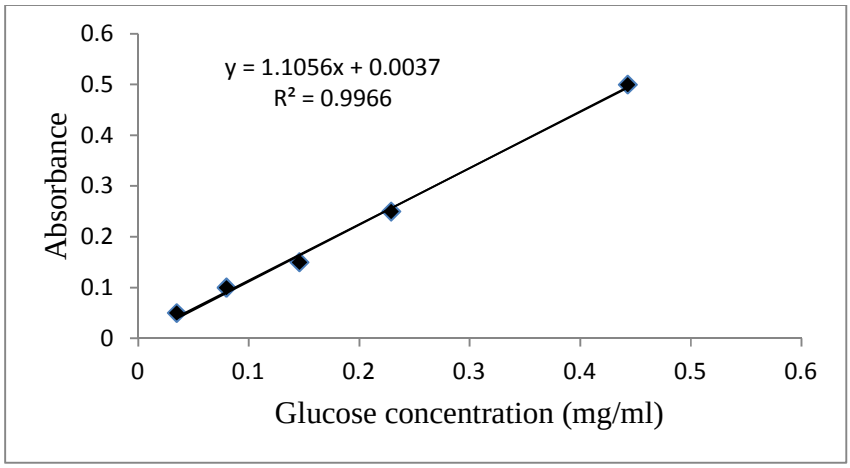


Figure 3.2.8.5.1: Calibration curve of glucose for quantification of TSS of enset leaves

3.2.7.6. Disease incidence

Disease incidence (DI) was evaluated 4 and 9 months after inoculation with Xcm suspension and treated with *A. salicifolia* leaf extract. DI was calculated separately for each treatment and clone under each plot and the average values were taken**.** Percentage of disease incidence was

calculated according to Haggag and El-Gamal, (2012) with little modification using the formula as follows:

$$\text{Disease incidence (\%)} = \frac{\text{number of dead plants}}{\text{total number of plants inoculated}} \times 100$$

3.3. Growth, Physiological and Biochemical Responses of *E. Ventricosum* Clones Induced by Leaf Extract and Infected With *X. campestris pv. musacearum*

3.3.1. Growth and physiological measurements

In the field experiment, growth and physiological data were collected from 3-5 individual plants per plot. Plants under each treatment were randomly selected and tagged for data collection. When tagged plants were lost (missed), equivalent plants of the same treatment were used for data collection. Growth data of the study enset clones were collected beginning a year after transplanting and before the application of treatments. Growth and physiological data, and leaf samples for biochemical assay were gathered 4, 9 and 12 months after the application of treatments.

3.3.1.1. Growth measurements

The effects of treatments on the growth of enset clones were evaluated in terms of plant height, psuedostem girth, leaf number, total leaf area and leaf area index. Plant height was measured from the soil surface to the tip of fully expanded youngest leaf (Fig. 3.3.1.1.1A). Pseudostem girth was measured at the widest region along the lower length. Green leaf number was counted and recorded. Leaf width and length of the 3rd leaf counted from the top were measured and values were used to calculate total leaf area (Fig. 3.3.1.1.1B and C).



Figure 3.3.1.1.1: Measurements of plant height (A), leaf width (B) and leaf length (C) of enset one year after transplanting and just before treatment application

3.3.1.1.1. Total leaf area

Leaf area is one of the parameters used to determine a plant's photosynthesis. Total leaf area of enset was determined according to Kumar *et al*. (2002) used for banana by counting the total number of leaves (N), measuring the length (L) and breadth (B) of the third leaf from the top and calculating the total leaf area (TLA) as:

$$TLA = L \times B \times 0.83 \times N \times 0.662$$

Where 0.83 is a correction factor for leaf shape differences and 0.662 is a correction factor for leaf size differences among enset plant leaves.

3.3.1.1.2. Leaf area index

Leaf area index (LAI) is the total area of green leaf per unit area of land and this was measured as follows:

$$LAI = TLA/A_g$$

Where LAI is leaf area index, TLA is total leaf area and A_g is the ground area occupied by each plant which was computed from the spacing between individual plants.

3.3.1.2. Physiological measurements

3.3.1.2.1. Leaf relative water content

Midday leaf relative water content (mRWC) was determined from 6-9 plants by punching out 3 cm x 3 cm (9 cm^2) leaf pieces from the mid region of the leaf blade. These pieces were immediately weighed to obtain fresh weight (FW). The pieces were placed in Petri dishes filled with distilled water for 24 hours, reweighed (TW) and then oven dried at 70°C for 24 hrs to get dry weight (DW) of the sample leaf pieces. Relative water content of the leaf was calculated as:

$$RWC = (FW - DW) / (TW - DW) \times 100$$

3.3.1.2.2. Gas exchange

Net assimilation rate (A), stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and transpiration rate (E) were measured simultaneously on the 3rd fully expanded leaf from the top (Fig. 3.3.1.2.2.1), using LCPro+ portable photosynthesis system (ADC, Bioscientific, Ltd. UK). This infrared gas analysis system is equipped with a leaf chamber covering 6.25 cm^2 leaf area and measurements were taken under field environmental conditions. Measurements were taken from 3-4 plants between 9:00 and 11:30 AM. Five recordings were taken from each plant at different positions when the CO_2 uptake was steady.

Water use efficiency (WUE) defined as mmol of net CO_2 uptake per mole of water transpired was calculated by dividing instantaneous values of A by E.

$$WUE = A / E$$



Figure 3.3.1.2.2.1: The simultaneous measurement of assimilation rate, stomatal conductance, intercellular CO_2 and transpiration rate of the 3rd enset leaf from the top

3.3.2. Biochemical responses

3.3.2.1. Leaf chlorophyll and carotenoid contents

The leaf samples were subjected to chlorophylls and carotenoids concentrations analysis. The amount of each pigment was quantified according to Lichtenthaler (1987) with little modification. Leaf samples were dried to constant weight and ground using mortar and pestle. The fine powder was sieved through 0.6 mm in diameter mesh and 20 mg from each treatment was taken in separate test tubes. The content in each test tube was moistened with 0.1mL of distilled water and left approximately for 10 minutes. Into each test tube, 8 mL of 96 % ethanol was added and thoroughly mixed in a vortex. The test tubes were wrapped in aluminum foil and incubated at room temperature overnight. The next day, the content of each test tube was mixed and centrifuged at 4000 rpm for 10 minutes. The absorbance of the extract was measured at 470, 649, and 664 nm using a spectrophotometer (NV202 spectrophotometer, Sunny). The absorbance of 96 % ethanol was used as a blank. The concentrations of chlorophylls and total carotenoids were calculated as follows:

$C_a = \frac{(13.36A_{664} - 5.19A_{649})*8.1}{DW}$	[mg g^{-1} DW]
$C_b = \frac{(27.43A_{649} - 8.12A_{664})*8.1}{DW}$	[mg g^{-1} DW]
$C_{a+b} = \frac{(5.24A_{664} + 22.24A_{649})*8.1}{DW}$	[mg g^{-1} DW]
$C_{x+c} = \frac{(4.785A_{470} + 3.657A_{664} - 12.76A_{649})*8.1}{DW}$	[mg g^{-1} DW]

Where: A649 =Absorbance at 649 nm

A664 =Absorbance at 664 nm

A470 = Absorbance at 470 nm

DW = dry weight of plant tissue extracted (mg) and 8.1 is volume of the solvent

3.3.2.2. Total nitrogen

Enset leaf samples were oven dried at 70°C for 24 hrs. These were milled, sieved in 0.6 mm mesh and subjected to total nitrogen analysis. Total nitrogen of leaf samples was estimated using kjeldahl method as described in (Baker and Thompson, 1992).

Principle of the method

Kjeldahl method involves the transformation of organic N to ammonium (NH_4^+) by digesting the sample with concentrated sulfuric acid (H_2SO_4) and then measuring the amount of NH_4^+ produced. Potassium sulfate (K_2SO_4) was used to increase the boiling point, and thus speed up the conversion reaction. The rate of organic matter oxidation was also increased by the addition of copper sulphate pentahydrate ($CuSO_4.5H_2O$) and selenium (Se) that serve as catalysts (Baker and Thompson, 1992).

$$\text{Organic nitrogen} + H_2SO_4 \xrightarrow[CuSO_4.5H_2O]{K_2SO_4} (NH_4)_2SO_4 + H_2O.$$

The NH_4^+ N created during the digestion procedure was determined by making the digest strongly alkaline with sodium hydroxide (NaOH), and collecting the volatilized ammonia (NH_3) into a boric acid indicator solution by steam distillation.

$$2(NH_4)_2SO_4 + 4NaOH \longrightarrow Na_2SO_4 + 4NH_3 + 4H_2O$$

The ammonia liberated was trapped by boric acid and titrated using HCl

$$NH_3 + H_3BO_3 \longrightarrow NH_4H_2BO_3$$

$$NH_4H_2BO_3 + HCl \longrightarrow NH_4Cl + H_3BO_3$$

Procedure

Leaf powder from each treatment, 0.3 g as described in Frew Kebede, (2012) for enset was weighed and added to Kjeldahl flasks containing 2 g of catalyst mixture composed of 100 g K_2SO_4, 10 g of $CuSO_4$ and 1 g of selenium powder. Into each flask, 7 mL of concentrated H_2SO_4 and 3 ml of 30 % H_2O_2 were added sequentially and immediately placed in the digestion block to initiate digestion. Similarly, the blank (without plant sample) was passed through all the steps like that of the samples to compensate for any contribution from the reagents used. Digestion was run for about 2 hrs. The digested samples were distilled with 40 % sodium hydroxide and received in 20 ml of 2 % boric acid containing 2.5 ml of bromocresol green-methyl red mixed indicator solution per litre of boric acid solution. The indicator solution was prepared from 0.01 g of bromocresol green and 0.07 g of methyl red dissolved in 100 mL of 90 % ethanol (Baker and Thompson, 1992). The distillate was then titrated with 0.1N HCl until the green color of the distillate changed to pink. Total percent (%) nitrogen present in the plant samples was calculated as follows:

$$\text{Total \% N} = \frac{(T-B) \times N \times 0.014 \times 100}{\text{Weight of sample in gram}}$$

Where, T = Titration volume of the sample

B = Titration volume of the blank

N = Normality of the titrating acid

3.3.2.3. Total protein

The total protein of the leaf sample was determined indirectly from the total nitrogen content of the leaf sample as:

Total protein = percent nitrogen * 6.25 (6.25 is conversion factor for most vegetables)

3.4. Statistical analysis

All data collected were subjected to analysis of variance using SPSS (Statistical Package for Social Sciences, version 20). This was done after carrying out test of homogeneity and normal distribution for each measured parameter. Means were compared by Tukey's Honestly Significant Difference (HSD) test at 95 % confidence interval.

CHAPTER FOUR

4. RESULTS

4.1. Evaluation of the Antibacterial Activity of Some Medicinal Plant Leaf Extracts and Antibiotics against *Xanthomonas campestris pv. musacearum*

4.1.1. Extract yield (%)

The result of the present study showed a wide variation in extract yield between medicinal plant species (Table 4.1.1.1). The highest extract yield was obtained from the leaves of *A. salicifolia* (55.3%) followed by *P. abyssinica* (45.2 %), while the lowest was obtained from *A. aspera* (7.8%) and *M. azedarach* (10.6%). The remaining species had an intermediate percent extract yield.

Table 4.1.1.1: Powder weight, extract weight and extract yield (%) of medicinal plant species (dry weight of methanol extract as % of powder weight of the leaf)

Medicinal plant species	**Powder weight (g)**	**Extract weight (g)**	**Extract yield (%)**
A. aspera	120	9.3	7.75
A. salicifolia	90	49.8	55.3
D. stramonium	120	21.73	18.11
M. azedarach	100	10.55	10.55
P. abyssinica	85	38.4	45.2
V. amygdalina	60	20.16	33.6

4.1.2. Antibacterial activity of leaf extracts

The identity of the pathogen was confirmed prior to antibacterial testing of plant extracts. Accordingly, results of the pathogenicity test showed development of bacterial wilt symptoms in inoculated plants of enset three weeks after inoculation (Fig. 3.1.1 and Appendex 1). The antibacterial activities of methanol crude leaf extracts of *A. salicifolia, P. abyssinica, A. aspera, D. stramonium*, *M. azedarach* and *V. amygdalina* against Xcm were tested *in vitro*. The test was carried out at 50, 100, 200 and 400 mg/mL concentrations. The extracts showed a wide variation in mean inhibition zone diameter of the test organism (Fig. 4.1.2.1). The inhibition zone between the test concentrations in extracts of *A. salicifolia* and *P. abyssinica* was significantly different at $P < 0.01$. Compared to others, extracts of these species produced the widest inhibition zone at all test concentrations. The inhibition zone decreased at lower test concentrations. In *A. aspera*, bacterial growth inhibition zone was significantly wider at 400 mg/mL as compared to 100 mg/mL. However, the antibacterial activity of *D. stramonium* and *V. amygdalina* leaf extracts was not significant among test concentrations (Fig. 4.1.2.1). Moreover, the result revealed that the leaf extract of *A. salicifolia* created the widest bacterial growth inhibition zone at all test concentrations. Extract of this species produced a significantly wider inhibition zone at all test concentrations as compared to other species ($P < 0.01$). Likewise, the leaf extract of *P. abyssinica* produced significantly higher inhibition zone than extracts of *A. aspera, D. stramonium* and *V. amygdalina* at 200 and 50 mg/mL test concentrations. At the highest test concentration (400 mg/mL), extract of *A. aspera* produced significantly wider inhibition zone than extract of *V. amygdalina.* However, the difference between extracts of *A. aspera, D. stramonium* and *V. amygdalina* at 200, 100 and 50 mg/mL test concentrations was not significant

(Fig. 4.1.2.1). The antibacterial susceptibility tests of *M. azedarach* extracts against Xcm didn't show inhibition at all test concentrations.

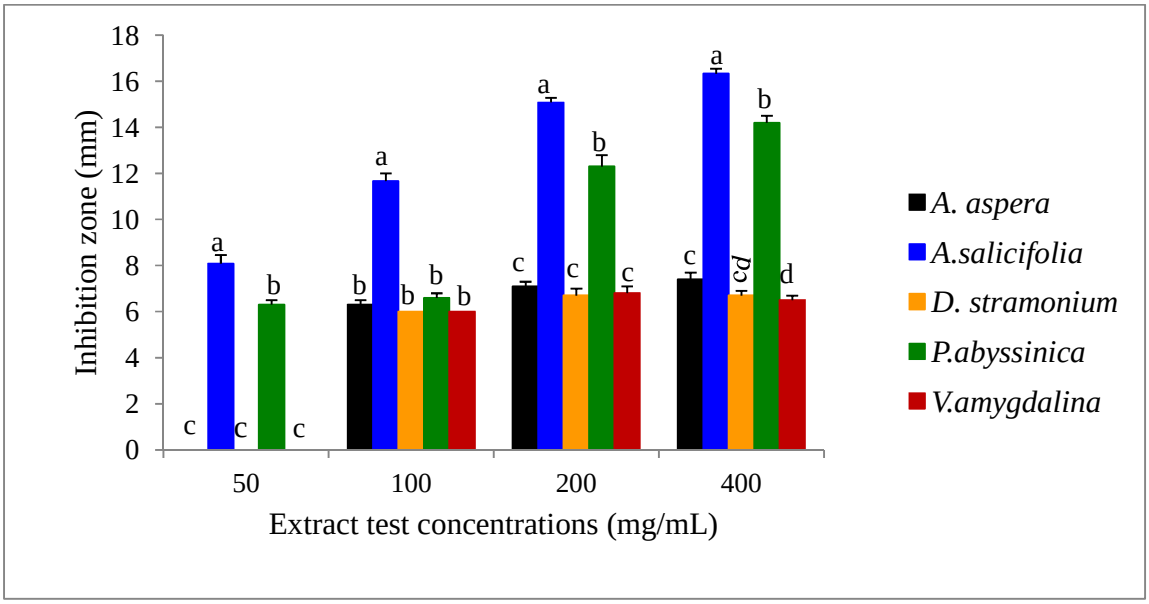


Figure 4.1.2.1: Growth inhibition diameters Xcm by medicinal plant leaf extracts at different test concentrations. Bars at each test concentration followed by different letters are significantly different at $P < 0.01$ ($n = 6$).

4.1.3. Antibacterial activity of antibiotics

The *in vitro* anti-Xcm activities of some commercially available antibiotics were qualitatively and quantitatively assessed by the presence or absence of inhibition zones, MICs and (MBCs). Visible variations were noticed between the tested antibiotics and test concentrations both within and between antibiotics (Table 4.1.3.1and Appendix 2). The differences between amoxicillin test concentrations (50, 25, 12.5 and 6.25 mg/mL) in inhibiting the growth of Xcm were insignificant at $P < 0.05$. The inhibition zone diameters of these test concentrations were significantly ($P < 0.05$) wider than lower test concentrations (0.78, 0.39, 0.195, 0.098, 0.049 and 0.02 mg/mL) except between 12.5 and 3.12 mg/mL. At lower test concentrations of amoxicillin below 0.195

mg/mL, the inhibition zone diameters became nil (Table 4.1.3.1). Unlike others, cephalexin inhibited the growth of Xcm only at higher concentrations (50, 25, 12.5 and 6.25 mg/mL) (Table 4.1.3.1). Besides, the differences between these test concentrations were insignificant ($P < 0.05$) except between 6.25 mg/mL and others. Chloramphenicol on the other hand, inhibited the growth of Xcm at lower concentrations that extend to 1.56 mg/mL. In chloramphenicol, significant ($P < 0.05$) differences were observed among test concentrations except between two consecutive concentrations in the serial dilution (50, 25, 12.5, 6.25, 3.12 and 1.56 mg/ml) test concentrations (Table 4.1.3.1).

Similar to amoxicillin, higher test concentrations of streptomycin sulphate showed wider inhibition zone diameter. However, the differences among these (50, 25, 12.5 and 6.25 mg/mL) test concentrations were insignificant at $P < 0.05$ (Table 4.1.3.1). The higher two (50 and 25 mg/ml) test concentrations had significantly ($P < 0.05$) wider inhibition zone diameter than the lower test concentrations (1.56, 0.78, 0.39, 0.195, 0.098, 0.049 and 0.02 mg/mL). The various test concentrations of tetracycline also inhibited the growth of Xcm at different levels. The highest inhibition zone diameter was recorded in the highest test concentration (50 mg/mL) followed by 25 mg/mL (Table 4.1.3.1). The difference between these two was insignificant at $P < 0.05$. Tetracycline showed inhibition up to the lowest test concentration (0.02 mg/mL) and significant differences were recorded between the higher (50, 25 and 12.5 mg/mL) and lower (3.12, 1.56, 0.78, 0.39, 0.098, 0.049 and 0.02 mg/mL) test concentrations (Table 4.1.3.1).

Comparison of tested antibiotics revealed the existence of marked variation in potency against Xcm; a plant pathogen. Accordingly, amoxicillin and streptomycin showed significantly ($P < 0.05$) wider inhibition zone diameter than cephalexin, chloramphenicol and tetracycline at 50, 25,

12.5 and 6.25 mg/mL test concentrations (Table 4.1.3.1). The difference between tetracycline and amoxicillin was insignificant in some of the lower (1.56 and 0.39 mg/ml) test concentrations. The significant variation between tetracycline and streptomycin was not restricted to the higher test concentrations. They also showed significant differences at lower (1.56, 0.78, 0.39 and 0.098 mg/mL) test concentrations. Cephalexin and chloramphenicol showed relatively narrow inhibition zone diameter as compared to others (Table 4.1.3.1). In general, the antibacterial activity of all antibiotics increased with increasing concentration.

Table 4.1.3.1: The antibacterial activity of antibiotics against Xcm showing inhibition zone diameters (mm) at various test concentrations (n = 6)

Test concentrations (mg/mL)	**Amoxicillin**	**Cephalexin**	**Chloramphenicol**	**Streptomycin sulphate**	**Tetracycline**
0.02	0.0±0.0f	0.0±0.0c	0.0±0.0d	12.0±1.5e	6.2±0.3fgh
0.049	0.0±0.0f	0.0±0.0c	0.0±0.0d	24.7±4.7d	7.2±0.2fgh
0.098	0.0±0.0f	0.0±0.0c	0.0±0.0d	22.0±1.7e	7.6±0.5fgh
0.195	6.0±0.0f	0.0±0.0c	0.0±0.0d	23.3±0.7de	13.2±0.3efg
0.39	23.7±0.9e	0.0±0.0c	0.0±0.0d	29.7±0.9d	14.1±0.7e
0.78	25.3±0.9e	0.0±0.0c	5.3±0.0d	33.0±0.6d	13.7±0.2e
1.56	31.7±2.7d	0.0±0.0c	5.4±0.0cd	35.0±0.6bcd	21.3±0.5d
3.12	41.0±0.6bc	0.0±0.0c	5.7±0.3cd	34.7±1.2bd	25.1±0.8d
6.25	38.7±2.4c	5.5±2.0b	16.7±0.9bc	43.3±1.7ac	28.2±1.5cd
12.5	46.3±0.9ab	13.7±1.2a	20.3±2.2b	43.7±1.9ab	31.5±1.9bc
25	48.0±1.0a	12.3±1.2a	25.7±3.2ab	46.0±0.6a	35.3±2.4ab
50	49.7±0.3a	15.7±1.8a	32.2±3.4a	50.3±3.2a	38.7±2.6a

Mean values in each column followed by similar letters are not significantly different at $P < 0.05$.

4.1.4. Minimum inhibitory and bactericidal concentrations of extracts and antibiotics

Minimum inhibitory and bactericidal concentration (MIC and MBC) values indicated that the effect of extracts on the growth of Xcm was variable among species (Fig.4.1.4.1). The highest MIC and MBC values were recorded by extracts of *A. aspera*, *D. stramonium* and *V. amygdalina* (Fig. 4.1.4.1). Extracts of *A. salicifolia* and *P. abyssinica* were potent that inhibited the growth of the test bacterium even at low concentrations as compared to others. The MIC and MBC values of the other extracts were 87.5 mg/mL higher than *A. salicifolia* and *P. abyssinica*. The extract of *M. azedarach* didn't exhibit any growth inhibition at 400, 200, 100 and 50 mg/mL concentrations and thus, MIC and MBC were not determined (Fig.4.1.4.1).

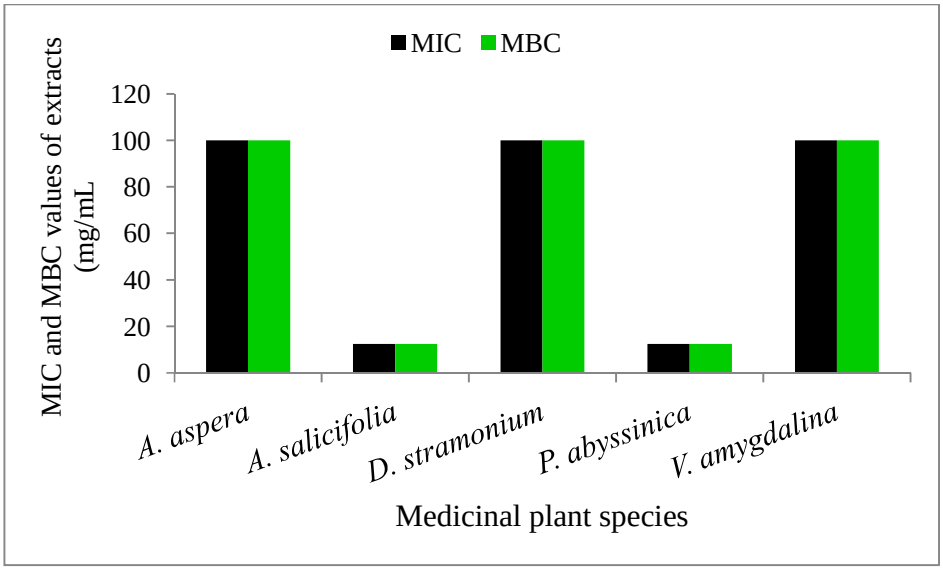


Figure 4.1.4.1: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of leaf extracts from medicinal plant leaves against Xcm.

Figure 4.1.4.2 shows the MIC and minimum bactericidal concentration (MBC) of antibiotics against Xcm. There was variation in both tests between antibiotics. In the assay, tetracycline showed the lowest MIC and MBC values as compared to others antibiotics (Fig. 4.1.4.2). The

highest MIC and MBC were recorded by test concentration of cephalexin, while the remaining antibiotics had moderate MIC and MBC (Fig. 4.1.4.2).

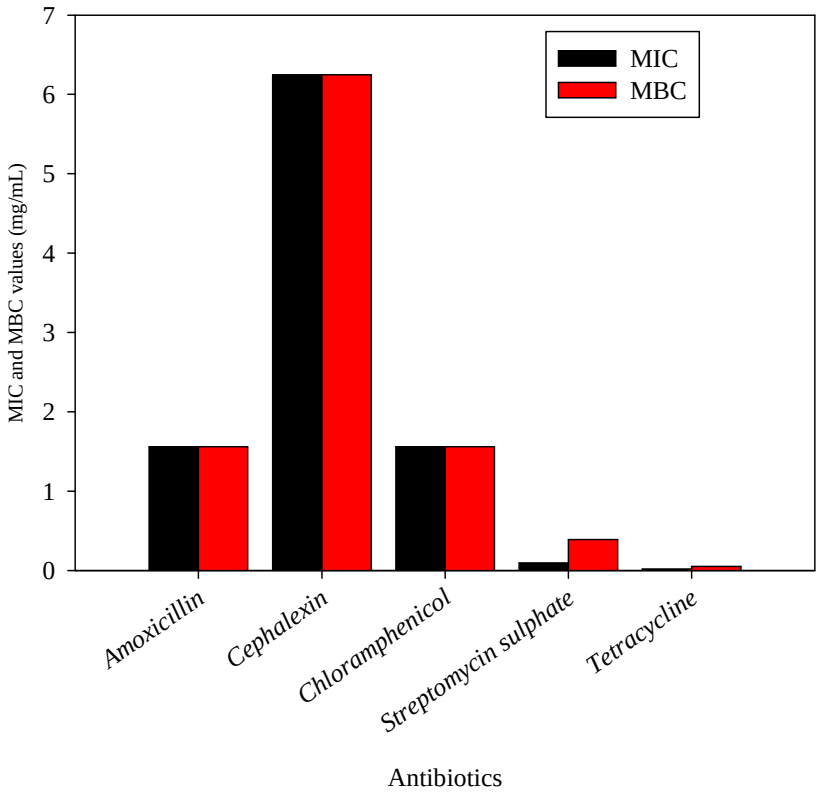


Figure 4.1.4.2: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of antibiotics against Xcm

4.1.5. Phytochemical screening and total phenolics content of extracts

Phytochemical screening of extracts

The preliminary phytochemical screening results revealed that the chemical constituents of extracts vary between species (Table 4.1.5.1). Accordingly, extracts of *A. salicifolia* and *P. abyssinica* had abundant amount of the tested secondary metabolites. However, glycosides were absent in the extract of *P. abyssinica*. Extract of *V. amygdalina* on the other hand had abundant

amount of phenols, terpenoids and tannins, and moderate amount of alkaloids, flavonoids and saponnins (Table 4.1.5.1).

Table 4.1.5.1: Results of the qualitative test of secondary metabolites in medicinal plant extracts

Medicinal plant species	Phytochemicals						
	Alkaloids	**Flavonoids**	**Phenols**	**Terpenoids**	**Saponnins**	**Tannins**	**Glycosides**
A. aspera	-	+	-	-	-	-	-
A salicifolia	++	++	++	++	++	++	++
D. stramonium	-	+	+	+	+	++	-
M. azedarach	-	-	-	+	-	-	-
P. abyssinica	++	++	++	++	++	++	-
V. amygdalina	+	+	++	++	+	++	-

++ = abundant; + = moderate; - = absent

Total phenolics content of extracts

The result of the quantitative chemical analysis of extracts showed that total phenolics content was significantly different between the studied plant species (Fig. 4.1.5.1). The highest total phenolics content was recorded by the leaf extract of *A. salicifolia.* It showed a 100, 85.7, 100, 61.2 and 12.3 % increment from *A. aspera*, *D. stramonium*, *M. azedarach*, *P. abyssinica*, and *V. amygdalina*, respectively. Extract of *V. amygdalina* ranked second and that of *P. abyssinica* ranked third in terms of total phenolics content. The total phenolics content of the remaining extracts was negligible (lower than the minimum concentration used to construct the standard calibration curve).

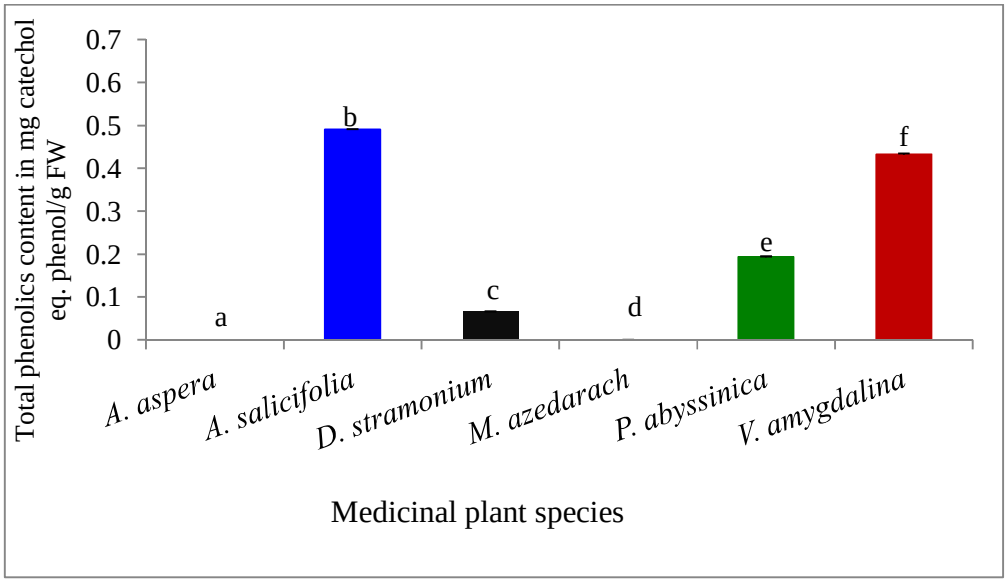


Figure 4.1.5.1: Total phenolics content (mg of catechol equivalent of phenol/g of FW) of medicinal plant leaf extracts. Bars followed by different letters are significantly different at $P < 0.01$. Values represent means of six replicates

4.2. Induction of Systemic Defense Responses in enset (*Ensete ventricosum (Welw.) Cheesman*) Clones by the Leaf Extract of *Agarista Salicifolia* against *Xanthomonas campestris pv. musacearum*

4.2.1. Phenylalanine ammonia lyase activity

Analysis of variance revealed that the activity of PAL was quickly elevated in leaf extract induced and infected control plants (Fig. 4.2.1.1A and B and Appendix 3). The activity of this enzyme measured at various time courses after treatment application was significantly higher ($P < 0.01$) in treated plants than untreated (0 h) ones (Fig. 4.2.1.1A and B). The difference between treatments in each clone was also statistically significant ($P < 0.05$). In the resistant clone, induced plants showed an 8, 4.6, 3, 13 and 4.6 fold increments in PAL activity compared to controls at 24, 48, 72, 96 and 120 h post treatment, respectively. Infected control plants on the other hand demonstrated a 7, 3.6, 2.3, 10.6 and 2.4 fold increments compared to controls. In this study, the resistant clone treated with *A. salicifolia* leaf extract showed the highest PAL activity 120 h followed by 96 and 72 h post treatment (Fig. 4.2.1.1A). Similarly, induced plants of the susceptible clone exhibited 1.5, 1.7, 1.4, 4.2 and 4.4 fold increments in PAL activity compared to controls at 24, 48, 72, 96 and 120 h post treatment, respectively. While infected control plants showed a 0, 1.3, 1.4, 3 and 2 fold increments in PAL activity compared to controls at similar sampling time. In this clone, the highest PAL activity was observed in leaf extract treated plants 120 and 96 h after treatment application. The activity of PAL declined after 96 h from treatment application in infected control plants of both clones (Fig. 4.2.1.1A and B). Higher PAL activity was recorded in induced plants of both clones compared to other treatments. Furthermore, PAL activity was increased

progressively up to 120 h post treatment (Fig. 4.2.1.1A and B). Induced and infected plants of the resistant clone illustrated significantly ($P < 0.01$) higher PAL activity than the corresponding plants of the susceptible clone (Fig. 4.2.1.1A and B).

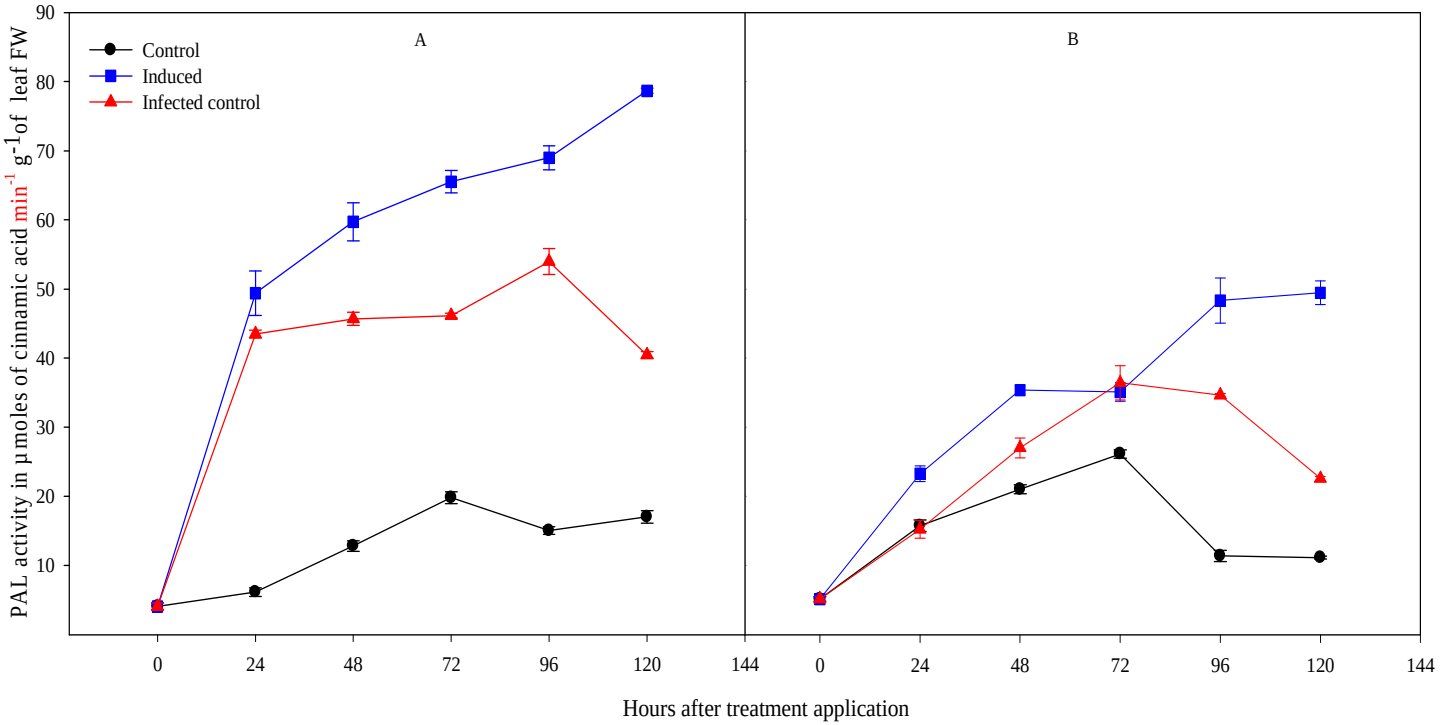


Figure 4.2.1.1: Time course activity of PAL in leaves of resistant (A) and susceptible (B) enset clones

Values represent means of six replicates. Bars indicate mean ± SE.

4.2.2. Peroxidase activity

The Post Hoc test for multiple comparisons of treatments revealed that the activity of peroxidase in treated resistant and susceptible plants were significantly ($P < 0.01$) increased with respect to untreated plants (Fig. 4.2.2.1A and B and Appendix 4). In the resistant clone, it showed a significant variation between treatments (Fig.4.2.2.1A). In control plants, peroxidase activity was boosted up 24 h and increased linearly till 120 h post treatment, but this activity of the enzyme was significantly lower than values of others treatments (Fig.4.2.2.1A). Infected control plants of the resistant clone attained the highest peroxidase activity 72 and 48 h post treatment followed by induced plants 96 and 120 h after treatment application, respectively (Fig.4.2.2.1A). The differences between the aforementioned values were significant ($P < 0.01$). Peroxidase activity measurements at the remaining sampling periods showed statistically significant variation between treatments (Fig.4.2.2.1A). In the susceptible clone, the activity of peroxidase in infected control plants was the highest at 48 and 72 h post treatment and these values were significantly ($P < 0.01$) different from the others measurements (Fig.4.2 2.1B) . Besides, induced plants had also higher enzyme activity at 96 and 120 h post treatment as compared to others treatments (Fig.4.2. 2.1B).

Moreover, there were no significant differences between untreated plants of the resistant and susceptible enset clones (Fig.4.2.2.1A and B). When treated, the clones showed a significant variation in peroxidase activity between treatments. Under the control treatment, the resistant clone had significantly higher peroxidase activity than the susceptible at 120 h post treatment. In addition, induced plants of the resistant clone attained significantly higher peroxidase activity than the susceptible one at 48 and 72 h post treatment. Conversely, induced plants of the

susceptible clone had significantly ($P < 0.01$) higher activity than the resistant clone at 96 and 120 h after treatment application (Fig. 4.2.2.1A and B). In both enset clones, peroxidase activity was elevated earlier and dropped lately in infected control plants than induced ones (Fig. 4.2.2.1A and B).

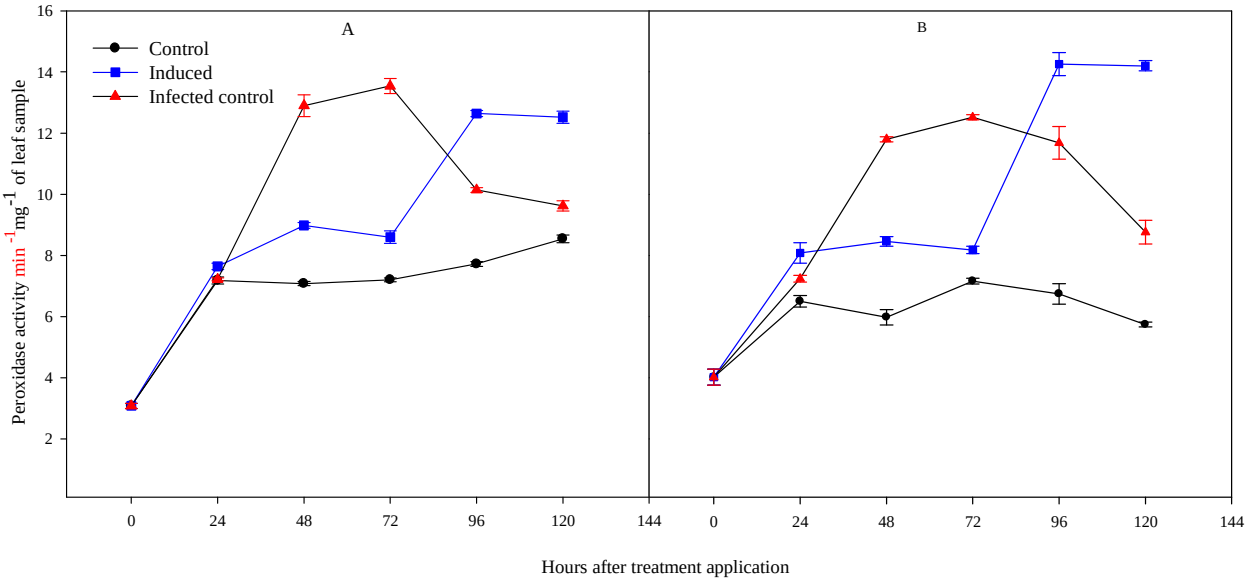


Figure 4.2.2.1: Peroxidase activity in leaves of resistant (A) and susceptible (B) enset clones. Values represent means of six replicates. Bars indicate mean ± SE.

4.2.3. Polyphenol oxidase activity

The studied enset (resistant and susceptible) clones showed an increased polyphenol oxidase activity post treatment with methanol, leaf extract and Xcm suspension (Fig. 4.2.3.1 and Appendix 5). The activity of the enzyme was significantly ($P < 0.05$) higher in treated plants of the resistant clone 24, 48, 72, 96 and 120 h after treatment application compared to untreated (0 h) plants (Fig. 4.2.3.1A). The highest polyphenol oxidase activity was recorded 72 and 120 h post treatment in induced and infected control plants of the resistant clone, respectively (Fig. 4.2.3.1A). In the susceptible clone, the activity of the enzyme in induced plants was higher than untreated (0 h) ones at 24, 48 and 96 h post treatment (Fig. 4.2.3.1B). However, significant differences were observed between induced and untreated plants 96 h after treatment application. The activity of the enzyme drastically declined 96 h post treatment in infected control plants of the susceptible clone (Fig. 4.2.3.1B). The activity of polyphenol oxidase in induced plants of the resistant and susceptible clones showed an increasing trend up to 72 and 96 h post treatment, respectively (Fig. 4.2.3.1A and B). The activity then after declined. On the other hand, the activity of polyphenol oxidase under untreated condition was insignificantly different between the two enset clones. Nonetheless, clear and significant ($P < 0.05$) differences were observed between the two clones after the application of treatments.

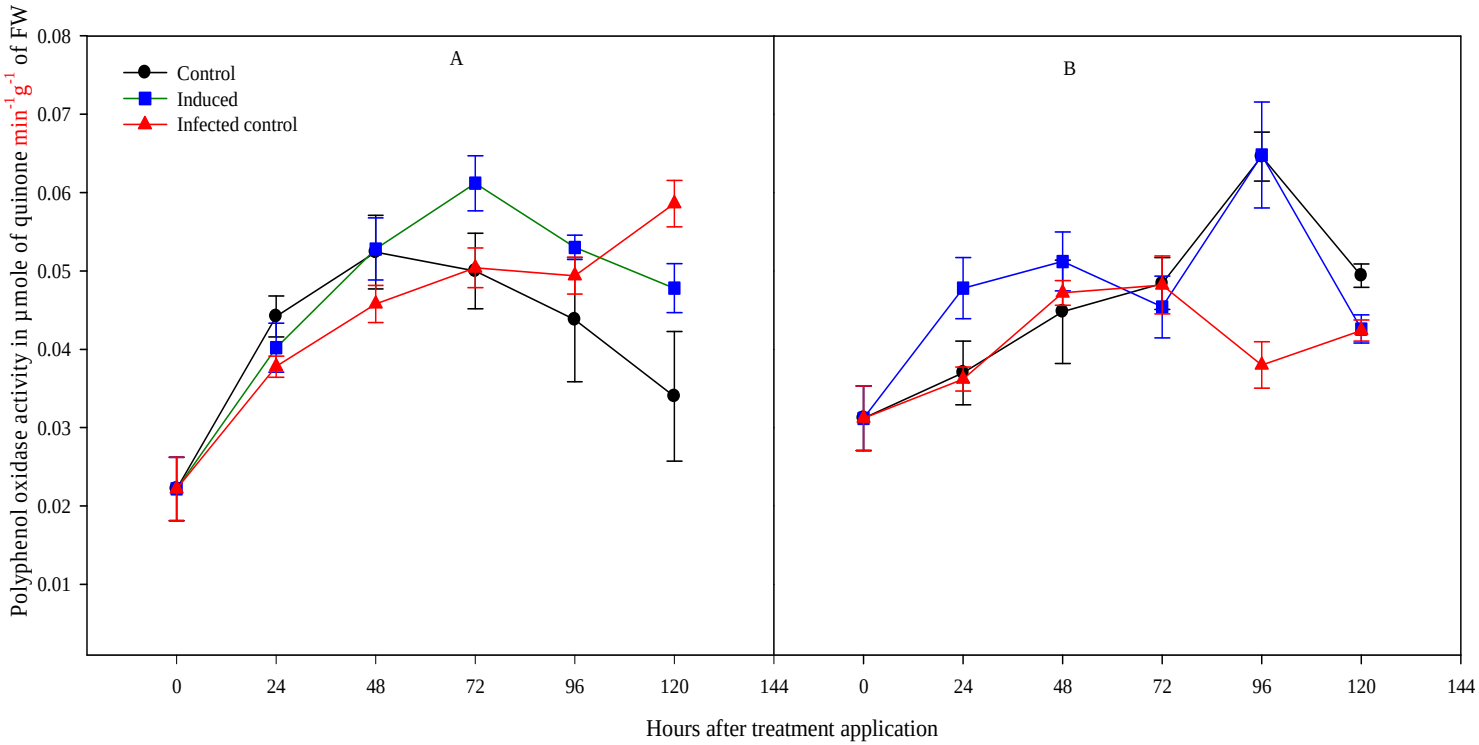


Figure 4.2.3.1: Induction of polyphenol oxidase activity in leaves of resistant (A) and susceptible (B) enset clones grown under field condition.Values represent means of six replicates. Bars indicate mean ± SE.

4.2.4. Total phenolics content

The analysis of variance showed that the total phenolics content (TPC) of enset clones was significantly ($P < 0.05$) higher in treated plants than untreated ones (Fig. 4.2.4.1 and Appendix 6). Statistically significant ($P < 0.05$) differences were recorded in the amount of TPC between the different sampling periods within treatments in both clones. The highest TPC in both clones was recorded at 120 days after treatment application (Fig.4.2.4.1A and B). Moreover, higher values of TPC were recorded in infected control plants of the resistant clone 3 and 4 days post treatment followed by induced plants of the same clone 4 days after treatment (Fig.4.2.4.1A). These values of TPC were significantly ($P < 0.05$) higher than others treatments (Fig.4.2.4.1A and B). Similarly, higher amount of TPC was produced by infected control plants of the susceptible clone as compared to other treatments within the clone (Fig.4.2.4.1B).

The difference in TPC between untreated plants of both clones was not significant. Control plants of the susceptible clone had higher TPC than control plants of the resistant clone at 2 and 3 days after treatment application (Fig. 4.2.4.1A and B). On the other hand, the TPC of infected control and induced plants of the resistant clone were significantly higher than the susceptible clone. The amount of TPC in the resistant clone was twofold greater than the susceptible (Fig. 4.2.4.1A and B). TPC was drastically boosted up one day post treatment and then increased slowly in infected control and induced plants of the resistant clone (Fig. 4.2.4.1A).

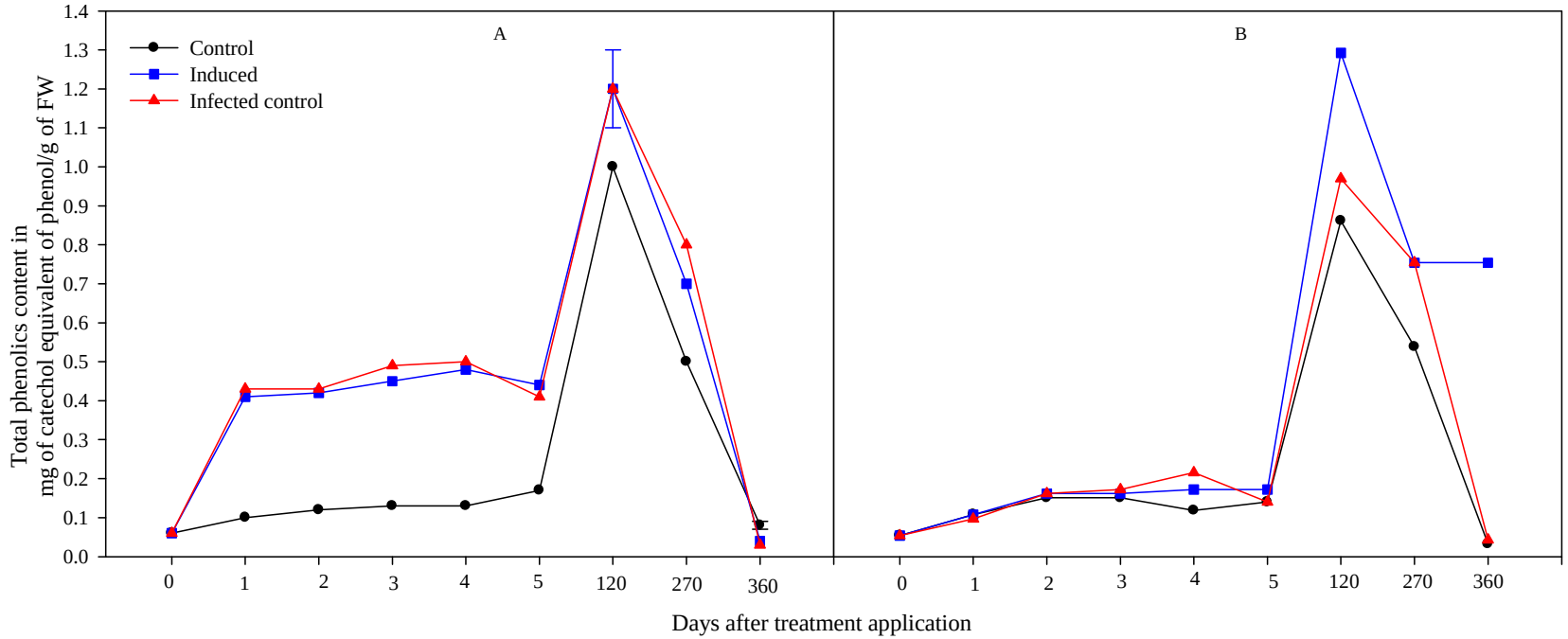


Figure 4.2.4.1: Time course changes in total phenolics content of resistant (A) and susceptible (B) enset clones

Values represent means of six replicates. Bars indicate mean ± SE.

The TPC of control plants was the lowest in both clones 9 months after treatment application. Induced and infected control plants of both clones had significantly higher TPC as compared to their respective controls (Fig. 4.2.4.1). Twelve months post treatment application, the effect of treatments on the TPC responses of the two enset clones was minimized. In this sampling period, the amount of total phenolic compounds in induced plants of the susceptible clone was significantly greater than control and infected control plants. Unlike others sampling periods, induced and infected control plants of the resistant clone had significantly lower total phenolic content than control plants (Fig. 4.2.4.1).

4.2.5. Total soluble sugars

Unlike other measured parameters, the amount of total soluble sugar (TSS) showed a significant ($P < 0.01$) difference between the two clones under untreated condition (Fig.4.2.5.1A and B). The resistant clone had higher TSS than the susceptible clone at most sampling periods (Fig.4.2.5.1A and B). Besides, resistant plants in the control treatment had significantly higher TSS in comparison to the susceptible and these plants achieved the successive maximum TSS values at 3, 4, 2 and 5 days post treatment, respectively (Fig. 4.2.5.1A and B). Infected control plants of the resistant clone produced lower TSS than the other treatments 3, 4 and 5 days after treatment application (Fig.4.2.5.1A). On the other hand, induced plants of the susceptible clone attained significantly higher TSS at 3, 4 and 5 days after treatment than other treatments (Fig. 4.2.5.1A and B).

Four months post treatment (120 days), control plants of the resistant clone had the highest concentration of total soluble sugars of all others treatments and the variation was significant at P

< 0.05. The variation between induced and infected control plants was also significant. Likewise, significant differences were also recorded among treatments in plants of the susceptible clone (Fig.4.2.5.1A). Nine months after treatment application, the highest total soluble sugar was recorded in control plants of the resistant clone and this value was significantly ($P < 0.05$) higher as compared to others treatments (Fig.4.2.5.1B).

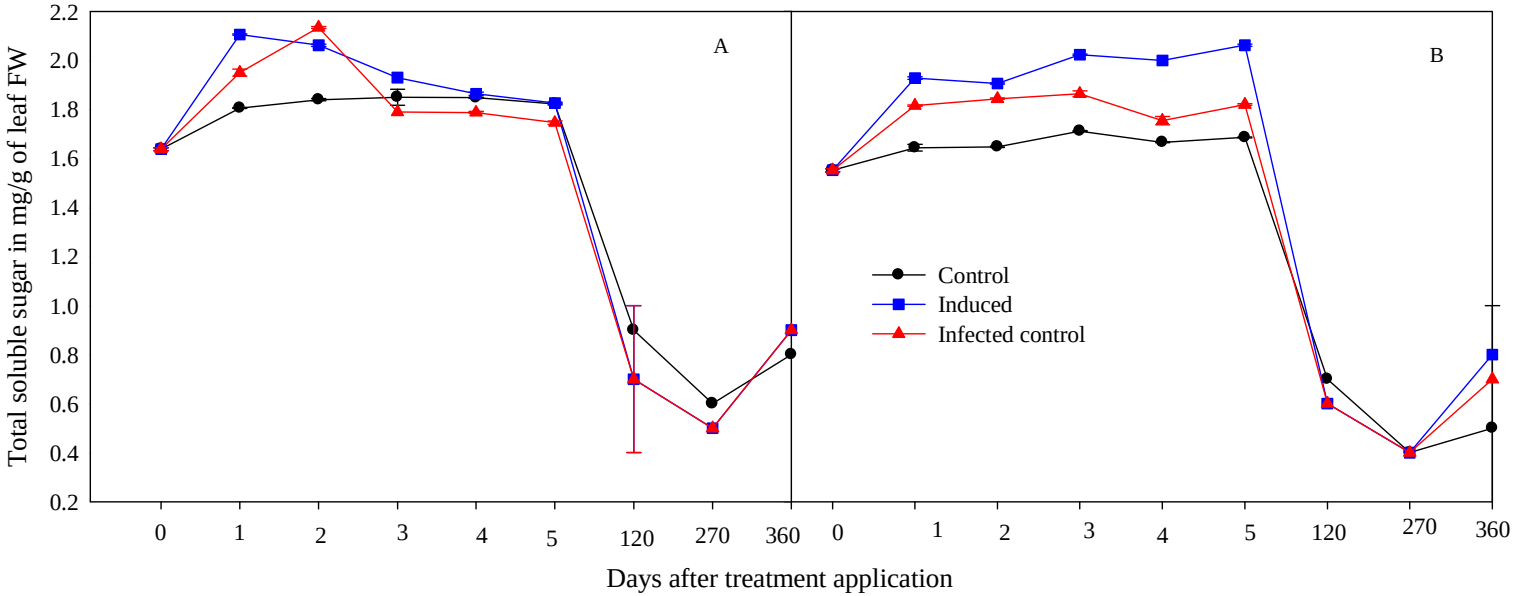


Figure 4.2.5.1: TSS content of leaves of resistant (A) and susceptible (B) enset clones under different treatments

Values represent means of six replicates. Bars indicate mean ± SE.

Twelve months post treatment application, the content of total soluble sugars in infected control plants of both clones was significantly higher than control plants (Fig.4.2.5.1A and B). Induced plants of the susceptible clone had higher total soluble sugars than infected control and control plants. The difference between induced and infected control plants of the resistant clone was not significant (Fig.4.2.5.1A and B). In addition, the chemical analysis result showed that the concentration of free proline under the various treatments in both enset clones at all sampling periods was negligible (data not shown). Overall, the performance of the resistant clone was better than the susceptible under most treatments.

The Pearson correlation analysis confirmed that a positive correlation exists between measured parameters (Table 4.2.5.1). The amount of TPC showed a significant positive correlation with PAL and peroxidase activities in the control plants of the resistant clone. It had also a positive correlation with TSS and polyphenol oxidase activity (Table 4.2.5.1). In induced resistant plants, PAL activity had a significant positive correlation with peroxidase, TPC and polyphenol oxidase. The TPC of infected control plants had a significant positive correlation with PAL, peroxidase and polyphenol oxidase activities. In the same way, the amount of TSS of control plants of the susceptible clone had positive correlation with PAL, peroxidase, polyphenol oxidase and TPC (Table 4.2.5.2). PAL activity revealed a significant positive correlation with peroxidase activity, TPC and TSS in induced plants (Table 4.2.5.1). PAL activity in infected control plants had a significant positive correlation with peroxidase activities and TSS (Table 4.2.5.1).

Table 4.2.5.1: The bivariate relationship between biochemical responses of the resistant and susceptible enset clones

Biochemical responses	Resistant clone														
	Control					Induced					Infected control				
	PAL	**POX**	**PPO**	**TPC**	**TSS**	**PAL**	**POX**	**PPO**	**TPC**	**TSS**	**PAL**	**POX**	**PPO**	**TPC**	**TSS**
PAL	1	.713	.551	.845*	.752	1	.936**	.915*	.954**	.485	1	.804	.835*	.991**	.536
POX	.713	1	.580	.905*	.927**	.936**	1	.758	.862*	.281	.804	1	.803	.830*	.534
PPO	.551	.580	1	.367	.829*	.915*	.758	1	.915*	.525	.835*	.803	1	.849*	.340
TPC	.845*	.905*	.367	1	.772	.954**	.862*	.915*	1	.673	.991**	.830*	.849*	1	.492
TSS	.752	.927**	.829*	.772	1	.485	.281	.525	.673	1	.536	.534	.340	.492	1
	Susceptible clone														
PAL	1	.758	.242	.670	.787	1	.939**	.629	.899*	.962**	1	.972**	.776	.693	.957**
POX	.758	1	.724	.857*	.754	.939**	1	.542	.792	.811	.972**	1	.879*	.757	.930**
PPO	.242	.724	1	.800	.584	.629	.542	1	.691	.682	.776	.879*	1	.866*	.648
TPC	.670	.857*	.800	1	.904*	.899*	.792	.691	1	.911*	.693	.757	.866*	1	.594
TSS	.787	.754	.584	.904*	1	.962**	.811	.682	.911*	1	.957**	.930**	.648	.594	1

*. Correlation is significant at the 0.05 level; **. Correlation is significant at the 0.01 level

PAL = Phenylalanine ammonia lyase; POX = Peroxidase; PPO = Polyphenol oxidase; TPC = Total phenolics content; TSS = Total soluble sugars

4.2.6. Disease incidence

The disease incidence assessment showed variation between treatments and clones (Fig. 4.2.6.1A and B and Appendix 7). Infected control plants of the susceptible clone showed the highest disease incidence at both assessment periods (Fig. 4.2.6.1A and B). Induced susceptible plants showed 33.3 % and 33.5 % disease incidence reduction at 4 and 9 MAT, respectively, as compared to infected control plants (Fig. 4.2.6.1A and B). All Xcm inoculated plants showed symptoms. Higher numbers of plants were died in infected control plants of the susceptible clone as compared to induced and the resistant clone (Appendix 8). However, most plants of the resistant clone endure the pathogen and continue to survive. In the resistant clone, induction prior to Xcm inoculation minimized disease incidence only by 3.2 % and 10.6 % at 4 and 9 months after treatment application, respectively.

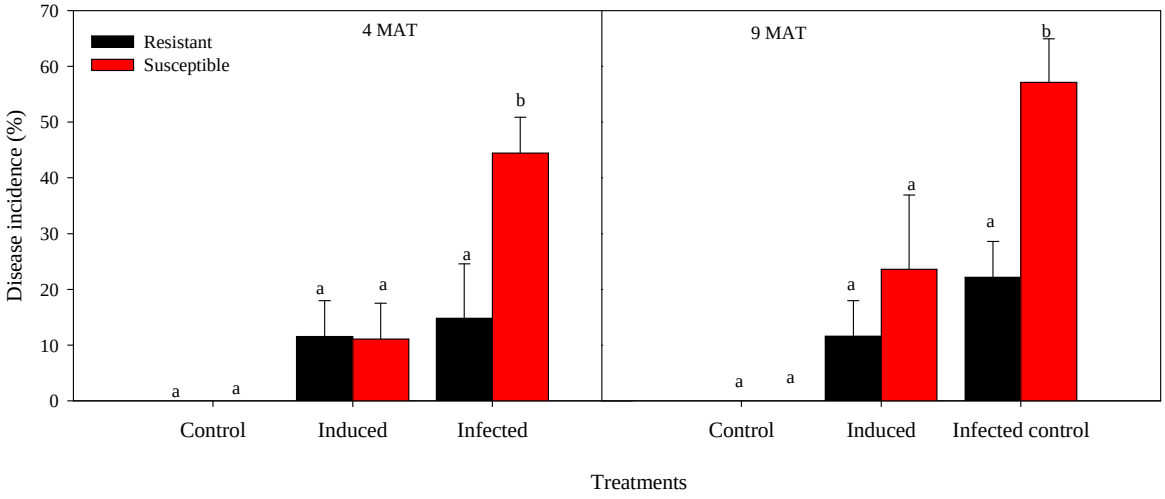


Figure 4.2.6.1: Percent disease incidence of resistant and susceptible enset clones estimated as number of dead plants divided by total number of plants treated multiplied by 100. Bars followed by different letters are significantly different at $P < 0.05$. Values represent means of three replicates. Bars indicate mean ± SE.

4.3. Growth, Physiological and Biochemical Responses of *E. ventricosum* Clones Induced by Leaf Extract and Infected With *X. campestris pv. musacearum*

4.3.1. Growth responses

The growth responses of the resistant and susceptible enset clones treated with methanol, leaf extract and Xcm suspension under field condition were evaluated 4 and 9 months after treatment application. Growth data were also taken before treatment application. From field observations, visible differences were seen in plant height, pseudostem girth, leaf length, width, total leaf area and leaf area index between plants of the two enset clones before treatment application. However, the differences were insignificant at $P < 0.05$ (Table 4.3.1.1 and Appendix 9). Similarly, insignificant differences in growth responses were recorded between treatments in the two enset clones four months post treatment application (Table 4.3.1.1). The only significant difference was recorded in plant height between control plants of the resistant and infected control plants of the susceptible clones. Nine months after treatment application, a significant difference was recorded in plant height between control and infected control plants of the susceptible clone. Control plants were longer than infected control plants by 26.7 % (Table 4.3.1.1). A significant difference was also observed between infected control plants of both clones. Infected control plants of the susceptible clone were shorter by 22.8 % than the corresponding plants of the resistant clone (Table 4.3.1.1). In plants of the resistant clone, there was an insignificant ($P < 0.05$) difference between treatments (Table 4.3.1.1).

Pseudostem girth, total leaf area and leaf area index showed a statistically significant ($P < 0.05$) difference between treatments nine months after treatment application (Table 4.3.1.1). Induced plants of the resistant clone had significantly lower pseudostem girth, total leaf area and leaf area

index than control plants. These growth responses showed a 22.2, 6.8 and 32 % reduction as compared to controls, respectively. However, the differences in the above growth parameters between control and infected control plants of the resistant clone were insignificant (Table 4.3.1.1). In the susceptible clone, control and induced plants had significantly ($P < 0.05$) larger pseudostem girth than infected control plants. Infected control plants showed a 29.4 % reduction in pseudostem girth when compared to the control (Table 4.3.1.1). On the other hand, control plants of the susceptible clone had significantly ($P < 0.05$) greater total leaf area and leaf area index than induced and infected control plants. The reduction in total leaf area was 6.9 and 37.3% in induced and infected control plants, respectively. Similarly, leaf area index was reduced by 29.9 and 37.3% in induced and infected control plants, respectively (Table 4.3.1.1). The lowest value for all growth responses was recorded by infected control plants of the susceptible clone. When infected control plants of the two clones are compared in terms of plant height, pseudostem girth, total leaf area and leaf area index, the resistant clone had significantly higher value than the susceptible clone. The number of green leaves before the treatments was greater than after the treatments. At all data collection periods, there was an insignificant ($P < 0.05$) difference between treatments and clones in third leaf length and width (Table 4.3.1.1). Plants of the resistant clone outperformed plants of the susceptible one in most measured growth parameters at all time courses (Table 4.3.1.1).

Table 3.1.1: Growth features of resistant and susceptible enset clones before treatment, four and nine months post treatment with methanol, leaf extract and Xcm. Mean ± SE (n = 9).

Time	enset clones	Treatments	Growth parameters						
			PH (m)	PSG (m)	GLN	TLL (m)	TLW (m)	TLA (m^2)	LAI (m^2)
Before	Resistant		3.4±0.2	1.2±0.0	25.1±1.0	2.5±0.1	0.7±0.1	2403.7±237.4	600.9±59.4
treatment	Susceptible		3.2±0.1	1.0±0.0	24.6±1.1	2.2±0.1	0.7±0.0	2133.5±165.5	533.4±41.4
4 months		control	5.2±0.2a	1.8±0.1	11.3±0.3	3.3±0.1	0.7±0.0	1720.2±90.8ab	430.1±22.7ab
post	Resistant	induced	4.8±0.2ab	1.9±0.1	11.3±0.4	3.1±0.1	0.7±0.0	1744.7±125.6a	436.2±31.4a
treatment		infected control	4.9±0.4ab	1.8±0.1	9.4±0.5	3.2±0.2	0.7±0.0	1414.9±157.6ab	353.7±39.4ab
		control	4.9±0.2ab	1.8±0.1	11.3±0.9	3.2±0.1	0.7±0.0	1436.3±185.3ab	359.1±46.3ab
	Susceptible	induced	4.3±0.3ab	1.6±0.1	11.0±0.8	2.8±0.2	0.7±0.0	1244.2±189.1ab	311.0±47.3ab
		infected control	4.1±0.1b	1.7±0.2	9.8±0.7	2.8±0.2	0.7±0.0	1062.0±171.6b	265.4±43.0b
9 months		control	6.2±0.2a	1.8±0.1a	9.2±0.2	3.7±0.1	0.9±0.0	2043.7±113.7a	510.7±28.4a
post	Resistant	induced	5.1±0.2ab	1.4±0.1bc	8.4±0.2	3.2±0.1	0.8±0.0	1389.1±87.7b	347.3±21.9b
treatment		infected control	5.7±0.3a	1.6±0.1ac	7.9±0.4	3.5±0.2	0.9±0.0	1608.1±159.5a	401.9±39.8a
		control	6.0±0.3a	1.7±0.1a	11.0±0.3	3.5±0.1	0.9±0.0	1861.4±56.8a	465.4±14.3a
	Susceptible	induced	5.6±0.3ab	1.6±0.1ac	9.2±0.3	3.1±0.2	0.8±0.0	1287.1±112.4b	321.8±28.1b
		infected control	4.4±0.4b	1.2±0.1b	7.7±0.5	2.6±0.2	0.7±0.1	694.8±149.5b	173.8±37.3c

PH = Plant height, PSG = Pseudostem girth, GLN = Green leaf number, TLL = Third leaf length, TLW = Third leaf width, TLA = Total leaf area and LAI = Leaf area index. At each data collection period, the differences between means of treatments within a clone in a column that share the same letter (s) are statistically significant at $P < 0.05$.

4.3.2. Physiological responses

Xcm is a xylem limited pathogen that produces abundant amount of xanthan which in turn clogs xylem and causes wilting. The effect of infection and treating enset clones with leaf extract and methanol on leaf relative water content was evaluated at different time courses. The relative water content of the leaf of both clones under the different treatments during the three periods was greater or equal to 90 %. The differences between treatments and clones were insignificant at $P < 0.05$ (Fig. 4.3.2.1).

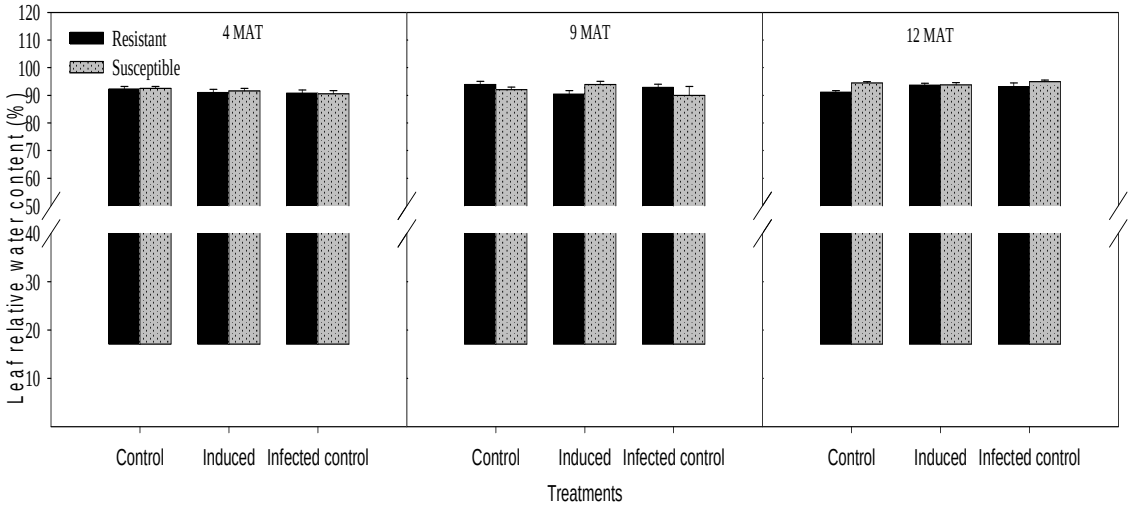


Figure 4.3.2.1: The relative water content of the leaf of resistant and susceptible clones. Bars indicate means ± SE, (n = 9).

The photosynthetic assimilation rate, transpiration rate, intercellular CO_2 concentration (C_i), stomatal conductance (g_s) and water use efficiency of the two enset clones were measured 4, 9 and 12 MAT. Four MAT, control plants of both clones showed significantly ($P < 0.05$) higher assimilation rate than infected controls (Fig. 4.3.2.2 and Appendix 10). Moreover, induced plants of the resistant clone had significantly ($P < 0.05$) higher assimilation rate than infected control plants (Fig. 4.3.2.2). The lowest assimilation rate was recorded in infected control plants of the resistant clone. In others treatments, plants of the resistant clone recorded a higher assimilation rate than plants of the susceptible clone. Similarly, stomatal conductance was significantly ($P < 0.05$) higher in control plants of the susceptible clone than infected control plants (Table 4.3.2.1A). Although the differences were insignificant, higher water use efficiency was recorded in infected control plants of both clones as compared to other treatments followed by induced plants. There were insignificant differences in transpiration rate and intercellular CO_2 concentration responses between treatments and clones at this data collection period (Table 4.3.2.1A).

Nine months after treatments application, induced plants of the resistant clone had a significantly ($P < 0.05$) higher assimilation rate than infected control plants (Fig.4.3.2.2). However, the variation with control plants was insignificant. In the same way, the variation in assimilation rate between treatments was not significant in plants of the susceptible clone (Fig.4.3.2.2). Unlike four months post treatment, infected control plants of the resistant clone were revived nine months after treatment application and recorded higher assimilation rate than plants of the susceptible clone. Concerning C_i, the concentration in control plants of the resistant clone was significantly lower compared to induced and infected control plants (Table 4.3.2.2B). In the

susceptible clone, the concentration of intercellular CO_2 in control plants was significantly lower than induced plants (Table 4.3.2.2B). Stomatal conductance was also significantly ($P < 0.05$) different between control and infected control plants of both the resistant and susceptible clones (Table 4.3.2.2B).

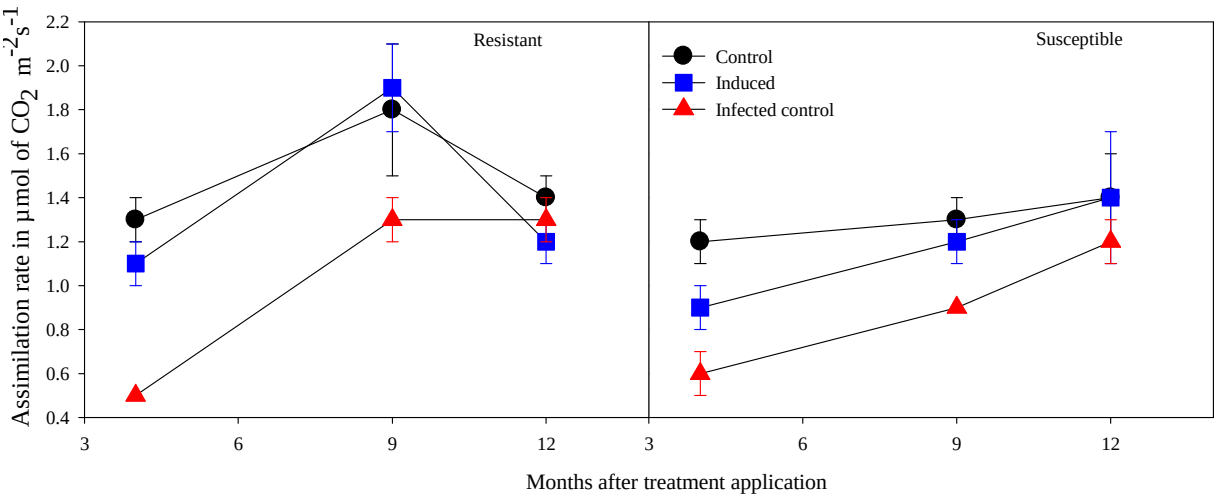


Figure 4.3.2.2: Time course change of assimilation rate of control, induced and infected control plants of two enset clones. Values are means of three replicates. Bars indicate SE.

Twelve months after treatment application, the stomatal conductance of the two enset clones was almost similar between treatments and thus significant differences in assimilation rate, transpiration rate and water use efficiency were not produced (Table 4.3.2.2C). The concentration of intercellular CO_2 in infected control plants of the susceptible clone was significantly greater than control and induced plants. However, it was not used to increase assimilation rate. This was evident from the lower assimilation rate of photosynthesis (Table 4.3.2.2C). On the other hand, the stomatal conductance of induced plants of the susceptible clone

was the slowest of all plants of others treatments and clones. Nonetheless, this was not directly related to photosynthetic assimilation rate (Table 4.3.2.2C).

The photosynthetic assimilation rate of the two enset clones exhibited an increasing pattern from the period of treatments application (Fig. 4.3.2.2). The rate of photosynthesis was comparatively lower 4 MAT than after 9 and 12 months. In plants of the resistant clone subjected to the three treatments, assimilation rate sharply increased 9 MAT and declined 12 MAT and went closer between treatments. In the susceptible clone, the rate of assimilation augmented sequentially up to 12 MAT. At this time, control and induced plants achieved almost similar assimilation rate. In both clones, the rate of assimilation was the slowest in infected control plants. Induced plants of the susceptible clone had an intermediate assimilation rate

Table 4.3.2.2: A physiological response of resistant and susceptible enset clones 4(A), 9(B) and 12 (C) MAT. Mean values that share similar letters in a column are not ssignificant at $P < 0.05$. Values are means of 5 readings per plant and three replicates. Bars indicate SE**.**

A

Enset clones	**Treatments**	**E** (mmol H_2O m^{-2} s^{-1})	**C_i** (µmol CO_2 mol^{-1})	**g_s** (mol H_2O m^{-2} s^{-1})	**WUE**
Resistant	control	3.3±0.4	355.0±7.7	0.1±0.1a	0.5±0.1
	induced	4.2±2.4	292.0±25.9	0.1±0.0a	1.4±0.8
	infected control	1.8±0.2	312.0±30.7	0.1±0.0a	2.1±0.8
Susceptible	control	3.1±0.4	336.0±5.6	0.1±0.0a	0.5±0.1
	induced	1.5±0.2	305.0±11.1	0.1±0.0a	1.0±0.3
	infected control	0.9±0.2	279.0±19.6	0.0±0.0b	1.6±0.4

B

Enset clones	**Treatments**	**E** (mol H_2O m^{-2} s^{-1})	**C_i** (µmol CO_2 mol^{-1})	**g_s** (mol H_2O m^{-2} s^{-1})	**WUE**
Resistant	control	1.4±0.2	305.5±18.8a	0.05±0.01bc	1.6±0.2a
	induced	1.3±0.1	367.6±3.7bc	0.09±0.01c	1.5±0.1ab
	infected control	1.7±0.1	394.6±2.6c	0.13±0.01a	0.9±0.1ab
Susceptible	control	1.4±0.2	286.6±17.3a	0.04±0.01b	1.3±0.3ab
	induced	1.9±0.2	356.9±6.7b	0.08±0.01c	0.8±0.1b
	infected control	1.3±0.2	329.1±10.3ab	0.05±0.01bc	1.0±0.2ab

C

Enset clones	Treatments	**E** (mol H_2O m^{-2} s^{-1})	**C_i** (µmol CO_2 mol^{-1})	**g_s** (mol H_2O m^{-2} s^{-1})	WUE
Resistant	control	0.71±0.1	276.0±29.9b	0.05±0.01ab	5.4±2.1
	induced	1.4±0.3	244.0±31.2b	0.07±0.02a	3.0±1.0
	infected control	0.8±0.1	268.0±12.9b	0.03±0.0ab	2.2±0.3
Susceptible	control	0.7±0.1	277.0±36.9b	0.07±0.01a	6.1±2.4
	induced	1.4±0.3	201.0±22.0b	0.02±0.01b	5.4±3.2
	infected control	0.9±0.1	360.0±26.7a	0.07±0.01a	2.5±0.8

E = Transpiration rate, C_i = Intercellular CO_2 concentration, g_s = Stomatal conductance and WUE = Water use efficiency

4.3.3. Biochemical responses of enset clones

Chlorophyll a, b, a+b, carotenoids x+c, total nitrogen and total protein content of leaf samples of the two enset clones grown under three treatments were estimated 4, 9 and 12 MAT. The chemical analysis performed 4 months after treatment application revealed that the concentration of chlorophyll a was significantly ($P < 0.05$) higher in control plants than infected control plants of the susceptible clone (Table 4.3.3.1B). The lowest chlorophyll a concentration was recorded in infected control plants. Leaves of induced plants had also significantly ($P < 0.05$) lower amount of chlorophyll a than controls (Table 4.3.3.1B). In the resistant clone, the concentration of chlorophyll a showed insignificant variation between control and infected control plants. However, the concentration in leaves of induced plants was significantly lower than control and infected control plants (Table 4.3.3.1A). Similarly, the highest concentration of chlorophyll b was recorded in control plants of the susceptible clone and it was significantly ($P < 0.05$) higher

than others treatments (Table 4.3.3.1A). Induced plants had also significantly greater concentration of chlorophyll b than infected control plants. Unlike the susceptible clone, the difference between control and infected control plants of the resistant clone in chlorophyll b concentration was insignificant (Table 4.3.3.1A). The amount of chlorophyll b in induced plants of the resistant clone was significantly lower than control and infected control plants (Table 4.3.3.1A). Regarding the concentration of chlorophyll a+b, control plants of the susceptible clone had significantly ($P < 0.05$) greater concentration than induced and infected control plants. The lowest concentration was recorded in infected control plants (Table 4.3.3.1A). Inversely, infected control plants of the resistant clone had higher chlorophyll a+b concentration than control and induced plants (Table 4.3.3.1A).

The concentration of total carotenoids in control plants of the susceptible clone was significantly ($P < 0.05$) higher than induced and infected control plants. The reverse was happened in the resistant clone. Accordingly, the content of total carotenoids in infected control plants was significantly ($P < 0.05$) higher than control and induced plants (Table 4.3.3.1A). Concerning total nitrogen and protein, the highest concentration was recorded in control plants of the susceptible clone of all others treatments ((Table 4.3.3.1A), followed by infected control plants of the resistant clone (Table 4.3.3.1A).

Nine months after treatment application, the biochemical responses of the two clones under the different treatments showed significant ($P < 0.05$) differences. In view of this, the concentrations of chlorophyll a, b and a+b were significantly higher in control plants of the susceptible clone as compared to induced and infected control plants (Table 4.3.3.1B). Nonetheless, the difference between treatments in the resistant clone was not significant (Table 4.3.3.1B). Moreover, significant differences in total carotenoids were not observed between treatments in both clones at this sampling period. The difference in total nitrogen and protein content between treatments in both clones was insignificant (Table 4.3.3.1B). At this sampling time, an increased amount of total nitrogen and protein was recorded in infected control plants of the susceptible clone (Table 4.3.3.1B).

Twelve months post treatment application, the effect of treatments on the biochemical responses of the two enset clones was minimized. Thus, the concentrations of chlorophyll a, b, a+b and total carotenoids in infected control plants of the susceptible clone were significantly higher than induced plants (Table 4.3.3.1C). The results of the above biochemical responses in control plants of the resistant clone were higher than infected control plants (Table 4.3.3.1C). However, the amount of total nitrogen and total protein in plants of both clones showed an insignificant difference between treatments (Table 4.3.3.1C).

Table 4.3.3.1: A biochemical response of two enset clones 4 (A), nine (B) and 12 (C) MAT Means are ± SE (n =5).

A

Clones	Treatments	Chl. a (mg/g)	Chl. b (mg/g)	Chl. a+b (mg/g)	TC (mg/g)	TN (%)
Resistant	control	7.2±0.0b	14.1±0.0c	8.6±0.0d	2.6±0.0a	8.2±0.1a
	induced	6.2±0.0c	11.4±0.1d	7.1±0.0b	2.5±0.0a	7.9±0.3a
	infected control	7.5±0.2ab	14.5±0.2c	8.9±0.1ad	2.7±0.1a	9.0±0.7a
Susceptible	control	7.7±0.1a	15.3±0.2a	9.3±0.1a	2.5±0.1a	12.3±0.8b
	induced	6.3±0.0c	12.1±0.2b	7.5±0.1b	2.2±0.0b	8.1±0.5a
	infected control	5.3±0.1d	9.9±0.1c	6.2±0.1c	2.1±0.1b	7.7±0.2a

B

Clones	Treatments	Chl. a (mg/g)	Chl. b (mg/g)	Chl. a+b (mg/g)	TC (mg/g)	TN (%)
Resistant	control	8.1±0.4ab	15.6±0.9ab	9.6±0.5ab	3.0±0.1a	10.4±1.4a
	induced	7.8±0.2b	15.7±0.6ab	9.5±0.3ab	3.0±0.1a	9.7±0.9a
	infected control	8.1±0.1ab	16.1±0.5a	9.8±0.2ab	3.1±0.0a	8.9±0.2a
Susceptible	control	9.4±0.4a	17.3±0.8a	10.8±0.5a	3.3±0.1a	10.7±1.9a
	induced	7.6±0.4b	13.6±0.8b	8.6±0.5b	2.9±0.2a	9.7±0.3a
	infected control	7.4±0.3b	12.9±0.4b	8.3±0.3b	3.1±0.1a	11.1±1.5a

C

Clones	Treatments	Chl. a (mg/g)	Chl. b (mg/g)	Chl. a+b (mg/g)	TC (mg/g)	TN (%)
Resistant	control	6.7±0.1c	17.5±0.4b	9.6±0.2b	2.7±0.1a	7.7±0.4a
	induced	5.4±0.1d	12.9±0.1d	7.3±0.1d	2.2±0.1b	7.1± 0.5a
	infected control	6.3±0.1c	15.4±0.1d	8.6±0.1c	2.7±0.1a	6.5±0.2a
Susceptible	control	7.8±0.1ab	18.0±0.1ab	10.3±0.1a	2.6±0.1a	6.5±0.9a
	induced	7.6±0.1a	16.9±0.2bc	9.9±0.1b	2.5±0.1ab	7.0±0.1a
	infected control	8.1±0.1b	18.6±0.3a	10.7±0.1a	2.6±0.1a	6.3±0.1a

Chl. b = chlorophyll b, Chl. a+b = chlorophyll a+b, TC = total carotenoids, TN = total nitrogen and TP = total protein

Mean values within a column that do not share similar letters are significantly different at $P < 0.05$.

CHAPTER FIVE

5. DISCUSSION

5.1. Evaluation of the Antibacterial Activity of Some Medicinal Plant Leaf Extracts and Antibiotics against *Xanthomonas campestris pv. musacearum*

The exploitation of higher plant products as novel chemical treatments in plant protection has gained emphasis recently and some plant products are being used globally as ecofriendly biocontrol agents (Gurjar *et al*., 2012). The present study was conducted to test the *in vitro* antibacterial activity of six medicinal plants against enset pathogen, Xcm. The tested plants are used in traditional medicine for the treatment of many diseases. Efficiency of extraction is an important step in the discovery of bioactive components from plant material. In the present study, *A. salicifolia* produced the highest extract yield followed by *P. abyssinica*. Extract yield of *V. amygdalina* was moderate. Similarly, Odey *et al.* (2012) found out 13.6 and 12 % extract yields of root and stem of *V. amygdalina*, respectively, which is twofold lower than the leaf extract yield in the present study. The differences in the extract yields might be ascribed to the different availability of extractable components, resulting from the varied chemical composition of plants (Sultana *et al*., 2009). Moreover, Zongo *et al.* (2011) and Moteriya *et al*. (2014) reported maximum percent extract yield when methanol is used as a solvent. Methanol is an all purpose solvent that dissolves most secondary metabolites in plants and enhances the release of these chemicals from cellular matrix (Visht and Chaturvedi, 2012; Moteriya *et al*., 2014).

The *in vitro* results indicated that methanolic leaf extracts exhibit antibacterial activity against Xcm. Similar antibacterial activity of methanolic extracts of other plant species against different plant and human pathogens have been reported by De Britto *et al. (*2011); Thippeswamy *et al*.

(2011) and Okoye *et al*., (2014). Thippeswamy *et al.* (2011) reported high antibacterial activity of methanolic leaf extract of *Samanea saman* against *Xanthomonas campestris* and other fungi plant pathogens. Similarly, methanol leaf extracts of *Acalypha indica, Aerva latana, phyllanthus amarus* at 100µg/mL test concentration have shown more than 11 mm inhibition zone diameter against *Xanthomonas campestris* (De Britto *et al.,* 2011). Moreover, Okoye *et al*. (2014) found out high antibacterial activity of *Alchornea cordifolia* leaf extract against human pathogens. In another study, fruit extracts of *Eucalptus globulus* found to have higher inhibition zone diameters against *Staphylococcus aureus* and *Bacillus subtilis* (Boulekbache-Makhlouf *et al*., 2013).

In the present study, extracts of most plant species inhibited the growth of the test bacterium *in* v*itro,* indicating the presence of antibacterial compounds in the extracts. These compounds are secondary metabolites that belong to various chemical categories in plant materials and work either individually or in combination to produce an antibacterial activity (Ghosh *et al*., 2011). This is evident from the results of the preliminary phytochemical screening that shows the presence of different secondary metabolites in the leaf extracts of the studied species. The variation is both in type and amount of chemical categories. The high antibacterial activity of methanolic extract of *A. salicifolia* and *P. abyssinica* may be due to the abundance of alkaloids, flavonoids, phenols, tannins, saponins and terpenoids. In addition, *A. salicifolia* extract contains glycosides. Each bioactive constituent exert antibacterial activity through different mechanisms (Kotzekidou *et al.*, 2008). Accordingly, alkaloids intercalate into cell wall and hinder its formation (Gurjar *et al*., 2012). Flavonoids are antimicrobial substances against a wide array of microorganisms *in vitro* and have the ability to bind and complex with bacterial cell walls and, to complex with extracellular and soluble proteins. This inactivates enzymes (Marjorie, 1999;

Ghosh *et al*., 2011 Gurjar *et al*., 2012). Terpenoids on the other hand are responsible for disbanding of the cell wall of microorganisms by deteriorating the membranous tissue (Marjorie, 1999; Ghosh *et al*., 2011; Jasmine *et al*., 2011; Gurjar *et al*., 2012). Similarly, saponins have the ability to cause leakage of proteins and certain enzymes from the cell (Marjorie, 1999). Moreover, Taguri *et al*. (2006), Rodriguez *et al*. (2009) and Boulekbache-Makhlouf *et al*. (2013) reported that hydrolysable tannins have potent antibacterial effects on various bacteria. They bind and form irreversible complexes with proline rich protein and cause inhibition of cell wall synthesis (Mamtha *et al*., 2004; Igbinosa *et al*. 2009; Ghosh *et al*., 2011; Gurjar *et al*., 2012). Overall, extracts that possess diversity of secondary metabolites in relatively large amount revealed high antibacterial activity against Xcm. This is evident from the low antibacterial activity of *A. aspera, D. stramonium, M. azedarach* and *V. amygdalina* leaf extracts that have less diverse and low amount of secondary metabolites.

The quantitative analysis on the other hand revealed that the leaf extract of *A. salicifolia* contains the highest amount of total phenolics content as compared to others and thus the maximum potency against the test bacterium. This is evident from the strong positive correlation between total phenolics content and bacterial growth inhibition zone. Similar correlation was observed in extracts of *P. abyssinica* at all test concentrations. Therefore, the antibacterial activity of the two species may be explained by the total phenolics content. Many research reports reveal that phenolic compounds contribute to the antimicrobial properties of plant extracts whereby the extent of inhibition depends on the concentration of these compounds (Taguri *et al*., 2006; Rhouma *et al*., 2009; Rodriguez *et al*., 2009; Tian *et al*., 2009; Zongo *et al*., 2011; Moteriya *et al*., 2014). The biological activities of phenolic compounds are associated to the presence of

hydroxyl groups and phenolic ring in their molecular structure. Membrane proteins of bacteria interact with hydroxyl groups of phenolics by hydrogen bonding. This interaction changes the membrane permeability that in turn causes loss of cellular constitutes and cell destruction (Marcucci *et al*., 2001; Kang *et al*., 2011). They also bind to adhesins, complex with cell wall and inactivate proteolytic macerating enzymes used by plant pathogens (Gurjar *et al*., 2012; Mohanta *et al*., 2007). From this it can be presumed that phenolic compounds act at two different points (cell membrane and cell wall) affecting the growth and metabolism of bacteria (Taguri *et al*., 2006; Stefanovic *et al*., 2012). Similar results have also been reported in other studies (Rajeshwar *et al*., 2005; Kuete *et al*., 2007). Extract of *V. amygdalina* contain high amount of total phenolics next to *A. salicifolia.* However, the antibacterial activity of the extract is low. This may be due to the moderate amount of flavonoids and saponins in the leaf extract of *V. amygdalina* and weak synergistic effect of all kinds of phenolic compounds on Xcm.

The minimum inhibitory concentration (MIC) is the lowest concentration value that inhibits the growth of microorganisms. On the other hand, minimum bactericidal concentration (MBC) is the lowest concentration of an antibacterial agent required to kill a particular bacterium. They are measures of assaying the effectiveness of antimicrobial plant extracts and predictive of their antibacterial potency. Methanolic extracts with low antibacterial activity against Xcm give high MIC and MBC, Viz. *A. aspera, D. stramonium, M. azedarach and V. amygdalina.* To the contrary, extracts with high antibacterial activity yield low MIC and MBC, Viz. *A. salicifolia* and *P. abyssinica.* Similarly, various MIC and MBC concentrations of methanol extracts of medicinal plants against both Gram positive and negative bacteria have been reported by Kang *et*

al. (2011). Unlike the present study, *M. azedarch* showed MIC against several human pathogens (Sen and Batra, 2012).

The results of the present study showed the potential use of antibiotics in controlling enset bacterial wilt disease. Antibiotics have been used to control certain bacterial diseases of high-value fruits, vegetables and ornamental plants since the 1950s in some developed countries (McManus *et al*., 2002). All antibiotics showed antibacterial activity against Xcm, but with varied potency. Streptomycin sulphate was found to be the most effective antibiotic in inhibiting the growth of Xcm. Similar effects of streptomycin have been reported on *Xylella fastidiosa* isolates that cause pierce's disease of grapevine (Kuzina *et al*., 2006) and citrus variegated chlorosis in citrus fruits (Lacava *et al*., 2001). According to Pawar (2015), treating *Xanthomonas campestris pv. mangiferaeindicae* (Xcmi) inoculated mango fruits with streptomycin sulphate inhibit the development of mango bacterial canker disease (MBCD). Conversely, SARI, (2013) reported streptomycin sulphate as the least effective antibiotic against Gurage and Hagere Selam Xcm isolates as compared to amoxicillin, chloramphenicol and tetracycline. Amoxicillin was the second effective antibiotic to inhibit the growth of Xcm at concentrations ranging from 50 – 0.39 mg/mL, which is in agreement with SARI, (2013) that reported higher antibacterial activity of amoxicillin against Gurage Xcm isolate.

Tetracycline on the other hand ranked third in its antibacterial activity against Xcm at higher test concentrations ranging from 50 – 0.39 mg/mL and second at lower concentrations below 0.39 mg/mL. It showed inhibition of Xcm growth at the lowest concentration. This result agrees with reports of Cooley *et al*. (1992), SARI, (2013) and Pawar, (2015). Oxytetracycline treatment; a

member of tetracycline antibiotics (Christiano *et al*., 2010), has significantly reduced the development of symptoms of X-disease caused by mycoplasma-like organism in peach and thus significantly enhance fruit yield 1 year after treatment (Cooley *et al*., 1992). It is also used as a foliar spray to control *Xanthomonas arboricola* pv. *pruni* on stone fruits and *Erwinia amylovora* on pome fruits (Christiano *et al*., 2010). Similarly, SARI, (2013) found out that tetracycline inhibits the growth of both Gurage and Hagere Selam Xcm isolates *in-vitro*. However, it is the most effective antibiotic on Hagere selam isolate. In an *in-vivo* study of antibiotics, tetracycline is found to be more effective to control mango bacterial canker disease than chloramphenicol, gentamicin and streptomycin (Pawar, 2015). Comparable to the present study, chloramphenicol has moderate effect on the growth of Xcm (SARI, 2013), *Xylella fastidiosa* (Kuzina *et al*., 2006) and *Xanthomonas campestris pv. mangiferaeindicae* (Pawar, 2015).

The MIC and MBC values of the present study especially tetracycline demonstrated the potential to use this antibiotic in controlling enset bacterial wilt. Tetracycline showed the lowest MIC (0.02 mg/ml) and MBC (0.049 mg/mL) against Xcm as compared to others. Similar results even lower MICs and MBCs of tetracycline have been reported by Lacava *et al*. (2001) against *Xylella fastidiosa*. Comparable result has been reported for streptomycin against *Xylella fastidiosa* (Lacava *et al*., 2001). Tao *et al*. (2011) also reported close MIC and MBCs of neomycin against *Xanthomonas campestris* pv. *citri, Erwinia carotovora* subsp. *carotovora, Xanthomonas oryzae* pv. *oryzae* and *Ralstonia solanacearum.* These antibiotics therefore, after optimizing the concentration and method of application, could be used as possible alternative controlling agents of enset bacterial wilt. This is because antibiotics have been used in USA to control crop diseases for more than 50 years without negative effects on human health or persistent impacts on the environment (Stockwell and Duffy, 2012). Moreover, direct toxic effects of antibiotics on plants,

soil microflora and fauna are improbable because of the low concentrations used in the process (Kumar *et al.,* 2005).

5.2. Induction of Systemic Defense Responses in enset (*Ensete* ventricosum *(Welw.) Cheesman*) Clones by the Leaf Extract of *Agarista salicifolia* against *Xanthomonas campestris pv. musacearum*

The results of our study demonstrate that the activity of defense related enzymes; PAL, peroxidase, polyphenol oxidase and compounds such as TPC and TSS increased in *Ensete ventricosum* clones upon inoculation with Xcm and treatment with the leaf extract and methanol. The level of induction significantly varies between treatments and clones. Similar results have been reported in *Hibiscus cannabinus* infected with mosaic disease (Chatterjee and Ghosh, 2008), *Phaseolus vulgaris* leaves and pods infected by *Anthracnose* (Gupta *et al*., 2010), *Solanum tuberosum* attacked by soft rot pathogens (Ngadze *et al.,* 2012). The induction has been also seen in *Arachis hypogaea* tissues infected with stem rot (Reddy and Sireesha, 2013), *Gossypium hirsutum* infected by cotton leaf curl Burewala virus (Siddique *et al*., 2014) and *Vicia faba* treated with plant growth promoting rhizobia inoculants and *Salix alba* extracts as folair and seed applications (Sofy *et al.,* 2014). It is a well established fact that induced enzymes such as PAL, peroxidase, polyphenol oxidase and compounds like total phenolics play an important role in crop disease resistance (Gupta *et al*., 2010; Ngadze *et al*., 2012; Reedy and Sireesha, 2013). In the present study, the magnitude of induction is evaluated in terms of increased activity of PAL, peroxidase and polyphenol oxidase, and elevated amount of TPC and TSS.

The activity of PAL increased in both clones in the control, leaf extract induced and infected control treatments as compared to the non-inoculated ones. It increased in time in the two enset clones. However, the increment in the resistant clone was greater and progressive than the susceptible clone. PAL activity was significantly higher in induced and infected control plants of the resistant clone at all time courses compared to control, indicating a possible means to develop disease resistance. In this respect, the works of Xu *et al.* (2011), Purwar *et al.* (2012) and Punithavalli *et al.* (2013) are congruent to the results of the present study. According to Xu *et al*. (2011), both resistant and susceptible lines of cotton showed an increased PAL enzyme activity after inoculation with *Verticillium dahlia* strain V991, but the increase was greater and faster in the resistant line. In wheat, the activity of PAL in resistant genotypes increased 2-3 folds higher than susceptible genotypes when infected with Karnal Bunt disease (Purwar *et al*., 2012). Similarly, the activity of PAL has been increased significantly in the resistant and wild genotypes of rice infested with *Cnaphalocrocis medinalis* as compared to susceptible ones (Punithavalli *et al*. 2013). Moreover, application of leaf extracts of *Adathoda vasica*, *Cymbopogen citrus* and *Ocimium sanctum* as seed treatment to control sheath blight disease of rice increased the activity of PAL (Govindappa *et al.*, 2011; Pal *et al.*, 2011). Several researches demonstrate that challenge treatments of plants with biotic and abiotic agents raise the activity of PAL earlier than other metaboilic changes (Kagale *et al*., 2004; Guleria and Kumar, 2006). The increase in PAL activity indicates the activation of phenylpropanoid pathway since PAL is the first key enzyme of the pathway that catalyzes deamination of *L*-phenylalanine into trans-cinnamic acid (Kagale *et al*., 2004). This acid serves as a precursor of various secondary metabolites, including phenols, phenylpropanoids, and monomers of lignin and salicylic acid

(SA), which might result in higher salicylic acid synthesis leading to induction of resistance (Basha *et al.,* 2006; Guleria and Kumar, 2006; Nafie and Mazen, 2008).

The activity of peroxidase increased almost equally in the resistant and susceptible clones under untreated conditions. Nonetheless, the activity of the enzyme significantly increased under induced and infected control plants of both clones. Similarly, Arun *et al.* (2010) reported increased peroxidase activity in pearl millet infected with *Sclerospora graminicola.* Cocoa plants induced by acibenzolar- *S*-methyl (ASM) against *Crinipellis perniciosa* and *Verticillium dahlia* showed a significant increase in peroxidase activity (Resende *et al.,* 2002). *Azadirachta indica* leaf extract treated sesame plants against *Alternaria sesami* (Guleria and Kumar, 2006) and rice leaves treated with *Datura metel* leaf extract and inoculated with *Rhizoctonia solani* or *Xanthomonas oryzae* pv. *oryzae* (Kagale *et al.,* 2004) showed a remarkable increase in peroxidase activity. According to the research reports mentioned above and others, increased expression of peroxidase is involved in the biochemical reaction necessary for lignification (Punithavalli *et al*., 2013). It participates in the oxidative polymerization of cinnamyl alcohol, a precursor for lignin synthesis, to yield lignin (Kuvalekar and Gandhe, 2010) and progressively incorporates phenolic compounds and structural proteins within the cell wall to produce cross-linkings during incompatible plant-microbe interactions or elicitor treatment (Odjakova and Hadjiivanova, 2001; Soylu *et al.,* 2003; Kagale *et al.,* 2004; Xu *et al*., 2011; Punithavalli *et al*., 2013). Increase in the polymerization activity of the enzymes can be taken as a response of the host plant to pathogen challenge (Kuvalekar and Gandhe, 2010). The cross-linking compounds act as barriers against pathogens invasion. Furthermore, induction of peroxidase has been associated with the increased production of superoxide radical and H_2O_2, a common feature of

defense responses in plants challenged by pathogens and elicitors and there is ample evidence indicating that H_2O_2 performs several important functions in disease resistance (Odjakova and Hadjiivanova, 2001; Govindappa *et al.*, 2011).

On the other hand, the response of polyphenol oxidase activity in the two *E. ventricosum* clones was linear under the various treatments and over time. Besides, the difference across treatments and between clones was insignificant. This result is against the works of Govindappa *et al*. (2011), Ngadze *et al.* (2012) and Jadesha *et al.* (2012) who observed discernible increase of polyphenol oxidase in rice induced with *Adathoda vasica* leaf extract, in potato tubers inoculated with various pathogen suspensions, and in banana peel and pulp dipped in leaf extract, respectively. Although there are no clear and definite ways about the potential anti-pathogen effects of polyphenol oxidase, Li and Steffens (2002) suggest several possibilities, including general toxicity of polyphenol oxidase-generated quinones to pathogens and plant cells accelerating cell death, alkylation and reduced bioavailability of cellular proteins to the pathogen, cross-linking of quinones with protein or other phenolics, forming a physical barrier to pathogens in the cell wall, and quinone redox cycling leading to H_2O_2 and other reactive oxygen species.

Associated with augmented activity of PAL and peroxidase, the amount of TPC was significantly increased upon treatment of *E. ventricosum* clones with methanol, leaf extract and Xcm during the different leaf sampling periods. In addition, TPC of the resistant clone was significantly higher than the susceptible one. Likewise, Arun *et al.* (2010), Ngadze *et al*. (2012) and Punithavalli *et al.* (2013) reported similar TPC responses of resistant and susceptible pearl millet, potato and rice cultivars, respectively. In this respect, Guleria and Kumar, (2006) reported that

sesame plants treated with *Azadirachta indica* leaf extract produce significantly high content of phenolic compounds. Likewise, rice leaves inoculated with *Cnaphalocrocis medinalis, a* pathogen that causes rice leaffolder yield significantly high content of phenolic compounds (Punithavalli *et al*., 2013). Abd El-Rahman *et al.* (2012) reported greater accumulation of phenolic compounds upon infection with the pathogen in induced and infected *Lupinus albus* seedlings compared to healthy plants. Moreover, Kagale *et al.* (2004) reported an increase in the level of phenols in rice leaves treated with *Datura metel* leaf extract and inoculated with *Rhizoctonia solani* or *Xanthomonas oryzae pv. oryzae*. These evidences might help us to infer that TPC has a key role in disease resistance development. Thus, accumulation of phenolic compounds after challenge inoculation has been correlated with the restriction of pathogen development, since such compounds are toxic to pathogens (Punithavalli *et al.,* 2013; Ojha and Chatterjee, 2012). Also, phenolic compounds may impede pathogen infection by increasing the mechanical strength of the host cell wall (Ojha and Chatterjee, 2012). These results agree with the general speculation that when plant cells are under infection, there is a switch from the normal primary metabolism to a multitude of the secondary defense pathway, and activation of novel defense enzymes and genes (Tan *et al.,* 2004).

In the present study, total soluble sugar responses of *E. ventricosum* clones exhibited variations between treatments. In both clones, significantly higher amounts of total soluble sugars were produced by leaves of induced plants up to 5 days after treatment application compared to others treatments. This corroborates with the results of Khallal, (2007) and Hao *et al.* (2013) on glucohexose treated tomato and arbuscular mycorrhizal fungi and/or jasmonic acid and salicylic acid treated cucumber plants, respectively. Both researchers reported increased production of TSS in plants treated with elicitors than plants infected with the respective pathogens. On the

contrary, greater rates of total soluble sugar decrease were noticed in cucumber and barley infected leaves than in healthy ones (Abood and Losel, 2003; Singh *et al.*, 2009).

Four months after treatment application, the content of total soluble sugars decreased in infected control and induced plants of both clones. Similar results have been reported in others studies. For instance, leaves of infected tea cultivars showed low contents of sugars (Ponmurugan *et al*., 2007). Scarpari *et al*. (2005) reported a reduction in the accumulation of total soluble carbohydrates in *Theobroma cacao* plants infected with *Crinipelis perniciosa*. Faba bean plants infected with *Botrytis fabae* resulted in noticeable decrease in total soluble sugars (Aldesuquy *et al*., 2014). Reduction in sugar content has been also observed in groundnut plants infected by *R. solani* (Lakshmi *et al*., 2011). Similar to Ponmurugan *et al*. (2007), the reduction was more prominent in susceptible than tolerant enset clone. Infected control plants of the resistant clone generated relatively higher amount of TSS than susceptible plants. Likewise, Misra *et al*. (2008) reported comparable results between resistant and susceptible taro genotypes. This shows that total soluble sugar responses of plants seem to be dependent on genotypes. The reduction might be attributed to the increase in the rate of consumption of sugars by the pathogen as respiratory substrate during pathogenesis (Ponmurugan *et al*., 2007). In addition, it could be due to increased invertase expression and microbial sucrolytic activity that degrade sucrose to hexoses in the cells. These changes consequently cause activation of defence and down-regulation of photosynthesis by delaying photosynthetic gene expression (Berger *et al*., 2007).

In contrast with the results of the present study at early stages, Arias *et al*. (2003), Scharte *et al*. (2005) and Lobato *et al*. (2009) reported increase in total soluble carbohydrates under infection both in the susceptible and tolerant clones. Sunflower plants infected with sunflower chlorotic

mottle virus showed an increase in total soluble sugars (Arias *et al.*, 2003). The same was observed in tobacco plants infected with tobacco mosaic virus (Scharte *et al.*, 2005). According to Lobato *et al.* (2009) both susceptible and resistant cultivars showed an increase in total soluble carbohydrate content in the pathogen-infected plants. Moreover, application of inducers slightly increased the content of total soluble sugars which is in agreement with the results of El-Khallal, (2007), Lakshmi *et al*. (2011), Aldesuquy *et al*. (2014) and Amin *et al*. (2015). Pre-treating faba bean plants with shikimic acid, salicylic acid alone or in combination could minimize the negative effects of *Botrytis fabae* on osmotic adjustment by increasing total soluble sugars that in turn improve the osmotic pressure as well as relative water content in leaves of infected plants (Aldesuquy *et al*., 2014). At the later stages of bacterial wilt development (9 and 12 months after treatment), the content of total soluble sugars increased and reached to the same or greater level as/than the controls under all treatments. This indicates that the adverse effect of the pathogen decreased as the time passed. The accumulation of total soluble sugars in bioagent or chemical induced and infected plants signifies the relationship between sugar regulation and activation of systemic resistance (Khallal, 2007). It is evident from several research reports that soluble sugars are involved in the responses of plants to abiotic and biotic stresses, and act as nutrient and metabolite signaling molecules that activate specific or hormonal-crosstalk transduction pathways, resulting in important modifications of gene expression (Couee *et al.*, 2006; Berger *et al*., 2007; Moghaddam and Ende, 2012).

There was a significant difference in disease incidence between the clones in infected controls. Disease incidence was significantly higher in the susceptible infected control plants as compared to other treatments. However, in plants pre-treated with leaf extract and consequently inoculated

by Xcm after 30 days, there were 33.3 % and 3.2 % disease incidence reduction in the susceptible and resistant clones, respectively. This shows that inducing the natural resistance increases the ability of susceptible plants to withstand pathogens in a non-genetic way (Ma and Cui, 2002). The result of the present study agrees with Resende *et al.* (2002) who reported 5-84 % reduction in disease incidence of witches' broom caused by *Crinipellis perniciosa* on cocoa seedlings sprayed with acibenzolar-*S*-methyl (ASM) 30 days before inoculation. Moreover, chemical inducing agents can cause disease incidence reduction ranging between 20 – 80 % (Soylu *et al*., 2003; Walters and Fountaine, 2009; Walters *et al*., 2013) and arbuscular mycorrhizal fungi plus jasmonic acid treatments can reduce disease incidence by 92 % (Khallal, 2007). The resistant clone showed the lowest disease incidence both in the induced and infected control plants. This might be related to the biochemical responses of the clone after treatment with the leaf extract and Xcm. Under these treatments, the resistant clone showed the highest PAL, peroxidase activity, and amount of TPC that had a strong positive correlation to each other. These might affect the growth of the pathogen directly as a toxic product and indirectly by strengthening the cell wall to hinder the further spread of the disease in the plant. The biochemical responses of the susceptible clone revealed induction of resistance against Xcm which was not as significant as the resistant clone. This is reflected in the reduction of disease incidence in induced plants as compared to infected control plants of the clone. Therefore, the result of the present study reveals that the biochemical changes occurring after application of the leaf extract and the pathogen can act as markers for systemic acquired resistance, triggering the crops' natural immunity. Exploiting systemic acquired resistance is a feasible disease controlling alternative strategy that diminishes the use of toxic chemicals.

5.3. Growth, Physiological and Biochemical Responses of *E. Ventricosum* Clones Induced by Leaf Extract and Infected With *X. campestris pv. musacearum*

Enset clones undertook changes in physiological and biochemical processes after inoculated with Xcm and treated with leaf extract. The changes varied depending upon the length of exposure to the treatments. Plant diseases that affect the photosynthetic leaf area and the roots or clog the xylem and phloem influence growth either by reducing the photosynthetic output or interfering with the translocation of water and inorganic or organic nutrients in the plant (Agrios, 2005). In the present study, results of growth parameters revealed that control, induced and infected control plants kept growing as the experiment progressed which resembles the report on *Zizania latifolia*, an aquatic plant infected with a smut fungus, *Ustilago esculenta* (Yan *et al.,* 2013). However, plant height, pseudostem diameter, leaf number, length, width and the consequent total leaf area and leaf area index were highly affected by the infection. The effect of infection was described by a clear reduction in plant height, pseudostem girth, leaf number, leaf length, leaf width, total leaf area and leaf area index in infected control plants as compared to induced and control plants of both clones. These findings are in agreement with results of others studies on stem mustard, *Brassica juncea var. tsatsai* (Guo *et al*., 2005), banana; *Musa acuminata* (Hooks *et al*., 2008) and faba bean, *Vicia faba* (Radwan *et al*., 2008). Stem mustard plants infected with turnip mosaic virus showed a marked reduction in plant height and leaf area due to the primary effect of the virus on the growth of leaves (Guo *et al*., 2005). Similarly, plant height, pseudostem diameter, canopy size and leaf area of banana plants infected with banana bunchy top virus (BBTV) were significantly smaller than those of control plants (Hooks *et al*., 2008). In faba bean, significant reductions in plant height, leaf area and leaf number were observed in response to bean yellow mosaic virus (BYMV) infection (Radwan *et al*., 2008). Moreover, Ahmed *et al*.

(2009) reported that *Meloidogyne javanica* that infects mungbean (*Vigna radiata*) plants affected growth and the reduction was severe as compared to controls. Similar reductions in plant height and leaf area were also found in *Capsicum annuum* varieties infected with tomato mosaic virus (Pazarlar *et al*., 2013).

On the other hand, plant height was significantly reduced in infected control plants of the susceptible clone than the resistance clone both four and nine months after treatment application. In agreement with this, plant height is reduced significantly more in susceptible cultivar TRI-2024 than in the tolerant cultivar TRI-2025 of *Camellia sinensis* infected with five pathogens namely; *Exobasidium vexans, Pestalotiopsis theae, Colletotrichum camelliae, Capnodium theae* and *Cephaleuros parasiticus* (Ponmurugan *et al*., 2007). In addition, growth responses such as plant height, leaf number and leaf area of induced plants of the susceptible clone were increased at both data collection periods compared to infected control plants. Similarly, spraying of high concentration of salicylic acid (SA) concentration (50 or 100 mM SA) on infected and uninfected faba bean leaves significantly increased plant height, leaf area and leaf number (Radwan *et al*., 2008). Furthermore, tomato plants sprayed with salicylic acid plus chitosan or chitosan alone 4 days prior to tomato mosaic virus (TMV) inoculation produced moderate plant height followed by salicylic acid application, while pathogen inoculation alone gave the lowest plant height (Abd El-Gawad and Bondok, 2015). The moderate performance of induced plants might be related to the short lag period before systemic resistance is expressed and allocation costs arising from the diversion of metabolites and energy from growth and other processes toward defenses (Walters *et al*., 2005). In general, induced resistance increases the ability of susceptible plants to withstand pathogens in a non-genetic way (Ma and Cui, 2002).

Pathogens affect the water balance of plants in one or more ways. Some interfere with the function of the roots; others with the translocation of water through the stem by growing in the xylem vessel and some others with the water economy of the plant by causing excessive transpiration through their effects on leaves and stomata (Agrios, 2005). In vascular wilts, the extent of reduction in water translocation varies from plant species to species and from pathogen to pathogen. Vessels may be filled with the bodies of the pathogen and substances secreted by the pathogen or by the host in response to the pathogen and become clogged. The affected vessels cease to function properly and allow little or no water to pass through them (Agrios, 2005). In the present study, the relative water content of the leaf in plants of both clones under the different treatments was relatively stable throughout the assessment period. The difference in relative water content between treatments was insignificant across all data collection periods. This is supported by the research reports of Solomon Tadesse *et al.* (2012) on grapevines.

In contrast to the present study, McElrone *et al.* (2003), Perez-Donoso *et al.* (2007), Pazarlar *et al*. (2013), Agamy *et al*. (2013) and Wang *et al*. (2015) found a decreased leaf relative water content in vine, grapevine, pepper, tomato and cucumber infected plants, respectively. According to McElrone *et al.* (2003), the effect of infection by *Xylella fastidiosa* on vine (*Parthenocissus quinquefolia*) leaf relative water content occurs due to reduced hydraulic conductance caused by clogging of the vessels. Similarly, the loss of xylem water transporting function in grapevines (*Vitis vinifera*) infected with *Xylella fastidiosa* was caused by a decrease in stem-specific hydraulic conductivity and the presence of tyloses in the lumens of obstructed water conduits, produced by grapevine's active responses to the pathogen (Perez-Donoso *et al.*, 2007). In

infected cucumber plants, Wang *et al*. (2015) reported a reduced stomatal conductance that resulted in a lower leaf transpiration rate. However, the leaf water content of infected plants was significantly lower than that of the healthy cucumber plants. This was caused by non-stomatal water loss induced by leaf cell membrane injury that leads to uncontrolled water loss from damaged cells to the apoplast (Wang *et al*., 2015). On the other hand, application of salicylic acid (SA) and Agrileen (AG) and bioactive materials significantly increased the leaf relative water content of tomato plants infected with *Alternaria tenuissima* (Agamy *et al*., 2013). This might be attributed to absence of toxic products from pathogens and increased production of osmolytes such as total soluble sugars during resistance induction (Agamy *et al*., 2013; Amin *et al*., 2015).

Plant-pathogen interaction alters primary metabolism either due to the fact that the induction of plant defence is cost-intensive that causes increased demand for assimilates in the plant or the pathogen tries to manipulate plant carbohydrate metabolism for its own. As a result, plants switch off photosynthesis and other assimilatory metabolism to initiate respiration and other processes required for defence (Berger *et al.*, 2007). In the present study, assimilation rate (A), transpiration rate (E) and stomatal conductance (g_s) were reduced in infected control plants of both clones four months post treatment application. The level of reduction in assimilation rate was twofold as compared to controls. The reduction was more prominent in the susceptible clone than the resistant, which is in agreement with the results of Ponmurugan *et al*. (2007) on tea cultivars infected with different diseases. Moreover, infection of rice genotypes with *Xanthomonas oryzae* pv. *oryzae* (*Xoo)* has produced a significant reduction in assimilation rate, transpiration and stomatal conductance in susceptible genotype PB1 when compared to resistant

IRBB21 and highly resistant *Oryza longistaminata* (Kumar *et al*., 2013), which supports the results of the present study.

Decrease in the gas exchange parameters such as A, E and g_s are also evident in others pathosystems. For instance, stem mustard plants infected with turnip mosaic virus revealed decreased photosynthetic rate, transpiration and stomatal conductance. The net photosynthetic rate was significantly reduced with the development of symptoms (Guo *et al*., 2005). Similar decreases are reported in grapevine plants infected with esca (Petit *et al*., 2006) and bois noir (Solomon Tadesse *et al*., 2012). A significant reduction in A, E and g_s was also found in infected leaves of tea cultivars as compared to healthy ones (Ponmurugan *et al*., 2007). Likewise, in *Bipolaris oryzae* infected rice plants, gas exchange parameters were reduced by about 65 % at 144 h after inoculation compared to non-inoculated plants (Dallagnol *et al*., 2011). Tomato plants infected with *Xanthomonas gardneri* also showed reduction in gas exchange parameters (Silveira *et al*., 2015). In infected control plants of the susceptible clone, the reduction in assimilation rate was closely associated with the decline in the concentrations of chlorophylls a and b. Similar results are reported on grapevine (Petit *et al*., 2006), tea cultivars (Ponmurugan *et al*., 2007) and rice plants under infection (Dallagnol *et al*., 2011). On the contrary, the low assimilation rate in infected control plants of the resistant clone was not related to the concentration of chlorophylls. It might be due to damage to the photosynthetic cells or down regulation of photosynthesis. Accordingly, Petit *et al*. (2006) reported that the low photosynthesis in esca infected grapevine could be due to the low activity of enzymes such as rubisco, carbonic anhydrase that catalyzes the conversion of CO_2 to HCO_3 or other enzymes involved in the utilization of photoassimilates. Moreover, the pathogen, *Bipolaris oryzae*

decreases photosynthesis of rice mainly through the damage to the cells basically the cell membrane (Dallagnol *et al*., 2011).

As mentioned earlier, leaf transpiration was low in infected control plants of both clones, but the effect of infection was more severe in the susceptible clone. The decrease in transpiration rate was closely related to stomatal conductance. Opposite to others gas exchange parameters, the variation in the concentration of intercellular CO_2 (C_i) was relatively stable, but higher C_i was recorded in infected control plants of the susceptible clone. This is supported by the research results of Guo *et al*. (2005) on mustard stem leaves, Petit *et al*. (2006) on infected grapevine and Dallagnol *et al*. (2011) on diseased rice leaves. The water use efficiency of infected control plants of both enset clones was higher than others treatments which might be due to the low transpiration rate. In contrast, Kumar *et al.* (2013) reported reduction of water use efficiency of infected susceptible rice genotypes as compared to resistant or highly resistant genotypes.

During each measuring time, the net carbon assimilation rate of plants pre-treated with leaf extract (induced) was significantly higher when compared to infected control plants. This indicates that in addition to inducing resistance in the plant, it may maintain the photosynthetic activity, transpiration and stomatal conductance at the level of healthy plants. These in turn increase the relative water content of the leaf via augmented absorption of water and nutrients from the soil. This is corroborated by the findings of Khan *et al*. (2003), Cavalcantia *et al*. (2006) and Vazirimehr and Rigi, (2014). Vazirimehr and Rigi, (2014) reported higher amount of pigments as well as enhanced photosynthesis in soybean plants treated with salicylic acid (SA). Similarly, foliar application of SA, acetyl salicylic acid (ASA) and gentisic acid (GTA) increased photosynthetic rate, stomatal conductance and transpiration in both soybean and maize plants (Khan *et al*., 2003). Cavalcantia *et al*. (2006) in their study found out that ‘St. Cruz Kada’

susceptible tomato is better protected against bacterial leaf spot caused by *X. vesicatoria* by pre-treating the plant with acibenzolar-S-methyl (ASM) and aqueous extract from dry necrotic tissue flour (VLA) of 'Lobeira' (*Solanum lycocarpum*) bush. ASM and VLA treated tomato plants have maintained the photosynthetic rate remarkably comparable to healthy plants (Cavalcantia *et al*., 2006). At the later stages of infection; 9 and 12 months after treatment application, the rate of carbon assimilation in infected control plants of both clones was progressively increased. Twelve months post treatment, the difference in assimilation rate between treatments was insignificant. This contradicts with the result of Guo *et al*. (2005) who reported a more dramatic decrease in net photosynthetic rate at the later times of infection of stem mustard with turnip mosaic virus.

The chemical analysis of the leaf samples of the two enset clones revealed different results with respect to the amount of photosynthetic pigments. Infection didn't affect the content of pigments in plants of the resistant clone. Unlike others studies, the concentration of pigments in induced plants of the resistant clone was lower than control and infected control plants. In the susceptible clone, the concentrations of chlorophyll a, b, a+b and carotenoids were lower in infected control plants than the control. The effect of the disease on pigments was minimized at the later stages of development (9 and 12 months after treatment application). Findings similar to responses of the susceptible clone have been found by Tan and Whitlow, (2001), Guo *et al*. (2005), Hooks *et al*. (2008), Radwan *et al*. (2008), Palanisamy *et al*. (2009), Labato *et al*. (2010), Kobra *et al*. (2011) and Pazarlar *et al*. (2013). Lower chlorophyll content was recorded in *Catharanthus roseus* plants infected with Ash yellow phytoplasms after 11 weeks (Tan and Whitlow, 2001). According to Guo *et al*. (2005) and Hooks *et al*. (2008), total chlorophyll, chlorophyll a and b

contents were significantly decreased in mustard stem and banana plants infected with turnip mosaic virus (TMV) and banana bunchy top virus (BBTV), respectively.

Similarly, decreased concentrations of chlorophyll a and b have been found in yellow vein mosaic virus infected genotypes of *Abelmoschus esculentus* leaves (Palanisamy *et al.*, 2009). In tobacco mosaic virus (TMV) infected varieties of pepper, reduced chlorophyll content was obtained compared to controls (Pazarlar *et al*., 2013). However, TMV infection did not affect the content of carotenoids (Pazarlar *et al*., 2013). In addition to reduced pigment contents in bean yellow mosaic virus (BYMV) infected *Vicia faba* leaves, most chloroplasts had increased stromal area, became spherical in shape and some lost their envelopes, either partially or totally. Chloroplasts were also fewer in numbers (Radwan *et al*., 2008). Diseased cotton plants had also lower chlorophyll content than controls (Kobra *et al*., 2011). Moreover, 80, 100, and 97 % reduction in chlorophyll a, b and carotenoids contents, respectively, have been observed in *Alternaria tenuissima* infected tomato plants (Agamy *et al*. (2013). The concentration of pigments in infected enset clones showed a progressive improvement at the later measuring periods. In contrast to this, the reduction in total chlorophyll has been progressive at the later stages of disease development in infected common bean plants (Labato *et al*., 2010).

In the studied enset clones, the effect of infection on the concentration of chlorophyll a was more pronounced than chlorophyll b which is in agreement with Lubaina and Murugan, (2013) findings on the response of *Alternaria sesami* infected Thilarani sesame cultivar. This might be due to higher degradation of chlorophyll a to b since chlorophyll b is synthesized from chlorophyll a and is catabolized after it is reconverted to chlorophyll a (Tanaka and Tanaka, 2011). According to Roca and Minguez-Mosquera, (2003) and Kariola *et al*. (2005), decreases in chlorophyll content are caused by increased chlorophyllase activity that participates in shaping

the plant defense response by causing a rise in reactive oxygen species that result in programmed cell death (Kariola *et al*., 2005; Agamy *et al*., 2013). The low chlorophyll content in infected plants might be also due to inhibition of chlorophyll synthesis (Kariola *et al*., 2005).

Inducing plants of the resistant clone didn't bring any improvement on contents of chlorophylls and carotenoids as compared to control and infected control. However, induced plants of the susceptible clone had higher photosynthetic pigments compared to infected control plants at later stages. In this regard, El-Khallal, (2007), Radwan *et al*. (2008), Agamy *et al.* (2013) and Amin *et al.* (2015) have found improved contents of chlorophyll and carotenoids in plants treated with different bioagents and elicitors before infection. Treating *Fusarium oxysporum* inoculated tomato plants with arbuscular mycorrhizal fungi, SA or Jasmonic acid (JA) alone or in combination, significantly decreases the effect of infection on the content of total chlorophyll (El-Khallal, 2007). Similarly, foliar application of 100 mM SA prior to pathogen inoculation restored the metabolism of infected faba bean leaves to the levels of healthy controls. It also increased plant resistance against bean yellow mosaic virus (BYMV) as evident from higher number and well developed chloroplasts with many starch grains and well organized cell organelles (Radwan *et al*., 2008). Comparable improvements in chlorophyll contents are reported in *Alternaria solani* infected tomato plants treated with SA (Faheed *et al*., 2005) and Headline (Pyraclostrobin 20% WG) treated groundnut plants grown on *Sclerotium rolfsii*-infested soil (Amin *et al*., 2015). The high chlorophyll content in elicitor and bioagent treated plants could be attributed to their stimulatory effect on rubisco activity and absence of toxic products from the pathogen due to prior application of the elicitor (Agamy *et al*., 2013).

The two enset (resistant and susceptible) clones showed variations in total nitrogen and protein content at the different sampling periods. Four and 9 months after treatment, an increased content of total nitrogen and protein was observed in infected control plants of the resistant and susceptible clones, respectively, compared to controls. Similar results of total nitrogen have been found in infected alfalfa leaves (Yardimci *et al*., 2007). Moreover, roots of turmeric (*Curcuma longa*) inoculated with *Fusarium solani* found to have higher concentration of total nitrogen than healthy plants (Reddy *et al*., 2005). In rice, inoculation with *Pyricularia oryzae* affects the overall nitrogen metabolism and resulted in an increased total nitrogen concentration compared to non-inoculated plants (Katsantonis *et al*., 2008). Decreased total nitrogen content was found in infected control plants of both clones 12 months after treatment. These results are in agreement with reports of Kumar and Shishodia, (2012) on infected leaves of *Cicer arietinum*. According to Etcbarian and Blackburn, (2000), greater concentration of total nitrogen has been found in *Puccinia hordei* infected leaves of barley especially in susceptible cultivars. This is consistent with total nitrogen content of infected control plants of the susceptible enset clone analyzed 9 months post treatment. Similar to infected control plants of the resistant enset clone 9 and 12 months post treatment, a decrease in total nitrogen has been seen upon infection of the resistant cultivar of barley (Etcbarian and Blackburn, 2000).

The changes in total protein content follow the same pattern to total nitrogen as it was derived from percentage total nitrogen multiplied by correction factor for horticultural crops. Contrasting results were recorded in both resistant and susceptible enset clones at different sampling periods. In some studies, infection had no effect on total protein for instance in infected mung bean plants (Ahmed *et al.,* 2009). In others, higher total protein content has been found in turmeric plants

inoculated with *Fusarium solani* and alfalfa plants infected with alfalfa mosaic virus (Reddy *et al*., 2005; Yardimci *et al*., 2007). Such increases are attributed to increased catabolic reactions or decrease in proteolytic degradation or both (Reddy *et al*., 2005). It might also be due to the increased level of viral proteins in the infected plant (Yardimci *et al*., 2007). To the contrary, decreased concentration of total protein has been reported in *Fusarium graminearum* infected wheat plants. This may be caused by denaturation or proteolysis of proteins and bound amino acids, resulting in an enhanced free amino acid content of the host tissue (Zhou *et al.,* 2005). Similar to the results of the present study, application of inducers such as salicylic acid (SA), Agrileen (AG), Headline and leaf extracts enhanced total protein content (Agamy *et al*., 2013; Amin *et al*., 2015).

6. CONCLUSION AND RECOMMENDATION

6.1. Conclusion

This study gives useful information about growth, physiological and biochemical responses of resistant and susceptible enset clones pretreated with *A. salicifolia* leaf extract and inoculated with Xcm including controls. In all the parameters studied, the resistant clone achieved better than the susceptible clone. Moreover, the study showed the potential role of medicinal plant extracts, commercially available antibiotics and induced resistance to control enset bacterial wilt. It also partially identified the types of responses in which resistant and susceptible enset clones basically differ during infection and induction and the inferred resistance mechanism of each clone. The findings of the present study could be summarized as follows:

- The leaf extracts of *A. salicifolia* and *P. abyssinica* showed high potency against Xcm which is attributed to the presence of abundant amount of alkaloids, flavonoids, phenols, terpenoids, saponins and tannins. The antibacterial activity of these extracts is positively correlated to the concentration of total phenolic compounds.

- Amoxicillin and streptomycin sulphate showed the highest anti-Xcm activity followed by tetracycline. However, the lowest MIC and MBC values were recorded by the test concentrations of tetracycline, indicating the potential use of this antibiotic in the control of enset bacterial wilt.

- *A. salicifolia* leaf extract induced-infected enset plants of both clones revealed augmented activity of disease resistance related enzymes such as PAL, peroxidase and polyphenol oxidase. The activity of PAL was highly significant in induced plants of the resistant clone. The resistant characteristics of the clone might be related to PAL as high PAL activity indicates the initiation of secondary metabolism. Moreover, the high concentration of total phenolic compounds post treatment is an evidence for the activation of phenylpropanoid pathway.

- Treating enset with medicinal plant extract prior to pathogen inoculation reduced disease incidence by about 33 % in plants of the susceptible clone and 3.2 % 4 MAT and 10.6 % 9 MAT in plants of the resistant clone. This implies that inducing innate immunity could be used as an alternative enset bacterial wilt controlling method.

- Overall, infection and induction of enset clones had no remarkable effect on growth and physiological parameters. However, 4 months after treatment application, the influence of infection on net photosynthetic rate was significant. This might be due to the low concentration of chlorophylls in plants of the susceptible clone and the diversion of primary metabolism to processes related to defense in plants of the resistant clone.

From these findings, it is possible to conclude that medicinal plant extracts, antibiotics and induction are feasible tools to control enset bacterial wilt in Ethiopia. Moreover, the biochemical responses are the major indicator characteristics that are responsible for the noticeable differences between the two enset clones.

6.2. Recommendations

We strongly believe that the results of the present study can be of use in alleviating the major constraint of enset cultivation; bacterial wilt disease. Unearthing an effective enset bacterial wilt controlling method will bring back the wide spread cultivation of enset in high bacterial wilt risk areas and contributes to ensure food security in the south and southwestern Ethiopia. However, a lot remains to be done to achieve the aforementioned goal and these are forwarded as recommendations as follows:

- Ethiopia is the center for plant diversity. Thus, many more screening researches should be conducted on the anti-Xcm activity of medicinal plants so that plant extracts with high potency could be found.

- The maximum extract yield, high potency against Xcm and low minimum inhibitory concentration of *A. salicifolia* and *P. abyssinica* leaf extracts could make the species better candidates for further processes that lead to biocide development. Therefore, further research on formulation development and commercialization of the extracts as biocides with broad spectrum activity should be done.

- The leaf extracts and usage as natural products to control plant diseases will minimize or avoid environmental and health hazards caused by synthetic pesticides. However, further researches into these extracts should be conducted to identify the active compounds responsible for such antibacterial activity.

- The *in vitro* tests revealed that extracts of *A. salicifolia* and *P. abyssinica* and antibiotics; amoxicillin, streptomycin sulphate and tetracycline had high anti-Xcm activity. However, their performance under field condition should be evaluated. In addition, the methods of application of these tools should be evaluated and the method in which the disease controlling agents achieve better should be identified.

- The leaf extract of *A. salicifolia* was applied to enset plants using mid vein injection method. This has induced systemic resistance in treated plants. However, the inducing and disease controlling capacity of the leaf extract should be evaluated in terms of other application methods such as foliar and soil application.

- Induction of resistance is a feasible alternative to control enset bacterial wilt. However, further researches should be undertaken to find out other elicitors from various sources that could result in high level of systemic resistance induction.

- Medicinal plant extracts can be directly used as biocides and also as elicitors to induce systemic resistance. This minimizes disease incidence in enset to a certain level. However, researches on the integrated use of leaf extracts, induced resistance and other biocontrol techniques should be conducted.

- In general, the results of the present study showed a green light about the potential of environmentally safe enset bacterial wilt controlling tools. Thus, many more other studies should be carried out on the potential of other biological control agents to control enset bacterial wilt.

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Appendix 1: ANOVA result of antibacterial activity of medicinal plant extracts

		Sum of Squares	df	Mean Square	F	Sig.
400mg/mL	Between Groups	341.800	4	85.450	423.719	0.000
	Within Groups	5.042	25	0.202		
	Total	346.842	29			
200 mg/mL	Between Groups	360.783	4	90.196	146.264	0.000
	Within Groups	15.417	25	0.617		
	Total	376.200	29			
100 mg/mL	Between Groups	144.383	4	36.096	183.538	0.000
	Within Groups	4.917	25	0.197		
	Total	149.300	29			
50 mg/ML	Between Groups	383.300	4	95.825	475.165	0.000
	Within Groups	5.042	25	0.202		
	Total	388.342	29			

Appendix 2: ANOVA result of anti-Xcm activity of antibiotic

		Sum of Squares	df	Mean Square	F	Sig.
Tetracycline	Between Groups	8651.122	11	786.466	76.817	0.000
	Within Groups	614.292	60	10.238		
	Total	9265.413	71			
Streptomycine	Between Groups	4297.639	11	390.694	33.172	0.000
	Within Groups	282.667	24	11.778		
	Total	4580.306	35			
Chloramphenicol	Between Groups	6735.312	11	612.301	26.112	0.000
	Within Groups	844.167	36	23.449		
	Total	7579.479	47			
Cephalexin	Between Groups	1279.639	11	116.331	49.856	0.000
	Within Groups	56.000	24	2.333		
	Total	1335.639	35			
Amoxacillin	Between Groups	12926.306	11	1175.119	276.499	0.000
	Within Groups	102.000	24	4.250		
	Total	13028.306	35			

Appendix 3: ANOVA result of PAL

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	75783.305	35	2165.237	251.171	0.000
Within Groups	1241.364	144	8.621		
Total	77024.669	179			

Appendix 4: ANOVA result of peroxidase activity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1829.545	35	52.273	233.970	0.000
Within Groups	32.172	144	0.223		
Total	1861.717	179			

Appendix 5: ANOVA result of polyphenol oxidase activity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.020	35	0.001	7.396	0.000
Within Groups	0.011	144	0.000		
Total	0.032	179			

Appendix 6: ANOVA result of TPC of enset clones

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.159	35	0.119	4235.818	0.000
Within Groups	.004	144	0.000		
Total	4.163	179			

Appendix 7:ANOVA result of disease incidence 9 MAT

		Sum of Squares	df	Mean Square	F	Sig.
Resistant	Between Groups	739.696	2	369.848	4.491	0.064
	Within Groups	494.147	6	82.358		
	Total	1233.842	8			
Susceptible	Between Groups	4945.662	2	2472.831	20.623	0.002
	Within Groups	719.447	6	119.908		
	Total	5665.109	8			

Appendix 8: Pictures showing the severity of enset bacterial wilt



Resistant- induced



Resistant- infected control



Susceptible- induced



Susceptible- infected control

Appendix 9: ANOVA of growth parameters 9 MAT

		Sum of Squares	df	Mean Square	F	Sig.
number of green leaves	Between Groups	66.315	5	13.263	13.202	0.000
	Within Groups	48.222	48	1.005		
	Total	114.537	53			
leaf length	Between Groups	6.139	5	1.228	6.840	0.000
	Within Groups	8.617	48	0.180		
	Total	14.756	53			
leaf width	Between Groups	.304	5	0.061	7.525	0.000
	Within Groups	.388	48	0.008		
	Total	.693	53			
plant height	Between Groups	19.838	5	3.968	5.254	0.001
	Within Groups	36.245	48	0.755		
	Total	56.083	53			
Pseudostem girth	Between Groups	1.904	5	0.381	10.856	0.000
	Within Groups	1.684	48	0.035		
	Total	3.588	53			
Total leaf area	Between Groups	10275032.815	5	2055006.563	16.257	0.000
	Within Groups	6067628.444	48	126408.926		
	Total	16342661.25	53			
Leaf area index	Between Groups	641302.593	5	128260.519	16.276	0.000
	Within Groups	378248.222	48	7880.171		
	Total	1019550.815	53			

Appendix 10: ANOVA results of photosynthetic responses of enset clones 9 MAT

		Sum of Squares	df	Mean Square	F	Sig.
assimilation rate	Between Groups	9.147	5	1.829	7.663	0.000
	Within Groups	20.053	84	0.239		
	Total	29.199	89			
Transpiration rate	Between Groups	4.164	5	0.833	1.794	0.123
	Within Groups	38.990	84	0.464		
	Total	43.154	89			
internal CO2	Between Groups	122774.722	5	24554.944	11.943	0.000
	Within Groups	172712.000	84	2056.095		
	Total	295486.722	89			
water use efficiency	Between Groups	8.257	5	1.651	3.343	0.008
	Within Groups	41.497	84	0.494		
	Total	49.755	89			
gas exchange	Between Groups	0.091	5	0.018	12.516	0.000
	Within Groups	0.123	84	0.001		
	Total	0.214	89			