Dynamics of Napier stunt phytoplasma between the cultivated and wild graminae in East Africa

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It all starts here

Dedication

To my late parents, may your souls rest in eternal peace

To my beloved wife Sharon A. Marongo for her constant encouragements and love

To our daughter Noella, for the new beginning

DECLARATION

I, George Ochieng Asudi, declare that the dissertation submitted for the degree of Philosophiae Doctor in Natural Sciences at the North-West University (Potchefstroom Campus), Potchefstroom, North West, South Africa, is my own independent work and has not previously been submitted by me at another university.

Approval by supervisors

The supervisors of this study give permission that the data generated during the study may be used for scientific publication.

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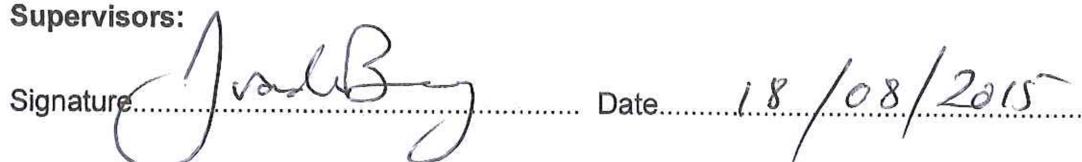

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Abstract

Cultivation of Napier grass, *Pennisetum purpureum*, the most important livestock crop in East Africa is severely constrained by Napier Grass Stunt (NGS) disease. The disease spreads via an insect vector or vegetative propagation of infected plant material and is caused by a phytoplasma. This necessitates the development of an integrated management approach for the disease. Therefore, objectives of this study were to assess the incidence of the disease and its severity, to identify its wild hosts and farmers' knowledge on these hosts, to assess the threat of NGS disease to cultivated grasses and to establish the role of wild inoculum sources in its spread. The study showed NGS incidence ranging from 33% in Uganda to 95% in Kenya with 49% of the farmers interviewed, being able to discern NGS disease by its symptoms. Most farmers cited roguing and use of alternative fodder grasses as control measures, making these strategies the likely components of an integrated management approach for the disease. Responders named Sedge grass (*Cyperus* spp.) and Star grass (*Cynodon dactylon*) as the likely hosts of diseases caused by phytoplasma. Phytoplasmas were detected in leaves of 11 of 33 wild grass species collected using polymerase chain reaction (PCR) based on the highly conserved phytoplasma-specific 16S ribosomal DNA fragment. Sequence determination of amplified PCR fragments revealed the presence of NGS-related phytoplasmas in 11 grass species, Bermuda grass white leaf (BGWL) phytoplasmas in three and goose-grass white leaf (GGWL) in two wild grass species, showing that the geographical distribution and diversity of phytoplasmas and their grass hosts are greater than previously thought. The relationships between NGS and *Hyparrhenia* grass white leaf (HGWL) phytoplasmas were determined using sequences based on *secA* gene and immunodominant protein (*imp*). Results showed a very low genetic diversity between NGS and HGWL and produced a phylogenetic tree congruent to that produced by the 16S, affirming the inclusion of HGWL in the 16SrXI group. NGS phytoplasma was transmissible to food crops through *Maiestas banda* Kramer (Hemiptera: Cicadellidae) under screen-house conditions. With 56.3%, *Saccharum officinarum* showed the highest infection level followed by *Eleusine coracana* with 50%, *Sorghum bicolor* with 43.8%, *Oryza sativa* with 31.3% and *Zea mays* with 18.8%. All the phytoplasma-infected plants were asymptomatic except *S. officinarum* plants, which showed mild to moderate symptoms consisting of foliar yellow leaves

and bright white or yellow midribs. This hints that besides wild hosts, food crops may also serve as alternative source of inoculum enabling a complex NGS disease cycle, which may add to challenges in the development of the disease control strategies. However, failure by *M. banda* to transmit HGWL and BGWL phytoplasmas back to Napier grass is an indication that it could be the exclusive vector of NGS. Therefore, there is need to initiate transmission trials using planthoppers and leafhoppers occurring on HGWL and BGWL phytoplasma-infected grasses to determine whether insect vectors capable of transmitting phytoplasmas from native grasses to Napier grass, are present in the region.

Keywords: Napier grass, fodder, NGS phytoplasma, incidence, transmission, wild grass hosts, East Africa, threats, food crops

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Chapter 1: General introduction

1.1 General background

Napier grass (*Pennisetum purpureum* Schumach) also known as Elephant grass, is a robust perennial forage indigenous to the Zambezi valley in Africa (Boonman, 1993). It grows in bamboo-like clumps and may reach 10 m in height (Farell *et al*., 2002). It was named after colonel Napier of Bulawayo, who in the early 20th century, championed its adoption as livestock fodder in the colonial Rhodesia, now Zimbabwe. European settlers introduced the grass into East Africa as mulch for coffee, but farmers found it more efficient as fodder for livestock (Boonman, 1993). Napier grass is the highest ranked fodder crop in East Africa. It is planted for environmental protection to help stabilize soils and to act as windbreaks (Jones *et al*., 2004; Orodho, 2006). Recently, a novel use of Napier grass has been discovered and exploited in a 'push–pull' strategy (PPS) for the management of the most injurious pests of cereals, stem borers (Cook *et al*., 2007; Pickett *et al*., 2014). The strategy involves intercropping a cereal crop such as maize (*Zea mays* L.) with a stemborer-repellent plant (push), usually *Desmodium* spp. with the trap crop, Napier grass planted as a border crop (pull) around this intercrop. Napier grass is more attractive to stemborer moths than maize for oviposition but supports only minimal survival of larvae. Therefore, when planted as a trap crop around a cereal crop it attracts more oviposition by stemborer moths than the main crop leading to a decrease in pest pressure and reduced yield losses (van den Berg, 2006; Khan *et al*., 2010; Midega *et al*., 2010).

There has been an escalated cultivation of Napier grass in the region in recent years because of the increased commercial dairying and uptake of the PPS. However, continued adoption of Napier grass farming in the region is under threat from a serious phytopathological constraint known as Napier grass stunt (NGS) or Napier stunt (NS) disease. The disease has been reported in Kenya, Uganda and Ethiopia and is caused by a phytoplasma. The symptoms of the disease include foliar yellowing of leaves, profuse tillering and severe stunted growth leading to loss of biomass and eventual death of plants (Jones *et al*., 2004; 2007; Nielsen *et al*., 2007). Phytoplasmas are uncultivable, obligate parasites and degenerate gram-positive prokaryotes. Globally, they are associated with numerous plant diseases of crops,

vegetables, fruits, grasses and ornamentals resulting into phytosanitary conditions and serious losses of world economies (Lee *et al*., 2000). Phytoplasmas lack a rigid cell wall and are only surrounded by a single cell membrane. Their genome is small ranging from 530 to 1350 kb with a low guanine plus cytosine (G+C) content. Together with acholeplasmas they form the family Acholeplasmataceae and order Acholeplasmatales within 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 similarity of their 16S rDNA sequences. Strains within a candidate species share at least 97.5% sequence identity of their 16S rDNA gene sequences. However, also phytoplasmas with more than 97.5% identity can be described as distinct species when vectors, host plants or ecological niches differ significantly (IRPCM, 2004). For routine identification and classification of phytoplasmas, 16S rDNA polymerase chain reaction (PCR) fragments are usually digested by restriction enzymes and fragments separated by polyacrylamide gel electrophoresis. The generated restriction fragment profile is typical for a phytoplasma group or sub-group. This system provides a rapid and reliable means for preliminary classification towards epidemiological studies on diseases associated with phytoplasma presence (Lee *et al*., 1998; Bertaccini, 2007). Currently, 37 '*Ca.* Phytoplasma' species have been described based on the 16S ribosomal sequence data (IRPCM, 2004) and new lists of species continue being published regularly (Win *et al*., 2013; Quaglino *et al*., 2013; Harrison *et al*., 2014). Besides the 16S, other less well-conserved genes such as s*ecY*, *tuf*, *secA*, *rp* (ribosomal protein) operon and the 16S–23S rRNA intergenic spacer region (ISR) are used as supplemental tools for finer phytoplasma differentiation and to support and subdivide the 16S groups into more distinct subclades (Bertaccini, 2007; Hodgetts *et al*., 2008).

Based on the 16S rDNA sequences, phytoplasmas associated with NGS in Kenya and Uganda belong to the 16SrXI group '*Ca.* Phytoplasma oryzae' or rice yellow dwarf (RYD), while those occurring in Ethiopia are known as African sugarcane yellow leaf (ASYL) phytoplasma, a member of the 16SrIII, '*Ca.* Phytoplasma pruni' or X-disease (Jones *et al*., 2004, 2007; Nielsen *et al*., 2007;). Two phytoplasmas closely related to the NGS were detected in other wild grasses in western part of Kenya. These are Bermuda grass white leaf (BGWL) detected in *Cynodon dactylon*

and *Hyparrhenia* grass white leaf (HGWL) found in *Hyparrhenia rufa*. HGWL is classified as a '*Ca*. Phytoplasma oryzae' strain and is closely related to NGS while BGWL belongs to the 16SrXIV group or '*Ca* Phytoplasma cynodontis' (Obura *et al*., 2010, 2011). In Ethiopia, phytoplasmas detected in *Medicago sativa* and *Cynodon dactylon* were classified as ASYL (Arocha *et al*., 2009). These studies suggested that *H. rufa*, *M. sativa* and *C. dactylon* could be alternative host plants for the NGS and could play a role in the spread of NGS disease in East Africa.

Since its first discovery in 1997 in the Bungoma district of Kenya (Orodho, 2006), NGS disease has spread to several districts in East Africa causing serious economic losses in the smallholder dairy industry in the region (Kabirizi *et al*., 2007; Khan *et al*., 2014). The disease also poses a significant threat to the cultivation of food crops such as cereals that depend on PPS for the control of cereal stem borers in the region. Despite this, no curative methods are available against these plant pathogenic agents. For this reason, management of phytoplasma-infected plants has mainly focused on controlling the insect vectors and on roguing infected plants from crops and weeds. Plant cultivars resistant to phytoplasma diseases do exist but are rare (Thomas & Mink, 1998; Kabirizi *et al*., 2007; Bisognin *et al*., 2008). Besides, most Napier grass cultivars selected and introduced lately in the East African region, are no longer immune to the phytoplasma probably due to more aggressive strains of the NGS (Mulaa *et al*., 2010; Kawube *et al*., 2014).

Phytoplasmal diseases are spread primarily by sap-sucking insect vectors belonging to the families Cicadellidae (leafhoppers), Fulgoridae (planthoppers) and Psyllidae (psyllids) (Weintraub & Beanland, 2006; Obura *et al*., 2009), through vegetative propagation of infected plant material (Boudon-Padieu, 2003) and also vascular connections made by parasitic plants such as dodder (*Cuscuta* spp.) between infected and uninfected host plants. These hemipteran insects feed on phloem tissues, where they acquire phytoplasmas and transmit them from plant to plant (Lee *et al*., 2000; Weintraub & Beanland, 2006; Obura *et al*., 2009). The family Poaceae (also called Gramineae or true grasses) has the largest number of plant species associated with phytoplasma diseases worldwide and is the one family on which the majority of the vector species, Delphacidae have been found (Arocha & Jones, 2010). In Kenya, *Maiestas banda* (Kramer) (Hemiptera: Cicadellidae), a leafhopper

in the tribe Deltocephalini (Satoshi, 1999; Webb & Viraktamath, 2009) was identified as the vector of NGS disease (Obura *et al*., 2009). In Ethiopia, a leafhopper *Exitianus* spp. (Hemiptera: Cicadellidae) and a planthopper *Leptodelphax dymas,* (Fennah) (Hemiptera: Delphacidae) have been suggested as potential vectors of the NGS phytoplasma (Arocha *et al*., 2009).

1.2 Description of the problem

Due to intensified dairy farming in East Africa region there is high demand for Napier grass. To address this need, landless farmers plant along highway verges and free land to cut and sell grass to animal owners. These limit natural pasturing and thus cattle are fed on crop residues and cultivated forage mainly Napier grass (Orodho, 2006), which is also used in the region as a trap crop for cereal stem borers, for soil and water conservation and as mulch in other farming systems (Jones *et al*., 2004; Cook *et al*., 2007). Recently, a new stunting disease of Napier grass named as NGS has emerged in the region, with devastating effects. The disease becomes visible in re-growth after cutting or grazing, with the affected shoots becoming pale yellow green and seriously dwarfed, with low biomass that is unable to sustain the feed requirements of dairy cows. Often the whole stool is affected with complete loss in yield leading to eventual death of the plant (Jones *et al*., 2004, 2007; Orodho, 2006; Kabirizi *et al*., 2007).

The disease, caused by 16SrIII-A and XI phytoplasma strains (Jones *et al*., 2004, 2007), is spreading fast in the region (Kabirizi *et al*., 2007; Khan *et al*., 2014) with potential to escalate to other areas in Sub-Saharan Africa with similar agro-ecologies. In Kenya, the disease is transmitted from infected Napier grass plant to healthy plants by an insect vector known as *M. banda* (Obura *et al*., 2009) and is controlled mainly by removal and burning of the infected plants (Kabirizi *et al*., 2007; Khan *et al*., 2014). However, these control measures are not efficient. Attempts to develop and introduce resistant Napier grass cultivars in the region in the recent past also failed when these cultivars became susceptible to the pathogen (Mulaa *et al*., 2010). The spread and increase in incidences of this disease in the region, therefore represent a real threat to the fodder and the cultivated plants such as sorghum, rice, sugarcane, finger millet and maize, which serve as staple and cash crops in the region.

Wild grasses such as *H. rufa*, *C. dactylon*, and *M. sativa* are vulnerable to phytoplasma infections (Arocha *et al.*, 2009; Obura *et al.*, 2010, 2011). It is therefore likely that many grasses could already be infected by NGS or other phytoplasmas in the wild habitat. These infected wild grasses might also be acting as reservoir to wild sources of inocula of phytoplasma and contributing to the spread of the disease in the region. Besides, the host range of the NGS phytoplasma, there is a shortage of information on the dynamics of this disease between cultivated and wild grasses. For instance, the potential dangers posed to cultivated grasses, principally cereals, other monocots and fodder grasses other than Napier grass are not fully understood.

1.3 Justification

Phytoplasma diseases limit the production of many crops including fruits, grasses, vegetables and ornamental plants. In general, the family Poaceae comprises the greatest number of plant species affected with these diseases (Lee *et al.*, 2000). Five 16Sr groups of phytoplasma including 16SrI, III, XI, XII and XIV are known to infect grasses (Arocha & Jones, 2010). Among these, 16SrIII and XI phytoplasma strains are the most important in East Africa causing NGS disease with severe losses in Napier grass and representing a threat to its cultivation. This 16SrXI strain is closely related to strains isolated from sorghum infected with grassy shoot, thatch grass infected with HGWL and sugarcane white leaf diseases, which are members of the 16SrXI group phytoplasma (Jones *et al.*, 2004; Obura *et al.*, 2009). This indicates the ability of the phytoplasma to infect grasses other than Napier grass and is a risk in eastern Africa where maize, rice, sorghum, millet and sugarcane serve as staple food and cash crops. The knowledge on the dynamics of the NGS disease between cultivated and wild grasses and its host range will contribute to the development of management approaches for phytoplasma diseases in cultivated grasses such as cereals, sugarcane and fodder grasses in East Africa.

1.4 Objectives

The objective of this study was to determine the dynamics of the Napier grass stunt phytoplasma between the cultivated and wild grasses as an important component in the development of an integrated management approach for the Napier grass stunt disease in East Africa.

1.4.1 Specific objectives

The specific objectives of this study are to:

- Assess the incidence of Napier grass stunt disease, its severity and the farmers' knowledge on its wild grass hosts.
- To detect, identify and classify phytoplasmas within wild grasses with potential to affect Napier grass and other monocots in East Africa
- To compare and test the identity of the *Hyparrhenia* grass white leaf and Napier grass stunt phytoplasmas and their phylogentic relation using genes based on other regions of the phytoplasma namely *secA* and immunodominant protein (*imp*).
- Assess the threat of Napier grass stunt disease to cultivated grasses, develop and implement an early detection and warning system.
- Establish the role of wild inoculum sources in the transmission of Napier grass stunt disease in East Africa.

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Chapter 2: Literature review

2.1 Main features of Phytoplasmas

Phytoplasmas, previously termed, as mycoplasma-like organisms (MLO), are non-cultivable degenerate gram-positive prokaryotes closely related to mycoplasmas, acholeplasmas and spiroplasmas. Although they were only described four decades ago by a group of Japanese scientists (Doi *et al*., 1967), the first phytoplasma (then called virus)-associated disease, aster yellows, was described in 1926 (Kunkel, 1926). The term 'MLO' was used to name phytoplasmas due to their morphological and ultrastructural similarity to mycoplasmas infecting animals. These prokaryotes lack a cell wall and are only surrounded by a single unit membrane (Doi *et al*., 1967; Lee *et al*., 2000), which appears to have suffered extreme genome reductions compared to their Gram-positive relatives like *Clostridium* or *Lactobacillus* spp.. Phylogenetic studies suggest that the common ancestor for phytoplasmas is *Acholeplasma laidlawii* Freundt in which the triplet coding for tryptophan (trp) is UGG, while in the other prokaryotes, including mycoplasmas and spiroplasmas, trp is coded by UGA (Bertaccini & Duduk, 2009).

Phytoplasmas differ from the mycoplasmas which infect animals by the existence of a spacer region (300 bp) between 16S and 23S ribosomal regions (Fig. 2.1) that codes for isoleucine tRNA and part of the sequences for alanine tRNA. Moreover, phytoplasmas and acholeplasmas lack functional phosphotransferase transport systems (PTS) (Oshima *et al*., 2004; Bai *et al*., 2006; Kube *et al*., 2008; Tran-Nguyen *et al.,* 2008) whereas mycoplasmas and spiroplasmas have PTSs (Razin *et al*., 1998) required for sugar importation. Furthermore, mycoplasmas and ureaplasmas encode all eight subunits of the F_0F_1-type ATPase catalytic core for ATPase synthase and utilize the transmembrane potential for ATP synthesis, but all phytoplasma genomes sequenced to date lack all eight subunits probably due to their genome reductions (Oshima *et al*., 2004; Bai *et al*., 2006).

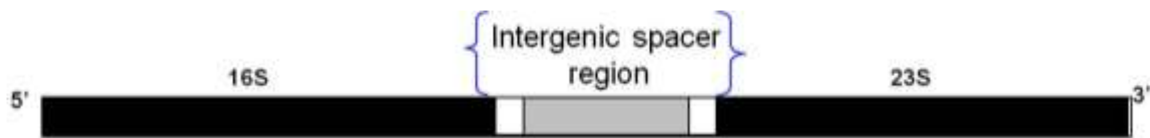

Figure 2.1: Diagrammatic representation of a phytoplasma rRNA operon, including the 16S and 23S rRNA genes and the intergenic spacer region (Smart *et al*., 1996).

On transmission electron microscopic images, phytoplasmas appear either as rounded pleiomorphic bodies or as short-branched filamentous forms with an average body size ranging from 80 to 800 nm in diameter (Doi *et al*., 1967; Bertaccini, 2007; Oshima *et al*., 2013). Phytoplasmas can survive and multiply only in the plant phloem and insect haemolymph. They are therefore strictly host-dependent (Lee *et al*., 2000; Bertaccini, 2007).

2.2 Phytoplasma genomes

Phytoplasma genomes are small and vary considerably, ranging from 530 to 1350 kbp. Bermuda grass white leaf (BGWL) phytoplasma represents the smallest genome size (~530 kbp) ever reported with the smallest chromosome known for any living cell (Lee *et al*., 2000; IRPCM, 2004). Phytoplasma chromosomes are also very small ranging from 680 to 1600 kb and consist of either a circular or a linear DNA molecule with short extra-chromosomal DNAs. The phytoplasma genome, however, consists of a low Guanine plus Cytosine content, which supports their phylogenetic affiliation to members of the class Mollicutes (Lee *et al*., 2000; Bertaccini, 2007). Phytoplasmas lack the genes that code for tricarboxylic acid cycle, pentose phosphate pathway, sterol biosynthesis, fatty acid biosynthesis, de novo nucleotide synthesis and biosynthesis of most amino acids (Oshima *et al*., 2004; Bai *et al*., 2006). Furthermore, analysis of the protein-coding genes show that glycolysis pathway supposed for '*Ca*. Phytoplasma asteris', is reduced in '*Ca*. Phytoplasma mali' and thus, maltose and malate are likely utilized as alternative carbon and energy sources (Kube *et al*., 2008). Generally, small-genome pathogenic bacteria have lost the genes for most biosynthetic pathways, most probably because many metabolites are obtainable within the host cell environment resulting into a lessened constraint on genes for biosynthetic potentialities (Hogenhout *et al*., 2008).

2.3 Life cycle of Phytoplasmas

As obligate parasites, phytoplasmas require diverse hosts, mainly plants and insects, for their replication, survival and spread. In plants, phytoplasmas reside mostly in the sieve elements of the phloem, including both mature and immature cells that still have nuclei (Doi *et al*., 1967; Hogenhout *et al*., 2008). In insects, phytoplasmas pass through insect gut cells and replicate in various body tissues. When the phytoplasmas reach the salivary glands, they are introduced into plants with the saliva that is transferred to the plant during the feeding process. In plant hosts, the highest concentration of phytoplasma has been found in mature sieve tubes. As phloem cells are live cells, this may be considered intracellular (Christensen *et al*., 2004, 2005; Hogenhout *et al*., 2008). However, in insects, phytoplasmas may be detected intra- and extracellularly in the insect tissues and therefore, they may be considered as intra- as well as extracellular pathogens or symbionts of plants and insects (Hogenhout *et al*., 2008).

After injection into plants, phytoplasmas negatively affect the fitness of their plant hosts. Infected plants develop symptoms such as stunting with abnormal leaves, flowers, fruits or seeds. However, in insects, phytoplasmas may or may not influence fitness and survival of insect vectors. Insect vectors may sometimes benefit from phytoplasma infection by living longer when deprived of a main food source and when exposed to lower suboptimal temperatures. For example, *Dalbulus* leafhoppers (Homoptera: Cicadellidae), when exposed for a long time to maize bushy stunt phytoplasma and *Spiroplasma kunkelii*, develop tolerance to these bacteria and become well adapted to each other (Hogenhout *et al*., 2008). In some cases, phytoplasmas may facilitate plants to become new hosts for leafhoppers that do not normally use certain plant species as hosts. For example, the leafhopper *Dalbulus maidis*, Delong and Wolcott (Homoptera: Cicadellidae), which is a maize specialist, can feed and survive on aster yellows phytoplasma (AYP)-infected plants, but not on healthy lettuce and China aster plants (Purcell, 1988).

2.4 Insect vectors of phytoplasmas

The single most successful order of insect vectors of phytoplasmas is the Hemiptera (=Rhynchota) (Weintraub & Beanland, 2006). The Hemiptera are a large and diverse

order of exopterygote insects that occur in all zoogeographic regions of the world and comprise of about 82,000 described species (Arnett, 2000). It consists of three suborders: Heteroptera (true bugs), Sternorrhyncha (scale insects, aphids, whiteflies and psyllids) and Auchenorrhyncha (spittlebugs, cicadas, leafhoppers, planthoppers and treehoppers) (Forero *et al.*, 2008; Weintraub & Wilson, 2010). Within the Auchenorrhyncha and Sternorrhyncha, over 200 leafhopper, planthopper and psyllid vector species of phytoplasmas, spiroplasmas, viruses and *Xylella* are known. While most Auchenorrhyncha feed from phloem tissue, two superfamilies (Cicadoidea: cicadas; Cercopoidea: spittlebugs) and a subfamily of the Cicadellidae (Cicadellinae) feed from xylem tissue. In addition, the majority of species in the leafhopper subfamily Typhlocybinae feed by removing the cell contents from mesophyll cells (Weintraub & Wilson, 2010).

The Hemiptera collectively possess characteristics that make its members efficient vectors of phytoplasmas (Weintraub & Beanland, 2006). (i) They are hemi-metabolous: thus, nymphs and adults feed similarly and are in the same physical location and often both immature and adults can transmit phytoplasma, (ii) they feed specifically and selectively on certain plant tissues in a nondestructive manner, promoting a successful inoculation of plant vascular systems without damaging conductive tissues and eliciting defensive responses; (iii) they have a propagative and persistent relationship with phytoplasmas; and (iv) they have obligate symbiotic prokaryotes that are passed on to the offspring through transovarial transmission, the same mechanisms that allow the transovarial transmission of phytoplasmas.

Within the groups of phloem feeding insects, primarily three taxonomic groups have been confirmed as vectors of phytoplasmas namely: the suborder Clypeorrhyncha (=superfamily Membracoidea) group containing the largest number of vector species confined to the family Cicadellidae, the Archaeorrhyncha (=Fulgoromorpha) group and the suborder Sternorrhyncha (Weintraub & Beanland, 2006). The most derived lineage is found within the superfamily Membracoidea, subfamily Deltocephalinae that includes more than 75% of all confirmed phytoplasma vector species (Webb & Viraktamath, 2009). Vector species in this family can be monophagous or polyphagous and can transmit one or more different phytoplasma taxa. Apart from the tribes Opsiini, Scaphtyopiini, Macrostelini and Scaphodeini, all other members of

the subfamily Deltocephalinae contain vector species that are confined to grass species. Little is known about the host relationships between the majority of species, but it is likely that they are narrowly oligotrophic (Weintraub & Wilson, 2010). The subfamily Macropsinae contains the second largest number of confirmed vector species. These vector species can be monophagous or oligophagous, but most feed on woody plants. Within the Auchaeorrhyncha, vector species are found in four families, i.e. Cixiidae, Delphacidae, Derbidae and Flatidae and transmit stolbur, coconut lethal yellows and AYP phytoplasmas. In psyllids (Sternorrhyncha: Psyllidae), phytoplasma vectors are found in two genera: *Cacopsylla* sp. (Hemiptera: Psyllidae) transmits apple proliferation phytoplasmas to pome and stone fruit trees, while in the other genus, *Bactericera trigonica* Hodkinson (Hemiptera: Psyllidae) transmits a stolbur phytoplasma to carrots (Weintraub & Beanland, 2006).

Two heteropteran families, Pentatomidae and Tingidae, have confirmed phytoplasma vector species. *Halyomorpha halys* Stål (=*H. mista* Uhler) (Heteroptera: Pentatomidae), transmits witches' broom phytoplasma to *Paulownia* spp. trees in Asia (Hiruki, 1999) while *Stephanitis typica* Distant (Heteroptera: Tingidae) transmits a root wilt phytoplasma to coconut palms in Southeast Asia (Mathen *et al*., 1990).

2.5 Phytoplasma acquisition and transmission

Phytoplasmas are obligate parasites of plants and insects restricted to the phloem from which they spread through the pores of the sieve plates (Lee *et al*., 2000). They are transmitted through vegetative propagation through grafting of infected plant material, cuttings, storage tubers, rhizomes or bulbs (Lee *et al*., 2000; Boudon-Padieu, 2003), vascular connections made between infected and uninfected host plants by parasitic plants and phloem-feeding insects most commonly Hemiptera, Auchenorrhyncha {leafhoppers (Cicadellidae) and planthoppers (Delphacidae)} and also by some psyllids (Psylloidea) (Weintraub & Beanland, 2006).

These insect vectors probe phloem tissues and passively ingest phytoplasma cells with the phloem-sap from the infected plants. The acquisition access period of the phytoplasmas by vectors can be as short as a few minutes and the longer the period the greater the chance of acquisition (Purcell, 1982). The time that elapses from initial acquisition to the ability to transmit phytoplasmas is known as the latent period

(LP) and is sometimes called the incubation period. The LP is temperature and host dependent and ranges from a few minutes to 80 days (Nagaich *et al*., 1974; Murral *et al*., 1996). During the LP the phytoplasmas move through and replicate in the competent vector's body. Phytoplasmas can move intracellularly via the epithelial cells in the midgut and replicate within a vesicle, or they can pass between two midgut cells and through the basement membrane to enter the hemocoel. Phytoplasmas circulate in the hemolymph, where they may infect other tissues including the Malpighian tubules, fat bodies and brain or reproductive organs. Replication in these tissues, albeit not essential for transmission, may be indicative of a longer coevolutionary relationship between host and pathogen. Lefol *et al*. (1993) demonstrated surface protein involvement, and some level of specificity, in attachment of phytoplasma particles to insect host cells. However, the molecular factors related to the movement of phytoplasmas through various insect tissues are still unknown.

To be transmitted to plants, phytoplasmas must penetrate specific cells and accumulate in high levels in the posterior acinar cells of the salivary glands (Kirkpatrick, 1992). At each point during this process, if the phytoplasmas fail to enter or exit a tissue, the insect becomes a dead-end host and is unable to transmit the phytoplasma. It has been shown that barriers including the basal lamina, the basal plasmalemma and the apical plasmalemma exist in the salivary glands that pathogens must cross before they can be ejected with the saliva (Wayadande *et al*., 1997). Therefore, although leafhoppers may be infected by phytoplasmas, they may be unable to transmit it to healthy plants (Lefol *et al*., 1993, Vega *et al*., 1993, 1994), most likely because of these salivary gland barriers (Wayadande *et al*., 1997). Under laboratory conditions, phytoplasma transmission from a plant host by a competent vector during feeding can be indirectly determined by using an electrical penetration graph monitoring (Backus *et al*., 2005) by observing different activities performed by insect stylets such as penetration of plant tissues.

Vector-host plant interactions play an important role in limiting or expanding the spread of phytoplasmas (Lee *et al*., 2003). Polyphagous vectors have the potential to inoculate a wide range of plant species, depending on the resistance to infection of each host plant. Additionally, insects that normally do not feed on certain plant

species can acquire and transmit phytoplasmas to those plants under laboratory conditions. This can also occur under field conditions. Hence, in many cases, the plant host range of a vector, rather than lack of phytoplasma-specific cell membrane receptors, will limit the spread of phytoplasmas by that species (Weintraub & Beanland, 2006).

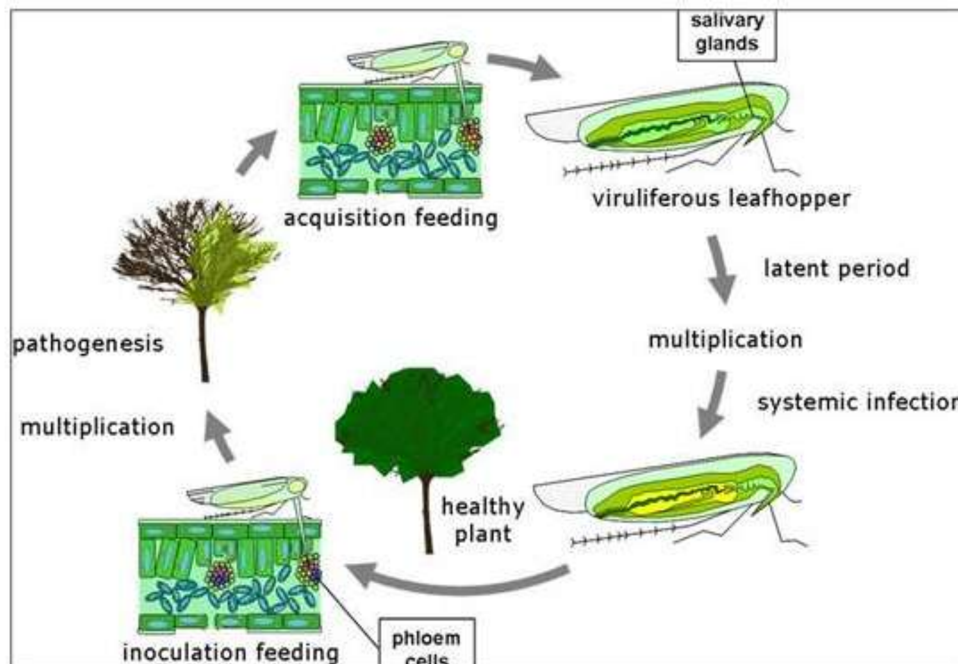

Figure 2.2: Schematic diagram of the life cycle of a phytoplasma (http://www.sporometrics.com).

2.6 Control of phytoplasma diseases and vectors

Until recently, management of phytoplasma diseases has focused on controlling the vector by insecticides (Weintraub & Beanland, 2006). Roguing is an alternative method for reducing vector host plants and/or reservoirs of the phytoplasma including weeds. In this method, phytoplasma-infected plants are entirely removed, or ratooned by removing only symptomatic shoots and it is quite effective following application of insecticide (Weintraub & Beanland, 2006).

The use of chemicals to control vectors will most likely continue for the foreseeable future. However, methods such as habitat management and the use of genetically modified crops are increasingly becoming popular for vector management or

management of phytoplasma spread within the plant. The vegetation composition surrounding crop plants or a field may have a profound effect on the presence and dispersal of phytoplasma vectors (Weintraub & Beanland, 2006). The habitat can be manipulated by use of organic or synthetic mulches to control the vectors. Use of such synthetic mulches, like plastic sheeting, can physically prevent the movement of vectors into the soil making them lay eggs at or just below soil surface while reflective mulches may repel them from the plants (Summers & Stapleton, 2002). Through genetic modification, genes present in plant species can be enhanced or foreign genes can be introduced in these species. These modifications provide protection from the insect vector or the pathogenic phytoplasma. Example includes the expression in rice of lectins highly toxic to planthoppers, that significantly reduced the survival, development and fecundity of the planthopper *Sogatella furcifera* Horvàth (Hemiptera: Delphacidae) and had substantial resistance against the other two planthoppers that affect rice (Powell *et al*., 1995; Nagadhara *et al.,* 2004; Weintraub & Beanland, 2006).

Leafhoppers and planthoppers are attacked by a range of predators such as spiders (Araneae) and true bugs (Miridae) which may in the grassland ecosystems prey on the eggs, nymphs and adult insect vectors with significant effect. The predators may reduce the natural population of the vectors below an economical threshold (Weintraub & Wilson, 2010). Unfortunately, the vegetation that can increase the incidence and abundance of natural enemies of vectors can also be favourable to those taxa that transmit phytoplasmas. More effort should therefore be made to determine those elements of the cropping environment that enhance the survival of natural enemies but do not increase vector numbers (Weintraub & Beanland, 2006). It is important to produce uninfected propagation material or phytoplasma resistant/tolerant varieties to prevent the outbreaks of phytoplasma diseases. However, the use of plant resistance can be done only under restricted and defined environmental conditions (Bertaccini, 2007).

2.7 Molecular detection and identification of phytoplasmas

Since their discovery as plant pathogens in 1967 (Doi *et al*., 1967), phytoplasmas have never been obtained in pure cultures, making their detection and identification difficult. The presence of characteristic symptoms in diseased plants and subsequent

observation of mycoplasma like bodies in ultrathin sections of diseased plants using electron microscopy were the main criteria used to diagnose diseases of possible phytoplasmal origin. Phytoplasma strains were differentiated and identified by their biological properties, such as the similarity and difference in symptoms they induced in infected plants, their plant hosts and insect vectors (Doi *et al*., 1967; Lee *et al*., 2000). For many years, the detection and the study on their morphology and ultrastructure relied on microscopic observations by staining with DNA dye 4´-6-diamidino-2-phenylindole (DAPI) or using transmission or scanning electron microscopy (Lee *et al.,* 2000; Bertaccini, 2007). In the 1980s, molecular-based tools such as mono- and polyclonal antibodies and cloned phytoplasma-specific DNA probes were developed. Serological tests provided relatively simple, sensitive and reliable means for the detection and identification of specific phytoplasma strains. Dot and Southern hybridizations using cloned phytoplasma-DNA probes and restriction fragment length polymorphism (RFLP) analyses of total genomic DNA permitted studies of genetic interrelationships among phytoplasmas, resulting in the recognition of several distinct phytoplasma groups (genomic strain clusters) and subgroups (sub-clusters) (Lee *et al.,* 1998; 2000).

In the late 1980s and early 1990s, PCR-based assays were developed for phytoplasmas. Initially, PCR primers were designed based on sequences of cloned phytoplasma DNA fragments (Lee *et al*., 2000; Bertaccini, 2007). Researchers later designed phytoplasma universal (generic) or phytoplasma group-specific oligonucleotide primers based on the highly conserved ribosomal operon. These PCR assays provided and facilitated detection of low titers of phytoplasmas that were not readily detected by serological or DNA-DNA hybridization assays. To date phytoplasmas diagnosis in host plants and insect vectors are largely done by molecular techniques such as PCR usually followed by RFLP or sequencing for assignation to a '*Candidatus* (*Ca*.) Phytoplasma' species or to a 16S rDNA group. The PCR-based assays have facilitated a much more sensitive means for phytoplasma detection and classification, and a more accurate and reliable tool than biological criteria long used for phytoplasma identification (Lee *et al*., 2000).

2.8 Phytoplasma classification

Due to their inability to grow *in vitro*, phytoplasmas were poorly characterized until the advent of molecular biology. It was also not possible to apply the traditional taxonomic criteria to phytoplasmas, based on phenotypic and biochemical characters. Lately, rRNA gene sequencing has provided evidence that these plant-pathogenic prokaryotes, closely related to spiroplasmas, mycoplasmas and acholesplasmas constitute a large unique monophyletic cluster within the class Mollicutes. A trivial name 'phytoplasma' and a new taxon '*Ca*. Phytoplasma', was proposed and adopted by the Phytoplasma Working Team of the International Research Project for Comparative Mycoplasmology (IRPCM) (2004), to identify and classify phytoplasma and its present composition. The name 'phytoplasma' emphasises the phylogenetic distance of these prokaryotes from the mycoplasmas infecting animals and humans while the '*Ca*.' suffix reflects their non-culturability (Murray and Schleifer, 1994). The IRPCM team also established the rules for defining new phytoplasma species. Thus, a new '*Ca*.' species is described when a 16S ribosomal (r) DNA sequence has less than 97.5% identity with any previously described '*Ca*. Phytoplasma' species. However, two phytoplasmas sharing more than 97.5% of the 16S sequence may be identified as separate '*Ca*.' species when they are transmitted by different vectors, they have different natural plant host (s) and there is evidence of a significant molecular diversity between them (IRPCM, 2004). Up to date 37 '*Ca*. Phytoplasma' species have been named following the 16S ribosomal grouping and the parameter is now commonly employed for identification of the phytoplasma-associated plant diseases (IRPCM, 2004; Win *et al*., 2013; Quaglino *et al*., 2013; Harrison *et al*., 2014).

Several hundred distinct phytoplasma 16S rDNA genes were sequenced. However, additional conserved DNA markers can be used as supplemental tools for finer phytoplasma differentiation (Bertaccini, 2007). Sequences from *tuf* gene, *rp* (ribosomal protein) operon, s*ecY* gene and the 16S–23S rRNA intergenic spacer region (16–23S ISR) have been used to support and subdivide the 16S groups into more distinct subclades than the 16S gene (Botti & Bertaccini, 2003; Streten & Gibb, 2005; Lee *et al*., 2006; Martini *et al*., 2007). The analyses conducted by RFLP or sequencing on *tuf* and/or s*ecY* genes also showed clear indications of phytoplasma

relationships (Bertaccini, 2007). Hodgetts *et al*. (2008), using *secA* gene sequences provided an improved resolution of groups and subgroups from a wide range of the 16S phytoplasma groups. The *secA* fragment also emerged as a promising marker for universal identification of phytoplasmas. Construction of a phylogenetic tree with a high resolution was achieved recently for different phytoplasma groups using a fragment of more than 2 kb of the s*ecY* gene (Lee *et al*., 2010), while a barcode system for '*Ca*. Phytoplasma' identification using *tuf* gene sequences was developed by Makarova *et al*. (2012). Phylogenetic analysis from this gene and the 16S rDNA alignments showed remarkable similarity in terminal taxa, implying that the *tuf* barcode is well linked to the existing 16S rDNA phytoplasma phylogeny. However, use of the 23S rDNA gene sequence was found not to be useful since it appears more or similarly conserved producing phylogenetic trees similar to those obtained using the 16S rDNA gene (Bertaccini, 2007).

2.9 Phytoplasmal diseases of the family Gramineae, a case study of '*Ca.* Phytoplasma oryzae' and '*Ca.* Phytoplasma cynodontis'

2.9.1 Economic importance of Napier grass

Napier grass, *Pennisetum purpureum* (2n=28) is a perennial feed crop with a vigorous root system, sometimes stoloniferous with a creeping rhizome. It is native to the Zambezi valley in Zimbabwe (Boonman, 1993) and grown in eastern and central Africa. Its natural habitat is damp grassland, forest margins and riverbeds. Mature plants usually grow to 3-5 m tall with up to 20 nodes. In riverbeds, however, Napier grass can reach 10 m high and produces 29 tonnes/ha of dry matter. It is mainly propagated from cuttings of 3-4 nodes in length or crown divisions and forms dense clumps of large flat leaves of 30-90 cm long and up to 3 cm wide. The grass is also highly heterozygous giving rise to a very heterogeneous population of seedlings, which are not "true to type" (Orodho, 2006). The grass has the advantage of withstanding repeated cutting, and four to six cuts per year can produce 50±150 tonnes green matter per hectare (Farell *et al*., 2002). If regularly fertilized, *P. purpureum* exhibits rapid regrowth and produces a high biomass which is very palatable in the leafy stage (van der Wouw *et al*., 1999), although it is best replanted every five or six years. This high performance has lead to the widespread use of the grass as a fodder crop. Economically, Napier grass constitutes between 40 and 80%

of forage in East Africa where it is used by smallholder dairy farmers practicing intensive (zero grazing) and semi-intensive dairy cattle production systems to meet the increasing demand for milk (Orodho, 2006). The grass is also widely used for environmental conservation for soil and water in hilly slope areas, and serves as mulch in other farming systems (Jones *et al*., 2004).

Napier grass is currently used in the region as a trap plant in the management of cereal stem borers (*Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) and *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae), the most deleterious insect pests of cereals and the main target pests in the 'push-pull' strategy (PPS) (Cook *et al*., 2007; Pickett *et al*., 2014). The strategy developed by the International Centre of Insect Physiology and Ecology (ICIPE) and partners is preferably rated by farmers in the simultaneous control of stem borers and *Striga* weed which are major constraints to efficient production of cereals, the most important staple food crops in Africa (Khan *et al*., 2010). The Lepidopteran stemborers cause between 10 and 80% yield losses (Kfir *et al*., 2002) while parasitic weeds in the genus *Striga* can cause up to 100% yield losses (Khan *et al*., 2010). PPS involves intercropping cereals with fodder legumes in the genus *Desmodium* and planting around this intercrop Napier grass as a trap plant (Cook *et al*., 2007; Pickett *et al*., 2014). Volatile chemicals released by *Desmodium* spp. repel stem borer moths (push component) while allelochemicals released by its roots suppress *Striga* weeds. Napier grass on the other hand releases chemicals attractive to stem borer moths (pull component). When planted around a cereal crop, it attracts more oviposition by stemborer pests than the main cereal crop whilst minimally supporting the development of the subsequent larvae leading to a suppression of the pest population and increased cereal grain yield (van den Berg, 2006; Khan *et al*., 2010; Midega *et al*., 2010).

2.9.2 Napier grass stunt (NGS) disease outbreak in eastern Africa

Napier grass stunt disease was first reported in Bungoma district of Kenya in 1997. It was later described in Uganda in 2001 and in Ethiopia in 2004 (Alicai *et al*., 2004; Jones *et al*., 2004, 2007; Orodho, 2006; Nielsen *et al*., 2007). In the field, NGS disease manifests in re-growth of Napier grass after cutting or grazing. The disease symptoms include foliar yellowing, small leaves, bushy appearance, yellow to purple streaking, proliferation of tillers and shortening of internodes to the extent that

clumps appear severely stunted with a low biomass yield. Affected shoots become pale yellow-green in colour and seriously dwarfed. Often the whole stool is affected with complete loss in yield contributing to the eventual death of the Napier grass plants (Jones *et al*., 2004; Orodho, 2006; Kabirizi *et al*., 2007). As a result, many smallholders have lost their entire Napier grass crop and are therefore impelled to reduce their number of animals or purchase fodder from the local market (Orodho, 2006; Arocha & Jones, 2010). According to phylogenetic analysis of 16S rDNA sequences, phytoplasmas associated with NGS in Kenya and Uganda belong to the 16SrXI group, '*Ca*. Phytoplasma oryzae' or rice yellow dwarf (RYD) (Jones *et al*., 2004; Nielsen *et al*., 2007). However, 16S rDNA sequences of NGS phytoplasma from Ethiopia belong to the African sugarcane yellow leaf (ASYL) phytoplasma, a member of the 16SrIII group, '*Ca.* Phytoplasma pruni' subgroup A or Western-X-disease (Jones *et al*., 2007; Arocha *et al*., 2009). Both 16SrXI and 16SrIII phytoplasmas cause identical symptoms in Napier grass in the different areas they occur, indicating the importance of geographic location and vector distribution for the NGS phytoplasma (Arocha & Jones, 2010).

2.9.3 Napier grass stunt phytoplasma vectors

The leafhopper *Maiestas banda* (Kramer) (Hemiptera: Cicadellidae) (Fig. 2.3), recently moved from genus *Recilia* (Webb & Viraktamath, 2009), was identified as the vector for NGS phytoplasma in Kenya (Obura *et al*., 2009). *Maiestas banda* has also been reported as the most abundant species found on Napier grass in the western part of Kenya, where NGS disease was first reported, suggesting its major role in the disease epidemics in the region (Koji *et al*., 2012). This hemipteran is a small insect with triangularly produced vertex and belongs to tribe Deltocephalini (subfamily: Deltocephalinae). The Deltocephalini tribe comprises insects, which are mostly phytophagous on plant species in the family Poaceae (Satoshi, 2009). In Ethiopia, no report is available on transmission of NGS phytoplasma. However, phytoplasmas detected in *Exitianus* sp. (Hemiptera: Cicadellidae) and *Leptodelphax dymas* (Fennah) (Hemiptera: Delphacidae), were identified as ASYL showing that they could be responsible for transmission of NGS in Ethiopia (Arocha *et al*., 2009).

Figure 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).

2.9.4 *Hyparrhenia* grass white leaf (HGWL) disease

Hyparrhenia grass also known as thatch grass is commonly found throughout the tropics where it serves as valuable cattle fodder, thatching and a border grass to prevent soil erosion (Skerman & Riveros, 1990). *Hyparrhenia* grass white leaf (HGWL) disease has been reported in Kenya in *H. rufa* causing symptoms similar to those found in NGS-infected plants, including white leaves, stunted growth and bushy appearance of the infected plants. Sequence analysis revealed highest 16SrDNA sequence identity (99%) of the HGWL phytoplasma with that of the NGS phytoplasma, confirming the HGWL phytoplasma as a member of the 16SrXI group phytoplasma. This study also suggested that *H. rufa* may be an alternative host plant for the NGS phytoplasma and might play a role in the epidemiology of NGS disease in the region (Obura *et al.*, 2011).

2.9.5 Bermuda grass wheat leaf (BGWL) disease

Bermuda grass (*Cynodon dactylon*) also known as star grass, is a perennial plant of the family Poaceae that is native to the African Savannah but is now widely distributed over many countries of the world (Marcone *et al.*, 1997). Bermuda grass is widely used as forage for livestock and wildlife and also provides turf to prevent soil erosion. Bermuda grass wheat leaf (BGWL) disease is a destructive phytoplasmal disease of Bermuda grass, occurring in several continents including Asia (Salehi *et al.*, 2009), Africa (Obura *et al.*, 2010), Australia (Tran-Nguyen *et al.*,

2000), Europe (Marcone *et al*., 1997) and America (Arocha *et al*., 2005). The phytoplasma infected *C. dactylon* plants exhibit extensive chlorosis of the leaves, whitening of leaves, bushy growing habit, small leaves, shortened stolons and rhizomes, stunting, proliferation of auxiliary shoots and death of the plant.

The phytoplasma associated with BGWL belongs to the 16SrXIV group, *'Ca*. Phytoplasma cynodontis'. A number of white leaf diseases of other grasses are associated with phytoplasmas that also fall within this group, including *Brachiara distachya* (Brachiaria grass), *Dactyloctenium aegyptum* (crowfoot grass) and *Poa annua* (annual blue grass) (Lee *et al*., 2000; Blanche *et al*., 2003). *'Ca*. Phytoplasma cynodontis' is distantly related to phytoplasmas associated with sugarcane, sorghum, rice and Napier grass which belong to the 16SrXI group. BGWL-related phytoplasmas have been identified in two dicotyledons namely: *Cirsium arvensis* (Canada thistle) and *Galactia tenuifolia*, and in the leafhopper *Psammotettix cephalotes* (Herrich-Schaeffer) (Marcone *et al*., 2004). *Exitianus capicola* (Stal), (Hemiptera: Cicadellidae), one of the insects of Bermuda grass fauna, is the vector of the BGWL agent in Iran (Salehi *et al*., 2009). This leafhopper was previously reported as a vector of a phytoplasma infecting *Limonium* hybrid crops in Israel (Weintraub *et al*., 2004).

2.9.6 Management of Napier grass stunt disease

There is no effective management approach for the NGS phytoplasma in East Africa. Roguing and burning of infected plants, and adoption of alternative fodder grasses have been suggested as control measures (Kabirizi *et al*., 2007; Khan *et al*., 2014). Roguing can help lower infection pressure by providing fewer phytoplasma-infected plants for vectors to feed on (Weintraub & Beanland, 2006). An alternative control strategy would be to identify Napier grass genotypes that are resistant to the pathogen or less attractive to vectors. Although some resistant Napier grass cultivars have been developed and introduced in the recent past in East Africa, recent observations show that most have lost resistance indicating that there is no effective control measure for this disease (Mulaa *et al*., 2010; Kawube *et al*., 2014).

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Chapter 3: Napier grass stunt disease in East Africa: farmer's perspectives on disease management

Abstract

Napier or Elephant grass (*Pennisetum purpureum*), the most important livestock fodder crop in East Africa, is under threat from Napier grass stunt (NGS) disease. This disease is caused by a phytoplasma, which is transmitted by the leafhopper, *Maiestas banda* (Hemiptera: Cicadellidae). After inoculation, the disease rapidly infects the whole plant causing extensive damage to Napier grass plants. There is therefore a need to develop an integrated management approach for the disease. A survey was conducted in three East African countries (Kenya, Uganda and Tanzania) during which 198 farmers were interviewed using semi-structured questionnaires. The questionnaire addressed the prevalence of NGS, source of planting material and Napier grass cultivars grown. Farmers were also questioned on how they perceived the severity of the disease, management options and on the knowledge of the existence of wild grass hosts of the disease and its vector. Disease prevalence in survey areas was expressed as a percentage of the total 198 fields assessed. Chi-square ($\chi 2$), *F* tests and one-way analysis of variance (ANOVA) were conducted to assess any differences between districts, gender and education levels with regards to the knowledge and perceptions of NGS in the three countries. The prevalence of NGS ranged from 33% in Uganda to 95.7% in Kenya with an average of 55.1%. Among the farmers interviewed, 49.5% were able to recognize NGS symptoms. Most farmers did not have effective management approaches for the disease but cited a variety of measures including roguing and introduction of alternative fodder grasses that could potentially form part of an integrated management approach for the disease. Sedge grass (*Cyperus* sp.) and Star grass (*Cynodon dactylon*) were listed by the respondents as the likely hosts of stunt diseases caused by the phytoplasma. It has been long suspected that weeds could play a role in the spread of the phytoplasma by acting both as reservoirs from which healthy plants could be re-infected and also as hosts for the vectors. Since there is no well-established control method for NGS, the majority of farmers uproot infected plants and replant with new ones to lower the infection pressure by providing fewer

infected plants for vectors to feed on. This pinpoints the need for farmers' awareness and the need to develop resistant Napier grass cultivars as a management option.

Keywords: Napier grass: Napier grass stunt disease; farmer perceptions; wild hosts; East Africa

3.1 Introduction

Napier or Elephant grass (*Pennisetum purpureum* Schumach) constitutes between 40 and 80% of forages in East Africa where it is used by smallholder dairy farmers in intensive (zero grazing) and semi-intensive dairy cattle production systems (Farrell *et al*., 2002a; Orodho, 2006). The grass is also widely used for soil and water conservation in hilly slope areas, and serves as mulch in banana-farming regions. It has also been reported as a prospective bio-fuel crop in the region (Jones *et al*., 2004; Orodho, 2006). Napier grass is currently used in the region as a trap plant in the management of cereal stem borers, *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) and *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae), the most injurious insect pests of cereals and the main target pests in the 'push-pull' strategy (PPS) (Cook *et al*., 2007; Pickett *et al*., 2014). The strategy involves intercropping a cereal crop such as maize (*Zea mays* L.) with a stemborer-repellent plant (push), usually *Desmodium* spp., with the trap crop, Napier grass planted as a border crop (pull) around this intercrop. Napier grass is more attractive to stemborer moths than maize for oviposition but supports only minimal survival of larvae. Therefore, when planted as a trap crop around a cereal crop it attracts more oviposition by stemborer moths than the main crop leading to a decrease in pest pressure and reduced yield losses (van den Berg, 2006; Khan et al., 2010; Midega et al., 2010).

Napier grass stunt (NGS) disease is caused by a phytoplasma, a cell wall-less bacterium of the new taxon '*Candidatus* (*Ca*.) Phytoplasma' (Class - Mollicutes; order - Acholeplasmatales) (IRPCM, 2004). Based on 16S rDNA sequences, phytoplasmas associated with NGS in Kenya and Uganda belong to the 16SrXI group '*Ca* Phytoplasma oryzae' or rice yellow dwarf, while those associated with NGS occurring in Ethiopia are known as African sugarcane yellow leaf (ASYL) phytoplasma, a member of the 16SrIII, '*Ca.* Phytoplasma pruni' or X-disease (Jones *et al*., 2004, 2007; Nielsen *et al*., 2007; Arocha *et al*., 2009). Two phytoplasmas

closely related to NGS were detected in other wild grasses in the western part of Kenya. These are Bermuda grass white leaf (BGWL) phytoplasma detected in *Cynodon dactylon* and *Hyparrhenia* grass white leaf (HGWL) found in *Hyparrhenia rufa*. HGWL is classified as a '*Ca*. Phytoplasma oryzae' strain and is closely related to NGS while BGWL belongs to the 16SrXIV group or '*Ca*. Phytoplasma cynodontis' (Obura *et al*., 2010, 2011). In Ethiopia, phytoplasmas detected in *Medicago sativa* and *C. dactylon* were classified as ASYL (Arocha *et al*., 2009). These studies suggested that *H. rufa*, *M. sativa* and *C. dactylon* could be alternative host plants for NGS and could play a role in the spread of the disease in East Africa.

Napier grass stunt was first reported in Kenya in 1997, in Uganda in 2001, in Ethiopia in 2004 and has been observed in Tanzania (Alicai *et al*., 2004; Jones *et al*., 2004, 2007; Orodho, 2006; Nielsen *et al*., 2007). In the field, NGS symptoms manifest in re-growth of Napier grass plants after cutting or grazing, with the affected shoots becoming pale yellow green and seriously dwarfed, with low biomass that is unable to sustain the feed requirements of dairy cows. Often the whole Napier stool is affected with complete loss in yield leading to eventual death of the plant (Alicai *et al*., 2004; Jones *et al*., 2004, 2007; Orodho, 2006; Kabirizi *et al*., 2007; Nielsen *et al*., 2007). The primary means of NGS spread is through the introduction of infected cuttings by farmers and/ or insect vectors carrying the phytoplasma (Orodho, 2006; Obura *et al*., 2009; Koji *et al*., 2012). In Kenya, *Maiestas banda* (Kramer) (Hemiptera: Cicadellidae), transmits NGS (Obura *et al*., 2009) while in Ethiopia, *Exitianus* sp. (Hemiptera: Cicadellidae) and *Leptodelphax dymas* (Fennah) (Hemiptera: Delphacidae) have been indicated as potential vectors in the transmission of ASYL (Arocha *et al*., 2009). Since Napier grass is vegetatively propagated (Orodho, 2006), NGS presents a serious phytosanitary problem. Although some resistant Napier cultivars were earlier developed and introduced in the region, recent observations report that they have also lost resistance (Mulaa *et al*., 2010; Kawube *et al*., 2014), most likely to more aggressive strains of the pathogen. There is, therefore, no effective control method for the disease.

The level of infection of a crop by phytoplasma is therefore dependent on the abundance of insect vectors and alternative host plants harbouring the pathogen (Lee *et al*., 2003; Sharon *et al*., 2005). Similar with other phytoplasmal diseases,

NGS is also exasperated under poor soil conditions and poor management of weeds in addition to poor harvesting of the plants (Orodho, 2006; Kabirizi *et al*., 2007). In particular, weeds and other plants provide a reservoir for the phytoplasmas and insect vectors (Weintraub & Beanland, 2006). As research into the epidemiology of NGS intensifies, there is need to provide more information on the existing farmers' knowledge and perceptions about the disease, which are currently lacking. Therefore, it is essential to identify the target farmers and further understand the farmers' needs and resources, their perception of the problem, as well as enlisting their support and collaboration in developing an appropriate management strategy. Previous studies have focused mainly on the farmers' knowledge and perception about NGS (Kabirizi *et al*., 2007; Khan *et al*., 2014) while neglecting knowledge on the existence of wild hosts. Therefore, the objectives of this study were to evaluate farmers' knowledge and perceptions of NGS and its wild grass hosts, establish farmers' current practices in managing the disease and identify NGS management challenges and intervention opportunities, aimed at developing an effective integrated management approach for the disease.

3.2 Materials and methods

3.2.1 The study area

This study was conducted in five districts in East Africa known to be NGS prone. These were Busia and Bungoma districts in western Kenya, Busia and Bugiri districts in eastern Uganda and Tarime district in northern Tanzania (Fig. 1). Altitudes at surveyed sites in Kenya ranged from 1211 to 1478 m above sea level (m a.s.l.), 1082 to 1243 m a.s.l. in Uganda and from 1232 to 1670 m a.s.l. in Tanzania. In total, 198 farmers and fields were surveyed with 57 in Uganda, 91 in Kenya and 50 in Tanzania. Of these, 30 farmers were in Busia district (Uganda), 27 farmers in Bugiri district, 44 farmers in Busia (Kenya), 47 farmers in Bungoma and 50 farmers in Tarime (Tanzania). The study focused on these areas because of the reported presence of NGS and the adoption of PPS (Jones *et al*., 2004).

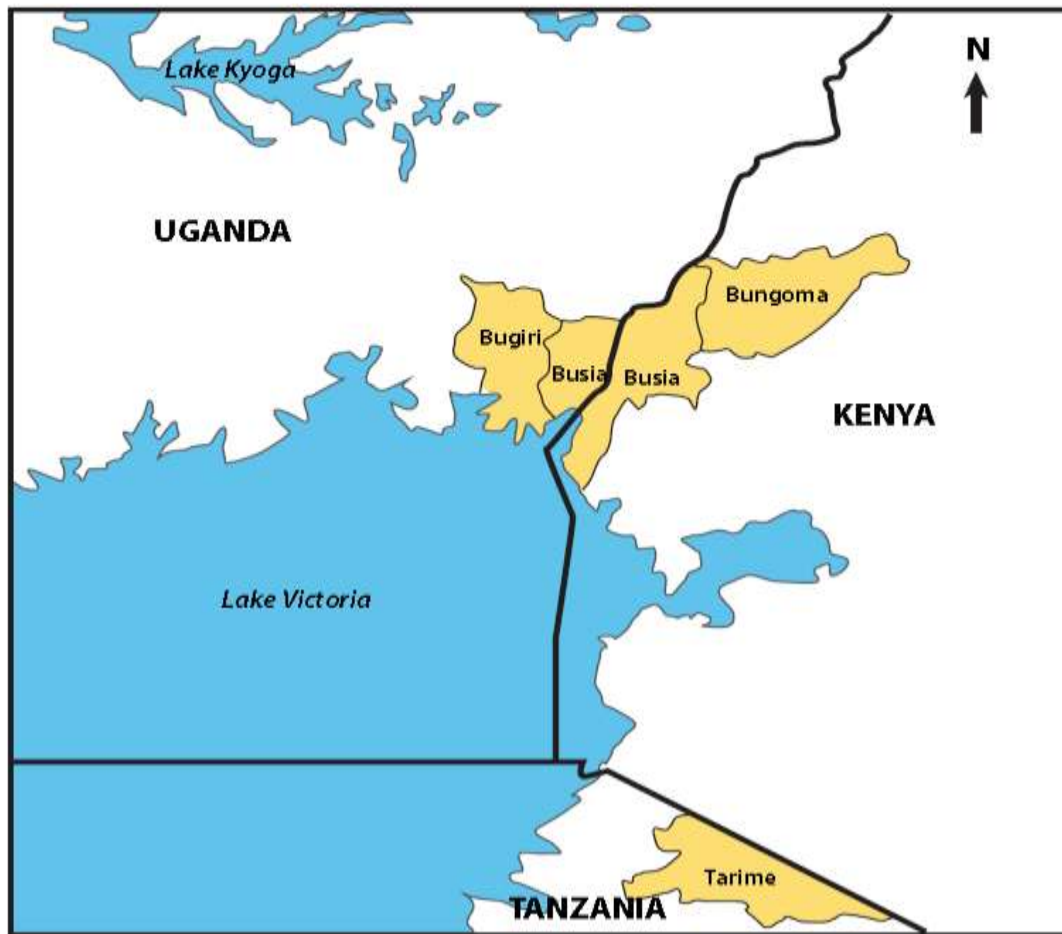

Figure 3.1: Map of East Africa showing the study areas.

3.2.2 Data collection

Surveys were carried out between March and July 2012. Purposive sampling was used. In this approach, farmers growing Napier grass for livestock production or practicing PPS were identified and interviewed. A semi-structured questionnaire administered through face-to-face interviews with the respondents was used to obtain information on the prevalence of NGS, first observation of the disease symptoms and its spread. This data was recorded in a series of binary responses (Khan *et al.*, 2014) by asking the farmers if they knew NGS and its wild hosts, if they were aware of the occurrence of the disease on their farms, and how they discern its symptoms. The responses to this were recorded as 1 and 0, where 1 was a 'yes' answer and 0 a 'no' answer. Fields planted with Napier grass were randomly selected for data collection and those containing one or more plants with observable NGS symptoms were considered as infested. These symptoms were recorded as

foliar yellowing (mentioned by farmers as yellow leaves), stunted growth, drying or death of plants. Subsequently, the farmers were asked to score the NGS severity on a scale range of 0 to 3, where 0 = no problem, 1 = low severity, 2 = medium severity and 3 = high severity. Farmers were also asked about their willingness to replace Napier grass with alternative fodder grasses, with responses treated as binary variables of 1 and 0, where 1 = yes and 0 = no. The other information addressed in the questionnaire included farmers' socio-economic characteristics namely age, gender and education level, rate of spread of NGS on their farms, presence and spread of the disease on their neighbours' farms, the source of planting materials, cultivars of Napier grown, existence of resistant Napier cultivars, seasonal prevalence of NGS symptoms and possible control measures.

To evaluate farmers' knowledge and awareness of the wild hosts of NGS phytoplasma, they were asked if they were aware of the existence of a phytoplasmal disease in wild grass, how they recognize it on their farms and how they depicted the symptomatic wild grass in the fields. The data was thus recorded in a series of binary responses as 1 and 0 where 1 = 'yes' and 0 = 'no'. Farmers were also asked to list wild grasses in which they had noticed symptoms similar to those observed on NGS infected plants. Similarly, severity of stunt disease in these wild grasses was scored using a scale range of 0 to 3, where 0 = no problem, 1 = low severity, 2 = medium severity and 3 = high severity. Additional information was recorded on the rate of spread of the disease and its causal agents in wild grasses, what was spreading the disease in the wild grasses; whether and how a phytoplasmal disease could spread from wild to cultivated grasses, and what neighbours and farmers themselves were doing to manage the disease in wild grasses if any. The rate of spread was scored using a scale range of 1 to 3, where 1 = slow, 2 = fast and 3 = very fast.

The distance between farms at which interviews were conducted was approximately 100 m within the same village and 10 km between villages. During the survey, Napier grass leaves were collected from each farm and tested for the presence of phytoplasma by nested PCR using universal primers based on the phytoplasma 16S rRNA gene (Deng & Hiruki, 1991; Obura, 2012). The sampling strategy consisted of taking five leaf samples on symptomatic or asymptomatic plants per farm in the five districts across the three countries. The leaves were cut off by means of a scissor

sterilized prior to use using 70% ethanol (ethyl alcohol). The sections of leaves were then placed in envelopes and stored at -20ºC before DNA was isolated (Doyle & Doyle, 1990). A reference phytoplasma strain, NGS maintained at the International Centre of Insect Physiology and Ecology (ICIPE) Mbita station, Kenya was used as the positive control while water in which no DNA was added was used as a negative control. The geographical co-ordinates of each surveyed location were recorded prior to data collection using a Global Positioning System (GPS) receiver.

3.2.3 Data analysis

Data were summarized using cross tabulations and processed descriptively using means, frequencies and percentages. Chi-square ($\chi 2$), *F* tests and one way analysis of variance (ANOVA) using the Statistical Package for Social Sciences (SPSS) version 18 (SPSS Inc. Chicago, USA) were conducted to assess any differences between districts, gender and education levels with regards to the knowledge and perceptions of NGS. Statistical significance was determined at three levels at 0.01, 0.05 and 0.1, and means separated by the Tukey test (Bechhofer *et al.*, 1995; Hsu, 1996) where applicable. Disease prevalence was expressed as a percentage, calculated as the proportion of fields with plants showing NGS symptoms to the total number of fields assessed multiplied by 100.

3.3 Results

3.3.1 Socio-economic and farm characteristics of the respondents

The age of the respondents ranged between 15 and 75 years with an average of 44.9. The age of farmers across the districts did not show any significant difference. However, it varied widely with majority of farmers (51.4%) being middle-aged (between 31-50 years), 29.7% being older than 50 years and only 18.92% being younger than 30 years. Among these, 54.5% were males while 45.5% were females. The majority of farmers had little education with 60.6% having primary education (8 years of basic education), 21.2% with secondary education (12 years of basic education) and 18.2% having received some form of tertiary education (Table 3.1).

Table 3.1: Socio-economic characteristics of the respondents

Variable	District					Mean	Significance	
	Bungoma N = 47	Busia-K N = 44	Tarime N = 50	Bugiri N = 27	Busia-U N = 30	N = 198	χ^2	F-test
Age (years)	43.1	45.7	45.1	40.3	50.6	44.9		2.34ns
Gender (%)								
Male	53.2	52.3	62.0	55.6	46.7	54.5	2.0ns	
Female	46.8	47.7	38.0	44.4	53.3	45.5		
Education levels (%)								
Primary	31.9	54.5	86.0	70.4	63.3	60.6	42.0***	
Secondary	44.7	13.6	4.0	22.2	23.3	21.2		
Tertiary	23.4	31.8	10.0	7.4	13.3	18.2		

***Statistically significant (Chi-square analysis and F-test) at $p < 0.01$; ns – not significant Key: Districts: Busia-K, Busia Kenya; Busia-U, Busia Uganda

3.3.2 Practice of crop cultivation

Farmers planted different crops including Napier grass, maize, sorghum, sugarcane, rice and millet. Among these, Napier grass and maize were cultivated by the majority of respondents at 94.4% and 93.9% respectively. Sorghum was grown by 13.1% of the farmers, sugarcane by 10.1%, millet by 8.6% and rice by 4%. Sources of Napier cuttings varied significantly across the districts ($\chi2 = 62.2$, df = 20, $P < 0.01$). The majority of respondents (84.3%) received the cuttings freely from the nearest neighbours, 9.1% cited research institutes such as Mogabiri Institute (Tanzania), ICIPE, Sang'alo Lake Basin Institute (Kenya) and National Agricultural Research Organisation (NARO)-Uganda from which they obtained the planting materials. The remaining 1% of farmers either bought from neighbours or reused the cuttings. The majority of farmers (83.3%) could not identify the Napier grass cultivars they were cultivating. However, a few of the farmers differentiated the cultivars descriptively as either small or large-leaved (10.1%) and smooth or rough-surfaced (1%) (Table 3.2).

Table 3.2: Crops cultivated by the respondents, Napier grass cultivars and source of cuttings

Variable	District					Mean N = 198	χ^2
	Bungoma N = 47	Busia-K N = 44	Tarime N = 50	Bugiri N = 27	Busia-U N = 30		
Crops grown by farmers (%)							
Maize	97.9	86.4	100	100	83.3	93.9	16.6***
Rice	–	–	–	25.9	3.3	4.0	39.3***
Sorghum	–	6.8	14.0	22.2	33.3	13.1	21.4***
Millet	–	2.3	22.0	14.8	3.3	8.6	20.5***
Sugarcane	17	2.3		18.5	20.0	10.1	16.4***
Napier	100	100	100	77.8	83.3	94.4	29.7***
Napier types cultivated by farmers (%)							
Not sure of the name	76.6	81.8	100	74.1	76.7	83.3	50.9***
small or large-leaved	21.3	18.2	–	–	6.7	10.1	
Smooth or rough-surfaced	2.1		–	3.7	–	1.0	
Source of Napier cuttings (%)							
From neighbours	97.9	97.7	79.2	70.4	66.7	84.3	60.2***
Research institute	2.1	–	20.8	3.7	16.7	9.1	
Reused cutting	–	–	–	3.7	–	0.5	
Bought	–	2.30	–	–	–	0.5	

**Statistically significant (Chi-square analysis) at $p < 0.01$. Key: Districts: Busia-K, Busia Kenya; Busia-U, Busia Uganda

3.3.3 Epidemiology of Napier grass stunt disease in East Africa

In total, NGS was recorded in 55.1% of the farms and symptoms were only observed on Napier grass. Disease prevalence ranged from 33.3% in Bugiri to 95.7% in Bungoma. Kenya had the highest NGS prevalence, which ranged between 88.6 and 95.7% while no prevalence was reported in Tanzania. Equally, the disease was more severe in the Bungoma (68.1%) and Busia (65.9%) districts of Kenya than in the other districts (Table 3.3). While all the interviewed farmers observed the disease to be spreading, the spread was faster in Kenya [Bungoma (53.2%), Busia (52.3%)] and in Uganda [Busia (30%)]. None of the farmers whose farms had infected plants (55.1%) had observed any Napier grass that was resistant to NGS. The remaining majority (39.4%) was not sure whether the grasses were resistant or not. Other constraints reported by the respondents included *Striga* weed mentioned by 76.8%, drought (94.4%) and infestation of cereals by stemborers (51%). An average of 43.4% of the farmers mentioned seeing NGS signs in other farms in nearby areas but only 2% of these farmers had recognised the symptoms after cutting the grass. However, the majority of respondents (41.4%) did not remember the particular time they had first seen the symptoms (Table 3.3).

Table 3.3: Incidence and severity of Napier stunt disease in East Africa

Variable	District					Mean	χ^2
	Bungoma N = 47	Busia-K N = 44	Tarime N = 50	Bugiri N = 27	Busia-U N = 30	N = 198	
Farmers with NGS disease on their farms							
Yes (%)	95.7	88.6	–	33.3	53.3	55.1	117.9***
Severity of NGS disease in the surveyed farms (%)							
High	68.1	65.9	–	14.8	33.3	37.9	125.8***
Low	14.9	20.5	–	11.1	13.3	11.6	
Moderate	12.8	2.3	–	7.4	6.7	5.6	
Is NGS disease spreading							
Yes (%)	95.7	88.6	–	33.3	53.3	55.1	117.9***
The rate of spread of NGS disease on farmers' fields (%)							
Very fast	53.2	52.3	–	3.7	30.0	29.3	131.7***
Fast	36.2	25.0	–	22.2	6.7	18.2	
Slow	6.4	11.4	–	7.4	16.7	7.6	
Occurrence of NGS disease in other farms in your area							
Yes (%)	93.6	84.1	–	–	16.7	43.4	145.7***
Severity of NGS disease in other farms in your area (Yes)							
High	70.2	63.6	–	–	10.0	32.3	147.0***
Low	14.9	15.9	–	–	3.3	7.6	
Moderate	8.5	4.5	–	–	3.3	3.5	

***Statistically significant (Chi-square analysis) at $p < 0.01$, Key: Districts: Busia-K, Busia Kenya; Busia-U, Busia Uganda

3.3.4 Farmers' knowledge and management strategies against NGS

Farmers (49.5%) were able to identify NGS symptoms including foliar yellowing of the leaves (mentioned by 52%), stunted plants with small leaves and bushy appearance (mentioned by 52.5%) and necrosis of leaves or plant death (mentioned by 20.2%) (Table 3.4; Fig. 3.2b). Some farmers (52%) observed that NGS occurs throughout the year and that none of the cultivated Napier cultivars was resistant to the disease. Some farmers also observed that NGS occurs and is more prevalent during the dry season (Table 3.4). Newly planted Napier grass was indicated to become affected mostly after the second harvesting (20.7% of the respondents), after first cutting (10.6%), before (5.1%), after the third (2.5%) or after sixth (0.5%) harvesting of Napier grass. The stage at which plants became affected varied significantly across the districts ($\chi 2 = 164.9$; df (24); $P < 0.01$). Some farmers (15.7%) were not sure at what stage the plants became affected with NGS. They did

however, indicate that affected plants degenerated mostly after the second harvesting of the crop (mentioned by 16.7% of the respondents) (Table 3.4).

Table 3.4: Farmers' knowledge of Napier stunt disease in East Africa

Variable	District					Mean	χ^2
	Bungoma N = 47	Busia-K N = 44	Tarime N = 50	Bugiri N = 27	Busia-U N = 30	N = 198	
Farmers aware of NGS disease							
(Yes) (%)	83.0	84.1	2.0	14.8	56.7	49.5	100.9***
How farmers discern NGS disease in Napier grass (%)							
Yellow leaves	80.9	84.1	–	11.1	40.1	45.5	116.5***
Stunted growth	80.9	79.5	–	11.1	50.0	46.0	106.8***
Drying and death	21.3	31.8	–	–	30.0	16.7	105.1***
Does the NGS disease occur throughout the year							
Yes (%)	95.7	88.6	–	33.3	33.3	52.0	156.9***
Plant cultivars resistant to NGS disease (%)							
Not resistant	95.7	88.6	–	33.3	53.3	55.1	151.9***
Not sure	4.3	11.4	100	44.4	30.0	39.4	
When is the disease more prevalent (%)							
During dry season	38.3	47.7	–	–	–	19.7	185.9***
During long rains	38.3	34.1	–	–	–	16.7	
Not sure	19.1	6.8	–	33.3	53.3	18.7	
When newly grown plants become affected Yes (%)							
Before cutting	–	11.4	–	7.4	10.0	5.1	164.9***
After 1st cutting	10.6	9.1	–	14.8	26.7	10.6	
After 2nd cutting	55.3	27.3	–	–	10.0	20.7	
After 3rd cutting	4.3	4.5	–	3.7	–	2.5	
After 6th cutting	2.1	–	–	–	–	0.5	
Not sure	23.4	36.4	–	7.4	6.7	15.7	
When affected plants degenerate (%)							
Before cutting	–	6.8	–	–	–	1.5	161.4***
After 1st cutting	8.5	9.1	–	14.8	26.7	10.1	
After 2nd cutting	42.6	22.7	–	–	10.0	16.7	
After 3rd cutting	19.1	11.4	–	3.7	–	7.6	
After 6th cutting	2.1	–	–	0.0	0.0	0.5	
Not sure	23.4	38.6	–	14.8	16.7	18.7	

***Statistically significant (Chi-square analysis) at P < 0.01, Key: Districts: Busia-K, Busia Kenya; Busia-U, Busia Uganda

Different measures were employed by farmers to manage the NGS in the affected surveyed districts. The most commonly used control method was roguing which was used by an average of 32.3% of the respondents. The number of farmers that used roguing differed significantly across districts, i.e. from 16.7% in Busia (Uganda) to 70.2% in Bungoma ($\chi 2 = 139.04$; df = 8; $p < 0.01$) (Table 3.5). Other methods of management, applied as general routine practices, included application of organic fertilizer (4%), replanting using new asymptomatic planting materials (4%), burning of

infected plants (1.5%), cutting (1%) and application of inorganic fertilizer (0.5%). Some farmers (15.7%) neither used nor were they aware of any of the above mentioned control measures. Of these control measures, the majority of the farmers (23.7%) reported roguing as the most effective, followed by replanting (7.1%), organic fertilizer (1%), with cutting and use of chemicals being mentioned by only 0.5% of the respondents. However, 8.6% of the respondents were of opinion that no control measures mentioned above were effective at all (Table 3.5).

Table 3.5: Percentage of the farmers practicing various disease management methods

Variable	District					Mean N = 198	χ^2
	Bungoma N = 47	Busia-K N = 44	Tarime N = 50	Bugiri N = 27	Busia-U N = 30		
Control measures used by farmers to manage NGS disease (%)							
No control	21.3	20.5	–	22.2	20.0	15.7	124.8***
Roguing	70.2	59.1	–	–	16.7	32.3	139.0***
Replanting	2.1	2.3	–	3.7	16.7	4.0	136.2***
Applying organic fertilizer	10.6	4.5	–	3.7	–	4.0	121.4***
Burning	2.1	2.3	–	3.7	–	1.5	119.9***
Cutting	–	4.5	–	–	–	1.0	123.6***
Applying inorganic fertilizer	2.10	–	–	–	–	0.5	113.5***
Most effective control method for NGS disease (%)							
Roguing	51.1	43.2	–	–	13.3	23.7	128.3***
Replanting	6.4	6.8	–	3.7	23.3	7.1	135.3***
Organic fertilizer	–	–	–	3.7	3.3	1.0	124.9***
Cutting	–	2.3	–	–	–	0.5	120.7***
Chemicals	2.1	–	–	–	–	0.5	120.2***
None	17.0	20.5	–	–	–	8.6	124.8***

***Statistically significant (Chi-square analysis) at $P < 0.01$, Key: Districts: Busia-K, Busia Kenya; Busia-U, Busia Uganda

3.3.5 Alternative fodder grasses that could be used to replace Napier grass

In case all Napier grass on a farm was affected, only 6.1% of the farmers would consider replacing it with an alternative fodder grass that is resistant to NGS. The response varied significantly across the districts from 2.3% in Busia-Kenya to 26.7% in Busia-Uganda ($\chi 2 = 38.2$; df = 8; $P < 0.01$). The alternative fodder grasses cited by the farmers included Guatemala grass (*Tripsacum laxum*) (2%), Amasanyi (*Panicum maximum*) (1.5%), Giant setaria (*Setaria sphacelata*) (0.5%), Kikuyu grass (*Pennisetum clandestinum*) (0.5%), Lucerne (*Medicago sativa*) (0.5%), Bermuda/star grass (*Cynodon dactylon*) (0.5%) and Rhodes grass (*Chloris gayana*) (0.5%) (Table 3.6). A few farmers grew giant setaria and Guatemala grass.

Table 3.6: Alternative fodder grasses that could be used as a replacement to Napier grass

Variable	District					Mean	χ^2
	Bungoma N = 47	Busia-K N = 44	Tarime N = 50	Bugiri N = 27	Busia-U N = 30	N = 198	
Farmers who would consider replacing Napier grass with other fodder grasses							
Yes (%)	4.3	2.3		3.7	26.7	6.1	38.2***
Alternative fodder grasses suggested by farmers (%)							
Tripsacum laxum	2.1	–	–	3.7	6.7	2.0	29.9***
Setaria sphacelata	–	–	–	–	3.3	0.5	28.3***
Pennisetum clandestinum	–	–	–	–	3.3	0.5	28.3***
Medicago sativa	2.1	–	–	–	–	0.5	33.7***
Panicum maximum	–	–	–	–	10.0	1.5	30.9***
Chloris gayana	2.1	–	–	–	–	0.5	33.7***
Cynodon dactylon	–	–	–	–	3.3	0.5	28.3***
Not sure of name	–	2.3	–	–	6.7	1.5	29.0***

***Statistically significant (Chi-square analysis) at $P < 0.01$, Key: Districts: Busia-K, Busia Kenya; Busia-U, Busia Uganda

3.3.6 Knowledge of stunt disease in wild grasses

Only an average of 6.1% of the farmers interviewed had observed symptoms of stunting diseases or diseases caused by phytoplasma in wild grasses. Of these, 14.9% of the respondents were from Bungoma and 11.4% from Busia in Kenya. None of the respondents from other districts had such knowledge. The diseased grass species observed by farmers inside fields were *C. dactylon* and *Cyperus* sp. mentioned by an average of 5.1% and 0.5% of the respondents respectively. Diseased *C. dactylon* plants were characterized by extensive chlorosis of leaves, bushy growing habit, small leaves, shortened stolons/rhizomes, stunting and proliferation of auxiliary shoots as shown in Fig. 3.3. The diseased *Cyperus* sp. mostly had yellow leaves, which later dried as observed by the respondents.

a. b.

Figure 3.2: a). Healthy Napier grass, b). Severely stunted bushy Napier grass with yellowing of leaves and reduced biomass.

Figure 3.3: Whitening, small leaves and bushy growth habit of Bermuda grass infected with Bermuda grass white leaf phytoplasma.

When asked when the disease was more prevalent, the majority of the respondents (5.6 %) had no idea when the stunting disease in wild grasses was most common (months). A small number of the respondents indicated the long rains season as the period when the disease was more prevalent. Equally, the severity of the disease in wild grasses in the fields bordering Napier grass farms was lower as reported by

3.5% of the respondents. However, the spread was rapid in the surrounding farms as mentioned by 3% ($P < 0.01$). Bacteria (0.5%), drought (0.5%) and lack of fertilizer (1%) were listed as some of the likely causes of the phytoplasmal disease symptoms in wild grasses. Equally, bacteria (0.5%), lack of organic fertilizer (0.5%) and wind (0.5%) were cited as factors that could contribute to spread of the disease to surrounding farms. Even though 5.6% of the farmers thought that the disease could spread from the wild to cultivated grasses, only a few of the respondents knew how this happened with some (0.5%) suggesting that it would occur through vegetative propagation (Table 3.7). Most farmers again reported roguing as the major method that could be used to control the disease in wild grasses both in the neighbourhood (0.5%) and in their farms (2.5%). Other farmers cited application of organic fertilizer (0.5%) and education or farm demonstrations (0.5%) as some of the ways that can be used to help cope with the disease.

Table 3.7: Farmers' knowledge and perception about stunt disease in wild grasses in East Africa

Variable	District					Mean	χ^2
	Bungoma N = 47	Busia-K N = 44	Tarime N = 50	Bugiri N = 27	Busia-U N = 30	N = 198	
Farmers aware of the stunt disease in wild grasses							
Yes (%)	14.9	11.4	–	–	–	6.1	15.5***
Wild grasses with stunt diseases							
Star grass	12.8	9.1	–	–	–	5.1	20ns
Sedge grass	2.1	–	–	–	–	0.5	
Not sure of the grass	–	2.3	–	–	–	0.5	
Symptoms of stunt disease identified by farmers in affected wild grasses (%)							
Drying of leaves	2.1	–	–	–	–	0.5	17.2***
Yellow leaves	12.8	11.4	–	–	–	5.6	
Stunted growth	12.8	11.4	–	–	–	5.6	
When the disease was identified in the wild grasses (%)							
During long rains	–	2.3	–	–	–	0.5	18.8***
Not sure	14.9	9.1	–	–	–	5.6	
Severity of the disease in the area surrounding your farm (%)							
Low	10.6	4.5	–	–	–	3.5	21.9***
Moderate	2.1	6.8	–	–	–	2.0	
High	2.1	–	–	–	–	0.5	
Is the stunt disease in wild grasses spreading							
(Yes) (%)	10.6	11.4	–	–	–	5.1	19.2***
Rate of spread of the disease (%)							
Fast	8.5	4.5	–	–	–	3.0	16.0***
Slow	2.1	6.8	–	–	–	2.0	
What farmers think might be causing the stunt disease in wild grasses (%)							
Bacteria	2.1	–	–	–	–	0.5	27.9ns
Drought	–	2.3	–	–	–	0.5	
Lack of inorganic fertilizer	–	2.3	–	–	–	0.5	
Lack of organic fertilizer	–	2.3	–	–	–	0.5	
Not sure	12.8	4.5	–	–	–	4.0	
What farmers think is spreading the stunt disease in wild grasses (%)							
Bacteria	2.1	–	–	–	–	0.5	21.6ns
Lack of organic fertilizer	–	2.3	–	–	–	0.5	
Not sure	10.6	9.1	–	–	–	4.5	
Wind	2.1	–	–	–	–	0.5	
Can disease spread from wild to cultivated grasses?							
Yes (%)	14.9	9.1	–	–	–	5.6	15.2***
How would this happen (%)							
Not sure	12.8	9.1	–	–	–	5.1	16.4***
Vegetative propagation	2.1	–	–	–	–	0.5	
What neighbours are doing to cope with the situation (%)							
Not sure	14.9	6.8	–	–	–	5.1	19.1***
Rouging	–	2.3	–	–	–	0.5	
What farmers think can be done to control stunt disease in wild grasses (%)							
Apply manure	–	2.3	–	–	–	0.5	20.3ns
Educate farmers	2.1	–	–	–	–	0.5	
Rouging	6.4	4.5	–	–	–	2.5	
Not sure	6.4	2.3	–	–	–	2.0	

***Statistically significant (Chi-square analysis) at $P < 0.01$, ns – not significant, Key: Districts: Busia-K, Busia Kenya; Busia-U, Busia Uganda

3.4 Discussion

Nearly all the farmers that participated in the surveys, regardless of age and gender, grew Napier grass. Other crops included sorghum, sugarcane, rice, millet and maize grown by a majority of the farmers as staple foods or cash crops. Cereals such as maize, wheat, rice, sorghum and millet are vitally important sources of food for humans and their livestock and contribute significantly to the local and national economies. According to Farrell *et al.* (2002a), almost all farmers obtain Napier grass planting material from neighbours or plants growing naturally within the farm and they usually do not know the names of the cultivars they cultivate. The present study corroborates previous research as the majority of farmers obtained cuttings freely from their neighbours and were not able to identify the different names of Napier grass cultivars they planted.

The study reported a high prevalence and severity of NGS in Bungoma and Busia counties of Kenya, and the neighbouring districts of Bugiri and Busia of Uganda, indicating a possibility of a very rapid spread of the disease over the last 18 years since its first report in Bungoma district (Orodho, 2006). This rapid spread and severity of the disease could also be attributed to the seemingly poor maintenance of Napier crops. Due to its perennial nature, farmers cut and allow Napier grass to re-grow for several years thus contributing to the spread of the disease. As Napier grass is vegetatively propagated through cuttings, exchange of cuttings could contribute to the spread of the disease within a farm when asymptomatic diseased cuttings are used (Orodho, 2006; Koji *et al.*, 2012). Long-distance spread of the disease from one place to another could also occur through exchange of planting materials obtained from other infested farms. However, the absence of NGS in Tanzania at the time of the study could be because most of the crops in the sampled fields had just been established. Besides, molecular detection using nested PCR confirmed 11.76% of the Napier grass collected in Tanzania to be positive with phytoplasma belonging to the 16SrXI group, which were identical to and shared a 100% similarity with sequences collected from Napier grass in Uganda and Kenya (GenBank accessions; EF012650, FJ862997, FJ862998, FJ862999, JQ868440, JQ868443) (Asudi *et al.*, 2015). Most of the Napier cuttings were obtained from

neighbours, which might have had the phytoplasma indicating why some of the plants were positive by nested PCR.

Phytoplasma disease affecting Napier grass is economically significant and has a negative impact on the smallholder dairy industry in the region where farmers depend on the grass as their principal source of feed for dairy animals (Orodho, 2006). The disease is even more severe during the dry season probably due to water stress (Kabirizi *et al*., 2007). Despite this, no curative methods are available against these plant pathogenic agents. For this reason, management of phytoplasma-infected plants has mainly focused on controlling the insect vectors and on roguing infected plants from crops and weeds. Resistant cultivars do exist but are rare (Thomas & Mink, 1998; Kabirizi *et al*., 2007; Bisognin *et al*., 2008). While, there is no current report on the control for the NGS vector, roguing was mentioned by a majority of the farmers as the most effective control method. This agrees with Kabirizi *et al*. (2007), who reported that about 90% of farmers practiced roguing to control NGS in the Masaka district in Uganda. Since there are no well-established control methods for NGS (Khan *et al*., 2014), roguing helps to lower the infection pressure by providing fewer inoculum sources for vectors to feed on. These infected plants are the likely source of secondary infection and the cause of spread of the pathogen (Weintraub & Beanland, 2006). Besides, a few of the respondents mentioned alternative fodder grasses that could be used to replace Napier grass. Some of these alternative fodder grasses may not be natural hosts to NGS phytoplasma and could be tolerant to drought. However, they do not perform well compared to Napier grass in milk production systems (Orodho, 2006). This shows the significance of Napier grass as a fodder crop. It also shows that not many farmers have alternative options for providing forage for their cattle.

Development of NGS management strategies is impeded by the lack of knowledge of its epidemiology. Not much is known about phytoplasma infection of wild plants that frequently serve as sources of inoculum for cultivated crops. Natural host plants may stay free from obvious disease symptoms due to a long co-evolution with their associated phytoplasmas (Caudwell, 1983). In this current study, a few respondents mentioned wild grasses that they regarded as the likely hosts of stunting disease caused by phytoplasma while majority did not. These were *C. dactylon* and *Cyperus*

sp. It has been long suspected that weeds could play an important role in the spread of phytoplasma by acting both as reservoirs from which healthy plants could be re-infected and also as hosts for the vectors (Weintraub and Beanland, 2006; Arocha *et al*., 2009; Obura *et al*., 2010, 2011). *Cynodon dactylon* is a well known host of white leaf diseases associated with phytoplasmas of groups 16SrIII and XIV (Arocha *et al*., 2005, 2009; Obura *et al*., 2010) and has also been found to host the 16SrXI phytoplasma (Asudi *et al*., 2015). This is informative especially in the development of an integrated management approach for NGS in East Africa. In addition, Napier grass often suffers a serious weed burden compared to sown pasture grasses (Farrell *et al*., 2002b). Plant stress caused by weed growth may predispose Napier grass to diseases or exacerbate disease symptoms (Orodho, 2006). Couch grass (*Digitaria scalarum*), star grass (*C. dactylon*), *Imperata cylindrica* and various sedges (*Cyperus* sp.) are the main weeds that invade and compete with Napier grass in East Africa (Boonman, 1993). Conversely, repeated cultivation encourages the spread of *Oxalis* sp. (Boonman, 1993) which is also a common weed in Napier grass plantings. Therefore, the weed control in and around the fodder crop may benefit disease management over the long term (Arocha *et al*., 2009). Given its high prevalence and severity, NGS could impact negatively on the smallholder dairy industry and the uptake of PPS in the region. There is therefore an urgent need to develop an integrated management strategy for the disease. Current management practices are mostly cultural and are largely not effective. Napier grass cultivars resistant to the pathogen or less attractive to the insect vector are also not available. Promoting the use of disease-free planting materials and implementing quarantine measures could help prevent the spread of NGS to areas that are not infested yet. Weed management should also be encouraged as weeds may harbour the phytoplasma and its insect vectors.

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Chapter 4: Detection, identification and significance of phytoplasmas in wild grasses in East Africa

Abstract

Plant pathogenic phytoplasmas found in wild grasses in East Africa could pose a serious threat to the cultivation of Napier grass, *Pennisetum purpureum*, the most important livestock fodder in the region. To asses this threat, leaves from plants of 33 grass species were sampled from Mbita, Bungoma and Busia districts in western Kenya, Tarime district in northern Tanzania and Busia and Bugiri districts in the eastern Uganda to determine which species host phytoplasmas, their identity and their relationship with disease symptoms. Phytoplasmas were detected using universal primers based on conserved phytoplasma-specific 16S rDNA sequence from 11 grass species collected. Sequence and phylogenetic analysis revealed the presence of Napier grass stunt (NGS)- related phytoplasmas in 11 grass species, '*Candidatus* Phytoplasma cynodontis' in three, and goosegrass white leaf (GGWL) phytoplasma in two wild grass species. This study showed that the geographical distribution, diversity of phytoplasmas and their grass host species in East Africa is greater than previously thought and that typical disease symptoms including white leaf or stunting alone are not reliable indicators of the presence of phytoplasma. It also shows the need to identify insect vectors responsible for phytoplasma transmission from native grasses to Napier grass or other cereals present in the region.

Keywords: Napier grass, phytoplasma, wild grass hosts, East Africa, fodder

4.1 Introduction

Phytoplasmas are cell wall-less bacteria belonging to the class *Mollicutes*, order *Acholeplasmatales* and the new taxon '*Candidatus* (*Ca.*) Phytoplasma' (IRPCM, 2004). Globally, phytoplasmas are associated with numerous plant diseases (Lee *et al.*, 2000). The inability to isolate and culture phytoplasmas axenically prevented their taxonomic and systematic classification for a long time. Phytoplasmas were therefore largely identified and classified based on symptoms, vector specificity and

host range (Lee *et al*., 2000; Bertaccini, 2007). A general and reliable system of phytoplasma detection and taxonomic classification was developed based on molecular tools such as polymerase chain reaction/restriction fragment length polymorphism (PCR-RFLP) of the conserved 16S ribosomal gene (16S rDNA). This system provided a rapid and reliable means for preliminary classification of phytoplasmas (Lee *et al*., 1998). Currently, 37 '*Ca.* Phytoplasma' species have been formally described based on the similarity of their 16S rDNA sequences with new species being documented regularly (IRPCM, 2004; Quaglino *et al*., 2013; Win *et al.,* 2013a; Harrison *et al*., 2014).

In East Africa, the most important livestock fodder affected by phytoplasmas is Napier grass (*Pennisetum purpureum* Schumach). Two phytoplasmas have been identified in *P. purpureum*, one designated Napier grass stunt (NGS), and the other, African sugarcane yellow leaf (ASYL) (Jones *et al.,* 2004, 2007; Nielsen *et al.,* 2007). These pathogens cause foliar yellowing, small leaves, proliferation of tillers and shortening of internodes with severely stunted clumps in Napier grass. Often the whole grass stool is affected with a complete loss in yield leading to eventual death of the infected plant. The disease has negatively impacted on the livelihoods of farmers that rely on the crop as their main source of feed for dairy animals. In some fields, the disease has caused up to 100% loss of the crop forcing the smallholder farmers to reduce the number of dairy cattle or purchase fodder from the local market (Orodho, 2006). NGS belongs to the 16SrXI group, '*Ca.* Phytoplasma oryzae' or rice yellow dwarf while ASYL belongs to the 16SrIII, '*Ca.* Phytoplasma pruni' or X-disease group (Jones *et al.,* 2004, 2007; Nielsen *et al.,* 2007; Arocha *et al.,* 2009). The leafhopper *Maiestas banda* (Kramer) (Hemiptera: Cicadellidae) transmits NGS to Napier grass in Kenya (Obura *et al.,* 2009), while the leafhopper *Exitianus* spp. (Hemiptera: Cicadellidae) and planthopper *Leptodelphax dymas* (Fennah) (Hemiptera: Delphacidae) have been suggested as potential vectors in the transmission of ASYL (Arocha *et al.,* 2009).

Two phytoplasmas closely related to the NGS group have been reported in other grasses in the western Kenya. These phytoplasmas are Bermuda grass white leaf (BGWL) found in *Cynodon dactylon* (Obura *et al.,* 2010), and *Hyparrhenia* grass white leaf (HGWL) found in *Hyparrhenia rufa* (Obura *et al.,* 2011). HGWL is

classified as a '*Ca.* Phytoplasma oryzae' strain and is closely related to NGS sharing a 99% 16S rDNA sequence identity, while BGWL displays a 100% identity with *Ca.* Phytoplasma cynodontis' strain LY-C1. The potential phytoplasma host characteristics suggested that *H. rufa* could be an alternative host plant for NGS phytoplasma and it could play an important role in the spread of this pathogen in East Africa (Obura *et al.,* 2011).

The basic epidemiological cycle of phytoplasma diseases consists of three components, the phytoplasma, a susceptible host plant and a suitable vector that feeds on the host plant (Lee *et al.,* 2003). This three-way interaction plays a significant role in determining the spread of phytoplasmas (Lee *et al.,* 2003; Obura *et al.,* 2009). Accordingly, the analysis of epidemiological systems of phytoplasmas with many host plants needs to take into account the occurrence of other natural plant hosts, and their role as alternative inoculum sources as well as the host range and feeding preferences of vector species or particular vector populations (Lee *et al.,* 2003; Weintraub & Beanland, 2006; Obura *et al.,* 2011). By extending previous survey work, the present study sought to discover whether there were other phytoplasmas and host grass species with the potential to affect Napier grass and other monocots in Kenya and neighbouring countries.

4.2 Materials and methods

4.2.1 Study area

Wild grasses were collected from Bungoma and Busia districts located in the western part of Kenya, Tarime district in the northern part of Tanzania and Busia and Bugiri districts in the eastern part of Uganda. Plant materials were collected between July 2012 and August 2013. The altitudes at surveyed areas ranged from 947 to 1697 meters above sea level (m a.s.l) in Kenya, 1079 to 1227 m a.s.l. in Uganda and between 1400 and 1700 m a.s.l in Tanzania. Surveys were also conducted in and along protected areas of Ruma National Park in the Lambwe Valley in the Mbita district. Other wild grass samples were collected in a grass nursery maintained at the International Centre of Insect Physiology and Ecology (ICIPE) Thomas Odhiambo Campus located at Mbita point, Mbita district, western Kenya (Fig. 4.1).

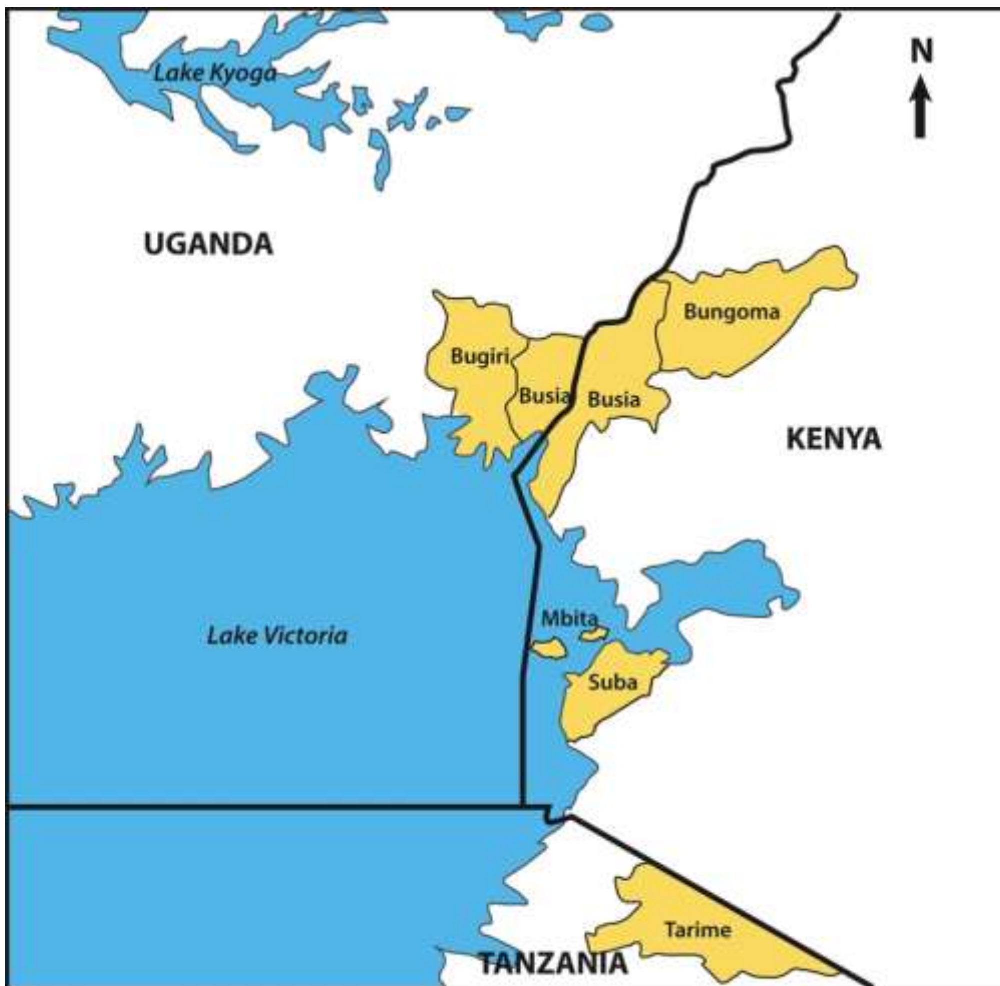

Figure 4.1: Map of East Africa showing the locations of the study.

4.2.2 Plant materials

Both, asymptomatic and symptomatic wild grasses were collected from randomly selected fields bordering Napier grass farms in Bungoma and Busia districts of western Kenya, Tarime district of northern Tanzania and Busia and Bugiri districts of eastern Uganda. In each of these fields, four quadrats (1m x 1m) were randomly thrown along demarcated transects and grass stands. Plant leaves were sampled from five different plants in each quadrat. For symptomatic grasses, leaves were collected from plant species exhibiting at least one of the following symptoms: yellow, white or creamy-coloured leaves, abnormal tillers, stunting or floral deformation. The grass species were collected with the inflorescence to facilitate species identification (Muyekho *et al.*, 2004). After collection, all plant materials were transported and stored at -20°C before isolating DNA. Collection sites were mapped

using the Global Positioning System (GPS) to provide accurate information on the locations.

4.3.3 DNA extraction, quantity and quality determination

DNA was extracted from leaves using methodologies adapted from Doyle & Doyle (1990). A standard weight of 0.3 g per sample was used for each sample. The leaf samples were frozen in liquid nitrogen and ground to powder using sterile pestles and then placed on ice to avoid melting and DNA degradation. Preheated CTAB buffer (65˚C) (600µl) (containing 2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris–HCl, pH8.0, 0.2% 2-mercapto-ethanol) was added to the crushed tissues and then gently vortexed to mix. The entire mixture was incubated at 65 ^{o}C for 1 hour (hr) in a water bath to solubilise cell membranes and separate them from nucleic acids. After incubation, the mixture was left to cool at room temperature and 600 µl of Chloroform: Isoamyl Alcohol (prepared in the ratio of 24:1) were added, vortexed gently and centrifuged at 4000 rpm for 10 minutes (min) to separate the aqueous and organic phase. The supernatant was carefully separated and placed in clean sterile and labelled 1.5 ml microcentrifuge tubes. A volume of 600 µl of ice-cold isopropanol was added to each microcentrifuge tube and incubated at -20°C for 2 hrs or overnight. The DNA was precipitated by centrifugation at 14,000 rpm for 30 min. The supernatant was discarded carefully and the pellet washed by 70% ethanol (1ml) and gently tilted. The pellet was left to settle for 1 min then centrifuged at 14,000 rpm for 4 min. The DNA pellet was air-dried at room temperature and reconstituted in 50 µl of sterile water. The quality of DNA was determined using 1% (w/v) agarose gel and quantified using a spectrophotometer. DNA samples were stored at –20°C until further use.

4.3.4 PCR amplification of phytoplasma DNA

Phytoplasma DNA was amplified using universal primer pair P1/P6 (Deng & Hiruki, 1991) in the first round PCR followed by primer pair NapF/NapR (Obura, 2012) (Table 4.1). The initial amplification was performed in a 25 µl PCR reaction mixture containing 10ng of genomic DNA, 10X PCR buffer (GenScript, USA Inc.), 2.5mM dNTPs, 1 unit *Taq* DNA polymerase (GenScript, USA Inc.) and 10 µM of each primer (Inqaba Biotech, South Africa Inc.). PCR reactions were carried out either in a PTC-

100 Thermal cycler (MJ Research Inc. USA) or Proflex PCR machine (Applied Biosystems) as follows: denaturation at 94 ºC for 5 min for 1 cycle; 35 cycles of 94 ºC for 30 s, 52 ºC for 60 s and 72 ºC for 2 min; and a final elongation step at 72 ºC 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 following conditions; denaturation at 94 ºC for 4 min for 1 cycle; 35 cycles of 94 ºC for 45 s, 55 ºC for 60 s and 72 ºC for 2 min; and a final elongation at 72 ºC for 10 min. Amplicons were then visualized by gel electrophoresis in a 1% agarose gel stained with ethidium-bromide using 1xTAE (40 mM Tris acetate, 1mM EDTA pH8.0) 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. The DNA from reference phytoplasma strain, NGS, maintained at ICIPE Mbita station was used as a positive control.

Phytoplasma DNA was also amplified using the P1/Tint primer pair (Smart *et al.*, 1996; Table 4.1). PCR was performed in a 25 µl final reaction mixture containing 20ng of genomic DNA, 10X PCR buffer supplied with $MgCl_2$ (KAPA Biosystems, Germany), 2.5mM dNTPs, 1.0 unit *Taq* DNA polymerase (KAPA Biosystems, Germany) and 10µM of each primer. PCR reactions were carried out using a Bio-Rad iCycler (Germany) as follows: denaturation at 94 ºC for 4 min for 1 cycle; 35 cycles of 94 ºC for 30 sec, 52 ºC for 1 min and 72 ºC for 90 sec; and a final elongation at 72 ºC for 5 min. The PCR products were directly purified using the Qiaquick purification kit (Qiagen Inc.) following the manufacturer's instruction.

Table 4.1: Sequences of the oligonucleotide primers used for PCR amplification in wild grasses and for sequencing

Primer	Location[a]	Sequences (5' to 3')	References
P1	16S	AAGAGTTTGATCCTGGCTCAGGATT	Deng & Hiruki, 1991
P6	16S	CGGTAGGGATACCTTGTTACGACTTA	Deng & Hiruki, 1991
Tint	SR	TCAGGCGTGTGCTCTAACCAGC	Smart *et al.*, 1996
NapF	16S	AGGAAACTCTGACCGAGCAAC	Obura, 2012
NapR	16S	ATTTTTCATTGGCAGTCTCGTTA	Obura, 2012

[a]Location of primer within the rRNA operon, i.e., within either the 16S rRNA gene or the 16S–23S intergenic spacer region (ISR) used in the amplification of phytoplasma. The product size of primer pairs P1/P6 = 1500 bp, P1/Tint = 1600 bp and NapF/R = 778 bp.

4.3.5 Sequence and phylogenetic analysis

The 16S rDNA amplicons of phytoplasma-positive grasses were purified using a QuickClean II PCR Extraction Kit (GenScript, USA Inc.) according to the manufacturer's instruction. DNA amplicons were sequenced directly using the NapF/NapR primers using Dye Terminator chemistry in a DNA automatic sequencer at the International Livestock Research Institute (ILRI), Nairobi Kenya. P1/Tint amplicons were ligated into pGEM-T easy vector system (Promega) and cloned using *Escherichia coli* NM522 strain (Promega). Successful clones were then purified using EasyPrep® Pro Plasmid Miniprep Kit (Biozym, Germany) according to the manufacturer's instruction. DNA sequences were assembled and edited using DNA Workbench (CLC bio, Aarhus, Denmark) software. The resulting consensus sequences are deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/). For phylogenetic analysis sequences of phytoplasma infecting grasses were obtained from the GenBank database (Table 4.3). The 16S rDNA and the ISR sequences were aligned using a progressive alignment algorithm (Feng & Doolittle, 1987) implemented in the DNA Workbench package (CLC bio, Aarhus, Denmark) with the default settings. The alignments were then exported in fasta format, converted to MEGA 6 format and used for distance and phylogenetic analyses in MEGA 6 software (Tamura *et al*., 2013). A dendrogram was then constructed using Neighbour-Joining method (Saitou & Nei, 1987) using 1000 replicates for bootstrap analysis (Felsenstein, 1985).

4.3.6 Transmission Experiments

The study was conducted at ICIPE Thomas Odhiambo campus, Mbita Point (0°25′ S, 34°12′ E) in Homabay county. Phytoplasma-infected *C. dactylon* (BGWL) and *H. rufa* (HGWL) grass species were used in the study as wild sources of inoculum. Diseased *P. purpureum* stunted with small yellow leaves and bushy in appearance were equally included in the trials as controls. Symptomatic grass plants identified in the surveyed areas were carefully removed from the ground, potted, labeled and maintained at the station and were confirmed to have phytoplasma by nested PCR (Fig. 4.3). Napier grass (bana variety) susceptible to NGS phytoplasma was used in the experimental trials. Cuttings were planted 2 cm deep and covered with a thin layer of soil in small cups and fertilized with phosphorus at the recommended rate.

The plants were germinated in a netted screen-house to prevent them from any movement of insects, for five weeks before subjecting to phytoplasma testing in cages in a different screen-house. No pesticides were applied to the plants. The plants were watered daily and other management practices done.

The insect vector used in the study was *M. banda*. The leafhopper colonies of gravid females were provided from the vector rearing screen-house at ICIPE. The insects were reared on potted NGS phytoplasma-free pearl millet plants in the screen house in wooden framed cages measuring 45x45x60 cm. The top and side openings of the cages were covered with fine white nylon mesh for aeration. Transmission tests were carried out as described by Obura et al. (2009). Because artificial inoculation with phytoplasma is not possible, the insect vector served as inoculum carrier to infect test plants. The trials were carried with each of the diseased plant species put in the middle of the cage and surrounded by three healthy Napier grass plants (Fig. 4.2), and repeated seven times. Another cage was added where only healthy Napier grass plants were included as negative controls. Using an aspirator, 50 gravid *M. banda* individuals from the rearing cages containing non-infected plants were introduced into each trial cages and the insects were allowed to feed back and forth for 30 days in order to sufficiently acquire and transmit the phytoplasmas. No insects were introduced into the cages containing healthy plants. After oviposition, the emerging nymphs were allowed to feed on the diseased plants and similarly acquire the phytoplasma. The insects were disturbed from time to time in the cages to redistribute the population.

a b)

Figure 4.2: Arrangement of potted trial plants in experimental cages during transmission experiments. The middle plant is the inoculum source (a). White-leaf infected *Cynodon dactylon* (b). White leaf-infected *Hyparrhenia rufa* surrounded by three healthy Napier grass plants. Fifty gravid *Maiestas banda* were released into the cage to act as phytoplasma vector.

After the 30 days, the inoculation setup was terminated and the exposed plants transferred to a separate screen house for phytoplasma testing and to evaluate disease symptom expression. The plants were transplanted into new large pots and phosphorus applied at the recommended rate. Exposed Napier grass plants were used as positive controls and unexposed Napier grass plants as negative controls. The trial plants were investigated for the presence of phytoplasma monthly after inoculation using nested PCR.

4.4 Results

4.4.1 Host plants

The grass species tested, collection sites, symptom description, number of samples and phytoplasma presence are listed in Table 4.2. A total of 2185 wild grass samples were collected comprising 830 from Bungoma and Busia districts of western Kenya,

400 from Tarime in northern Tanzania, 850 from Busia and Bugiri districts in eastern Uganda and 105 wild grass samples from Mbita district. Samples from Mbita district comprised of 65 grasses from the ICIPE grass nursery and 40 samples collected in Ruma National Park. Wild grasses collected in the five districts comprised 28 species in 21 genera while those from Mbita district comprised 33 species in 22 genera. Ninety-three grass samples could not be identified since they lacked inflorescences (Table 4.2). Phytoplasma infection was confirmed by the amplification of a 778 bp 16S rDNA fragment by nested PCR (Fig. 4.3) from 270 out of 2185 wild grass samples tested (Table 4.2).

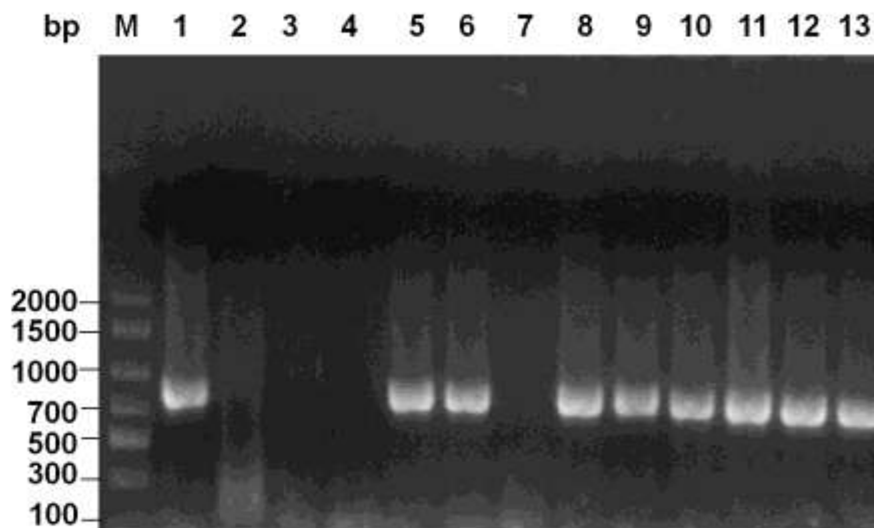

Figure 4.3: Electropherogram of nested-PCR products amplified with P1/P6 and NapF/NapR primers from wild grasses. M: 1 kb DNA marker (GenScript, USA Inc.); lane 1: Reference NGS phytoplasma, 2: negative control (water). Representative wild grass plant samples negative for phytoplasma: lanes 3, KW2 (*Cyperus rotundus*); 4, KW21, (*Panicum maximum*); 7, KW275, (*Eragrostis curvula*). Phytoplasma-infected wild grass plant samples: lanes 5, KW709 (*Cynodon dactylon*); 6, KW22 (*Cynodon dactylon*); 8, KW261 (*Sporobolus pyramidalis*); 9, TW429 (*Brachiaria brizantha*); 10, TW348 (*Sporobolus pyramidalis*); 11, TW384 (*Hyparrhenia rufa*); 12, UW522, (*Sorghum versicolor*); 13, UW61 (*Cynodon dactylon*).

Of all the grass samples, 81 of 850 sampled in Kenya (Bungoma and Busia districts), 61 of 400 from Tarime district and 74 of 830 of grasses from Uganda (Busia and

Bugiri districts) were phytoplasma-positive. Fifty five out of 105 samples from Mbita district were positive for phytoplasma. In the Busia and Bungoma districts of Kenya, phytoplasmas were detected in 1 of 74 of *Brachiara brizantha,* 2 of 47 of *Eleusine indica*, 55 of 132 of *C. dactylon*, 21 of 51 of *H. rufa*, 1 of 33 of *Sporobolus pyramidalis* and 1 of 1 of *Coix laryma-jobi* samples collected. In the Tarime district of Tanzania, phytoplasmas were detected in 6 of 40 of *B. brizantha*, in 41 of 107 of *C. dactylon*, in 1 of 12 of *E. indica*, in 9 of 24 of *H. rufa* and in 2 of 13 of the *S. pyramidalis* plants. Phytoplasmas were also detected in five other grass species found in Uganda comprising 4 of 98 of *B. brizantha*, 62 of 124 of *C. dactylon*, 3 of 89 of *Digitaria scalarum*, 2 of 29 of *H. rufa* and 3 of 82 of *Sorghum versicolor*. In Mbita district, 1 of 4 of *Chloris gayana* plants tested positive for phytoplasma, 30 of 30 of *C. dactylon*, 1 of 5 of *D. scalarum*, 1 of 2 of *Enteropogon macrostachyus*, 1 of 2 of *H. cymbaria*, 15 of 20 of *H. rufa* and 2 of 6 of *B. brizantha*. Of the 198 *P. purpureum* samples collected, 45 of 91 collected in Kenya tested positive for phytoplasma, 26 of 57 from Uganda and 6 of 50 from Tanzania.

Table 4.2 Grass species screened for the presence of phytoplasmas from East Africa

Grass species	Common name	Location	Symptoms	Phytoplasma	No. positi No. teste
Andropogon gayanus	Gamba grass	Mbita	None	-	0/1
Bothriochloa bladhii	Bluestem	Mbita	None	-	0/1
Bothriochloa insculpta	Sweet pitted grass	Bungoma, Busia-K, Tarime, Bugiri, Busia-U, Mbita	None	-	0/19
Brachiaria brizantha	Signal grass	Bungoma, Busia-K, Tarime, Bugiri, Busia-U, Mbita	Yellow leaves	+$^{\beta}$	13/218
Brachiaria sp.	Signal grass	Bungoma, Busia-K, Tarime	None	-	0/126
Cenchrus ciliaris	African foxtail	Mbita	None	-	0/1
Chloris gayana	Rhodes grass	Bungoma, Busia-K, Tarime, Bugiri, Busia-U, Mbita	None	-	1/17
Chloris roxybarghiana	Horsetail grass	Bungoma, Busia-K, Tarime, Mbita	None	-	0/7
Coix laryma-jobi	Otiro (Luo)	Busia-K	None	+$^{\beta}$	1/1
Cymbopogon citratus	Lemon grass	Mbita	None	-	0/2
Cymbopogon nardus	Blue citronella	Bungoma, Busia-K, Tarime, Mbita	None	-	0/12
Cynodon dactylon	Bermuda grass	Bungoma, Busia-K, Tarime, Bugiri, Busia-U, Mbita	White leaves, stunting	+	192/39
Cyperus rotundus	Sedge	Bungoma, Busia-K, Tarime, Bugiri, Busia-U	Yellow leaves	-	0/123
Danthonia sp.	Poverty grass	Bungoma, Busia-K, Bugiri, Busia-U	None	-	0/33
Digitaria scalarum	Couch grass	Bungoma, Busia-K, Tarime, Bugiri, Busia-U, Mbita	Yellow leaves	-	4/351
Echinochloa pyramidalis	Antelope grass	Bungoma, Busia-K, Mbita	None	-	0/3
Eleusine indica	Crowfoot grass	Bungoma, Busia-K, Tarime, Bugiri, Busia-U	None	+$^{\beta}$	3/86
Enteropogon macrostachyus	Bush rye	Mbita	None	+$^{\beta}$	1/2
Eragrostis curvula	Weeping love grass	Bungoma, Busia-K, Tarime	None	-	0/8
Eragrostis superba	Maasai love grass	Bungoma, Tarime, Mbita	None	-	0/9
Heteropogon contortus	Bunch spear grass	Mbita	None	-	0/2
Hyparrhenia cymbaria	Thatch grass	Mbita	White leaves	+	1/2
Hyparrhenia filipendula	Fine wood grass	Bungoma, Busia-K, Tarime, Bugiri, Busia-U	None	-	0/20
Hyparrhenia hirta	Thatch grass	Mbita	None	-	0/1
Hyparrhenia pilgerana	Thatch grass	Busia-K, Tarime, Bugiri, Busia-U	None	-	0/8
Hyparrhenia rufa	Thatch grass	Bungoma, Busia-K, Tarime, Bugiri, Busia-U, Mbita	White leaves, stunting	+$^{\beta}$	52/125
Hyparrhenia sp.	Thatch grass	Mbita	None	-	0/1
Imperata cylindrica	Spear/sword grass	Busia-K, Tarime, Bugiri, Busia-U, Mbita	None	-	0/59
Loudetia kagerensis	'Buoywee' (Luo)	Busia-K, Bugiri, Busia-U	None	-	0/6
Melinis minutiflora	Molasses grass	Mbita	None	-	0/1
Panicum antidotale	Perennial Sudan grass	Bugiri, Busia-U	None	-	0/4
Panicum deustum	Broadleaf Panicum	Mbita	None	-	0/1
Panicum maximum	Guinea grass	Bungoma, Busia-K, Tarime, Bugiri, Busia-U, Mbita	None	-	0/161
Pennisetum mezianum	Fountain grass	Mbita	None	-	0/1
Pennisetum polystachion	Thin Napier grass	Tarime, Bugiri, Busia-U, Mbita	None	-	0/55
Pennisetum purpureum	Napier/ Elephant grass	Bungoma, Busia-K, Tarime, Bugiri, Busia-U, Mbita	Stunting, yellow leaves	+$^{\beta}$	77/198
Pennisetum sphacelatum	Fountain grass	Mbita	None	-	0/1
Rottboellia cochinchinensis	Itch grass	Busia-K, Tarime, Bugiri, Busia-U	None	-	0/66
Setaria incrassata	Purple pigeon Grass	Busia-K, Bugiri, Mbita	None	-	0/3
Setaria sphacelata	African bristle grass	Bungoma, Busia-K, Bugiri, Busia-U, Mbita	None	-	0/7
Sorghum arundinaceum	Wild sorghum	Bungoma, Busia-K, Tarime, Mbita	None	-	0/5
Sorghum sudanensis		Mbita	None	-	0/1
Sorghum versicolor	Wild sorghum	Bugiri, Busia-U, Mbita	None	+$^{\beta}$	3/83
Sporobolus pyramidalis	Drop-seed grass	Bungoma, Busia-K, Tarime, Bugiri, Busia-U, Mbita	None	+$^{\beta}$	3/77
Sporobolus (CS) consimilis		Mbita	None	-	0/1
Themeda triandra	Red oat grass	Mbita	None	-	0/2
Chrysopogon zizanioides	Vetiver	Mbita	None	-	0/1
Unknown		Bungoma, Busia-K, Bugiri, Busia-U	None	-	0/93
Total				**270**	**2185**

Presence or absence of phytoplasmas indicated by + and –, respectively. Not all individuals displayed symptoms listed for the species. Plant names are according to those described by Muyekho *et al.* (2004). $^{\beta}$At least one symptomless sample tested positive. $^{\#}$Includes individuals with and without symptoms. Busia-K = Busia Kenya, Busia-U = Uganda.

4.4.2 Relationship between phytoplasma dectection and disease symptoms

Phytoplasma infected Bermuda grass (*C. dactylon*) plants showed whitening of leaves, a bushy growing habit, small leaves, shortened stolons/rhizomes, stunting and proliferation of auxiliary shoots (Fig. 4.4a). These symptoms were similar in all locations surveyed. Symptomatic thatch grasses (*H. rufa*) appeared stunted and bushy with small white leaves (Fig. 4.4b). These symptomatic thatch grasses were mainly found in grass veld at several locations of Lambwe valley in western Kenya and in the wild grassland of the Nyamongo village in the Tarime district in northern Tanzania. Some of the infected *B. brizantha* (Fig. 4.5) and *S. pyramidalis* plants showed yellow leaf symptoms while the remaining phytoplasma-positive species did not show symptoms (Tables 4.2, 4.3). Nine of the eleven wild grass species that tested positive for phytoplasma, i.e. *B. brizantha*, *C. gayana*, *C. laryma-jobi*, *E. indica*, *D. scalarum*, *E. macrostachyus*, *H. cymbaria*, *S. pyramidalis* and *S. versicolor*, are newly recorded phytoplasma hosts in the region. The *H. rufa* and *C. dactylon* plants that tested positive for phytoplasmas in Busia and Bugiri districts of Uganda and Tarime district of Tanzania are newly reported phytoplasma hosts in these areas.

(a) (b)

Figure 4.4: (a) Whitening, small leaves and bushy growing habits associated with BGWL phytoplasma-infected Bermuda grass that were common in all studied districts, (b) stunting, bushy growing habits and small white leaves associated with HGWL phytoplasma-infected thatch grass found in Mbita and Tarime districts.

Figure 4.5: White-yellow leaves on GGWL phytoplasma-infected signal grass found in Mbita district.

The relationship between phytoplasma presence in leaves and symptoms varied according to plant species. There was a clear association between phytoplasma and white leaf symptoms in *C. dactylon*. All symptomatic *C. dactylon* plants were phytoplasma-positive and asymptomatic plants were negative. There was also a clear association between a phytoplasma infection and stunting, bushy-growth habits and white leaf symptoms in *H. rufa*. However, in *D. scalarum* and *Cyperus rotundus*, yellow leaf symptoms were not associated with phytoplasma presence. Some of the *B. brizantha* and *S. pyramidalis* plants had yellow leaf symptoms while the remaining phytoplasma-positive species were asymptomatic. Symptom descriptions are provided in Tables 4.2 and 4.3.

Table 4.3: Diversity of phytoplasmas in grasses and relationship between symptoms and phytoplasma detection

Plant Host	Host Symptoms	Phytoplasma identified*	Accession number		16S rRNA group	No. detected / tested
			16S rRNA	ISR		
Brachiaria brizantha	Asymptomatic	NGS-Bb(Tz)	KM875525		16SrXI	1/40
	Asymptomatic	BGWL-Bb(Tz)	KM875526		16SrXIV	2/40
	White leaves	GGWL-Bb(Tz)	KM875519	KM924296	-	2/40
	Asymptomatic	BGWL-Bb(Ug)	KM875528		16SrXIV	3/98
	Asymptomatic	RYD-Bb(Ug)	KM875529		16SrXI	1/98
	White leaves	GGWL-BrX3(Ke)	KM875533	KM924298	-	1/6
	Yellow leaves	BGWL-BrX5(Ke)	KM875534		16SrXIV	1/6
	Asymptomatic	RYD-Br(Ke)	KM875535		16SrXI	1/74
Chloris gayana	Asymptomatic	RYD-Cg(Ke)	KM879878		16SrXI	1/1
Enteropogon macrostachyus	Yellow leaves	RYD-Em(Ke)	KM879875		16SrXI	1/2
Coix laryma-jobi	Asymptomatic	RYD-Cl(Ke)	KM879877		16SrXI	1/1
Cynodon dactylon	White leaves, stunted growth	RYD-Cd(Tz)	KM875527		16SrXI	1/107
Cynodon dactylon	White leaves, stunted growth	BGWL-K1(Ke)	KM875511		16SrXIV	112/393
	White leaves, stunted growth	BGWL-K2(Ke)	KM875512		16SrXIV	12/132
	White leaves, stunted growth	BGWL-Ug1	KM875513		16SrXIV	13/132
	White leaves, stunted growth	BGWL-Ug2	KM875514		16SrXIV	27/124
	White leaves, stunted growth	BGWL-Ug3	KM875515		16SrXIV	21/124
	White leaves, stunted growth	BGWL-Tz1	KM875516		16SrXIV	13/107
	White leaves, stunted growth	BGWL-Tz2	KM875517		16SrXIV	20/107
	White leaves, stunted growth	BGWL-Tz3	KM875518		16SrXIV	4/107
	Asymptomatic	RYD-Cd(Ke)	KM875539		16SrXI	1/393
Digitaria scalarum	Asymptomatic	RYD-Ds(Ug)	KM875530		16SrXI	3/89
Digitaria scalarum	Yellow leaves	RYD-Ds(Ke)	KM879876		16SrXI	1/5
Eleusine indica	Asymptomatic	RYD-Ei(Ke)	KM975504		16SrXI	2/47
Eleusine indica	Asymptomatic	BGWL-Ei(Tz)	KM875524		16SrXIV	1/12
Hyparrhenia rufa	White leaves, stunted growth	RYD-Hr(Tz)	KM875523		16SrXI	8/24
Hyparrhenia rufa	Asymptomatic	BGWL-Hr(Tz)	KM875522		16SrXIV	1/24
	White leaves	RYD-Hr(Ug)	KM875531		16SrXI	2/29
	White leaves, stunted growth	RYD-Hr(Ke)	KM875538	KM924299	16SrXI	31/72
Hyparrhenia cymbaria	White leaves	RYD-Hc(Ke)	KM875537		16SrXI	1/2
Sporobolus pyramidalis	Asymptomatic	RYD-Sp(Ke)	KM875536		16SrXI	1/33
Sporobolus pyramidalis	Asymptomatic	RYD-Sp(Tz)	KM875521		16SrXI	1/13
Sporobolus pyramidalis	Asymptomatic	GGWL-Sp(Tz)	KM875520	KM924297	-	1/13
Sorghum versicolor	Asymptomatic	RYD-Sv(Ug)	KM875532		16SrXI	3/82
Pennisetum purpureum	Asymptomatic	NGS- Pp1(Tz)	KM924303		16SrXI	2/50
Pennisetum purpureum	Asymptomatic	NGS- Pp2(Tz)	KM924304		16SrXI	4/50
Pennisetum purpureum	Yellow leaves, stunted growth	NGS-Pp1(Ug)	KM924305		16SrXI	10/57
Pennisetum purpureum	Yellow leaves, stunted growth	NGS- Pp2(Ug)	KM924306		16SrXI	16/57
Pennisetum purpureum	Yellow leaves, stunted growth	NGS-Pp(Ke)	KM924300		16SrXI	16/91
Pennisetum purpureum	Yellow leaves, stunted growth	NGS-Pp2(Ke)	KM924301		16SrXI	12/91
Pennisetum purpureum	Yellow leaves, stunted growth	NGS-Pp3(Ke)	KM924302		16SrXI	8/91

*The names of the phytoplasmas are given according to the type of phytoplasma followed by abbreviation of the species' name and country code, RYD = Rice yellow dwarf, NGS = Napier grass stunt, BGWL = Bermuda grass white leaf, GGWL = goose grass white leaf, Tz = Tanzania, Ke = Kenya and Ug = Uganda.

4.4.3 Genetic relatedness of phytoplasmas infecting wild grasses

Comparison of the sequences obtained indicated that phytoplasmas from the wild grass species were different. Thus, among the grasses collected in Kenya, phytoplasmas found in *C. gayana, C. laryma-jobi*, *D. scalarum*, *E. macrostachyus*, *E. indica*, *H. rufa*, *H. cymbaria* and *S. pyramidalis* were closely related to the 16SrXI group of phytoplasmas. They shared sequence similarities that ranged from 98.43 to

98.95% with the 16SrXI phytoplasma sequences of NGS phytoplasmas reported previously in Uganda and Kenya (GenBank accession No. AY377876, EF012650, FJ862997, FJ862998, FJ862999, JQ868440, JQ868443). In *C. dactylon* phytoplasmas from two 16S groups were found. One accession had the NGS phytoplasma strain while the remaining accessions had their highest sequence similarity with BGWL strains, which belong to the 16SrXIV group (accession No. EU409293, AJ550984, AJ550986, AJ550985, EF444485, HE599391, HE599395, AF248961, AB052871, EU234510, AB642601, AB741630, HE599392, HE599393 and KF234570). The sequence similarity with reference strains ranged from 99.47% to 99.6%. In *B. brizantha* plants sampled in Mbita district, three phytoplasma strains were detected. One strain had a close similarity with NGS (99%), the second belonged to BGWL (99.6%) while the third strain was identified as a goose-grass white leaf (GGWL) phytoplasma having a 99.6% sequence similarity to GenBank accession AB741629. All phytoplasmas were detected in separate plants.

In Tanzania, phytoplasmas closely related to BGWL strains were common and were detected in the majority of *C. dactylon* plants sampled. Only one sample contained a phytoplasma strain closely related to NGS. '*Ca*. Phytoplasma cynodontis' was also detected in one *H. rufa* and one *E. indica* plant. These phytoplasmas were closely related to a BGWL strain from India, which belongs to the 16SrXIV group with a 99.6% sequence similarity. Two different phytoplasmas were detected in *S. pyramidalis* species collected in Tanzania. One had a close resemblance to GGWL (GenBank accession No. AB741629) with 99.85% sequence similarity, while the other was similar to NGS isolates from Uganda and Kenya sharing a 98.83% similarity with sequences in databases (GenBank accession No. AY377876, EF012650, FJ862997, FJ862998, FJ862999, JQ868440, JQ868443). Eight of the nine phytoplasma-infected *H. rufa* plants collected from Tarime were classified as NGS phytoplasma with a 98.95% sequence similarity. In individual *B. brizantha* accessions, three different phytoplasmas were detected, namely: GGWL, NGS and BGWL, sharing a 99% (AB741629), 98.95% (JQ868443) and 99.6% (KF234570) similarity with sequences in the database, respectively.

Since GGWL was not previously reported in East Africa, a larger fragment was amplified for sequence comparison using ISR primers (Table 4.1; Fig. 4.6). All ISR

sequences (KM924296, KM924297, KM924298) from Mbita and Tarime districts were classified as a GGWL phytoplasma from Myanmar (GenBank accession No. AB741629) sharing a 99.25% identity confirming results from the 16S sequences. Other phytoplasmas amplified by the ISR primers were HGWL phytoplasmas (GenBank accession No. KM924299). HGWL strains shared a 98.71% similarity with NGS phytoplasma (GenBank accessions JQ868443, JQ868440) and a 97.35% sequence similarity with a Psammotettix cephalotes' flower stunt phytoplasma strain BVK isolated from Germany (GenBank accession No. HQ589192). HGWL also shared a 96% sequence similarity with Bermuda grass white leaf phytoplasma (GenBank accession No. AF248961).

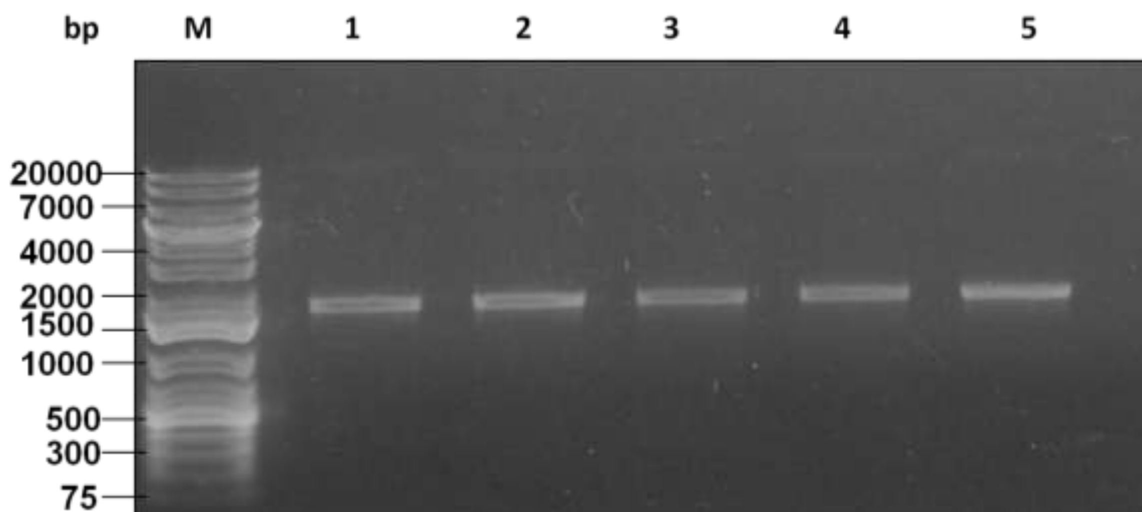

Figure 4.6: Electropherogram of PCR products amplified with P1/Tint primers from infected GGWL and HGWL phytoplasmas. M: 1 kb plus DNA marker (Thermo Scientific).

The resulting 16Sr DNA sequences indicated that phytoplasmas in all *C. dactylon* species collected in Busia and Bugiri districts of Uganda were indistinguishable. These phytoplasmas were nearly identical to BGWL strains of group 16SrXIV phytoplasma (accession No. EU409293, AJ550984, AJ550986, AJ550985, EF444485, HE599391, HE599395, AF248961, AB052871, EU234510, AB642601, AB741630, HE599392, HE599393 and KF234570) and shared a sequence similarity ranging from 99.34% to 99.6%. Phytoplasmas detected in *S. versicolor*, *H. rufa*, and *D. scalarum* collected from Uganda were closely related to the NGS strains formerly reported in Kenya and Uganda (GenBank accessions No. AY377876, EF012650,

FJ862997, FJ862998, FJ862999, JQ868440, JQ868443). Two phytoplasmas were found in *B. brizantha* sampled in Uganda. One of these had a close identity to NGS phytoplasma with 98.95% sequence similarity while the other phytoplasma had a 99.6% sequence similarity with BGWL phytoplasma.

Sequence analysis indicated that phytoplasmas in *P. purpureum* were identical and shared a sequence similarity of between 99.21–100% with the 16SrXI phytoplasma strains reported previously from Uganda and Kenya (GenBank accessions; AY377876, EF012650, FJ862997, FJ862998, FJ862999, JQ868440, JQ868443).

Table 4.4: Acronyms and NCBI accession numbers of phytoplasma 16S rDNA sequences used for phylogenetic analyses

Strain	Phytoplasma	16S rRNA group	Host plant	Origin	EMBL Accession#
BGWL	Bermuda grass white leaf	16SrXIV	Bermuda	India	KF234570
BGWL	Bermuda grass white leaf	16SrXIV	Bermuda	Turkey	HE599393
BGWL-LY-C1	Bermuda grass white leaf	16SrXIV	Bermuda	China	EU409293
GGWL	Goose-grass white leaf	-	Goose-grass	Myanmar	AB741629
SGSVarI	Sorghum grassy shoot VarI	16SrXI-B	Coast button grass	Australia	AF509324
SGSVarII	Sorghum grassy shoot VarII	16SrXI-B	Purpletop Rhodes	Australia	AF509325
NGS	Napier grass stunt	16SrXI	Napier	Kenya	JQ868443
NGS	Napier grass stunt	16SrXI	Napier	Kenya	JQ868440
NGS	Napier grass stunt	16SrXI	Napier	Kenya	FJ862999
NGS	Napier grass stunt	16SrXI	Napier	Kenya	FJ862998
NGS	Napier grass stunt	16SrXI	Napier	Kenya	FJ862997
NGS	Napier grass stunt	16SrXI	Napier	Uganda	EF012650
NGS	Napier grass stunt	16SrXI	Napier	Kenya	AY377876
NGS	Napier grass stunt	16SrIII	Napier	Ethiopia	DQ305977
BVK	‘Psammotettix cephalotes' flower stunt	16SrXI-C	Periwinkle	Germany	HQ589192
SUK22-4	Sugarcane green grassy shoot phytoplasma	-	Sugarcane	Thailand	KF908793

EMBL Accession number

4.4.4 Phylogenetic analysis

A phylogenetic analysis of the 16S rDNA sequences obtained in this study and from GenBank database entries listed in Table 4.3 and 4.4 was performed. When compared, accessions from Tanzania and Kenya clustered into three discrete clades: the 16SrXI group, GGWL group and the 16SrXIV group. The analysis revealed that all NGS-related phytoplasmas belonged to the 16SrXI phytoplasma group but were distinct from the NGS phytoplasmas that infect Napier grass. The group was subdivided into main groups comprising phytoplasmas from Napier grass

in one branch and those from the wild grasses in the other branch (Fig. 4.7). All the BGWL related phytoplasmas clustered together as one distinct group. GGWL-related phytoplasmas were also grouped together in one phylogenetic clade differing from those of the 16SrXI or 16SrXIV phytoplasma groups. Phylogenetic analysis of phytoplasma sequences from Uganda showed two main distinct phytoplasma groups comprising of the 16SrXI and 16SrXIV groups (Fig. 4.7). Analysis of phytoplasma sequences from infected Napier grass grouped these into one full group comprising the 16SrXI phytoplasma group.

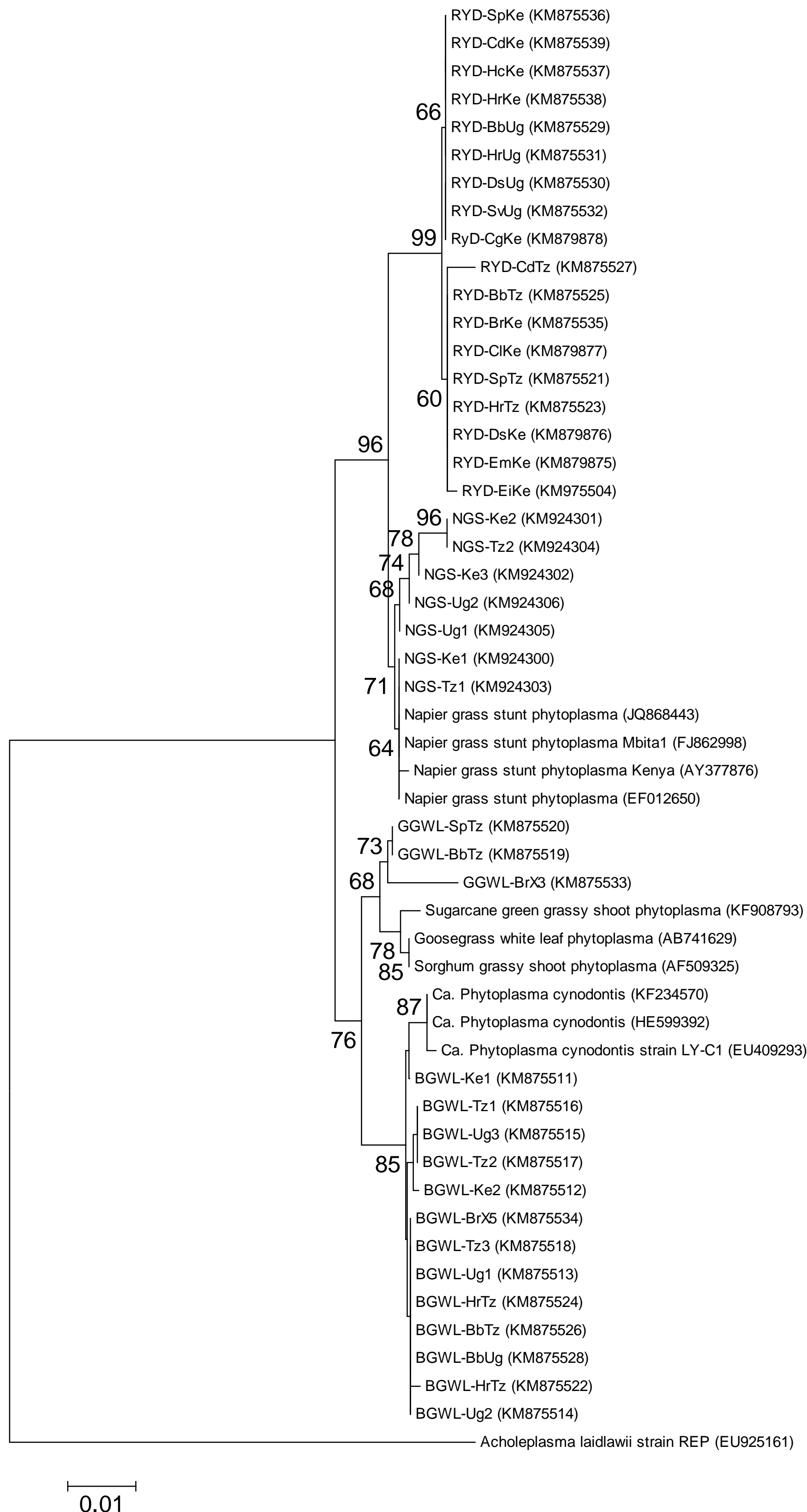

Figure 4.7: Phylogenetic dendrogram of the 16S rRNA gene sequences of 51 phytoplasmas generated by Mega6 as described in the text. Bootstrap values were based on 1000 replicates. Phytoplasma names and sequence accession numbers are provided in Table 4.3.

4.4.5 Vector transmission

All the samples of *M. banda* were phytoplasma negative when tested by nested PCR. In the first experiment done using diseased wild grasses, *C. dactylon* and *H. rufa* plants and surrounded each by healthy plants, the nested PCR done using the NAPF/NAPR primers did not amplify the expected 778 bp of the 16Sr DNA fragment from any of the test plants following 30 days of post-inoculation. Therefore, the test plants were maintained and observed for another period of 60, 90 and 120 days post-inoculation. All the test plants remained negative for phytoplasma transmission from BGWL and HGWL indicating the inability of the *M. banda* to transmit either BGWL or HGWL back to Napier grass. All Napier grass remained negative regardless of the phytoplasma group used. However, Napier grass infected with NGS had foliar yellow leaves after the first cut and later became stunted with bushy appearance at the end of the study. The survival of *M. banda* on healthy Bermuda and thatch grasses was tested. The results showed that most of the insects did not survive for long on these grasses. For example, on healthy Bermuda grass all *M. banda* in the first and second cage died on third day and fourth day of incubation respectively. However, in the third cage these insects survived for five days. A similar result was achieved on healthy thatch grasses, with most insects dying on the fourth and the fifth day. In one cage, however, one female insect survived up to the eighth day.

4.5 Discussion

In East Africa, grasses represent a significant proportion of the vegetation, populating most habitats (Muyekho *et al.*, 2004). Like other angiosperms, they are infected by phytoplasmas (Jones *et al.*, 2004, 2007; Nielsen *et al.*, 2007; Arocha *et al.*, 2009; Obura *et al.*, 2010, 2011) that are transmitted mainly by phloem-sucking insects (Weintraub & Beanland, 2006; Obura *et al.*, 2009). This infection often leads to reduced plant growth and biomass (Jones *et al.*, 2004, 2007; Nielsen *et al.*, 2007). Cultivated grasses like Napier grass and related monocots are important fodder plants for livestock and consequently for human nutrition (Orodho, 2006). Additionally, Napier grass also plays a significant role in the region as a trap plant in the ‘push-pull’ strategy used for management of cereal stem borers (Pickett *et al.*,

2014). Therefore, phytoplasmas pose a potential threat for these economically important plants.

During a survey to assess the prevalence and genetic relationship of phytoplasmas in wild grasses, more than 2,000 samples from 33 wild grass species were collected and tested. Besides the known phytoplasma host species *C. dactylon*, *H. rufa* and *P. purpureum* (Jones *et al*., 2004; Obura *et al*., 2010, 2011), nine grass species were found as new hosts in East Africa. Based on sequence similarities and phylogenetic analysis based on 16S rDNA sequences, these phytoplasmas were divided into NGS, GGWL and the BGWL-related groups. The sequences from infected Napier grasses and *H. rufa* were definitively affiliated with the 16SrXI group , distinct from those associated with NGS in Ethiopia, which belongs to 16SrIII-A group '*Ca*. Phytoplasma pruni' or X-disease (Jones *et al*., 2007; Arocha *et al*., 2009). BGWL and HGWL phytoplasmas have been reported previously in studies from western Kenya. The studies suggested that, because of their close similarity to NGS phytoplasma, these phytoplasmas were important and posed a threat to the cultivation of Napier grass in the East African (Obura *et al*., 2010, 2011). The current study has not only found evidence of a wider geographic distribution of these phytoplasmas, but has also shown that these plant pathogens occur in more grass species than previously thought.

The goose-grass white leaf (GGWL) phytoplasma has never been reported in the region in either wild or cultivated Poaceae. In this current study, GGWL phytoplasma was detected in Mbita and Tarime in two wild grasses namely *B. brizantha* and *S. pyramidalis*. The *B. brizantha* infected plants were white with reduced leaf size and stunted while the phytoplasma infected *S. pyramidalis* plant showed no symptoms. This phytoplasma has been reported previously in Myanmar in *Eleusine indica* (goose grass) showing similar disease symptoms like in *B. brizantha* including white small leaves, bushy growth habit, stunting and death of plants (Win *et al*., 2013b). The phytoplasma was shown to be distantly related to sugarcane white leaf (SCWL) phytoplasma, which belongs to the 16SrXI group or and other phytoplasma agents in the 16SrXIV group, but was closely related to sorghum grassy shoot phytoplasma (SGS) '*Ca*. Phytoplasma oryzae'. This study showed that GGWL phytoplasma possessed other distinct features and therefore could not be identified into a

previously described 16Sr groups (Win *et al.*, 2013b). The current study has revealed similar findings and shown that GGWL found in symptomless and symptomatic plant species in East Africa possess similar features as that found in Asia.

Apart from a few species, most of the wild grass hosts from which phytoplasmas were detected were indistinguishable from healthy conspecific grasses. However, phytoplasma-infected *C. dactylon* plants found in western Kenya, northern Tanzania and eastern Uganda showed stunting with small white leaves as observed in western Kenya (Obura *et al.*, 2010). Likewise, HGWL infected *H. rufa* had similar symptoms as those reported in the previous study in Kenya displaying stunted growth with white leaves. This phytoplasma was named HGWL to depict the plant species from which it was detected (Obura *et al.*, 2011). The findings from the current study confirm earlier reports that symptoms alone are not always reliable indicators of phytoplasma presence or identity in plant hosts (Blanche *et al.*, 2003; Bertaccini *et al.*, 2005; Seemüller and Schneider, 2007). The inability to recognize a symptomless plant harbouring a phytoplasma could also result in inadvertent exposure of cultivated plants in East Africa to a potential inoculum source as has been suggested before in Australia (Blanche *et al.*, 2003).

Besides, detecting phytoplasmas in symptomless plants (Blanche *et al.*, 2003; Bertaccini *et al.*, 2005; Seemüller & Schneider, 2007), the location of a phytoplasma in a plant may vary over time (Blanche *et al.* 2003). For example, Blanche *et al.* (2003) showed variation in the occurrence of SGS phytoplasma in different parts of *Whiteochloa cymbiformis* and *Sorghum stipoideum* by searching for the phytoplasma at different times of the year. Therefore, it is possible that the location of phytoplasmas in wild grass species identified here is also variable, within plants and over time. It also shows that the potential reservoir of phytoplasmas in East African grasses may be larger than these surveys suggest. The movement, distribution and colonization of phytoplasmas in plants are theoretically systemic, however, in reality it is not. Particularly grasses can grow quickly in a short time and negative PCR results might often be encountered when fresh leaf samples are taken because they have not yet been colonised. It might therefore be useful in future studies to examine other parts of the plant including stems, shoot apex and roots as have been

examined in other phytoplasma-infected plants (Wei *et al.*, 2004; Saracco *et al.*, 2006) to indicate more reliable locations for phytoplasma detection.

Several studies have shown the role of wild grasses and weeds as alternative phytoplasma hosts and implicated them in disease epidemiology (Blanche *et al.*, 2003; Arocha *et al.*, 2009; Obura *et al.*, 2010, 2011). This may be true, as many wild host plants may remain free from obvious phytoplasma disease symptoms due to co-evolution with their phytoplasmas (Caudwell, 1983). However, a new phytoplasma disease may emerge directly when recently introduced plant becomes a suitable feeding host for a naturally occurring vector or indirectly when a new competent vector is introduced into a habitat where both the natural and the cultivated host exist. This therefore may lead to disease outbreaks in cultivated crops enabling the phytoplasma to exploit a new ecological niche. In such cases, genetically distinct phytoplasma strains may result if the secondary epidemiological cycle is isolated from the original system (Caudwell, 1983; Lee *et al.*, 1998). Therefore, the current findings are not only informative, but suggest that these wild grass hosts are important and could be acting as wild sources of inocula for nearby fields planted with Napier grass, cultivated crops and other monocots. An introduction of a competent vector into the habitat therefore may initiate a three-way phytoplasma epidemiological system (Lee *et al.*, 2003; Obura *et al.*, 2009) resulting in the emergence of phytoplasma diseases with significant impacts on both wild and cultivated plants in the East African region.

While some wild grasses host only one strain of phytoplasma, others host multiple strains. For instance, two phytoplasmas were found in *C. dactylon*, *E. indica*, *H. rufa* and *S. pyramidalis* while three of them were detected in *B. brizantha*. However, there was no indication of multiple simultaneous infections. It is likely that double or multiple infections also occur in the plants examined. However, they could not be detected by the methods employed. Additionally, NGS-related phytoplasmas were detected in ten different plant species, BGWL in three plant species and GGWL in two plant species. This is possible since the specificity of the phytoplasma for the insect host is usually stricter compared to the plant host ranges, which are usually broader. The wild grasses identified here as phytoplasma hosts often grow copiously in fields bordering those planted with Napier grass. However, phytoplasmas that are

closely related to BGWL and GGWL have not yet been transmitted to Napier grass. Similar findings have been reported in Australia where SCWL was not found in sugarcane plants tested (Blanche *et al.*, 2003) despite wild host plants that occurred nearby. Because these phytoplasmas were not detected in Napier grass collected, the infection of wild grasses with the 16SrXIV or GGWL phytoplasma may involve another, as yet unknown vector, suggesting their disassociation with NGS. While, the vector responsible for the transmission of NGS from phytoplasma-infected Napier grass to a healthy plant was reported recently in Kenya (Obura *et al.*, 2009), there is no record of the vectors responsible for the transmission of phytoplasmas in many wild grasses reported in this study. In addition, *M. banda* failed to transmit HGWL and BGWL phytoplasmas to healthy Napier grass plants and survived on their healthy hosts just for a few days indicating that it may be a sole transmitter of the NGS. The preference of *M. banda* for Napier grass could also explain the lack of transmission to other grasses in these experiments. Despite repeated agitation, the insects could move back to the healthy Napier grass before transmission or acquisition could have occurred. The leafhopper *M. banda* has been reported as the most abundant insects occurring on Napier grass in the western part of Kenya, supporting its role in the transmission of NGS in the region (Koji *et al.*, 2012) but its role as a vector of HGWL and BGWL has not been identified.

However, phytoplasma disease transmission is probably through sap-sucking insect vectors mostly by leafhoppers and planthoppers as with other plant species (Weintraub & Beanland, 2006). Therefore, further studies are needed to identify insect vectors and examine the likelihood of phytoplasma transmission to wild and cultivated monocots. For instance, Salehi et al. (2009) collected four leafhopper and one planthopper species on white leaf-affected Bermuda grass, and showed the leafhopper *Exitianus capicola* Stål (Hemiptera: Cicadellidae) as the only natural and experimental vector of the BGWL agent in Iran. Even though the other insects were incubated with the phytoplasma-infected Bermuda grass for a period of up to four months, they did not acquire nor did they transmit the disease to healthy plants. Similar results were reported by Obura et al. (2009), when they tested the NGS-phytoplasma transmission ability of five leafhopper and three planthopper species and identified the leafhopper *M. banda* as a vector of NGS-phytoplasma in Kenya. This shows the importance of host-specificity. Phytoplasmas differ with respect to

host specificity, which is usually higher for vectors than for the plant hosts (Hogenhout *et al.*, 2008; Lee *et al.*, 1998). For example, whereas phytoplasmas of the aster yellows (16SrI) phylogenetic group infect various plant species and are transmitted by a wide range of leafhopper species, phytoplasmas of the apple proliferation group (16SrX) are restricted to only one or a few closely related plant hosts and vectors (Lee *et al.*, 1998). The reasons for plant host specificity are still not completely understood, but the existence of resistant plant taxa and the variation of phytoplasma titres in different host plants indicate that plant susceptibility is not only a question of successful inoculation by vectors but also a result of complex phytoplasma-plant host interactions. Therefore, phytoplasmas of different phylogenetic groups show considerable differences with respect to their plant host range and their vector specificity (Weintraub & Beanland, 2006; Hogenhout *et al.*, 2008).

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Chapter 5: Phylogenetic analysis of Napier grass stunt (NGS) and *Hyparrhenia* grass white leaf (HGWL) phytoplasmas based on the *secA* and immunodominant protein (*imp*) gene

Abstract

Phytoplasma classification has focused primarily on ribosomal sequences with the 16S rRNA gene being the most important. In phytoplasmas, this gene is highly conserved and present in two, sometimes slightly different copies. The conservativeness of the gene poses a challenge especially for defining sub-groups within a species or closely related phytoplasmas not yet validly described. In East Africa, phytoplasmas associated with Napier grass stunt (NGS) and *Hyparrhenia* grass white leaf (HGWL) diseases are strains of '*Candidatus* Phytoplasma oryzae' classified in the 16SrXI group which share 99% 16S rDNA sequence identity. To examine the genetic divergence of NGS and HGWL, other more variable genes were explored. The less-conserved genes *secA* and the immunodominant membrane protein (*imp*) were selected for differentiation. New sets of primers to amplify the fragments were derived from database entries and fragments obtained were sequenced. The information was used to conduct an alternative phylogenetic analysis for these two related phytoplasmas and to provide insights into their geographical contribution. Phytoplasmas used in this study were obtained from our previous work from phytoplasma-infected Napier grass (*Pennisetum purpureum*), and Rhodes (*Chloris gayana*) and thatch (*Hyparrhenia rufa*) grasses collected in Kenya, Uganda and Tanzania. The presence of phytoplasma in the samples was confirmed by using a combination of the 16S rDNA primer P1 and the intergenic spacer region (ISR) primer Tint. Phylogenetic analysis was carried out by using *secA* gene primers designed previously and the *imp* primers designed by aligning *imp* gene sequences with other phytoplasma sequences available in the database. The results showed that the two phytoplasmas were closely related with very low genetic diversity. The two gene sequences produced a phylogenetic tree congruent to that produced previously by the 16S rDNA sequences, thereby supporting the inclusion of HGWL in the 16SrXI group, '*Candidatus* Phytoplasma oryzae'. The close relationship between HGWL and NGS also raises the possibility of the transmission of phytoplasma from thatch to Napier grass in the future with a devastating effect.

Keywords: Napier, thatch grass, phytoplasma, East Africa, NGS, HGWL, *secA*, *imp.*

5.1 Introduction

Phytoplasmas are important insect transmitted bacterial pathogens, which cause diseases, many with high economic impact in hundreds of plant species (Lee *et al.*, 2000; Weintraub & Beanland, 2006). Globally, the family Poaceae has the largest number of plant species associated with phytoplasmas while majority of their vector species belong to the hemipteran family Delphacidae (Arocha & Jones, 2010). Phytoplasmas have a simple structure and cannot be distinguished morphologically. They are wall-less and surrounded by a single unit membrane. During their evolution, they suffered extreme genome reductions compared to their Gram-positive relatives like *Clostridium* or *Lactobacillus* spp., most likely because of their parasitic lifestyle (Bertaccini, 2007; Hogenhout *et al.*, 2008). Phytoplasmas have a small genome that ranges from 530 to 1350 kb and a low guanine plus cytosine (G+C) content ranging from 21.4 to 29.5 mol% (IRPCM, 2004).

Due to the inability to grow phytoplasmas *in vitro,* they were poorly characterized until the advent of molecular biology. Therefore, it was also not possible to apply the traditional taxonomic criteria, based on phenotypic and biochemical characters (Lee *et al.*, 2000). Lately, ribosomal (r) DNA gene sequencing has provided evidence that these plant-pathogenic prokaryotes, closely related to acholeplasmas, spiroplasmas and mycoplasmas constitute a large unique monophyletic cluster within the class *Mollicutes*. Together with *Acholeplasma* species, they form the family *Acholeplasmataceae* within the order *Acholeplasmatales*. The genus name 'Phytoplasma' (IRPCM, 2004) was proposed for these plant pathogenic organisms to replace the provisional name mycoplasma-like organisms or MLO (Doi *et al.*, 1967). The new taxon '*Candidatus* (*Ca.*)' Phytoplasma', was adopted by the Phytoplasma Working Team of the International Research Project for Comparative Mycoplasmology (IRPCM), to identify and classify non-helical prokaryotes causing plant diseases vectored by phloem feeding insects (IRPCM, 2004).

The IRPCM team also established the rules for defining new phytoplasma species. This system of phytoplasma classification is defined primarily on the basis that strains within a candidate species share at least 97.5% sequence identity within their

16S rDNA gene sequences. In addition, organisms with more than 97.5% identity can be described as distinct species when vectors, host plants or ecological niches differ significantly (IRPCM, 2004). For rapid classification, amplified 16S rDNA PCR products are usually digested by enzymes such as *Alu*I, *Hae*III or *Rsa*I and then separated by polyacrylamide gel electrophoresis giving characteristic restriction fragment length polymorphism (RFLP) patterns (Lee *et al.*, 1998). The parameter is now commonly used for identification of the phytoplasma-associated plant diseases and new phytoplasma groups are continuously being published. Up to date 37 '*Ca.* Phytoplasma' species have been validly described based on the 16S ribosomal sequence data (IRPCM, 2004; Win *et al.*, 2013; Harrison *et al.* 2014).

In phytoplasmas, the 16S rDNA gene exists in two copies and has no coding function. The gene is highly conserved among these plant pathogenic agents but sometimes the copies differ slightly (Liefting *et al.*, 1996). This therefore presents a challenge in the characterization of phytoplasmas especially in defining sub-groups within a phytoplasma group (Streten & Gibb, 2005). Apart from the 16S sequences, additional conserved markers can be used as supplemental tools for finer phytoplasma differentiation (Bertaccini, 2007). Classification using the 23S rDNA gene sequences is useful but the gene appears to be similarly conserved producing phylogenetic trees similar to those obtained using the 16S gene (Bertaccini, 2007). Use of the 16S–23S intergenic spacer region (ISR) shows more sequence variation than 16Sr RNA gene. However, besides the conserved tRNA-ile the spacer region comprises only about 160 bases for comparison. Phylogenetic trees generated based on this region are also poorly supported by bootstrap values and place certain isolates in wrong groups (Hodgetts *et al.*, 2008).

Other less conserved genes of the phytoplasma genome have also been exploited as additional tools to satisfactorily discriminate between and among phytoplasmas. The *tuf* gene, encoding the elongation factor Tu (EF-Tu) is a well-conserved gene with a central role in translation (Schneider *et al.*, 1997). The 16SrI ('*Ca.* Phytoplasma asteris'-related) group has been sub-divided using *tuf* gene, ribosomal protein (*rp*) gene and the 16S–23S rRNA intergenic spacer region (16–23S ISR) (Botti & Bertaccini, 2003), along with the *secY* gene (Lee *et al.*, 2006). The *tuf* gene and *rp* operon have been used to support the sub-groups in the 16SrXII group

(Streten & Gibb, 2005). Martini *et al*. (2007) used primers that amplified a larger fragment of the *rp* operon from a wide range of phytoplasmas which produced a tree similar to those derived from the 16S gene sequences but subdivided the 16S groups into more distinct subclades. Construction of a phylogenetic tree with a high resolution was also achieved from different phytoplasma groups using a fragment of the *secY* gene (Lee *et al*., 2010). Recently, Makarova *et al*. (2012) developed a barcode system for '*Ca*. Phytoplasma' identification using *tuf* gene sequences. Phylogenetic analysis from this gene and the 16S rDNA alignments showed remarkable similarity in terminal taxa, implying that the *tuf* barcode is well linked to the existing 16S rDNA phytoplasma phylogeny. Hodgetts *et al*. (2008) using *secA* gene sequences provided an improved resolution of groups and subgroups from a wide range of the 16S groups. The *secA* fragment also emerged as a promising marker for universal identification of phytoplasmas.

Phytoplasmas associated with Napier grass stunt (NGS) disease in Napier grass (*Pennisetum purpureum* Schumach) in Kenya, Tanzania and Uganda are classified in the 16SrXI group and are strains of '*Ca*. Phytoplasma oryzae' (Jones *et al*., 2004; Nielsen *et al*., 2007). *Hyparrhenia* grass white leaf (HGWL) disease has also been detected in thatch grass (*Hyparrhenia rufa*) in Kenya and Tanzania and found to induce symptoms similar to those found in NGS-infected plants. Sequence analysis revealed highest 16SrDNA sequence identity (99%) of the HGWL phytoplasma with that of the NGS phytoplasma, confirming HGWL as a member of the 16SrXI phytoplasma group (Obura *et al*., 2011). Other phytoplasmas closely related to NGS have also been reported in the region and pose a great threat to the cultivation of Napier grass. Despite their relatedness, HGWL and NGS have only been described based on the 16S rDNA sequences. Therefore, the aim of the study was to compare and test the identity of the NGS and HGWL phytoplasmas and their phylogenetic relation using alternative markers based on the *secA* gene and the immunodominant membrane protein (*imp*).

5.2 Materials and methods

5.2.1 Phytoplasma strains and nucleic acid preparation

The phytoplasmas used in this study were obtained from our previous work from infected *P. purpureum*, infected *H. rufa* and other plants with phytoplasmas belonging to the 16SrXI group (Table 5.1). Total DNA was extracted from leaf samples using the CTAB extraction method according to Doyle and Doyle (1990). Leaf samples weighing approximately 0.3 g were powdered into liquid nitrogen and mixed with 600µl of preheated CTAB buffer (65˚C) (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris–HCl, pH8.0, 0.2% 2-mercapto-ethanol). DNA was extracted with one volume of chloroform: isoamyl alcohol (24:1). After centrifugation at 13000rpm for 10 minutes, the supernatant was transferred to a new microcentrifuge tube, mixed with an equal volume of isopropanol and precipitated overnight at -20°C. This was followed by centrifugation at 13,000 rpm and rinsing of the DNA pellet in 70% Ethanol. The DNA was air dried at room temperature and dissolved in 50µl Tris-EDTA (10 mM Tris, 1 mM EDTA; pH8.0) buffer. The quality of isolated DNA was verified using 1% (w/v) agarose gel and quantified using a spectrophotometer. All plant samples are listed in Table 5.1.

Table 5.1: Grass samples used for the study

Grass species	Common name	Country	Phytoplasma strain*	No. positive/ No. tested
Brachiaria brizantha	Signal grass	Kenya, Tanzania	GGWL	4/6
Chloris gayana	Rhodes grass	Kenya	NGS-related	2/2
Cynodon dactylon	Bermuda	Kenya, Uganda	BGWL	19/32
Digitaria scalarum	Couch grass	Kenya	NGS-related	1/1
Enteropogon macrostachyus	Bush rye	Kenya	NGS-related	2/2
Hyparrhenia rufa	Thatch grass	Kenya, Tanzania	HGWL	13/20
Pennisetum purpureum	Napier grass	Kenya, Uganda, Tanzania	NGS	9/31
Sporobolus pyramidalis	Dropseed grass	Tanzania	GGWL	3/4

*Phytoplasma strain name is according to the plant host. NGS = Napier grass stunt, HGWL = Hyparrhenia grass white leaf, BGWL = Bermuda grass white leaf.

5.2.2 Preparation of bacterial growth medium (LB)

Luria broth (LB) is a nutrient-rich medium used to culture bacteria in the laboratory. The broth (1L) was prepared by mixing 10g NaCl, 10g tryptone, 5g yeast extract. For preparation of solid medium 15g agar was added. The preparation was mixed by swirling and then autoclaved for 20 minutes. The agar solution was then cooled to

55°C in a water bath. Ampicillin (50µg/ml), X-gal (40mg) and 100 µM IPTG were added to the agar solution and swirled gently. Approximately 25mL of LB agar was poured on a 10cm polystyrene Petri dish under a lamina flow hood under sterile conditions. The lids were placed on the plates and then allowed to cool for 30-60 minutes until solidified. The plates were inverted and kept at 4°C for future use.

5.2.3 Phytoplasma DNA amplification and cloning

PCR was performed in a 25 µl final reaction mixture containing 20ng of genomic DNA, 1X PCR buffer supplied with magnesium (KAPA Biosystems, Germany), 100µM dNTPs, 1.0 unit *Taq* DNA polymerase (KAPA Biosystems, Germany) and 100nM of each primer. The PCR reactions were carried out using a Bio-Rad iCycler as follows: denaturation at 94 ºC for 4 min for 1 cycle; 35 cycles of 94 ºC for 30 sec, 52 ºC for 1 min and 72 ºC for 90 sec; and a final elongation at 72 ºC for 5 min for P1/Tint. Denaturation at 94 ºC for 4 min for 1 cycle; 35 cycles of 94 ºC for 30 sec, 53 ºC for 1 min and 72 ºC for 90 sec and a final extension at 72 ºC for 15 min for SecA primers. Denaturation at 94 ºC for 4 min for 1 cycle; 35 cycles of 94 ºC for 30 sec, 52 ºC for 30 sec and 72 ºC for 45 sec and a final extension at 72 ºC for 15 min for F_NGS/R_NGS. All primers used are listed in Table 5.2.

Table 5.2: Sequences of the oligonucleotide primers used for PCR amplification in wild grasses and sequencing

Primer	Sequences (5' to 3')	Location[b]	References
P1	aagagtttgatcctggctcaggatt	16S	Deng & Hiruki, 1991
Tint-short	tcaggcgtgtgctctaaccaac	SR	Smart *et al.*, 1996
NGSsecfor1	tatacwacwaatagtgaaatwgg´	*SecA*	Bekele *et al.*, 2011
NGSsecfor2	cgatgaagtwgattctgtc	*SecA*	Bekele *et al.*, 2011
NGSsecrev1	gataagtaatagtagcagcaatttcag	*SecA*	Bekele *et al.*, 2011
NGSsecrev2	agcttctaaagcttgatgtaatcc	*SecA*	Bekele *et al.*, 2011
F_NGS	atgcaaaatgaaaatttttgg	*imp*	Wambua, unpublished
R_NGS	tttaatatttaaatcagattttaaagcag	*imp*	Wambua, unpublished

[b]Location of primer within the rRNA operon, i.e., within either the 16S rRNA gene, the 16S–23S intergenic spacer region (ISR), *secA* or *imp* of the phytoplasma.

The PCR products were visualized on a 1% (w/v) agarose gel stained with ethidium bromide using 1xTAE (40mM Tris acetate, 1mM EDTA; pH8.0) as a running buffer, and photographed. The amplicons were directly purified using a QIAquick® PCR

purification kit (Qiagen, Inc) according to the manufacturer's instruction. The purified PCR products were then ligated using the pGEM-T easy vector system (Promega) following the manufacturer's instructions in a 10 µl final reaction mixture containing 5 µl of the rapid ligation buffer (2X), 0.5 µl of pGEM®-T Easy Vector (50ng) and 1 µl of the T4 DNA Ligase (3 Weiss units/µl). The reaction mixture was incubated overnight at 4 ºC for the maximum number of transformants. The ligation mixture was desalted against water for 1 hour on a VSWP membrane filter (Merck Millipore Ltd).

The desalted DNA was pipetted carefully from the membrane filters into a sterile 0.5 ml microcentrifuge tube. High efficient competent cells (*Escherichia coli* NM522 strain - Promega) previously frozen at -80 ºC were thawed and then mixed with the DNA by flicking the tube. The mixture of cells and the DNA was transferred into a pre-chilled 0.2 cm electoporation cuvette and the suspension tapped to the bottom. The cuvette was then placed in the shock-pod and closed by pushing the chamber lid down. The mixture of cells and DNA was pulsed once at 2.5 kV using a BIO-RAD Gene Pulser™. The cuvette was then removed from the chamber and the mixture was resuspended immediately in 0.5 ml SOC solution to increase recovery of *E. coli* transformants. The cell suspension was transferred into a 5 ml polypropylene tube and then incubated for 1 hour at 37 ºC with shaking at 130 rpm. The transformation culture was plated in three different quantities of 1 µl, 10 µl and 100 µl to LB agar plates containing ampicillin, IPTG and X-Gal. The plates were then incubated overnight at 37 ºC. The white clones were selected and the presence of inserts verified by PCR as illustrated above. The insert containing clones were picked by sterile micropipette tips placed into universal tubes with 5ml LB plus ampicillin (final concentration 100 µg/ml) media, and then grown overnight at 37 ºC at 230 rpm.

5.2.4 Sequence and phylogenetic analysis

Plasmid DNA was purified from positive clones using EasyPrep® Pro Plasmid Miniprep Kit (Biozym, Germany) according to the manufacturer's instruction. The *imp* and *secA* inserts were sequenced using M13 primers in a DNA automatic sequencer at Eurofins Genomics, Germany. DNA sequences were assembled and edited using DNA Workbench (CLC bio, Aarhus, Denmark) software. The *imp* and *secA* gene sequences were aligned using a progressive alignment algorithm (Feng & Doolittle, 1987) implemented in the DNA Workbench package (CLC bio, Aarhus, Denmark)

with default settings. The alignments were then exported in fasta format, converted to MEGA 6 format and used for distance and phylogenetic analyses in MEGA 6 software (Tamura *et al*., 2013). Average intra and inter-group evolutionary divergences were calculated using the Maximum Composite Likelihood model (Tamura *et al*., 2004). BLAST searches were performed at the NCBI website (http://www.ncbi.nlm.nih.gov/). A dendrogram was then constructed using Neighbour-Joining method as illustrated by Saitou & Nei (1987) using a 1000 replicates for bootstrap analysis (Felsenstein, 1985). *imp* and *secA* gene sequences of phytoplasma infecting other plants for comparison were obtained from the GenBank database (Table 5.3). The *imp* sequence of a reference NGS phytoplasma strain maintained at ICIPE Mbita station was used for comparison (sequence kindly provided by Dr. Lillian Wambua, unpublished).

5.3. Results

5.3.1 PCR-amplification of phytoplasma DNA from infected plants

A total of 98 grass samples were tested comprising 59 samples from Mbita and Bungoma districts of western Kenya, nine from Tarime district of northern Tanzania and 30 grass samples from Bugiri and Busia districts of eastern Uganda (Table 5.1). Phytoplasma infection in the grass samples was confirmed by the amplification of a 1600 bp sized fragment (Fig. 5.1) comprising the 16S rDNA and part of the ISR using P1/Tint primers (Deng & Hiruki, 1991; Smart *et al*., 1996) from 53 out of 98 grass samples tested (Table 1). Of the samples collected in Kenya, seven out of 16 *P. purpureum*, 12 of 17 *H. rufa*, 2 of 2 *E. macrostachyus*, 1 of 1 *D. scalarum*, 2 of 2 *C. gayana*, 14 of 16 *C. dactylon* and 2 of 4 *B. brizantha* tested positive for phytoplasma. In the Tarime district of Tanzania, phytoplasmas were detected in 3 of 4 of *S. pyramidalis*, 2 of 2 *B. brizantha* and 1 of 3 of *H. rufa*. Among the samples collected in Bugiri and Busia districts of eastern Uganda, 2 of 15 of *P. purpureum* and 5 of 15 of *C. dactylon* tested positive for phytoplasma.

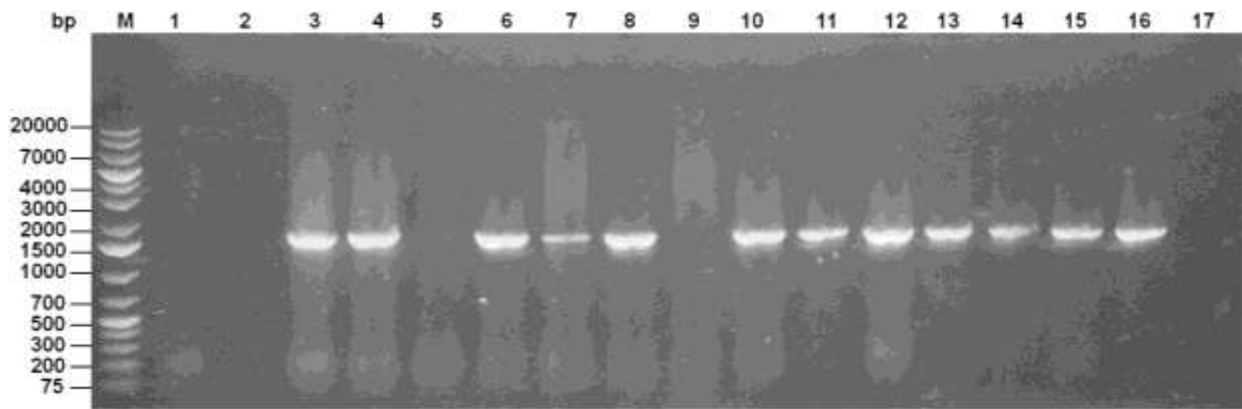

Figure 5.1: Electropherogram of PCR products comprising the 16S rDNA and intergenic spacer region amplified with P1/Tint primers. M: 1 kb plus DNA marker (Thermo Scientific, CA); lane 1-15: Samples from symptomatic Napier and thatch plants, 16: Reference phytoplasma (NGS), 17: negative control (water).

DNA of the 53 phytoplasma-positive samples confirmed using P1/Tint primers were then subjected to PCR amplification using primers based on phytoplasma-specific fragments of the *secA* and *imp* gene. For *secA* gene analysis a phytoplasma-specific 400 bp fragment (Fig. 5.2) was amplified from leaves of the 53 grass samples in a nested PCR assay using primers NGSsecfor1 and NGSsecrev1 in the first round followed by NGSsecfor2 and NGSsecrev2 primers in the second PCR reaction. PCR fragments of about 500 bp were already amplified in the first round using primers NGSsecfor1/NGSsecrev1 from leaves of infected Napier grass but not from other phytoplasma-infected grasses. For the *imp* gene analysis, a phytoplasma-specific 500 bp fragment was amplified from all the 53 grass samples (Fig. 5.3).

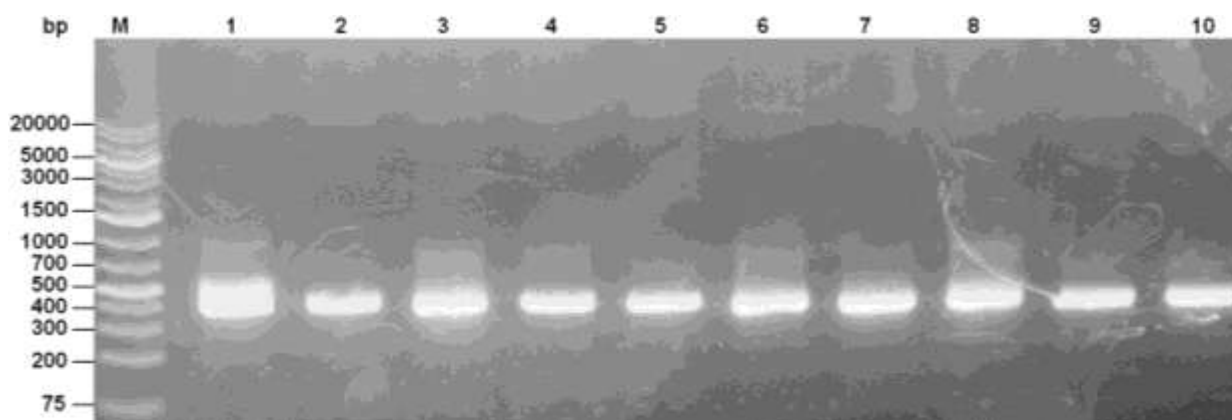

Figure 5.2: Electropherogram of *secA* gene-PCR products amplified in a nested-PCR using NGSsecfor2/ NGSsecrev/2 primers from grass samples. M: 1 kb plus DNA marker (Thermo Scientific, CA).

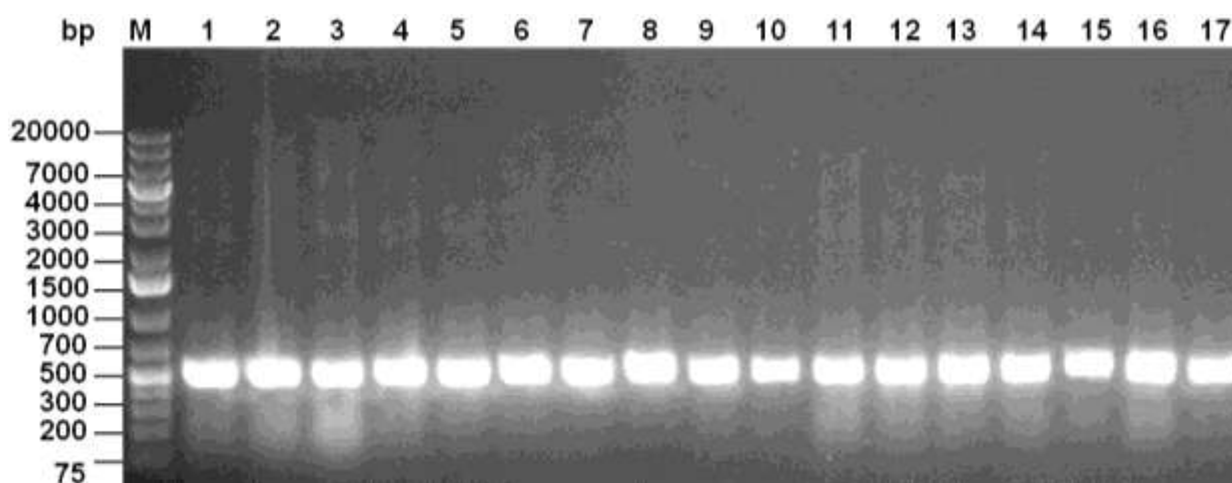

Figure 5.3: Electropherogram of *imp* gene-PCR products amplified using F-NGS/R-NGS primers from grass samples. M: 1 kb plus DNA marker (Thermo Scientific, CA).

5.3.2 Sequence and phylogenetic analysis of phytoplasmas based on *imp* gene sequences

Following amplification of the phytoplasma DNA, *imp* gene sequence analysis was conducted on PCR fragments from 12 grass samples comprising five *P. purpureum*, five *H. rufa* and two *C. gayana* accessions. Of the five *P. purpureum* samples, three were collected in Kenya and two were from Uganda. Four of the *H. rufa* samples were from Kenya and one was collected from Tanzania while all the *C. gayana*

samples were from Kenya. Multiple alignment of the sequences from *imp* gene showed that the 12 phytoplasma sequences were nearly identical (Fig. 5.4). Out of the 472 base pairs (bp) of the amplified *imp* gene fragment, there were only two variable sites at positions 211 and 294 relative to the start codon. In some sequences, the 'C' was changed to a 'T' at position 211 while at the position 294 the 'G' was replaced by an 'A'. Therefore, the *imp* gene sequences from the five phytoplasma-infected Napier grass plants had two nucleotides in difference to the four sequences of phytoplasma-infected thatch grass samples. However, the *imp* gene sequence from one phytoplasma-infected thatch grass (TW384) sample was identical to those from phytoplasma-infected Napier grass samples from Uganda and Kenya. The translated 157 amino acid sequences differed only at position 71 (relative to the start codon) amongst the sequence variants where proline (P) was changed to serine (S). The second nucleotide change did not cause a change in the amino acid sequence.

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      *** ********************************************************************************** **
HR16  ACGCCTGTGTCTAATCAAAAAGTAAGTGCTTTAAAACAAGGTATCAGTGATTCAATAGAAAAAATTAAGAAACATAATGAAGTAGCGTC
HR7   ACGCCTGTGTCTAATCAAAAAGTAAGTGCTTTAAAACAAGGTATCAGTGATTCAATAGAAAAAATTAAGAAACATAATGAAGTAGCGTC
HR3   ACGCCTGTGTCTAATCAAAAAGTAAGTGCTTTAAAACAAGGTATCAGTGATTCAATAGAAAAAATTAAGAAACATAATGAAGTAGCGTC
HR15  ACGCCTGTGTCTAATCAAAAAGTAAGTGCTTTAAAACAAGGTATCAGTGATTCAATAGAAAAAATTAAGAAACATAATGAAGTAGCGTC
NGS   ACGTCTGTGTCTAATCAAAAAGTAAGTGCTTTAAAACAAGGTATCAGTGATTCAATAGAAAAAATTAAGAAACATAATGAAGTAGCATC
TW384 ACGTCTGTGTCTAATCAAAAAGTAAGTGCTTTAAAACAAGGTATCAGTGATTCAATAGAAAAAATTAAGAAACATAATGAAGTAGCATC
NAP14 ACGTCTGTGTCTAATCAAAAAGTAAGTGCTTTAAAACAAGGTATCAGTGATTCAATAGAAAAAATTAAGAAACATAATGAAGTAGCATC
NAP7  ACGTCTGTGTCTAATCAAAAAGTAAGTGCTTTAAAACAAGGTATCAGTGATTCAATAGAAAAAATTAAGAAACATAATGAAGTAGCATC
MAS1  ACGTCTGTGTCTAATCAAAAAGTAAGTGCTTTAAAACAAGGTATCAGTGATTCAATAGAAAAAATTAAGAAACATAATGAAGTAGCATC
UG20  ACGTCTGTGTCTAATCAAAAAGTAAGTGCTTTAAAACAAGGTATCAGTGATTCAATAGAAAAAATTAAGAAACATAATGAAGTAGCATC
UG29  ACGTCTGTGTCTAATCAAAAAGTAAGTGCTTTAAAACAAGGTATCAGTGATTCAATAGAAAAAATTAAGAAACATAATGAAGTAGCATC
CG1   ACGTCTGTGTCTAATCAAAAAGTAAGTGCTTTAAAACAAGGTATCAGTGATTCAATAGAAAAAATTAAGAAACATAATGAAGTAGCATC
CG2   ACGTCTGTGTCTAATCAAAAAGTAAGTGCTTTAAAACAAGGTATCAGTGATTCAATAGAAAAAATTAAGAAACATAATGAAGTAGCATC
```

Figure 5.4: *Imp* nucleotide sequences alignment for the comparison between the phytoplasma accessions showing the variable sites at positions 211 and 294 relative to the start codon indicated by boxes and bold. Asterisks indicate absence of mutation or nucleotide substitutions.

No Blast hit resulted when BLASTN were performed. Therefore, the translated protein query had to be subjected to BLASTP. The *imp* sequences from the accessions had their highest similarity with the *imp* gene sequence reported for '*Ca*. Phytoplasma oryzae' sequence from Japan (GenBank accession BAH24241) sharing a 34% identity.

Table 5.3: Acronyms and GenBank accession numbers of phytoplasma *imp* and *secA* sequences retrieved from the database for phylogenetic analyses

Strain	Phytoplasma	*imp*/ *secA**	16S rRNA group	Host plant	Origin	EMBL Accession#
RYD	Rice yellow dwarf	*imp*	16SrXI	Tsuwabuki	Japan	BAH24241
SCGS	Sugarcane grassy shoot	*secA*	16SrXI	Sugarcane	Sri-Lanka	JF754453
NGS	Napier grass stunt	*secA*	16SrXI	Napier grass	Ethiopia	EU168750
CRW	Coconut root wilt	*secA*	16SrXI-B	Coconut palm	India	JX394030
BGWL	Bermuda grass white leaf	*secA*	16SrXIV	Bermuda grass	Malaysia	FJ755004
AYL	Arecanut yellow leaf	*secA*	16SrXI	Areca palm	India	JX394029
PWY	Periwinkle yellows	*secA*	16SrV	Periwinkle	China	JQ911772
SCGS	Sugarcane grassy shoot	*secA*	16SrXI	Sugarcane	India	KC347005
SCWL	Sugarcane white leaf	*secA*	16SrXI	Sugarcane	India	JF754450
CCLY	Coconut lethal yellowing	*secA*	16SrIV	Coconut	Tanzania	KJ776697
SCGS	Sugarcane grassy shoot	*secA*		Sugarcane	India	DQ459440
BGWL	Bermuda grass white leaf	*secA*	16SrXIV	Bermuda grass	Sri Lanka	JF754454
BGWL	Bermuda grass white leaf	*secA*	16SrXIV	Bermuda grass	Sri Lanka	JF754456

#EMBL Accession number, *accessions available in the databases for *imp* and *secA* gene sequences retrieved for phylogenetic analysis

The phylogenetic analysis based on the nucleic acid *imp* gene sequences divided the phytoplasma sequences in two branches. The four HGWL phytoplasma sequences from Mbita (HR15, HR3, HR7, and HR16) formed a single branch, whereas the five NGS phytoplasma sequences from Kenya (NAP14, NAP7, MAS1) and Uganda (UG20, UG29) together with one HGWL phytoplasma (TW384) from Tanzania formed the second branch (Fig. 5.5). Sequences from the two *C. gayana* accessions also grouped with the sequences from Napier grass accessions.

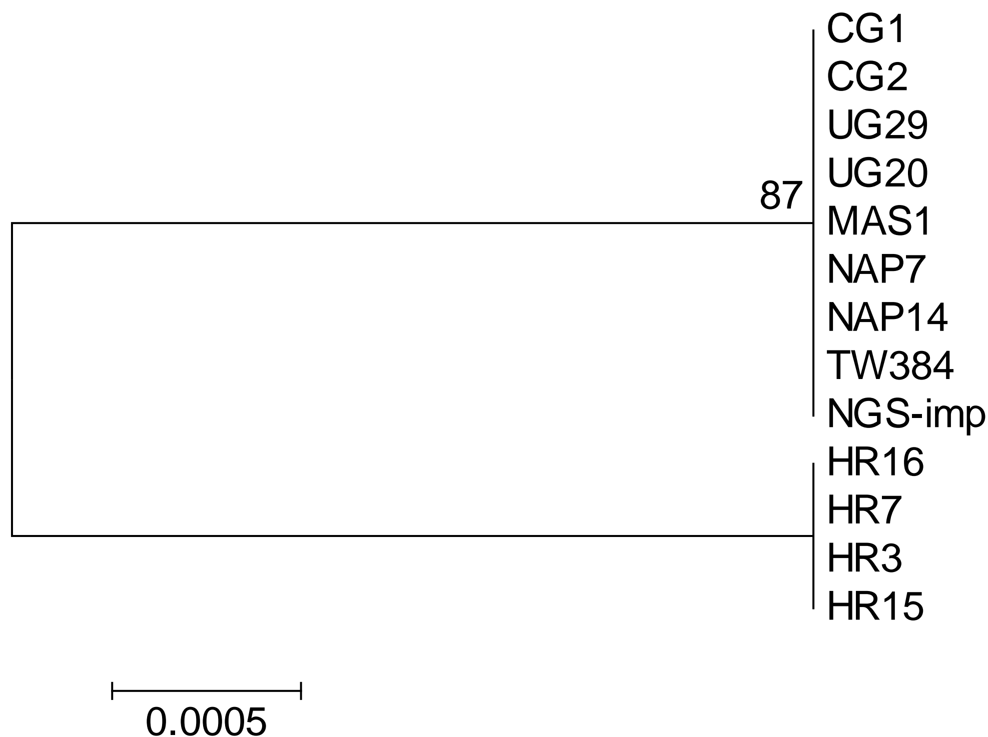

Figure 5.5: Phylogenetic dendrogram of the *imp* gene sequences of phytoplasmas from NGS and HGWL infected plants generated by Mega 6. The evolutionary history was inferred using the Neighbour-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Bootstrap values greater than 50% (expressed as percentages of 1000 replications) are shown.

5.3.3 Sequence and phylogenetic analysis using *secA* gene sequences

Sequence analyses on the *secA* gene fragment were conducted on ten grass samples comprising five *P. purpureum* and five *H. rufa* samples (Fig. 5.5). The *secA* sequences were almost identical differing only in a few nucleotide positions.

```
          ********** *************************************
HR15     ATTCGATGAAGTTGATTCTGTCTTAATAGATGAGGCTAGAACACCTTTAA
HR3      ATTCGATGAAGTTGATTCTGTCTTAATAGATGAGGCTAGAACACCTTTAA
HR7      ATTCGATGAAGTTGATTCTGTCTTAATAGATGAGGCTAGAACACCTTTAA
TW384    ATTCGATGAAGTTGATTCTGTCTTAATAGATGAGGCTAGAACACCTTTAA
HR16     ATTCGATGAAGTAGATTCTGTCTTAATAGATGAGGCTAGAACACCTTTAA
MAS1     TATCGATGAAGTAGATTCTGTCTTAATAGATGAGGCTAGAACACCTTTAA
NAP14    TATCGATGAAGTTGATTCTGTCTTAATAGATGAGGCTAGAACACCTTTAA
NAP7     TATCGATGAAGTTGATTCTGTCTTAATAGATGAGGCTAGAACACCTTTAA
UG20     TATCGATGAAGTTGATTCTGTCTTAATAGATGAGGCTAGAACACCTTTAA
UG29     TATCGATGAAGTTGATTCTGTCTTAATAGATGAGGCTAGAACACCTTTAA
```

Figure 5.5: *secA* nucleotide sequences alignment of the phytoplasma accessions examined. The variable sites are boxed and bold. Asterisks indicate absence of mutation or nucleotide substitutions.

BLASTN showed that *secA* gene sequences from the accessions had their highest similarity with that of the *secA* gene sequence reported for '*Ca*. Phytoplasma oryzae' (GenBank accession EU168750) (Table 5.3) sharing 99% identity. The sequences also shared 87% sequence similarity with sugarcane grassy shoot phytoplasma sequences (GenBank accessions JK54450, JK54451, JK54452, JK54453) and 83% similarity with Bermuda white leaf phytoplasma sequences (JF754454, JF754455, JF54456). However, the sequences were distantly related with the coconut lethal yellowing phytoplasma strain TZ1 (KJ776697) whose country of origin is Tanzania, sharing about 77% sequence identity. *SecA* gene sequences were translated and BLAST search performed. The translated *SecA* sequences differed only in one amino acid position.

A phylogenetic tree using the neighbor-joining method separated the nucleic acid sequence of the phytoplasma accessions from this study into two main branches. One branch consisted of NGS-infected accessions while the other branch had HGWL-infected accessions (Fig. 5.6a). The branch consisting of HGWL-infected accessions was further split into two sub-branches consisting of four accessions on one side and one on the other. Similarly, the branch comprising NGS-infected accessions was also divided in two main branches consisting of four accessions on one side and one on the other. These two samples (HR16 and MAS1) which fell apart from their main branches had an extra variable site at positions 13 relative to the start codon. However, when a phylogenetic tree was constructed using the

GenBank accessions, all the NGS and HGWL accessions formed a single clade together with the GenBank NGS *secA* gene sequence (EU168750) (Fig. 5.6b).

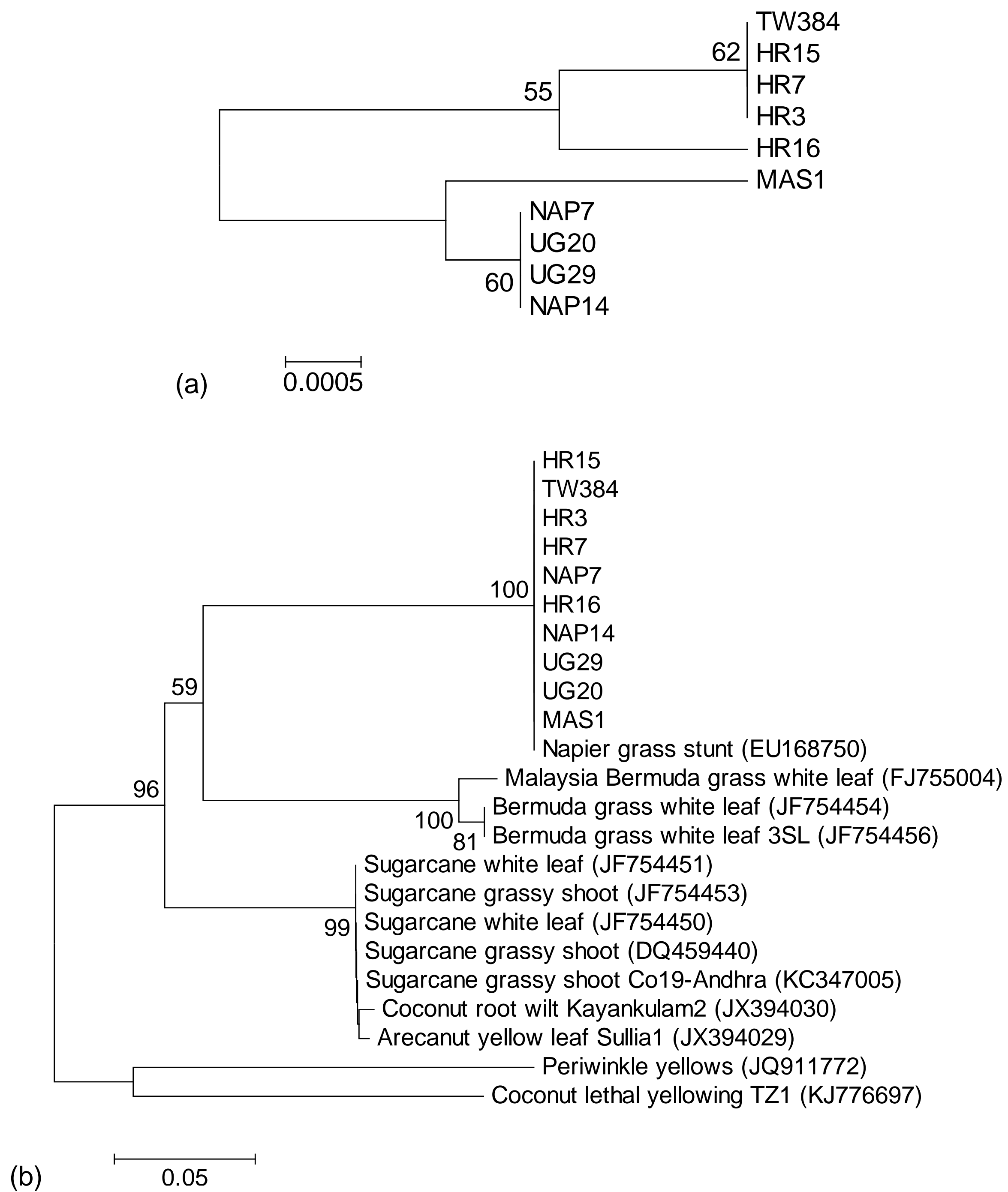

Figure 5.6: a) A dendrogram of the *secA* gene sequences of accessions used in this study. b) Phylogenetic tree computed on the basis of a 400bp *secA* nucleotide gene sequence of 23 phytoplasmas generated by the neighbor-joining method. Bootstrap values were based on 1000 replicates. Phytoplasma names and sequence accession numbers are provided in Table 5.3.

5.4 Discussion

Based on the available 16S rDNA sequences of phytoplasmas from infected Napier- and thatch grass both seem to be closely related (Obura et al. 2011). To examine their relatedness in more detail molecular markers like the *secA* and *imp* gene fragments were investigated. Since little sequence information is available for NGS and HGWL phytoplasmas, primers based on the *imp*- (Wambua, unpublished) and *secA* gene (Bekele *et al.*, 2011), designed previously were used to analyze the phylogenetic relationships between the two phytoplasmas. The results showed that based on the immunodominant protein and *secA* gene sequences, phytoplasmas from the two phytoplasma-infected grasses were closely related and differed by just a few bases. In some cases, using the *imp* gene sequences NGS and HGWL sequences were identical as exemplified for HGWL accession TW384, which was identical to NGS accessions. HGWL has been characterized previously on the 16S rDNA sequence level (Obura *et al.*, 2011). According to the 16S rDNA sequence comparison, HGWL was closely related to NGS phytoplasma and shared a 99% sequence identity. In addition to the 16S rDNA sequences, recent studies based on the sequences generated from the ISR, showed that HGWL was similarly close to NGS phytoplasma and shared up to 98.7% similarity (Asudi *et al.*, 2015). Therefore, results based on sequence information of the *imp* and *secA* genes support these findings and corroborate the close relationship of HGWL and NGS phytoplasmas with a very low diversity between them.

Despite their close similarity, comparison of the two phytoplasmas using *secA* sequences generated a tree that divided the HGWL and NGS phytoplasmas into two separate branches. This comparison also showed a clear separation of HGWL TW384 accession from NGS. Therefore, *secA* gene sequences could be more suited for fine differentiation of these phytoplasmas, although on a very high level.

Phytoplasma taxonomy has been largely based on a single criterion, mainly on ribosomal DNA sequences and to a minor degree on other genes. Other criteria include the host range and the vector specificity (Bertaccini, 2007). Additionally, evidences of significant molecular diversity, either achieved by hybridization to cloned DNA probes, serological reaction or PCR-based assays contribute to the classification (IRPCM, 2004). The little molecular diversity between HGWL and NGS

evidenced by the 16Sr, ISR, *secA* and *imp* gene sequences, indicate that these phytoplasmas belong to the same '*Ca*. Phytoplasma' species.

A comparison of all *secA* gene sequences from HGWL, NGS and other NGS-related phytoplasmas showed a similarity of 99% with the deposited NGS *secA* sequence (EU168750). However, when BLASTP search was carried out, all the *imp* gene sequences from NGS and HGWL accessions showed only 34% identity with the *imp* sequence from '*Ca*. Phytoplasma oryzae', (GenBank accession BAH24241). The reason for the high similarity of the *secA* gene to the '*Ca*. Phytoplasma oryzae' gene and the low similarity of both on the *imp* protein level prompts further research as both groups belong to the same species according to their 16S rDNA homology. Phytoplasmas have single, abundant membrane proteins, which are immunodominant and are located on the external surface of the cell (Milne *et al*., 1995; Keane *et al*., 1996). Since mollicute membrane proteins play an important role in the attachment of the phytoplasma to the host cell, immunodominant membrane proteins (IDPs) are candidates for the involvement in the host–pathogen interactions (Boonrod *et al*., 2012; Neriya *et al*., 2014). When compared within a type, IDPs are highly variable, indicating that they have been subjected to strong divergent selective pressures (Hogenhout *et al*., 2008). Therefore, the *imp* gene could be under much more adaptive to stress than the *secA* gene. The *imp* is most likely in contact with insect and plant proteins and needs to interact with those proteins. Therefore, the conformation has to be adapted for optimal fitness.

HGWL phytoplasma has been reported previously in Kenya (Obura *et al*., 2011) and has been observed in the wild grassland in Tarime in Tanzania and detected in Uganda (Asudi *et al*., 2015). Symptoms observed on HGWL-infected plants include stunting, bushy appearance and whitening of plant leaves. HGWL affects thatch grass, whereas NGS cause economically important lethal disease of Napier grass characterised by similar symptoms that include stunted growth, leaf discolouration and decline (Jones *et al*., 2004; Obura *et al*., 2011).

The plants affected by a certain phytoplasma might largely depend on the host range of the transmitting insect vector. Some phytoplasmas might have a potentially large plant host range but in practice, they are restricted to one or a few, because of feeding preferences of the vector (Lee *et al*., 1998; Weintraub & Beanland, 2006).

There are no reports on the identity of the insect vector responsible for the transmission of HGWL. However, the leafhopper *Maiestas banda* (Kramer) (Hemiptera: Cicadellidae) has been identified recently as a vector capable of transmitting NGS from infected Napier grass plants to healthy plants (Obura *et al*., 2009). The same vector has been tested for transmission of the HGWL phytoplasma from infected thatch grasses to Napier grass. However, no significant results have been achieved suggesting the possibility of the existence of a different vector in the wild. Although, the number of transmission trials was high, the failure of transmission may have been due to either the lack of acquisition by or multiplication of the HGWL phytoplasma in *M. banda* (Asudi *et al*., unpublished, Chapter 4).

Use of 16S rDNA sequences remain the most important tool for identifying and characterizing phytoplasmas and to date several hundreds of phytoplasma 16S rDNA have been sequenced (Bertaccini, 2007; Lee *et al*., 2007). However, the tool does not provide clear resolution of all the 16Sr groups and usually does not resolve subgroups clearly without the use of a wide range of restriction endonucleases (Wei *et al*., 2007). Therefore, additional markers have been developed to distinguish the various 16Sr groups and subgroups and to support the classification of phytoplasmas in the 16Sr groups (Bertaccini, 2007; Hodgetts *et al*., 2008). Comparison of the *imp* and *secA* gene sequences between HGWL and NGS phytoplasma accessions using multiple sequence analysis showed a very low genetic diversity. The use of *imp* and *secA* gene sequences therefore affirm the resemblances of these two phytoplasmas and suggest that there is the possibility of the transmission of HWGL phytoplasma to Napier grass in the future with a potentially devastating effect. This also emphasises the need to find the vector of HGWL and conduct future transmission experiments.

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Chapter 6: The significance of Napier grass stunt phytoplasma and its transmission by the leafhopper species *Maiestas banda* (Hemiptera: Cicadellidae) to cereals and sugarcane

Abstract

Production of Napier grass, *Pennisetum purpureum*, the most important livestock crop in East Africa is severely constrained by Napier Grass Stunt (NGS) disease. The disease is spread via an insect vector or through vegetative propagation of infected plant material. Wild grasses and weeds have been identified as alternative hosts to NGS but there is no record regarding the ability of this phytoplasma to infect cultivated food crops in the family Poaceae. However, since many food crops are susceptible to phytoplasma infections, it is possible that they could be infected by NGS phytoplasma and act as reservoir, assisting its spread in the regions where these crops are cultivated. Therefore, the aim of this study was to assess the transmission of NGS phytoplasma to food crops using a vector and to establish any threats posed by the phytoplasma to these crops. Five food crop plant species, i.e. *Eleusine coracana*, *Sorghum bicolor*, *Oryza sativa*, *Zea mays* and *Saccharum officinarum* were used in the study. Napier grass (Bana variety), susceptible to NGS disease, was included as a control treatment. Polymerase chain reaction (PCR), based on the highly conserved 16S gene, primed by P1/P6-NapF/NapR nested primer sets was used to diagnose phytoplasma on test plants, before and after transmission experiments. Food crop plants were placed adjacent to phytoplasma-infected Napier grasses with the vector *Maiestas banda* (Hemiptera: Cicadellidae) in cages for a period of 30 days. The crop plants were then observed for another three months after removal from the cages for disease development. With 81.3%, *P. purpureum* showed the highest infection level followed by *S. officinarum* with 56.3%, *E. coracana* with 50%, *S. bicolor* with 43.8%, *O. sativa* with 31.3% and *Z. mays* with 18.8%. However, all the test crop plants remained symptomless except sugarcane plants, which exhibited mild to moderate symptoms consisting of foliar yellow leaves and bright white or yellow midribs. Napier grasses, however, had yellow leaves and were stunted. The study showed that food crops cannot only be infected but may also serve as source of inoculum for this phytoplasma. It suggests that there could

exist a complex epidemiological system for NGS disease and also highlights the challenges to development of the disease control strategies.

Keywords: Napier grass stunt, phytoplasma, *Maiestas banda*, trasnmission, threat, food crops.

6.1 Introduction

Phytoplasma infections restrain the production of many important plants including grasses, vegetables, fruits, trees and ornamentals from temperate to tropical regions worldwide leading to phytosanitary constraints and considerable losses for world economies. These wall-less bacteria cause diseases in plant species belonging to more than 700 plant species (Lee *et al.*, 2000; IRPCM, 2004). Of these, phytoplasmas infecting plant species in the family Poaceae are diverse and strains belonging to five 16Sr '*Candidatus* (*Ca.*) Phytoplasma' groups namely 16SrI, III, XI, XII and XIV have been reported to cause contagions in these plants (Arocha & Jones, 2010). Among them, phytoplasma strains belonging to the 16Sr groups III and XI play a significant role in East Africa causing Napier grass stunt (NGS) disease in Napier grass (*Pennisetum purpureum* Schumach) with devastating effect (Jones *et al.*, 2004, 2007; Nielsen *et al.*, 2007). The disease is often lethal and has impacted negatively on the livelihoods of farmers relying on the crop as their main source of feed for dairy animals (Orodho, 2006; Arocha & Jones, 2010). Characteristic symptoms of the disease include foliar yellowing, small leaves, tiller proliferation and shortening of internodes, to the extent that clumps appear severely stunted. Often the whole grass stool is affected with a complete loss in yield leading to eventual death of the infected plant (Jones *et al.*, 2004, 2007; Orodho, 2006; Kabirizi *et al.*, 2007; Kawube *et al.*, 2014). The disease has been reported in Kenya, Uganda and Ethiopia and has been recently detected in Tanzania, posing a serious negative effect on both dairy and cereal production in the predominantly mixed crop and livestock farming systems (Jones *et al.* 2004, 2007; Kabirizi *et al.*, 2007; Nielsen *et al.* 2007; Khan *et al.*, 2014, Asudi *et al.*, 2015).

According to the phylogenetic analysis, the phytoplasma strains infecting Napier grass plants in Uganda and Kenya are closely related to phytoplasma strains isolated from sorghum grassy shoot (SGS), *Hyparrhenia* grass white leaf (HGWL)

and sugarcane white leaf (SCWL) diseases. These phytoplasma strains are members of the 16SrXI group, '*Ca*. Phytoplasma oryzae' or rice yellow dwarf group (Jones *et al*., 2004; Nielsen *et al*. 2007; Obura *et al*., 2011) which differ from the phytoplasma strains which infect Napier grass in Ethiopia which are members of the 16SrIII-A group or X-disease group (Jones *et al*., 2007).

As obligate parasites, phytoplasmas are adapted to live in two ecological niches, plant phloem and insects. They are transmitted from time to time from infected to healthy plants through vegetative propagation of infected plant material, vascular connections made by parasitic plants such as dodder (*Cuscuta* spp.) between infected and uninfected host plants (Boudon-Padieu, 2003) and mostly by sap-sucking insect vectors belonging to the families Cicadellidae (leafhoppers) and Fulgoridae (planthoppers) (Weintraub & Beanland, 2006). In Kenya, NGS disease is spread by a sap-sucking insect vector known as *Maiestas* (= *Recilia*) *banda* (Hemiptera: Cicadellidae) (Obura *et al*., 2009) which belongs to the tribe Deltocephalini. This insect tribe comprises of insects that are mostly phytophagous on plant species belonging to the family Poaceae (Satoshi, 1999; Webb & Viraktamath, 2009). These leafhoppers probe phloem tissues and passively ingest phytoplasma cells with the phloem-sap from the infected plants. The acquisition access period of the phytoplasmas by vectors can be as short as a few minutes and the longer the period the greater the chance of transmission (Purcell, 1982). Once acquisition occurs, the ingested phytoplasmas pass through the insect midgut cells and move into the insect haemocoel, where they are transported throughout the insect body with haemolymph (Lefol *et al*., 1993; 1994). Phytoplasma reach the salivary glands and penetrate the gland cells, where they can multiply (Kirkpatrick, 1992). The incubation period is believed to be a few to 80 days, during which the phytoplasma particles invade the insect tissues and multiply after which the insect releases phytoplasma with saliva which is secreted when feeding in the phloem of a plant host. This period of time that elapses from initial acquisition to the ability to transmit the phytoplasma is known as the latent period (LP). This period is highly variable and is temperature and host plant dependent (Nagaich *et al*., 1974; Murral *et al*., 1996). The phytoplasma takes up residence in the plant host and begins to multiply. After the LP, the plant begins to develop symptoms of the disease. Once the titre of phytoplasma is sufficiently high, a plant can serve as an acquisition host

for any vector species feeding upon it (Lee *et al*., 2000; Weintraub & Beanland, 2006).

The three-way phytoplasma-vector-plant host interaction plays an important role in the spread of phytoplasma diseases worldwide. The knowledge of potential plant hosts or host range is also essential to assess the possible threat of foreign phytoplasmas to native or economically important plants grown in the country (Lee *et al*., 2003; Weintraub & Beanland, 2006; Obura *et al*., 2009). However, this is only possible through transmission experiments, which can prove the capability of candidate vector species to transfer phytoplasmas to healthy plants. Like other phytoplasma diseases, NGS is spreading fast in the East African region (Kabirizi *et al*., 2007; Khan *et al*., 2014) with potential to escalate to other areas with similar agro-ecologies in Sub-Saharan Africa. In some fields, the disease has caused up to 100% loss of the crop forcing the smallholder farmers to reduce the number of dairy cattle or purchase fodder from the local market (Orodho, 2006; Arocha & Jones, 2010). All over the world, several methods have been tried in controlling these phytoplasma diseases including weeding and roguing (Kabirizi *et al*., 2007; Arocha *et al*., 2009), insecticide application (Boudon-Padieu, 2003), planting and development of resistant plant cultivars (De La Rue *et al*., 2003) or by use of certified seeds or seedlings (Mannini, 2007). While most of these measures are not yet applicable to manage NGS phytoplasma, the disease is controlled mainly by removal and burning of infected plants (Kabirizi *et al*., 2007; Khan *et al.,* 2014). This reduces disease pressure, by lowering density and infestation levels of vector populations and decreases the amount of the phytoplasma inoculum (Weintraub & Beanland, 2006). Since NGS spreads through vegetative propagation when infected cuttings are used as planting materials (Orodho, 2006; Koji *et al*., 2012), development of efficient and affordable diagnostic tests are needed to reduce the spread. Other control measures include the selection of resistant Napier grass cultivars. However, many of these cultivars selected and introduced in the recent past have been found to be susceptible to the disease in several locations in western Kenya and Uganda. Besides, the selected cultivars have been found to be less fruitful than the existing Napier grass varieties and hence do not fulfill farmers' needs (Mulaa *et al*., 2010; Kawube *et al*., 2014).

The spread and increase in incidences of NGS disease in the region represent a real threat to the cultivation of Napier grass, which is a predominant fodder crop in East Africa (Orodho, 2006). The disease also poses a significant threat to other cultivated crops (Obura *et al*., 2009) such as sorghum, maize, sugarcane, finger millet and rice, which are major food and cash crops in the region. Since cultivated crops in the family Poaceae are vulnerable to phytoplasma infections (Arocha & Jones, 2010), it is likely that these crops could be infected by NGS phytoplasma in future and they could act as hosts, assisting its spread in the region. Therefore, the aim of this study was to assess the potential danger of the NGS phytoplasma to food crops and the vectoring capability of *M. banda* in order to develop and implement an early detection and warning system.

6.3 Materials and methodology

6.3.1 Study area

The study was conducted at the International Centre of Insect Physiology and Ecology (ICIPE) Thomas Odhiambo campus, at Mbita Point (0°25′ S, 34°12′ E) located at the shores of Lake Victoria in Homabay county, Kenya.

6.3.2 Experimental plants

Napier grass plants infected by the NGS phytoplasma were used as source of inoculum in the study. Diseased *P. purpureum* plants were stunted with small yellow leaves and bushy in appearance (Fig. 6.2). The grasses were carefully removed from the ground, potted, labeled and maintained at the ICIPE Mbita research station. The presence of phytoplasmas in diseased plants was confirmed by using a nested PCR procedure (Fig. 6.3). Plants of cultivated crops were used in order to assess whether infection with phytoplasma was possible and to study symptomatology. These crop plant species were *Zea mays* (hybrid cultivar 505), *Eleusine coracana* (finger millet) (local cultivar), *Sorghum bicolor* (Gadam), *Saccharum officinarum* (sugarcane) and *Oryza sativa* (Narica). The *Z. mays* hybrid cultivar 505 was sourced locally from a commercial supplier from the Western Seed Company (Kitale, Kenya). Napier grass (Bana variety) susceptible to NGS disease (Obura *et al*., 2009) was included in the experiment as a control treatment. Seeds and cuttings, were sown in

pots and maintained in a netted screen-house for 3–5 weeks before subjecting to phytoplasma testing in cages in a different screen-house.

6.3.3 Rearing of insects

The insect vector *M. banda* was used in the study. Gravid female leafhoppers, recognized by their abdomen, were collected from the vector rearing screen house at ICIPE-Mbita research station where they were reared on potted disease-free pearl millet plants in wooden framed cages measuring 45 x 45 x 60 cm. The top and side openings of these insect rearing cages were covered with fine white nylon mesh for aeration. Only insects that hatched from eggs in these cages (F1 generation off spring) were used because they are phytoplasma free.

6.3.4 Transmission tests

Transmission tests were carried out as described by Obura *et al*. (2009). A diseased Napier grass plant was put in the middle of the cage surrounded by six healthy phytoplasma-free potted food crops (Fig 6.1). Each set up was then repeated 21 times. Using an aspirator, 50 gravid *M. banda* individuals from the rearing cages containing non-infected plants were introduced into each inoculation cage and the insects were allowed to feed back and forth for 30 days as described by Obura *et al*. (2009). After oviposition, the emerging nymphs were allowed to feed on the diseased plants and similarly acquire the phytoplasma. The insects were disturbed from time to time inside the cages to redistribute the population. After 30 days, the inoculation setup was terminated and the exposed plants transferred to a separate screen-house for phytoplasma testing to evaluate disease symptom expression. The plants were transplanted in new big pots using phosphorus at recommended rate. Exposed Napier grass plants were used as positive controls and unexposed Napier and other unexposed crop plants as negative controls. Surviving insects from each cage were also tested for phytoplasma.

Figure 6.1: Arrangement of potted trial plants in cages during transmission experiments. The plant in the centre represents the inoculum source surrounded by six healthy test plants. Fifty gravid *Maiestas banda* were released into the cage to act as phytoplasma vectors.

6.3.5 Evaluating the threats of Napier grass stunt disease to food crops

Following termination of the exposure period, food crops were screened monthly three times (for sugarcane, rice, Napier grass and sorghum) and two times (for finger millet and maize) for the presence of NGS-phytoplasma using nested PCR assays. Individual plants were assessed on the parameters of plant growth as presented by plant height, leaf length, disease symptom development and severity, mortality rates and biomass yield. Plant height was measured from the soil surface to the tip of the youngest growing leaf. The leaf length represented the average length of five mature leaves measured from the ligula to the tip of the longest leaves per plant. To determine disease symptom development and severity, an overall visual rating of symptoms was done using a scale ranging between 0 and 3, where 0 = asymptomatic (no visible symptoms on leaves), 1 = foliar yellowing, 2 = stunted growth or dwarfed plants with small leaves and bushy in appearance and 3 = necrosis of leaves or plant death. Number of live tillers and the tiller length were also determined where relevant. The number of tillers was recorded by counting all live

tillers sprouting from the base of the main plants. Tiller length was measured from the soil surface to its tip. Sugarcane, rice, sorghum and Napier grass plants were cut back every month to allow for the re-growth mimicking a common farmers' harvesting practice for Napier grass and for the development of symptoms such as yellowing and retarded growth (Kawube *et al.*, 2014). However, maize and finger millet were not cut back.

At trial completion, root and above ground plant mass were determined. Plants were cut off at soil level and fresh weights of each determined by means of an analytical balance. The root mass with adherent media was soaked in water overnight before roots were carefully detached from the media and blotted with absorbent paper prior to determination of root fresh weight. Each of the respective plant tissues were subsequently dried in an oven at 70°C for 48 hours before determining dry weight. Relative growth indices were determined based upon measurements of growth and biomass of each treatment. Data were summarized and percentage of exposed plants that tested positive for phytoplasma calculated. Percentage of plants that developed NGS disease symptoms was also calculated in a similar manner. The data collected on plant growth parameters (plant length, leaf length, tiller number and biomass) was analyzed using paired samples t-tests to compare performance of the exposed plants to that of their respective controls. The Statistical Package for Social Sciences (SPSS) version 18 (SPSS Inc. Chicago, USA) was used and statistical significance was determined at 99% confidence level.

6.3.5 DNA extraction, quantity and quality determination

The plants were tested for the presence of NGS phytoplasma every 30 days after their removal from the transmission trial cages. Older leaves from the plants in the screen-house were sampled systematically and placed in well-labelled 1.5 ml microcentrifuge tubes. A standard weight of 0.3 g per sample was obtained and the DNA extracted using methodologies adapted from Doyle and Doyle (1990). The leaf samples were frozen in liquid nitrogen and ground to powder using sterile pestles and then placed on ice to avoid melting and DNA degradation. Preheated CTAB buffer (65˚C) (600µl) (containing 2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris–HCl, pH8.0, 0.2% 2-mercapto-ethanol) was added to the crushed tissues and then gently vortexed to mix. The entire mixture was incubated at 65 ^{o}C for 1 hour in a

waterbath to solubilize cell membranes and separate them from nucleic acids. After incubation, the mixture was left to cool at room temperature and 600μl of chloroform: isoamyl alcohol (prepared in the ratio of 24:1) were added, vortexed gently and centrifuged at 4000 rpm for 10 minutes (min) to separate the aqueous and organic phase. The supernatant was carefully separated and placed in clean sterile and labeled 1.5 ml microcentrifuge tubes. A volume of 600 μl of ice-cold isopropanol was added to each microcentrifuge tube and incubated at -20°C for 2 hours or overnight. The DNA was precipitated by centrifugation at 14,000 rpm for 30 minutes. The supernatant was discarded carefully and the pellet washed with 70% ethanol (1ml) with gentle tilting. The pellet was left to settle for 1 min then centrifuged at 14,000 rpm for 4 min. The DNA pellet was air-dried at room temperature and reconstituted in 50 μl of sterile water. The quality of isolated DNA was determined using 1% (w/v) agarose gel and quantified using a spectrophotometer. DNA samples were stored at –20°C until further use.

6.3.6 Phytoplasma DNA amplification

Phytoplasma DNA was amplified using universal primer pair P1/P6 (Deng & Hiruki, 1991) in the first round PCR followed by primer pair NapF/NapR (Obura, 2012) (Table 6.1). The initial amplification was performed in a 25 μl PCR reaction mixture containing 10ng of genomic DNA, 1X PCR buffer (GenScript, USA Inc.), 100 μM dNTPs, 1.0 unit *Taq* DNA polymerase (GenScript, USA Inc.) and 100 nM of each primer (Inqaba Biotech, South Africa Inc.). PCR reactions were carried out either in a PTC-100 Thermal cycler (MJ Research Inc. USA) or Proflex PCR machine (Applied Biosystems) as follows: denaturation at 94 ºC for 5 min for 1 cycle; 35 cycles of 94 ºC for 30 s, 52 ºC for 60 s and 72 ºC for 2 min; and a final elongation step at 72 ºC 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 following conditions: denaturation at 94 ºC for 4 min for 1 cycle; 35 cycles of 94 ºC for 45 s, 55 ºC for 60 s and 72 ºC for 2 min; and a final elongation at 72 ºC for 10 min. Amplicons were visualized by gel electrophoresis in a 1% agarose gel stained with ethidium-bromide using 1xTAE (40 mM Tris acetate, 1mM EDTA pH8.0) 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. The reference phytoplasma strain, NGS, maintained at ICIPE Mbita station was used as a positive control.

Table 6.1: Sequences of the oligonucleotide primers used for PCR amplification

Primer	Target gene[a]	Sequences (5’ to 3’)	References
P1	16S	AAGAGTTTGATCCTGGCTCAGGATT	Deng & Hiruki, 1991
P6	16S	CGGTAGGGATACCTTGTTACGACTTA	Deng & Hiruki, 1991
NapF	16S	AGGAAACTCTGACCGAGCAAC	Obura, 2012
NapR	16S	ATTTTTCATTGGCAGTCTCGTTA	Obura, 2012

[a]Location of primer within the rRNA operon, i.e., within the 16S rRNA gene used in the amplification of phytoplasma.

6.4 Results

6.4.1 Vector transmission experiments

All test plants raised in a netted screen-house were negative for phytoplasmawhen tested by nested PCR prior to transmission studies. All Napier grass plants with typical yellows and stunting symptoms (Fig. 6.2) used as source plants for the acquisition-access feeds tested positive for phytoplasma (Fig. 6.3).

Figure 6.2: Napier grass plants infected with Napier grass stunt phytoplasma showing typical disease symptoms

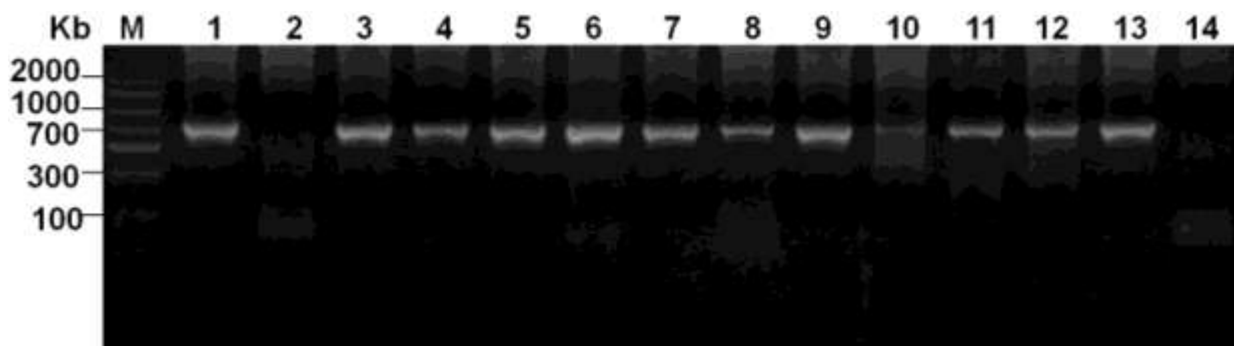

Figure 6.3: Electropherogram of nested-PCR products amplified with P1/P6 followed by NapF/NapR primers. M: 1 kb DNA marker, (GenScript, USA Inc.) lane 1: Reference Napier grass stunt phytoplasma, 2: negative control (water), 3–13: diseased Napier grass plants, lane, 14: healthy Napier grass plant.

Sixty days after exposing healthy plants to phytoplasma transmitting insects, a 778 bp DNA fragment was amplified from 40.04% of plants exposed to the leafhopper vector and the positive controls indicating that the food crops acquired NGS-phytoplasma. With 56.3% infected plants, *S. officinarum* showed the highest infection level followed by *E. coracana* with 50%, *S. bicolor with* 43.8%, *O. sativa with* 31.3% and *Z. mays* with 18.8%. Napier grass used as control in the experiment had the highest infection rate with 81.3%. A second screening was performed after two months of incubation. *Pennisetum purpureum* showed the highest proportion of infected plants with a notable increase from 81.3% to 87.5%. All the other test plants maintained the proportion of infection overtime and did not die. At the third stage of screening, maize and finger millet plants were excluded from screening because plants were too old and started to senesce. However, sorghum, sugarcane and Napier grass plants were tested and were found to maintain their proportion of infection.

6.4.2 Symptom development in food crops

Plants were assessed for change in leaf colour and retarded growth as an indication of symptom development. The experiment showed that except for Napier grass and sugarcane, none of the test plants showed typical yellowing of leaves and stunted growth usually associated with NGS phytoplasma. For instance, all sorghum plants had healthy green leaves with dotted marks 30 days post-inoculation. All exposed

and NGS phytoplasma-infected finger millet leaves were healthy and green but with some yellow dotted lines but dried towards the end of the trial. Likewise, all maize and rice plants that tested positive for NGS phytoplasma maintained their healthy green colouration from the start until the end of the experiment. Young phytoplasma-infected sugarcane plants, however, had pale green leaves and/ or foliar yellowing which turned to bright yellow with white midribs at during later stages (Fig. 6.4a and b). The change in leaf characteristics was maintained for sugarcane plants even after cutting back the plants. However, the sugarcane with yellow leaf symptoms did not develop any stunting symptoms similar to those observed on NGS phytoplasma-infected Napier grasses (Fig. 6.5).

a b

Figure 6.4: Potted sugarcane (*Saccharum officinarum*) plants in the screen house with pale green to yellow leaves that are similar to those observed on Napier grass stunt phytoplasma-infected plants; b) a sugarcane plant with bright yellow midrib.

Figure 6.5 Comparison of Napier grass plants without (top) and with Napier grass stunt phytoplasma symptoms (below) after 4 months of incubation.

6.4.3 Effect of Napier grass stunt phytoplasma on plant yield related parameters

Paired T-tests showed that there were no significant differences in plant height and leaf length between the healthy and phytoplasma-infected food crops. However, plant height and leaf length varied significantly between the infected and healthy Napier grass plants ($P<0.01$) (Table 6.2). Thus, healthy Napier grass plants were more elongated in height than phytoplasma infected plants. The unexposed healthy Napier grass plants also had longer leaves compared to their respective exposed phytoplasma-infected plants. Data on fresh weight and the dry weight did not show a significant variation between the phytoplasma-infected and non-infected plants from sugarcane, maize, sorghum, rice and finger millet. However, the variation was significant ($P<0.01$) between the exposed and unexposed Napier plants, with the unexposed plants weighing 83.8% more compared to the exposed Napier plants

(Table 6.2). A paired sample t-test showed that tiller numbers recorded from sugarcane, maize, finger millet, sorghum and rice were not significantly different between the exposed and unexposed plants. However, the tiller numbers recorded from Napier plants showed significant variation between the exposed and unexposed plants. The tiller numbers for control Napier plants averaged 8.0 while for the exposed plants averaged 40.4. Similarly, tiller length varied greatly between control and the samples positive for phytoplasma in Napier plants with a mean difference of 19 cm. However, there were no significant differences in the tiller length between the unexposed and exposed plants from sugarcane, rice, finger millet, maize and sorghum (Table 6.2).

Table 6.2: Impact of NGS phytoplasma on food crops' growth related parameters

Plant species	Plant height (cm)		t	p	Leaf length (cm)		t	p
	Exposed	Unexposed			Exposed	Unexposed		
Finger millet	99.5±6.6	99.9±6.3	-0.06	0.96	50.4±3.2	48.85±3	0.56	0.59
Maize	108.8±4.8	109.5±5.4	-0.13	0.89	52.0±4.5	52.1±4.8	-0.02	0.99
Rice	63.6±4.4	63.9±5.2	-0.04	0.97	22.3±1.8	22.1±1.8	0.13	0.90
Napier	90.2±7.3	143.1±8.9	-3.89	0.001	65.2±4.0	87.4 ±5.9	-3.58	0.002
Sugarcane	123.96±6.9	120.31±7.0	0.39	0.70	79.6±4.6	86.6±6.20	-1.035	0.317
Sorghum	90.75±8.04	92±9.32	-0.11	0.92	40.9 ±4.8	41.5±4.50	-0.142	0.89
Plant species	**Tiller Number (cm)**		**t**	**p**	**Tiller length (cm)**		**t**	**p**
	Exposed	Unexposed			Exposed	Unexposed		
Finger millet	0.00±0	0.00±0			0.00±0	0.00±0		
Maize	0.00±0	0.00±0			0.00±0	0.00±0		
Rice	1.8±0.6	1.8±0.4	0	1	17.2±2.5	19±3.3	-0.79	0.48
Napier	40.4±4.6	8±0.9	6.68	0	63.9±5.7	82.9±4.7	-3.033	0.007
sugarcane	4±0.46	4.56±0.47	-0.84	0.42	11.8±2.4	10.2±1.2	0.737	0.472
Sorghum	0.92±0.26	0.92±0.19	0	1	51.4±8.9	48.8±7.5	0.307	0.767
Plant species	**Fresh weight (g)**		**t**	**p**	**Dry weight (g)**		**t**	**p**
	Exposed	Unexposed			Exposed	Unexposed		
Finger millet	63.3±8.9	65.4±10.7	-0.26	0.81	11.9±2.1	12.7±2.1	0.43	0.68
Maize	35.7±3.0	34.8±3.9	0.07	0.94	5.9±0.5	5.9±0.6	-0.05	0.96
Rice	11.3±1.61	11.1±1.46	0.35	0.74	1.57±0.1	1.51±0.1	0.27	0.80
Napier	27.34±4.0	86.22±20.16	2.922	0.009	4.69±0.7	27.9±14.0	-1.55	0.14
Sugarcane	76.7±9.30	79.3±5.5	-0.22	0.83	12.5±1.4	12.7±0.9	-0.12	0.91
Sorghum	49.46±9.31	50.37±8.04	-0.082	0.936	6.83±1.3	9.04±1.41	-1.496	0.163

Exposed – food crops subjected to Napier stunt phytoplasma transmission using *Maiestas banda* as inoculum carriers. Unexposed – food crops not inoculated with Napier stunt phytoplasma but maintained as controls. All t-values are associated with p-values at 0.01

Table 6.3: Morphological changes on food crops infected by NGS phytoplasma

Species name	Cultivar	Symptoms
Zea mays	Hybrid cultivar 505	Asymptomatic
Eleusine coracana	Finger millet	Asymptomatic
Oryza sativa	Narica	Asymptomatic
Sorghum bicolor	Gadam	Asymptomatic
Saccharum officinarum	Sugarcane	Yellow leaves, white-yellow midribs
Pennisetum purpureum	Bana	Yellow leaves, stunted growth

6.5 Discussion

The epidemiology of phytoplasma-associated diseases is per se connected to the biology of their insect vectors. In order to understand the epidemiology of these diseases, it is vital that specific vectors are identified, their behaviours studied and their preferred host plants taken into consideration (Weintraub & Beanland, 2006). Obura *et al*. (2009) identified *M. banda* as a vector for NGS phytoplasma among a group of leafhoppers and planthoppers collected from phytoplasma-infected Napier grass fields. Later, Koji *et al.* (2012) while using malaise traps, showed that these insects were the most abundant species found on Napier grass in the western parts of Kenya, where NGS disease was first reported (Orodho, 2006), supporting its role in the disease epidemics in the region. In this current study, the potential threats of this phytoplasma to cultivated crops and their significance as alternative hosts were investigated.

The study showed that all trial crop plant species used in the transmission experiments could be infected by the NGS phytoplasma originating from infected Napier grass. However, most of the plants including rice, sorghum, maize and finger millet plants did not develop symptoms despite them being diagnosed with NGS phytoplasma. Phytoplasma-infected sugarcane plants, however, had both weak and moderately strong symptoms comprising yellowing of leaves and yellow mid-rib characteristics but were not stunted like phytoplasma-infected Napier grass plants. In most plant species, phytoplasma infections induce a range of symptoms suggesting profound disturbances in the normal balance of plant growth regulators (Lee *et al*., 2000). Usually, plant species develop disease symptoms such as yellowing, stunting, virescence, phyllody, witches'-broom, leaf-roll, fasciations of stems and flower stalks, and generalized decline (Lee *et al*., 2000; Bertaccini *et al*., 2005; Jones *et al*., 2004, 2007; Nielsen *et al*., 2007). However, in some plant species, phytoplasmas can exist

without causing an apparent disease or apparent impact as is the case of the ash yellows phytoplasma in velvet ash trees (Sinclair *et al*., 1994) and other phytoplasmas in alder (Lederer & Seemüller, 1991), apricot (Kirkpatrick *et al*., 1990), almond (Uyemoto *et al*., 1992) and apple trees (Seemüller & Schneider, 2007). Other examples include the detection of aster yellows in asymptomatic lilies (Bertaccini *et al*., 2005) and the transmission of NGS phytoplasma to sorghum plants without any symptoms (Wamalwa, 2013). Such associations between phytoplasmas and their hosts may suggest that those plants are tolerant to the phytoplasma infection.

Due to their long incubation time in plants, phytoplasma diseases are often detected too late (Hogenhout *et al*., 2008). In Napier grass, NGS disease symptoms usually manifest after several cuttings or grazing by animals (Jones *et al*., 2004) indicating the possibility that by then insect vectors have already disseminated the bacteria to other plant hosts. The transmission of NGS phytoplasma to other crop species without causing any visible symptoms, also show challenges present in the disease control. The study suggests that the insect vectors could acquire NGS phytoplasma and transmit it to a plant species, which becomes a new host and inoculum source for the nearby farms planted with Napier grass. This might add an additional disease cycle, which makes it more difficult to control.

In natural agro-ecosystems, both insect vectors and alternative hosts that serve as reservoirs of infection play a fundamental role in phytoplasma disease spread (Lee *et al*., 2003). Five-food crop plant species used in this trial could be infected by NGS phytoplasma. These crops can be considered as alternative host plants and could act as unrecognized sources of inoculum or new reservoirs of the NGS phytoplasma in the region. It has been suggested that the existence of a broad plant and insect host range may make phytoplasma disease outbreaks unpredictable (Hogenhout *et al*., 2008). Therefore, just like natural hosts (Caudwell, 1983; Lee *et al*., 1998), cultivated crop plant hosts identified in this study could enable additional epidemiological cycles in the spread of NGS phytoplasma directly or indirectly. If the experimental data obtained in this study are confirmed in the field, these complex epidemiological systems may hamper the identification of proper targets for control strategies, or the biology of the species involved may complicate development of

appropriate management measures (Maixner, 2010). The efficient control of NGS disease in future, might therefore, be hindered by its suspected distribution on both cultivated and wild host species and movement between these different species.

Artificial inoculation of monocots with phytoplasmas through graft transmission is not possible. Therefore, the insect vector *M. banda* (Obura *et al.*, 2009) provides the only way of transmitting NGS phytoplasma from infected plants to test plants. In the experimental set-up, the insects were able to inoculate the cultivated plants quite efficiently with the NGS phytoplasma during their regular feeding activity. The long inoculation access period of one month and high proportions of infected insects, seem to have created a scenario, which favoured transmission. However, the scenario is also given in the field and therefore likelihood of transmission exists there too.

Apart from *P. purpureum* and *M. banda*, the current study reports an additional five plant species, which may be considered alternative hosts and may harbour the NGS phytoplasma. This information can be used to develop management strategies aimed at controlling the vector, alternative or preferred hosts for the vector and/or alternative hosts for the phytoplasma, and subsequent spread of disease. It is worth noting that even though the crop plants are able to acquire NGS phytoplasma, we didn't test if vectors could acquire phytoplasma from them. There is therefore need for further studies including field surveys to determine if these particular plants are regular or occasional NGS phytoplasma hosts in the region.

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Chapter 7: General discussion, conclusions and recommendations

The spread and progress of diseases infecting plants are determined by inoculum density and the presence of the vectors. Control of these vectors in plants is different in annual and in perennial crops, as impacts in annual crops are usually seasonal, while in perennial plant systems, losses may occur over many years (Christensen *et al*., 2005; Weintraub & Beanland, 2006; Bertaccini, 2007; Weintraub & Wilson, 2010). Napier grass stunt (NGS) disease is the most destructive diseases of Napier grass, the most important forage crop in the East African region. The disease is spread by *Maiestas banda* (Hemiptera: Cicadellidae) and is caused by a phytoplasma (Jones *et al*., 2004; 2007; Orodho, 2006; Nielsen *et al*., 2007; Obura *et al*., 2009). The development of a management strategy for this disease is still in its early stages (Arocha & Jones, 2010). Therefore, it is managed primarily by weeding and rouging of infected plants, application of organic and inorganic fertilisers and planting cultivars resistant to the disease (Orodho, 2006; Kabirizi *et al*., 2007; Khan *et al*., 2014; Asudi *et al*., 2015). However, most of the selected Napier cultivars have succumbed to the disease (Mulaa *et al*., 2010; Kawube *et al*., 2014). Therefore, the development of resistant clones should desirably constitute a defence mechanism against the disease and the insect vectors that spread the phytoplasma (Kawube *et al*., 2014).

In order to develop a management approach for the NGS disease, this study evaluated its incidence, severity and the farmers' knowledge on its wild grass hosts. The study also identified alternative phytoplasma hosts and determined the dynamics of NGS phytoplasma between wild and cultivated Poaceae. The study showed that apart from Napier grass, several crop species were grown by a majority of the farmers as basic foods. The study showed an eminent incidence of NGS disease in Kenya followed by Uganda and then Tanzania suggesting a rapid spread of the disease. This depicts that the disease is rife and could perhaps spread to other areas with the same environmental conditions. Among the interviewees, 49.5% were able to identify NGS disease by its symptoms. However, since there is no remedy for the disease, most respondents removed morbid Napier grass plants and burnt them. They also suggested use of other alternative forage crops to reduce dependency on Napier grass. Not many farmers were cognisant of the wild grass

plants that harbour NGS-phytoplasma, but a small percentage of them alluded to *Cynodon dactylon* and *Cyperus* spp. as the likely hosts.

Results of the evaluations done with alternative plant hosts demonstrated the presence of substantial number of wild grass species, i.e. ten more species, which harbour NGS-related phytoplasmas. These wild grass plant species could serve as alternative hosts for NGS and could be its source of inoculum acting as reservoirs. Hypothesis for the emergence of phytoplasma diseases is the branching, through vector feeding of a natural pathway from native to cultivated plants, enabling the mollicute to manipulate a new ecological niche (Lee *et al*., 1998). Therefore, it is possible that NGS-related phytoplasmas may become exposed to a new group of insect vectors in a new epidemiological system and serve as alternative source of phytoplasma for Napier grass, cereals and other monocots, which serve as basic food and cash crops in the region. Besides, nested PCR based on the universal 16S rDNA fragment revealed the presence of additional phytoplasma strains including Bermuda grass white leaf (BGWL) phytoplasma which is a member of the 16SrXIV group, '*Candidatus* Phytoplasma cynodontis' and goose-grass white leaf (GGWL) phytoplasma which was not assigned to a specific 16Sr phytoplasma group (Win *et al*., 2013). It is the first time GGWL-phytoplasma has been detected in East Africa in Kenya and Tanzania. It is also the first report of the BGWL phytoplasma and *Hyparrhenia* white leaf (HGWL) phytoplasma in Tanzania and Uganda. Therefore, further studies should be done to determine the insect vectors and investigate any threats of these phytoplasmas to the wild and cultivated plants.

Phytoplasmas are minute plant pathogenic agents responsible for numerous crop diseases worldwide (Lee *et al*., 2000). The discovery of molecular biology made it possible to correctly identify and characterize them as *Mollicutes* (Lee *et al*., 1998; IRPCM, 2004). In this study, nested PCR flanked by P1/P6 and NAPF/NAPR primers (Deng & Hiruki, 1991; Obura, 2012) representing the 16S rDNA fragment were used to detect the phytoplasmas from wild grasses. As supporting tools, sequences of the 16-23S intergenic spacer region (ISR) (Smart *et al*., 1996) and other primers based on *secA* (Bekele *et al*., 2011) and immunodominant protein (*imp*) (Wambwa, unpublished) were exploited. The use of 16-23 ISR sequences confirmed the presence of GGWL-phytoplasmas in wild grasses with a sequence similarity of 99%

and showed the close resemblance of HGWL phytoplasma to NGS phytoplasma when they shared a 98.7% similarity. In addition, sequence analysis based on the *secA* and *imp* gene fragments validated the closeness between HGWL and NGS phytoplasmas. Use of *secA* was able to discriminate between the HGWL from NGS accessions and proved to be the best markers that could be used for delineation of these phytoplasmas.

The role of wild plants as source of inoculum in the spread of NGS phytoplasmas was also investigated. Using *M. banda* as the insect vector, identified HGWL and BGWL-infected plants were put together with the healthy Napier grass plants. The study showed that the healthy plants remained negative when tested for presence of the phytoplasmas, indicating that no transmission occurred and that the insects did not acquire the two phytoplasmas. Host specificity plays a fundamental role in the spread of phytoplasma diseases. Usually, this is greater for vector than for the plant hosts (Lee *et al.*, 1998). However, there are no explanations for these strict host-relations, but the failure of *M. banda* to transmit both HGWL and BGWL phytoplasmas to Napier grass plants show that indeed there exist different natural vectors of these bacteria in the wild. It also shows that *M. banda* might be the exclusive vector of the NGS phytoplasma. However, because of the close similarity of HGWL and NGS phtoplasmas, the role of thatch grass in the spread of NGS-disease should be considered in future studies. These studies should focus first on the identity of the insect vector for the HGWL and disease host range. Once identified, transmission trials can be initiated.

Under screen-house conditions, NGS-phytoplasma was transmissible to cereals and sugarcane plants. Hence, the plant host range of *M. banda,* rather than lack of phytoplasma-specific cell membrane receptors, will limit the spread of NGS phytoplasma to these monocots. However, all plant species remained symptomless except sugarcane plants, which had moderate symptoms. This also shows that these monocots could harbour NGS and could be acting as extra source of this phytoplasma. An extensive plant and insect host range can make the occurrence of phytoplasma diseases very difficult to ascertain (Hogenhout *et al.*, 2008) and this could occur for NGS. Just like emergence of new phytoplasma diseases (Caudwell, 1983; Lee *et al.*, 1998), this study indicated that the plant species identified here as

new hosts could alter the normal NGS-disease cycle and initiate a new alternative disease cycle leading to a continuous spread of the disease. There is no report of previous NGS disease outbreaks in cultivated grasses in East Africa. The study therefore predicts that phytoplasma infection may occur in cereals and other plants through opportunistic feeding by the vector when Napier grass is cut and the insects move to nearby fields planted with these plants. Even in this case, the long incubation period of phytoplasmas in plants (Weintraub & Beanland, 2006; Hogenhout *et al*., 2008) will ensure that cereals mature before disease development. In case of sugarcane plants, there is need to do a field survey to determine if NGS-phytoplasma infected sugarcane plants exist. This information can be used to develop a management strategy for the NGS disease for the control of the vector and other preferred plant hosts.

There is no successful control measure for phytoplasma diseases. Therefore, to control NGS disease, infected Napier and thatching grasses and potential wild grass hosts should be removed from the fields. Use of certified clean seedlings and application of chemicals to kill insect vectors in combination with roguing might help reduce the spread of the disease. The study also suggests that quarantine measures (Weintraub & Beanland, 2006) should be enforced to prevent the spread of NGS disease from one region to the other. The phytoplasma host range and the feeding behaviour of the insect vector determine the geographical distribution and impact of phytoplasma diseases (Lee *et al*., 2003). Napier grass stunt disease is caused by the same phytoplasma in Tanzania, Kenya and Uganda indicating the possibility of being vectored by a common leafhopper in the three countries. Thus, knowledge of the migration patterns of *M. banda* in the region may help in future prediction of the disease outbreak and spread and might lead to the development of an efficient management strategy for the disease. Farmers should be made aware of the disease symptoms so that they can eliminate diseased plants in time. However, other technologies including manipulation and enhancement of genes and development of phytoplasma resistant plants or plants that are not liked by the insect vectors (Weintraub & Beanland, 2006) should be tried for control of the NGS.

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The results discussed in chapter 4 have been published as Detection, identification and significance of phytoplasmas in wild grasses in East Africa'. *Plant Disease*, http://dx.doi.org/10.1094/PDIS-11-14-1173-RE.

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Appendix 1

Appendix 1: Questionnaire used to gather information regarding farmers' perceptions on the wild hosts of the Napier stunt phytoplasma in East Africa

Farmer's personal information

1. Name of the farmer __________________________Contacts _______________
2. Age _________________________ Gender ____________________________
3. Education level ____
4. Area of residence Sub-location_____________ Location___________________

   Division _________________________ District __________________________

   GPS coordinates ____
5. Which crops do you grow on your farm? Maize ( ) Rice ( ) Sorghum ( ) Millet ( ) Sugarcane ( ) Napier grass ( ) Others (specify) ___________________

   ____
6. What are the major production constraints for the different crops?

Constraint	Maize	Rice	Sorghum	Millet	Sugarcane	Napier grass
Napier grass stunt disease						
Stem borers						
Striga weed						
Drought						
Cold						
Nutritional diseases (quote symptoms)						
Others (specify)						

Farmer's knowledge of Napier stunt disease and crops affected

7.1 Do you know a disease called Napier grass Stunt Disease? Yes ( ) No ( )

7.2 If Yes, in what crops have you seen it? ___________________________________

____

7.3 What are the symptoms of the disease? ________________________________

____

8. Do you have NSD on your farm? Yes ( ) No ( )

8.1 If **Yes**, how severe is the disease in your farm? Low ( ) moderate ( ) high ( )

8.2 If **Yes**, is it spreading on your farm? Yes ( ) No ( )

8.3 If **Yes**, how fast is it spreading? Slow [ ] Fast [ ] Very fast [ ]

8.4 Does the disease occur throughout the year? Yes ( ) No ( )

8.5 When is the disease more prevalent? During dry season [ ] During cold season [ ] During long rains [ ] During short rains [ ]

8.6 Which crops are mainly affected by Napier stunt disease? _______________

____

9. When are the newly grown plants affected? After 1st cutting ( ) After 2nd cutting( )
10. When do the affected plants degenerate? After 1st harvest ( ) 2nd harvest ( ) 3rd harvest ( )
11. Answer appropriately

Crop	Cultivars grown	Resistant to NSD	Source of planting material
Maize			
Rice			
Sorghum			
Millet			
Sugarcane			
Napier grass			

12 What control measures do you use to manage the disease? Rouging () Burning () Chemicals ( ) Replanting () Others ____________________________________

13 Which among these methods is more effective in controlling the disease? ____

14 Would you consider replacing Napier grass with any other fodder grasses resistant to NSD? Yes ( ) No ( )

If **Yes**, what grasses would you prefer, and give reasons for your preference ____

____

15 Have signs of Napier stunt disease (NSD) been reported in other farms in your area? Yes ( ) No ( )

If **Yes**, when was the disease first seen in your area? ______________________

Please rate the seriousness of NSD in your area Low ( ) Moderate ( ) High ( )

Farmers' knowledge of NSD in wild hosts

10. In what uncultivated grass(es) have you seen NSD? ______________________

____

11. a). What symptoms do the affected grasses show?_________________________

____

(*Probe the respondent: How do you know it is NSD that causes these symptoms?*)

11. b). When did you first notice NSD in the wild grasses? ____________________

____

12. How severe is the disease in the area surrounding your farm? Low ( ) moderate ( ) high ( )

13. a). Is it spreading in your area ? Yes ( ) No ( )

b). If **Yes**, how fast is it spreading? Slow [ ] Fast [ ] Very fast [ ]

14. What do you think caused/is causing NSD? ______________________________

15. What do you think is spreading it? _____________________________________

16. Do you think NSD can spread from wild grasses to your cultivated crops?

Yes ( ) No ( )

If **Yes** , how would this happen? _______________________________________

____

17. How can NSD be stopped? ____

18. What are you doing to cope with the situation? ____________________________

19. What are your neighbours doing to cope with the situation? _________________

20. What do you think can be done to control NSD in your area? ________________

APPENDIX 2

Concise recommendations for the management of the Napier grass stunt disease in East Africa

Background

Napier grass, *Pennisetum purpureum* (Poaceae), is an important crop that significantly contributes to livelihoods of small-scale farmers in East Africa by supporting the smallholder dairy and cereal production systems in the region. It is the main fodder for the dairy industry, in addition to its use as a trap plant for management of cereal stemborer pests in 'push–pull' strategy (PPS). One of the most important threats to the productivity of Napier grass is Napier grass stunt disease (NGS) associated with phytoplasmas.

Symptoms and distribution of Napier grass stunt disease

The disease occurs in Kenya, Uganda, Ethiopia and Tanzania. This disease spreads quickly and covers several districts of western Kenya, causing serious economic losses in the smallholder dairy industry. NGS symptoms include foliar yellowing, little leaves, bushy appearance, yellow to purple streaking, proliferation of tillers and shortening of internodes, to the extent that clumps are severely stunted and have a low biomass yield. Affected shoots become pale yellow-green in colour and seriously dwarfed. Often the whole stool is affected, with complete loss in yield and eventual death (Fig. 1).

Figure 1: Severely stunted bushy Napier grass with yellowing of leaves and reduced biomass.

Napier grass stunt disease vectors

The leafhopper *Maiestas banda* (Hemiptera: Cicadellidae) (Fig. 2) transmits NGS to Napier grass in Kenya, while the leafhopper *Exitianus* spp. (Hemiptera: Cicadellidae) and planthopper *Leptodelphax dymas* (Hemiptera: Delphacidae) have been suggested as potential vectors in the transmission of NGS in Ethiopia.

Figure 2: Dorsal view of *Maiestas banda* (Hemiptera: Cicadellidae), a vector of Napier grass stunt phytoplasma in Kenya.

Management of Napier grass stunt disease

The disease is controlled mainly by removal and burning of the infected plants. However, these control measures are not efficient. Attempts to develop and introduce resistant Napier grass cultivars in the region in the recent past also failed when these cultivars became susceptible to the pathogen. The spread and increase in incidences of this disease in the region represent a real threat to the fodder and the cultivated plants such as sorghum, rice, sugarcane, finger millet and maize, which serve as staple and cash crops in the region.

Since there is no remedy for the disease, most respondents remove diseased Napier grass plants and burn them. Farmers also suggested use of other alternative forage crops such as Guatemala grass (*Tripsacum laxum*), Amasanyi (*Panicum maximum*), Giant setaria (*Setaria sphacelata*), Kikuyu grass (*Pennisetum clandestinum*), Lucerne (*Medicago sativa*), Bermuda/star grass (*Cynodon dactylon*) and Rhodes grass (*Chloris gayana*). Additionally, use of disease-free planting materials and implementing quarantine measures could help prevent the spread of NGS to areas that are not infested yet.

Wild host plants of the Napier grass stunt phytoplasma

Eleven grass species have so far been found to harbour NGS-related phytoplasmas (Table 1). These wild grass plant species could serve as alternative hosts for NGS and could be its source of inoculum acting as reservoirs. Apart from a few species, most of the wild grass hosts from which phytoplasmas were detected were indistinguishable from healthy conspecific grasses. Therefore, weeding and roguing could help control the disease spread.

Table 1: Wild grasses which are alternative hosts to NGS phytoplasma

Grass species	Common name	Host Symptoms
Brachiaria brizantha	Signal grass	Asymptomatic
Chloris gayana	Rhodes grass	Asymptomatic
Enteropogon macrostachyus	Bush rye	Yellow leaves
Coix laryma-jobi	Otiro (Luo)	Asymptomatic
Cynodon dactylon	Bermuda grass	Asymptomatic
Digitaria scalarum	Couch grass	Asymptomatic
Eleusine indica	Crowfoot/ goosegrass	Asymptomatic
Hyparrhenia rufa	Thatching grass	White leaves, stunted growth
Hyparrhenia cymbaria	Thatching grass	White leaves, stunted growth
Sporobolus pyramidalis	Drop-seed grass	Asymptomatic
Sorghum versicolor	Wild sorghum	Asymptomatic

Figure 3. stunting, bushy growing habits and small white leaves associated with HGWL phytoplasma-infected thatch grass found in the surveyed districts.

Disease threat to food crops

Sugarcane, rice, maize, sorghum and finger millet are able to acquire NGS via the insect vector, *M. banda*. However, except for sugarcane, all these species remain symptomless. This shows that these monocots could harbour NGS and could be acting as extra source of this phytoplasma.

The following factors should be taken into account in areas where there is a disease threat:

a. Infected Napier and thatching grasses and potential wild grass hosts should be removed from the fields.
b. Use of certified clean seedlings and application of chemicals to kill insect vectors in combination with roguing might help reduce the spread of the disease.
c. Quarantine measures should be enforced to prevent the spread of NGS disease from one region to the other.
d. Knowledge of the migration patterns of *M. banda* in the region may help in future prediction of the disease outbreak and spread and might lead to the development of an efficient management strategy for the disease.
e. Farmers should be made aware of the disease symptoms so that they can eliminate diseased plants in time.
f. Technologies including manipulation and enhancement of genes and development of phytoplasma resistant plants or plants that are not liked by the insect vectors should be tried for control of the NGS.

Appendix 3

COLLABORATIVE CROP RESEARCH PROGRAM
THE McKNIGHT FOUNDATION

icipe
African Insect Science for Food and Health

Solving Napier Stunt Disease to save the smallholder dairy sector in East Africa – a success story

The International Centre of Insect Physiology and Ecology (*icipe*) in Kenya, working with national partners in East Africa and Rothamsted Research (UK), and funded by the McKnight Foundation (USA), has found varieties of Napier grass that are resistant to Napier Stunt Disease (NSD).

> *"Napier Stunt Disease interferes with animal fodder, soil erosion control, incomes and rural employment."*
>
> Boniface Aono

Napier grass is the most important fodder crop in smallholder dairy production systems in East Africa. It is a vital component of the intensive crop–livestock management system which sustains the livelihoods of dairy smallholders.

Since the late 1990s, Napier grass has been hit by the increasingly rapid spread of a disease which stunts its growth, often killing the plant. NSD is a considerable and growing threat to livelihoods and the future of the smallholder dairy sector.

In 2000, *icipe* and Rothamsted Research scientists Professors Zeyaur Khan, John Pickett and Lester Wadhams observed for the first time a stunting disease in Napier grass in Teso, western Kenya. They had a special interest in Napier grass as pioneers of push–pull technology, a cereal crop protection system in which it is planted as a trap plant to attract insect pests. Concerned about the threat posed not only to increased uptake of push–pull but also to the smallholder dairy sector, they began to track the spread of the disease.

By 2002, they observed that the stunting was spreading rapidly in the region, affecting about a quarter of Napier grass. In response, they initiated research into the causes and transmission of NSD, in order to develop a sustainable disease management approach. The team's labours bore fruit in 2013 when two NSD-resistant cultivars passed on-farm trials, and participating farmers were given the go-ahead to multiply them for wider distribution.

But ongoing work is still needed to develop an integrated management system, including the introduction of resistant cultivars, building farmers' knowledge about how NSD spreads, the proper disposal of diseased plants, the potential role of other grasses as reservoirs of NSD, and diversification of fodder sources.

Continuing scientific research is also essential to deepen understanding of the biology and epidemiology of the disease, particularly its potential to spread to food crops.

Above: Boniface Aono contrasts healthy and infected Napier grass plants on his farm in Kisumu, Kenya.

Below: Dr Khan shows EU Ambassador Lodewijk Briët the insect vector of NSD at the *icipe* field station at Mbita Point, where it was first identified by his team of scientists.

Napier grass at the heart of intensified smallholder dairy production

East Africa has a long tradition of mixed smallholder farming, combining the production of crops and livestock on the same farm. In the last thirty years, several contextual changes have driven the adaptation and intensification of this traditional livelihood system.

- **Population increase** has led to more land being used for cultivation, at the expense of grazing land.
- **Fragmentation of farm land** is widespread, as existing family holdings are split for inheritance by the next generation.
- **Liberalization of the dairy sector** in 1992 allowed smallholder farmers to produce, process and market their own milk for the first time.
- **Improved breeds of dairy livestock** have been introduced through both widespread government and non-governmental organization (NGO) programmes, and private enterprise. While improved cows and goats produce more milk, they also demand more fodder, and must be stall-fed to protect them from diseases.

These drivers of change have resulted in a steady increase in the number of improved and cross-breed dairy cattle and goats kept in zero-grazing units on small farms, and rising demand for cultivated fodder to provide an alternative to purchased animal feeds. Napier grass – high-yielding and easy to manage and propagate – is the fodder crop most commonly grown to meet this demand. It is also widely planted for environmental protection, stabilizing soil and acting as a windbreak.

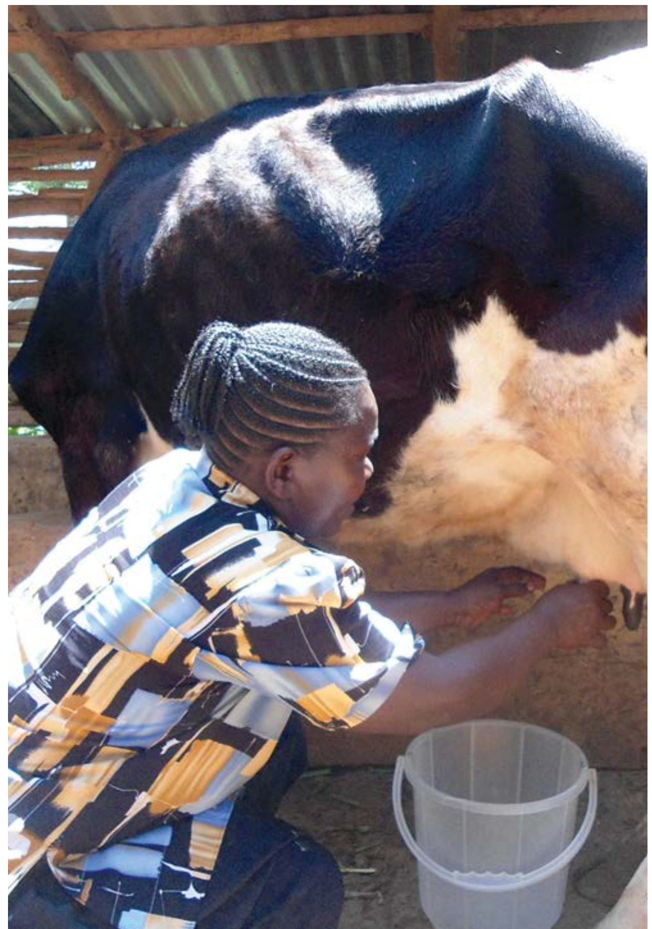
Pascal and Ruth Otieno's experience of intensifying their dairy production is typical of many. They have been push–pull farmers since 2006, and they received a Friesian dairy cow from the NGO Heifer International when their fodder production rose. Pascal says that the most visible impact of intensifying his milk and fodder production has been to increase the family's cash income.

Napier grass is cut and fed to stall-reared or tethered dairy animals, goats as well as cattle. Kenya has the largest smallholder dairy sector in sub-Saharan Africa.

Napier Stunt Disease threatens livelihoods

Whether grown in a single stand or as a border crop, Napier grass has become an integral part of the improved livelihoods that can result from intensive smallholder dairy production. For many farmers, milk production improves household income, helps meet the costs of educating children and provides much-needed dietary protein. Zero-grazing units facilitate the collection and processing of farmyard manure to improve soil fertility.

But these development gains have been eroded since the inexorable spread of NSD. Milk production has dropped, and instead of gaining income from milk sales, many farmers are forced to find cash to buy the fodder needed by their improved cattle. Some have had no choice but to sell their animals.



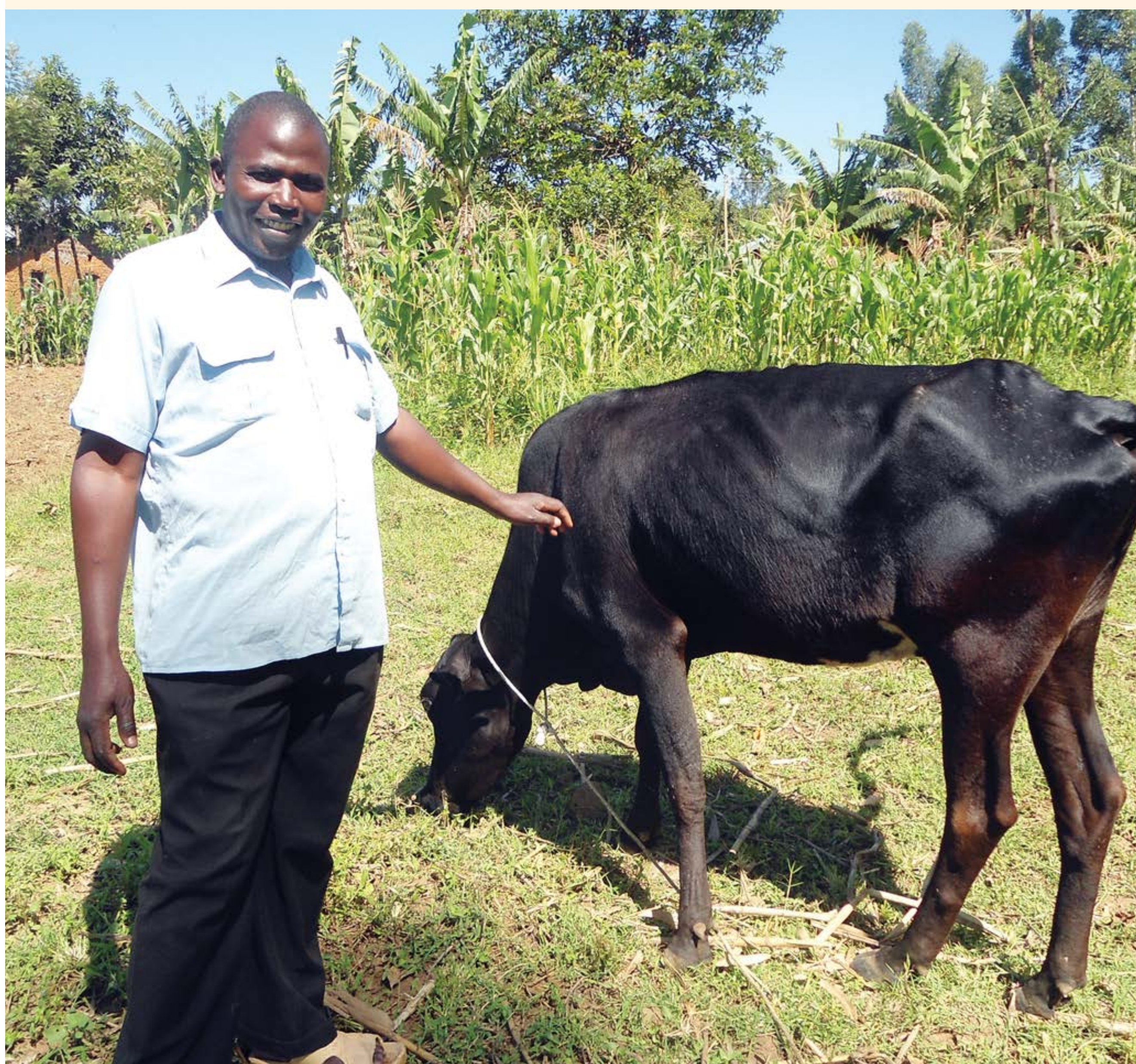
Ruth Otieno milks the Friesian dairy cow which gives 20 litres every day. Most of this is sold to pay school fees for the family's six children.

Napier Stunt Disease makes agricultural intensification riskier for farmers

When George Kane and other members of his Farmer Field School group first noticed diseased Napier grass on their plots in 2007, they had no idea what caused it. Some tried adding more manure to the soil or rotating the crop, while others uprooted the diseased grass and began again with new plant material. But no method proved effective and the disease spread steadily.

NSD struck at a crucial time on George's farm. He had decided that the carrying capacity of his one acre smallholding meant that he was better suited to a single, productive stall-fed dairy animal than his three local-breed grazing cattle. He sold his local cattle and replaced them with a Friesian cross-breed. His gamble will pay off only if a viable solution to NSD is found, assuring him of enough fodder to adequately feed his cow.

> *"Milk is gold, but its production is affected by NSD. More than 90 percent of Napier grass in Bungoma district is affected."*
>
> George Kane



Milk sales are the main source of income for George's family of eight. Since NSD spread across his farm, his cross-breed cow does not get enough to eat and is rapidly losing weight.

Dr Khan and fellow agricultural entomologist Dr Charles Midega supervise the extraction of DNA from plants to detect NSD in the *icipe* laboratory at the Thomas Odihambo Campus, Mbita.

Evolving research on Napier Stunt Disease

Having observed the rapid spread of the stunting disease in 2002, the *icipe* team set out to learn more about the problem. This was achieved over several years of multidisciplinary research to uncover the complex biological, chemical and ecological interactions between plants, insects, bacteria and people, which would shed light on the origins and spread of the disease.

The *icipe* team first contacted Rothamsted plant pathologist Dr Phil Jones. He recommended inviting his colleague Dr Latunde-dada Akiwunmi to collect Napier cuttings and culture them in a laboratory at *icipe* in Nairobi, in order to identify the disease. Dr Akiwunmi discovered that it was caused by bacteria. Further DNA analysis by Dr Jones identified phytoplasma, a tiny parasitic bacteria, as the culprit. Phytoplasma are known to cause around 200 plant diseases, often spread by insects that feed on plant sap.

The *icipe* scientists turned to finding out whether any insects were spreading the NSD phytoplasma. They collected live samples of 20 different species of sap-sucking insects associated with Napier grass and reared them in cages, feeding them on diseased Napier grass to acquire the phytosplasma. When the insects laid eggs on the diseased grass, the emerging nymphs acquired phytoplasma in the same way. After 30 days, healthy Napier plants were introduced and exposed to the insects and nymphs for 60 days. Samples of surviving insects and plants were then tested for phytoplasma. This process led to the identification of a common leafhopper as the insect vector of the disease.

Carrying out this kind of study demands the DNA analysis of many thousands of plant and insect samples, to detect the presence of the disease. Early in the research, screening for phytoplasma was carried out using a Polymerase Chain Reaction machine. But this method is laborious, costly and technically demanding. During their research on NSD, the *icipe* team has used a new simpler phytoplasma diagnostic tool, Loop Mediated Isothermal Amplification of DNA (LAMP), which makes screening for NSD cheaper and faster.

Phytoplasma transmission experiments like this one led to the identification of the insect vector of NSD.

FACTS ABOUT NAPIER STUNT DISEASE

What is Napier grass?

Napier grass (*Pennisetum purpureum*) is a high-yielding fodder grass which tolerates frequent cutting. These qualities make it the most important fodder grass in East Africa. It is grown by the majority of the region's smallholder dairy and cereal farmers.

Healthy Napier grass for sale at Luanda market, near Maseno, Kenya.

In push–pull technology, adopted by more than 70,000 of the region's farmers, Napier grass is used as a trap plant to attract insect pests.

What is Napier Stunt Disease?

NSD is a disease that affects Napier grass. Its symptoms are visible in the re-growth that happens after the grass has been cut or grazed. Affected plants are recognized by severe stunting and yellowing, and a profuse growth of shrivelled, unhealthy new plant shoots. Often the whole stool is affected, and dies. NSD also attacks other fodder grasses such as *Cynodon dactylon* and *Hyparrhenia rufa*.

What causes it?

NSD is caused by a specialized bacteria called phytoplasma, which stops the grass from taking up the nutrients it needs to grow. The phytoplasma that causes NSD is a member of a phytoplasma group, 16SrXI, already known to cause stunting in rice and Bermuda grass.

A specimen of stunted Napier grass, showing yellowing and biomass loss.

How is it transmitted?

The phytoplasma are carried from plant to plant by the leafhopper *Maiestas banda* Kramer, which draws its food from the part of the Napier grass which is infected by phytoplasma. High population densities of *Maiestas banda* Kramer on field sites in western Kenya confirmed the identification of the leafhopper as the principal insect vector for NSD.

The phytoplasma are also spread through the common practice of propagating split Napier grass roots for multiplication.

Maiestas banda Kramer, a tiny leafhopper, spreads the disease from plant to plant.

For over 35 years, Aloice Ouma preserved a Napier grass variety on his farm in Busia, Kenya. He shared it with *icipe* to use in their research. It turned out to be phytoplasma-resistant, and now bears his name – Ouma 2.

Searching for an integrated management solution

In 2008, social scientists from the *icipe* team interviewed farmers to find out more about their perceptions of NSD and its effects on their livelihoods. They surveyed a random sample of 150 farmers in western Kenya.

- 87% were aware of the disease and its rapid spread, but none knew what caused it or had a strategy for managing it.
- The majority did not produce enough Napier grass or other fodder on their farm to feed their livestock.
- Milk production had gone down by an average of 65%.

Although the news that NSD was caused by a phytoplasma and carried by a leafhopper began to spread to farmers through *icipe* scientists and field technicians, it was clear that an NSD management strategy was urgently needed. The *icipe* team turned to searching for varieties of Napier grass that would resist the disease.

But this search had to be undertaken with extreme caution. There are different mechanisms of resistance to disease. Some plants and varieties can host the phytoplasma but not develop symptoms of the disease, while others escape infection because they are unattractive to the leafhopper. Introducing a variety with high resistance to the leafhopper would risk forcing the insect to seek new hosts, possibly spreading the disease to previously unaffected plants, including food crops.

The team obtained funding from the McKnight Foundation to develop a sustainable management strategy for control of NSD. This included identifying Napier varieties with a low level of resistance to the leafhopper but a high level of durable resistance to the phytoplasma. With support from the International Livestock Research Institute (ILRI), *icipe* scientists collected germplasm – parent planting material – of fifty Napier grass cultivars and obtained 70 new accessions from the Kenya Agricultural Research Institute (KARI). In addition, hundreds of varieties were collected from farmers' fields. All of these were screened over a two-year period. The team also screened several alternative fodder grasses for their resistance to NSD.

This process led to the selection of three resistant varieties with slightly different resistance mechanisms. These were cultivated for two years at *icipe*'s Mbita Point field station to test the durability of their resistance, before being subjected to on-farm trials in 2013. Two varieties, Ouma 2 and South Africa, are particularly attractive to *Maiestas banda* but resistant to its negative effects. As well as providing farmers with reliable productivity, these varieties will also help control the spread of the disease to non-resistant but less attractive varieties. In addition, an alternate fodder grass, *Brachiaria* cv Mulato II was also identified as resistant to NSD. *Brachiaria* is used as a drought-tolerant trap crop in climate-smart push–pull technology.

Brachiaria cv Mulato II, a drought-tolerant fodder grass, is resistant to NSD. It is widely used in climate-smart push–pull technology in eastern Africa.

Building on the foundations of farmer participation

Bungoma farmer Peter Waboya remembers the moment in 2008 when he first learned about the phytoplasma causing NSD from *icipe* field technicians. It was just one episode in his long relationship with *icipe*. He adopted push–pull in 2006 and has become a great champion of the technology, heading the Simana Push–Pull Farmer Field School. This teaches groups of farmers about the technology and how it works, and supports them in implementing it.

Peter is one of a network of experienced farmer leaders and peer educators that has been built up to disseminate push–pull technology. It was to this network that *icipe* turned for hosting on-farm trials of the new resistant varieties of Napier grass. The knowledge, skills and relationships already in place meant that these farmers were poised to multiply and share new plant material as soon as it had passed the necessary screening procedures.

When Peter was invited to Mbita Point to share his experiences of NSD and learn about on-going research, he used his training in making participatory videos to film parts of his visit. This footage became part of the short film he has made to train other farmers on the causes and management of NSD.

Opportunities and challenges in extending stunt-resistant Napier grass

In Butere district, Kenya, David Omurumba and his neighbour Elizabeth Atieno are members of the Waaminifu self-help group. Both hosted on-farm trials of the resistant Ouma 2 and South Africa Napier grass varieties. In September 2013, they got the go-ahead to multiply the plants for distribution to other farmers. The group gathered for a field day on David's farm, and all 26 members will plant push–pull and single stand Napier fodder using cane cuttings from the plot.

Sadly, the fate of Elizabeth's trial crop has not been so positive. Such is the desperation for fodder in this area that one night thieves came and harvested the lush new growth of Napier grass. This loss of vital plant material serves to demonstrate the severity of NSD's impact on rural livelihoods.

Rampant NSD left David Omurumba so disillusioned that he uprooted his entire Napier grass crop in 2012. He hopes that the South Africa (in the background) and Ouma 2 varieties that have been field-tested on his farm will end the severe shortage of fodder in Butere district.

On-going research is essential

The identification of the phytoplasma, *Maiestas banda* Kramer, and two NSD-resistant Napier grass varieties are important achievements, milestones on the road towards an effective and robust management strategy for the disease.

A priority now is to ensure that the widest possible extension of the resistant varieties is undertaken in the context of adequate training. There is a need to introduce proper hygiene practices, and to fingerprint the resistant varieties to avoid any future mixture with susceptible varieties.

But on-going research into NSD remains urgent. It is vital that strategies to reduce stunt in Napier grass must not cause the shift of the phytoplasma to other crops. Many cereal crops – maize, millet and rice – are in the grass family, potential hosts to *Maiestas banda* Kramer. Further analysis of NSD phytoplasma DNA in rice and millet has shown that it can indeed infect these important food crops.

Participants in on-farm trials of South Africa and Ouma 2 host a visit from Dr Linnet Gohole (centre, with handbag), the Regional Representative of the McKnight Foundation, which funded the NSD research, and Dr Francis Muyeho of the Kenya Agricultural Research Institute (standing third from left).

Diseased and healthy specimens of *Cynodon dactylon* (left) and *Hyparrhenia rufa* (right) show clearly how NSD also affects other grasses. This means that the spread of the disease could lead to the infection of food crops in the *Poaceae* (*Gramineae*) family.

The research needed to avert this risk is into the epidemiology of the disease in the context of the agro-ecosystems where it is found. This includes searching for wild grasses in the field which are susceptible to infection with NSD, but also those which may host the leafhopper and the phytoplasma without developing the disease, becoming a source of infection for valuable crops.

The team of scientists at *icipe* will continue working on these challenges in research and extension, in partnership with the farmers who face a daily struggle with the pests and diseases of their crops. Together, they will ensure that the focus of the research agenda is on farmers' real needs.

Acknowledgements

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