ADDIS ABABA UNIVERSITY SCHOOL OF GRADUATE STUDIES DEPARTMENT OF MICROBIAL, CELLULAR & MOLECULAR BIOLOGY

Culturable endophytes of enset (*Ensete ventricosum*): diversity, plant growth promotion, and bacterial wilt disease control potentials

A Thesis submitted to the School of Graduate Studies of the Addis Ababa University in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Biology (Applied Microbiology)

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Abstract

Enset (*Ensete ventricosum* (Welw) Cheesman) is a perennial root crop used as a staple and co-staple food in Ethiopia. Enset cultivation is one of the sustainable agricultural systems in the country and offers significant ecological benefits by reducing soil erosion, resistance to different diseases and pests and relative tolerance to drought. Although enset agriculture is a good example of sustainable agriculture, the crop is largely affected by bacterial wilt disease caused by *Xanthomonas campestris pv. musacerum.* Because the pathogen resides in the vascular tissue of host plant, chemical based control strategies cannot be employed to control the disease.

Studies showed that endophytes, symbiotic microorganisms (both bacteria and fungi) that live inside healthy plants have properties of biological control against different plant diseases. Having these properties endophytes might be one component of integrated disease management programs on enset. This study was initiated with the aim of investigating the diversity of culturable endophytic bacteria and fungi from enset and assesses the presence of phytobeneficial properties and their roles in protecting the plant against enset wilt disease.

Endophytic bacteria and fungi were isolated from surface sterilized samples of leaf, stem and root of healthy enset plants. A total of 446 bacterial and 105 fungal endophytes were identified indicating the presence of high diversity of bacterial and fungal endophytes associated with enset. The bacterial endophytes were grouped to four phyla and 53 genera. Proteobacteria were the most frequently isolated phylum from all the plant parts followed by gram positive *Actinobacteria*, *Firmicutes*, and non-proteobacteria gram negative *Bacteroidetes.* The fungal isolates were grouped in to two phyla and 42 species. Ascomycota was more detected phylum compared to Zygomycota.

The *in vitro* investigation of plant growth promotion (PGP) characteristics of 105 bacterial and 44 fungal isolates showed that majority of the isolates were positive to one or more plant growth promoting characteristics. IAA production is a more common property among enset endopytes. Moreover, there were isolates that produce siderophore and able to grow on nitrogen free medium. Phosphate solublisation was detected in bacterial isolates but none of the tested fungal endophytes show phosphate solublization.

The potential of enset endophytes to control bacterial wilt of enset was also assessed *in vitro* and *in vivo*. In the *in vitro* test bacterial and fungal endophytic isolates belonging to different genera showed marked inhibition of the growth of *Xanthomonas campestris pv. musacerum* with the highest observed for bacterial isolates belonging to the genera *Pseudomonas*, *Bacillus*, and *Rhizobium.* And from the fungal endophytes two isolates identified as *Mycosphaerella coacervata* and *Plectosphaerella cucumerina* inhibited the growth of the pathogen. Plants treated with a mixed culture of bacteria showed a mean disease severity of 30.7% as compared to a disease severity of 47.4% for the control plants.

In the endophytic metabolite analysis for potential antimicrobial compounds, hundreds of VOC and NVDOC were identified. The compounds produced include phenols, lactone, cyanide, pyrazine and dimethyl disulfide. Some of the compounds were reported to have antimicrobial activity against different fungal and bacterial pathogens in previous studies. Moreover, other unknown compounds were also detected

In conclusion, this study showed high occurrence, diversity, plant growth promoting characteristics and the biocontrol potential of enset endophytes. Furthermore, the results also indicated enset endophytes as a potential alternative increase crop productivity.

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List of abbreviations

CAS	Chrom azurol S
CTAB	Hexadecyl trimethyl ammonium bromide
EECC	Enset endophyte culture collection
GC-MS	Gas chromatography mass spectrometry
ITS	Internal transcribed spacer
IAA	Indole-3-acetic acid
EECC	Enset endophyte culture collection
LC-MS	Liquid chromatography-mass spectrometry
NA	Nutrient agar
NVDOC	None volatile organic compond
NFM	Nitrogen free medium
NCBI	National center for biotechnology information
PDA	Potato dextrose agar
PGP	Plant growth promotion
pv.	Pathovar
SNNP	Southern Nations, Nationalites and Peoples
VOC	Volatile organic compound

Chapter 1

1. Introduction

1.1. General introduction

Bacteria and fungi that colonize the internal tissues of plants without causing any harm to the host are known as endophytes (Saikkone et *al*., 1998; Ulrich *et al*., 2007; Mano, and Morisaki, 2008; Magnani *et al*., 2010). Endophytic association is very common in several plant species and endophytes are isolated from all plant organs including, reproductive structures (Rosenblueth *and* Martínez-Romero, 2006; Compant *et al*., 2011). The entry of these endophytes to the host plant is known to be either through natural openings on leaves and roots, or through plant wounds (Cohen, 2006; Giri and Dudeja, 2013).

The relationship between endophytes and their plant host is known to be mutualistic (Cankar and Kraigher, 2005; Aliferis *et al*., 2013). Thus, several endophytes benefit the host plant by producing plant growth hormones or increase nutrient availability, protect the plant from disease and pests through induction of systemic resistance and/or by directly attacking the pathogen or pest that attack the plant (Rosenblueth *et al*., 2006; Park., *et al*., 2007; Mendes *et al*., 2007; Khan and Doty, 2009; Kelemu, *et al*., 2011).

Artificial inoculation of bacterial or fungal endophytes that are antagnostic to the pathogen helps the plant defend itself from attack by disease and pests through mechanisms such as production of diffusible and volatile antimicrobials, through competition for nutrient and space and by inducing systemic resistance (Taghavi *et al*., 2010).

Several studies demonstrated the potential of endophytes in suppressing plant pathogens. For example, endophytic *Bacillus subtilis* showed a significant supperessive effect of bacterial wilt disease of mulberry (Ji *et al*., 2008). Similarly, endophytic *Pseudomonas* suppressed wilt disease of egg plant (Ramesh *et al*., 2009). Another challenge study on the infection of the fungal pathogen *(*Rhizoctonia solani AG3) and inoculation with endophytic fungi (*Trichoderma atroviride* and *Epicoccum nigrum)* showed drastic decrease of disease severiety on potato plants (Rachid and Mohamed, 2010). Significant reduction in disease severity of coffee leaf rust caused by the pathogen *Hemileia vastatrix* was also observed by inoculating seedlings of coffee with endophytic bacteria (Silva *et al*., 2012)

In recent years particular attention has been given to the study of the diversity and the beneficial roles of endophytes on different agricultural crops (Hallmann, *et al*., 1997; Hardoim, *et al*., 2008; Aravind, *et al* 2009). High endophyte diversity has been reported from such crops as rice, maize, banana, sweet potato, and wheat (Rosenblueth, *et al*., 2006; Mano, *et al*., 2008; Liu *et al*., 2012; Pious, *et al*., 2013; Sessitsch, *et al*., 2012). The existence of a diverse group of endophytes from different plant species and geographic locations is expected to be source of microbial bioresources for different agricultural applications.

Enset is an important crop in Ethiopia which plays an enormous role in ensuring food security in the centeral and southern parts of the country. According to different authors, more than 15 million people use enset as source of staple food (Brandt *et al.,* 1997; Temesgen, 2005; Welde-Michael *et al*., 2008). The crop tolerates many biotic and abiotic stresses and considered as one of the economically viable and sustainable agricultural systems in the country (Tsegaye and Struik, 2002).

Although the crop is tolerant to different diseases and pests, reports also showed that the crop is repeatedly affected by bacterial wilt disease caused by *Xanthomonas campestris* pv. *musacerum (*Dereje Ashagari, 1985; Admasu Tsegaye and Struik, 2001). The disease is also a major problem in banana farming system in East and Central Africa (Adriko *et al*., 2012). To our knowledge today, no scientifically based method for the control of the disease has been developed. Currently research efforts aimed at developing scientific control methods is underway in different laboratories. These efforts include selection of tolerant variety (ies) through breeding, transformation of enset using disease resistant genes from other plants, and looking for natural enemies that antagonize the pathogen and serve as biocontrol agents.

Dispite the crop's desirable agronomic properties to tolerate biotic and abiotic stresses, very limited studies were conducted on the role of beneficial microbes to promot plant health and and control the wilt disease caused by *Xanthomonas campastris* pv. *musacerum.* This study, enset endophytes were studied for different plant beneficial properties. Given the properties of endophtes in colonizing internal plant parts, sharing similar nich with pathogens and PGP and disease suppression; they might be useful in integrated disease management strategies.

Objectives

The overall aim of this study was to characterize culturable bacterial and fungal endophytes from enset plant and investigate their potential for disease suppression and plant growth promotion.

The specific objectives were to:

1. Isolation and molecular identification of bacterial and fungal endophytes of enset.
2. Test the *in vitro* antagonistic property and the *in vivo* disease suppression properties of endophytes on *Xanthomonas campestirs* pv. *musacerum*.
3. *In vitro* characterization of selected endophytes for growth on nitrogen free medium (NFM), indole-3-acetic acid (IAA) production, siderophore production, phosphate solublization and cellulose and pectinase production.
4. To study the inhibitory effect of nonvolatile diffusible organic compounds (NVDOCs) and volatile organic compounds (VOC) produced by selected endopytes o*n* *Xanthomonas campestris* pv. *musacerum.*

1.2. Literature review

1.2.1. Endophytes as plant associated organisms

Several studies revealed the presence of a plethora of microbes and their symbiotic interactions with plants (Cankar and Kraigher , 2005; Aliferis *et al*., 2013; Brader *et al.,* 2014). In these symbiotic interactions, the microbes gain nutrition and protection from harsh environmental conditions while the plant gets protection from a number of biotic and abiotic stresses (Rosenblueth and Martínez-Romero, 2006, Rodriguez et al., 2009). In 1866, De Barry for the first time use the term endophyte to describe fungi that live in the tissues of symptom free plants. Since then, the definition has been modified to better explain these organisms in terms of location and the type of plant microbe interaction (Wilson, 1995, Mano and Morisaki, 2008).

The present understanding is that groups of fungi and bacteria that colonize internal parts of plants without causing apparent disease symptom are considered as endophytes (Hallmann *et al*., 1997; Khan and Doty, 2009; Lahlali & Hijri, 2010; Taghavi *et al*., 2010). However, it has been noted that some endophytes can also adapt dual type of life style interaction, like endophytic epiphytic or endophytic rhizospheric at least for parts of their life cycle (Hardoim *et al*., 2008; Whipps *et al*., 2008; Mano and Morisaki, 2008; Hunter *et al*., 2010). In addition, some endophytes can change to pathogenic lifestyle on a different plant species and a different set of environmental conditions (Saikkone et *al*., 1998; Faeth and Fagan, 2002; Kogel et *al*., 2006; Rakotoniriana *et al*., 2008; Shittu *et al*., 2009; Wang *et al*., 2009).

The endophytic infection of plants can be through transmission via seeds, germinating radicles, vegetative planting material, roots, stomata, flowers, or through plant wounds (Zinniel *et al*., 2002; Rosenblueth *et al*., 2006; Lacava and Azevedo, 2013). In addition, infection pattern by endophytes can range from highly localized colonization of a specific

tissue to a widely distributed colonization of endophytes (Saikkone *et al*., 1998). Observations made on different grasses, medicinal plants, fruit crops and other plants showed that the endophyte-plant interaction is mediated by many chemical signals and metabolites originating from both the plant and the microbe (Saikkone *et al*., 1998; Mano *et al*., 2006; Kharwar *et al*., 2009; Kelemu *et al*., 2011).

So far, a wide range of bacterial taxa was identified as endophytes in different plant species grown in different environments. Both bacterial and fungal endophytes were detected in almost all types of plants studied including grasses, medicinal plants, crops, rubber tree, and other plants (Abraham *et al*., 2013; Saikkone et *al*., 1998; Faeth and Fagan, 2002; Tian *et al*., 2004; Amirita *et al*., 2012; Gazis and Chaverri, 2010). Several of them seem to show some degree of preference for a certain plant species but many didn't show specificity in host plant (Cohen, 2006; Weilharter *et al*., 2011; Giri and Dudeja, 2013). Moreover, the taxonomic groups identified as endophytes are common soil microbes (Hallmann and Berg, 2006).

1.2.2. Plant colonization property

Unlike pathogenic microorganisms, healthy plant colonization is the distinctive characteristics of beneficial endophytic microorganisms. Thus, research on the ability of endophytes to colonize plants regarding is becoming an important aspect to understand endophytic organisms. Although the knowledge of bacterial traits that determine internal plant colonization is limited (Mittler *et al*., 2013), some of the factors that influence the interaction are, rhizosphere bacteria, chemotactic response of bacteria in the rhizosphere, bacterial lipopolysaccharides, high bacterial growth rate, and ability to synthesize vitamin B1 and exude NADH dehydrogenases, having type IV bacteria pili, flagella and ability to

produce plant polymer degrading enzymes (Compant *et al*., 2005; Ryan et al 2007; Hardoim *et al*., 2008; Reinhold-Hurek and Hurek, 2011; Weilharter *et al*., 2011; Wang *et al.,* 2015).

Artificial inoculation and tracking of endophytes in different plant species showed variability in the extent of spread and occurrence of endophytic microbes in different plant species and plant tissues. Some can spread through the whole plant and others might have localized association (Zinniel *et al*., 2002). For example, varibality was observed in rice where, colonization was more formed by endopytic *Pantoea* sp. than endophytic *Ochrobactrum* sp. (Rosenblueth and Martínez-Romero, 2006). Although, endopytes are detected from all above ground and below ground tissues, the root is the most colonized plant part (Rosenblueth and Martínez-Romero, 2006). Studies correlate this high colonization in the below ground plant part with the high plant microbe interaction and higher nutrient availability in the rhizosphere environment.

In many cases, biocontrol properties of endophytes have has been shown to suppress pathogens of higher plants. This correlation between colonization and pathogen suppression attracted a lot of interest in studying plant colonization pattern of a potential biocontrol and plant growth promoting endophytes (Chi *et al*., 2005; Compant *et al*., 2005; Weilharter *et al*., 2011).

1.2.3. Methods of endophyte detection and identification

Both culture dependent and culture independent methods are employed to study endopytes associated with a certain host plant. In the culture dependent analysis of endophytic organisms, a range of methods from morphological to biochemical identification and functional study of bacterial and fungal endophytes is available (Bayman, 2007). Culture

dependent method of isolation of endophytes combined with and 16S rDNA based identification of bacteria is widely used in culturable endopytic community studies. For fungal endopytic community study, ITS and 18S rRNA sequenceing are commonly employed (Reiter, 2002; Mano *et al*., 2006; Arnold *et al*., 2007; Bayman, 2007; Weyens *et al*., 2012; Li *et al*., 2010). Moreover, endophytic community structure and functional study based on 16S rRNA terminal restriction fragment length polymorphism and clone liberaries, pyrosequencing of ITS genes and analyses of protein-encoding gene fragments are also very useful (Rasche *et al*., 2006; Sessitsch *et al*., 2012 Peršoh, 2013).

Whole genome sequencing of endophytic isolates is also becoming popular. This method is used to get a detailed understanding of plant beneficial characteristics and prediction of specific genes that are potentially involved in adapting endophytic lifestyle (Weilharter et al., 2011; Martínez-García et al., 2015; Wang *et al*., 2015).

Although not effective in identification of endophytes, microscopic visualization is used for *in situ* observation of endophytic microbes. Its use is limited mainly for identification of hyphal structures of fungi for lack of spore-forming structures inside the plant (Sun and Guo, 2012). Application of fluorescent proteins in microscopy to study the plant colonization pattern, disease control and plant growth promotion by endophytes is another method widely applied in such studies (Ryan *et al*., 2007). This method is only used for endophytic bacteria and fungi which are fluorescently tagged before plant inoculation and can be visualized in the intercellular, intracellular and vascular tissues and within leaves (Chi *et al*., 2005; Weyens *et al.,* 2012).

Moreover, successful detection of introduced bacteria and fungi was made possible by labelling of the endophytes before inoculating to different plant species and microscopic visualization (Rosenblueth *and* Martínez-Romero, 2006). Labelling and visualization of endophytes need cloning of flourcent proteins such as green flourcent protein (GFP) b-glucuronidase (GUS) using appropriate types of cloning vectors (Compant *et al*., 2005b, Ryan *et al*., 2007).

Different types cloning vectors are designed to carry out cloning and transformation of green fluorescent protein in different cell types. Broad host range plasmids like a set of pBBR1and its modified forms are used by many colonization studies (Miller *et al*., 2000). Successful tagging tracking of gram positive and gram negative endophytic bacteria in maize, bean, and rice was reported (Verma *et al*., 2004; Chi et al., 2005; Ji *et al*., 2008; Rosenblueth and Martínez-Romero, 2006; Hsieh *et al*., 2005). Developing antibiotic resistant derivative isolates of a target endophyte is another method of measuring the colonization ability of endophytes in artificial inoculation (Andreote *et al*., 2009).

1.2.4. Plant growth promoting endophytes

The lack of sufficient nutrients in many agricultural soils on one hand and ecological and economic concerns over the impact of increased use of chemical fertilizers and pesticide chemicals on the other hand have necessitated the need for better alternatives to increase crop productivity (Mittler *et al*., 2013, Marella, 2014). The use of endphytes, which are plant associated, growth promoting bacteria and fungi (Glick, 2012; Compant *et al*., 2005a, b; Ryan *et al*., 2007), is one area under consideration for increased crop productivity.

Endophytes can increase plant growth with direct and indirect mechanisms. Direct mechanisms include production of phytohoromons such as auxin or cytokinin and lowering of plant ethylene levels, increasing nutrient availability and iron acquisition, or by inducing systemic resistance (Algam *et al*., 2005; Long *et al*., 2008; Badri *et al*., 2009). On the other hand the indirect mechanisms include protection from pest and pathogen attacks (Glick, 2012).

From *in vitro* and *in vivo* phenotypic studies, it was shown that many endophytes have one or more of the following properties. These include plant growth promotion such as nitrogen fixation, phosphate solublization, siderophore production, plant hormone production plant pathogen and pest suppression, or many others (Compant *et al*., 2005a, b; Ryan *et al*., 2007). Despite its abundance in the atmosphere, the available form of nitrogen is a limiting plant nutrient in many soils. Nitrogen fixing endophytes that belong to *Acetobacter, Herbaspirillum, Pseudomonas, Burkholderia* were detected from different plants (Kirchhof *et al*., 1997).

Next from nitrogen, phosphorus is another important element that limits plant growth (Jasim *et al*., 2013; Ji *et al*., 2014). Many microorganisms can solubilise phosphate by releasing organic acids that chelate the phosphate bonded cation and convert the phosphate to soluble forms (Keneni et *al*., 2010; Lacava and Azevedo, 2013).

Another element required for normal plant growth is iron. To acquire sufficient iron from the environment, plant associated microorganisms produce siderophores, small molecular weight compounds involved in chelating iron and transport it into the cell (Kajula *et al*., 2010). In addition to the growth promoting effect, siderophore producing microbes are considered as disease suppressing agents. This is due to the effect of making the iron unavailable for pathogen normal growth.

Plant growth promotion through production of phytohormons is another plant growth promoting property detected in many endopytes. Approximately 80% of bacteria associated with plants produce auxin phytohormone IAA (Glick, 2012; Lacava and Azevedo, 2013). Microbial production of auxins is known to trigger an increase in cell elongation, cell division, and differentiation in various plants. Besides its effect on growth, IAA production has been shown to increase tolerance to different abiotic stresses (Khan and Doty, 2009).

The above and many other mechanisms of plant growth promotions are examples of the beneficial roles of endophytes for plant growth. Moreover studies supplemented by genome sequencing of endophytic bacteria and fungi showed the presence of several new gene clusters that might be involved in direct and indirect plant growth promotion. Genome studies of genera *Pseudomonas*, *Burkholderia* and *Enterobacter* can be mentioned as an example in this regard (Taghavi *et al*., 2010; Weilharter *et al*., 2011; Kwak *et al*., 2012; Wang *et al*., 2015). Furthermore functional metagenomic studies of endophytes of rice roots also showed that, apart from plant growth promotion and plant growth stress resistance, there are also protein domains that are involved in nitrogen fixation, denitrification and nitrification (Sessitsch *et al*., 2012).

1.2.5. Endophytes as biological control agents

Plants interact with both deleterious and beneficial microbes in the below and above ground environment. Among the beneficial microbes, disease and pest suppressive bacteria and fungi were detected in the rhizosphere, phyllosphere, and endosphere of plant environment (Weller, 1998; Baker, 1968; Yu *et al*., 2010; Raaijmakers and Mazzola, 2012; Humphrey *et al*., 2014).

Compared with the endosphere and phyllosphere, the rhizosphere is nutrient, competitor and predator rich environment. As a result, microflora in the rhizosphere are considered to play a role in disease suppression properties that help them to adapt harsh environmental conditions (Gunatilaka, 2006; Raaijmakers & Mazzola, 2012). On the other hand the endosphere is less competitive and less nutrient rich environment. However, microbes in the endosphere might be equipped with specific metabolic potential needed to interact, colonize and adapt in the internal plant environment (Yu *et al*., 2010, Brader *et al*., 2014).

The widely recognized mechanisms of plant protection by beneficial bacteria and fungi are competition for an ecological niche or a substrate, production of inhibitory allelochemicals, and induction of systemic resistance (ISR) in host plants (Compant *et al*., 2005). Several studies were focused on screening of a potential biocontrol agent against bacterial and fungal phytopathogens. However, the screening of an efficient antagonist is one of the several steps in development of a biocontrol agent that can be effective in the field. Moreover, an effective disease control strategy requires the consideration of cropping system; disease epidemiology; the biology, ecology, and population dynamics of biocontrol organisms; and the interactions among these variables (Lo, 1998).

Antimicrobial secondary metabolites produced both by the plant and the microorganisms is one of the mechanisms of pathogen suppression (Demain, 1998; Berdy, 2005; Badri *et al*., 2009; Raaijmakers *et al*., 2009; Junker and Tholl, 2013). As part of plant disease control mechanisms, different antimicrobials produced by plant associated bacteria and fungi were identified (Yu *et al*., 2010; Ju et *al*., 2013). Bacterial endophytes have been exploited more extensively than fungal endophytes for control of plant diseases (Azevedo *et al*., 2000).

Plant disease suppression properties are more frequently expressed in the bacterial taxa group *Pseudomonas*, *Burkholderia, Bacillus, Streptomyces and fungal taxa Trichoderma, Gliocladium* and nonpathogenic *Fusarium oxysporum*. This might show the metabolic potential of these groups to produce diverse secondary metabolites that can be used to target diverse pathogenic agents (Berdy, 2005; Brader *et al*., 2014; Weller, 2007; Raaijmakers *et al*., 2009). A certain organism can produce a compound with antimicrobial activity for a purpose of antagonism or other functions (Yu *et al*., 2010; Demain & Fang, 2000). The actual action of a certain antibiotic can be confirmed by comparing with antibiotic-deficient mutants (Weller, 1998).

Recent genome wide studies of different plant associated microbes indicate the presence of more potential to discover new active metabolites with new mechanism of actions (Brader *et al*., 2014; Raaijmakers & Mazzola, 2012; Wang *et al*., 2015). Studies with high throughput genetic based techniques of natural compounds have shown the possibility of description of over one million products out of which 50, 000 are microbial origin. Moreover, more than 22, 000 have showed a sign of bioactivity. Among these 17% are metabolites produced by bacteria mainly *Bacillus* and *Pseudomonas* spp.; 45% from actinomycetes and about 38% are of fungal origin (Solecka *et al*., 2012; Brader *et al*., 2014, & Berdy, 2005).

Several studies showed endophytic microbes produce a number of secondary metabolites that inhibit different human and plant pathogens (Gunatilaka, 2006; Raaijmakers & Mazzola, 2012; Saraf *et al*., 2014). These endophytes are found in crops, medicinal plants, plants in special environments and other types of plants (Strobel &Daisy, 2003; Kharwar *et al*., 2009; Yu *et al*., 2010) of which more emphasis was given on medicinal and crop plants (Zhang *et al*., 2006; Yu *et al*., 2010; Brader *et al*., 2014).

1.2.6. Antimicrobial microbial metabolites

Antibiotics are a chemically heterogeneous group of organic, low-molecular weight compounds produced by microorganisms that kill or inhibit the growth or metabolic activities of other microorganisms (Thomashow *et al.*, 1997). The major modes of action of such compounds involve interference in cell wall, DNA, RNA, or protein synthesis in the target microbe (Raaijmakers & Mazzola, 2012). After the discovery of diverse classes of antimicrobial compounds, the discovery of novel metabolite has declined in the last two decades (Brader *et al*., 2014; Raaijmakers & Mazzola, 2012).

Common chemical structures like cyclopeptide/cyclopeptolide, depsipeptide, various macrocyclic lactone/lactam ring systems are detected in many antibiotics (Demain & Fang, 2000; Yu *et al*., 2010). Diverse peptide type compounds account the largest group of antibiotics. This include from the simple amino acid derivatives b -lactams, to the high molecular proteides, covering about 5000 compounds.

The second largest group is the diverse macrolactones, polylactones and ansalactones including simple lactones, macrolides, other macrocyclic lactones with 18 to 60 membered rings, and various acrolactones. Various quinone derivatives, simple or complicated sugar derivatives, *N*- and *O*-heterocycles, benzene, aromatic derivatives, various alicyclic and aliphatic compounds and various fatty acid derivatives are the common structures (Berdy, 2005; Zhang *et al*., 2006; Yu *et al*., 2010; Kanchiswamy *et al*., 2015). Some of these structures are unique in antibiotics isolated from microbes (Berdy, 2005).

The action of a certain antimicrobial could be specific to one type of pathogen or broad range. For example a phenolic compound, 4-(2, 4, 7-trioxa-bicyclo (4.1.0) heptan-3-yl) phenol isolated from an endophytic fungus was inhibitory against different bacteria and fungi (Subban

et al., 2012). In general different mechanisms of action are reported. Morphological alterations and cytoplasm agglutinations were demonstrated on *Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* by antibiotic (Subban *et al*., 2012). The inhibitory effect of Kakadumycin A is shown to be on RNA synthesis of different plant pathogens (Zhang *et al*., 2006). According to Zeriouh *et al*., 2011, lipo peptides, lturin A and bacillomycin were cytotoxic compounds on pathogens *Xanthomonas campestris* pv. *cucurbitae* and *Pectobacterium carotovorum* sub sp. *Carotovorum.* Disintegration of the plasma membrane lysis of the bacterial cell is the mechanism of action by the specific antimicrobial compound.

In terms of their form, inhibition of phytopathogen can occur through volatile or diffusible or combinations of the two forms of antimicrobial compounds. Volatile Organic Compounds (VOCs) are low-molecular weight (100–500 Daltons), high vapour pressure, low boiling point, and lipophilic compounds (Kanchiswamy *et al*., 2015; Schmidt *et al*., 2015). Plant associated bacteria and fungi produce diverse groups of VOCs which are involved in promoting plant growth, induce disease resistance, and inhibit plant pathogens. VOCs play a significant role in the communication between microorganisms and microbes with their plant host (Bailly and Weisskopf, 2012).

Although study on volatiles is at its early stage, some reports are available on their mechanisms of action. For example volatile released by *Bacillus subtilis* was reported to induce changes in gene expression and affect motility and biofilm formation of the exposed *Escherichia coli* (Kim *et al*., 2013). The other mechanism mentioned was change in membrane permeability and leakage of intercellular compounds which is caused by perturbation of the lipid fraction of the plasma membrane in *Escherichia coli* and

Staphylococcus aureus that were exposed to monoterpenes (Schmidt *et al*., 2015). Transcriptional microarray studies on *Bacillus subtilis* suggest the mode of action by VOCs from *Muscodor albus* is DNA damage of bacterial cells by a naphthalene derivatives (Mitchell *et al*., 2010).

1.3.7 Enset plant

Enset (*Ensete ventricosum* (Welw) Cheesman) is a perennial plant in the family *Musaceae* under the order *Schistaminae.* Different species of enset are widely distributed in different parts of the world as wild plants. However in Ethiopia, the plant was domesticated for more than 10,000 years and used as a staple or co-staple food for more than 15 million people (Brandt *et al*., 1997). The area of enset cultivation in Ethiopia is estimated to be over 263,000.00 ha or land, centeral statistics authority (CSA), 2001/2002).

Enset grows in altitudes between 1,600 to 3,100 m.a.s.l, average annual rainfall of 1,100 to 1,500 mm, a mean temperature of 8-27^{0}C and a relative humidity of 63-80% (Taye and Asrat, 1966; Asnaketch Woldetensaye 1997; Admasu Tsegaye, 2002). The ideal soils for enset cultivation are moderately acidic to alkaline (pH of 5.6 to 7.3). The crop is propagated vegetatively and harvested right before the onset of flowering.

Kocho, bulla and amicho are the main starchy foods from the underground corm and pseudostem. In addition to its food value, enset is important for its medicinal values due to its high calcium and iron content. Moreover its leaf and different parts are used as source of high quality fibre and animal forage. Although assessment of yield of enset is difficult due to its complicated processing procedures, it was reported that its yield is higher compared to other food crops (Admasu Tsegaye and Struik, 2001). Enset growing regions in Ethiopia rarely

face the problem of land degradation and starvation during the time of drought or rain shortage.

Although studies show enset is a crop with high agronomic quality and can tolerate different biotic and abiotic stresses, there are diseases and pests that attack the crop. Diseases such as root-knot lesions, nematode, black leaf streak, fungal leaf spot, bacterial wilt, bacterial leaf sheath rot and heart leaf rot are identified as production constraints of enset agriculture (Genet Birmeta, 2004; Temesgen Addis, 2005). Among these, bacterial wilt disease caused by *Xanthomonas campestris* pv. *musacerum* is the major challenge that devastated the crop on several occasions (Dereje, 1985; Welde-michael *et al*., 2008).

1.3.8 Bacterial wilt disease of Enset

Bacterial wilt disease of enset is a systemic disease caused by a bacterial pathogen *Xanthomonas campestris* pv. *musacerum*. The disease was first reported in Ethiopia in 1968 (Yirgou & Bradbury, 1968). In addition to enset the pathogen become a major threat to banana production in other African countries. The disease was first reported on banana farms in Uganda in 2001 (Tushemereirwe *et al*., 2004). Since then, farmers in East Africa experienced a total crop yield loss where the disease is established (Aritua *et al*., 2008; Adriko *et al*., 2012). The disease has also been reported from the northern Rwanda, the eastern Democratic Republic of Congo and Tanzania (Adriko *et al*., 2012).

The pathogen spreads through vascular system and an infected plant shows yellowing and wilting of infected leaves and finally dying of the whole plant (Yirgou & Bradbury, 1968, Dereje, 1985). The same pathogen manifests itself as premature fruit ripening and wilting of leaves and the dying of the whole plant in banana (Ssekiwoko et al. 2006). The pathogen mainly moves through the vascular system by passive movement and spreads in all parts of the plant. Studies on host range of the disease identified enset, banana and *Canna indica* as

the only hosts (Ssekiwoko et al. 2006). Contaminated farm tools, infected soil and plant debris, inset vectors, farm animals, rain water can transmit the disease.

Although different clones have different disease tolerance levels, all banana and enset clones are susceptible to the disease (Biruma *et al*., 2007; Weldemichael *et al*., 2008). The systemic nature of the disease is making it more difficult to control the disease as infected plants can only show the symptom after the bacteria spreads well and difficult to remove it from the infiected plant. So far, the sanitary control method such as use of disease free planting material, burning of contaminated farm tools, and burning and burying of diseased plants is the only control method applied.

1.3.9 **The pathogen *Xanthomonas campestris* pv. *musacerum*.**

The pathogen has yellow, convex, mucoid colonies on yeast extract sucrose or nutrient agar (Yirgou & Bradbury, 1968). It is a gram negative, rod, aerobic, motile with a single polar flagellum cellular structure. The pathogen produces Xanthan gum which is a polysaccharide that causes significant blockage of vessels in infected plant tissues (Biruma et al., 2007). High diversity of the pathogen was reported at strain level and different strains of the pathogen showed different level of virulence.

There are biochemical and phylogenetic methods that are proposed for identification of the bacterium (Yirgou & Bradbury, 1968; Adriko *et al*., 2012). However, the biochemical methods are less specific; need several confirmatory steps and consum time espically if screening of large samples is needed. On the other hand, the phylogenetic methods, using sequencing of the 16s rDNA and gyrase B gene are not giving sufficient variability to distinguish the pathogen from closely related species (Hauben *et al*., 1997; Aritua *et al*.,

2008). Similarly, *hrpB* operon of the *hrp* gene cluster is confirmed to lack specificity to amplify target regions from *Xanthomonas campestris* pathovars and strains (Ivey *et al*., 2010; Adikini *et al*., 2011; Adriko *et al*., 2012). Therefore, PCR methods tarteging specific gene sequences in the pathogen DNA are continuously developing.

Currently a specific primer to amplify a gene encoding the general secretion pathway protein D (GspD) is used for specific identification of the pathogen from other gram positive and negative bacteria (Adriko *et al*., 2012). The whole genome sequence of the pathogen is also available and might be helpful to increase our understanding of virulence mechanism and development of disease resistance by the host plant (Wasukira *et al*., 2012).

Chapter 2

Diversity of culturable bacterial and fungal endophytes of enset

Abstract

The aim of this study is to assess the occurrence and diversity of culturable endophytes in different parts of enset plants. Plant samples were collected from farmer's field's in eight sampling sites. A total of 446 bacterial and 105 fungi were isolated from a surface-sterilized plant samples and identified by sequencing 16S rRNA and ITS gene sequencing of bacteria and fungi respectively. The bacterial sequences were grouped in to 4 phyla and 53 genera. Proteobacteria were the most frequently isolated phylum from all the plant part followed by Actinobacteria, Firmicutes, and Bacteroidetes. The fungal sequences were grouped in to two phyla and 42 species. Ascomycota is the most frequently detected phylum and few isolates were identified in the phylum Zygomycota. Moreover, 16S rRNA and ITS gene sequence showed the similarity of enset endophytes with other endophytes detected from other plant species from a different geographic region. In relation to the distribution of endopytes in different plant parts, higher percentages of endopytes were detected in the belowground plant part than above ground plant part. In conclusion enset harbours diverse groups of endophytic microbes. Endophytes are commonly reported for their plant beneficial properties. Thus, the presence of diverse groups of endophytes in enset might indicate the beneficial plant microbe interaction existing in enset plant. The specific potentials that enset endophytes might confer are subject of the next chapters.

Key words: Endophyte, microbial diversity, endophyte host colonization, *Ensete ventricosum*

Introduction

Both bacteria and fungi are reported as endophytes from many plant species. They are detected from several plants using culture dependent and culture independent methods (Sessitsch *et al*., 2012). The diversity and abundance of endophytic bacteria and fungi in host plants depends on environmental factors, bacterial species, plant genotype, developmental stage of the plant, infection frequency, cultivation conditions, soil type, seasonal variations, and plant physiology such as plant hormone levels and type of root exodate(Rosenblueth and Martínez-Romero, 2006; Bayman, 2007; Taghavi *et al*., 2010).

From the above factors, the effect of root exudates were demonstrated in a study that detected distinct rhizosphere microbial communities of different plants that were grown on the same soil (Long *et al*., 2009). In another study, specific research on the effect of plant hormone level in relation to endophytic composition showed that elevated salicylic acid (SA), and jasmonic acid affected abundance and diversity of endophytes in *Arabidopsis thaliana* (Rosenblueth and Martínez-Romero, 2006). Moreover, levels of soluble carbohydrates, calcium, and phenolic compounds (which have long been associated with plant responses to biotic stress) were found to significantly influence bacterial community structure (Hunter *et al*., 2010). In addtion to natural factors, method of isolation, the disinfectant used during surface sterilization , and inoculum density could also vary the diversity and abundance of endophytes (Rosenblueth and Martínez-Romero, 2006; Hunter *et al*., 2010).

In many endophyte diversity studies, bacterial phyla that belong to *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* are predominantly detected in plant species such as potato, banana, sugarcane, bamboo and other plants (Sessitsch *et al*., 2002; Lian *et al*., 2008; Velázquez *et al* 2008 ; Han *et al*., 2009; Reinhold-Hurek and Hurek, 2011;

Sessitsch *et al*., 2012; Lacava and Azevedo, 2013). Moreover, endophytic bacteria studies showed that, members of the bacterial genera *Pseudomonas, Bacillus, Enterobacter, Burkholderia, Agrobacterium, Pantoea, Mythlobacterium, Rhizobium, Xanthomonas* are most commonly found as endophytes (Hallmann *et al*., 1997; Velázquez *et al.,* 2008; Mittler *et al*., 2013).

Fungal endophytes are the other groups of endophytes commonly detected in several plant species. These endopytes were isolated from roots, stems, leaves, bark and floral organs (Gazis and Chaverri, 2010; Jin *et al*., 2013). Fungal endophytes are also detected in all plant species studied so far and the diversity varies from plant to plant where woody plants, may contain very high number of species (Faeth and Fagan, 2002). According to Rodriguez *et al*. (2005), endophytic fungi can be classified in to two major classes based on their transmission to host plant species. In category one, a relatively small number of fastidious species that are detected in a few monocot hosts, and in the other category, a large number of species with broad host ranges, including both monocots and dicots. So far, very little is known about the later groups of endophytes. Fungal endophytes transmission can be vertically by hyphae that grow in to seeds or horizontally with host colonization by contact with the surrounding environment (Saikkone *et al*., 1998; Park *et al*., 2012).

So far, endophytic fungi that belong to diverse taxonomic groups were identified (Wang *et al*, 2009). They are Ascomycota, Basidiomycota, Zygomycota Oomycota, Deuteromycota of which Ascomycota are the dominant groups (Saikkone et *al*., 1998; Wang *et al*.2009; Gazis and Chaverri, 2010; Sun and Guo, 2012). With the predominant genera of *Fusarium Phoma*, *Alternaria, Phomopsis* sp. (Tian, *et al*., 2004, Whipps *et al*., 2008; Park *et al*., 2012; Scholtysik *et al*., 2013; Jin *et al*., 2013).

Although diverse groups of endophytes were reported, studies on endophytic plant association for many crop and wild plants in different ecological conditions are scarce. Therefore, understanding the diversity of endophytes associated with different plants might lead to identification of novel isolates which might have special ecological adaptations and agricultural applications.

Enset can be cultivated in a wide range of environmental conditions and is known for its tolerance to different harsh environmental conditions. Although several studies were undertaken on its important agronomic qualities, studies on the the occurrence of benifical microbs like endophytes is very limited. Understanding the diversity of enset associated endophytes could initiate study on the potential microorganisms that can improve and growth and health against bacterial wilt disease.

Material and method

Sample site and sample collection

Enset samples were collected at farmer’s fields from eight different locations in Southern Nations, Nationalites and Peoples’ regional state (SNNPRS) (Fig 2.1). Sampling was carried out during the dry season from November 2011-January, 2012. Although farmers at each sampling site give different vernacular names to enset landrace, there is no compiled data showing if these landraces are genetically distinct. The vernacular names of the samples is listed in Appendix-I. Therefore, in this study endophytic association was analyzed on the basis of plant parts and sampling sites. Elevation of the sampling location and its coordinates were indicated using GRAMIN GPS-60 instrument.

Table 2.1 Plant sampling sites with corresponding geographic coordinates and altitudes

Site name	Altitude m.a.s.l	Geographic Coordinate
Atat	1,943	N 08^{0} 10’ 47.1’’ E 037^{0} 47’ 18.6’’
Alechowerero	3,200	N 08^{0} 00'1. 3" E 038^{0} 13' 11.1"
Howeleso	1,841	N 06^{0} 52’ 25.9’’ E 038^{0} 27’, 13. 4”
Limu	2,320	N 07^{0} 34’ 03.2’’ E 037^{0} 52’, 32.7’’
Telo	2,049	N 07^{0} 07’ 35.5’’ E 036^{0} 21’8’’
Marka	2,384	N 07^{0} 03’48’’ E 037^{0} 9’ 29.6’’
Chencha	2,775	N 06^{0} 15’ 41.5’’ E 037^{0} 33’ 53.5’’
Gedeb	2,435	N 05^{0} 57’ 49.7’’ E 038^{0} 15’, 37.1’’

At each sampling site four healthy and mature enset plants of 3 to 4 years old were selected to collect root, leaf, and pseudostem samples. The oldest leaf and pseudostem samples were free of any physical damage were cut using a sterile knife wheras approximately 50 to 70 cm

piece was cut from undamaged root samples. All samples were placed in an icebox and transported to the laboratory and kept at 4^0C for further processing.

Sample treatment and isolation

Sample surface sterilization and bacterial endophyte isolation were carried out by modified a modified protocol by Sun *et al*, (2008). Approxmitely 200 mm root and 200x200 mm leaf samples were washed with water and the leaf and pseudostem samples were socked in 75% ethanol for 1 minute, then in 2.5% sodium hypochlorite for 3 minute and, in 96% ethanol for half a minute. Whereas, root samples were first treated with 75% ethanol for 3 minutes, followed by in 2.5% hypochlorite solution 10 miutes and in 96% ethanol for 2 minutes. After these treatments samples were rinsed with 500 ml sterile distilled water three times. After surface sterilization samples were trimmed at the edges to remove tissue that might have absorbed residue of the disinfectants.

For bacterial isolation, the rest of the samples were homogenised using mortar and pestle in 5ml 0.85% NaCl. The homogenate was serially diluted using 0.85% NaCl in the range of 10^{-1} to 10^{-7}. The solution was vigorously mixed using a vortex mixer from which 100 µl of the solution was spread plated in duplicate on nutrient agar plates and incubated at 28^0C for five days. Control plates were prepared by spreading the final rinse water.

Distinct colonies were picked, purified through repeated streaking, and stored at -80^0C. For fungal isolation, three small pieces of each sample of leaf, pseudo stem and root were cut and stacked on potato dextrose agar media (Oxoid) and incubated at 28^0C for 8 days. Fungal colonies were purified and stored on PDA slants at 4^0C. Bacteria and fungal isolates were

desiginated with abbrevation EECC (Enset endophyte culture collection) followed by their specific identification numbers.

Bacterial DNA extraction

Genomic DNA was extracted by modifying freez thaw method described by Tsai and Betry, (1992) where DNA purification steps by gel filtration were ommited. One- two colonies of the bacteria were picked from an overnight culture of the bacteria and added into an eppendroff tube containing 50 µl of sterile 10 mM Tris 1mM EDTA buffer, pH 8.0. Then the whole mixture was incubated in boiling water for ten minutes and immediately transferred to -20^0C freezer for fifteen minutes. This procedure was repeated twice. The whole content was centrifuged at 2555xg for 5 min to settle the suspended cell material and the supernatant was transferred to sterile tubes. The concentration of DNA was adjusted to 30 ng/µl for subsequent PCR reaction.

PCR amplification and sequencing of 16s rRNA

Univirsal bacterial 16S rRNA primers published by Galkiewicz, and Kellogg, (2008) and Thermo scientific manfactures details with modified anneling temperature were used to conduct the PCR reaction. The mixture consisted of 1 µl of 30 ng template DNA, 1 µl 5 pmol/ µl from each forward 63f (59-CAGGCC TAA CAC ATG CAA GTC-39) and reverse H1542r (5'AAG GAG GTG ATC CAG CCG CA 3') universal 16S RNA bacterial primers, 0.2 µl of 1.25u Dream Taq DNA polymerase, 2.5 µl of 10x Dream Taq PCR buffer, 16.8 µl ddH2O. The PCR condition was as follows: 94^0C for 3 min, followed by 30 cycles at 94^0C for 1 min, 65^0C for 1 min, 72^0C for 2 min and final extension at 72^0C for 10 min.

PCR products were separated using 1% agarose gel run in 0.5X Tris Acetate - EDTA (TAE) electrophoresis buffer at 80 V for 40 min and stained with 10,000 X gel red. PCR amplicons were purified using Fermantas PCR product cleaning kit (Fermantas manf details) as described by the manufacturer. Partial sequencing of the PCR amplicons using the forward primer was conducted with the BigDye terminator cycle chemistry (Applied Biosystems, Foster City, CA, USA) and sequence analyzed using ABI 3130 Genetic Analyzer (Applied Biosystem) at the Sequencing and Genotyping unit at the BecA-ILRI Hub.

Fungal DNA extraction

Genomic DNA of fungal isolates was extracted using hexadecyl trimethyl ammonium bromide (CTAB) buffer. Fresh fungus cultures were freez dried using liquid nitrogen and fine powdered by shaking for 10min using GenoGrinder- 2000 at a speed of 500 strokes per minute. The samples were homoginized with CTAB extraction buffer (200mM Tris, Ph 7.5, 50mM EDTA, Ph 8.0, 2M NaCl; 2% CTAB; 1% beta-mercaptoethanol). The homogenate was incubated at 65^0C water bath for 30min with shaking, cooled for 10min and centrifuged at 3500rpm with eppendrof centrifuge 5427 for 10min.

The supernatant was transferred to new eppendrof tube and mixed with 500µl of chloroformisoamylalcohol (24:1) and centrifuged at 3500rpm for 10min. The upper aqueous layer was transferred to new tube and the chloroform-isoamylalcohol step was repeated. The upper aqueous layer was transferred in to fresh tube and gently mixed with 600µl of 100% cold isopropanol for about 5min. The whole content was centrifuged and the pellet was resuspended gently with 70% ethanol, centrifuged for 10min. The washing with ethanol and centrifugation steps was repeated to discard the supernatant.

The pellet was allowed to air dry until the ethanol was completely evaporated. On the dried pellet 100 µl of double distilled water was added and incubated at 45^0C water bath for 60min with gentle shaking. The pellet was then mixed with 5 µl of RNase-A and incubated at 37^0C water bath for 2 hours. The RNase was denatured by incubating it at 65^0C for 15mins to recover the genomic DNA of the fungus (Martini *et al*., 2009).

PCR amplification and sequencing of fungal ITS

For PCR amplification of the internal transcribed spacer region of the fungal DNA was undertaken using fungal universal primers and by reducing the time in each PCR step and increasing the number of cycles (Neubert *et al*., 2006). The forward primer ITS1f (5'-CTTGGTCATTTAGAGGAAGTAA-3') and reverse primer ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') were used. The PCR mix constitutes, 2.5 µl 10xbuffer, 0.5 µl of 10mM dNTP, 1 µl of 10pm ITS1f and of 10pm ITS4, 0.2 µl Taq polomarase, 18.8 µl water and 1 µl of 50ng template DNA. The PCR condition was; 94^0C for 5 min, followed by 40 cycles at 94^0C for 20sec, 55^0C for 20sec, 72^0C for 1 min and final extension was done at 72^0C for 10 min (Neubert *et al*., 2006).

PCR products were separated by electrophoresis using 1.8% agarose gel run in 0.5X Tris Acetate - EDTA (TAE) electrophoresis buffer at 80 V for 40 min and stained with 10,000 X gel red. PCR amplicons were purified using Fermantas PCR product cleaning kit (manf details) as described by the manufacturer (Fermantas). Sequencing of the PCR amplicons using the forward and reverse primers was conducted with the BigDye terminator cycle chemistry (Applied Biosystems, Foster City, CA, USA) and sequencing was done with ABI 3130 Genetic Analyzer (Applied Biosystem) at the Sequencing and Genotyping unit at the BecA-ILRI Hub.

Data analysis and sequence accession numbers

Partial 16s rRNA sequences were edited with MEGA 5.05. Identification of the sequences was done using BLAST local alignment search tool. Dendrogram for depicting the diversity of bacterial isolates was generated with MEGA 5.05 software. The 16S rDNA sequences generated in this study were deposited in NCBI database under accession numbers, JX867279-JX867329 and JX908910-JX909265.

Fungal ITS sequences were edited and assembled using CLC main work bench 6.6.2 version. Sequence similarity was checked on BLAST local alignment search tool NCBI. Dendrogram for depicting diversity of fungal isolates was generated with MEGA 5.05 software. The ITS sequences for fungal isolates were deposited in NCBI under accession numbers, KP942851-KP942954. Heat map showing the relative abundance of bacterial and fungal genera was generated on an online tool CIMminer. http://discover.nci.nih.gov/cimminer/

Results

Isolation and identification of bacterial and fungal isolates

In this study, a total of 446 bacterial isolates were identified and grouped in to 53 genera (Table 2.1). Some of the preserved cultures were not identified because of the bias caused during PCR amplification and sequencing of the 16S rRNA. Likewise a total of 105 fungal isolates were identified using ITS sequence similarly of which a few original fungal isolates were not amplified during in the PCR and others didn't gave a clean sequences for identification (Table 2.1).

Table 2.2 Enset sampling site vs number of endophytes isolated

Site	Altitude	Total no of fungal isolates	Total no of bacterial Isolates
Atat	1,943	11	52
Alechowerero	3,200	10	62
Howeleso	1,841	10	65
Limu	2,320	9	50
Telo	2,049	23	56
Marka	2,384	11	53
Chencha	2,775	14	46
Gedeb	2,435	17	62
Total		105	446

Diversity and distribution of bacterial endophytes

The overall diversity of bacterial endophytes isolated in this study is depicted on a dendrogram generated using the partial sequence of 16S rRNA gene (Fig. 2.1). The sequences were approximately 600 bp each and all sequences showed 99-100% similarity with the reference sequences in the NCBI database.

Table 2.3 Distribution of bacterial isolates in taxanomic groups and respective palnt parts

Phylum	Total % of the isolates	Genera	Total % of the isolates	Total % in plant parts		
				Leaf	Pseudostem	Root
Proteobacteria	88.1	*Pseudomonas*	30.0	9.0	6.1	15.0
		Variovorax	8.3	0.2	0.7	7.4
		Acinetobacter	6.7	2.5	2.0	2.2
		Xanthomonas	5.6	2.5	1.6	1.6
		Herbaspirillum	4.5	1.1	1.8	1.6
		Rhizobium	4.0	0.2	0.7	3.1
		Stenotrophomonas	3.4	0.4	0.4	2.5
		Enterobacter	3.1	0.7	0.7	1.8
		Pantoea	2.9	1.6	0.4	0.9
		Rahnella	2.5	0.7	0.9	0.9
		Erwinia	2.2	0.2	0.4	1.6
		Serratia	2.0	0.9	0.9	0.2
		Raoultella	1.3	0.7	0.2	0.4
		Agrobacterium	1.3	0.2	0.4	0.7
		Acidovorax	1.3	0.7	0.0	0.7
		Dyella	0.9	0.4	0.2	0.2
		Ensifer	0.7	0.0	0.0	0.7
		Sphingomonas	0.7	0.0	0.4	0.2
		Burkholderia	0.7	0.0	0.0	0.7
		Lysobacter	0.7	0.0	0.0	0.7
		Klebsiella	0.4	0.0	0.4	0.0
		Duganella	0.4	0.4	0.0	0.0
		Escherichia	0.4	0.2	0.2	0.0
		Ochrobactrum	0.4	0.0	0.0	0.4
		Delftia	0.2	0.0	0.0	0.2
		Achromobacter	0.2	0.0	0.0	0.2
		Divosa	0.2	0.0	0.0	0.2
		Bosea	0.2	0.0	0.0	0.2
		Mitsuaria	0.2	0.0	0.0	0.2
		Kluyvera	0.2	0.0	0.0	0.2
		Aurantimonas	0.2	0.0	0.2	0.0
		Acidisoma	0.2	0.0	0.2	0.0
		Novosphingobium	0.2	0.0	0.0	0.2
		Citrobacter	0.2	0.2	0.0	0.0
		Simplicispira	0.2	0.0	0.0	0.2
		Hafnia	0.2	0.0	0.0	0.2
		Caulobacter	0.2	0.0	0.0	0.2
		Paracoccus	0.2	0.0	0.0	0.2
		Ewingella	0.2	0.0	0.0	0.2
Firmicutes	5.2	*Staphylococcus*	2.0	1.1	0.0	0.9
		Exiguobacterium	0.2	0.0	0.0	0.2
		Bacillus	2.9	1.3	0.2	1.3
Actinobacteria	3.8	*Streptomyces*	0.4	0.2	0.0	0.2
		Arthrobacter	0.9	0.0	0.0	0.9
		Agrococcus	0.2	0.0	0.2	0.0
		Rathayibacter	0.2	0.0	0.2	0.0
		Plantibacter	0.2	0.0	0.0	0.2
		Curtobacterium	0.2	0.0	0.2	0.0
		Agromyces	0.2	0.0	0.0	0.2
		Microbacterium	1.3	0.2	0.0	1.1
Bacterioidetes	2.9	*Pedobacter*	1.1	0.0	0.0	1.1
		Chryseobacterium	0.7	0.0	0.0	0.7
		Flavobacterium	1.1	0.4	0.0	0.7
		TOTAL	100.0	26.2	20.0	53.8

The bacterial isolates were classified in to 53 bacterial genera which were clustered in four phyla. Proteobacteria account 88.11% of the total number of isolates followed by firmicutes, actinobacteria and bacterioidetes with 7.17, 3.81 and 2.91 percentages respectively (Table 2.3). Out of the 53 genera isolated from enset, 4 genera were the most abundant accounting for more than 50% of all the isolates. *Pseudomonas* accounted for 30% of the total number of the isolates and those belonging to the genera *Variovorax, Acinetobacter*, and *Xanthomonas*, were the next most abundant accounting for 8.3%, 6.7% and 5.6%, of the total isolates, respectively (Table 2.3).

Higher number of endophytes was detected from below ground plant part than from above ground plant part. Isolates from the root account 53.8% followed by 26.2% and 20% of the isolates from leaf and pseudostem respectively. Analysis of the distribution of bacterial genera in leaf, pseuostem and root samples showed 16 of genera (*Pseudomonas, Variovorax, Acinetobacter, Xanthomonas, Herbaspirillum, Rhizobium, Stenotrophomona, Enterobacter, Pantoea, Rahnella, Erwinia, Serratia, Raoultella Agrobacterium, Dyella and Bacillus)* were distributed in all the leaf, pseudostem and root samples while the rest of the genera were detected in one or two plant tissues (Fig 2.2, Table 2.3).

Twenty one out of the 53 genera were represented by only one isolate where *Achromobacter, Paracoccus, Bosea and Curtobacterium are* among them (Table 2.3). Overall, more diverse bacterial endophytes were isolated from the root than from the shoot parts of the plants while leaf and pseudostem contained equal number of genera but with difference in in relative abundance of each genus (Fig 2.2). Clustering of the plant parts showed pseudostem and leaf are more similar in terms of the type of bacterial taxa detected and the relative abundance of the isolates (Fig 2.2).

Figure 2.1 Dendrogram showing diversity of bacterial endophytes isolated from *Enset ventricosum.* 16S rRNA gene partial sequences were used for identification and diversity analysis. Diversity was computed using Neighbor-Joining method with the bootstrap test (2000 replicates) is shown next to the branches. The Diversity analysis was conducted in MEGA 5.05

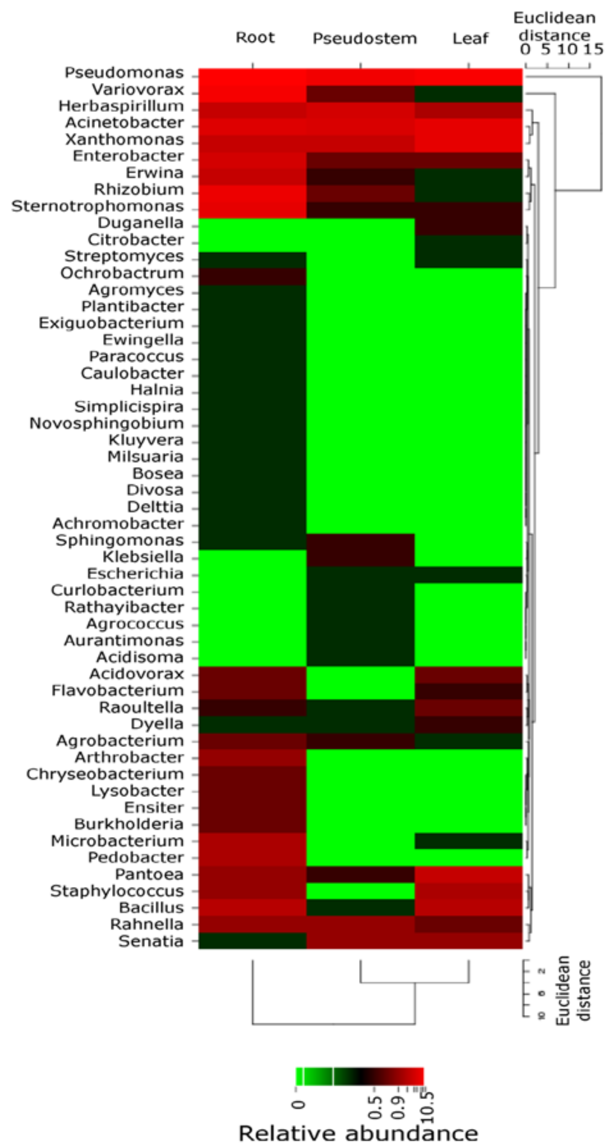


Figure 2.2 Heat map showing relative distribution and abundances of bacterial genera in leaf, pseudostem and root samples.

From each sampling site a total of 46 to 65 isolates belonging to 14 to 26 genera were detected (Table 2.2). Out of the total 53 bacterial genera identified, 21 were found to be unique to a specific site. Two bacterial genera, *Pseudomonas* and *Xanthomonas* which account 35.6% of the isolates were isolated from all the sampling sites (Fig 2.3).

Bacterial isolates in genera *Variovorax,, Acinetobacter, Herbaspirillum, Rhizobium, Stenotrophomonas, Enterobacter, Bacillus, Pantoea, Rahnella, Erwinia, Staphylococcus, Serratia, Raoultella, Agrobacterium, Acidovorax, Microbacterium, Flavobacterium, Pedobacter, Dyella, Arthrobacter Ensifer, Sphingomonas, Burkholderia, Chryseobacterium, Lysobacter, Klebsiella, Streptomyces, Duganella, Escherichia , Ochrobactrum* which account 59.64 % of the isolates were detected from more than one sampling sites.

On the other hand isolates in the genera, *Rathayibacter, Plantibacter, Bosea, Mitsuaria, Curtobacterium, Kluyvera, Aurantimonas, Acidisoma , Novosphingobium , Citrobacter , Exiguobacterium, Simplicispira ,Hafnia Agromyces, Caulobacte, Paracoccus, Ewingella, Delfti, Achromobacter, Divosa Agrococcus* that account 4.7 % of the isolates were detected in one sampling site only(Fig 2.3).

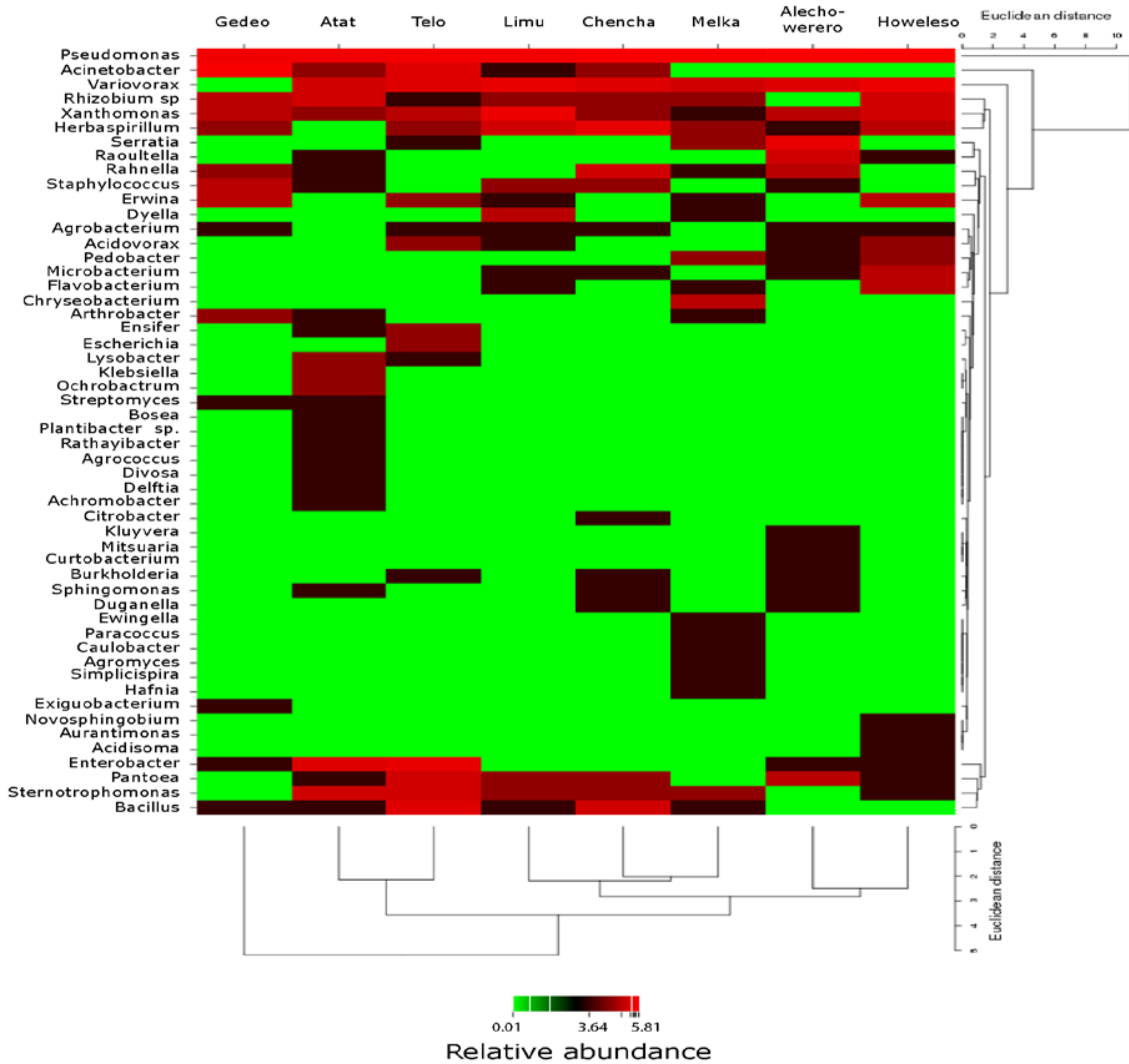


Figure 2.3 Heat map showing relative distribution and abundance of bacterial genera in the enset sampling sites.

Blast search for similar 16S rRNA sequence indicated some of the isolates from enset were highly similar with other endophytic bacterial reported from other crop and non crop plant species. Among these, *Rahnella aquatilis* from banana, *Herbaspirillum sp* from maize, *Pseudomonas sp*. were isolated from medicinal plants (Table 2.4).

Table 2.4 Bacterial endophyte 16S rRNA sequences that showed similarity with other endophytic bacterial sequences in the NCBI database.

Isolate code	Accession number	Reference organism	Percent similarity	Source plant of the reference organism
EECC-127	X908936	Pseudomonas sp. B45 [JQ390103]	100	Medicinal plants (Miller *et al*., 2012)
EECC-290	JX909073	Xanthomonas [FJ416608]	100	Rice (NCBI)
EECC-671	JX908955	Burkholderia sp. [JQ061271]	100	Sugar cane (NCBI)
EECC-698	JX908969	Herbaspirillum sp. EMA 117 [HQ872002]	99	Maize (Montanez et al., 2012)
EECC-699	JX908970	Kluyvera intermedia [JF938989]	100	Nepenthes sp (Bhore *et al*.,2013)
EECC-184	JX909017	Microbacterium foliorum [JQ660074]	99	Jatropha (NCBI)
EECC-191	JX909022	Pseudomonas oryzihabitans [JX067899]	99	Tobacco (NCBI)
EECC-192	JX909023	Pseudomonas sp. XjGEB-1 [JQ320089]	99	Gramineae (NCBI)
EECC-235	JX908993	Pantoea vagans strain Ka11 [JF460764]	99	Tobacco (NCBI)
EECC-258	JX909047	Dyella marensis [FN796824]	99	Cytisus striatus (Beccerra-Castro et al., 2011)
EECC-299	JX909078	Stenotrophomonas maltophilia [HQ224659]	100	Taxus globosa (NCBI)
EECC-304	JX909083	Bacillus simplex [JQ693815]	100	Lucerne (NCBI)
EECC-318	JX909096	Microbacterium phyllosphaerae [DQ365561]	100	Ginseng (Cho et al., 2007)
EECC-324	JX909100	Bacillus sp. [JQ821363]	100	Maize (NCBI)
EECC-327	JX909102	Bacillus aryabhattai [JQ659928]	100	Jatropha (NCBI)
EECC-401	JX909138	Erwinia billingiae [HQ224645]	100	Taxus globosa (NCBI)
EECC-402	JX909139	Acinetobacter johnsonii [GU188942]	100	Taxus globosa (NCBI)
EECC-403	JX909140	Rahnella sp. [JN887799]	100	Switchgrass (NCBI)
EECC-444	JX909166	Acinetobacter sp. [HQ413275]	100	Sugarcane (NCBI)
EECC-574	JX909230	Enterobacter cowanii [JN835540]	100	Lantana camara (NCBI)
EECC-576	JX909232	Caulobacter sp. [JQ659583]	100	Jatropha (NCBI)
EECC-602	JX909260	Pantoea rodasii [JQ236631]	99	Medicinal plants (NCBI)
EECC-491	JX909189	Variovorax sp. [GQ861460]	100	Alpine grass (NCBI)

Diversity and distribution of fungal endophytes

Based on the fungal internal transcribed spacer (ITS) sequence similarity 105 fungal isolates were classified in to two phyla and 42 species. The phylogenetic relationship of the representative isolates including reference sequences in the data base is depicted on Fig 2.4. The phylum Ascomycota comprise of many fungal representatives (97.14%) compared to the

three isolates representing the phylum Zygomycota. The group *Fusarium oxysporum was* relatively abundant endophytic fungus which accounted for 20.95% of the total number of fungal isolates. Other fungal species belonging to the groups *Plectosphaerella cucumerina*, *Epicoccum nigrum*, *Penicillium chrysogenum* were also relatively dominant with 12.38, 5.71, 5.71 percentages of the total number of isolates, respetively. Twenty four out of the 42 genera were represented by one isolate only.

Analysis on the basis of type of plant sample such as leaf, pseudostem and root, showed there are fungal taxa that were unique and common to the three plant parts. Higher diversity of fungi was found in root samples with a total of 23 fungi species. Countrary to our result, Ghimire *et al*. (2010) found significantly higher fungal diversity from the shoot tissue of switchgrass than root tissue. In their study, *Fusarium oxysporum* was the most frequently isolated with a total of 38.63% of the total number of the isolates. On the other hand, fungal species such as *Acremonium kiliense*, *Chaetomium globosum*, *Cladosporium tenuissimum* were represented by only one isolate and were found in root samples only.

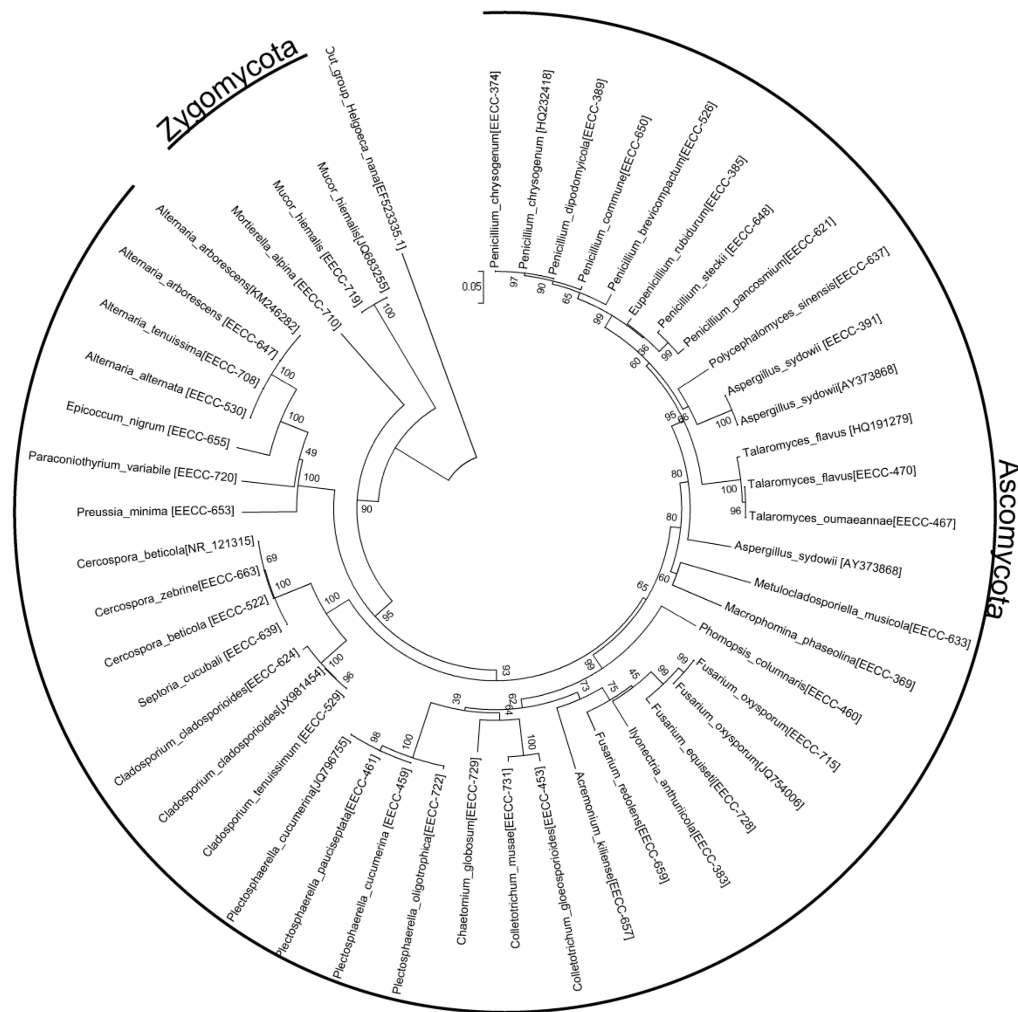


Figure 2.4 Dendrogram showing diversity of fungal endophytes isolated from *Enset ventricosum*. ITS gene sequences were used for identification and diversity analysis. Diversity was computed using Neighbor-Joining method with the bootstrap test (2000 replicates) is shown next to the branches. The evolutionary distances were computed using the p-distance method. Evolutionary analyses were conducted in MEGA 5.05

The leaf and pseudostem samples contained the same number of fungal taxa but they differ in the type of taxa observed. Fungal species such as *Alternaria alternate*, *Aspergillus versicolor*, *Colletotrichum musae* were found in the pseudostem while, *Alternaria tenuissima*, *Cercospora beticola*, *Colletotrichum gloeosporioides* were localized to leaf samples (Fig 2.5).

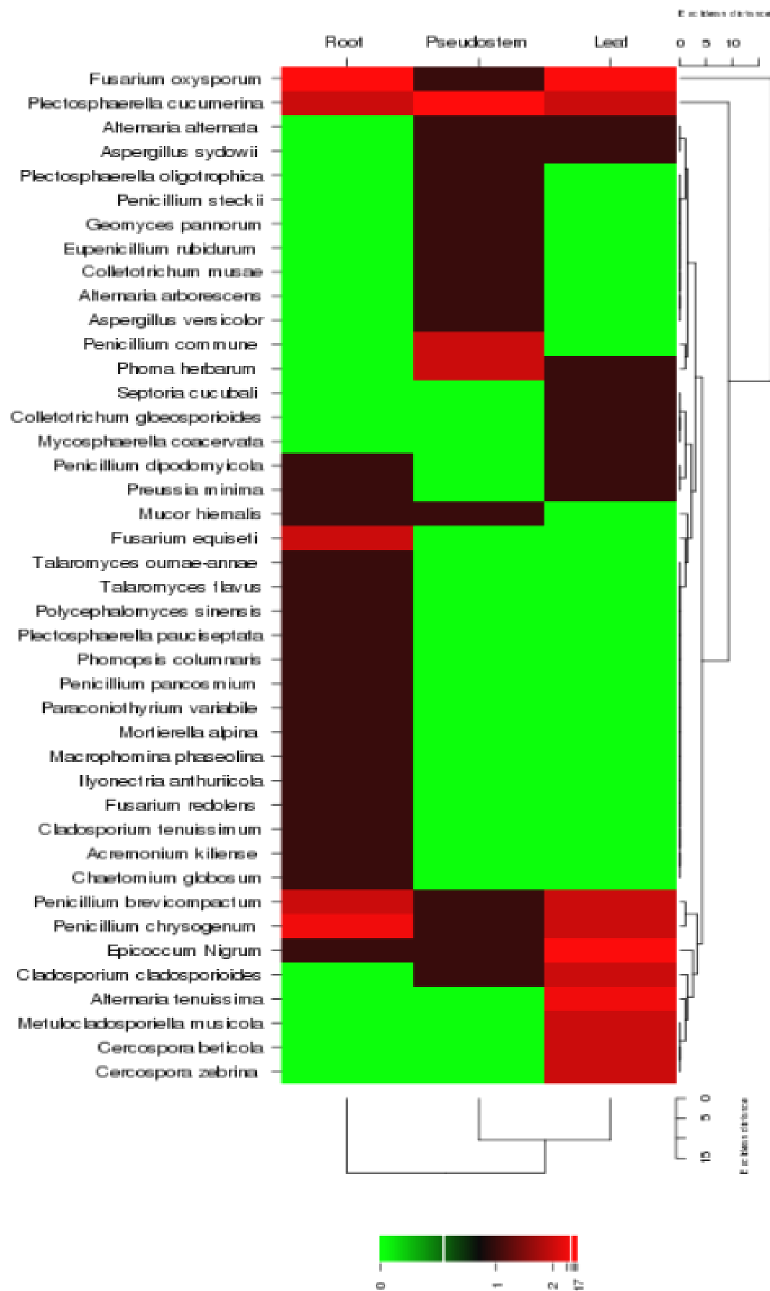


Figure 2.5 Heat map showing relative distribution and abundances of different fungal species in leaf, pseudostem and root samples.

Fungal diversity and abundance across sampling sites showed that *Fusarium oxysporum and Plectosphaerella cucumerina* were distributed across seven sites except that they were not detected at Chancha and Atat sites respectively (Fig 2.6).

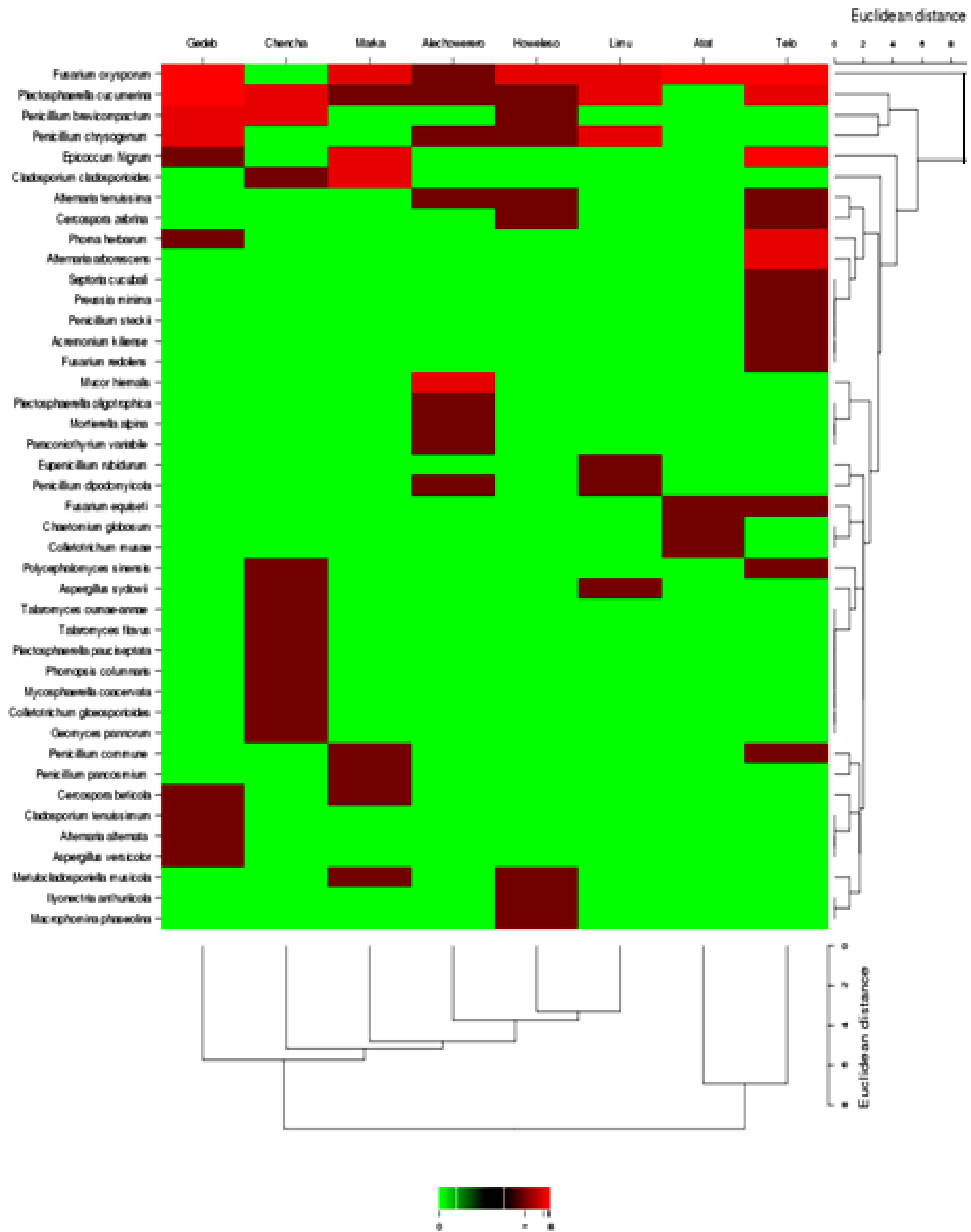


Figure 2.6 Heat map showing relative distribution and abundance of fungal species in the enset sampling sites.

Higher diversity was detected in Telo site with a total of 15 fungal species while Atat showed the lowest diversity represented by only four fungal species (Fig 2.6). On the other hand, cluster analysis

gave two major clusters of the sites interms of the type of taxa they share and Atat and Telo were two sites that share a fungal species which was not detected in other sites and formed one cluster while the other six sites formed another major cluster.

As it was observed for bacterial isolates, there were fungal isolates that showed 99 and 100% ITS sequence blast similarity with endophytes isolated from crop and none crop plants in different geography regions (Table 2.5).

Table 2.5 Fungal endophyte ITS sequences that showed similarity with other endophytic fungal sequences in the database.

Fungal Isolate code	Accession number	Reference organism	Similarity	Source plant of the reference organism
EECC-372	KP942913	Plectosphaerella cucumerina [KP068972]	99	Plants of semiarid sandy areas [NCBI]
EECC-379	KP942914	Fusarium oxysporum [JX500732]	99	Medicinal plants [NCBI]
EECC-530	KP942917	Alternaria alternata [KP127978]	100	Wild Echinacea purpurea [NCBI]
EECC-728	KP942954	Fusarium equiseti [JQ936262]	100	Soybean [NCBI]
EECC-523	KP942920	Fusarium oxysporum [JQ754006]	100	Common bean [NCBI]
EECC-636	KP942949	Alternaria arborescens[KM246282]	100	Agricultural crops [NCBI]
EECC-715	KP942933	Fusarium oxysporum[JQ754006]	100	Common bean [NCBI]
EECC-652	KP942879	Phoma herbarum [JQ936331]	100	Soybean [NCBI]
EECC-661	KP942865	Plectosphaerella cucumerina [KP068972]	98	Fruit trees [NCBI]
EECC-726	KP942921	Fusarium oxysporum[JQ754006]	99	Common bean [NCBI]
EECC-371	KP942852	Penicillium chrysogenum[HQ232418]	99	[NCBI]
EECC-462	KP942857	Cladosporium cladosporioides[JX981454]	99	Early diverged plant lineages [NCBI]
EECC-660	KP942861	Fusarium equiseti [KM246255]	99	Agricultural crops [NCBI]
EECC-637	KP942860	Polycephalomyces sinensis[EU272527]	99	[NCBI]
EECC-729	KP942862	Chaetomium globosum[KM268675]	99	Tobacco [NCBI]
EECC-532	KP942867	Aspergillus versicolor[KF381083]	99	Tea [NCBI]
EECC-534	KP942899	Phoma herbarum [JQ936331]	99	Soybean cultivars [NCBI]
EECC-628	KP942869	Epicoccum nigrum[JQ676202]	99	Medicinal plant [NCBI]
EECC-654	KP942870	Fusarium oxysporum[KF313101]	99	Pinus thunbergii [NCBI]
EECC-658	KP942878	Alternaria tenuissima[KJ082100]	100	Bupleurum scorzonerifolium Willd [NCBI]
EECC-655	KP942876	Epicoccum nigrum[KC867291]	99	Puccinellia distans [NCBI]
EECC-652	KP942879	Phoma herbarum [JQ936331]	99	Soybean [NCBI]
EECC-624	KP942880	Cladosporium cladosporioides[JX981454]	99	Early diverged plant lineages [NCBI]
EECC-664	KP942891	Epicoccum cf. Nigrum[JQ676202]	99	Medicinal plant [NCBI]
EECC-647	KP942903	Alternaria arborescens [KM246282]	99	Agricultural crops [NCBI]
EECC-708	KP942908	Alternaria tenuissima[KJ082100]	100	Bupleurum scorzonerifolium Willd [NCBI]
EECC-713	KP942909	Fusarium oxysporum [JN859433]	100	Plants of semiarid sandy areas (Knapp et al., 2012)
EECC-520	KP942937	Epicoccum nigrum[EU715663]	99	Mexican yew [NCBI]
EECC-629	KP942947	Cladosporium cladosporioides [KM246242]	100	Agricultural crops [NCBI]
EECC-529	KP942866	Cladosporium tenuissimum[JQ246357]	100	[NCBI]

Discussion

This study revealed the presence of diverse groups of bacteria and fungi that exist as endophytes in enset plant (Table 2.2). The eight sampling sites that are located in the Western and Eastern Highlands of Ethiopia and are separated by the East African Rift Valley with one sampling site found within the Rift Valley and are different in altitudinal range (3,200 m.a.s.l and the lowest at 1,841 m.a.s.l) were condidered. The difference in altitude is known to cause significant variation in the mean annual temperature, rainfall pattern, and other environmental factors that could determine the distribution of endophytes.

The factors that are considered to affect the establishment of endophytes in the host plant are duration of plant growth, method of propagation, environmental factors, and whether the plant is annual or perennial are important (Faeth and Fagan, 2002). Enset being a perennial plant it might have created ample time for different microbes from the soil and phylosphere to come to contact with the plant and colonize. Moreover, the crop is propagated through vegetative means. Therefore, compared to seed propagated crops more number of endophytes might be trensfered to the newely propagated plant.

Diverse groups of bacterial and fungal endophytes were detected from enset. Bacteria belonging to the phylum *Proteobacteria* were the most dominant across all sampling sites followed by *Firmicutes* and *Bacteroidetes* (Table 2.3). Previous studies using culture dependent and independent methods have also shown that phylum *Proteobacteria* account 87% of the proportion in the re-inoculation of naturally occurring endophytes and other studies also showed that the *genus Pseudomonas* were predominant in bacterial endophyte diversity studies in banana, potato, rockcress and pearl millet (Jie *et al.,* 2009; Gupta *et al*., 2013; Li, *et al*., 2010).

As it is indicated in the results, all the fungal isolates were grouped only in to two fungal phyla and *Ascomycota* comprise many fungal species isolated in this study. Although previous studies were more focused on mycorrhizas and root endophytes that belong to *Basidiomycetes*, many of studies also showed that *Ascomycota* represent a significant proportion of fungal endophytic diversity studies (Wang *et al*., 2009).

In study by Yuan *et al*, (2009) on medicinal orchid, *Ascomycota* were the dominant phylum and fungal groups such as *Xylariaceae Colletotrichum, Phomopsis and Fusarium* were more frequent. Similar results were also obtained on the total diversity of endophytic fungi from foliage and sapwood of *Hevea brasiliensis,* where *Ascomycota* comprises almost 97% of the isolates followed by *Basidiomycota* and *Zygomycota* in 1 and 2 percentages respectively and *Penicillium, Pestalotiopsis and Trichoderma* were the most frequently detected genera (Gazis and Chaverri, 2010).

The distribution of endophytes on the different parts of enset plants showed that bacterial and fungal endophytes isolated from the root were more diverse than those isolated from the shoot parts (Fig 2.2 and Fig 2.5). Moreover pseudostem and leaf cluster together in terms of the diversity of bacterial and fungal groups they contain and their relative abundance (Fig 2.2 and 2.5). Although rhizosphere community is not included in our study, similar results were obtained by Jin *et al*, (2014) where principal component analysis clustered rhizosphere and roots microbial communities together whereas leaf and stem endopytes formed another cluster.

The underground part of plants is in direct contact with diverse groups of microorganisms existing in the rhizosphere which is reported as the major route for plant microbe interaction

and endophytic colonization (Rosenblueth and Martínez-Romero, 2006). This phenomenon is explained by the microbes attracted by plant root exudates that serve as plant microbe communication media as well as nutrient source for many microbes (Rosenblueth and Martínez-Romero, 2006).

Variable endophytic colonization pattern were observed in different parts of enset plant when some common and some unique endophytic bacteria and fungal groups were detected from leaf, pseudostem and root samples. *Pseudomonas* was distributed equally in the above and belowground parts. On the other hand the genus *Variovorax* was found predominantly in the roots while the genera *Acinetobacter, Xanthomonas,* and *Herbaspirillum* were predominantly found in the leaf and pseudostem. Such variable pattern of colonization ranging from tissue specific to bacterial endophytes that can be detected from any parts of the host plant was also observed in stem and roots of potato plants and higher endophytic fungi detected from leaves than steam and root of medicinal orchid (Sessitsch *et al*., 2002; Yuan *et al*., 2009).

Studies showed that, the variability in plant colonization ability of the endophytes could be determined by the original plant part the endophyte comes in contact with, the metabolic property of the endophyte and the morphological and chemical nature of different plant tissues. Difference in the availability of nutrients in the immediate environment that are required by a certain group of microbes for survival or symbiotic interaction with the host plant might also determine the distribution of endophytes in the different parts of a plant. For example, a study conducted on the phyllosphere microbial community of perennial plants showed that 38% of the microbial population variation was caused by the leaf chemical content, such as phosphorus, nitrogen, and soluble sugar and water content in different leaf samples

In this study, no altitude-based distribution pattern of bacterial endophytes was observed. Thus, members of the genera *Pseudomonas, Variovorax*, *Acinetobacter*, *Xanthomonas*, *Herbaspirillum, Rhizobium* accounting for about 60% of the total bacterial isolates, were found in at least seven of the sampling sites with variable altitudinal range. Although similar pattern of fungal distribution were observed by genera *Fusarium* and *Plectosphaerella* which account 38.09% of the total fungal isolates at phylum level, the phylum Zygomycota showed site specific distribution where all the isolates were detected only from Alechowerero which has the highest altitude in this study which might show altitudinal based pattern of distribution by fungal endophytes.

Interestingly, most of the sites examined in this study had one or more unique bacterial taxa occurring only in that geographic location. Out of the eight sampling sites examined in the enset growing regions, Atat, located in Central Ethiopia, showed the highest diversity of endophytes with a total of 26 genera detected out of which *Delfitia, Achromobacter, Divosa, Agrococcus, Rathayibacter, Plantibacter, Bosea, Klebsiella* and *Ochrobactrum* were unique to the site (Fig 2.3). Moreover these isolates were also represented by only one or two isolates which could be an indication of their rare number in the specific environment.

On the otherhand, the fungal diversity observed in Atat site is the lowest where only four fungal species were detected and *Fusarium oxysporum* constituted 72.7% of the isolates. The highest fungal diversity was observed in Telo site where 15 fungal species were detected out of which *Alternaria arborescens, Septoria cucubeli, Preussia minina, Penicillium steckii, Acremonium kiliense, and Fusarium redolens* were unique species to the site. In terms of bacterial diversity this site doesn’t have any unique genera detected.

Although several abiotic and biotic factors, such as plant variety, soil type, nutrient composition, pH, and agronomic practices could determine endophyte diversity, which of these factors contribute to the high diversity of endophytes at the Atat and Telo sampling site remains to be determined. In general, identification of unique bacterial and fungal endophytes in a given location could be interesting as it might help to identify potentially useful but rare microbial strains useful for plant growth and survival. However, those bacterial and fungal taxa which are ubiquitous in all environmental conditions might also have a better advantage in field applications that target biocontrol and plant growth promoting potentials.

Based on 16S rRNA sequence similarities, several of the enset endophytes isolated in this study showed close similarity with other endophytes isolated from other cultivated plant species like banana, maize, legumes, palm, and others. As these reports are from geographically distant locations on the globe, the similarity may indicate that there are bacteria and fungi that can adapt different types of environmental conditions and might colonize a wide range of plant species. Similarly, the ITS sequence similarities of enset associated fungal endophytes showed the detection of fungal isolates such as *Alternaria, Cladosporium, Acremonium, Aspergillus, Colletotrichum, Fusarium, Penicillium and Phomopsis.* Endophytes of similar fungal groups were detected from soybean, medicinal plants (Pimentel *et al*., 2006; Huang *et al*., 2008; Yuan *et al*., 2009).

Diverse groups of culturable endophytic bacterial and fungal community detected in this study might indicate the presence of plethora of beneficial plant microbe interaction. As endophytes are known for their plant beneficial functions, further phenotopic and genetic study of the endopytes is also useful to unravel specific benificaial properties they might confer to the host plant.

Chapter 3

Plant growth promotion and bio-control potential of enset endophytes

Abstract

Enset is largely affected by a bacterial wilt disease caused by *Xanthomonas campestris* pv. *musacerum.* Endophytic bacteria and fungi are known to protectect their host from attack by pathogens and insect pests; increase availability of nutrients and enhanced growth through the production of plant hormones. The aim of this study was to test if enset endophytes play any of these roles. Selected isolates were tested for *in vitro* and *in vivo* antagonistic effect against *Xanthomonas campestris* pv. *musacerum,* for their growth on NFM, solubilise phosphate and produce siderophore and IAA. In the *in vitro* test, different bacterial different genera inhibited the growth of the pathogen with average clear zones diameter of 0.5-3.0cm. The highest inhibito was observed for isolates identified as *Pseudomonas*, *Bacillus*, and *Rhizobium.* Moreover, two fungal isolates identified as *Mycosphaerella coacervata* and *Plectosphaerella cucumerina* inhibited the growth of the pathogen by 3 and 3.2cm diameter respectively. When selected strains were tested *in vivo*, different levels of disease severity was observed in the different treatments. Plants treated with a mixed culture of bacterial isolates EECC-550(*Pseudomonas sp.*), EECC-679(*Pseudomonas sp.*), EECC-572 (*Pseudomonas sp.*), EECC-12(*Bacillus sp.*), EECC- 562(*Acinetobacter sp.*), EECC-53 (*Rahnella sp.*), EECC-574(*Enterobacter sp.*), EECC-543(*Variovorax sp.*), EECC-37(*Ensifer sp.*), EECC-682, EECC- 484(*Arthrobacter sp.*), EECC-55, EECC-671(*Bulkoholderia sp.*). showed a 30.7% mean disease severity compared 47.4% severity for the control plants. Moreover, majority of the isolates showed one or more plant growth promoting properties. The *in vitro* and *in vivo* results indicate enset endopytes have PGP and biocontrol properties.

Key words: Endophytes, PGP, *Xanthomonas campestris* pv. *musacerum*, *Ensete ventricosum*

Introduction

Microbial mediated crop improvements have been in use for the past several years and many bacterial and fungal biocontrol agents and plant growth promoters are identified. However, due to the need to control newely emerging plant pathogens and the need to continuously improve crop productivity, the effort of finding new and improved microbial agents is a continuouing process. Among the strategies to increasing crop health and growth, increasing the availability of nitrogen and phosphrous, production of plant hormones, inhibition of plant pathogens through mechanisms like siderophore production, synthesis of lytic enzymes and disease and pest management are the subjects of many studies (Dong-Sung *et al*., 2007).

Nitrogen and phosphorus are the two major nutrients that limit plant growth. Soil deficient in these nutrients can be supplemented using chemical fertilizers or through the addition of bacterial and fungal inoculum that can increase the availability of these nutrients (Chen *et al*., 2005). In relation to increasing nitrogen, apart from the most studied *Rhizobia* species, nitrogen fixing ability were detected in free living endophyte of species *Herbaspirillum*, *Enterobacter*, *Azospirillum* , *Bacillus*, *Pseudomonas*, *Serratia, Klebsiella* and others (Dong *et al*., 1994; Elbeltagy *et al*., 2001; Chi *et al*., 2005, Glick *et al*., 2012; Ngamau *et al*., 2012).

Moreover, nitrogen fixing endophytes were isolated from different tissues of non leguminous plants such as sugarcane, rice, banana, and grass (Dong *et al*., 1994; Elbeltagy *et al*., 2001; Kelemu *et al*., 2011; Ngamau *et al*., 2012). Similarly, several endophytic bacteria and fungi showed phosphate solublization ability (Vitorino *et al*., 2012; Mendes *et al*., 2014). Endophytic bacteria belonging to the genera *Enterobacter, Pseudomonas* and arbuscular mycorrhizal fungi are some of the endophytes that showed characteristics of phosphate solubilisation (Ngamau *et al*., 2012; Vitorino *et al*., 2012).

Siderophore production is another plant growth promoting property exhibited by endophytic bacteria and fungi. Siderophores can indirectly promote plant health by sequestering iron from the soil and making it unavailable to phytopathogens (Kajula *et al*., 2010; Jasim *et al*., 2013). In addition, some groups of siderophores were reported to facilitate uptake of Zn and Cu (Ji *et al*., 2014). Plant hormone production is another property which is commonly detected in endophytic bacteria and fungi. Endophytes can also produce IAA which is a plant hormone that is directly needed for plant growth. The direct role of the production of IAA by endophytic bacteria and fungi for increased plant growth was demonstrated by Khan *et al*. (2012).

In relation to plant disease control, endophytes colonize an ecological niche similar to phytopathogens, and it is believed that they could be equipped with different mechanisms of pathogen suppression (Bressan and Borges, 2004; Ryan *et al*., 2007). In addition since they are protected from environmental stress and microbial competition they might have advantage over other rhizospheric and phylospheric microbes (Ji *et al*., 2008). The endophytes might suppress the pathogen activity through mechanisms such as competition for space and nutrients, production of lytic enzymes, induction of systemic resistance in the host plant and antibiosis (Compant *et al*., 2005a; Hsieh *et al*., 2005). Moreover, variable levels of disease reduction were observed in endophyte mediated disease control studies (Raaijmakers *et al*., 1995; Ting *et al*., 2008).

Successful disease control potentials were observed in several studies. For example, *Bacillus subtilis* was shown to be effective in controlling mycotoxin production by *Fusarium* in corn, and its use has been patented (Bayman, 2007). Recently, it has been reported that inoculation

of banana with endophytes protected the host plant from infection by *Fusarium oxysporum* f. sp. *cubense* race 4 fungal pathogen and resulted in better plant growth (Jie *et al*., 2009). In another study, *Curtobacterium flaccumfaciens* was shown to be highly promising for control of citrus variegated chlorosis (Bayman, 2007). It was also observed that soacking seeds of bean in suspension of endophytic bacteria *Pantoea agglomerans* isolate LRC 8311 decreased disease severity of bacterial wilt of bean caused by *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (Hsieh *et al*., 2005). A study conducted by Sapak *et al*, (2008) demonstrated the potential of *Pseudomonas aeruginosa* as a biocontrol agent against *Ganoderma boninense* resulting in decreased disease incidence levels on palm plants.

Similarly, the presence of the endophyte *Bacillus* in mulberry tissues had positive effects on suppression of infection by *Ralstonia solanacearum*, resulting in reduced incidence of bacterial wilt in a green house experiment. Introduction of naturally-occurring endophytes into tissue culture banana plantlets led to a 67% suppression rate of wilt disease at the fifth month after pathogen infection on plantlets in the greenhouse (Ji et *al*. 2008). In addition to disease suppression, growth of host plantlets was also promoted with the inoculation of endophytes (Lian *et al*., 2009).

Production of hydrolytic enzymes by endophytic microbes is also another property which is studied as an indicator for successful colonization of a potential plant growth promoting and/or a biocontrol agent. Amylase, cellulase, laccase, lipase, and protease are some of the hydrolytic enzymes produced by endophytic bacteria and fungi (Dai *et al*., 2010; Amirita *et al*., 2012; Kalai-Grami *et al*., 2014). Robl *et al*, (2013) reported the production of xylanase and pectinase by endophytic fungal isolates of *Aspergillus niger, Trichoderma atroviride,* and, *Alternaria sp.* In another study, pectinase cellulase and xylanase were produced by

bacterial endophytes of genera *Acetobacter, Acinetobacter, Methylococcus, Bacillus, Micrococcus* and *Planococcus* (El-Deeb *et al*., 2012). Moreover the hydrolytic enzymes produced by endophytic microbes are also studied for their industrial applications as well (Corrêa *et al*., 2014).

These and other successful trials generated a lot of interest to study the potential use of endophytes to improve productivity of several crops. In our study, plant growth promoting properties such as nitrogen fixation, phosphate solubilisation, siderophore and indole acetic acid production of endophytic isolates were determined by *in vitro* and biocontrol properties *Xanthomonas campestris* pv. *musacerum* were carried out in *in vivo* methods.

Material and method

Indole acetic acid (IAA) production by bacterial isolates

IAA production was detected as described by Jasim *et al*, (2013) with some modification. Bacterial cultures were grown for 24 h in nutrient broth medium transferred to fresh nutrient broth amended with 3mg/ml tryptophan and incubated at 30°C for 5 days in a shaking incubator. The cultures were centrifuged at 3000 rpm for 30 min on Centurion K280 model centrifuge. Two ml of the supernatant was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution) Development of pink colour indicated IAA production.

Growth on nitrogen free medium

Growth of bacterial endophytes on nitrogen free Ashib's medium was considered as property of fixing atmospheric nitrogen. The Ashib's medium and trace element was prepared following the protocol on Doty *et al*. (2009) and containing (g/l) 20g sucrose; 0.2g K_2HPO_4; 0.2 $MgSO_4.7H_2O$; 0.2 NaCl; 0.1g K_2SO_4; 5gCa_2CO_3; 15g purified Agar. The composition of the trace was 0.0625 g $CuSo_4$, 0.012 $ZnSo_4.7H_2O$, 0.14g H_3BO_3, $Na_2MoO_4.2H_2O$ and 0.15 $MnSO_4.H_2O$ all in 100ml ddH_2O. Vitamin solution contained 10mg and 20mg Biotine and Pyridoxol mixed in 100ml ddH_2O (Kirchhof *et al*., 1997). Ten ml of the vitamin solution and 2 ml trace element were mixed in to the Ashib's medium to which fresh bacterial cultures were streaked and incubated at 28^0C for 7 days. Growth of culture was considered as positive for nitrogen fixation.

Phosphate solublisation by bacterial isolates

Bacterial cultures were spot inoculated on Pikovskaya (PVK) medium containing per litre: glucose, 10 g; Ca_3 $(PO_4)_2$, 5 g; $(NH_4)_2SO_4$, 0.5 g; NaCl, 0.2g; $MgSO_4.7H_2O$, 0.1 g; KCl, 0.2 g; yeast extract, 0.5 g; $MnSO_4.H_2O$, 0.002 g; and $FeSO_4.7H_2O$, 0.002 g. The plates were incubated at 28^0C for 7 days. Formation of clear halo zone around the colonies indicates phosphate solubilisation activity (Jasim *et al*., 2013).

Siderophore production by bacterial isolates

Siderophore production was tested by modifying the protocol Jasim *et al*. (2013) on Chrom azurol S (CAS) containing agar medium. All the glasswares were cleaned with 6N HCL before preparation of any component of the medium to avoid any trace of Fe. Solution A: CAS indicator was prepared by dissolving 60.5mgCAS/50ml dd H_2O, then solution B: 27mg$FeCl_3.6H_2O$ was mixed with 100ml ddH_2O containing 83.3conc HCL then solution C: was prepared by dissolving 72mg HTDMA (hexadecyltrimethylammonium bromide) in 40ml

ddH_2O. Then 50 ml from solution was mixed with 10 ml from solution B and 40ml solution C autoclaved separately from the media.

Basal medium was prepared from 3.24g PIPES(piperazine-N, N-bis 2- ethanesulfonic acid), 0.05g NaCl,0.03 g KH_2PO_4, 0.01gNH_4Cl and 1.5% purified agar was mixed in 100ml and autoclaved separately from which 2ml from 50% glucose and 3ml of 10% filter sterilized casaminoacid was added on the medium from the indicator was used as 10ml in 100ml medium. Pure isolates were streaked on the plates and incubated at 28^0C in dark. After 48hours formation of yellow to orange hallo around the grown colonies was an indication of siderophore production.

Cellulase production by bacterial isolates

Qualitative test for cellulase activity of selected endophytic bacteria was checked by following the protocol by Elbeltagy *et al*, (2000). The media composition was (A) NaCl 0.25 g, K_2HP0_4 1.5 g, carboxy methyl cellulose 2.5 g, distilled water 400 mL; (B) 1 M aqueous solution of $MgS0_4$. $7H_20$; (C) $Na_2HP0_4.12H_20$ 3.0 g, NH_4Cl 5.0 g, glycerol 2.5 g, yeast extract 0.5 g, purified agar (Oxoid) 6.5 g, distilled water 100 mL; (D) 7.5% (w/v) $CaC1_2$. After autoclaving solutions A and C were mixed together to which 1.0 mL each of solutions, B and D was added. The culture was spot inoculated on the plates and incubated at 28^0C for five days. Cellulase activity was detected by flooding the plates with 0.1% (w/v) Congo red for 15 to 30 min followed by bleaching the plates with 1 M NaCl. Formation of yellowish hallo is indication of cellulase production (Elbeltagy *et al*., 2000).

Pectinase production by bacterial isolates

Bacterial endophytes were screened for pectinase production by growing them on nutrient agar supplemented with 0.5% pectin. After incubating at 28^0C for 5 days the plates were overlaid with 2% CTAB solution for 30 min. CTAB solution was then discarded and the plate surface was washed with 1 M NaCl. The presence of clear zone around bacterial growth is indicated pectinase production (Ma *et al*., 2011).

Phosphate solublization by fungal isolates

Phosphate solublization was detected by growing each isolate on Pikovskaya (PVK) medium containing per litre of glucose, 10 g; Ca_3 $(PO_4)_2$, 5 g; $(NH_4)_2SO_4$, 0.5 g; NaCl, 0.2g; $MgSO_4.7H_2O$, 0.1 g; KCl, 0.2 g; yeast extract, 0.5 g; $MnSO_4.H_2O$, 0.002 g; and $FeSO_4.7H_2O$, 0.002 g and incubated at 28^0C for 7 days and formation of clear halo zone around the colonies indicated phosphate solubilisation activity (Nath *et al*., 2012).

Siderophore productions by fungal isolates

Similar composition of CAS agar that was used by Mahmoud and Abdalla, 2001 for screening different fungi for production of siderophore was also used in this study. All the glassware were cleaned with 6N HCL before preparation of any component of the medium this was to avoid any trace of Fe. Solution A: CAS indicator was prepared by dissolving 60.5mgCAS/50ml dd H_2O, then solution B: 27mg$FeCl_3.6H_2O$ was mixed with 100ml ddH_2O containing 83.3conc HCL then solution C: was prepared by dissolving 72mg HTDMA (hexadecyltrimethylammonium bromide) in 40ml ddH_2O. Then 50 ml from solution A then 10 ml from solution B and 40ml solution C was mixed and gave dark blue solution which was autoclaved separately from the media. Basal medium was prepared from 3.24g PIPES(piperazine-N, N-bis 2- ethanesulfonic acid), 0.05g NaCl,0.03 g KH_2PO_4,

$0.01gNH_4Cl$ and 1.5% purified agar was mixed all per 100ml and autoclaved separately then 2ml from 50% glucose and 3ml of 10% filter sterilized casaminoacid was added on the medium from the indicator was used as 10ml in 100ml medium. Pure isolates were streaked on the plates and incubated at 28^oC in dark. After 48hours formation of yellow to orange hallo around the grown colonies was an indication of siderophore production.

Cellulase production by fungal isolates

Screening of the fungal isolates for cellulose activity was detected with yeast extract peptone agar medium (0.1g yeast extract, 0.5g peptone, 16g agar, and 1000ml distilled water) amended with 0.5% Na-carboxymethyl cellulose and the culture was incubated at 28^0C for 7days. The plates were flooded with0.1% congo red and destained with 1M sodium chloride for 15 min. Appearance of yellowish area around the fungal growth is indication of cellulose production (Amirita *et al*., 2012).

Pectinase production by fungal isolates

Pectinolytic activity was determined by growing the fungi in Pectin Agar medium (Pectin - 5g, yeast extract-1g, agar- 15g pH 5.0 in 1L distilled water). After 7 days of incubation the whole plate with fungal growth was flooded with 1% aqueous solution of CTAB. A clear zone formed around the fungal colony indicated pectinolytic activity (Sunitha, *et al*., 2013).

Preparation of Endophytic and pathogen culture

Prior to starting the *in vitro* and *in vivo* experiments, the pathogen culture used in this study was identified by sequencing the 16S rDNA and amplifying the Gsp D protein gene using the pathogen specific primers according to Adriko *et al*, (2012) and the results are found in Appndix-II. Out of the total 446 bacterial isolates, 105 were screened for *in vitro* inhibition of the pathogen growth. The selection criteria was, the 16S rDNA sequence similarity with other

endophytic sequences in the NCBI database, isolates of the Maze (relatively wilt disease tolerant variety) or isolates from the wild variety were considered. For fungal endophytes, out of the total 104 fungal isolate, 44 were screened for their antagonistic activity against *Xanthomonas campestris* pv. *musacerum*. The selection criteria was, the fungal ITS sequence similarity with other endophytic sequences in the data base, isolates of the Maze (relatively tolerant variety) or isolates from the wild variety were considered.

***In vitro* co-culturing of pathogen and potential biocontrols**

For bacterial endophytes, fifty ml of nutrient broth was prepared for each bacterial isolate culture and inoculated with 1ml of a 10^8 cfu/ml of endophytic culture suspension and incubated at 28^0C for 48hour. The broth culture was used to test the inhibitory effect of the endophytes on *Xanthomonas campestris* pv. *musacerum* by well diffusion method. The pathogen culture suspension was prepared in 10^8 cfu/ml (OD 0.1 at 600nm) in 0.85% NaCl. Then 100 µl was spread plated on Nutrient agar plates prior to inoculation of the antagonist in the well. Wells were made using 2mm diameter cork borer. Ten micro liter of a 48 hour liquid culture of the endophytes were inoculated in the wells. The plates were inoculated at 28^0C for 5days. Formation of clear zone around the colonies was considered as positive for inhibition of the pathogen growth.

For fungal endophyts, fresh cultures of fungi grown on potato dextrose agar medium were used to test the antagonistic effect of the fungal endophytes on *Xanthomonas campestris* pv. *musacerum*. The pathogen culture was spread plated at concentration of 100 µl of 10^8 cfu/ml. Then agar blocks of the fungal cultures that grew for 7 days was placed on the top of the pathogen spread on nutrient agar. The plates were incubated at 28^0C for 7 days and formation of clear zone around the fungal agar blocks is an indication of inhibition of pathogen growth.

Plant material and soil and pot preparation for *in vivo* test

Tissue cultured enset plants of Badedete variety was kindly provided by the Holleta Research Centre and National Biotechnology Research Lab, Ethiopian Institute of Agricultural Research. The greenhouse experiment was conducted at International Live stock Research Institute. The tissue cultured plant material was acclimatized before transferring them to the actual green house experiment. All the pots used in this experiment were cleaned with detergent and disinfected with 70% ethanol and 3% sodium hypochlorite. The soil used for acclimatization and planting of the experimental plants was autoclaved forest soil. Before transferring the enset plants to actual experimental pots, the tissue cultured plant lets were transferred to small pots containing sterile forest soil and acclimatized for one month.

Experimental design and monitoring of glasshouse experiment

The *in vivo* experiment was conducted from Sept-December, 2013 at ILRI Nairobi in a contained but none-controlled glasshouse conditons**.** A total of 13 bacterial endophytes and two fungal endophytes that gave higher inhibition, that were isolated from different plant part and/or that belong to different taxa were included (Table 3.1). A total of 18 treatments with six replicates were included in a complete randomized block design experiment. The experimental setup was different combinations of single and mixed cultures of eighteen treatments in six replicates in a randomized block design (RCBD) (Table 3.1).

Table 3.1 Combination of inoculum for 18 treatments for *in vivo* experiment

Treatment No	Culture combinations inoculated in the specific treatment
1	EECC-550 (*Pseudomonas sp.*), EECC-679 (*Pseudomonas sp.*), EECC-572 (*Pseudomonas sp.*), EECC-12 (*Bacillus sp.*), EECC- 562(*Acinetobacter sp.*), EECC-53(*Rahnella sp.*), EECC-574 (*Enterobacter sp.*), EECC-543 (*Variovorax sp.*), EECC-37(*Ensifer sp.*), EECC-682 ((*Arthrobacter sp.*), EECC- 484 (*Pedobacter sp.*), EECC-55 (*Rhizobium sp.*), EECC-671 (*Bulkoholderia sp.*)
2	EECC-454(*Mycosphaerella coacervata*), EECC-528(*Plectosphaerella cucumerina*)
3	EECC-550 (*Pseudomonas sp.*), EECC-679 (*Pseudomonas sp.*), EECC-572 (*Pseudomonas sp.*), EECC-12 (*Bacillus sp.*), EECC- 562(*Acinetobacter sp.*), EECC-53(*Rahnella sp.*), EECC-574 (*Enterobacter sp.*), EECC-543 (*Variovorax sp.*), EECC-37(*Ensifer sp.*), EECC-682, EECC- 484 (*Arthrobacter sp.*), EECC-55 (*Rhizobium sp.*), EECC-671 (*Bulkoholderia sp.*), EECC-454(*Mycosphaerella coacervata*), EECC-528(*Plectosphaerella cucumerina*)
4	EECC-550 (*Pseudomonas sp.*)
5	EECC-572 (*Pseudomonas sp.*)
6	EECC-679 (*Pseudomonas sp.*)
7	EECC-454 (*Mycosphaerella coacervata*)
8	EECC-528(*Plectosphaerella cucumerina*)
9	Sterile dd water
10	*Xanthomonas campestris* pv. *musacerum*
11	EECC-550 (*Pseudomonas sp.*), EECC-679 (*Pseudomonas sp.*), EECC-572 (*Pseudomonas sp.*), EECC-12 (*Bacillus sp.*), EECC- 562(*Acinetobacter sp.*), EECC-53(*Rahnella sp.*), EECC-574 (*Enterobacter sp.*), EECC-543 (*Variovorax sp.*), EECC-37(*Ensifer sp.*), EECC-682 (*Arthrobacter sp.*), EECC- 484 (*Pedobacter sp.*), EECC-55(*Rhizobium sp.*), EECC-671 (*Bulkoholderia sp.*) + ***Xanthomonas campestris* pv. *musacerum***
12	EECC-454(*Mycosphaerella coacervata*), EECC-528(*Plectosphaerella cucumerina*) + ***Xanthomonas campestris* pv. *musacerum***
13	EECC-550 (*Pseudomonas sp.*), EECC-679 (*Pseudomonas sp.*), EECC-572 (*Pseudomonas sp.*), EECC-12 (*Bacillus sp.*), EECC- 562(*Acinetobacter sp.*), EECC-53(*Rahnella sp.*), EECC-574 (*Enterobacter sp.*), EECC-543 (*Variovorax sp.*), EECC-37(*Ensifer sp.*), EECC-682, EECC- 484 (*Arthrobacter sp.*), EECC-55 (*Rhizobium sp.*), EECC-671 (*Bulkoholderia sp.*), EECC-454(*Mycosphaerella coacervata*), EECC-528 (*Plectosphaerella cucumerina*) + ***Xanthomonas campestris* pv. *musacerum***
14	EECC-550 (*Pseudomonas sp.*) + ***Xanthomonas campestris* pv. *musacerum***
15	EECC-572 (*Pseudomonas sp.*) + ***Xanthomonas campestris* pv. *musacerum***
16	EECC-679 (*Pseudomonas sp.*) + ***Xanthomonas campestris* pv. *musacerum***
17	EECC-454(*Mycosphaerella coacervata*) + ***Xanthomonas campestris* pv. *musacerum***
18	EECC-528(*Plectosphaerella cucumerina*) + ***Xanthomonas campestris* pv. *musacerum***

Preparation of the endophyte and pathogen cultures and inoculations

For bacterial endophytes, 50 ml of nutrient broth was prepared for each bacterial isolate culture and inoculated with 1ml of a 10^8 cfu/ml of endophytic culture suspension and incubated at 28^0C for 48hour. The 48 hour culture of the endophyte culture was inoculated in

30ml total volume in respective treatments. For the treatments with mixed culture, the total volume of 30ml was divided proportionally to the culture broths included in the mix.

Fungal isolates that were grown on potato dextrose broth for 15 days was used as inoculum culture (Chen, 2005). The volume of the inoculums was 30ml for single isolate and proportional mix of single cultures for mixed cultures that made the final volume to 30ml. The endophyte culture was inoculated 15 days prior to pathogen inoculation. Watering was done every other days. Pathogen suspension at concentration of 10^8 cfu/ml was prepared in 0.85% NaCl solution and 1ml was inoculated on the second youngest leaf (Adriko *et al*., 2012). The inoculation of the pathogen was done15 days late than the endophyte inoculation.

Monitoring and data analysis

Plants were monitored for a total of four months. Morphological data on the number of wilted leaf, the number of healthy leaves, wilt on the central leaf and death of a plant were recorded. Disease severity for the treatments was calculated as follows.

Disease severity was assessed using a scale of 0–5: 0, healthy; 1, partial wilting of one lower leaf; 2, wilting of two to three lower leaves; 3, wilting of all but the top two to three leaves; 4, wilting of all leaves; or 5, dead. Disease severity was calculated using the following formula.

Disease severity = (5A+4B+3C+2D+E)/5Nx100

A=number of plants on scale 5; B=number of plants on scale 4; C=number of plants on scale 3; D=number of plants on scale 2; E=number of plants on scale 1; N=total number of plants Hyakumachi *et al*. (2013).

Analysis of variance on the mean disease severity between the treatments was calculated with GenStat Release 14.1.

Result

Plant growth promoting properties of bacterial endophytes

Plant growth promoting properties of selected 105 bacterial and 44 fungal isolates were tested *in vitro* (Appendix-III). Majority of the bacterial endophytes produced one or more plant growth promoting characteristics (Fig 3.1). More than 75 % of the endophytes tested produced the plant growth hormone indole acetic acid (IAA) and 56% produced siderophore (Fig 3.1). Interestingly, about 47% of the isolates tested were able to grow on NFM, indicating their ability to fix atmospheric nitrogen. About 29% of the isolates were also capable of phosphate solubilisation (Fig 3.1).

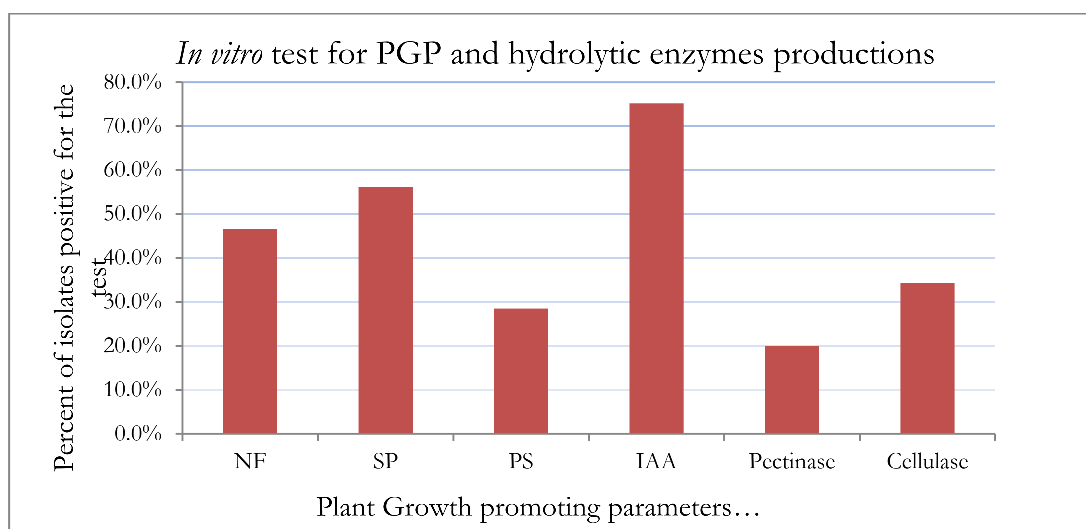


Figure 3.1 *In vitro* PGP NF: Growth on NFM, SP: Siderophore production, PS: Phosphate solubilisation, IAA: Indole acetic production, pectinase and cellulase enzyme production

In addition to plant growth promoting properties, production of pectinase and cellulase were determined to check the possibility of production of plant cell wall degrading enzymes as mechanism of entry and colonization by the endophytes. Qualitative determination of the presence of the enzymes showed that 34.3% and 20% of the bacterial isolates were able to produce cellulase and pectinase, respectively (Fig. 3.1) indicating that the majority are negative for these hydrolytic enzymes.

Table 3.2 *In vitro* PGP characteristics of bacterial isolates that showed sequence similarity with endophytes sequences in the NCBI.

Isolate code	Identification	Nitrogen free media	Siderophore	Phosphate solubilisation	IAA	Pectinase	Cellulase
EECC-9	*Enterobacter sp.*	++	-	+	+	-	-
EECC-20	*Stenotrophomonas sp.*	-	-	-	+	+	-
EECC-21	*Rhizobium sp.*	+++	-	-	+	-	+
EECC-24	*Achromobacter sp.*	-	-	-	+	-	-
EECC-37	*Ensifer sp.*	+++	-	-	+	-	-
EECC-48	*Pseudomonas sp.*	-	+	-	+	-	-
EECC-53	*Rahnella sp.*	+	+	+	-	-	+
EECC-107	*Pseudomonas sp.*	+	+	+	+	-	+
EECC-127	*Pseudomonas sp.*	-	-	-	-	-	-
EECC-128	*Pseudomonas sp.*	-	-	-	+	-	-
EECC-129	*Pseudomonas sp.*	-	+	-	+	-	-
EECC-145	*Pseudomonas sp.*	-	-	-	+	-	+
EECC-163	*Pseudomonas sp.*	+	+	-	+	-	+
EECC-169	*Pseudomonas sp.*	-	-	-	+	-	+
EECC-184	*Microbacterium sp.*	-	-	-	-	-	-
EECC-191	*Pseudomonas sp.*	+	+++	+	+	-	-
EECC-192	*Pseudomonas sp.*	-	-	-	+	-	-
EECC-193	*Pseudomonas sp.*	-	-	-	+	-	-
EECC-195	*Pseudomonas sp.*	+	-	-	+	-	-
EECC-206	*Novosphingobium sp.*	-	+	-	-	-	+
EECC-216	*Variovorax sp.*	+++	+	++	+	-	-
EECC-220	*Pseudomonas sp.*	+	-	++	+	-	-
EECC-225	*Raoultella sp.*	+++	-	-	+	-	-
EECC-235	*Pantoea sp.*	-	-	+	+	-	-
EECC-240	*Pseudomonas sp.*	-	++	-	-	+	-
EECC-258	*Dyella sp.*	-	++	-	+	-	-
EECC-259	*Dyella sp.*	-	+	-	-	-	-
EECC-260	*Dyella sp.*	+	-	-	+	-	+
EECC-266	*Pseudomonas sp.*	+	+++	-	+	-	-
EECC-290	*Xanthomonas sp.*	+++	++	-	+	+	+
EECC-298	*Pseudomonas sp.*	-	-	-	+	-	-
EECC-299	*Stenotrophomonas sp.*	-	+	-	+	+	-
EECC-310	*Stenotrophomonas sp.*	-	+	-	-	+	-
EECC-318	*Microbacterium sp.*	-	-	-	+	-	-
EECC-324	*Bacillus sp.*	+	-	-	+	-	+
EECC-326	*Rahnella sp.*	+	+	-	+	+	+
EECC-327	*Bacillus sp.*	+	+	-	+	+	+
EECC-329	*Stenotrophomonas sp.*	-	-	-	+	+	-
EECC-351	*Pseudomonas sp.*	+	+	-	-	-	-
EECC-352	*Staphylococcus sp.*	-	-	-	+	-	-
EECC-353	*Pseudomonas sp.*	-	+	-	+	-	-
EECC-354	*Herbaspirillum sp.*	-	++	-	+	-	-
EECC-355	*Pseudomonas sp.*	+++	+++	+	+	-	-
EECC-401	*Erwinia sp.*	-	+	++	+	-	-
EECC-402	*Acinetobacter sp.*	-	-	-	+	-	-
EECC-403	*Rahnella sp.*	+	+	-	+	-	+
EECC-444	*Acinetobacter sp.*	+	+++	+++	+	+	-
EECC-507	*Stenotrophomonas sp.*	-	+	-	+	-	-
EECC-514	*Pseudomonas sp.*	-	+	+	-	+	-
EECC-548	*Pseudomonas sp.*	-	++	-	-	+	-
EECC-551	*Stenotrophomonas sp.*	+	-	-	-	-	-
EECC-556	*Acinetobacter sp.*	+++	+++	++	+	-	-
EECC-562	*Acinetobacter sp.*	+++	+++	+++	+	-	-
EECC-567	*Pseudomonas sp.*	+	++	-	+	-	-
EECC-572	*Pseudomonas sp.*	-	+++	+	+	-	-

EECC-574	*Enterobacter sp.*	+	+	-	-	+	-
EECC-579	*Pseudomonas sp.*	+	+++	+++	-	+	-
EECC-602	*Pantoea sp.*	+	++	+++	+	-	+
EECC-606	*Pseudomonas sp.*	+++	-	-	+	+	-
EECC-612	*Acinetobacter sp.*	-	-	-	-	-	+
EECC-671	*Burkholderia sp.*	+++	+++	-	+	-	+
EECC-673	*Pseudomonas sp.*	-	++	+++	+	+	-
EECC-679	*Pseudomonas sp.*	+	++	+	+	-	+
EECC-682	*Arthrobacter sp.*	+	+	++	+	-	-
EECC-683	*Plantibacter sp.*	-	-	-	+	-	+
EECC-685	*Bosea sp.*	+	-	-	+	-	+
EECC-699	*Kluyvera sp.*	-	+	+	-	-	-
EECC-701	*Raoultella sp.*	+	+	-	+	+	+
EECC-705	*Rahnella sp.*	+	+	-	+	-	+

Plant growth promoting potential rating for nitrogen, siderophore and phosphate is: + weak positive, ++ positive, +++ strong positive and - Negative. For IAA and Enzyme: +, positive and – Negative.

Some of the endophytes in the group, *Pseudomonas*, *Variovorax*, *Acinetobacter*, *Pantoea* and *Xanthomonas* were positive to multiple plant growth promotion properties tested (Table 3.2). On the other hand no plant growth promoting property was detected in few isolates that belong to *Microbacterium* (EECC-318), *Acinetobacter* (EECC-612), and *Pseudomona*s (EECC-127).

Diversity of bacterial and fungal isolates in this study was analzed based on sampling location and plant part. However, farmer's information about disease response of the enset samples was considered during selection of isolates for their beneficial functions like antagonistic properties. As a result, isolates from the Maze clone which is widely considered to have much better tolerance to bacterial wilt disease and isolates from wildly grown enset samples which again are considered as resistant to the disease were screened for plant growth promotiom and antagnostic properties. Plant growth promotion were detected from isolates of wild, Maze and those that had 16S rRNA sequence similarity with antagnostic bacterial sequences in the data base (Table 3.3).

Table 3.3 *In vitro* PGP characteristics of bacterial isolates that showed sequence similarity with antagonistic bacteria sequences in the NCBI, isolates of Maze and wild enset varieties.

Isolate group	Isolate code	Identification	Nitrogen	Siderophore	Phosphate	IAA	Pectinase	Cellulase
Similar with antagnositc bacteria	EECC-12	*Bacillus subtilis*	++	+++	-	+	-	+
	EECC-40	*Lysobacter sp.*	-	+	-	-	-	+
	EECC-57	*Rathayibacter sp.*	-	-	-	+	-	-
	EECC-70	*Lysobacter sp.*	-	-	-	+	-	+
	EECC-108	*Rahnella sp.*	-	+++	-	+	-	+
	EECC-140	*Pseudomonas sp.*	-	+	++	+	-	+
	EECC-147	*Sphingomonas*	++	-	-	+	-	-
	EECC-162	*Pseudomonas sp.*	-	-	-	+	-	-
	EECC-669	*Pseudomonas sp.*	-	+	++	+	-	+
Isolates from (Maze) variety	EECC-356	*Burkholderia sp.*	+	-	-	-	-	+
	EECC-357	*Pseudomonas sp.*	++	+++	-	+	-	+
	EECC-359	*Variovorax sp.*	+++	++	++	+	-	+
	EECC-361	*Herbaspirillum sp.*	-	+	-	+	-	-
	EECC-362	*Herbaspirillum sp.*	-	++	-	-	-	-
	EECC-363	*Staphylococcus sp.*	-	-	-	+	+	-
	EECC-365	*Xanthomonas sp.*	-	-	-	+	-	+
	EECC-366	*Variovorax sp.*	-	+	-	+	+	-
	EECC-480	*Erwinia sp.*	-	+++	-	-	+	-
	EECC-482	*Pseudomonas sp.*	+++	-	-	+	-	-
	EECC-483	*Pseudomonas sp.*	-	++	-	-	-	-
	EECC-484	*Pedobacter sp.*	-	+	++	+	-	-
	EECC-485	*Flavobacterium sp*	-	+	-	-	-	-
	EECC-490	*Variovorax sp.*	++	-	-	+	-	-
Isolates from wild enset	EECC-541	*Xanthomonas sp.*	+++	-	-	+	+	+
	EECC-542	*Pantoea sp.*	+	+	+	+	-	+
	EECC-543	*Variovorax sp.*	-	-	+	-	-	+
	EECC-547	*Rhizobium sp.*	++	-	+	-	-	-
	EECC-549	*Herbaspirillum sp.*	+	-	-	+	-	-
	EECC-550	*Pseudomonas sp.*	-	+	+	-	-	-
	EECC-599	*Xanthomonas sp.*	+	++	++	+	-	-
	EECC-600	*Serratia sp.*	+	++	+	+	-	+
	EECC-601	*Agrobacterium sp.*	+++	-	-	+	-	-
	EECC-603	*Bacillus sp.*	-	-	-	-	-	+
	EECC-608	*Xanthomonas sp.*	-	-	-	-	-	-
	EECC-610	*Bacillus sp.*	-	-	-	+	-	+
	EECC-613	*Variovorax sp.*	-	-	-	+	-	+

Plant growth promoting potential rating for nitrogen, siderophore and phosphate is: + weak positive, ++ positive, +++ strong positive and - Negative. For IAA and Enzyme: +, positive and - Negative

Plant growth promoting properties of fungal endophytes

Fungal endophytes were characterized for siderophore production and phosphate solubilisation activities.

Table 3.4 PGP characteristics and hydrolytic enzyme production by fungal isolates

Blast similarity category	Isolate Identification		Plant growth promotion	Hydrolytic enzymes	
	Isolate code	Taxanomy	Siderophore	Pictnase	Cellulase
Isolates similar with endophytes	EECC-371	*Penicillium chrysogenum*	+	+	+
	EECC-372	*Plectosphaerella cucumerina*	+	+	+
	EECC-379	*Fusarium oxysporum*	+	+	+
	EECC-462	*Cladosporium cladosporioides*	+	+	+
	EECC-520	*Epicoccum nigrum*	+	+	+
	EECC-523	*Fusarium oxysporum*	-	-	-
	EECC-529	*Cladosporium tenuissimum*	+	-	-
	EECC-530	*Alternaria alternata*	-	+	+
	EECC-532	*Aspergillus versicolor*	+	+	+
	EECC-534	*Phoma herbarum*	+	+	+
	EECC-536	*Fusarium oxysporum*	+	+	+
	EECC-624	*Cladosporium cladosporioides*	-	-	-
	EECC-628	*Epicoccum nigrum*	-	-	-
	EECC-629	*Cladosporium cladosporioides*	-	-	+
	EECC-635	*Fusarium oxysporum*	+	+	+
	EECC-636	*Alternaria arborescens*	+	+	+
	EECC-637	*Polycephalomyces sinensis*	+	+	+
	EECC-647	*Alternaria arborescens*	+	-	-
	EECC-649	*Epicoccum nigrum*	-	-	-
	EECC-652	*Phoma herbarum*	+	+	+
	EECC-708	*Alternaria tenuissima*	+	+	+
	EECC-713	*Fusarium oxysporum*	+	+	+
	EECC-715	*Fusarium oxysporum*	+	+	+
	EECC-726	*Fusarium oxysporum*	+	-	+
	EECC-727	*Fusarium oxysporum*	-	-	-
	EECC-728	*Fusarium equiseti*	-	+	+
	EECC-729	*Chaetomium globosum*	+	+	+
	EECC-654	*Fusarium oxysporum*	+	+	+
	EECC-655	*Epicoccum nigrum*	-	-	-
	EECC-656	*Fusarium oxysporum*	-	+	+
	EECC-657	*Acremonium kiliense*	-	-	-
	EECC-658	*Alternaria tenuissima*	+	+	+
	EECC-660	*Fusarium equiseti*	+	+	+
	EECC-661	*Plectosphaerella cucumerina*	+	+	+
	EECC-664	*Epicoccum cf. Nigrum*	+	+	+
Isolates from Maze Variety	EECC-456	*Preussia minima*	-	-	-
	EECC-457	*Plectosphaerella cucumerina*	+	+	+
	EECC-469	*Aspergillus sydowii*	+	+	+
	EECC-470	*Talaromyces flavus*	-	-	+
	EECC-621	*Penicillium pancosmium*	+	+	+
	EECC-643	*Fusarium oxysporum*	-	-	-
Isolates from wild variety	EECC-658	*Alternaria tenuissima*	-	-	-
	EECC-659	*Fusarium redolens*	+	+	+
	EECC-663	*Cercospora zebrina*	+	+	+

+: Posititive for the test; - : Negative for the test.

Out of the total 44 fungal isolates tested, 29 isolates that belonging to different taxa produced siderophore. On the other hand, ability to solubilize tricalcium phosphate was not observed in any of the fungal isolates. Unlike the bacterial endophytes, majority of fungal endophytes were positive to production of both hydrolytic enzymes of which 72.7 and 66 % of the fungal isolates producing cellulase and pectinase, respectively (Table 3.4).

In vitro antagonistic effect of endophytes

Bacterial and fungal endophytes listed in Table 3.2, 3.3 and Table 3.4 were screened for *in vitro* inhibition of *Xanthomonas campestris* pv. *musacerum.* Different type of growth properties were observed on the well diffusion test. Some isolates were able to cover most of the space on the plate others showed small growth but surrounded by clear zone around the wells while others didn't show any growth or clear zone. Those isolates that showed clear zone around the wells as direct inhibition of the pathogen through production of inhibitory metabolites were considered as antagonistic isolates (Fig 3.2).

The isolates which showed clear zone around the wells are listed on Table 3.5. A total of 26 bacterial and two fungal isolates inhibited growth of the pathogen *in vitro*. Although most of the isolates were members of the genus *Pseudomonas*, isolates belonging to *Bulkoholderia, Bacillus, Ensifer, Rhizobium* and others inhibited the growth of *Xanthomonas campstris* pv. *musacerum* (Table 3.5). *The two fungal pathogen that showed inhibition were identified as Mycospharella coacervata and Plectosphaerella cucumerina.*

Table 3.5 *In vitro* inhibition activity of endophytes against *Xanthomonas campestris* pv. *musacerum.*

Isolate code	Isolated from	Genus	Clear zone diameter (cm)	Enset variety
	Bacterial isolates			
EECC-12	Stem	*Bacillus sp.*	2.00	Nechewe
EECC-37	Root	*Ensifer sp.*	1.95	Yesherakinke
EECC-53	Root	*Rahenella sp.*	0.50	Yesherakinke
EECC-55	Root	*Rhizobium sp.*	2.10	Yesherakinke
EECC-144	Root	*Pseudomonas sp.*	1.60	Aegeremer
EECC-162	Root	*Pseudomonas sp.*	1.90	Ado
EECC-163	Root	*Pseudomonas sp.*	1.65	Ado
EECC-240	Leaf	*Pseudomonas sp.*	0.65	Gimbo
EECC-298	Leaf	*Pseudomonas sp.*	2.30	Oreda
EECC-355	Leaf	*Pseudomonas sp.*	2.10	Maze
EECC-357	Root	*Pseudomonas sp.*	2.30	Maze
EECC-358	Root	*Pseudomonas sp.*	2.00	Maze
EECC-399	Stem	*Bacillus sp.*	1.35	Genetech
EECC-434	Leaf	*Pseudomonas sp.*	0.50	Astara
EECC-484	Root	*Pedobacter sp.*	2.05	Maze
EECC-514	Stem	*Pseudomonas sp.*	1.35	Boza
EECC-543	Root	*Virovorax sp.*	0.50	Wild
EECC-550	Root	*Pseudomonas sp.*	3.00	Wild
EECC-562	Root	*Acitnobacter sp.*	0.50	Nobo (wild)
EECC-567	Root	*Pseudomonas sp.*	2.35	Butecho(wild)
EECC-572	Stem	*Pseudomonas sp.*	2.00	Butecho(wild)
EECC-574	Stem	*Enterobacter sp.*	0.80	Butecho(wild)
EECC-579	Stem	*Pseudomonas sp.*	2.00	Bocho(wild)
EECC-671	Root	*Bulkoholderia sp.*	1.50	Tereye
EECC-679	Leaf	*Pseudomonas sp.*	2.30	Bishamerat
EECC-682	Root	*Artherobacter sp.*	1.00	Bishamerat
	Fungal isolates			
EECC-454	Leaf	*Mycosphaerella coacervata*	3.2	Ketise
EECC-528	Stem	*Plectosphaerella cucumerina*	3.0	Torech

In vivo test of selected endophytes for disease suppressive properties

Endophytes were inoculated in test plants two weeks prior to the inoculation of the pathogen. There were a total of 18 treatments divided in to four major categories (Table 3.1). 1) Plants inoculated with pathogen + different combinations of antagonistic organisms, 2) Control plants inoculated with pathogen only, 3) Plants inoculated with endophyte only, 4) control plants inoculated with sterile water. The virulence property of the pathogen was manifested

on plants inoculated with the pathogen showing typical symptom of wilt ten days after inoculation while control plants remained healthy.

Infected plants were monitored for two months and an increase in disease severity was observed in all inoculated plants. Four measurements of the disease severity were taken in eight weeks and analysis of variance was calculated for the mean disease severity of the four measurements (Table 3.6). Overall analysis of variance for the means of disease severity for eight weeks showed plants inoculated with mixed culture of bacteria (Treatment 11) has significantly differerent lower disease severity at ($P< 0.05$).

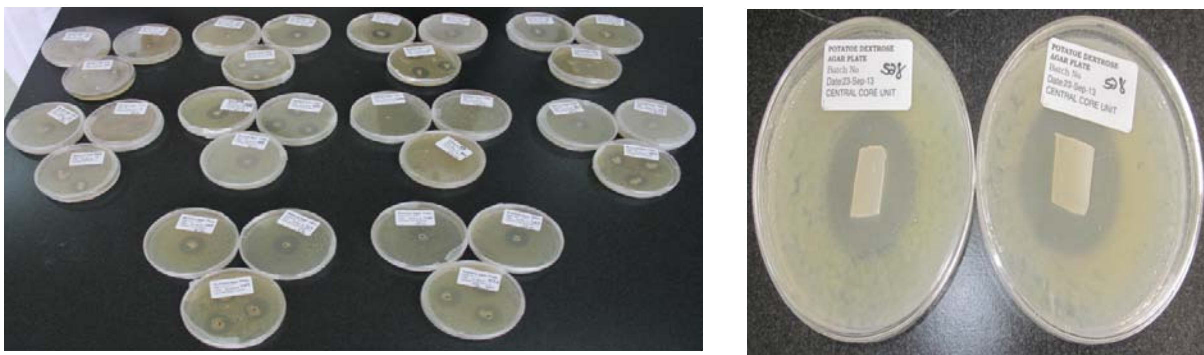


Figure 3.2 Pictures showing *in vitro* inhibition of the pathogen by endophytic bacteria and fungus.

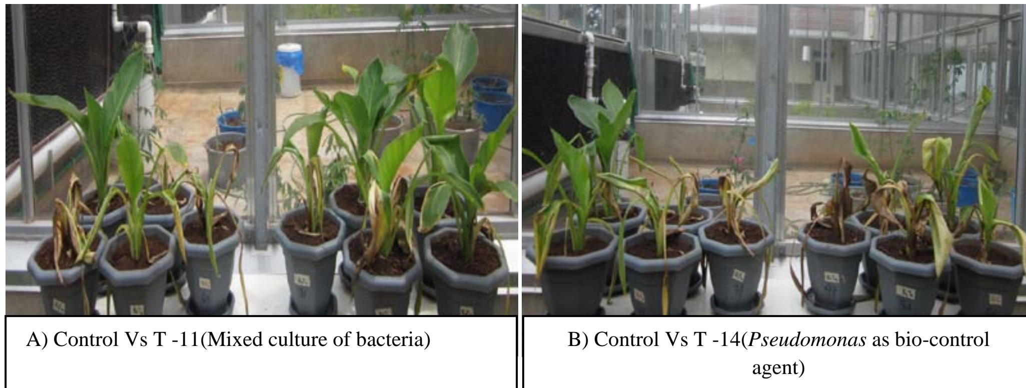
On visual observations, the plants treated with a mixed culture of bacteria had lesser disease symptoms manifested on the leaves (Fig 3.3). The mean disease severity was lowered by 30.7% after eight weeks of pathogen inoculation while control plants showed disease severity of 47.4%. On the other hand, in treatment 14 where a single culture of pseudomonas was inoculated disease severity was higher than the control (47.4% vs 52.3%).

Table 3.6 Analysis of variance for means of disease severity

ANOVA for the overall mean disease severity		
Treatment	Inoculum culture/s	Mean disease severity %
10	*Xanthomonas campestris* pv. *musacerum*	47.4
11	EECC-550, EECC-679 , EECC-572 , EECC-12 , EECC- 562, EECC-53, EECC-574 , EECC-543, EECC-37, EECC-682, EECC- 484, EECC-55, EECC-671 + Pathogen	30.7
12	EECC-454, EECC-528	50
13	EECC-550, EECC-679, EECC-572 , EECC-12, EECC- 562, EECC-53, EECC-574, EECC-543, EECC-37, EECC-682, EECC- 484, EECC-55, EECC-671, EECC-454, EECC-528 + Pathogen	42.6
14	EECC-550 + Pathogen	52.3
15	EECC-572 +Pathogen	44.8
16	EECC-679 + Pathogen	39.9
17	EECC-454 + Pathogen	38.2
18	EECC-528 + Pathogen	42.2
	Grand Mean	43.1
	LSD	10.9
	CV	17.3
	F pr	0.016

Treatments 1-8 were inoculated with the corresponding endophyte cultures in the treatments 11-18 without the pathogen as controls if any of the endophytes cause disease like symptoms and treatment 9 was inoculated with sterile water as a control for any environmental contaminant that might cause wilt symptoms. Pathogen: *Xanthomonas campestris* pv. *musacerum*, the identification of each culture in the treatments is found in table 3.1

Moreover, variable disease severity and rate of symptom development was observed among treatments. The symptom development in the treatment which was inoculated with mixed culture of bacteria was slower than other treatments. Faster disease progress was observed in treatments inoculated with single isolate of pseudomonas as bio-control agent (Fig 3.4). As it is shown in Fig 3.4 treatments 1-9 which were not inoculated with pathogen didn’t show any disease symptoms and this confirms any of the endophytes inoculated as biocontrol agents did not have any pathogenic property on the plant and any disease symptom development in the experimental plants is due to the inoculated pathogen.



A) Control Vs T -11(Mixed culture of bacteria)

B) Control Vs T -14(*Pseudomonas* as bio-control agent)

Figure 3.3 Picture showing diseased plants in the control, treatment with mixed culture of bacteria and fungus, treatment with pure culture of *Pseudomonas* isolate.

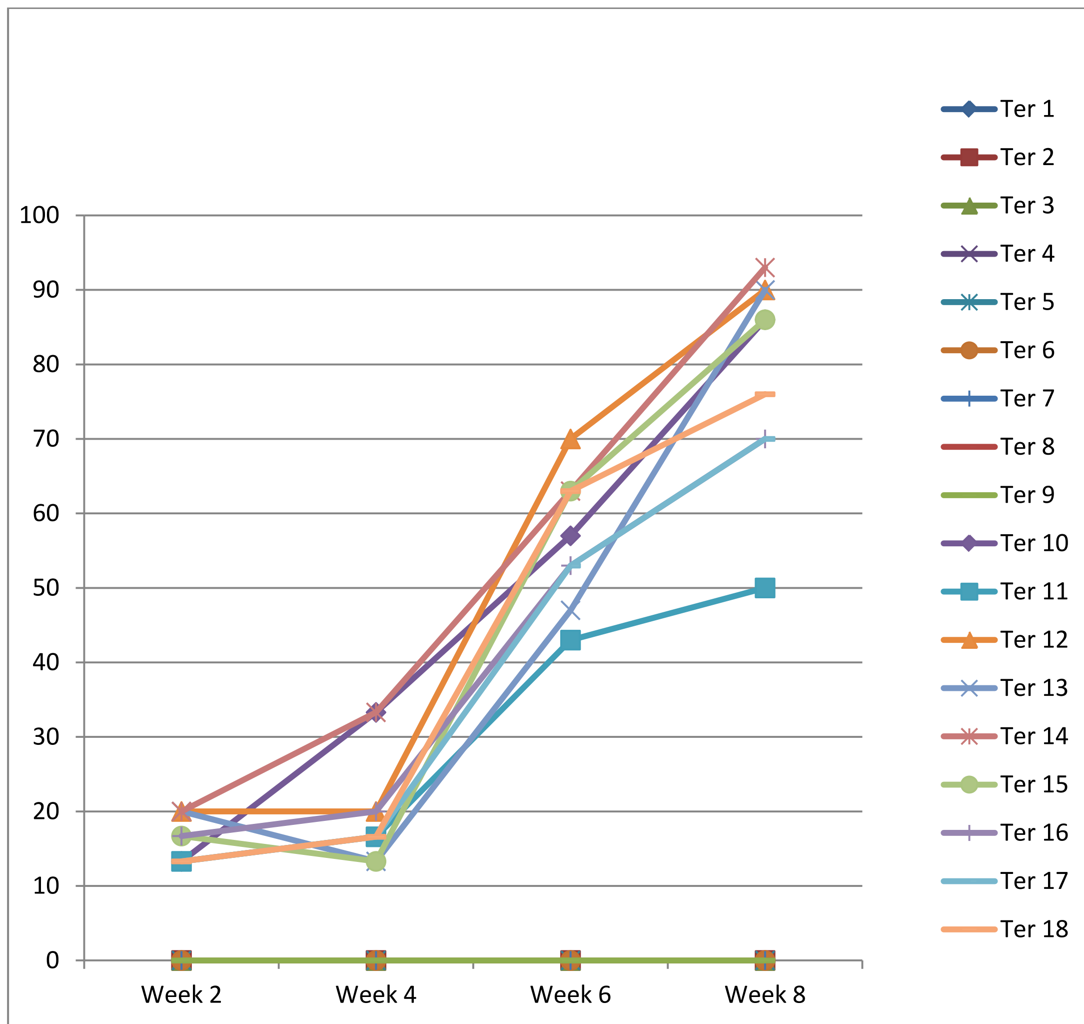


Fig 3.4 Disease progress curve showing percent disease severity at different weeks

Discussion

Enset is a crop mainly known for its high productivity and tolerance to harsh biotic and abiotic environmental conditions in different agro-ecologies. However, the factors that might have increased its productivity or the mechanisms of tolerance to harsh environmental conditions have not been well invistigated. Studies carried out on disease tolerance and increased productivity of several crops showed that endophytes can help their hosts to resist biotic and abiotic stresses and promote plant growth and health (Reinhold-Hurek and Hurek, 2011).

The majority of endophytes isolated from enset produced plant hormone IAA, which might be indicative of their potential involvement in promoting the growth of enset. Similarly, studies reported that production of IAA is a common benifical trait among many plant beneficial microorganisms (Etesami *et al*., 2013; Jasim *et al*., 2013).

The fact that a significant number of enset endophytic isolates were able to grow on NFM suggest that they have the capacity to fix atmospheric nitrogen. In most agricultural soil nitrogen is one of the most important limiting nutrients. The fact that nearly 50% of the enset endophytes were capable of growing on NFM might suggest that these organisms help the plant to grow in nitrogen poor soil. Thus availability of nitrogen by the help of these microbes might have contributed to the characteristics of the crop related to high productivity. Phosphate is another limiting nutrient for plant growth. Although it was not widely detected among the tested isolates, phosphate solublizing property was observed in some of the enset endophytes. In many agricultural soils, phosphate is available as an insoluble phosphate where plants cannot easily take it up (Vitorino *et al*., 2012).

Different studies reported that plant associated endophytes have the capacity to solubilize insoluble phosphate. In our study, the proportion of bacterial endophytes capable of solubilising phosphate was relatively small (29%) compared to the 71% of bacterial endophytes that are not solublizing phosphate. Moreover, none of the fungal isolates were able to solublize phosphate. Similar observations were made by Khan *et al*, (2007) that phosphate-solubilising bacteria constitute 1–50% and fungi 0.5%–0.1% of the total respective population in soil sample and generally indicated that phosphate-solubilizing bacteria outnumber phosphate-solubilizing fungi by 2–150 times. Similarly Vitorino *et al* (2012) reported that none of the fungal endophytes in their study were able to solubilise $CaHPO_4$ while they have the properties of solubilising $FePO_4$.

Another important PGP characteristic that was manifested by both bacterial and fungal isolates was the production of siderophores compounds that bind iron. Plant associated microbes are known to produce these compounds and chelate iron from the surrounding environment. This property has dual effect on the microbe and plant interaction (Jasim *et al*., 2013). The direct effect is to increase the availability of iron for growth of the beneficial microbes and the host plant and the indirect effect is collection of iron from the surrounding environment and makes it unavailable to pathogenic microbes. As a result, siderophore production is one of the many characteristics of biocontrol agents.

Production of hydrolytic enzymes like cellulase, xylanase and pectinase is commonly linked to ability of an endophyte to enter and colonize inter and intracellular compartment of plant tissue (Dai *et al*., 2010; Mittler *et al*., 2013; Kalai-Grami *et al*., 2014). Thus the production of pectinase and cellulase enzymes by enset endophytes in this study might indicate that these hydrolytic enzymes are useful to degrade plant material for intercellular and intracellular colonization of the host plant.

In a study by Quadt-Hallmann et al, 1997, degradation of cell wall-bound cellulose by endophytic *Enterobacter asburiae* was considered as mechanism of entry to cotton plants. In addition, other mechanisms like invagination of the root hair cell wall, by penetration of the junction between root hair and adjacent epidermal cells, passive plant uptake were discussed as general entry and colonization mechanisms. In this study also, not all isolates produced the hydrolytic enzymes, and other mechanisms mentioned here might be used by the endophytes to enter and colonize their host. Apart from the mentioned functions, some studies consider the production of hydrolytic enzymes with induction of systemic resistance.

The *in vitro* inhibition effect of the endopytes on the pathogen and the presence of other anti pathogen properties like siderophore production by the same isolates, EECC-12 (*Bacillus sp*), EECC-572(*Pseudomonas sp*), EECC-671(*Bulkoholderia sp*.) and. EECC-679 (*Pseudomonas sp*) showed the endophytes might be equipped with combination of indirect plant pathogen suppressive traits that include antibiosis cell wass degrading enzymes and induced systemic resistance and some of the properties are indedeciated in (Table 3.2, Table 3.3 and Table 3.4).

In the green house study, thirteen bacterial and two fungal isolates were inoculated in single and mixed cultures. Majority of the isolates that showed growth inhibition *in vitro* were members of the genus *Pseudomonas (*Table 3.5). Many of the biocontrol and plant growth promoting agents studied in previous studies also belong to this genus (Compant *et al*., 2005; Dong-sung *et al*., 2007, Rashid et *al*., 2012; Gupta *et al*., 2013). Moreover, bacteria in the group *Pseudomons* is known to survive both in soil and plant environment and considered as good candidate as biocontrol agents (Jousset *et al*., 2006).

The presence of *Pseudomonas* isolates in a relatively large numbers, the presence of more number of antagonistic *Pseudomonas* against the wilt pathogen and the presence of PGP *Pseudomonas* isolates can be a very interesting combination to further focus on *the group* to use them as biocontrol agents against *Xanthomonas campestris* pv. *musacerum*. In addition to *Pseudomonas*, Bacillus, Rhizobium and *Bulkoholderia sp* showed *in vitro* inhibition of the pathogen. Previous biocontrol studies also showed the potential of *Pseudomonas* as biocontrol agents and the wide range of antimicrobial metabolites produced by this group (Abraham *et al*., 2013).

Most of the isolates that showed inhibition of the pathogen were isolated from root samples. As endophytes enter in to the plant from the surrounding environment, the detection of higher proportion of the antagonistic endophytes detected from the root might indicate that the original source of these antagonistic organisms could be the rhizosphere. Furthermore, as most microbes which adapt the rhizosphere are living in a competitive environment and are considered as good candidates as bio-control agents, those endophytes originated from the rhizosphere might have a better advantage to adapt the rhizosphere and endosphere environment for further application of the endophytes in controlling the pathogen.

In microbial antagonism experiments, the straight forward approach to select an antagonist is to choose those ones which give clear zone as indication of inhibition of the growth of the test pathogen. Thus, some of the endophytes like EECC-550 (*Pseudomonas sp*.), EECC-679 (*Pseudomonas sp*.), EECC-572(*Pseudomonas sp*.) and EECC-12 (*Bacillus sp*.) were selected based on the wider clear zone detected during agar well diffusion test. However, since a successful endophytic biocontrol agent needs to have additional characteristics like efficient plant colonization property, isolates with smaller inhibition diameter but different taxonomic

identity were included with the assumption that they might have variable efficiencies in their plant colonization ability (Table 3.5).

Better suppression of disease was observed in the treatment where plants were inoculated with mixed culture of bacteria. This might be due to several factors that include a better plant colonization, increased antagonistic potential or synergetic effect of microbe to microbe interaction. In addition some of the interaction might also induce systemic resistance. Since there could be complex form of interactions that exist in the plant, extensive investigation on the plant and microbial community interactions is needed to pin point the mechanisms that improved disease suppression by the mixed culture of bacteria.

Similar results were observed in a study by Jie *et al*, (2009) where inoculation of tissue cultured banana plants with naturally occurring endophytes gave better resistance to fusarium wilt disease and also promoted plant growth. Their work also discussed the higher disease suppression observed in studies that used mixed culture of biocontrol agents compared to using a single biocontrol agent.

On the other hand, there was also treatment 14 which was inoculated with a single culture of *Pseudomonas sp* (EECC-550) and had higher disease severity (Table 3.6). However, during metabolomics study, the unique property of the isolate (EECC-550) was production of cyanide containing compounds. Although cyanide is one of the antimicrobial compounds that suppress plant pathogens, study by Blom *et al*, 2011 suggested that the production of cyanide by *Pseudomonas aeruginosa* was also observed to affect the plant health and resulted in a weaker plant physiology. Similarly, production of cyanide by EECC-550 might be toxic to enset platelets and resulted in a higher disease severity in treatment 14 where the isolate was inoculated as a biocontrol agent.

In general the *in vitro* and *in vivo* studies of the endophytes from enset showed promising results to further study them for their both direct and indirect plant growth promotion properties. Since this is the first attempt on enset endophyte mediated plant growth promotion and bacterial wilt disease control many of the experimental factors needs to be further optimized for a better understanding of the microbe-microbe, microbe-plant and microbe environment interaction of the endophytes in order to use them as plant health and growth promotors.

Chapter 4

Metabolite profiling of antagonistic bacterial endophyte of enset

Abstract

Plant associated microbes produce vast diversity of metabolic products that include important antimicrobial compounds. *Xanthomonas campestris* pv. *musacerum* is a bacterial pathogen that causes bacterial wilt disease on enset and banana. This study was conducted to investigate the effect of (VOCs) and (NVDOCs) on the growth of *Xanthomonas campestris* pv. *musacerum.* Fourteen endophytic isolates were screened for their inhibition of the pathogen both VOCs and NVDOCs. On split plate assay seven isolates were able to inhibit the pathogen completely by VOCs. Comparative study of VOCs of two *Bacillus* isolates that had inhibitory and none inhibitory effect indicated the presence of unique cluster of metabolites that potentially contain the inhibitory compound/s. Compounds like Eicosane, 2, pentanone, 3-methyl, dimethyl disulfide, Dimethyl pentasulfide, Hexanone and other compounds including unknowns were identified as unique to the isolate EECC-324. The NVDOC study of the two isolates also revealed the presence of a unique cluster of compounds in EECC-12 which showed inhibition through NVDOC. The metabolomics study of the isolates indicated that there are diverse known and unknown compounds which might have antimicrobial effect on the pathogen and further screening is needed to know the potential ecological role. In general, the diverse groups of compounds produced by the endophytes might indicate the untapped metabolic potential which can be exploited to extract and identify antimicrobials to control *Xanthomonas campestris* pv. *musacerum.*

Key words: Microbial metabolites, VOC, NVDOC, *Xanthomonas campestris* pv. *musacerum*

Introduction

Microbial bioactive secondary metabolites are produced both as VOCs and NVDOCs. Until recently, most studies on endophytes were focused on the search for NVDOC antimicrobials (Garbeva *et al*., 2014). But, currently there is an increasing attention given to volatile antimicrobials for the management of plant pathogenic microorganisms (Raaijmakers & Mazzola, 2012; Hernández-León *et al*., 2015).

Since the first report of the plant growth promotion effect of bacterial volatile 2,3-butanediol and acetoin on *Arabidopsis thaliana*, VOC producing bacteria and fungi are attracting wider interest in the search of compounds that are plant growth promoting and antagonistic to plant pathogens and pests (Ryu *et al*., 2003; Fernando *et al*., 2005). These compounds can act across long distance and pass through soil particles and also can act in the aerial parts of plants. Hence, they are considered to be more efficient than liquid fungicides (Zhou *et al*., 2014). Thus, VOC from plant associated and soil bacteria and fungi are becoming the subject of many studies in search of biocontrol agents to suppress different fungal and bacterial plant pathogens.

VOCs produced by bacteria include; fatty acids and their derivatives (e.g. hydrocarbons, aliphatic alcohols and ketones), aromatic compounds (e.g. alcohols and phenols), nitrogen containing compounds, terpenes, pyrazines, and sulphur compound (Schmidt *et al*., 2015). In general, a wide variety of VOCs with known and unknown functions are produced by bacteria. According to Kai *et al*., 2009, the classification of bacterial volatiles has revealed 75 fatty acid derivatives, 50 aromatic compounds, 74 nitrogen-containing compounds, 30 sulfur compounds, 96 terpenoids, and 18 halogenated selenium, tellurium, or other metalloid

compounds. On the other hand volatiles from fungus are mostly alcohols, benzenoids, aldehydes, alkenes, acids, esters and ketones (Morath *et al*., 2012; Schmidt *et al*., 2015).

Most of the works done so far are focused on the action of beneficial bacteria and fungi VOCs against fungal pathogens (Zhou *et al*., 2014). In this regard, several volatile compounds have proven to have a potential antimicrobial activity. The phenol compound 2-acetamidophenol produced by of *Pseudomonas fluorescens* strain 2-79 (NRRL B-) was shown to suppress the fungal pathogen responsible for take-all disease of wheat (Saraf *et al*., 2014). Wang *et al*., (2013) also showed that VOCs produced by *Streptomyces* could inhibit conidiophores and hyphae in fungi.

Inhibition was observed on mycelia growth, spore and conidial germination of *Fusarium moniliforme* after exposure to volatiles of *Streptomyces alboflavus* TD. Mitchell *et al*., 2010 also reported potential blend of antimicrobial VOCs, from *Muscodor crispans* that are active against different plant fungal and bacterial pathogens as well as a number of human pathogens. Ethylene, hydrogen cyanide, dimethyl disulfide, cyclohexanal, 2-ethyl, 1-hexanol, pyrazines and phenols are some of the well studied volatile organic compounds with antimicrobial activities against bacterial and fungal plant pathogens (Kai *et al*., 2009; Yu *et al*., 2010; Saraf *et al*., 2014, Schmidt *et al*., 2015).

In the effort of finding NVDOC antimicrobial agents against plant pathogens, diffusible phenazines, pyoluteorin, and pyrrolnitrin are among the well-characterized antibiotics that play an important role for plant protection and produced by the Gram-negative bacteria that are used as biocontrol agents (Haas and Défago, 2005). Lipopeptides that have antibacterial and antifungal activity are another widely studied class of diffusible antibiotics (Haas and

Défago, 2005; Raaijmakers *et al*., 2010; Zeriouh et *al*., 2011). In additon some recent studies indicated that the actual microbial-inhibitory activity could probably be caused by a mixture of antimicrobial metabolites (Traxler *et al*., 2013; Zhou *et al*., 2014; Schmidt *et al*., 2015).

Other studies also showed that diverse group of antimicrobials produced by several plant beneficial microbiome including endophytic bacteria and fungi. The major NVDOC produced by endophytes that protect the host plant include alkaloids, peptides, steroids, terpenoids, quinones, flavonoids, aliphatic compounds, phenols, polyketides and chlorinated metabolites (Gunatilaka, 2006; Yu *et al*., 2010, and Mousa & Raizada, 2013). Most of these compounds are active against bacterial and fungal pathogens as well as against insect and nematode pests (Tan & Zou, 2001; Firakova *et al*., 2007).

The production of antimicrobials by endophytic bacteria and fungi was demonstrated in several studies and among these, deposition of antimicrobial crystals of 2, 4-diacetylphloroglucinol (DAPG) by endophytic *Pseudomonas fluorescens* strain FPT 9601 around the roots of tomato plant, i*n vitro* production of bioactive compounds Kakadumycins, Coronamycin from different isolates of endophytic Streptomyces , isolation of Pentaketide antifungal agent CR377 from endophytic *Fusarium* sp. can be mentioned (Aino *et al*., 1997 and Saraf *et al*., 2014; Firakova *et al*., 2007; Subban *et al*., 2012; Strobel & Daisy, 2003).

In addition, Oocydin A is an antioomycetous compound isolated from *Serratia marcescens*, an endophytic bacterium associated with *Rhyncholacis penicillata* (Gunatilaka, 2006). Trichodermin isolated from endophytic fungus *Trichoderma harzianum*, Ilexcornuta, is reported to give protection against the Solanaceous plant pathogens *Alternaria solani* and *Rhizoctonia solani in vitro* (Mousa & Raizada, 2013). A phenol compound 2-Methoxy-4-

hydroxy-6-methoxymethylbenzaldehyde, produced by endophytic fungus *Pezicula* sp. strain 553, was inhibitory against phytopathogen *Cladosporium cucumerinum* (Tan & Zou 2001).

Material and methods

Two compartment (split-plate) assay to study the effect of VOCs on XCM growth

Selected bacterial endophytes (EECC-550 (*Pseudomonas sp.*), EECC- 574 (*Enterobacter sp.*), EECC-572 (*Pseudomonas sp.*), EECC-671 (*Burkoholderia sp.*), EECC-484(*Pedobacter sp.*).) with inhibitory activity during well-diffusion assay and other isolates (EECC-705(*Rahnella sp.*), EECC-9(*Enterobacter sp.*), EECC-356(*Burkoholderia sp.),* EECC-48 (*Puseudomons sp.*), EECC-324 (*Bacillus sp.)*, EECC-216 (*Variovorax sp.*).) with out inhibiton activity on well diffusion assay were tested for the effect of their VOCs on pathogen growth.

Bacterial cultures were refreshed on nutrient agar plates at 28°C for 48 hrs and suspension of each isolates were made using 0.85% NaCl solution and the OD was adjusted to 0.1 at 600 nm (10^8 cfu/ml). Fifty microliter of the suspension was spread-plated on one side of the two compartment plates and the other compartment was left empty. In case of the mix culture, equal proportion of eight isolates was mixed in a sterile eppendorf tube and 50 µl aliquot was inoculated on the respective plates. The plates were incubated at 28^0C for 24 hrs.

The growths of the potential antagonists were checked on all plates before inoculating the pathogen. Then, *Xanthomonas campestris* pv. *musacerum* culture was refreshed on nutrient agar plates to prepare a suspension in 0.85% NaCl. The pathogen cell suspension was inoculated on three spots where each spot contains 30 cfu/2 µl. Control plates were prepared by spot inoculating the pathogen suspension on plates without any endophytic inoculum. Each isolates was tested in four replicates.

The plates were sealed and incubated at 28^0C until visible growth was observed on the control plates. Growth of the pathogen colonies was scored five days after inoculation. The inhibition by endophyte originated VOCs was scored based on the level of inhibition of Xcm growth, the isolates were classified into three groups; complete inhibitors (CI) where no growth of pathogen colonies detected, partial inhibitors (PI) where there is growth but vusually less growth of the pathogen compared to the control and non-inhibitors (NI) where the growth of pathogen colonies is equivalent with the control plates (Table 4.1).

Trapping of volatile metabolites from endophytic isolates

Each isolate was refreshed on nutrient agar plates to make suspensions in 0.85% NaCl to bring the OD to 0.1 at 600 nm. One hundred µl of the suspension was spread-plated on special petri dishes with leads to which a steel trap containing 150 mg Tenax TA and 150 mg Carbopack B (Markes International Ltd., Llantrisant, UK) was fixed and used for volatile trapping (Garbeva *et al*., 2014). The petri dishes were sealed and incubated at 28^0C for five days. Then the Tenax steel traps were removed at day 5 capped and stored at 4^0C until GC-QTOF-MS analysis. All treatments were inoculated in triplicates.

GC-QTOF-MS for volatile metabolite profiling

Volatiles were desorbed from the traps using an automated thermodesorption unit (model UnityTD-100, Markes International Ltd., Llantrisant, UK) at 210^0C for 12 min (He flow 50 mL/min) and trapped on cold trap at -10^0C. The trapped volatiles were introduced into the GC-QTOF (model Agilent 7890B GC and the Agilent 7200A QTOF, Santa Clara, USA) by heating the cold trap at 280^0C for 3 min with split ratio set to 1:20. The column used was a 30×0.25 mm ID RXI-5MS, film thickness 0.25 µm (Restek 13424-6850, Bellefonte, PA, USA).

Temperature was adjusted to 39^0C for 2 min, from 39 ^{0}C to 95^0C at 3.5 ^{0}C/min, then to 165 ^{0}C at 4 ^{0}C/min, to 280 ^{0}C at 15 ^{0}C/min and finally to 325^0C at 40 ^{0}C/min, hold 20 min. The VOCs were detected by the MS operating at 70 eV in EI mode. Mass spectra were acquired in full scan mode (40–400 amu, 4 scans/s). MassHunter Qualitative Analysis Software V B.06.00 Build 6.0.633.0 (Agilent Technologies, Santa Clara, USA) was used to control the instrument and for data acquisition and analysis (Schmidt *et al*., 2015).

Assay for diffusible inhibitory metabolites

Fresh culture of each isolate was used to prepare a suspension of 0.1 OD at 600 nm for inoculating broth culture needed for well-diffusion assay 2 ml/100 ml of the suspension were inoculated in nutrient broth and incubated at 28^0C for 48 hrs. Then, bacteria containing cultures were used to conduct assay for their inhibition on *Xanthomonas campestris* pv. *musacerum.* Nutrient agar plates were first spread-plated with 100 µl of a 10^8 cfu/ml suspension of the pathogen and subsequently inoculated with 30 µl of endophyte broth culture in well-diffusion method. Control plates were inoculated with the pathogen suspension only. Plates were incubated at 28^0C until the growth of the pathogen is visible on the control plates.

In parallel with conducting the well diffusion assay with a broth containing the bacteria, the endophyte cultures were filtered using 2 µm syringe filters from which 30 µl the filtrate were inoculated on nutrient agar plates spread plated with 100 µl of 10^8 cfu/ml suspension of the pathogen and incubated at 28^0C until the pathogen growth was observed on the control plates. The presence of inhibition from the broth culture was scored qualitatively by checking clear zone formation around the wells.

Extraction of metabolites

Ethyl acetate extraction

Nutrient agar plates were prepared and spread-plated with 100 µl of each isolate and in four replicates and incubated at 28^0C for three days. After three days, the agar zone carrying bacteria was cut into small pieces and transferred to 100 ml red-screwcap bottles to which 20 ml of 80% (v/v) acetone was added. The whole content was shaken at 200 rpm for 1 hr. The liquid part of the content was transferred to new falcon tube and centrifuged for 10 min at 4800 rpm, at 4^0C. The supernatant was transferred to new 100 ml bottle and the acetone was evaporated using air blower until the water fraction was left.

The water fraction was acidified with trifluoroacetic acid (0.1% (v/v)), mixed with 2 volumes of ethyl acetate and shaken vigorously at 200 rpm for 5min. The extract was stored at -20^0C overnight to freeze the water fraction. On the next day, the unfrozen (ethyl acetate) fraction was transferred into falcon tube and dried under air flow. The dried extract was dissolved in 400 µl of 50% (v/v) methanol while keeping it on ice and stored at -20^0C until further use (Raaijmakers *et al*., 1999). The extract samples were tested for their bioactivity against the pathogen using disc-diffusion method (Ho *et al*., 1998).

Methanol extraction

Each isolate was grown in liquid nutrient broth culture for three days. Then, the whole content was centrifuged and the supernatant was further filtered using 0.2 µm inorganic membrane filter and the filtrate was subjected for freeze-drying. Semi-polar secondary metabolites were extracted from the freeze-dried filter samples. Briefly, 2 ml of 75% Methanol containing 0.1% formic acid was added to the freeze dried samples and vigorously mixed on vortex mixer and sonicated for 15 min. The sonicated samples were centrifuged at

20,360 x g and the supernatant was transferred to new Eppendorf tube. Samples were tested for their bioactivity using disc-diffusion method (Raaijmakers *et al*., 1999).

LC-MS for non-volatile diffusible organic compounds

LC-MS profiling of the crude extracts of the freeze-dried-culture-filtrate was performed using an Q Exactive™ Hybrid Quadrupole-Orbitrap FTMS Mass Spectrometer (LC-Q-Orbitrap-FTMS-MS) instrument consisting of a Dionex UltiMate 3000 U-HPLC system for fast chromatographic separation with a PDA (photodiode array) detection interfaced to a combined quadruple precursor ion selection with high-resolution, accurate-mass (HRAM) and Orbitrap detection (Thermo Fisher Scientific) equipped with an ESI source. The online scan-to scan polarity (+/-) switching capability at high mass resolution (280, 000 at *m/z* 200) allows the most comprehensive metabolomics profiling of compounds present in complex samples.

The sample injection volume was 5 µL. A Luna RP-C18 (2) analytical column (Phenomenex, USA) was used for chromatographic separation. The mobile phase consisted of a binary eluent solvent system of degassed ultra-pure water (solvent A) and acetonitrile (solvent B), both containing 0.1% v/v FA, while keeping the flow rate of the eluents at 0.19 $ml.min^{-1}$ and maintaining the column temperature at $40^{0}C$. The HPLC gradient started at 5% B and linearly increased to 35% B across a period of 45 min. The column was re-equilibrated for 15 min following the separation of each sample (Vos *et al*., 2007; van der Hooft *et al*., 2011).

Data processing

|Mass chromatograms obtained from GC-Q-TOF-MS and LC-Q-Orbitrap-FTMS-MS analysis were processed (peak picking, baseline correction and peak alignment) in untargeted manner using the MetAlign software package (Lommen, 2009). Extraction and reconstitution of

compound mass spectra were performed according to the method described by Tikunov et al. (2012). For VOCs identification, an automated NIST-MS Search combining spectra matching, accurate mass information and retention indices was performed using NIST 2014 V2.20 (National Institute of Standards and Technology, USA, http://www.nist.gov) and Wiley 9th edition spectral libraries.

Multivariate statistical analysis

Gene Maths XT (Applied Maths, Belgium) was used for hierarchical clustering analysis (HCA). While performing the HCA Pearson's correlation coefficients were used to calculate the distance or similarity between two entries. The resulting clusters were summarized using a complete linkage algorithm. To compare metabolite levels, their relative intensity values obtained from the LC-MS data processing were log2 transformed and scaled by using the average as an offset and the standard deviation for scaling ((x-average (offset)) / SD (scale)).

Results

Inhibition assay through volatile organic compounds on split plate assay

In the split plate assay, the effect of VOCs produced was assayed by the fifteen different isolates and the mixed culture on the growth of Xcm.

Table 4.1Qualitative scoring of enset endophytes on inhibition of the pathogen through VOC

Culture code EECC-	Level of inhibition	Identification	Sampling site	Enset variety for isolation	Plant part	Clone rating by farmers on disease response
550	CI	Pseudomonas	Telo	Wild	Root	Tolorant
705	"	Rahnella	Alechowerero	Benzena	Root	Moderate
9	"	Enterobacter	Atat	Nchewe	Root	Tolerant
574	"	Enterobacter	Telo	Wild	Stem	Tolerant
572	"	Pseudomonas	Telo	Wild	Stem	Tolerant
356	"	Burkoholderia	Cenecha	Maze	Root	Highely tolerant
671	"	Burkoholderia	Alechowerero	Tereye	Root	Moderate
484	PI	Pedobacter	Mareka	Maze	Root	Highely tolerant
48	"	Pseudomonas	Atat	Nechewe	Root	Tolorant
216	"	Variovorax	Howelso	Wankore	Root	Moderate
543	"	Variovorax	Telo	Wild	Root	Tolorant
324	"	Bacillus	Chencha	Chemo	Leaf	Moderate
Mix	"	Mix of 8 isolates		Different	Different	---
12	NI	Bacillus	Atat	Nechewe	Stem	Tolerant
562	"	Acenitobacter	Telo	Wild	Root	Tolerant

Mix: Proportiional mixture of isolates; EECC-550, EECC-679, EECC-572, EECC-12, EECC-562, EECC-574, EECC-543, EECC-484, EECC-671.

Seven isolates EECC-550 (*Pseudomonas sp.*), EECC-705 (*Rahnella sp.),* EECC-9 (*Enterobacter sp.*, EECC-574 (*Enterobacter sp.*), EECC-572 (*Pseudomonas sp.*, EECC-356 (*Burkoholderia sp.*), EECC-671(*Burkoholderia sp.*) which didn't show any type of the

pathogen growth was scored as complete inhibitors, five isolates and the mix culture with very stunted growth of the pathogen colonies were scored as partial inhibitors and two isolates with the visible growth of the pathogen spots were scored as non-inhibitors (Table4.1 and Figure 4.1).

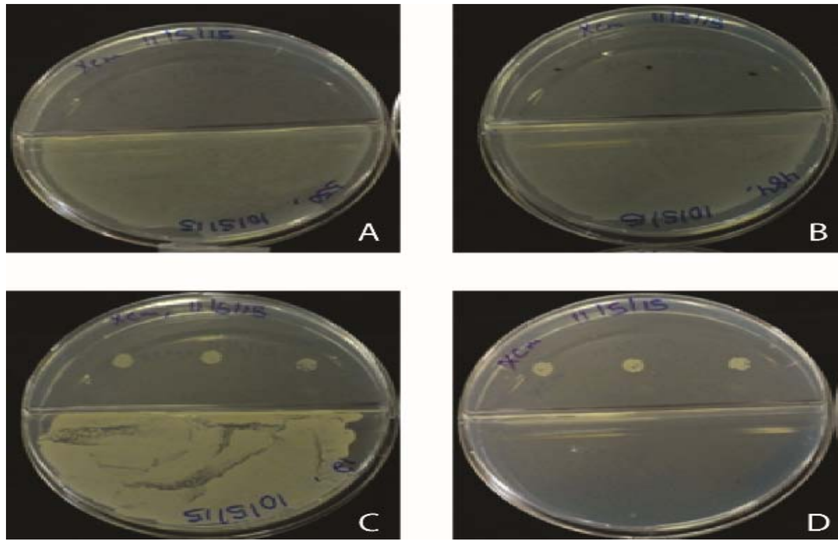


Figure 4. 1 Inhibition of XCM growth by enset endophyte-associated VOCs in split plate assay. A) Assay where the pathogen growth is completely inhibited. B) Assay where the stunted pathogen was not easily visible. C) Assay where the pathogen growth was visible. D) Control plates with pathogen spots only.

To unravel the "*Xanthomocidal*" VOCs produced by the isolates, untargeted metabolomics using high resolution GC-QTOF-MS was performed. A total of 336 VOCs were detected and out of which 308 were significantly different at least between two isolates and were used to perform the hierarchical cluster analysis (HCA) (Figure 4.1(A)). The clustering of the isolates was based on the VOCs profile that were predominantly reflects the phylogenetic relationship between the isolates. Moreover, the HCA based on the VOCs indicated complementarily between the VOCs profile and the partial 16S rRNA gene sequencing-based classification of the isolates.

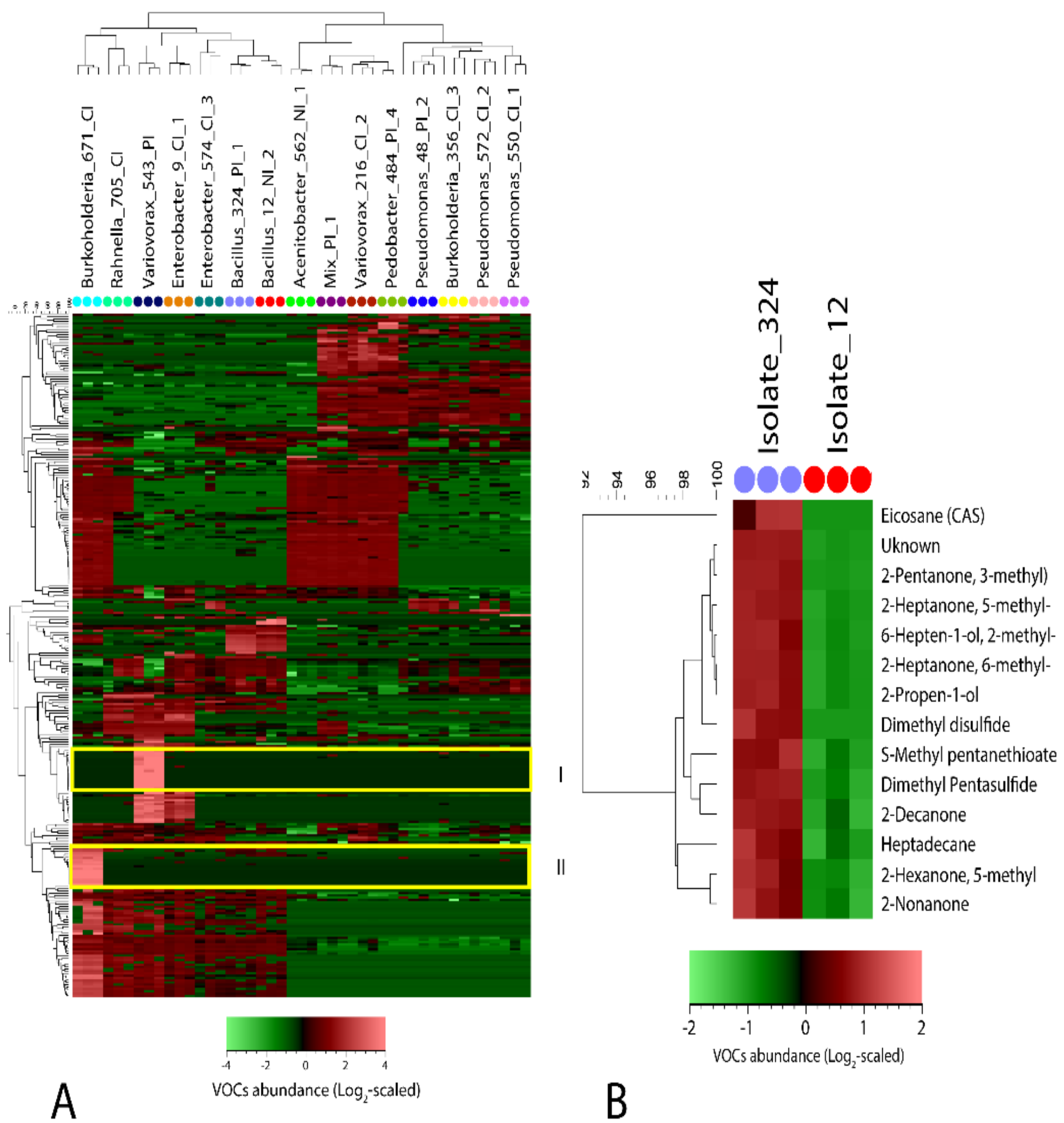


Figure 4.2 Hirarchical cluster analysis (HCA) of enset endophytes based on VOCs. (**A**) Heat map showing the abundance and distribution of VOCs in different enset endophytes. VOCs that were abundantly produced by the isolates are represented by the red patches and lowly abundant one are represented by the green patches (B) Volatile organic compounds that showed higher emission from isolate EECC-324 that showed partial inhibition against the pathogen in the split-plate assay.

Isolate EECC-324 *Bacillus sp* and EECC-12 *Bacillus sp*. were classified as *Bacillus* based on the partial 16S rRNA gene sequence similarity and VOCs profile. In the split plate assay, VOCs from isolate EECC-324 showed partial inhibition (PI) against the pathogen while isolate EECC-12 showed no inhibition (NI). The VOC profile revealed that there were 22 VOCs that were significantly different between the two isolates, out of which 14 showed significantly higher abundance in isolate EECC-324 that partially inhibited the pathogen (Figure 4.2(B)).

3.2 Inhibition assay through nonvolatile diffusible organic compounds (NVDOCs)

The inhibition from NVDOCs was carried out by using broth cultures, culture filtrates and extracts of the culture filtrate (Table 4.2). Most of the isolates were positive for antagonistic effect when the broth cultures were used (Table 4. 2). However, the culture filtrate from most of the isolates didn't show any inhibitory effect except for isolate EECC-12**.** Based on this, isolate EECC-12 and EECC- 324 that were categorized as *Bacillus sp.* based on the partial 16S rRNA sequencing were considered for the comparative analysis of X*anthomocidal* NVDOCs.

Table 4. 2: Inhibition of *Xanthomonas campestris* pv. *musacerum* by diffusible compounds.

Isolate	Inhibition from cultue broth			Inhibition from filtrate			Inhibition from ethylacetate extract			Inhibition from Methanol extract			Remark
EECC-12	+	+	+	+	+	+	+	+	+	-	-	-	Growth on filter & ethyl acetate extract
EECC-572	+	+	+	-	-	-	-	-	-	-	-	-	
EECC-671	+	+	+	-	-	-	-	-	-	-	-	-	
EECC-9	+	+	+	-	-	-	-	-	-	-	-	-	
EECC-356	+	+	+	-	-	-	-	-	-	-	-	-	High growth on the plates
EECC-574	+	+	+	-	-	-	-	-	-	-	-	-	
EECC-543	+	+	+	-	-	-	-	-	-	-	-	-	
EECC-550	+	+	+	-	-	-	-	-	-	-	-	-	
EECC-705	+	+	+	-	-	-	-	-	-	-	-	-	
EECC-324	-	-	-	-	-	-	-	-	-	-	-	-	High growth on the plates
EECC-216	-	-	-	-	-	-	-	-	-	-	-	-	
EECC-48	+	+	+	-	-	-	-	-	-	-	-	-	
EECC-562	+	+	+	-	-	-	-	-	-	-	-	-	
EECC-484	+	+	+	-	-	-	-	-	-	-	-	-	
Mix	+	+	+	-	-	-	-	-	-	-	-	-	
Control	-	-	-	-	-	-	-	-	-	-	-	-	

To analyze the semi-polar metabolite profile of the isolates, untargeted metabolomics using Q Exactive™ Hybrid Quadrupole-Orbitrap FTMS Mass Spectrometer (LC-Q-Orbitrap-FTMS-MS) instrument was performed. In the analysis that was done in both positive and negative ionization mode, a total of 679 semi polar secondary metabolites were detected in the MeOH extract of the culture filtrate. Out of the 679 semi-polar secondary metabolites 200 of them were significantly different between isolate EECC-12 and the control isolate EECC-324.

The HCA that was based on the 200 metabolites was characterized by the presence of 6 metabolite clusters that showed different pattern of accumulation between the control

samples and the isolates (**Fig 3**). Metabolites in cluster I were abundantly present in the *Xanthomocidal* isolate EECC-12. Interestingly, metabolites in cluster IV showed higher accumulation showing the isolate could potentially use metabolites in cluster I to synthesize metabolites shown in cluster IV. The comparative analysis of NVDOC in Fig 4.3 shows that metabolites in cluster IV are only detecected in the *Bacillus sp* (EECC-12) and not in the control or in the other *Bacillus sp*. (EECC-324) that didn't show inhibiton effect. Therefore, the observed inhibitory effect by NVDOC from EECC-12 could be on or more of metabolites present in cluster IV.

Further works that include LCMS/MS and NMR analysis is required to identify metabolites that are abundantly found in cluster IV. Metabolites in cluster III that were highly utilized by isolate 324 also could potentially be used to synthesize metabolites in cluster V. Similarly, metabolites in cluster II that were highly utilized by both isolates might be used to synthesize metabolites in cluster VI.

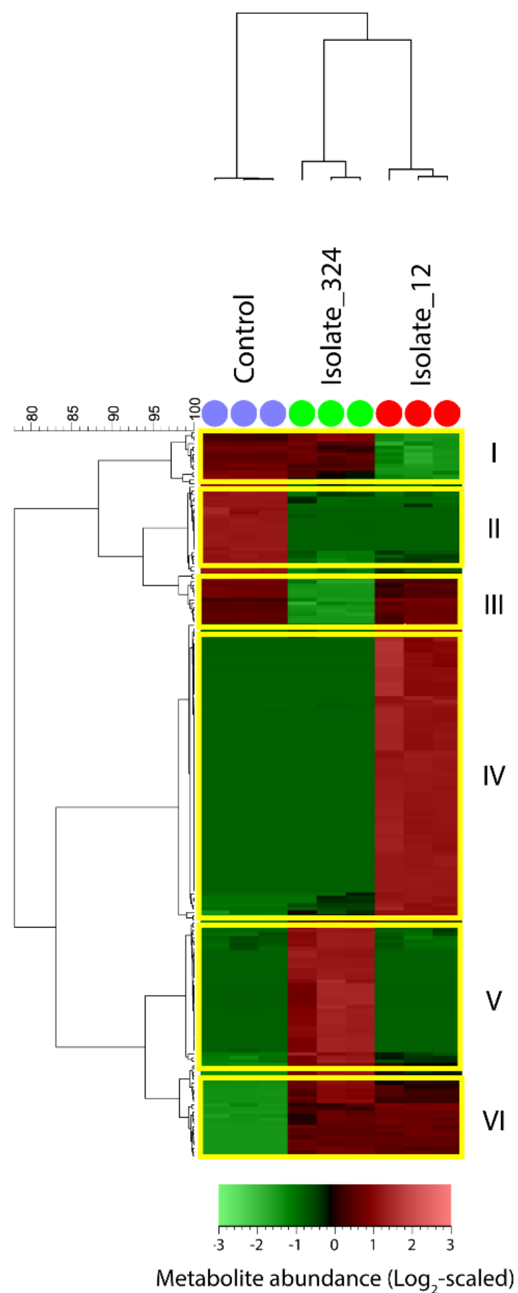


Figure 4. 3 Hierarchical cluster analysis (HCA) of enset endophytes based on nonvolatile diffusible organic compounds (NVDOCs). Each clusters marked by yellow line represents metabolites that have similar pattern of accumulation across different samples. Culture filtrate and ethyl acetate extract of the culture filtrate from isolate 12(EECC-12) showed inhibition against XCM in well-plate assay.

Discussion

The effect of microbial VOC's on plants is attracting the focus of many plant health and growth studies. To date several volatile and non-volatile compounds from plant beneficial microbes are identified. In this study, hundreds of volatile and NVDOC were identified from selected endophytic isolates. The compounds produced include phenols, lactone, cyanide, pyrazine and dimethyl Disulfide and these compounds were reported to have antimicrobial activity against different fungal and bacterial pathogens (Elkahoui et *al*., 2014, Schmidt *et al*., 2015). These compounds might have diverse plant beneficial effects.

It was also interesting to note that each isolate had compounds, which are uniquely produced by the specific isolate, and compounds commonly produced by two or more isolates (Fig 4.2 (A)). Furthermore, the variation in the level of inhibition observed during in vitro tests shown in Table 3.4 and Table 4.1. In general the results indicate the production of variable inhibitiory substance and/or concentrations which might inhibit the pathogen by single compound or by synergetic effect of more than one compound.

Comparative analysis of volatile metabolites produced by two *Bacillus* isolates, EECC-12 (none inhibitory *in vitro*) and EECC-324 (partially inhibitory *in vitro*), showed the production of 14 VOCs compounds belonging to alkanes, ketones (6 out of 14), alcohols, and sulfur containing compounds. These compounds could be the potential antimicrobial agents produced by isolate EECC-324. Moreover, the presence of more than one compound in this distinct cluster might indicate a cocktail of VOCs might be responsible for the inhibition of the pathogen growth (Figure 4.2B). Isolate 671 (complete inhibitor) and 543 (partial inhibitor) are characterized by the presence of a peculiar cluster of VOCs that showed higher accumulation in these isolates.

Interestingly, the *Xanthomocidal* property of most of the enset endophytes seems to be related to VOCs. This was indicated through checking none inhibitory effect of the cell free filter broth on the growth of the pathogen. As it is shown on table 4.2, the broth culture of the endophytes inhibited the pathogen. However, the cell free filtrate of the cultures was not able to inhibit *Xanthomonas* growth. These results partially indicated the presence of the bacterial cell is important enhance the inhibition by releasing VOC's.

Unlike other isolates, the cell free filtrate of the *Bacillus* isolate EECC-12 was able to inhibit the pathogen, and the comparative analysis of diffusible metabolites with isolate EECC-324 indicated one cluster of unique compounds n isolate EECC-12 was likely to be responsible for the pathogen growth inhibition. In general, compared to NVDOCs, VOCs showed the ability to travel longer distances to exert their action on the pathogen.

Moreover studies also showed that, volatile compounds have showen to have a synergistic effect when combined with antibiotic. For example, hydrophilic antibiotics such as vancomycin and β-lactam antibiotics, which have a marginal activity on the Gram-negative bacteria *E. coli* and *Listeria monocytogenes*, exhibit an enhanced antibacterial activity when pre-treated with the volatile eugenol synergistic effects of terpenes and penicillin on multi-resistant strains *S. aureus* and *E. coli* have also been reported (Schmidt et al., 2015). Therefore the indication of the inhibition of *Xanthomonas campestris* pv. *musacerum* by volatile organic compounds could be a more desired property to control the disease.

Finally, metabolomics screening of the function of microbial metabolites needs several steps of investigation. This work revealed several metabolomic information, which can be useful to design the way forward to continue the effect of the potential compounds on bacterial wilt disease control.

On the other hand some of the metabolites like the sulfur containing compounds detected in enset endophytes are known to have fungicidal effect (Schmidt *et al*., 2015). Therefore further study of the disulfide dimethyl compound might be useful to understand the wider antimicrobial potential of the metabolites produced by enset endophytes.

Chapter 5

Conclusions and recommendations

The problem of low crop productivity could be caused by diseases, pests and poor soil conditions. Exploiting microbial inoculums as bio-control agents and plant growth promoters are one of the strategies to overcome this problem. This research was undertaken to evaluate the existence of endophytes in enset and their potential roles to improve enset health and growth. The results showed that many more culturable bacteria than fungi exist as endophytic microbes in enset plant. Amongest the endophytes *Pseudomonas* and *Fusarium* were detected more frequently.

In the phenotypic studies of PGP, the endophytes showed one or more plant beneficial properties. Furthermore *Pseudomonas* isolates showed relatively more beneficial characteristics which might be indicative of their potential to be considered as interesting candidates in further investigating them as potential plant inoculum. The *in vitro* antagonistic properties of the endophytes against *Xanthomonas campestris* pv. *musacerum* showed indigenous endphytic bacteria and fungi have the property to antagonise the pathogen.

The ability of the endophytes to suppress the pathogen was also observed in the *in vivo* study where low levels of disease severity were observed in most of the treatments. Furthermore, the treatment 11 that was inoculated with a mixed culture of *Pseudomonas, Bacillus, Ensifer, Rhizobium, Rahenella, Pedobacter, Virovorax, Acitnobacter, Enterobacter, Bulkoholderia*, as biocontrol agents had significantly lower disease severity compared tothe control. Although it was not statistically significant, highest disease severity was observed in a treatment 14 where pure culture of *Pseudomonas* was inoculated as bio-control agent and

In relation to study on the compounds that potentially contributed to the effect of inhibition of the pathogen. It was observed that diverse group of volatile and none volatile compounds were produced by the endophytes. Among these phenols, lactone, cyanide, pyrazine and dimethyl disulfide compounds might have caused the inhibition of the pathogen. Moreover, as the endophytes showed different levels of inhibiton and produced both unique and common compounds, the mode of inhibition could be by a single compound and/or mixture of compounds.

Since this is primary screening of the compounds further screening and extraction and analysis is needed to pin point specific compounds that caused the inhibition. Moreover, the metabolomics profiling also showed that the endophytes could be source of compounds like sulphur containing compounds which are known for their antifungal activity. In conclusion, the multiple modes of actions with respect to wilt disease control and plant growth promotion were manifested by enset endophytes.

The following recommendations are given based on the results in this study.

- Single type of growth condition and only culture dependent methods were used in this study, therefore studies with diversified culture conditions and studies supported with culture independent methods will be important to get a better understanding of the actual endophytic microbial community associated with enset.
- Studies assisted with endophyte labelling and microscopic visualization is recommended to understand colonization property and biocontrol potential of the isolates.

- Further screening of the isolates with different inoculation methods, inoculum amount, on different clones of enset, and in the field is essential to know their optimum disease suppression conditions.
- Similarly the *in vitro* plant growth promoting potential of the endophytes in this study should be tested *in vivo*.
- Further study on endophytes integrated with other control methods such as antagnostic rhizospheric microbes might be a better disease control strategy.
- Future work on study on the potential of the enset endophytic metabolites to control other phytopathogens might also be important.

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Appendices

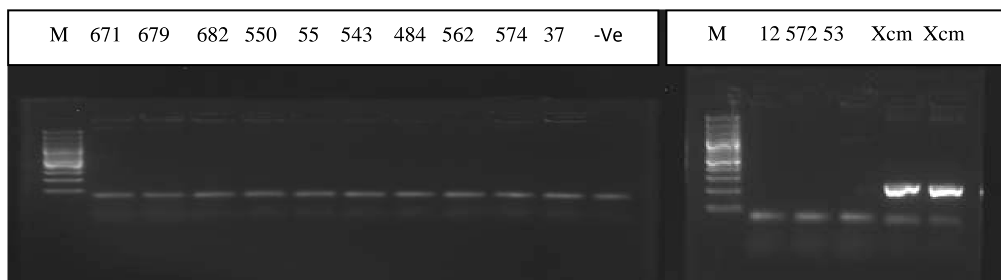
Appendix-I

Table showing enset sample information. Vernacular names were recorded for the samples collected in this study and the source variety of each isolate is known, however sequences of the isolates were majorly analyzed based on sampling site than based on the enset vernacular names.

Altitude (masl)	Study site	Farmers enset clone/variety (Vernacular names in each region)
1,841	Shebedino (Howeleso)	Ado, Diberamo, Wankore, Sediso
1,943	Gurage (Atat)	Nechewe, Yesherakinke, Kemenar, Bishamerat
2,049	Kefa (Telo)	Nobo, Bocho, Butecho, Utino (Wild sample)
2,320	Hadyia (Limu)	Gimbo, Sisikela, Oreda, Sipara
2,384	Dawero (Marka)	Maze, Amia , Shodedine, Boza
2,435	Gedio (Gedeb)	Genetche, Nifo, Torecho, Astara
2, 775	Gamo (Chencha)	Maze, Chemo, Ketise, Bora
3,200	Alcho Werero (Silte)	Beneze, Ageremer, Tereye , Benzena

Appendix-II

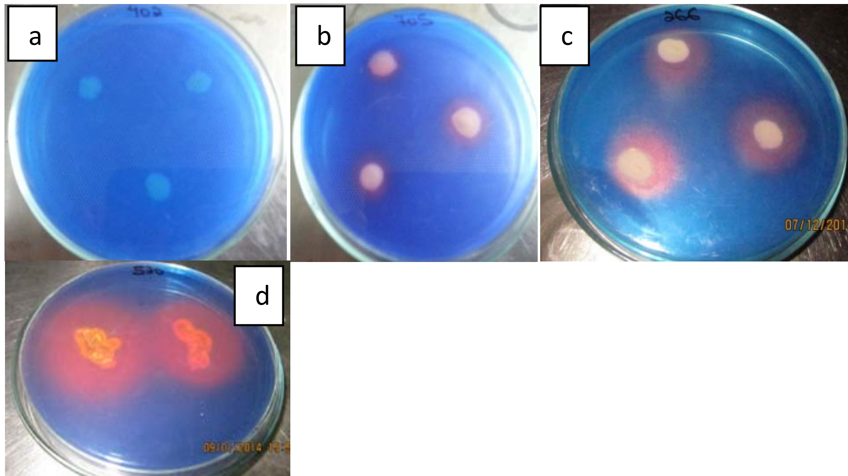
Gel picture PCR amplification of endophytes and Xcm isolates by primers Gsp Dm- F2 and Gsp Dm R3 to confirm the isolate used as pathogen inoculum is *Xanthomonas campestris pv. musacerum* and the isolates used as endophytes are not *Xanthomonas campestris pv.musacerum.*



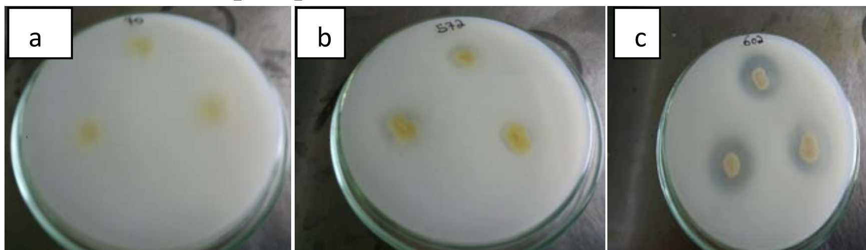
Appendix-III

Phenotypic characterization of endophytic isolates for different plant growth promoting properties.

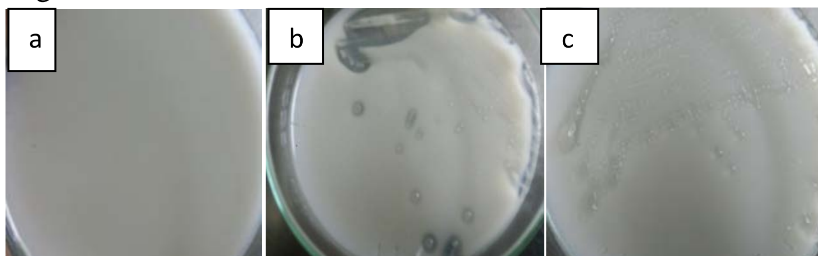
A) Siderophore production test by endophytic isolates on chrom azurol S containing media. a) Negative to production, b) with ++ level of production, c) with +++ level of production, d) Fungal endophyte positive for siderophore



B) Tri calcium phosphate solubilisation test on Pikovskaya (PVK) medium. a) Negative for phosphate solubilisation property, b) with + level of phosphate solubilisation, c) with +++ level of phosphate solubilisation.

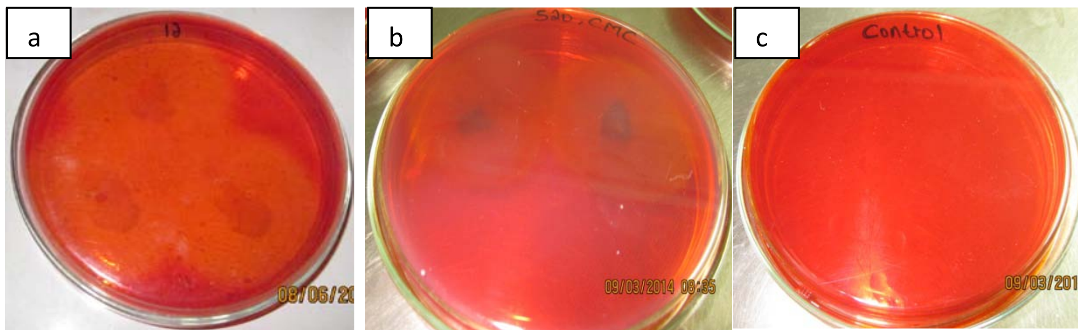


C) Growth on Ashib's nitrogen free medium (NFM). a) Isolate with negative growth on NFM, b) Isolate with ++ level of growth on NFM medium, c) Isolate with +++ level of growth on NFM.



D) Indol acetic acid by endophytes isolated from enset showing control (cont), strain negative for IAA production (14), positive for IAA production (13).

E) Cellulase production on CMC containing media. a) Bacterial endophyte positive for cellulase production b) Fungal endophyte positive for cellulase production c) Control.



Appendix-IV Green house experimental procedures.

A) Table showing different combinations of the cultures used to inoculate enset plants in the green house.

No	Name in CRBD 6 replicates	Isolate code
1	Representative antagonistic Bacteria (Mixed culture-1)	679, 572, 550, 12, 562, 53, 574, 543, 37, 682, 484, 55, 671
2	Fungus all positive (Mixed culture-2)	454, 528
3	Fungus all positive + representative antagonistic bacteria (Mixed culture-3)	454,528,679, 572, 550, 12, 562, 53, 574, 543, 37, 682, 484, 55, 671
4	Pseudomonas single isolate 1	550
5	Pseudomonas single isolate 2	572
6	Pseudomonas single isolate 3	679
7	Fungus single isolate 1	454
8	Fungus single isolate 2	528
9	Nothing inoculated	Sterile water
10	Pathogen inoculated	*Xanthomonas campestris pv musacerum*
11	Mixed culture 1 + pathogen	679, 572, 550, 12, 562, 53, 574, 543, 37, 682, 484, 55, 671,324
12	Mixed culture 2 + pathogen	454, 528
13	Mixed culture 3 + patogen	454,528,679, 572, 550, 12, 562, 53, 574, 543, 37, 682, 484, 55, 671
14	Pseudomonas single isolate 1+ pathogen	550
15	Pseudomonas single isolate 2 + pathogen	572
16	Pseudomonas single isolate 3 + pathogen	679
17	Fungus single isolate 1+ pathogen	454
18	Fungus single isolate 2+ pathogen	528

B) Table and pictures showing green house experimental layout.

CRBD experimental layout						
	Replications					
	1	**2**	**3**	**4**	**5**	**6**
Plots						
1	6	3	17	8	6	4
2	4	5	16	18	12	16
3	12	6	7	16	9	5
4	7	15	9	3	1	12
5	16	2	8	4	13	7
6	14	7	6	13	10	3
7	8	18	1	2	15	11
8	13	4	18	5	11	9
9	18	13	12	6	4	14
10	3	9	5	10	17	18
11	17	16	4	1	7	13
12	2	10	13	14	8	17
13	1	11	10	9	18	15
14	10	12	15	12	2	1
15	5	17	14	11	16	8
16	9	8	11	15	3	10
17	15	14	3	7	5	2
18	11	1	2	17	14	6

C) Pictures showing wilt symptoms at different stages of the experiment. a) Picture showing initial symptom development on the inoculated leaf, b) pictures showing disease spread to other leaves than infected, c) pictures showing healthy, survived and died plants at the end of the experiment.