DECLARATION

This is my original work and has not been presented for a degree or other awards in any other University.

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DEDICATION

This thesis is dedicated to my family for their immense support towards my education.
ACKNOWLEDGEMENTS

I greatly appreciate Kenyatta University for giving me the opportunity to further my studies. I express my special appreciation to Professor Dr. Ralf Oelmüller of Friedschiller University for allowing me to carry out my research in his laboratory and Dr. Alexandra C.U. Furch of the same university for hosting me during the whole period of my research. I acknowledge my supervisors Dr. George Ochieng’ Asudi and Dr. Titus Obidi Magomere for their inspiration, mentorship and tireless effort in guiding me to complete my research study.

I owe gratitude to the Department of Biochemistry and Biotechnology (Kenyatta University) and her staff for the close companionship and cooperation during the entire period of carrying out my studies. I also acknowledge my colleagues and laboratory technologists in the Institute of General Botany and plant physiology (Friedrich-Schiller University) for their support and ample coordination in the laboratory. I also owe a big thank you to DAAD (Deutscher Akademischer Austauschdienst) for scholarship to enhance my studies.

Most importantly, words cannot express my gratitude to my parents Mr. and Mrs. Omenge and siblings for all of their support. Foremost, without my husband, Stephen Kimei by my side through the most challenging times, this achievement would have not been easy. Following your footsteps was the best choice. To God be the glory.
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ABSTRACT

The Napier grass stunt (NGS) phytoplasma is a phloem limited bacterium that is transmitted by insect vector, Maiestas banda (Hemiptera: Cicadellidae). The phytoplasma causes NGS disease in Napier grass resulting into huge forage yield losses hence impacting negatively the economy of smallholder dairy farmers in East Africa. Phytoplasma infections causes massive changes in phloem physiology including the sieve elements which may lead to reduced rate of phloem mass flow that interfere with translocation of food in host plants. Hormonal imbalance also occurs in infected plants and is a defense mechanism used by the host plants to counteract the pathogen attack. Therefore, the structures of vascular tissues were examined using fluorescent microscopy and phytoplasmas were visualized by confocal microscopy using 4',6-diamidino-2-phenylindole (DAPI) dye while the rate of phloem mass flow determined using 5,6 carboxyfluorescein diacetate (CFDA) dye in healthy and phytoplasma infected Napier grass plants. The cells of phytoplasma were detected and quantified by real-time qPCR while the levels of phytohormones were investigated using liquid chromatography tandem-mass spectrometry (LC-MS/MS) method. The areas of all vascular tissues in phytoplasma-infected Napier grass leaves were significantly reduced as follows; vascular bundles (34.8%), xylem vessels (42.6%), phloem (27.2%) and sieve elements (53.5%). The individual and aggregates of phytoplasma cells were detected on the membranes of the sieve elements that demonstrated high yields an average of 2.29×10^5 cells/μg of total DNA extracted in the plant leaves by qPCR results. The rates of phloem mass flow were significantly reduced by 40.7% in the phytoplasma-infected plants. With the exception of SA and ABA, all measured phytohormones were affected by phytoplasma infection. The concentrations of cis-12-oxo-phytodienoic acid, jasmonic acid and jasmonic acid isoleucine were significantly reduced 3, 2 and 8 folds, respectively. This study sheds light on the significance and mechanisms of phytoplasma infection.
CHAPTER ONE

INTRODUCTION

1.1 Background information to the study

Napier grass (*Pennisetum purpureum* Schumach) also known as elephant grass is the most important fodder crop in East Africa supporting immensely the small-holder dairy industry in the region. The grass is widely accepted and grown by majority of small scale farmers because it can be easily propagated and managed. Moreover, the crop also has high yield and wide ecological range (Orodho, 2006). In addition, the fodder grass is also utilized as a trap plant in the push-pull strategy (PPS) practised in the region to control the most injurious cereal pests, stem borers (Khan *et al*., 2010; Midega *et al*., 2010). Regardless of its great economic importance, continued production of Napier grass is under a serious threat of Napier grass stunt (NGS) disease in many areas in East Africa (Pallangyo *et al*., 2008; Asudi *et al*., 2015; Kawube *et al*., 2015).

NGS-disease is caused by phytoplasma, uncultivable, cell wall-less obligate parasite that existed through degenerate evolution from gram-positive prokaryotes. Globally, phytoplasmas cause numerous plant diseases of grasses, crops, fruits, vegetables and ornamentals resulting into phytosanitary conditions and serious losses of world economies (Lee *et al*., 2000; IRPCM 2004; Asudi *et al*., 2015). In Napier grass, the pathogen induces characteristic symptoms such as small leaves, foliar yellowing, proliferation of tillers and reduced internodes. These symptoms often become apparent following regrowth of the infected-plant after animals grazing or several cuttings which
usually lead to the death of the infected plants (Alicai et al., 2004; Orodho, 2006; Jones et al., 2004, 2007; Kabirizi et al., 2007; Nielsen et al., 2007; Pallangyo et al., 2008; Asudi et al., 2015; Kawube et al., 2015). In NGS phytoplasma-infected sugarcane plants, foliar yellowing of young plant leaves have been reported which are replaced with bright yellow midribs in late stages of plants’ growth. However, in cultivated crops including finger millet, maize, sorghum, rice and pearl millet, the NGS disease can occur without necessarily causing symptoms (Asudi et al., 2016a). Napier grass stunt disease has been detected and reported in many regions in East Africa and has impacted negatively on many dairy farmers’ livelihoods (Orodho, 2006; Pallangyo et al., 2008; Arocha and Jones, 2010; Asudi et al., 2015; Kawube et al., 2015). Primarily, NGS is spread from one place to another through propagation of infected root splits, plant material or cuttings (Orodho, 2006; Koji et al., 2012). The NGS-phytoplasma is then transmitted to phloem sieve tube elements from infected to healthy plants by insect vectors mainly the leafhopper Maiestas banda (Kramer) (Hemiptera: Cicadellidae) (Obura et al., 2009).

Since their discovery as plant pathogens five decades ago, phytoplasmas have not been cultured in vitro and this complicates the process of detection and identification. Therefore, the existence of distinctive symptoms in infected plants was the major criterion to diagnose diseases caused by phytoplasmas (Doi et al., 1967) including NGS disease. Varying primer combinations based on the 16S ribosomal (r) gene were developed and used to detect and characterize phytoplasma in plants and insects. These primers are universal, generic or species-specific (Lee et al., 1998; 2000). For NGS disease, routine detection and characterization have been mainly based on the amplification of the 16Sr DNA in a nested (n) PCR assay consisting of two rounds of
PCRs, loop-mediated isothermal amplification of DNA assay and also on real time quantitative PCR (Obura et al., 2011a; Obura, 2012; Wambua et al., 2017). Based on the 16S rDNA sequences, phytoplasmas belonging to the 16SrXI group, ‘Candidatus (Ca.) Phytoplasma oryzae’ or rice yellow dwarf (RYD) cause NGS disease in Tanzania, Uganda and Kenya (Jones et al., 2004, 2007; Obura et al., 2009; Asudi et al., 2016b) while the pathogen in Ethiopia is a member of the ‘Ca. Phytoplasma pruni’ (Nielsen et al., 2007; Arocha et al., 2009). Serological methods including enzyme-linked immunosorbent assay (ELISA) has also been applied for the detection of phytoplasmas for more than three decades (Lee et al., 2000). However, these were not applied in the detection of NGS phytoplasma until recently. Therefore, two specific monoclonal antibodies designed to detect the immunodominant membrane protein (IMP) in phytoplasma-infected Napier grass were developed (Wambua et al., 2017). Besides, a recombinase polymerase amplification technique based on the IMP, which is more rapid, sensitive and easy to use and is applicable to field testing and detection of NGS-phytoplasma has been developed (Wambua et al., 2017).

Despite the achieved progresses on the detection, differentiation and characterization of NGS-phytoplasma, there remain no studies on the possible mechanisms of the plant-NGS-phytoplasma interactions and the explanation to the varied responses of host plants to NGS-phytoplasma infections. Phytoplasmas reside strictly in the phloem sieve tube elements of the host plants and from there they systemically spread to the whole plant through the sieve plate pores of the phloem tissue causing impairment of sieve tube functions (Pagliari et al., 2016, Fig.1.1). Therefore, the phloem transport system in phytoplasma-infected plant is inhibited resulting into a build-up of unusual huge amounts
of carbohydrates in the source leaves and reduced amounts in sink organs (Lepka et al., 1999; Lee et al., 2000; Christensen et al., 2004; Musetti et al., 2013).

Fig. 1.1: Semithin sections of midribs from healthy and infected *Arabidopsis thaliana* plants. (a) Healthy plants vascular bundles have a regular collateral pattern and no cell alteration. (b) In infected leaf tissues many phloem components show plasmolysis, collapse or necrosis. Moreover, massive production of new phloem components causes phloemhyperplasia. Amber segments indicate phloem thickness measuring lines. Bars correspond to 25 µm. c: cambium; p: phloem; x: xylem (Pagliari et al., 2016).

Phytoplasma infections have been reported to also cause hormonal imbalance in host plants (Leon et al., 1996; Das and Mitra, 1998; Sánchez-Rojo et al., 2011; Mardi et al. 2015) and evolve mechanisms, which modify the phytohormone-signaling pathways to suppress or evade host immunity. For example, phytoplasma SAP11 effector produced by Aster Yellows phytoplasma strain Witches’ Broom (AY-WB) manipulate plant development and jasmonic acid (JA) biosynthesis in Arabidopsis (Gai et al., 2014; Sugio et al. 2011). Generally, phytohormones regulate plant growth and immune responses towards attack by pathogens and insect vectors (Sugio et al., 2011; Pieterse et al., 2012; Mardi et al., 2015). The knowledge of the effects of NGS phytoplasma on phloem mass
flow and changes in the phloem morphology and occurrence of phytohormones in host plants is essential in understanding the phytoplasma–plant host interactions and the mechanism of pathogenicity of NGS-phytoplasma. Therefore, the objectives of this study included use of microscopy to determine phloem morphology and the mass flow in phytoplasma-infected Napier grass leaves and changes in phytohormones in phytoplasma-infected plant leaves using liquid chromatography tandem-mass spectrometry technique.

1.2 Statement of problem and justification

Production of Napier grass in East Africa is severely threatened by NGS-disease. The disease is caused by NGS-phytoplasma and affects Napier grass plants in most smallholder farms causing between 40 and 90% loss in forage yield (Jones et al., 2004; Orodho, 2006; Nielsen et al., 2007; Kawube et al., 2015; Asudi et al., 2016b). In the phloem tissues, phytoplasmas are exclusively limited to the sieve elements (Lepka et al., 1999; Lee et al., 2000; Christensen et al., 2004; Musetti et al., 2013) whose terminal ends are modified as perforated sieve plates, which allow the flow of sap from one element to the next. Osmotic pressure gradient inside the sieve tubes drives the mass flow between source and sink that distributes carbohydrates, amino acids, proteins, phytohormones, vitamins, and other signaling molecules throughout the whole plant (Knoblauch et al., 2016). However, the study on ultrastructural modifications, in terms of the areas of the vascular tissues, due to phytoplasmas infection has not been conducted in any plant species and because phloem mass flow is influenced by the structural modifications of the sieve elements anatomic studies on the structures of vascular bundles was conducted.
Various phytohormones play key role in stimulating the immune signaling pathways (Pieterse et al., 2012). These hormones were initially known to be growth and development regulators (Santner and Estelle, 2009). Jasmonic acid and salicylic acid (SA) with its derivatives are reported as the major hormones that are involved in defense (Browse, 2009, Vlot et al., 2009). This complex defense and signalling system in phytoplasma-infected plants is unknown in Napier grass. Therefore study on the phytohormones level and mass flow rates in the phytoplasma-infected Napier grass was conducted to understand the biochemical defense mechanism involved in infected plants.

1.3 Hypotheses

i. There is no significant change in vascular tissue morphology between the healthy and phytoplasma-infected Napier grass.

ii. There is no significant variation in the rate of phloem mass-flow between the healthy and phytoplasma-infected Napier grass.

iii. There is no significant variation in phytohormone levels between healthy and phytoplasma-infected Napier grass.
1.4 Objectives

1.4.1 General objective

To determine the impact of phytoplasma infection on phytohormone levels and phloem mass flow in Napier grass.

1.4.2 Specific objectives

i. To determine the morphological differences of vascular tissues between healthy and NGS-phytoplasma-infected Napier grass.

ii. To assess the variation in the rate of phloem mass flow between healthy and NGS-phytoplasma-infected Napier grass.

iii. To determine changes in the occurrence of phytohormones between healthy and NGS-phytoplasma-infected Napier grass.
2.1 Economic importance of Napier grass

Napier grass is a fodder crop that has a dynamic stoloniferous root system with creeping rhizome. It was originally found at the Zambezi valley in Zimbabwe and it has since been grown in the wider central and eastern Africa (Farell et al., 2002). It thrives in damp grassland, riverbeds and at the margins of the forests. The mature plants normally grow to between 3 and 5 m tall with a maximum of 20 nodes though it can reach up to 10 m high producing 29 tonnes/ha of dry matter in riverbeds. The main propagation method of Napier grass is through cuttings with 3-4 nodes in length. The propagated cuttings form thick clumps of 3 cm wide and 30-90 cm long flat leaves (Orodho, 2006). Napier grass can withstand repeated cuttings of which 4-6 cuts per year produces 50-150 tonnes green matter per hectare. When Napier grass is regularly fertilized, it produces high biomass that is very palatable during the leafy stage and it is best replanted every five to six years. These high performance attributes have led to the increased usage of the grass as a fodder crop (Farell et al., 2002).

Napier grass constitutes 40 to 80 % of total forage utilized by smallholder dairy farmers in East Africa who practice intensive and semi-intensive dairy production systems. A part from forage usage, Napier grass is also used for conservation of water and soil in hilly slope areas (Orodho, 2006) and is currently used to control cereal stem borers (Chilo partellus Swinhoe), (Lepidoptera: Crambidae) and Busseola fusca (Fuller) (Lepidoptera:
Noctuidae), (Pickett et al., 2014) in the push and pull strategy (PPS). The PPS strategy
developed by the International Centre of Insect Physiology and Ecology (ICIPE) and its
partners, is preferred by farmers for stem borers and Striga weed management which
significantly reduce cereal production where? The sentence is hanging? (Khan et al.,
2010). The Lepidopteran stem borers cause cereal yield loss of between 10 and 80 %
(Kfir et al., 2002) while Striga can cause up to total yield loss (Khan et al., 2010). The
strategy involves planting Napier grass (trap plant) around the intercrop of legumes in the
genus Desmodium with cereals (Pickett et al., 2014). Allelochemicals released by
Desmodium spp roots suppress Striga weeds while its volatiles released repel (push) stem
borer moths. Conversely, Napier grass releases chemicals that lure (pull) stem borer
moths. Napier grass will then attract more stem borer pests oviposition than the main
cereal crop consequently creating unfavorable conditions for larvae development leading
to a reduced population of stem borer and hence increased cereal performance (Khan et
al., 2010; Midega et al., 2010).

2.2 Phytoplasmas and Napier grass stunt disease in Eastern Africa

Phytoplasmas are uncultivable, cell wall-less obligate parasites that existed through
degenerate evolution from gram-positive prokaryotes. Globally, they cause numerous
crop diseases, vegetables, fruits, grasses and ornamentals resulting into phytosanitary
conditions and great economic loss (Lee et al., 2000). Phytoplasma genome is small
ranging from 530 to 1350 kb with high AT content. Together with acholeplasmas, they
form the family Acholeplasmataceae and order Acholeplasmatales in the class
Mollicutes. The trivial name of the agents, which reflected their host and most
pronounced disease symptom was replaced by the new taxon *Candidatus* (*Ca.*) phytoplasma. The system of phytoplasma classification is based on the identity of their 16S rDNA sequences. Strains that are within a candidate species share sequence identity of at least 97.5% of their 16S rDNA gene sequences. (IRPCM, 2004).

In Kenya NGS disease was reported in 1997 for the first time in Bungoma district, and afterwards in Uganda and Ethiopia in 2001 and 2004 respectively (Jones *et al.*, 2004; 2007; Orodho, 2006; Nielsen *et al.*, 2007) and has also been detected in Tanzania (Pallangyo *et al.*, 2008; Asudi *et al.*, 2016b). In the field, the NGS symptoms are observed in the grass after re-growth of several grazing by animals or cuttings. Mostly the whole plant is affected with a complete loss in yield which contributes to the ultimate death of the Napier grass plants (Jones *et al.*, 2004; Orodho, 2006; Kabirizi *et al.*, 2007; Asudi *et al.*, 2015) (Fig. 2.1). The NGS disease has spread to several locations in East Africa causing serious economic losses in smallholder dairy industry and poses a significant threat to cereal production in the region (Orodho, 2006; Kabirizi *et al.*, 2007; Khan *et al.*, 2014; Asudi *et al.*, 2015; Asudi *et al.*, 2016b). Besides, many smallholder farmers have lost their entire Napier grass plantation and are forced to purchase fodder from the local market or reduce their number of animals (Arocha and Jones, 2010).
Fig. 2.1: (a) Healthy Napier grass, (b) phytoplasma infected Napier grass which is severely stunted and bushy with foliar yellowing of leaves (Asudi et al., 2015)

2.3 Transmission of Napier grass stunt phytoplasma

Diseases caused by phytoplasma are spread mainly by sap sucking insect vectors which belongs to the families Cicadellidae (leafhoppers), Fulgoridae (planthoppers) and Psyllidae (psyllids). Napier grass stunt disease can also be transmitted by propagation of infected cuttings and through vascular connections between uninfected and infected host plants done by parasitic plants such as dodder (Cuscuta spp.). These heteropteran insects acquire phytoplasmas by feeding on phloem tissues and then transmit them from one plant to another as shown in Fig. 2.2 (Lee et al., 2000; Weintraub and Beanland, 2006; Obura et al., 2009).
The most common means of spread of NGS is through the planting of infected cuttings by farmers or insect herbivore having the phytoplasma (Orodho, 2006; Koji et al., 2012). In Kenya, *M. banda* (Kramer) (Hemiptera: Cicadellidae) (Fig. 2.3), a leafhopper in the tribe Deltocephalini was look for a better termto be the vector for NGS-disease (Obura et al., 2009). In Ethiopia, a leafhopper *Exitianus* spp. (Hemiptera: Cicadellidae) and a plant hopper *Leptodelphax dymas*, (Fennah) (Hemiptera: Delphacidae) were reported to be the potential vectors of the NGS phytoplasma (Arocha et al., 2009). A number of suspected alternative plant hosts also exist. This includes thatch grass (*Hyparrhenia rufa*) in which Hyparrhenia grass white leaf phytoplasma was detected and which is closely related to NGS phytoplasma sharing 99 % identitiy (Obura et al., 2011b; Asudi et al., 2016b).
Fig. 2.3: Dorsal view of *Maiestas banda* (Hemiptera: Cicadellidae), a vector of Napier grass stunt phytoplasma in Kenya (not drawn to scale) (Obura et al., 2009).

2.4 Phloem involvement in mass flow

The plant vascular transport system translocates photoassimilates from source to sink tissues through phloem tissue that is made up of companion cells, parenchyma cells and sieve elements in higher plants (van Bel, 1996; Schulz, 1998; Hafke et al., 2005). The sieve elements are observed to be elongated and are parietally lined with a mictoplasmic layer which is composed of plastids, endoplasmic reticulum, phloem-specific proteins and few inactive mitochondria. A huge mass of pore-plasmodesmata and endoplasmic reticulum connects the sieve elements while the companion cells underline the symplastic connection across the boundary and maintain the viability of the sieve elements (Kempers et al., 1998; van Bel, 2003; Martens et al., 2006). Specific walls that exist and connect individual sieve elements are converted into sieve plates and are perforated with plasmodesmata which are transformed into sieve pores whose diameter measure to a maximum of 2.5 μm (Behnke and Sjolund, 1990; Schulz, 1998; van Bel, 2003; Mullendore et al., 2010). These adaptations explain existence of very long sieve tubes from individual sieve element module which forms a tube-like symplastic continuum that enables transportation of photoassimilates (van Bel and Knoblauch, 2000).
The xylem and phloem together form the vascular system which conducts the long-distance transportation of heterogeneous components in higher plants which is as a result of tension and pressure gradients that build up in xylem vessels and sieve elements and usually there is a balance of the negative hydrostatic potential in between xylem vessels and sieve elements in intact plants (Zimmermann et al., 2013). Two forces drive translocation in higher plants namely longitudinal pressure gradient within xylem and phloem, and lateral pressure gradient that exists between xylem and phloem (Will and van Bel, 2006). This longitudinal pressure gradient in the xylem results in uptake of water in the root region and water loss by the process of transpiration. On the other hand, during the process of translocation in the phloem tissues, assimilates are normally amassed in the sieve tubes of source areas and are released into the sink regions (Münch, 1930). The resultant turgor variation between source and sink drives the mass flow (Fig. 2.4) (Dinant and Lemoine, 2010).
Fig. 2.4: Demonstration of mass flow pressure theory as proposed by Münch (1930)

Phloem sap or photo-assimilates contains amino acids, proteins and carbohydrates which makes sieve elements a target for pests and pathogens. Pests and pathogens access to the sieve elements leads to various impairments such as interruption of the translocation process, microbial pathogen infections and loss of nutrients (Dinant et al., 2010; Giordanengo et al., 2010). Therefore, plants have developed various defense mechanisms to counter pathogens and pests.

2.5 Phytohormones

The symptoms expressed by phytoplasma-infected plants are suggestive of changes in phytohormone balance (Pieterse et al., 2009). Phytohormones are small molecules that
work synergistically and/or antagonistically to regulate plant growth, reproduction, development and plants’ response to environmental conditions (Pieterse et al., 2009; Santner et al., 2009; Jaillais and Chory, 2010). Earlier studies show hormonal imbalance in phytoplasma-infected plants including decreased levels of cytokinin and abscisic acid (ABA) in mature leaves and roots and an increase in flowers (Kirkpatrick, 1991; León et al., 1996; Lee et al., 2000; Tan and Whitlow, 2001). Though it is not clear whether phytoplasmas synthesize their own plant growth regulators as some walled plant-pathogenic bacteria do or change the normal levels of endogenous plant hormones. Additionally, phytohormones are cellular signal molecules with the major functions in the immune response regulation process to insect herbivores and beneficial microbes (Pieterse et al., 2012). For instance, JA and SA-mediated signaling pathways in pathogen resistance are documented (Robert-Seilanianantz et al., 2011a). Mostly, plant defense against biotrophic pathogens is regulated positively by SA signaling while resistance to necrotrophic pathogens and also to herbivorous pests are regulated through JA pathways (Berrocal-Lobo et al., 2002; Glazebrook, 2005; Bari and Jones, 2009; Robert-Seilanianantz et al., 2011a). Antagonistic crosstalk between JA and SA signaling pathways have also been explained with positive regulator of JA-related genes as a suppressor of SA pathway reported (Gimenez-Ibanez and Solano, 2013). The induction of SA contributes to repression of JA-signaling increasing the susceptibility of plants to necrotrophic pathogens and insect vectors (Birkenbihl et al., 2012; Sánchez-Vallet et al., 2012).

Other phytohormones including ABA and auxins that were initially described for their roles in the response to abiotic stresses and in the processes of regulating plant growth have come up to be critical players in plant immune response (Mauch-Mani and Mauch,
2005; Kazan and Manners, 2009; Ton et al., 2009; Fu and Wang, 2011). Pathways that are involved in hormonal signaling are interlinked in a complex network and ABA, cytokinins, gibberellins, auxin and ethylene pathways are often regarded to be the modulators of the backbone of JA–SA signaling (Pieterse et al., 2012).

2.6 Detection and quantification of phytoplasma

Conventional microscopic technique has been used in the past to explain phytoplasmas colonization behavior through sieve tubes (Lee et al. 2010). The confocal laser scanning microscopes have been used in the detection of phytoplasmas in freshly sectioned petioles and stems of Euphorbia pulcherrima and Catharanthus roseus using DNA dyes such as DiOC7 and SYTO 13 that revealed phytoplasmas as dense fluorescent masses in sieve elements (Christensen et al., 2004). Other microscopic dyes such as the 4, 6-diamidino-2-phenylindole (DAPI) and aniline blue stains are usually used for quick phytoplasma diagnosis (Lee et al., 2000; Christensen et al., 2004; Arismendi et al., 2010). Because the genomes of most phytoplasma strains are composed of higher AT sequences with a correspondingly low GC content ranging from 19.3% in ‘Ca. Phytoplasma oryzae’ to 28% in ‘Ca. Phytoplasma asteris’ (Hogenhout and Seruga, 2010; Fischer et al., 2016), this feature makes phytoplasmas amenable to staining techniques such as the DAPI staining. 4, 6-diamidino-2-phenylindole is a fluorescent stain that has the ability to pass through cell membranes and binds strongly to AT-rich regions of DNA and has been used for detection of phytoplasmas in many infected plants (Eriksson et al., 1993; Lee et al. 2000).
The interaction between DAPI with DNA has been the subject of a number of studies since this aromatic compound was synthesized (Jansen et al., 1993). Formerly, several diamidine compounds were synthesized for use as a trypanocide agent (Jansen et al., 1993). However, the DAPI special spectral properties have made it a preferred DNA probe rather than for medical purpose. It is extensively used in phytopathology for detection of phytoplasmas and spiroplasmas in different plant species (Bricker and Stutz, 2005; Fletcher et al., 2006; Arismendi et al., 2010). Upon application of DAPI staining, phloem cells of infected material show a strong fluorescence, brighter than of a typical nuclei of parenchymal cells. The infected tissues mostly show bright phytoplasma-like spots in the phloem sieve tubes that are not visible in healthy tissues (Arismendi et al., 2010). The DAPI staining method is considered a precise and rapid method of studying localization of phytoplasmas in the phloem sieve tubes of different tissues (Arismendi et al., 2010).

Phytoplasmas are unequally distributed in the phloem tissues of diseased plants and vary in titres according to the seasons and plant organs with the lowest concentrations detected in some woody hosts (Firrao et al., 2007). These reasons have made phytoplasma detection and identification challenging. In the past, phytoplasma diagnosis relied on characteristic symptoms induced in diseased plants and later on molecular techniques such as nested PCR (Lee et al., 1998; 2000). Complete diagnostic protocol is laborious requiring several post-amplification steps. In order to overcome these challenges, various approaches including universal and group-specific quantitative real-time PCR (qPCR) protocols were proposed and have since replaced the traditional nPCR due to increased
sensitivity and speed of detection for phytoplasmas mass screening (Galetto and Marzachi, 2010).

During a real-time PCR run, amplicons are visualized using fluorescently labelled probes, which bind to a target sequence or stain the double-stranded DNA (Christensen et al., 2004; Galetto and Marzachi, 2010). In the event of low phytoplasma DNA, concentration in the mRNAs of the host especially the highly expressed ribosomal ones may offer the best target for diagnosis (Firrao et al., 2007). Thus the pathogen-specific amplicon is directly detected in a reverse transcription RT-PCR using group-specific primers and the TaqMan Probes (Margaria et al., 2008). The RT-PCR technique enables quantification of phytoplasmas within the plant organs, an important aspect of understanding pathogenic effects and complex phenomena like disease management (Galetto and Marzachi, 2010).
3.1 Plant materials

Napier grass (cultivar, cv. ‘Ouma 2’) cuttings infected with ‘Ca. Phytoplasma oryzae’ strain NGS, were obtained from diseased Napier grass plants with yellow to purple streaking and stunted growth characteristics at the International Centre of Insect Physiology and Ecology (ICIPE), Mbita research station, Kenya (0°25’ 34°12’E). Cuttings from Napier grass of the (cv. ‘Ouma 2’), not infected with NGS were also obtained from the same station. Grass cuttings were packaged and transported to Germany (Matthias-Schleiden-Institut for Genetic, Bioinformatic and Molecular Botanic) in paper bags and were sown in pots and maintained in a screen-house under 8 hours of darkness and 16 hours light (25 °C, 65 % relative humidity). After the 10th week, the plants were cut back 5 cm above the ground (first cutback) and allowed to regrow for 4 weeks after which sampling were done.

3.2 Isolation and amplification of phytoplasma DNA

Genomic DNA was extracted from leaf samples of Napier grass culms by the CTAB extraction method according to Doyle and Doyle (1990). About 0.3 g of each sample was placed in liquid nitrogen and homogenized with sterile pestles in 0.6 ml of preheated CTAB buffer (65°C) (containing 2% CTAB, 20 mM EDTA, 1.4 M NaCl 100 mM Tris–HCl, 0.2% 2-mercapto-ethanol, pH 8.0). The homogenate was then extracted with an equal volume of chloroform: isoamylalcohol (24:1). The organic and aqueous phases
were separated by centrifugation. DNA was precipitated from the aqueous phase by adding an equal volume of isopropanol and the pellet washed twice with absolute ethanol and then air-dried and reconstituted in a 50 μl of sterile distilled water. The concentrations of DNA were determined by Nanovue™ Plus Spectrophotometer (GE Healthcare, UK) and quality verified using 1% (w/v) agarose gel electrophoresis and visualized with ethidium bromide then stored at -20°C for subsequent analysis. The extracted DNA samples were then diluted to approximately 70 ng/μl for subsequent qPCR analysis.

The 16S rRNA gene of the rRNA operon was amplified using universal primer pair P1 (AAGAGTTTGATCCTGGCTCAGGATT)/P6(CGTTAGGGATACCTTGTTACGACTTA) (Deng and Hiruki, 1991) in the first round PCR followed by primer pair NapF (AGGAAACTCTGACCGAGCAAC)/NapR(AGGAAACTCTGACCGAGCAAC) (Obura, 2012) (Table 1). The initial amplification was performed in a 10 μl PCR reaction mixture containing genomic DNA, 10X PCR buffer (Thermo Scientific™, Lithuania), 0.2 mM dNTPs, 5 unit DreamTaq DNA polymerase (Thermo Scientific™, Lithuania) and 400 μM of each primer (Eurofins Genomics, Germany Inc). PCR reactions were carried out in a Proflex PCR machine (Applied Biosystems) as follows: 94 °C for 3 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 90 s and extension at 72 °C for 90 s; and a final elongation step at 72 °C for 10 min. DNA amplified in the initial PCR was vortexed gently to mix and 0.6 μl used as a template in a nested PCR with the same conditions except for annealing which was at 56 °C. Amplicons were then visualized by gel electrophoresis in a 1% agarose gel stained with ethidium-bromide using 1xTBE (40 mM Tris acetate, 1mM EDTA pH 8.0) as running buffer, and
photographed. In all the experiments, water controls were included in which no plant nucleic acid was added to the PCR reaction mix as negative controls and the DNA from reference phytoplasma strain, NGS, as a positive control.

3.3 Oligonucleotide primers and probes

Real time quantitative PCR assay was done according to Christensen et al. (2004) with a few modifications. PCR reactions were carried out in triplicates in 10 µL reaction mixture using Dream Taq (Thermo Scientific™, Lithuania), 0.2 mM dNTPs and 400 nM of each forward and reverse primers targeting phytoplasma 16S rDNA in a CFX96 Touch™ Real-Time PCR (Bio-Rad Laboratories, Inc). The fluorogenic probe for phytoplasma DNA was synthesized by Eurofins Genomics, Germany and was 5’ labelled with the reporter dye FAM (6-carboxyfluorescein) and 3’ labelled with the quenching dye TAMRA (6-carboxytetramethylrhodamine). The probe for plant DNA for a normalization of PCR results was labelled with HEX (hexachloro-fluoresceine) and TAMRA at the 5’ and 3’ ends, respectively.

Two standard curves were made, one for phytoplasma quantification and the other for quantification of plant DNA. DNA for the phytoplasma standard curve was obtained from phytoplasma-infected Napier grass following amplification with primer pair P1/P6 and NapF/NapR (Deng and Hiruki, 1991; Obura, 2012). For the standard curves, the obtained amplicon was diluted 10-fold in a series (10⁰, 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵ and 10⁻⁶).
3.4 Determination of rate of mass flow and anatomical changes in vascular tissues

The phloem-mobile dye, 5, 6 carboxyfluorescein diacetate (CFDA) (containing 2 mg/L KCl, 1 mg/L CaCl₂, 1 mg/L MgCl₂, 50 mg/L mannitol and 2.5 mg/L MES/NaOH buffer, pH 5.7) was used to investigate phloem mass flow in Napier grass leaves. Potted plants were laid down horizontally and leaf tip of each leaf studied removed using a fresh razor blade. The leaf tip with the cut part was immediately immersed into a freshly prepared 1 μM CFDA solution and incubated for 30 minutes at room temperature. After incubation in the dye, rates of mass flow were determined by the furthest distance the fluorescent dye had moved from the tip of the leaf by examining thin cross sections made with fresh razor blades and mounted on a fluorescent microscope slide (AXIO Imager.M2, Zeiss, Jena, Germany). The physiological state of the phloem tissue was then assessed using a microscope with a 40x buffer immersion objective and the phloem tissue was examined at 488 nm. Measurements of the area of the vascular bundles, phloem, xylem and sieve elements were recorded and the digital images taken by a colour 164 camera (AXIOCAM 503 colour Zeiss, Jena, Germany).

The breadths and the lengths of every third leaf were measured in cm with an aid of a ruler from one side/tip of the leaf to the other side/stalk in cm. The rates of mass flow between the phytoplasma-infected and healthy plant leaves were recorded after 30 minutes of incubation in CFDA solution and expressed as distance (cm) over time (h). The whole area of the vascular tissue represented the area containing the two xylems, phloem and the sieve elements measured in μm². The areas of the two xylems and the
phloem were also measured in µm$^2$ while the area of the sieve elements represented an average of five visible sieve elements measured in µm$^2$.

### 3.5 Bioimaging of phytoplasma and occlusion events

Phytoplasmas were visualized in infected Napier grass plant leaves using DAPI which was prepared in dimethyl sulfoxide (1 mg/ml). For confocal microscopy, cross sections of the leaves of Napier grass were taken using a fresh razor blade and stained with 0.3 µM DAPI. After 30 min of incubation, DAPI stained cross sections of Napier grass were imaged using a laser scanning microscope 880 (Zeiss Microscopy GmbH, Jena, Germany) with the 405 nm laser line. Images were taken with a 40x objective (Plan-Apochromat 40x/0.8). Lambda stacks were created using the 32-channel GaAsP detector followed by Linear Unmixing with ZEN software (Zeiss, Jena, Germany). Z-stacks were taken from specific areas of the sample and Maximum Intensity Projections were produced with ZEN software.

### 3.6 Phytohormones analysis

To concentrate the leaf samples, 200 mg powdered freeze dried plant was first acidified by adding 15 µL formic acid (0.5% v/v final concentration) to a mix of internal phytohormone standards (Vadassery et al., 2012). Subsequently, the sample was loaded onto a reversed-phase column (Oasis HLB 3cc; Waters, Eschborn, Germany) that was first pre-conditioned by the addition of 2 ml methanol (MeOH) followed by 2 ml water. After washing with 2 ml 5% (v/v) MeOH/water solution containing 0.5% (v/v) formic acid, the column was eluted with 2 ml MeOH. The eluate was completely dried in a
speed-vac, resolved in 0.1 ml MeOH and subsequently subjected to liquid chromatography–mass spectrometry analysis according to Vadassery et al. (2012).

3.7 Data analysis

Quantitative data obtained from determination of phytoplasma titre was derived from the standard curve of the plant DNA was constructed from one of the healthy sample extract and was diluted 10-fold in a series of six dilutions. The amount of input DNA in both plant and phytoplasma standard curves was estimated to 1 ng/µL and the number of copies calculated using an established formula (Falentin et al., 2010) as shown below.

\[
\text{Number of copies} = \frac{\text{Amount (ng)} \times 6.022^{23}}{\text{length of genome (bp)} \times 1 \times 10^9 \times 650}
\]

Standard curves obtained were used to determine C_T values which are normally inversely proportional to the phytoplasma DNA copy number of each sample. In principal, phytoplasmas contain two copies of DNA per cell, therefore, the phytoplasma DNA copy number was divided by two to obtain the number of phytoplasma cell per µg of total DNA extracted. The number of phytoplasma cell per µg of total DNA extracted were analysed using the Statistical Package for Social Sciences (SPSS version 20.0) to compare between phytoplasma-infected and healthy plants using independent sample t-tests. These results were expressed as mean ± standard error of mean and the statistical significance determined at 95% confidence level.
The rate of phloem mass flow and the ratios of the areas of the vascular tissues (µm²) to the width (cm) of the cross sections were calculated by dividing the areas of the vascular tissues with the width of the cross sections examined. The phytohormones concentrations of the sampled leaves that were obtained from LCMS/MS were divided by the fresh weight of each leaf sample and expressed in ng/g FW. The results of rate of phloem mass flow, areas of the vascular bundles and phytohormones concentrations obtained were also analysed using the Statistical Package for Social Sciences (SPSS version 20.0) to compare between phytoplasma-infected and healthy plants using independent sample t-tests. Data was expressed as mean ± standard error of the mean and the statistical significance determined at 95% confidence level.
CHAPTER FOUR

RESULTS

4.1 The rate of phloem mass flow of healthy and phytoplasma-infected leaves

A 778-bp DNA fragment was amplified from six plants, however, there was no phytoplasma DNA amplification from four plants which were considered to be healthy. This assay confirmed the healthy and phytoplasma-infected plants that were subjected to subsequent tests (Fig. 4.1).

Fig. 4.1.1: Electropherogram of the nested PCR products that were amplified with P1/P6 followed by NapF/NapR primers. M: 1 kb DNA marker (Thermo Scientific™), lane 1 through 6 diseased Napier grass plant leaves samples; 7: negative Napier grass while lane 8: positive control.

After 30 minutes the phloem-mobile 5,6 carboxyfluorescein (CF) was observed to have been translocated through the phloem sieve tubes (Fig. 4.2) and was observed to have
moved faster and further in the phloem tissues of healthy plant leaves than in phytoplasma-infected plant leaves.

**Fig. 4.1.2:** Microscopic images of vascular tissues of Napier grass leaves after application of the 5, 6 carboxyfluorescein diacetate dye. A: shows image of the vascular tissues of intact leaf after fluorescence of 5, 6 carboxyfluorescein through the sieve tubes. B: shows no fluorescence observed at the same length in another leaf.

The rate of mass flow varied significantly (Fig. 4.1.3; P < 0.05) between the phytoplasma-infected and healthy plant leaves and ranged between 36 and 43 cm/h with an average rate of 37.67 cm/h in healthy Napier grass plant leaves and between 20 and 26 cm/h with an average of 22.33 cm/h in phytoplasma-infected plant leaves (fig. 4.1.3).
Figure 4.1.3: The rate of phloem mass flow of healthy and phytoplasma-infected plant leaves investigated using 5, 6 carboxyfluorescein diacetate buffer solution. The rate of phloem mass flow (n = 6) are expressed as the mean ±SE. Different letters on the error bars represent significant differences, t test, P values <0.05.

4.2 Effects of phytoplasma infection on vascular morphology of Napier grass leaves

The microscopic images of the anatomy of Napier grass leaf were taken as shown in figure 4.2.1.
Figure 4.2.1: Semi-thin cross-sections of midribs from healthy (a) and phytoplasma-infected (b) Napier grass leaf. In infected leaf tissues some phloem components show necrosis, collapse or plasmolysis. The areas of vascular bundles, xylem vessels (XV), phloem tissue (PT) and sieve elements (SE) of both healthy and phytoplasma-infected leaves were measured by fluorescence microscopy. For each plant, at least three cross-sections from n= 6 observed. Scale bar: 20 μm.

Independent sample t-tests showed that there were significant differences in the areas of vascular bundles, xylem, phloem and sieve elements between the healthy and phytoplasma-infected Napier grass leaves (Fig. 4.2.2; P < 0.05). Thus, the vascular bundles, xylem, phloem and sieve elements occupied larger areas in healthy than in phytoplasma-infected plants. The mean area of the vascular bundles ranged between 6068.51 and 14185.94 μm² in healthy plant leaves with an average of 9389.26 μm² and between 3265.26 and 7982.23 μm² with an average of 6117.62 μm² in phytoplasma-infected leaves (Fig. 4.2.2A). Larger areas were also recorded for the xylem tissues in healthy than in phytoplasma-infected Napier grass leaves. These areas varied
significantly (Fig. 4.2.2B) and ranged from 411.87 to 1151.65 µm² in healthy plants and between 295.55 and 658.23 µm² in phytoplasma-infected Napier grass plant leaves.

The areas of the phloem tissues varied widely and ranged from 799.65 to 1455.19 µm² with an average of 1135.33 µm² in healthy plant leaves and from 552.41 to 1039.8 µm² with an average of 827.13 µm² in phytoplasma-infected leaves (Fig. 4.2.2C). The variation in areas of the sieve elements in phytoplasma-infected were quite different from those of the healthy Napier grass plants. The areas of the sieve elements were significantly larger in healthy Napier grass plants than in phytoplasma-infected plants. This varied greatly ranging between 49.22 and 58.48 µm² with an average of 54.74 µm² in healthy Napier grass plants and between 23.36 and 31.04 µm² with an average area of 25.45 µm² in phytoplasma-infected leaves (Fig. 4.2.2D).
Figure 4.2.2: Representation of areas of vascular bundle (A), xylem (B), phloem (C) and sieve elements (D) of healthy and phytoplasma-infected Napier grass leaves. The areas of the vascular tissues (n=6) are expressed as the mean ±SE. Phytoplasma-infected leaves were characterized by significantly smaller areas of vascular bundles, xylem, phloem and sieve elements than the respective areas of the healthy plants. Different letters on the error bars represent significant differences, t test, P values <0.05.

4.3 Localization of phytoplasma cells in Napier grass leaves

In phytoplasma-infected Napier grass leaf cross sections, DAPI fluorescence nearly filled the lumen of most sieve tubes (arrows) while in the healthy (control) there is no fluorescence in the sieve elements (Fig.4.3.1A). In the longitudinal sections of phytoplasma-infected leaves, the fluorescent dots and aggregated spots were accumulated predominantly on the membrane of the sieve plates (arrows) while the control sections of...
the healthy Napier grass showed no DAPI-specific fluorescence on the membrane of the sieve tubes (Fig. 4.3.1B) apart from fluorescence that was due to autofluorescence.

![Image of cross-sections and longitudinal sections](image)

**Figure 4.3.1:** Thin cross-sections (A and B) of midrib phloem tissues and longitudinal sections (C and D) of healthy and phytoplasma-infected Napier grass stained with 4, 6-diamidino-2-phenylindole (DAPI) dye. After DAPI staining, observation was done under confocal microscope. In the sieve elements (SE) of phytoplasma-infected plants, blue fluorescence (arrows- phytoplasma) mainly aggregate on both sides of the sieve plate. The sieve elements of healthy plants remained unlabelled, the fluorescence observed in the controls (A and C) was due to autofluorescence. SP represents the sieve plates. Bars correspond to 10 and 5 μm as indicated on each image.
4.4 Quantification of phytoplasma in infected Napier grass

The sensitivity of the qPCR assay was assessed by plotting the cycle threshold (C_T) values for dilution series of phytoplasma-infected Napier grass tissue against their relative concentration which resulted into a regression line with regression coefficient of 0.995 (Fig. 4.4.1; Fig. 4.4.2). The limit of detection of the assay was examined using the similar dilution series, the assay detected to at least 10^{-6}. The quantity of phytoplasma titre in the plant leaves varied from 6.96×10^2 in P23 culm 1 to 1.17×10^6 in P19 culm 1 with an average of 2.29×10^5 cells/μg of total DNA extracted (Table 4.1).

![Figure 4.4.1: Quantitative real-time PCR of a dilution series (10^0 to 10^{-6}) of phytoplasma-infected Napier grass leaf.](image)

The threshold numbers of PCR cycles (CT value; means of triplicates) are plotted against the log number of DNA copies. Relatively large error bars occur in dilution 10^{-2} only, reflecting a larger variability in CT. Linear regression coefficient is provided in the graph.
Figure 4.4.2: Quantification of phytoplasma in infected plant tissue; a real-time polymerase chain reaction assay of total DNA extracted from leaves of diseased shoot tissue from Napier grass (a) while (b) represents negative controls.

Table 4.1: Infection level in individual Napier grass plants analyzed with TaqMan assay

<table>
<thead>
<tr>
<th>Plant name</th>
<th>Culm number</th>
<th>Cultivar</th>
<th>$C_T$,16S (phytoplasma)</th>
<th>$C_T$,18S (plant)</th>
<th>Normalized mean quantity (cells/μg of total DNA extracted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P19</td>
<td>1</td>
<td>Ouma 2</td>
<td>21.09±0.18</td>
<td>13.90±0.37</td>
<td>1.72X10^4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Ouma 2</td>
<td>18.03±0.52</td>
<td>11.43±0.46</td>
<td>1.57X10^5</td>
</tr>
<tr>
<td>P23</td>
<td>1</td>
<td>Ouma 2</td>
<td>25.53±0.08</td>
<td>16.27±0.13</td>
<td>6.96X10^2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Ouma 2</td>
<td>23.75±0.29</td>
<td>12.27±0.26</td>
<td>2.52X10^3</td>
</tr>
<tr>
<td>P20</td>
<td>1</td>
<td>Ouma 2</td>
<td>20.60±0.33</td>
<td>14.87±0.06</td>
<td>2.45 X10^4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Ouma 2</td>
<td>15.25±0.37</td>
<td>11.65±0.09</td>
<td>1.17X10^6</td>
</tr>
<tr>
<td>P14</td>
<td>1</td>
<td>Ouma 2</td>
<td>18.89±0.41</td>
<td>13.66±0.28</td>
<td>5.91X10^6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Ouma 2</td>
<td>16.92±0.06</td>
<td>13.47±0.50</td>
<td>2.10X10^7</td>
</tr>
</tbody>
</table>
4.5 Impacts of phytoplasma infection on the levels of phytohormones

Abscisic and salicylic acids were not differentially influenced by phytoplasma infection hence, the concentrations of ABA and SA in healthy and phytoplasma-infected Napier grass plants were similar (Fig. 4.9a; P > 0.05). Three different jasmonates – jasmonic acid, cis OPDA and jasmonic acid isoleucine conjugate (JA-Ile) were studied as representatives of the JA pathway. In contrast to SA and ABA concentrations, the concentration of OPDA was 3 folds lower in phytoplasma-infected Napier grass leaves (Fig. 4.9c) while JA level was reduced by 2 folds in phytoplasma-infected Napier grass leaves (Fig. 4.9d). Interestingly, the level of JA-Ile was reduced by 8 folds in phytoplasma-infected Napier grass leaves (Fig. 4.9e).
Figure 4.5.1: Phytohormone concentrations in reaction to NGS phytoplasma infection. For each phytohormone, three healthy and three infected plants were analysed in triplicates. Different letters on the error bars represent significant differences, t test, P values <0.05.
CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

In East Africa, only Napier grass and other wild grasses are known to be affected by NGS phytoplasma. Other cultivated crops could also be affected in artificial transmission trials. Napier grass stunt disease impairs the size and quality of the crops reducing their vigour and longevity (Alicai et al., 2004; Orodho, 2006; Jones et al., 2004, 2007; Kabirizi et al., 2007; Nielsen et al., 2007; Pallangyo et al., 2008; Kawube et al., 2015; Asudi et al., 2015, 2016a,b). Because phytoplasmas are obligate parasites living in the phloem systems, impairment of the phloem by NGS-phytoplasma is very likely and has been supported by the presence of NGS phytoplasma in the phloem saps (Wambua et al., 2017) and in leaves.

To determine the rate of phloem mass flow in healthy and phytoplasma-infected plants, CFDA dye was used because it permeates the cell membrane in acetate form. The dye is then cleaved by cytosolic enzyme particularly intracellular esterase generating membrane-impermeant CF which fluoresces and is transported by mass flow within the sieve elements (Knoblauch and van Bel, 1998). Therefore, the presence of carboxyfluorescein enabled the detection of phloem mass flow in the sieve elements. However, there was a reduced rate of flow in phytoplasma-infected than in healthy leaves. Besides, the phloem in healthy Napier grass leaves contained twice as much sieve tube cross-sectional area as phloem in diseased plants. Phytoplasmas reside exclusively in
the sieve tube elements which are involved in the long-distance transportation of water and photoassimilates. In many host plants, phytoplasmas damage the sieve tube functions and inhibit the phloem transport in infected plants causing accumulation of carbohydrates in large quantities in mature leaves leading to the sealing of the sieve elements (Lepka et al., 1999). In addition, being a universal mode of sieve-plate occlusion, callose may also be plugged by proteins causing constriction of the sieve tube elements as has been observed in *Vicia faba* sieve tubes infected by Stolbur phytoplasma where sieve tubes were occluded with thick callose deposits. This deposition of callose may result into the intercellular transport regulation of photoassimilates through the sieve pores which may also limit phloem mass flow (Furch et al., 2007; Musetti et al., 2013).

Furthermore, it has been documented that sealing of sieve elements may also prevent an invasion and manifestation of pathogens including phytoplasma. The sealing of these elements may also occur to counteract the release of phytoplasma effectors and elicitors and also to redirects nutrient flows in plants. In this case, sealing of sieve tube elements in diseased plants is seen as a general response to pathogen attack by the plant immune systems. The slower rate of phloem mass flow in phytoplasma-infected leaves could also be explained by the small areas of the sieve tube elements observed in phytoplasma-infected leaves as a result of mass flow resistance. This resistance to phloem mass flow could also have led to the slower translocation of photoassimilates in phytoplasma-infected plants. Moreover, the xylem tissues of phytoplasma-infected Napier grass leaves were negatively affected with regard to their area probably due to the functional association between xylem and phloem. Both xylem and phloem tissues are engaged in the long-distance transportation of water and photoassimilates in the vascular bundles of
angiosperms through the water circulation system with high rates of water intake by roots and water loss from the aerial parts, respectively (Will and van Bel, 2006). The areas of the xylem and phloem tissues also decreased proportionally with the vascular area and this may indicate that phytoplasma infection causes an adaptation of the leaf size (little leaves) due to limited the transport capacity of the sieve tubes and this explains the observed reduction of the areas of the vascular tissues of the plants infected with phytoplasmas.

The bioimaging method enables the detection phytoplasmas in the living plant tissue and also identified sieve elements, sieve pores, companion cells and the symplasmic contacts that links them. The DNA-specific dye, DAPI dye that requires fixation for deep penetration in plant tissues (Schulz, 1987) was used and confocal microscopic technique was used to avoid tissue damage and to achieve high resolution level in three dimensions. The study showed formation of dense masses of phytoplasmas in the sieve tubes of phytoplasma-infected plants. The DNA presence in mature sieve elements was a sufficient evidence of phytoplasmas since mature sieve elements lack nucleus confirming the presence of high AT content (80.7 %) in the genome of NGS phytoplasma, the highest reported value for phytoplasma species sequenced to date (Fischer et al., 2016). Because, they are enucleated, sieve element cells consist of only a reduced cytoplasm which helps to form a low resistance passageway for the photoassimilates enhancing the spread and multiplication of phytoplasmas in diseased plants (Christensen et al., 2005).

DAPI has been used before to detect and identify different phytoplasma strains in the sieve elements in many infected plant species (Lee et al., 2000). For instance, the
colonization patterns of apple (*Malus* spp.) and pear (*Pyrus* spp.) trees infected with the ‘*Ca. Phytoplasma mali*’ and ‘*Ca. Phytoplasma pyri*’, respectively were monitored over a period of several years with fluorescence microscopy using the DAPI test and periodic transmission grafting (Arismendi *et al*., 2010). The ‘*Ca. Phytoplasma fraxini*’ (16SrVII-A) has also been detected in Murta (*Ugni molinae*) and common chaura (*Gaultheria phillyreifolia*) by DAPI staining. These results indicated that phytoplasma cells concentrate around the sieve elements of the roots and the aerial parts (Schaper and Seemüller, 1982; Seemüller *et al*., 1984a, b; Arismendi *et al*., 2010). The results of this study corroborate previous findings as most phytoplasmatic cells were found located on the cell lumen of most sieve tube elements. The evidence that Flavescence dorée phytoplasma adhere to insects’ tissues (Lefol *et al*., 1993), also suggest that phytoplasmas could also adhere and grow on the cell membrane of the sieve elements against the assimilate flow (Christensen *et al*., 2005), and this could explain the slow phloem mass flow in phytoplasmatic infected plants. Besides, the current study showed a marked individual and aggregations of cells on the cell membranes of the conductor tissue confirming previous research findings (Arismendi *et al*., 2010).

Detection of phytoplasmatic bodies in infected plant species through DAPI staining is rapid and inexpensive and allowed the detection of phytoplasmas earlier before the invention of molecular techniques (Schaper and Seemüller, 1982; Seemüller *et al*., 1984a, b). However, use of DAPI stain alone for phytoplasma identification is not sufficient, due to its nonspecificity with occasional DNA stains contamination from other microorganisms or organelles including mitochondria and chloroplasts (Fránová *et al*., 2007). For that reason, DAPI staining is used mainly as a preliminary diagnostic tool and
is usually complemented by PCR (Arismendi et al., 2010). The use of nPCR and qPCR allowed for detection and absolute quantification of the phytoplasma cells in the infected plant leaves. The qPCR results successfully demonstrated high yields of phytoplasma cells in the plant leaves indicating the possibility of rapid multiplication and spread in the Napier grass. Similar variation in the phytoplasma titres have been found in phytoplasma-infected plants including Napier grass leaves (Christensen et al., 2004; Wambua et al., 2017) indicating that phytoplasma quantities differ in individual plants. This could also be an indication of many chances of phytoplasma transmissions through insect feeding.

A slight increase in the concentrations of ABA and SA was observed in the phytoplasma-infected than in healthy Napier grass plants. Many studies have implicated the role of phytohormones in the pathogenicity of phytoplasma indicating that phytoplasma infection may be the cause of hormonal imbalances in infected plants. For instance, brinjal plants infected with little-leaf phytoplasma exhibited higher IAA and cytokinin in the stems and floral tissues than in the healthy tissues (Das and Mitra, 1998). Leon et al. (1996) studied the levels of ABA and ethylene in coconut palms (Cocos nucifera L.) infected with lethal yellowing phytoplasma and found insignificant changes in ABA in the early stages of disease development. However, the researchers observed an increase of 2.5 times in ABA in more advanced stages of the disease development indicating that stage of disease development affects the levels of ABA produced in phytoplasma-infected plants. NGS phytoplasma-infected plants show little or no symptoms in the early stages of plant growth (Asudi et al., 2015; 2016a; Wamalwa et al. 2017) and this could account for the insignificant levels of ABA and SA in the current study. Generally, ABA accumulation promotes stomata closure and decreases the rate of gas exchange.
consequently reducing photosynthetic activity and transpiration (Cutler et al., 2010; Brandt et al., 2012; Mittler and Blumwald, 2015). This negatively impacts the plant growth which is manifested by reduced size of plant organs including vascular bundles and may results in an early senescence (Wehner et al., 2015).

Increased SA concentrations have been implicated in reduced plant growth as it influences the lignin content production (Gallego-Giraldo et al., 2011) suggesting the reasons for the stunted and proliferation of little tillers in phytoplasma-infected Napier grass. In addition, the increase in the production of endogenous SA and its conjugates is key signal to plant defense responses (Dempsey et al., 2011) and usually precedes the induction of pathogenesis-related genes and onset of disease resistance (Conrath et al., 2006). Ameliorative effect has also been demonstrated endo- and exogenously in plants infected with other pathogens including fungi, bacteria and phytoplasma indicating that SA activates plant defense responses against pathogen attack enhancing plant recovery from infection (Mauch et al., 2000; Verberne et al., 2000; Sánchez-Rojo et al., 2011). Ameliorative effect has also been observed when potato infected with potato purple top phytoplasma was sprayed with low concentrations of SA indicating that SA activates plant defense responses against phytoplasma attack enhancing plant recovery from infection (Sánchez-Rojo et al., 2011).

Interestingly, Napier grass produces high levels of SA comparable to that found in rice (5000–30 000 ng/ g fresh weight) but exceeds elevated levels in infected tobacco and Arabidopsis tissues (500–2000 ng/ g fresh weight) (Raskin et al., 1990; Silverman et al., 1995; Chen et al., 1997) suggesting high SA levels may be a preformed chemical barrier
against pathogen infection or may be serving other biological functions in Napier grass as has been proposed in rice. The insignificant changes in SA levels in between the healthy and infected Napier grass shoots, however, indicates that SA may be a poor activator of pathogen resistance gene expression and induced resistance in Napier grass.

Salicylic acid and jasmonic acid defence pathways are either mutually synergistic or antagonistic (Mur et al., 2006). Because plants have to cope with multiple attackers and diverse abiotic stresses in natural environments, a complex regulatory mechanism has evolved. How plants prefer one response over the other is not yet known (Bari and Jones, 2009). An impressive decrease in cis-OPDA, JA and JA-Ile concentrations was observed in phytoplasma-infected Napier grass plants suggesting a downregulation of JA defense machinery.

Similar results have been found showing that phytoplasma infection is associated with reduced levels of JA biosynthesis in AY-WB phytoplasma infected Arabidopsis. This has been explained by the destabilisation of the class II TCPs by the SAP11 leading to a decreased synthesis of JA in phytoplasma infected plants. Phytoplasma infection also decreases the abundances of enzymes lipoxygenase and allene oxide synthase which are involved in the JA biosynthesis (Sugio et al. 2011; Mardi et al. 2015). Because JA is involved in the plant defence response against insect herbivores including the AY-WB leafhopper vector Macrosteles quadrilineatus (Sugio et al. 2011), decreased levels in infected plants increase the production and fitness of insect vectors. Therefore, based on the downregulation of JA synthesis by NGS phytoplasma infection, the current findings suggest that phytoplasmas decreases the endogenous JA levels by effector proteins to
attract their insect vectors and this could account for the continued spread of NGS disease in the East Africa.

5.2 Conclusions

i. The NGS phytoplasma infection negatively affected the areas of the vascular tissues which impairs the physiological activity of the vascular tissues.

ii. Napier grass stunt phytoplasma infection impairs phloem mass flow and this may be regarded as protective measures of the host plant against phytoplasma spread to uninfected sieve elements which in turn results in constrained distribution of nutrients and signalling compounds throughout the plant which may account to yellow leaf symptom that are expressed by Napier grass stunt diseased plants.

iii. Napier grass stunt phytoplasma-infected plants show little and sometimes no symptoms in the early stages of plant growth because of the insignificant levels observed in ABA and SA concentration between healthy and phytoplasma-infected plants.

iv. Napier grass stunt phytoplasma also inhibits biosynthesis of jasmonates in infected Napier grass, which results in compromised the plant’s immune system that is achieved through the modification of plants transcription factors by effector proteins secreted by phytoplasma in order to make Napier grass a suitable host to phytoplasma insect vectors and hence enhancing insect vector fitness.
5.3 Recommendations for further studies

i. Considering the fact that sieve element protein agglutination and plugging by sieve element proteins is a plant response to phytoplasma infection, the effect of potential sieve element occlusion proteins in response to phytoplasma infection should be investigated in Napier grass since this could have contributed to the impaired phloem transport.

ii. Napier grass plants also expressed high SA concentrations. There is need, therefore to study SA metabolism in Napier grass to understand the biochemical mechanisms that allow Napier grass to maintain exceptionally high levels of salicylic acid and if salicylic acid could be probably performing other roles in Napier grass.

iii. The mechanism utilized by phytoplasma to maximize their fitness by manipulating host plant development as suggested by JA suppression in infected Napier grass is still unknown. There is therefore a need to study different phytoplasma effector proteins expressed in infected Napier grass.

iv. Accumulation of abscisic acid level in plants has been reported to negatively affect plant growth which is manifested at different stages of plant development and results in an early senescence. Therefore, there is need to investigate the levels of ABA in different stages of development.
REFERENCES


APPENDICES

Appendix 1: Mean analysis of effect of phytoplasma infection on the areas of vascular bundles (VB), xylem vessels (XV), phloem tissue, sieve elements (SE) and on the rate of phloem mass flow (mass flow) morphology and phloem mass flow.

<table>
<thead>
<tr>
<th></th>
<th>F</th>
<th>df</th>
<th>Sig. (2-tailed)</th>
<th>Mean Difference</th>
<th>Std. Error</th>
<th>Difference (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VB</td>
<td>0.85</td>
<td>10</td>
<td>0.038</td>
<td>3271.83</td>
<td>1368.63</td>
<td>2.56</td>
</tr>
<tr>
<td>XV</td>
<td>2.75</td>
<td>10</td>
<td>0.037</td>
<td>298.67</td>
<td>123.87</td>
<td>0.10</td>
</tr>
<tr>
<td>Phloem</td>
<td>0.36</td>
<td>10</td>
<td>0.024</td>
<td>308.17</td>
<td>116.10</td>
<td>-0.14</td>
</tr>
<tr>
<td>SE</td>
<td>1.06</td>
<td>10</td>
<td>0.00</td>
<td>29.33</td>
<td>1.90</td>
<td>-8269.23</td>
</tr>
<tr>
<td>Mass flow</td>
<td>0.84</td>
<td>10</td>
<td>0.00</td>
<td>15.33</td>
<td>1.54</td>
<td>-1.61</td>
</tr>
</tbody>
</table>

Appendix 2: Mean analysis of effect of phytoplasma infection on the levels of phytohormones

<table>
<thead>
<tr>
<th>Phytohormones</th>
<th>F</th>
<th>df</th>
<th>Sig. (2-tailed)</th>
<th>Mean Difference</th>
<th>Std. Error</th>
<th>Difference (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis-OPDA</td>
<td>4.89</td>
<td>8</td>
<td>0.007</td>
<td>9.59</td>
<td>3.05</td>
<td>2.56</td>
</tr>
<tr>
<td>JA</td>
<td>1.25</td>
<td>8</td>
<td>0.019</td>
<td>0.60</td>
<td>0.22</td>
<td>0.10</td>
</tr>
<tr>
<td>JA-Ile</td>
<td>54.61</td>
<td>8</td>
<td>0.039</td>
<td>1.03</td>
<td>0.51</td>
<td>-0.14</td>
</tr>
<tr>
<td>SA</td>
<td>3.74</td>
<td>8</td>
<td>0.267</td>
<td>-1861.89</td>
<td>2778.55</td>
<td>-8269.23</td>
</tr>
<tr>
<td>ABA</td>
<td>1.01</td>
<td>8</td>
<td>0.25</td>
<td>-0.369</td>
<td>0.54</td>
<td>-1.61</td>
</tr>
</tbody>
</table>