THE PATHOSYSTEM OF NAPIER STUNTING DISEASE IN WESTERN KENYA

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EGERTON UNIVERSITY

DECLARATION AND RECOMMENDATION

1. DECLARATION

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

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2. RECOMMENDATION

This thesis has been submitted with our recommendation as supervisors.

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DEDICATION

To my dear Dad Mr. Joseph Obura for his hard work, the role of a model and an inspiring father. My beloved mother Monica, my siblings: Caleb, Herine, Ruth, Tereza, Hellen, Daniel and Walter; my cousin Patrick and my uncle Philip, thanks for your support.

ABSTRACT

Napier stunting (Ns) disease, caused by Rice Yellow Dwarf (RYD) phytoplasma, is a big threat to Napier grass production and food security in Kenya. The disease destroys fodder completely, with a devastating impact on livestock farmers. To control the disease, this study described the four components of Ns-disease pathosystem; phytolasma itself as the causative pathogen, the host plant susceptible to phytoplasma, the insect as a competent vector, and the alternative sources of phytoplasma inoculum in western Kenya. The study also developed quicker, more economic and robust methods for diagnosis of Ns-disease. The vector was identified through laboratory transmission experiments with field collected insects, while the alternative host grasses were discovered by molecular detection and characterization of natural phytoplasmas in symptomatic grasses. The local Napier grass germplasm characteristics were studied for genetic diversity and Ns-disease response using Amplified Fragment Length Polymorphism (AFLP) technique and laboratory transmission experiments. Molecular diagnosis of Phytoplasma in test plants and insects was performed by nested polymerase chain reaction (PCR) and loop mediated isothermal amplification of DNA (LAMP), based on phytoplasma 16S and ITS gene sequences. The study discovered that Ns-disease is transmitted by insect vector *Maiestas (=Recilia) banda* (Homoptera: Cicadellidae). The vector showed feeding preference to closely related pennisetum species: Napier grass and Pearl Millet. The RYD phytoplasma was transmissible to Cereals under laboratory conditions. It was also discovered in wild Thatching grass *Hyparrhenia rufa,* where it is pathogenic. A closely related phytoplasm; Bermuda grass white leaf (BGWL), was also discovered in pasture grass *Cynodon dactylon.* The phytoplasma therefore circulates between Napier grass and other wild grasses corresponding to a polycyclic epidemic. AFLP analysis divided the local Napier grass germplsm into 4 genetically distinct groups; however, the quality of this germplasm as source of resistance to Ns-disease was poor, and none of the varieties showed resistance to phytoplasma infection, which explains the high prevalence of Ns-disease in western Kenya. Based on Ns-disease pathosystem, this study recommends the following disease control strategies: elimination of infected plants (Napier grass, Bermuda grass and Thatching grass), certification of planting material and control of the insect vector *M. banda*. There is also need to extensify resistance screening to a much wider germplasm, both wild and improved varieties.

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ACRONYMS & ABBREVIATIONS

16S rRNA	16S ribosomal RNA, a component of the 30S subunit of prokaryotic ribosomes
23S rRNA	23S ribosomal RNA, a component of 30S subunit of prokaryotic ribosomes
AAP	Acquisition access period
AFLP	Amplified Fragment Length Polymorphism
AFLP	Amplified fragment length polymorphism
AFRNET	African Feeds Resources Network
ANOVA	Analysis of variance
BGWL	Bermuda grass white leaf
BLAST	Basic Local Alignment Search Tool
BOLD	Barcode of life database
bp	Base pairs, unit for measuring DNA size
BSU	Biosystematics support unit
Ca. P.	*Candidatus* Phytoplasma, or the genus of phytoplasmas
cDNA	Complementary DNA
COI	cytochrome c oxidase I gene
CTAB	Cyltrimethylammonium bromide
ddH_2O	Distilled and deionized water
DIG	Digoxygenin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Dinucleotides
DSR	Daily survival rate
EDTA	Ethylenediaminetetracetic acid
EMBL	European Molecular Biology Laboratory
FB-HWT	Farm based hot water treatment
GS	Genetic similarity
HCl	Hydrochloric acid
HGWL	Hyparrhenia grass white leaf
HSD	Highest significant difference
HWT	Hot water treatment
ICSB	lnternational Committee on Systematic Biology
ILRI	International Livestock Research Institute
IRPCM	International Research Program on Comparative Mycoplasmology
KARI	Kenya Agricultural Research Institute
LAMP	Loop mediated isothermal amplification of DNA
mtDNA	Mitochondrial DNA
mM	Milli Molar
MSV	Maize streak virus
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
Ns	Napier stunting
NPV	Negative predictive value
PCA	Principal component analysis
PCO	Principal coordinates

PCR	Polymerase chain reaction
PPV	Positive predictive value
REPs	Repetitive palindromes
RH	Relative humidity
SD-HWT	Short duration hot water treatment
SDS	Sodium Dodecyl Sulphate
SSC	Tri-sodium citrate
T_a	Annealing temperature of an oligonucleotide
TAE	Buffer made of Trizma base Acetate and EDTA salt
T_m	Melting temperature of an oligonucleotide
Tris–EDTA	Buffer made of Trizma base and EDTA salt
Trp	Trypophan
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
µl	Microlitre
µM	Micromolar

CHAPTER ONE

INTRODUCTION

1.1 Background to the problem

Napier or Elephant grass *Pennisetum purpureum* Schumacher, is a robust perennial forage originating from Africa. It grows in bamboo-like clumps (Anderson et al. 2008) and may reach 10 m in height (Boonman, 1997). It was named after colonel Napier of Bulawayo, who in the early last century, championed its adoption as livestock fodder in the colonial Rhodesia, now Zimbabwe (Boonman, 1993). The grass was introduced in East Africa by European settlers as mulch for Coffee, but farmers found it more efficient as fodder for livestock. Currently, the grass is the principal fodder crop in all small holder livestock production systems in East Africa (Staal et al. 1998), and the demand is raising, mostly among the poor households in densely populated areas of Central, Western, Nyanza, Rift Valley and Coast provinces. These are areas where demographic pressure has lead to the degradation or extinction of communal resources. These areas are common in the most fertile highland and midland agro-ecological zones of Sub Saharan Africa (SSA), of which western Kenya is probably one of the most conspicuous examples (Braun et al. 1997). The challenge of achieving agricultural sustainability in the densely-populated regions poses strong demands to design small holder agriculture. In western Kenya, the farmers grow cereals intercropped with legumes and keep dairy cows of exotic breeds. Farms are small, with an average holding size of 0.9-2.0 ha (Gitau et al. 1994), and the animals are confined in stalls and fed on cultivated fodder, mainly Napier grass (Potter, 1987).

1.2 Statements of the problem

Napier grass has been known to have high susceptibility to the snow mold *Beniowskia sphaeroidea* (Boonman, 1993), and smut fungus *Ustilago kamerunensis* (Orodho, 2006). However, the two fungal diseases have very low incidence in western Kenya, and therefore do not threaten the overall productivity of Napier grass in the region. Recently, another disease of Napier grass called: Napier stunting (Ns) disease, caused by a phytoplasma, appeared in the farmers fields in western Kenya (Jones et al. 2004). The disease leads to severe stunting and lethal yellowing of affected grasses. It leads to reduction in fodder yield by up to 90% in affected

farms (Lusweti et al. 2004; Mulaa et al. 2004), and the disease is a threat to Napier grass production and food security in western Kenya, where the grass is the main fodder for smallholder livestock farmers. The disease is also a threat to the continued utility of Napier grass as a trap plant in the 'push-pull' strategy to control Cereal stem-borer insect pest (Khan et al., 2008). The phytoplasma causing Ns-disease is closely related to those that cause Rice Yellow Dwarf, Sorghum grassy shoot, Sugarcane white leaf and Sugarcane Yellow leaf (Jones et al. 2004, 2006). Therefore, there is risk that the disease will spread to infect Sugarcane, Rice, Sorghum and other wild grasses in the ecosystem leading to food insecurity in Kenya.

1.3 Objectives

The main objective of this study was to describe the pathosystem of Ns-disease in western Kenya. The specific objectives were:

i. To identify the insect vector(s) of Napier stunting disease,
ii. To determine the vector(s) host grass preference,
iii. To develop simple, rapid and sensitive Napier stunting disease diagnostic tool(s),
iv. To identify the alternative host grasses of Napier stunting disease,
v. To determine the genetic diversity of local Napier grass germplasm,
vi. To describe the response of local Napier grass varieties to phytoplasma disease,

1.4 Justification of the study

Napier grass is the principal livestock fodder for smallholder livestock farmers (Orodho, 2000). In 2004, Napier stunting (Ns) disease, caused by phytoplasma, appeared in Napier grass farms in western Kenya. The disease destroys fodder completely, with a devastating impact on farmers (Lusweti et al. 2004; Mulaa et al. 2004), Like other phytoplasma diseases, Ns-disease is maintained by natural disease cycle consisting of four components: the phytoplasma itself as the causative pathogen, the host plant susceptible to the causative pathogen, the insect as a competent vector feeding on the host plant, and alternative grasses which are significant as reservoir of phytoplasma inoculum. For Ns-disease, the insect vector, and wild reservoir grasses were unknown. The threat of Ns-disease to cereals was also unknown. The genetic diversity of local Napier grass germplasm, and its potential as source of Ns-disease resistance was also

unknown. There was also need to develop robust diagnostic tools to monitor active and latent phytoplasma infections in host plants and insect vector.

1.5 Research Questions

i. Which insect vector(s) are involved in Napier stunting disease pathosystem?
ii. Is the vector a polyphagus insect, capable of transmitting phytoplasma to many grasses in the ecosystem?
iii. Do alternative molecular diagnostic tools exist for Napier stunting disease?
iv. Are wild grasses and cereals involved in the pathosystem of Ns-disease?
v. Does diversity exist in the local Napier grass germplasm?
vi. How do the local verities respond to Napier stunting disease?

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CHAPTER TWO

LITERATURE REVIEW

2.1 The dairy sub-sector in Kenya

Kenya's smallholder dairy system, with an estimated 3 million dairy herds, is the largest in Sub-Saharan Africa (Orodho, 2006). These smallholder dairies in Kenya contribute over 80 percent of the total marketed milk output (Thorpe et al. 2000). They are self sufficient, as milk produced is sold for cash, and the manure from the dairy units is used to fertilize food crops. They are concentrated in the high dairy potential areas, also characterized by high human population. Farms are small, with an average holding size of 0.9-2.0 ha (Gitau et al. 1994), and the animals are confined in stalls and fed on cultivated fodder (Fig. 1), mainly Napier grass (Orodho, 1990). Many of the stall feeding units are found among the poor households in densely populated areas of central, western, coastal regions and in the peri-urban centers (Smith and Orodho, 2000). The demand for Napier grass fodder has intensified in these areas, with some plots established by the road sides. Other miscellaneous feeds include maize crop residues, compound feeds, milling by-products, sugarcane tops, banana pseudo-stems, as well as other grasses and weeds (Staal et al. 1999).

2.2 Napier grass

2.2.1 Origin and Characteristics

In East Africa, Napier grass (Fig. 2) was promoted by European settlers in Uganda for soil conservation and for mulching coffee (Acland, 1971), but farmers found it more useful as livestock fodder. It is a robust perennial grass, which grows in bamboo-like clumps (Anderson et al. 2008) and may reach 10m in height (Boonman, 1997). By 1983, about 240,000 ha or 4 percent of the arable land of Kenya was under Napier grass production. It is the principal fodder for smallholder livestock systems in East Africa (Lekasi, 2000). The grass is valued by farmers for its high biomass yield, perennial nature, high leaf nutritive value and pest resistance (Bhandari et al. 2006). The dry matter yields surpass most tropical grasses (Humphreys, 1994). It also withstands defoliation, and under good weather conditions it can be cut in every 6-8 weeks giving up to 8 cuts in a year. Apart from fodder, the grass is grown widely in the region to

control soil erosion (Acland, 1971), and as trap plant in the widely adopted 'push-pull' technology to control cereal stem borer insects (Khan et al. 2008).

2.2.2 Napier grass germplasm in Kenya

Napier is a shy breeding grass, and the seeds have low genetic stability and viability (Humphreys, 1994), and effort to develop Napier grass seeds for farmers failed (van Gastel, 1978), therefore seeds are not available. The grass is established vegetatively from stem cuttings or crown divisions. Napier can form a hybrid with closely related pennisetum species. It has been hybridized with the Pearl millet *P. glaucum* to produce the Pakistani Napier hybrid, sometimes called 'Bajra' (Orodho, 2006). In Kenya, several Napier grass varieties have been collected locally, introduced from other African countries or improved through selection. The varieties have been selected for the number of tillers produced, plant geometry, dry matter yield, plant height, hairiness of leaf and stem, flowering and resistance to fungal disease. Most of the varieties look morphologically similar; this has lead to serious confusion of varietal names, with several varieties circulating under more than one name (Orodho, 2006).

2.2.3 Pests and diseases

Napier grass is susceptible to snow mold and smut fungus. These two fungal diseases were contained by introduction of resistant varieties (Orodho, 2006). In 2004, a new disease, Napier stunting (Ns) disease (Fig. 3), caused by phytoplasma pathogen, appeared in the farmers fields in western Kenya (Jones et al. 2004). The disease is caused by the Rice yellow dwarf (RYD) phytoplasma, a member of the 16SrXI group. The closest relatives to Ns-phytoplasma are those associated with sugarcane white leaf (SCWL), sugarcane grassy shoot (SCGS), annual blue grass white leaf (ABGWL), Bermuda grass white leaf (BGWL) and Brachiaria grass white leaf (BraWL) phytoplasmas (Tran-Nguyen et al. 2000), therefore, there is a potential threat of phytoplasma epidemic in these grasses. Infected Napier grass turns yellow, stunted and gradually starts to die. Although a number of factors determine disease progression, plants infected early in the planting, express symptoms in the second cutting, and die by the first year (Orodho et al. 2006). Ns-disease was early reported in eastern Uganda (Tiley, 1967), and has spread eastwards to Kenya (Jones et al. 2004) and southwards to Tanzania (Nielsen et al. 2007).

Figure 1: A zero-grazing livestock unit in Bungoma, western Kenya.

Figure 2: Napier grass plot in Busia, western Kenya.

2.3 Phytoplasmas

2.3.1 Phytoplasma and their relatives

The genus *Candidatus* Phytoplasma, abbreviated as *Ca.* P. is a group of pleomorphic bacteria in the class Mollicutes, which inhabit the phloem of many plant species (IRPCM, 2004). Phytoplasma were first observed in ultrathin sections of Mulberry plants showing yellows symptoms (Doi et al. 1967). Among the Mollicutes, they are closely related to *Acholeplasma laidlawii*, in which the triplet coding for tryptophan (trp) is UGG. Moreover they are genetically distinguishable from other Mollicutes because they have a spacer region (about 300bp) between the 16S and 23S ribosomal regions (McCoy et al. 1989).

2.3.2 Economic importance of phytoplasmas

Phytoplasmas are associated with more than 600 distinct plant diseases worldwide (McCoy et al. 1989). They severely affect grasses, herbaceous and woody plants (Bertaccini, 2007), and are the primary limiting factor to the production of many important crops worldwide. In grasses, phytoplasma infection results to yellowing and bushy growing habit. It is hypothesized that yellowing is due to inhibition of photosynthesis, especially photosystem II, and breakdown of chlorophyll and carotenoids (Bertamini and Nedunchezhian, 2001). The bushy or witches' broom appearance of many infected grasses is due to changes in normal growth patterns related to the loss of apical dominance. Studies on phytoplasma genome reveals that they lack the known virulence genes (such as *hrp*) found in other pathogenic bacteria (Oshima et al. 2004). However, they are known to lower phloem translocations, and because they lack most common metabolic pathways, they are highly host dependent with detrimental effects. They also deplete sugars (Maust et al, 2003), photosynthetic pigments and soluble proteins (Musetti et al. 2005) in affected plants. Their infections also lead to alterations in the hormone balance (Maust et al. 2003).

Figure 3: Diseased Napier grass: leaf yellowing, bushy appearance and stunting.

2.3.3 Transmission of phytoplasmas

They are naturally transmitted by phloem feeding insects in the order Hemiptera, mainly leafhoppers, planthoppers and Psyllids (Fig. 4). Some insects may transmit phytoplasma trans-ovarially to their off springs (Tedeschi et al. 2006). The pathogen is also spread through parasitic plant dodder (*Cuscuta* sp.), grafting of infected plants, and vegetative propagation (Razin, 2007). More recently, the pathogen was found to be disseminated via the seed in coconut *Cocos nucifera* (Cordova et al. 2003), alfalfa *Medicago sativa*, lime *Citrus aurantiaca* and tomato *Lycopersicum esculentum* (Botti and Bertaccini, 2006).

2.3.4 Movement in plants

Phytoplasmas lack genes coding for cytoskeleton elements such as flagella or cilia (Christensen et al. 2005), therefore, active movement by these organisms is unlikely. In plants, phytoplasmas are thought to move systemically using the sieve tube system. Their small pleomorphic sizes make them pass freely through the sieve pores together with assimilate. Phytoplasmas never settle in the meristems, and they are unevenly distributed in host plants. Generally, levels are lower in roots (sink organ) and moderate in the stems. The highest titer is found in source organs (mature leaves) (sometimes with a titer ≈40 times higher than that of the roots) (Bertaccini & Duduk, 2009).

2.3.5 Diagnosis and identification

Preliminary diagnosis of phytoplasma is by observation of symptoms, and electron microscopy on ultra-thin sections of the phloem tissue. Currently, nested polymerase chain reaction (Nested-PCR) is the gold standard for detection of phytoplasma. It is sensitive and specific even in samples with unusually low phytoplasma titers or inhibitors (Gundersen et al. 1994). Nested-PCR is performed by preliminary amplification using a universal primer pair followed by a second amplification using a second universal primer pair (Lee et al. 1994; 1995). Many primers based on 16S rRNA gene (Fig. 5), ribosomal protein gene operon, *tuf* and *SecY* genes have been designed for the detection, identification, and classification of phytoplasmas (Wei et al. 2004). Because they are uncultivable, phytoplasmas are characterized by phylogenetic analysis of 16S rRNA gene sequences, and a strain can be recognized as a novel *Ca.* P. species if its 16S rRNA

gene sequence has <97.5% similarity to that of any previously described *Ca.* P. species. So far, 33 *Ca.* P. species have been proposed (Zhao et al. 2009).

2.3.6 Genes and genomics properties

Phytoplasmas have small genomes with G and C content as low as 23%, which is the threshold for a viable genome (Razin, 2007). They lack many genes for standard metabolic functions. Their genome comprises two rRNA operons, large numbers of transposon genes, insertion sequences and a unique family of repetitive palindromes (REPs) called PhREPS which may play a role in transcription termination or genome stability (Jomantiene et al. 2007). Phytoplasmas also have plasmids. Some small plasmids may be of viral origin, as some of them share significant sequence similarity with genes in plant geminiviruses (Nishigawa et al. 2002). The genes encoded in plasmids are known to play important roles in the pathogenicity of many plant pathogenic bacteria.

2.3.7 Control of phytoplasma diseases

Many phytoplasmas have been managed by spraying insecticides to eradicate the insect vectors. However, the efficacy of insecticidal spraying is quite low, and severity of phytoplasma diseases intensify, despite extensive use of insecticides (Firrao et al. 2007). Phytoplasma free plants can be raised by meristem tip culture, use of antibiotics (Bertaccini, 2007), and by hot water treatment (HWT) (Caudwel et al. 1997). In the ecosystem, phytolasma inoculum can be removed, more so for the phytoplasmas transmitted by monophagous vectors, feeding on infected plants. Antibiotic therapies have been suggested. However, the antibiotics are too costly, prohibited in several countries, and do not always provide long-time control. Some work has gone into the production of transgenic plants producing antibodies with minimal success (Chen and Chen, 1998; Le Gall et al. 1998). A more practical approach is by using clean plant material or by finding phytoplasma-resistant varieties.

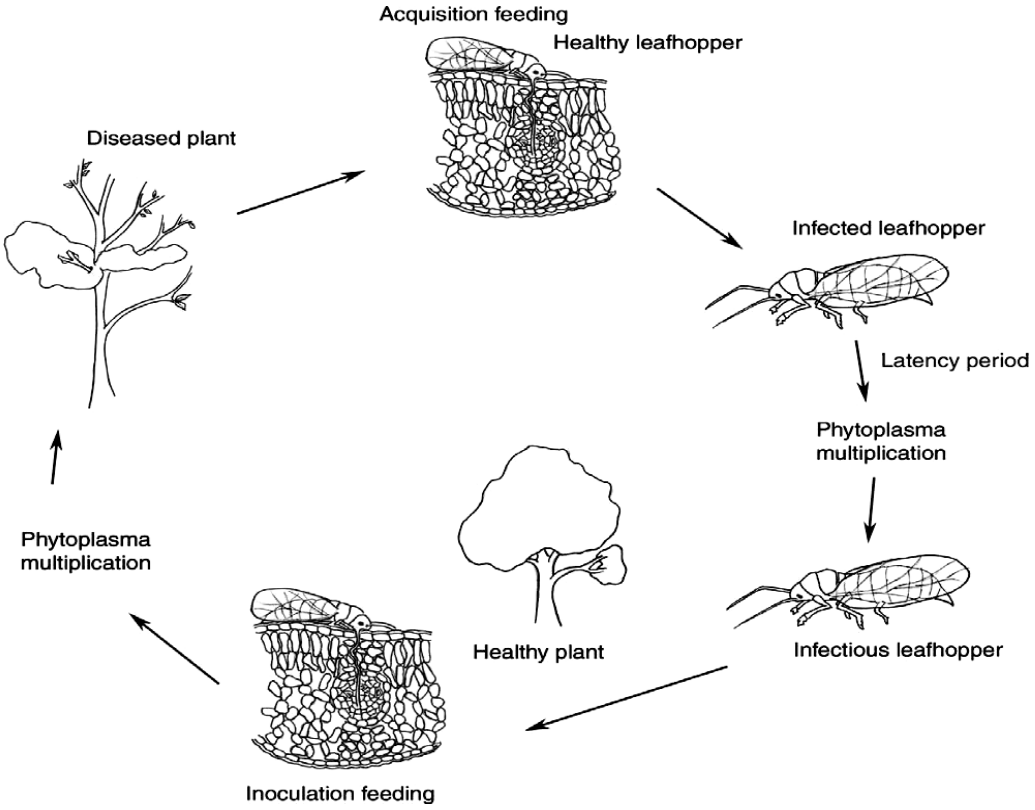

Figure 4: Natural cycle of phytoplasma diseases (Weintraub and Beanland, 2006)

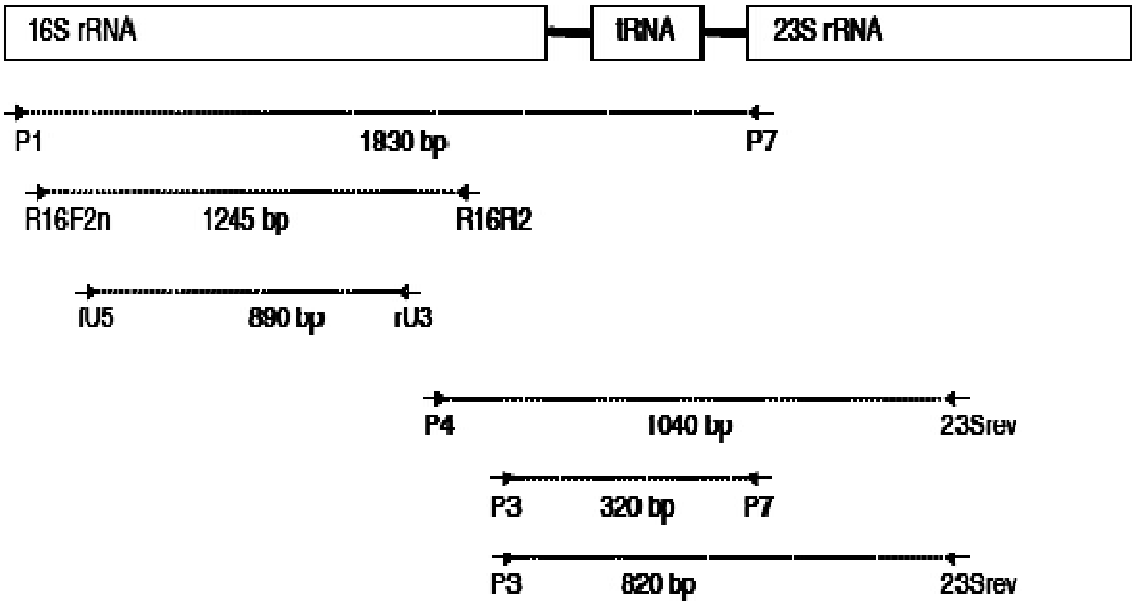

Figure 5: Ribosomal RNA operon showing the 16S, tRNA region and 23S. The primer names, their annealing sites and fragment sizes amplified are shown (Lee et al. 1993).

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CHAPTER 3

INSECT VECTOR OF NAPIER STUNTING DISEASE

3.1 Introduction

Phytoplasmas are naturally transmitted by sap-sucking insect vectors in order Hemiptera, mainly the leafhoppers, plant hoppers and Psyllids, which transmit the pathogen in a persistent propagative manner (Weintraub and Beanland, 2006). The pathogen is also disseminated by vegetative propagation of infected plant material, by parasitic plants and by grafting of infected material (Dale and Kim 1969). Since there was no parasitic plant associated with Napier grass, the epidemic was spreading through infected Napier grass and/or through the insect vectors. With assistance from the laboratory of Entomology, Tokyo University of Agriculture in Japan and the National Museum of Wales in the UK, ICIPE had identified five leafhopper (*Glossocratus afzelii* (Stäl), *Cofana polaris* Young, *Cofana spectra* (Distant), *Maiestas banda* Kramer, and *Cicadulina mbila* Naude) and three planthopper species (*Leptodelphax dymas* Fennah, *Thriambus strenuus* Van Stalle, and *Sogatella manetho* Fennah) to be closely associated to Napier grassin western Kenya. Using Safranine dye technique developed by Khan and Saxena (1984), all the insects probed phloem and could potentially transmit Ns-disease.

The chapter describes the use of natural transmission experiment to identify the vector of Ns-disease among these plant and leafhoppers closely associated with Napier grass. The vector competence was further confirmed by artificial feeding experiment on Sucrose TE-media. The vector was also tested for vertical transmission of phytoplasma through the ovary to the egg and finally to the offsprings of infected female insects. It was also genetically coded for taxonomic identification. Additional study on the vector was further conducted to determine its interaction with the common grasses in western Kenya, especially whether it was a generalist or a specialist feeder. This was done by investigating the vector survival, population build-up, and growth on 32 common grasses in western Kenya.

3.2 Materials and methods

3.2.1 Insects and Plants

Eight plant and leafhoppers (Table 1) were reared on potted stunting-free 40–50-day-old Napier grass (Bana variety) in the screen house, at 20–28°C and 65–70% RH, in separate cages (25×25×60 cm) made of wooden frame. Top and side openings of the cages were covered with fine nylon mesh for aeration. Periodically, these colonies were infused with insects collected from the field to minimize inbreeding. Diseased Napier grass (Bana cultivar) accessions susceptible to Ns-disease were obtained from Mabanga (0°45′ S, 34°34′ E). Diseased plants were provisionally identified by foliar symptoms, carefully removed from the ground, potted, labeled, and maintained in separate sections of similar insect-free screen house. Healthy Napier grass (Bana cultivar) were obtained from Kitale (1°0′ N, 35°7′ E), Trans Nzoia district, from where the incidence of Ns-disease had not been reported. The healthy plants were also maintained in a separate screen house away from the cultured insects. All plants were tested for the presence of phytoplasma by nested polymerase chain reaction (nPCR). For the studies on vector host-plant preference, the seeds of cereals: *Sorghum bicolor, Oryzae sativa* and *Pennisetum glaucum* were obtained from the local farmers, while the other grasses tested (Table 5) were collected from Napier grass agro-ecology and kept in the screen house until the experiment. For experiments, the grasses were established in 12 cm diameter plastic pots and used for the experiments at the 4–6 leaf stage.

3.2.2 Natural transmission experiments

Using an aspirator, a total of ten gravid females of each insect species were obtained from the healthy colonies in cages and reared on diseased Napier grass for 30 days to acquire stunting phytoplasma (acquisition feeding) (Weintraub & Beanland, 2006). The insects oviposited on the diseased Napier grass and the emerging nymphs were allowed to feed on the same plants and similarly acquire the phytoplasma. After the 30 days, four pots of healthy Napier plants were introduced into the same cage and exposed to the adult insects and their nymphs for additional sixty days. Surviving insects were sampled from each cage after 60 days, fed on healthy Napier grass for 4 days and then kept at −20°C, after which two insects were pooled at a time for phytoplasma detection by nested PCR as described in protocol 3.2.3. The nested PCR products

were characterized by DNA sequencing and sequence analysis. The exposed plants were then pruned for the appearance of re-growth and incubated for another 30 days. Their leaf cuts were then sampled for DNA isolation and phytoplasma detection by nested PCR. The plants were quarantined until the appearance of well-developed stunting symptoms. The experiment was repeated three times for each insect species. A setup of unexposed healthy plants was used as a control setup.

3.2.3 Phytoplasma diagnosis in plants and insects

Phytoplasma was diagnosed in test plants and insect vectors by nested PCR. DNA template from plant and insect tissue was prepared for PCR analysis using the protocol of Doyle and Doyle (1990) with slight modifications. 300mg of leaf tissue was powdered in liquid nitrogen, 600µl cetyltrimethylammonium bromide (CTAB) buffer at 65°C was added (CTAB buffer, 2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris–HCl, pH8.0, 0.2% 2-mercaptoethanol), then the mixture incubated at 65°C for 30 min. DNA was extracted with equal volume of chloroform: Isoamyl alcohol (24:1) and precipitated overnight in a −20°C freezer. Following centrifugation at 14,000 rpm, the DNA pellet was rinsed twice with 70% ethanol, dried and dissolved in 50µl Tris–EDTA pH8 buffer. The quantity and quality of the isolated DNA was estimated by electrophoresis on ethidium bromide stained, 0.8% agarose gel. DNA from the insects was similarly extracted but the buffer contained 3% CTAB. Nucleic acid (20ng/µl) was used for amplification by nested PCR. The PCR and nested PCR reactions were performed according to published protocols (Lee et al. 1998), using primer pair P1/P6 (Deng and Hiruki 1991) and nested PCR with R16F2n/R16R2 (Lee et al. 1993) primers, in a PTC 100 programmable thermocycler (MJ Research). DNA amplified in PCR primed by P1/P6 was diluted 1:50 with sterile water and vortexed to mix, and 1µl used as a template in a nested PCR. PCR products were detected in a 1% ethidium-bromide stained agarose gel using 1× TAE (40 mM Tris acetate, 1 mM EDTA pH8.0) as running buffer, and photographed.

3.2.4 Phytoplasma classification

The identities of phytoplasma isolated from the insects and Napier grass were determined by direct sequencing and phylogenetic analysis. The phylogeny of the isolates was based on sequence homology with the sequences of phytoplasma previously isolated from Napier grass in

eastern Africa. Nested PCR products (1.2 kb in size) from infected Napier grass and insect vector species were gel purified using QIAquick PCR purification kit (Qiagen, Valencia) and sequenced directly (SegoliLab, ILRI). The 16S rRNA gene sequences were aligned for phylogenetic analysis using Mega version 4 (Koichiro et al. 2007). Genetic distances among the nucleotide sequences were calculated using DNA neighbor-joining method (Saitou and Nei 1987) and the tree viewed using Java 6.0 software.

3.2.5 Vector host plant preference

The vector identified in 3.2.2 above was tested for survivorship, population buildup and growth on 32 common grass species in western Kenya. For survivorship studies, each of the 32 grass species was planted in eight pots each, and then enclosed individually in insect tight cages before being infested with ten female adults newly emerged from diseased Napier grass. The setup was monitored daily for ten days. At day ten, total mortality was determined by counting the number of dead insects in each cage. The adult mortality data was used to calculate the vector Daily Survival Rate (DSR) according to the method of Small and DeMaster (1995), where DSR=1-(#Deaths/#Animal Days), where Deaths is the total number of animals which died at day 10, and Animal days referred to the reporting period which was 10 days in this study.

For growth studies, each grass species was replicated in ten pots, and then each potted grass was transferred to insect tight cage and infested with ten first-instar nymphs emerging from diseased Napier grass diet. Growth was measured by the number of nymphs that became adults and the time taken to reach the adult stage. Growth index was also calculated based on the ratio of nymphs developing into adults to the mean growth period in days (Saxena et al. 1974).

Analysis of variance was used to examine the effect of grass species on vector survival rates, population build-up, and growth index. Pairwise differences in population buildup, survivorship and growth index among the grasses were determined using Student's Newman Keuls (SNK) test. Significance was determined at $P<.05$. All statistical analyses were performed by using R 2.13.0 (2008)

3.2.6 Transovarial transmission

To determine whether Ns-phytoplasma is transmitted vertically by infected female insects, through the egg to their off-springs, ten phytoplasma infected and gravid females were obtained from diseased colony in cages and introduced into a cage of a healthy host plant for 12 hours to lay eggs. After 12 hours, the gravid insects which had laid eggs were removed, and then the plants incubated in insect tight cage, and monitored daily until nymphs appeared. After nymphal appearance, the first instar nymphs were then transferred to a second healthy host plant to develop to adult stage. At adult stage, 12 female insects were sampled from the cage for phytoplasma inoculation by membrane feeding experiment. Membrane feeding was conducted using the original method of Zhang et al. (1998). White microcentrifuge tubes (1.5 ml) were used as insect chambers. Their cylindrical cups were filled with 200 µl of 5% sucrose in TE (10 mM Tris (pH 8.0), 1 mM EDTA) and sealed with Parafilm. The parafilm was tightened to simulate the normal phloem pressure in the plant. The bottom ends of the microcentrifuge tubes were cut, then one insect was placed in each tube, and the cut end was sealed with perforated Aluminium foil. Each tube, containing an individual leafhopper, was kept at 23 to 25°C for 48 hrs in a horizontal position with the cap facing a light source to attract the insects to the feeding medium. The insects feed by probing the parafilm and sucking the diet in the same way in which they normally probe plant tissues and suck phloem sap. DNA was extracted from each feeding medium ration and analyzed by nested PCR for the presence of Ns-phytoplasma using NapF and NapR primers (Table 7). Template was prepared for PCR as follows: The phytoplasma cells were pelleted out of the feeding solution by centrifugation at 12,000 rpm for 15 mins. The liquid phase was poured off, and then 50µl of ddH_2O was added and boiled at 95°C for 5 mins before PCR. After membrane feeding, the insects were tested for phytoplasma infection using the analysis as described in experiment 3.2.3 above. A similar setup on diseased plants served as control experiment.

3.2.7 Vector barcoding

One hind leg of each of the eight insects associated with Napier grass was cleaved, and total DNA isolated by the CTAB protocol of Doyle & Doyle (1990) as described in 3.2.3. Mitochondrial gene; cytochrome *c* oxidase I (COI) was amplified by polymerase chain reaction using primers LCO-1490 (5'-GGTCAACAAATCATAAAGATATTGG-3'), and LCO-2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994). The reaction mixture contained 1µl of template DNA, 0.5µM of each primer, 200µM of each dNTPs, 0.4U of Phusion DNA polymerase (New England Biolabs), 1x PCR buffer (New England Biolabs) and 3% DMSO (New England Biolabs). PCR was performed for 35 cycles in a PTC-100 Thermal cycler (MJ Research, Inc.). Reaction conditions were as follows: 2 min at 98°C, 1 cycle; 1 min at 98°C, 1 min at 45°C, 1 min at 72°C, 35 cycles; and 10 min at 72°C, 1 cycle. Double-distilled water (ddH_2O) was used as a negative control. Amplification was confirmed by electrophoresis on a 1% Agarose gel, stained with ethidium bromide. The COI specific bands were gel purified using QuickClean Gel purification kit (Genescript, Valencia), according to manufacturer's protocol and sequenced directly (Segolilab, ILRI, Nairobi), and the sequences were viewed and edited in Mega version 4. Reference specimens were submitted to the insects' repository (BSU, ICIPE, Nairobi).

3.3 Results

3.3.1 Transmission experiments

After 60 days of exposing healthy plants to phytoplasma transmitting insects, a 1.2 kb DNA fragment was amplified in seven out of 12 plants (58.3%) exposed to a leafhopper *M. banda* and the positive control (Fig. 6). These plants later simultaneously developed Ns-disease symptoms and dried. The unexposed plants in the control setup (Fig. 7), and those exposed to the 7 other insect species (Table 2) remained Ns-disease negative.

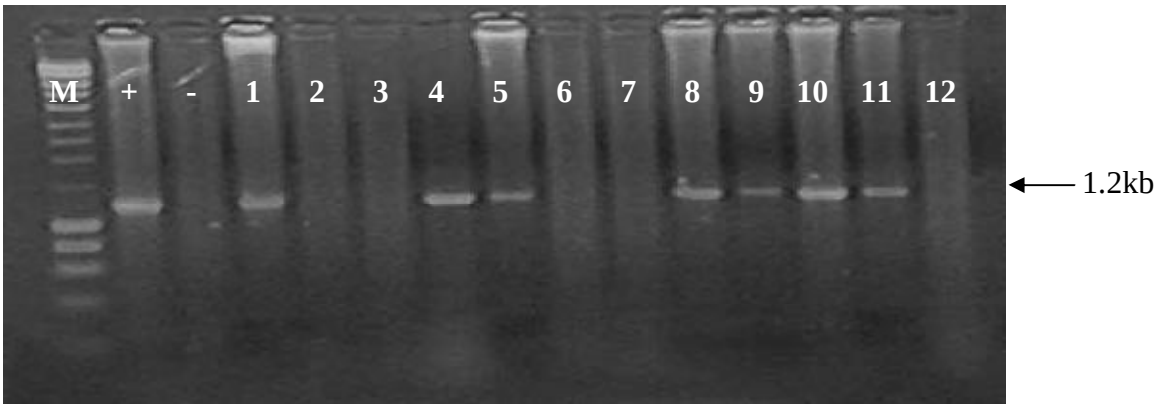

Figure 6: Ethidium-bromide stained agarose gel of nested PCR on DNA from plants 90 days after exposure to *Maiestas (=Recilia) banda*, Lanes: M, HyperLadder1 (Bioline); (+), positive control; (−), negative control; (1–12), healthy plants after exposure to *M. banda*. Note the 1.2 kb specific amplification in seven out of 12 samples.

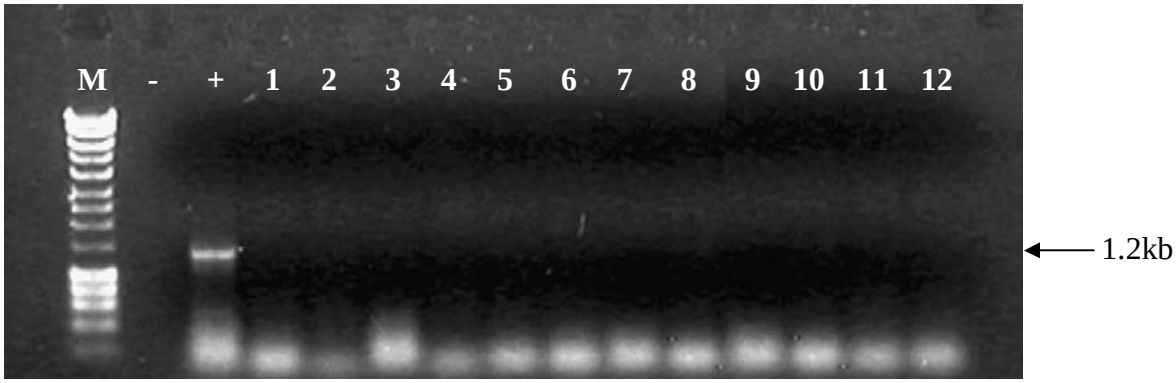

Figure 7: Ethidium-bromide stained agarose gel of nested PCR products of control plants. Lanes: M, HyperLadder1 (Bioline); (−), negative control; (+), positive control; (1–12), healthy plants before exposure to *Maiestas (=Recilia) banda*

Similarly, 1.2kb phytoplasma-specific DNA fragments were amplified from three out of five *M. banda* templates (Fig. 8, Table 2). No phytoplasma-specific amplification (1.2 kb) was observed in the templates of the other 7 insect species (Table 2), implying that the pathogen was non-persistent in these insects. Date of exposing plants to the vector was known, therefore, we determined how long the plants stayed before showing Napier stunting disease symptoms. All the plants that were positive for phytoplasma developed symptoms after an average of 120 days from the date of exposure, showing that the disease incubates in the host plants for about 4 months before symptoms appear.

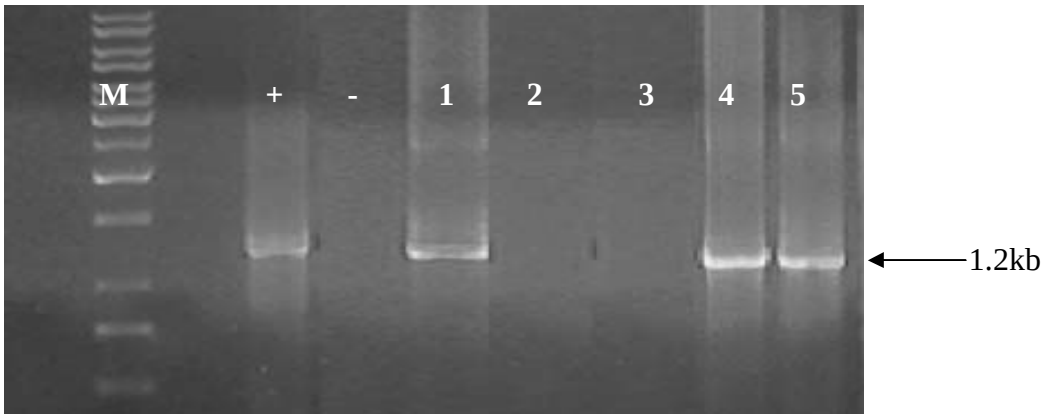

Figure 8: Ethidium-bromide stained agarose gel electrophoresis showing results of nested PCR on DNA isolated from *Maiestas (=Recilia) banda* samples used in the transmission experiment. Lanes: M, 1 kb molecular weight marker (Fermentas); (+), positive control; (−), negative control; (1–5), *M. banda* samples.

Table 1: Disease status of Napier grass and insects before and after exposure to phytoplasma.

Insect species	Napier grass		Insects	
	Before Exposure	After Exposure	Exposed	After exposure
Cofana spectra (Distant)	(12)(-)	(12)(-)	(5)(+)	(5)(-)
Cofana Polaris Young	(12)(-)	(12)(-)	(5)(+)	(5)(-)
Glossocratus afzelii (Stäl)	(12)(-)	(12)(-)	(5)(+)	(5)(-)
Leptodelphax dymas Fennah	(12)(-)	(12)(-)	(5)(+)	(5)(-)
Maiestas banda (Kramer)	(12)(-)	(12)(7+)	(5)(-)	(5)(+3)
Thriambus strenuus Van Stalle	(12)(-)	(12)(-)	(5)(+)	(5)(-)
Sogaiella manetho Fennah	(12)(-)	(12)(-)	(5)(+)	(5)(-)
Cicadulina mbila Naude	12(-)	(12)(-)	(5)(+)	(5)(-)

(#), number of plants or insects; (+) phytoplasma positive; (-), phytoplasma negative

3.3.2 Phytoplasma classification

Figure 10 shows the leafhopper vector *M. banda*. The 16S rDNA sequences of phytoplasma isolates from the vector and Napier grass are available in GenBank under accessions: FJ862999, FJ862998, and FJ862997. Figure 9 shows a phylogram constructed based on the isolates and three other sequences of phytoplasma previously isolated from Napier grass (Table 3). The phytoplasmas (GenBank acc. FJ862997, FJ862998 and FJ862999) formed a monophyletic clade

together with 2 phytoplasmas previously isolated from Napier grass in Kenya (Acc. AY377876) and Uganda (Acc. EF012650) showing that they belong to the same species. The phytoplasma isolated from Napier grass in Ethiopia (Acc. DQ305984) clustered differently, showing that it belonged to a different species.

Table 2: Phytoplasma 16S rDNA sequences used for phylogenetic analyses.

Name	Phytoplasma strain designation	Origin	Accession numbers	16Sr group	Reference
Napier grass stunting	Ca. P. oryzae	Kenya	AY377876	16SrXI	Jones et al., 2004
Napier grass stunting	Ca. P. oryzae	Uganda	EF012650	16SrXI	Nielsen et al., 2007
Napier grass stunting	Ca. P. pruni	Ethiopia	DQ305984	16SrIII	Jones et al., 2006

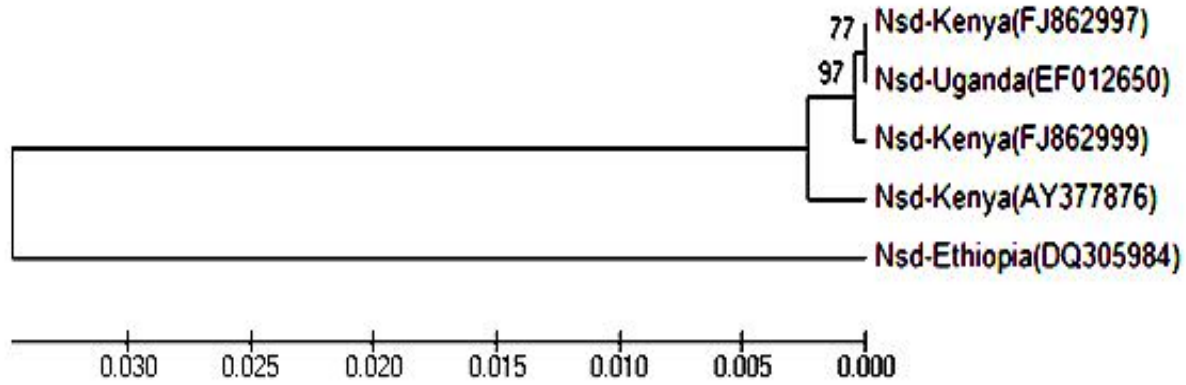

Figure 9: Phylogram showing the similarities between 16S rDNA sequences of phytoplasma isolated from Exposed (treated) Napier grass (acc. FJ862998), *Maiestas (=Recilia) banda* (acc. FJ862999), Diseased Napier grass [(inoculums source) (acc. FJ862997)], and Napier stunting phytoplasma sequences in the GenBank [Kenya: Acc. AY377876; Ethiopia: Acc. DQ305984 and Uganda: Acc. EF012650.

Figure 10: Dorsal view of *Maiestas banda* (Hemiptera: Cicadellidae), a vector of Napier stunting phytoplasma in Kenya

3.3.3 DNA barcoding of insects associated with Napier grass

A 750-bp COI sequence was amplified from the eight insects associated with Napier grass. These COI sequences are shown in Appendix 2, and a phylogram based on these sequences is shown in Figure 11. The reference specimens and other insect information are available at the insects' repository (BSU, ICIPE, Nairobi). The data information includes: Date of collection, collector, collection site and the full phylogeny of the insect species.

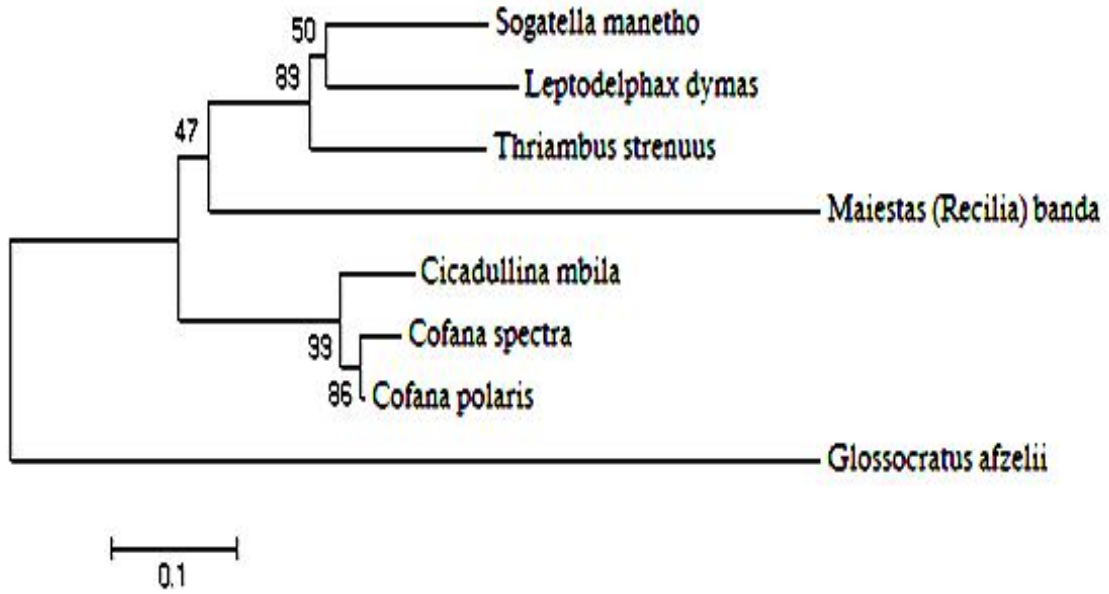

Figure 11: COI DNA phylogenetic tree of insects associated with Napier grass**.**

3.3.4 Transovarial transmission and Membrane feeding experiments

Pearl millet *P. glaucum*, as a suitable host plant was used for egg laying by infected *M. banda* and to raise a colony of phytoplasma free insects. The plant was selected because it is established from the seed making it free from latent phytoplasma infections undetectable by PCR but acquirable by the insect during feeding. Nymphs were observed on the 11th day in both Pearl millet (treatment) and diseased Napier grass (control) setups. However, the nymphs developed faster in pearl millet (15 days) than on diseased Napier grass (19 days) ($F=46.04$; $df=17$; $p=3.18e-066$). Results show that 75% of the insects raised on diseased plants were inoculative (Fig. 12, Table 4).

Table 3: Transovarial transmission and membrane feeding experiment of *Maiestas banda.*

Test plants	Egg period (Days)	Nymphal period (Days)	Insects		Feeding media	
			Positive/ Tested	Infection rate	Positive/tested	Inoculation efficiency
Diseased Napier grass (Control)	11 days	18.8(0.49)	12/12	100%	9/12	75%
Pearl Millet (Treatment)	11 days	15.4(0.16)	12/12	0%	0/12	0%

Phytoplasma analysis of the whole insect specimens after membrane feeding showed that all of them were infected (Fig. 12B). In the treatment setup, whole insect specimens were phytoplasma negative by PCR (Fig. 12D). Likewise, Sucrose-TE media fed to these insects remained devoid of phytoplasma (Fig. 11C, Table 4). The study therefore determined that Ns-phytoplasma is not transovarially transmitted.

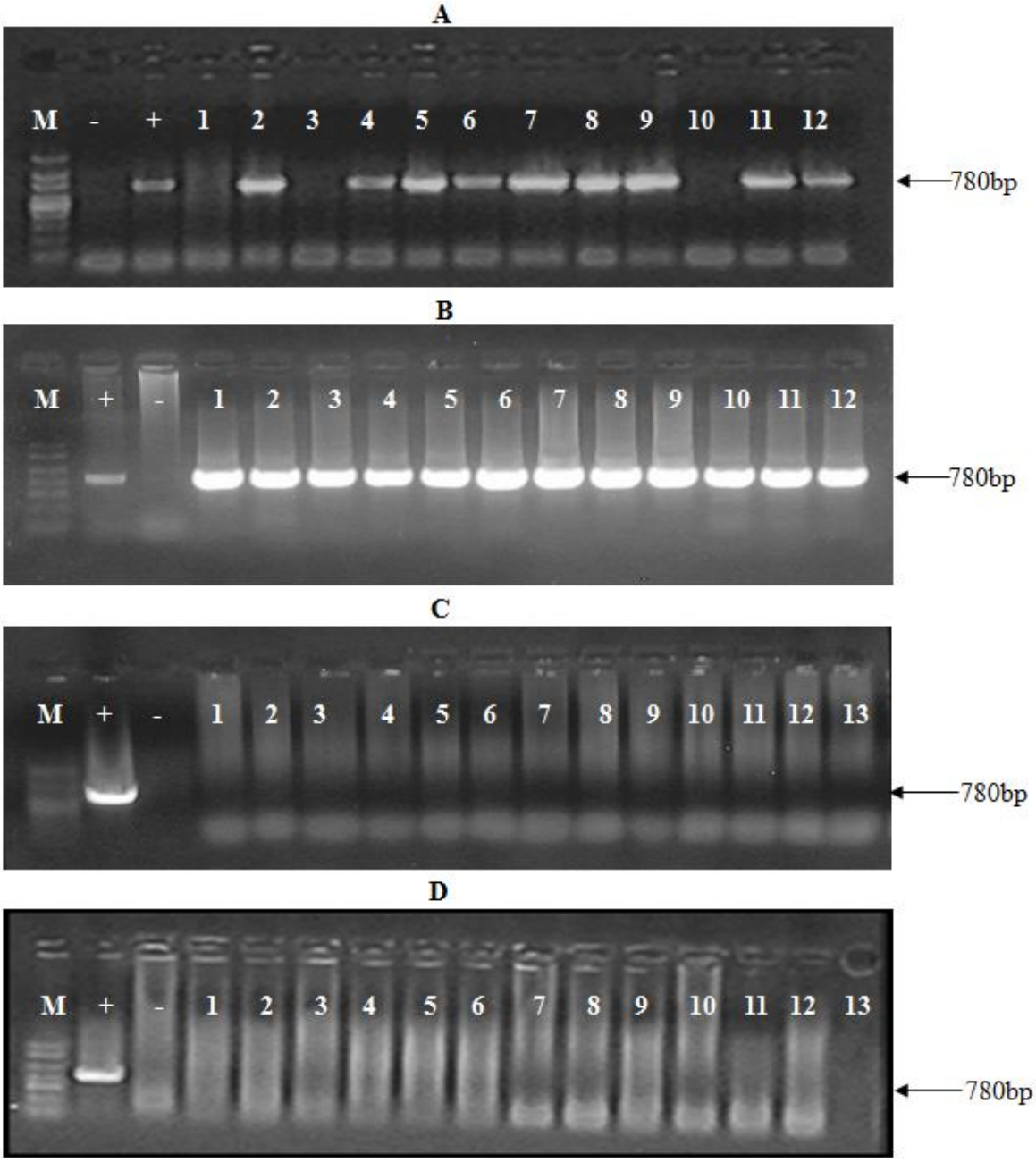

Figure 12: Agarose gel electrophoresis showing results of Gel A: membrane feeding of insects reared on diseased Napier grass; Gel B: PCR analysis of whole insects reared on diseased Napier grass; Gel C: Membrane feeding of insects reared on Pearl Millet; Gel D: PCR analysis of whole insects reared on pearl millet. Lanes: M, I kb DNA ladder (Genscript); (+), Positive control; (-), Negative control; (1-13), Samples.

3.3.5 Host plant preference

The differences in daily survivorship of *M. banda* on grass species were significant ($P < 0.05$), with the insects surviving best on *P. glaucum* and *P. purpureum* (Table 4) than all other grasses tested. However, the survival rate was higher on *P. glaucum* (74%) than on Napier grass (37%) ($f = 4.27$, $df=32$; $p = 4.40e-10$). There was zero *M. banda* survival on three grass species: *C. ciliaris, D. scalarum,* and *S. versicolor* while other grasses tested showed *M. banda* survivorship of less than 0.24. Results showed that only nymphs reared on *P. glaucum* and *P. purpureum* developed to form adults. However, the insect development was 3 days shorter on *P. glaucum* (15.4 days), than on *P. purpureum* (18.2 days). The mean growth index was also twice higher in *P. glaucum* (0.43), than on *P. purpureum* (0.21) ($f = 43.07$; $df=1$; $p = 3.64e-06$) (Table 5). *P. glaucum* was therefore more adequate than *P. purpureum* for the nymphal development of the insect because it shortened the nymphal period and increased the viability of these insects. There were absolute nymphal mortalities in all the other grasses tested.

Table 4: Survivorship and growth of *Maiestas banda* on common grasses.

S/N	Grass species	Common Name	Survival rate	Gr. index
1	*Pennisetum glaucum*	Pearl millet	0.74(0.24)a	0.43(0.06)a
2	*Pennisetum purpureum*	Napier grass	0.38(0.16)b	0.25(0.06)b
3	*Cymbopogon nardus*	Citronella grass	0.27(0.25)c	0
4	*Sorghum sudanensis*	Sudan grass	0.243(0.26c	0
5	*Oryzae sativa*	Rice	0.23(0.17)c	0
6	*Sorghum arundinaceum*	Wild sorghum	0.20(0.10)c	0
7	*Themeda triandra*		0.17(0.29)c	0
8	*Brachiaria sp*	Mulato	0.17(0.30)c	0
9	*Hyparrhenia rufa*	Thatching grass	0.17(0.29)c	0
10	*Sorghum bicolor*	Sorghum	0.13(0.10)d	0
11	*Hyparrhenia cymbaria*	Thatching grass	0.13(0.04)d	0
12	*Panicum maximum*	Guinea grass	0.125(0.21)d	0
13	*Cynodon dactylon*	Bermuda grass	0.12(0.21)d	0
14	*Brachiaria brizantha*	Signal grass	0.12(0.16)d	0
15	*Melinis minuteflora*	Molasses grass	0.11(0.30)d	0
16	*Eragrostis superba*	Heart seed grass	0.10(0.20)d	0
17	*Rottboellia*	Itch grass	0.10(0.17)d	0
18	*Hyparrhenia filepindula*	Thatching grass	0.10(0.20)d	0
19	*Sporobolus pyramidalis*	Giant rat`s tail	0.09(0.28)e	0
20	*Eragrostis curvula*	African love	0.07(0.12)e	0
21	*Bothriochloa insculpta*	Pinhole grass	0.07(0.13)e	0
22	*Eleusine indica*	Wire grass	0.06(0.11)e	0
23	*Bothriochloa bladhii*	Blue grass	0.0571 (0.15)e	0
22	*Eleusine indica*	Wire grass	0.06(0.11)e	0
23	*Bothriochloa bladhii*	Blue grass	0.0571 (0.15)e	0
24	*Hyparrhenia hirta*	Thatching grass	0.05(0.10)e	0
25	*Pennisetum clandestinum*	Kikuyu grass	0.04(0.10)e	0
26	*Echinochlora pyramidalis*	-	0.03(0.05)e	0
27	*Heteropogon contortus*	Wild oats	0.03(0.06)e	0
28	*Phragmites karka*	Tall reed	0.03(0.06)e	0
29	*Setaria incrassate*	Pigeon grass	0.03(0.06)e	0
30	*Pennisetum polystachion*	Thin Napier	0.01(0.03)e	0
31	*Digitaria scalarum*	Couch grass	0.00(0.00)	0
32	*Cenchrus ciliaris*		0(0.00)	0
	P		4.4	2.20E-16
	F		4.27	156.58

Means in column with the same letter are not significantly different (P > 0.05 (HSD).

3.4 Discussion

3.4.1 Vector of Napier stunting disease

This study identified *Maiestas (=Recilia) banda* as the insect vector of Ns-disease in western Kenya. The insect is a small leafhopper with triangularly produced vertex. It is differentiated from other members of the genus *Maiestas,* based on the presence of yellowish brown bands on its tegmina, and a distinct transverse band on the pronotum of the insect. The specific name '*banda'* originated from these bands. *M. banda* is a leafhopper in the family Cicadellidae, subfamily Deltocephalinae, and tribe Deltocephalini. Members of this tribe alone comprises 75% of all confirmed phytoplasma vectors (Weintraub and Beanland, 2006) showing that they have long evolutionary association with phytoplasmas. Deltocephalini are widely distributed in the world from the tropical to the semi-polar regions, and 23 species have been reported in tropical Africa (Satoshi, 1999). They are mostly phytophagous on Cereals, Sugarcane, in addition to other wild grasses (Satoshi, 1999).

DNA barcoding is a taxonomic method that uses a short genetic marker in an organism's DNA to identify it as belonging to a particular species (Herbat et al. 2003). It differs from phylogeny in that the main goal is not to determine classification but to identify an unknown sample in terms of a known classification (Kress et al. 2005). In insect DNA barcoding, like other animals, the cytochrome c oxidase I gene (COI) is used because of two important advantages. Firstly, the universal primers for this gene are very robust. The COI also possesses a greater range in phylogenetic signal than any other mitochondrial gene. Together with the distinct morphology (banding pattern) of *M. banda,* this study has provided genetic code information as a reference library for identification of the vector, more so for the immatures without distinct banding characteristic of adults. Identification will be done by comparing sequences of unknown insect with those published in this work (Appendices).

Other species in this genus have been identified as vectors of plant pathogens. The zigzag leafhopper *Maiestas dorsalis* (Motschulsky) is a vector of rice dwarf phytoreovirus (Takata, 1985), rice gall dwarf phytoreovirus (Brunt et al., 1990), and rice orange leaf phytoplasma (16SrXI group; Rivera et al. 1963) in Asia. Another genus of *Maiestas; M. mica* has been reported to vector a phytoplasma causing the blast disease in oil palm seedlings in West Africa

(Desmier de Chenon, 1979). Ritthison (2004) also detected 210-bp phytoplasma DNA fragment associated with sugarcane white leaf disease (16SrXI) in *M. distinctus* and *M. dorsalis*, showing intricate interaction between this genus with phytoplasma and other plant pathogens. The vector acquire the phytoplasma by feeding on infected plants for 1–3 h, and, after a latent period of 20–39 days, the phytoplasma passes from the gut to the salivary gland of the insect. Leafhoppers are then capable of inoculating healthy plants in usually less than 1 h of feeding (Weintraub and Beanland, 2006). The latent period in Napier grass is about 6 months in western Kenya.

Similarity between phytoplasmas is defined at 97.5% homology between their 16S rDNA sequences (ICSB, 1997). In this study, the phytoplasmas isolated from the vector and Napier grass showed highest sequence homology with phytoplasma sequences previously isolated from Kenya and Uganda. The insect might be the vector of Ns-disease in Uganda. This can be confirmed through vector surveys in Uganda followed by transmission experiments to confirm vector competence.

3.4.2 Vector host plant preference

The studies on vector interaction with other grass species were conducted under same screen house conditions, thus the differences in survivorship, and growth of *M. banda* were attributed to the plant factor alone, and reflect the field behavior of this insect. In all the studies, insects were obtained from diseased Napier grass to simulate field conditions where the insects are restricted on diseased Napier grass. The survivorship rate, and growth index is a measure of adult feeding, optimal nymphal and adult survival. There is experimental confirmation that *M. banda* has narrow host range and that it has distinct preference hierarchy in its food choice with Pearl millet becoming the most preferred host plant species followed by Napier grass. Reasons for the preference towards the pennisetums are not understood. High nutritional value could be the reason for the high preference towards pennisetums (Bernays and Chapman, 1994). Analysis of plant physical and chemical traits is necessary to reach conclusions (West and Cunningham, 2002). Nutritional similarity between Pearl millet and Napier grass is predictable because compared to other pennisetums, the millet and Napier grass are closely related species (Brunken (1977), with Pearl millet being the progenitor of Napier grass (Ingham et al. 1993). They form a monophyletic group that share a basic chromosome number of x = 7 (Martel et al. 2004).

In western Kenya where cultivation of Pearl millet is not intensive, it is likely that *M. banda* is wholly restricted to Napier grass host diet. The preference of *M. banda* to Napier grass diet, and the perennial nature of Napier grass restricts the phytoplasma pathogen to Napier grass, and reduces the risk of phytoplasma spreading to infect cereals and other grasses in the ecosystem. Several studies have shown that insect vectors that normally are not supported by certain grass species could transmit phytoplasma to these grasses under laboratory conditions. Hence, the plant host range of *M. banda*, rather than lack of phytoplasma-specific cell membrane receptors, will limit the spread of Ns-phytoplasma to other grasses in the ecosystem. The narrow host range of the insect enables it to process information about the environment more efficiently, increases the rate of host plant location/utilization (Dukas and Clark, 1995; Bernays and Funk, 1999), process more information about that species by the females, and detects the variation in the quality of individual plants more efficiently (Janz and Nylin, 1997). Likewise, the overspecialization of *M. banda* on the perennially available Napier grass host plant boosts its survival chance. However, the narrow host range denies the insect access to a greater resource base, a more nutritionally balanced diet (Egan and Funk, 2006).

The perennial availability of Napier grass by intensive cultivation in western Kenya and the distinct feeding preference of *M. banda* on Napier grass over other grasses is the reason for the high abundance of *M. banda* in the region. It could have been difficult to control *M. banda* when wild reservoir plants were sources of contamination as happens in polyphagous leafhoppers such as stolbur/*Hyalesthes obsoletus*/bindweed/nettle, or when reservoirs are unknown. *M. banda* will be easier to control since it reproduces on affected crops, and control strategies against the insect will specifically target the crop. The insect will also serve as the source of natural phytoplasma inoculum in screening Napier grass varieties for pytoplasma resistance.

3.4.3 Transovarial transmission and membrane feeding

The present work has demonstrated that the leafhopper. *M. banda* cannot transmit Ns-phytoplasma transovarially. This simplifies the control of Ns-disease as the offsprings of an infected leafhopper remains un infective until it feeds on a diseased plant. It was determined that the leafhopper *M. banda* oviposited in both Pearl millet and diseased Napier grass. The egg duration does not depend on host plant as the egg period was 11 days in both pearl millet and diseased Napier grass. It was determined that up to 75% of infected *M. banda* transmitted

phytoplasma to the Sucrose-TE media. Transmission rate is a measure of vector competence, as up to 75% of insects in an infected colony were phytoplasma inoculative. The negative transmission in 25% of viluferous insects was due to phytoplasma in the gut but not in the salivary glands. Even though males were not tested in this experiment, previous studies have shown that male leafhoppers transmit phytoplasma as their female counterparts, however, the transmission efficiency among the males is lower than females (Beanland et al. 1999). The different movement and feeding behaviors of males and females may explain, in part, differences observed. The higher transmission efficiency of female hoppers accounts for why only female insects were used in all the experiments of the insect vector. Through the Sucrose-TE feeding media, this study has demonstrated Ns-phytoplasma passage through the *M. banda* body which is an important factor indicating the species as a competent vector. It allowed for significant reduction of time compared with transmission to host plants. It was also non-invasive and minimized the effect of the insect–plant interaction in the inoculation phase. The assay can also be used to collect pure copies of phytoplasma for genome sequencing and for raising antibodies.

3.5 Conclusions

The study identified the *M. banda* as the insect vector of Ns-disease and established the grasses that support the vector population in western Kenya. The vector was found to have narrow host range of two closely related pennisetum species; *P. glaucum* and *P. purpureum,* with *P. glaucum* being the most preferred host plant. Because *P. glaucum* is not common in western Kenya, it was concluded that it is *P. purpureum* which supports the population of *M. banda* in the region. The narrow host range means that actions on the vector such as sampling, insecticide spraying, mass trapping etc should consider only Napier grass vegetation. The feeding behavior is also a weak point in vector evolution as the removal of Napier grass alone in western Kenya is enough to debilitate *M. banda* population in the region. The study determined that Ns-phytoplasma is not transmissible vertically through the egg to offsprings of infected females. The life history of *M. banda,* its dis-association to wild host plants and its restricted feeding to Napier grass is a breakthrough to effective control of this species. Insecticide treatments, which are a routine measure to control the vector of many phytoplasmas will be effective against *M. banda.* Phytoplasmas differ with respect to host specificity, which is usually higher for vectors than for the plant hosts (Lee et al. 1998). Many phytoplasmas are known to be vectored by more than one

insect species. For example, the known vectors of stolbur phytoplasma include the cixiid planthoppers *Hyalesthes obsoletus* Signoret, *Pentastiridius leporinus* (L.) and *Reptalus panzeri* (Löw) (Garnier, 2000). It is likely to discover other vectors of Ns-disease in eastern Africa. Vector studies should continue in all the countries of eastern Africa to determine the role of *M. banda* and other sap feeding insects in the region plays in disseminating phytoplasma pathogen.

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CHAPTER 4

MOLECULAR DIAGNOSIS OF NAPIER STUNTING DISEASE

4.1 Introduction

Phytoplasmas are uncultivable. They also occur in low copies in host tissues, and are unevenly distributed, which make their detection difficult, even in apparently diseased plants (Maliyakal, 1992). Ns-disease induces distinct symptoms in Napier grass as the infection leads to yellowing of plant foliage, reduced plant size, bushy growing habit and general stunting (Jones et al., 2004). These observable changes in plant morphology can be used for preliminary diagnosis of Ns-disease. However, the symptoms are delayed for up to 6 months under laboratory conditions, or until the grass is cut for re-growth in the field setup. These symptoms also have high similarity to those for drought stress and certain mineral deficiencies, making disease diagnosis based on symptoms very difficult and unreliable.

Phytoplasmas can also be observed on ultra-thin sections of phloem tissue, using Electron Microscope (Chen et al. 1989), however, the specificity of Electron microscopy is low. Diagnostic protocols based on Enzyme Linked Immunosorbent Assays (ELISA) emerged in 1980s, however, the production of monoclonal antibodies against a single phytoplasma epitope was very difficult (Lin and chen, 1985) and most ELISA based protocols failed. Polymerase chain reaction (PCR) as phytoplasma diagnostic tool began in early 1990s. PCR proved to be a highly sensitive, relatively simple and rapid method of detecting phytoplasma (Davis and lee, 1993). Currently, phytoplasma detection and characterization are based predominantly on PCR amplification of rRNA genes (rDNAs), especially the 16S rDNA (Bertaccini et al. 1995, 1997; Lloyd-Macglip et al. 1996). The 16S rRNA genes, 16S-23S rRNA intergenic spacer regions and 23S rRNA genes have been widely used as targets to detect and identify many different types of phytoplasma (Gundersen et al. 1996).

In nested PCR, two pairs of PCR primers are used to amplify 16S rRNA gene locus. The first primer pair (usually P1/P6, Deng and Hiruki, 1990) amplifies a bigger region within the 16S rRNA gene locus, while the second pair (nested primers, usually R16F2n/R16R2, Lee et al. 1993) binds within the PCR products of the first primer pair, to specifically amplify the gene loci of the genus *Candidatus* Phytoplasma. In the nested PCR strategy, if a wrong gene locus was

amplified by first primer pairs, the probability is zero that it would be re-amplified by the second set of primers. Nevertheless, amplification by the first set of primers increases concentration of 16S rRNA gene loci, usually undetectable in an agarose gel, but resolved quite well after the second amplification. This two round PCR assay procedure increases both sensitivity and specificity of nested PCR technique.

P1/P6 and R16F2n/R16R2 nested PCR primers for phytoplasma diagnosis were designed based on the gene sequences of other phytoplasma and bacteria, and are reactive to all phytoplasma groups. Due to point mutation in primer binding sites, it is essential to design primers based on the gene sequence of regional phytoplasmas. I this study, primers were designed from Ns-phytoplasma 16S rRNA gene sequence and optimized for diagnosis of Ns-disease by nested PCR. However, nested PCR is laborious, costly and rely on highly trained personnel. A simple diagnostic tool is necessary for monitoring Ns-disease epidemic in western Kenya. Recently, a DNA diagnostic tool called loop-mediated isothermal amplification (LAMP) was developed (Notomi et al. 2000; Nagamine et al. 2002). The tool has been applied for diagnosis of pathogenic viruses (Poon et al. 2005), bacteria (Iwamoto et al. 2003), fungi (Endo et al. 2004), *Plasmodium falciparum* (Daniel et al. 2007; Poon et al. 2006), *Mycoplasma pneumoniae* (Saito et al. 2005), *Candidatus Liberibacter asiaticus* (Okuda et al. 2005), African Trypanosomiasis (Njiru et al. 2004) and some phytoplasmas (Tomlinson et al. 2010) in host tissues. LAMP is isothermal DNA synthesis tool with high specificity. DNA synthesis is achieved using DNA polymerase with strand displacement activity, and a set of inner and outer primers. The amplification is completed within 30–60 min, at isothermal temperature of 60–65°C in a simple water bath or heating block. The DNA products are “stem-loop DNAs with several inverted repeats of the target gene (Notomi et al. 2000). The products are detectable by agarose gel electrophoresis or simply by visual observation of turbidity in positive reactions (Mori et al. 2001). This study developed isothermal diagnosis of Ns-disease in host plants and the insect vector.

4.2 Materials and methods

4.2.1 Test plants

Eight symptomatic and asymptomatic grass samples each were collected from the field at ICIPE's Mbita point field station in western Kenya. The collected samples were transferred to the laboratory in sealed plastic bags.

4.2.2 Design and synthesis of PCR primers

Phytoplasma 16S rRNA gene sequence (Acc. AY377876, Fig. 14) was downloaded from the Genbank and used to design a nested PCR primer pair. Primer design was done on the basis of the sequence using OLIGO software (Wojciech, 2007). After comparison of the sequence with the plant sequences or other plant pathogenic bacteria, and confirmation of dissimilarity between them, the forward and reverse primer pair was designed for more robust and specific detection of phytoplasma in Napier grass using nested PCR. After design, the primer sequences were submitted to Bioneer (South Korea) for synthesis.

4.2.3 Design and synthesis of LAMP primers

Two gene fragments, available in the GenBank, were targeted for isothermal amplification. 16S (Acc. AY736374, Fig. 15), a gene highly conserved among phytoplasma groups was targeted for broad-based isothermal phytoplasma diagnosis. , while the internal transcribed spacer (ITS) sequence of the rRNA gene (EU168784, Fig. 21), a region of high nucleotide variability, was targeted for specific diagnosis of Ns-disease. In both sequences, six distinct regions: F1-F2-F3 and B1–B2–B3 were designated (Fig. 13), and a set of primers were developed to hybridize to the six regions. For the ITS gene sequence, loop primers (FL and BL, Nagamine et al. 2002) were added to increase the specificity. All the primers were designed using Primer Explorer software, version 3, and synthesized (Bioneer, Seoul, South Korea).

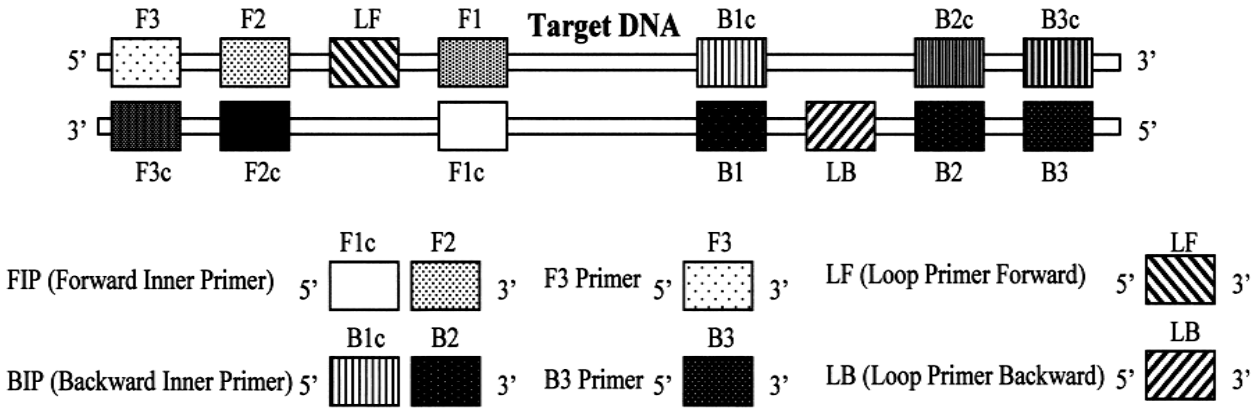

Figure 13: Target DNA and the design of isothermal primers.

4.2.4 DNA template preparation

To obtain template for nested PCR and isothermal diagnosis, DNA was extracted from leaf samples (8 symptomatic & 8 asymptomatic) using the CTAB extraction protocol of Doyle and Doyle (1990). Leaf tissue (300 mg) was powdered in liquid Nitrogen, and incubated in preheated buffer (CTAB buffer: 2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris–HCl, pH8.0, 0.2% 2-mercaptoethanol). DNA was extracted with 1 volume of Chloroform:Isoamyl Alcohol (24:1) and precipitated overnight in a −20 freezer using isopropanol. Following centrifugation at 14,000 rpm (Centrifuge 5415 C, Eppendorf), the DNA pellet was rinsed twice with 70% Ethanol, dried and dissolved in 50 µl Tris-EDTA (20 mM Tris, 1 mM EDTA pH 8) buffer.

4.2.5 Nested polymerase chain reaction

Nested PCR reaction mix and cycling conditions followed the protocol published in Obura et al. (2009), using primer pair P1/P6 (Deng & Hiruki, 1991) for first round amplification, and nested PCR with R16F2n/R16R2 primers (Lee et al. 1993) and NapF/NapR primers synthesized from the 16S rRNA gene sequence of Ns-phytoplasma (Fig. 14). The reaction mixture contained 50 ng of each primer, 125 µM of each dNTPs, 1U of Taq DNA polymerase (Gene- script, USA), 1x PCR buffer with 1.5 mM $MgCl_2$ (Genescript, USA). 0.7 µl of P1/P6 PCR product was used as template in nested PCR. The reaction mixture was adjusted to 25 µl by sterile distilled water. PCR was performed for 35 cycles in a PTC-100 Thermal cycler (MJ Research, Inc.). Reaction conditions were as follows: 2 min at 94 °C, 1 cycle; 1 min at

94°C, 2 min at 52°C (55°C for R16F2n/R16R2 and NapF/NapR), 1 min at 72°C, 35 cycles; and 10 min at 72°C, 1 cycle. The PCR water was used as negative control. The PCR products were then resolved on ethidium bromide stained 1% agarose gel using 1x TAE (40 mM Tris acetate, 1 mM EDTA pH8.0) as running buffer, and photographed. Because primers can react probably with sequences of plant genome or dimers and false positives could be observed, a DNA template of corresponding healthy plant was used as control.

4.2.6 Loop mediated isothermal amplification of DNA

Isothermal reaction was conducted as described by Nagamine et al. (2002), with some modifications. The reaction was performed in a total 25 µl reaction mixture containing 0.8 µM each of FIP and BIP, 0.2 µM each of the outer primers (F3 and B3), and 0.4 µM each of loop primers (FL and BL). Other components were: 1 M Betaine (Sigma), 1x Thermopol buffer (New England Biolabs), 8U of Bst DNA polymerase large fragment (New England Biolabs), 400 µM of each dNTPs and 2 µl of template DNA. The reaction mixture was adjusted to 25 µl using sterile distilled water. The mixture was heated at 95 °C for 5 min then chilled on ice to prevent re-annealing of the DNA. Then 8U of Bst DNA polymerase large fragment was added followed by incubation at 65°C for 1 h in a water bath. The reaction was terminated by heating at 80 °C for 10 min. Double distilled water was used as negative control while DNA extracted from diseased Napier grass was used as positive control. The amplified products were analyzed by electrophoresis on a 2% ethidium bromide stained agarose gel using 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA), and photographed. Positive Predictive Value (PPV) of LAMP assay was calculated as: a/(a+b), and Negative Predictive Value (NPV) was also calculated as: d/(c+d). The sensitivity of the assay was determined as: a/(a+c), where a=true positive, b=false positive, c = false negative, d = true negative.

4.2.7 Detection limit of LAMP diagnosis.

Template DNA extracted from diseased grass sample was serially diluted 1:20, three times, and tested for phytoplasma presence by the isothermal diagnosis and nested PCR. Products of the 2 assays were resolved side by side on ethidium bromide 1% (nested PCR) and 2% agarose gels using 1x TAE as running buffer.

4.2.8 Specificity of LAMP diagnosis

LAMP diagnosis was tested on 11 Phytoplasma groups (Table 5). Bermuda grass white leaf-BGWL and Ns-disease phytoplasma samples were obtained from the field at ICIPE's Mbita point field station in western Kenya. The other phytoplasma groups were obtained from Nottingham University (School of Biosciences, UK). Template preparation for LAMP diagnosis was done by the method of Doyle and Doyle (1990). Prior to diagnosis, the concentration of template DNA was standardized at 200 µM.

Table 5: Phytoplasma groups tested for specificity by LAMP diagnosis.

Phytoplasma isolate	Disease caused/Host plant & origin	16Sr group
SGP	Strawberry green petal, Thailand	I-C
SOYP	Soybean phyllody, Thailand	II-C
TBB	Tomato big bud, Australia	II-D
PYLV	Peach Western X, Canada	III-A
CSPWD	Cape St Paul wilt of coconut, Ghana	IV
BLL	Brinjal little leaf, India	VI-A
BGWL-K	Bermuda grass white leaf, Kenya	XIV
NSD	Napier stunting disease, Kenya	XI
PD	Pear decline, Germany	X-C
STOL	Stolbur, USA	XII-A
HMLO	Hydrangea MLO	I-B

4.2.9 Molecular typing of LAMP products

LAMP products (10 µl) were digested with BsrI endonuclease (New England Biolabs) according to the manufactures instructions. The digested fragments were resolved on a 2% ethidium bromide stained agarose gel using 1x TAE as running buffer and photographed under UV trans-illuminator. The restricted fragments on the agarose gel were evaluated for specificity by Southern blot (Sambrook et al. 1989). The gel was blotted to Hybond-N+ (Amersham, USA) overnight by capillary elution with alkaline buffer (0.4 M NaOH, 1 M NaCl). The membrane was cleaned briefly in 2×SSC buffer and baked in an oven for 2 h at 80 °C. Prehybridization was

done in 4 ml of hybridization buffer (2% blocking reagent, 5×SSC, 0.02% SDS 0.1% Sarcosyl) at 42 ℃ for 2 h. A 1200-bp DNA probe was labeled with DIG-11-dUTP by nested polymerase chain reaction using R16F2n/R16R2 primers and PCR DIG Probe Synthesis Kit (Roche, Mannheim, Germany), according to the manufacturers protocol. Prior to hybridization, the probe DNA was boiled at 100 ℃ for 10 min, then immediately cooled on ice. Denatured probe DNA (4µl) was added to the hybridization bag containing the membrane and hybridization solution, and sealed. The membrane was then incubated in a hybridization oven at 42 ℃ overnight. Following hybridization, the membrane was washed twice for 15 min in 30 ml of 2×SSC, 0.1% SDS, then washed twice for 15 min in the 2nd buffer (0.1×SSC, 0.1% SDS), pre-warmed at 68 ℃. The membrane was then briefly transferred to a buffer (0.1 M Maleic acid, 0.15 M NaCl) before detection. Detection of positive hybridizations was performed with the non-radioactive DIG system according to the recommendations of the manufacturer (Roche). The results were documented by photographing the membrane.

4.2.10 Specificity of PCR primers

The specificity of newly developed PCR primers was compared to nested PCR using R16F2n/R16R2 primers and 8 symptomatic grasses. Molecular typing of the nested PCR products, using the new primers, was performed by DNA sequencing and sequence analysis. The products were gel purified on GeneJET Gel Extraction Kit (Fermentas) according to manufacturer's protocol and sequenced directly (SegoliLab, ILRI, Nairobi). Sequences were viewed and edited using Genedoc software version 2.7, and deposited in the EMBL GenBank database. Identity of the gene sequence was determined by BLAST search at NCBI database (http://www.ncbi.nlm.nih.gov). Specificity was further confirmed by testing the reactivity of the new nested PCR primers on 8 phytoplasma groups (Table 6).

Table 6: Phytoplasma groups tested for specific diagnosis using NapF/NapR primers.

Phytoplasma name	Disease caused
1. 16SrI AY	Aster Yellows
2. 16SrII SPLL	Sweet potato little elaf
3. 16SrIII CSP	Poinsettia branching factor
4. 16SrV EP-2	Elm Yellows
5. 16SrVI EY	Potato witches broom
6. 16SrX PWB	Apple proliferation
7. 16SrXII STOL	Stolbur
8. 16SrXXII CSPWD	Cape St Paul wilt of coconut

4.3 Results

4.3.1 Specificity of NapF/NapR nested PCR primers

The newly developed nested PCR primer names (NapF and NapR) and their sequences are presented in Table 7. The primers targeted a short gene target of 778 bp (Fig. 14). The lengths of forward and reverse primers were set at 21 and 23 bp respectively, which was optimal for PCR. The melting temperature (T_m) difference between the primers was 1^0C to maximize PCR product yield. The GC contents were also set at 52% and 35% for the forward and reverse primers respectively. Di-nucleotide repeats were avoided in all the primers. In nested PCR, the annealing temperature (T_a) of NapF/.NapR primer pair was optimized at 55^0C, which was not too high to produce insufficient primer-template hybridization, and not too low to lead to non-specific products. Primer sequences exhibited zero to chloroplast sequences, plastid 16S sequences or to any published plant sequences. Database searches in the NCBI Blast Search showed full primer homology to phytoplasma sequences.

Diagnosis of phytoplasma by nested PCR using P1/P6 primers, followed by re-amplification in nested PCR with the NapF/NapR primers yielded 778 bp on the agarose gel(Fig. 15B), similar to the predicted DNA size (Fig. 14).

TCTTTTAATTTTTAAAAGACCTTTTTCGAAAGGTATGCTTATTCAGGGGATTGCGACACATTAG

TTAGTTGGTAGGGTAAAAGCCTACCAAGACTATGATGTGTAGCTGGACTGAGAGGTCGAACAGC

CACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATTTTCGGCAAT

GGAGGAAACTCTGACCGAGCAACGCCGCGTGAACGATGAAGTATTTCGGTATGTAAAGTTCTTT

NapF

TATTGAAGAAGAAAAAATAGTGGAAAAACTATATTGACGCTATTCAATGAATAAGCCCCGGCAA

ACTATGTGCCAGCAGCCGCGTAATACATAGGGGGCAAGCGTTATCCGGAATTATTGGGCGTAAA

GGGTGCGTAGGCGGTTTAATAAGTCTGTAGTTTAATTTCAGTGCTTAACACTGTCCTGCTATAG

AAACTATTAGACTAGAGTGAGATAGAGGTAAGCGGAATTCCATGTGTAGCGGTAAAATGCGTAA

ATATATGGAGGAACACCAGAGGCGTAGGCGGCTTACTGGGTCTTTACTGACGCTGAGGCACGAA

ACCGTCCCGACCAAACACGATTACATACCCTCCTAGTCCACCCCCTAAACCATCACTACTAAGT

GTCGGGGAAACTCGGTACTGAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAG

TATGAAACTTAAAGGAATTGACGGGACTCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAG

ATACACGAAAAACCTTACCAGGTCTTGACATACTCTGCAAAGCTATAGCAATATAGTGGAGGTT

ATCAGGGATACAGGTGGTGCATGGTGGTCGTCAGCTCGTGTCGTGAGATGTTAGGTTAAGTCCT

AAAACGAGCGCAACCCTTATCGTTAGTTACCAGCATGTTATGATGGGGACTTTAACGAGACTGCC

AATGAAAAATTGGAGGAAGGTGAGGATCACGTCAAATCATCATGCCCCTTATGATCTGGGCTAC

NapR

AAACGTGATACAATGGCTGTTACAAAGAGTAGCTAAAACGCGAGTTTATAGCCAATCTCAAAAA

AACAGTCTCAGTTCGGATTGAAGTCTGCAACTCGACTTCATGAAGTTGGAATCGCTAGTAATCG

CGAATCAGCATGTCGCGGTGAATACGTTCTCGGGGTTTGTACACACCGCCCGTCAAACCACGAA

AATCCCTAATACTCCAAAGCCGTCCCCTAACTTCTTCCCAACACCGCAACCGTCTAACGTAGCAT

TGATGATTGGGGTTAAGTCGTAACAAGGTATCCCTACCGGAAGGTGGGGATGGATCACCTCCTT

TCTAAGGAAAAGTTTTTAAATTTTCATCTTCAGTTTTGAAAGACTTAGTTCTTATAAGTTTTTC

CTTTTTT

Figure 14: The 16S sequence of phytoplasma (GenBank Acc. AY377876). The primer annealing sites are highlighted in bold/underlined.

Table 7: Sequences of NapF and NapR nested PCR primers developed for diagnosis of Ns-disease. The conventional P1/P6 and R16F2n/R16R2 nested PCR primer pairs are also shown.

Primer	Sequence (5'-3')	Expected size	Reference
P1	AAGAGTTTGATCCTGGCTCAGGATT	1500 bp	Deng and Hiruki, 1991
P6	CGGTAGGGATACCTTGTTACGACTTA		
R16F2n	GAAACGACTGCTAAGACTGG	1200 bp	Lee et al. 1993
R16R2	TGACGGGCGGTGTGTACAAACCCCG		
NapF	AGGAAACTCTGACCGAGCAAC	778 bp	
NapR	ATTTTTCATTGGCAGTCTCGTTA		

The sensitivity of NapF/NapR primers was higher. Only 62% of diseased Napier grass samples were positive by R16F2n/R16R2 nested PCR (Fig. 15A), while all the 8 diseased grasses tested by the NapF/NapR primers were positive (Fig. 15B). Detection of phytoplasma by NapF/NapR primers was robust. This was demonstrated by the thick DNA bands on the agarose gel (Fig. 15A and 15B). NapF/NapR primers were reactive to all the 16S rRNA gene of other phytoplasma groups tested (Fig. 16).

The DNA sequence amplified by NapF/NapR primers are available in the GenBank (Acc. No. FJ744596). The sequence exhibited 100% homology to other Ns-phytoplasma sequences in the GenBank. BLAST results showed that the sequence had zero homology to plant sequences and other phytopathatogenic bacteria.

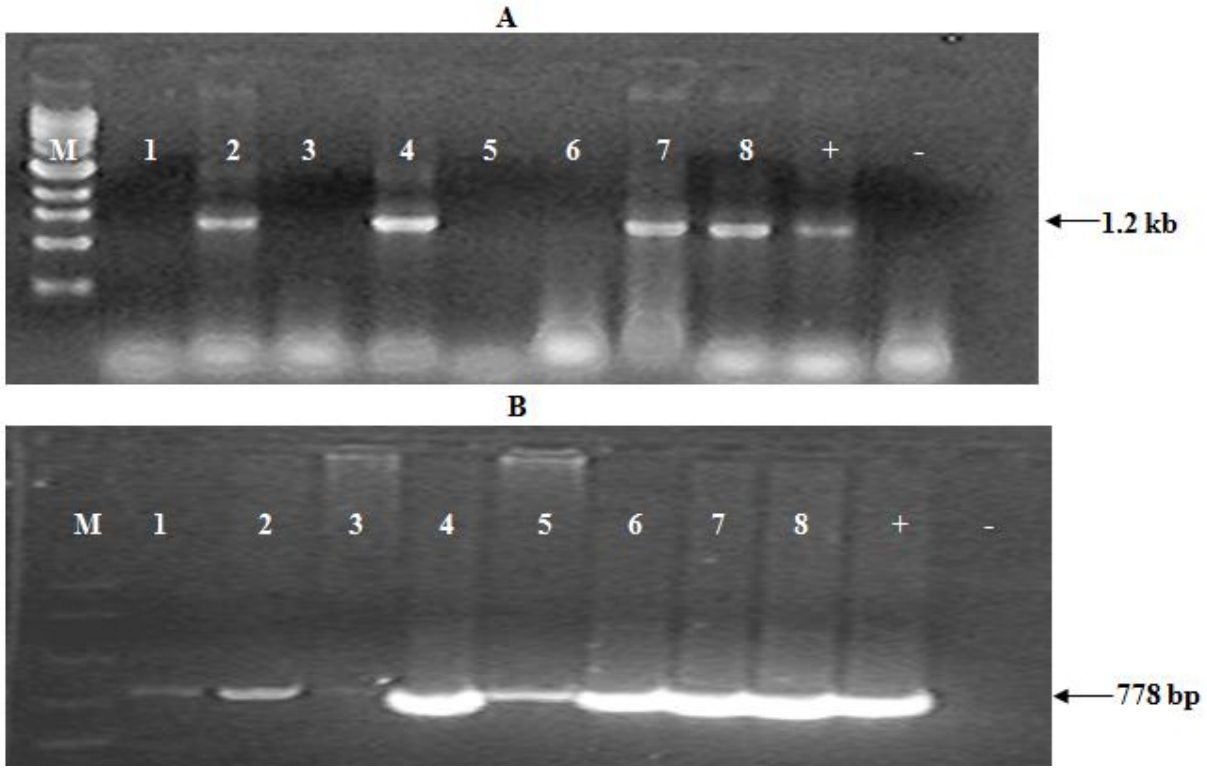

Figure 15: Agarose gel electrophoresis of DNA extracted from diseased Napier grass and amplified by R16F2n/R16R2 (Gel A) and NapF/NapR (Gel B) primers. Lanes: M, PCR DNA ladder (Genscript); (+), Positive control; (-), Negative control; (1-8), Samples extracted from diseased Napier grass.

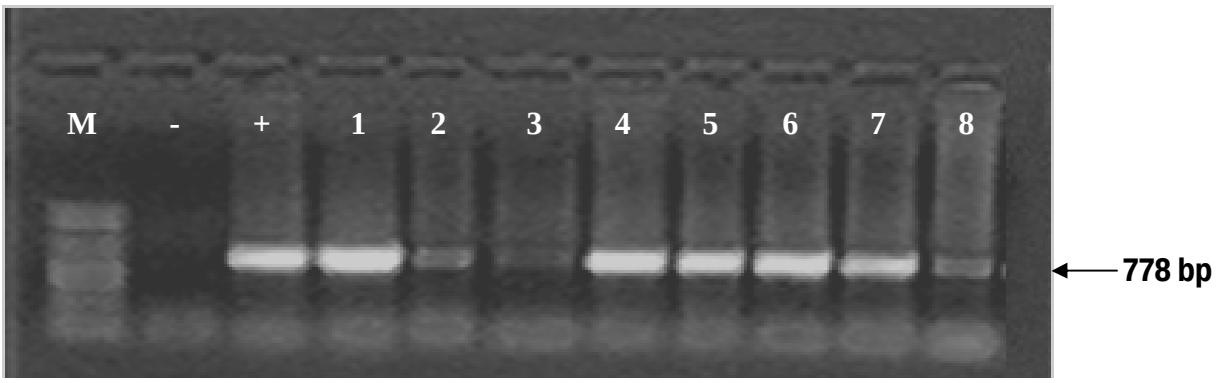

Figure 16: Agarose gel electrophoresis of DNA from 8 Phytoplasma groups tested for amplification by NapF/NapR phytoplasma nested primers. Lanes: M, 100 bp DNA ladder (Genscript); (+), Positive control; (-), Negative control; (1-8), Phytoplasma group samples (Table 7).

4.3.2. LAMP diagnosis of 16S gene

The 4 LAMP primers for amplification of 16S gene are presented in Table 8. The primers targeted 6 DNA regions: F3, F2, F1, B1, B2, and B3 targeted for LAMP amplification are presented in Figure 17. FIP and BIP, hybrid primers, targeted F1c/F2, and B1c/B2 regions respectively. The F1c and B1c sequences were complementary to F1 and B1 regions. The melting temperature (T_m) values of outer primers (B3 and F3) were set lower than those of inner primers (FIP and BIP) to ensure that DNA synthesis occurred earlier from inner primers than from outer primers. The concentration of the inner primers (FIP and BIP) was set to be 8 times higher than the outer primers (B3 and F3) because only the inner primers are used for DNA synthesis during the isothermal amplification. Strand displacement DNA synthesis is a limitation in isothermal reactions, the target gene was set at 184bp (Fig. 17).

Table 8: Primer name and sequences for LAMP diagnosis of 16S gene.

Primer	Type	Sequence (5'-3')
PhytoBIP	Backward inner ($B1c+TTTT+B_2$)	CGTGGGGAGCAAACAGGA-TTTT-TAGTACTCATCGTTTACG GC
PhytoFIP	Forward inner ($F1_C+TTTT+F_2$)	TCAGCGTCAGTAAAGACCCAGTAA-TTTT-TATATGGAGGAA CAC
PhytoB3	Backward outer	ACTTCAGTACCGAGTTTCC
PhytoF3	Forward outer	CGGAATTCCATGTGTAGCG

Figure 18 summarizes isothermal diagnosis of phytoplasma in 8 symptomatic and 8 asymptomatic grass samples. All (8/8) of symptomatic grasses tested positive for phytoplasma by isothermal diagnosis. The sensitivity and positive predictive values were both 100%. There was complete agreement between symptoms and isothermal diagnosis of phytoplasma in the symptomatic grass samples (Fig. 18A). In asymptomatic grass samples, 3 of the 8 grass samples tested positive for phytoplasma by isothermal diagnosis (Fig. 18B), while the rest (5/8) were negative. The negative predictive value for isothermal diagnosis was 63%. Amplicons of isothermal diagnosis were ladder-like DNA fragments, with the smallest fragment about 184-bp (Fig. 18A and 18B).

TCTTTTAATTTTTAAAAGACCTTTTTCGAAAGGTATGCTTATTCAGGGGATTGCGACACATTAG

TTAGTTGGTAGGGTAAAAGCCTACCAAGACTATGATGTGTAGCTGGACTGAGAGGTCGAACAGC

CACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATTTTCGGCAAT

GGAGGAAACTCTGACCGAGCAACGCCGCGTGAACGATGAAGTATTTCGGTATGTAAAGTTCTTT

TATTGAAGAAGAAAAAATAGTGGAAAAACTATATTGACGCTATTCAATGAATAAGCCCCGGCAA

ACTATGTGCCAGCAGCCGCGTAATACATAGGGGGCAAGCGTTATCCGGAATTATTGGGCGTAAA

GGGTGCGTAGGCGGTTTAATAAGTCTGTAGTTTAATTTCAGTGCTTAACACTGTCCTGCTATAG

AAACTATTAGACTAGAGTGAGATAGAGGTAAG**CGGAATTCCATGTGTAGCG**GTAAAATGCGTAA
F3

A**TATATGGAGGAACAC**CAGAGGCGTAGGCGGC**TTACTGGGTCTTTACTGACGCTGA**GGCACGAA
F2 **F1**

AG**CGTGGGGAGCAAACAGGA**TTAGATACCCTGGTAGTCCAC**GCCGTAAACGATGAGTACTA**AGT
B1 **B2**

GTCGGGG**AAACTCGGTACTGAAGT**TAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAG
B3

TATGAAACTTAAAGGAATTGACGGGACTCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAG

ATACACGAAAAACCTTACCAGGTCTTGACATACTCTGCAAAGCTATAGCAATATAGTGGAGGTT

ATCAGGGATACAGGTGGTGCATGGTGGTCGTCAGCTCGTGTCGTGAGATGTTAGGTTAAGTCCT

AAAACGAGCGCAACCTTATCGTTAGTTACCAGCATGTTATGATGGGGACTTTAACGAGACTGCC

AATGAAAAATTGGAGGAAGGTGAGGATCACGTCAAATCATCATGCCCCTTATGATCTGGGCTAC

AAACGTGATACAATGGCTGTTACAAAGAGTAGCTAAAACGCGAGTTTATAGCCAATCTCAAAAA

AACAGTCTCAGTTCGGATTGAAGTCTGCAACTCGACTTCATGAAGTTGGAATCGCTAGTAATCG

CGAATCAGCATGTCGCGGTGAATACGTTCTCGGGGTTTGTACACACCGCCCGTCAAACCACGAA

AATCGGTAATACTCGAAAGCGGTGGCCTAACTTCTTCGGAAGAGGGAACCGTCTAAGGTAGGAT

TGATGATTGGGGTTAAGTCGTAACAAGGTATCCCTACCGGAAGGTGGGGATGGATCACCTCCTT

TCTAAGGAAAAGTTTTTAAATTTTCATCTTCAGTTTTGAAAGACTTAGTTCTTATAAGTTTTTC

CTTTTTT

Figure 17: 16S gene (GenBank acc. AY736374). LAMP primer annealing sites are underlined.

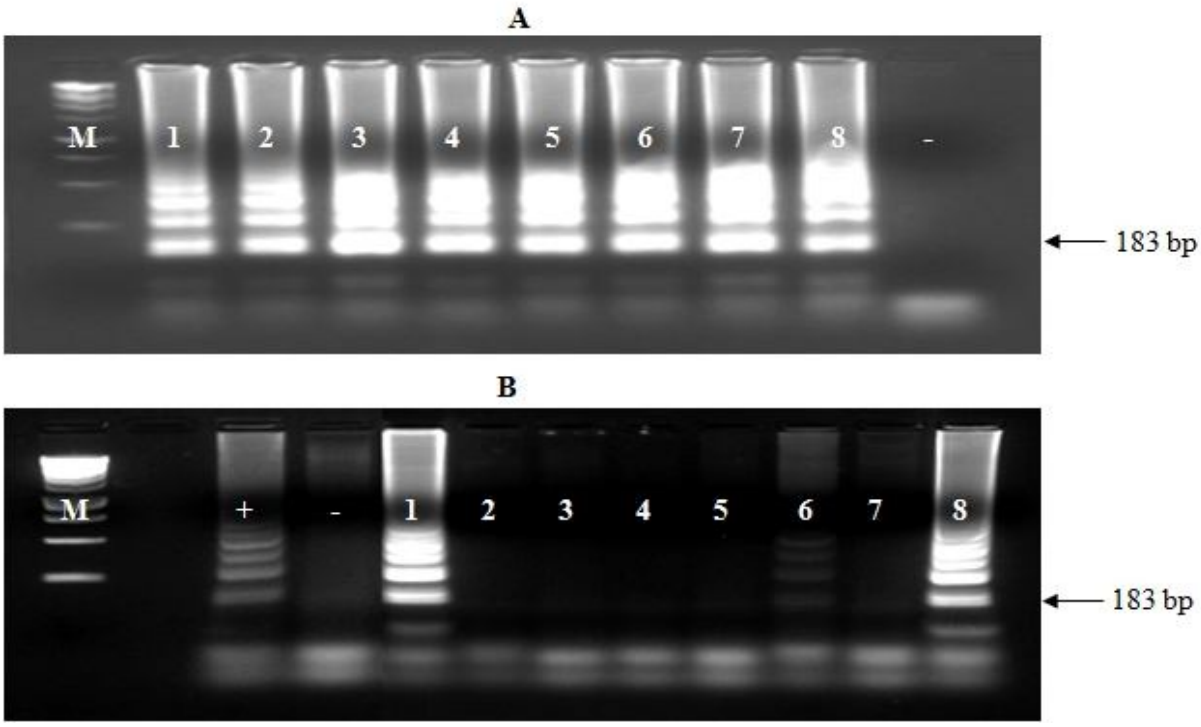

Figure 18: 2% Agarose gel electrophoresis of 8 symptomatic (Gel A) and 8 asymptomatic (Gel B) Napier grass samples analyzed for phytoplasma by Loop mediated isothermal amplification of DNA (LAMP). M, 1000 bp DNA ladder (New England Biolabs); (1 –8), Napier grass samples. Ladderized DNA fragments represent phytoplasma positive samples. (-) & (+), Negative (ddH_2O) and positive controls (Diseased Napier grass) respectively.

The detection limit of isothermal diagnosis on serially diluted samples is presented in Figure 19. The initial DNA concentration by spectrophotometer reading was 3 ng/µl. The DNA concentrations in the serial dilutions were therefore: Stock DNA=3 ng/ul; Dil 1 (1:20) = 0.15 ng/ ul; Dil 2 (1:20)=7.5 pg/ul and Dil 3 (1:20)=0.375 pg/ul. The limit of nested PCR was dilution 1, while isothermal tool detected phytoplasma up to dilution 2, 20-fold higher than for nested PCR.

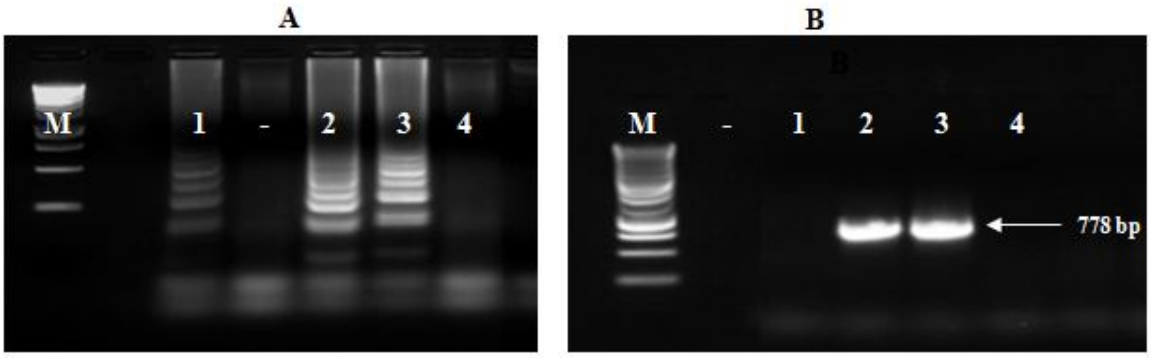

Figure 19: Agarose gel electrophoresis comparing the detection limit of LAMP (gel A, 2% agarose) and nested PCR (gel B, 1% agarose). Lanes: M, 1-kb DNA ladder (Promega); (-), negative control (ddH_2O); 1, Initial DNA; 2, 1:20 dilution; 3, 1:40 dilution; 4, 1:60 dilution.

Digestion of LAMP products with the BsrI endonuclease yielded two specific DNA fragments (Fig. 20) of 66 and 113 bp, similar to the predicted sizes (Fig. 17). In Southern blot analysis, there was specific binding of probe DNA to the fragments produced by isothermal diagnosis (Fig. 21). This was molecular evidence of similarity between probe DNA and isothermal DNA products.

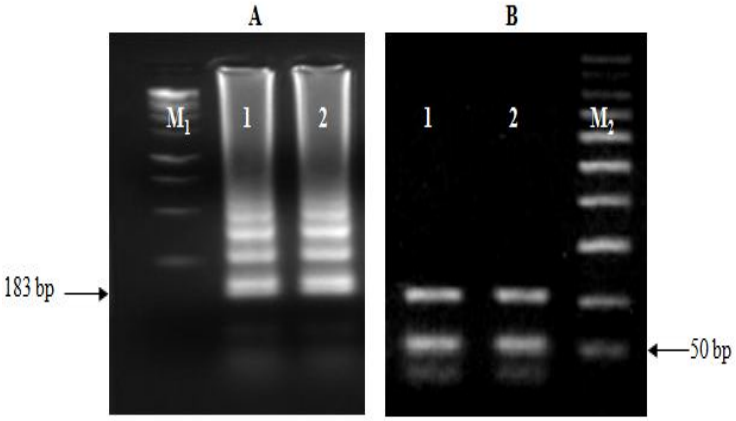

Figure 20: BsrI restriction endonuclease of DNA products generated Loop mediated isothermal amplification of DNA (LAMP). Gel A, 2% Agarose gel electrophoresis of LAMP products (1 & 2) before BsrI endonuclease digestion; B, 2% Agarose gel electrophoresis of LAMP products (1 & 2) after BsrI endonuclease digestion. Lanes: M_1, 1 kb DNA ladder (Promega); M_2, 50-bp DNA ladder (Fermentas).

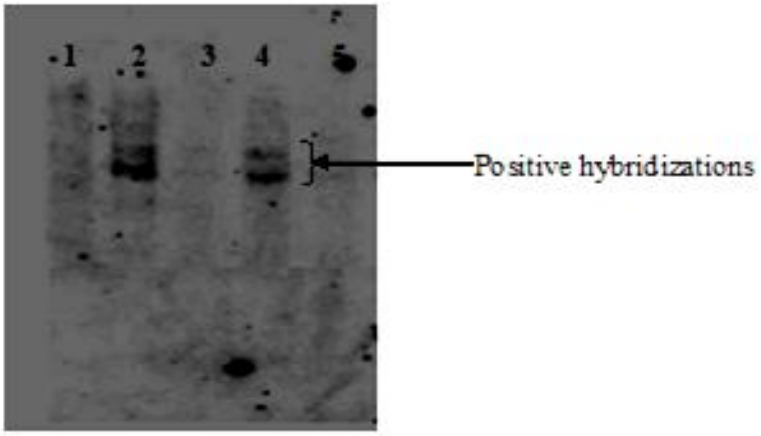

Figure 21: Southern blot analysis of BsrI digested isothermal DNA fragments. Lanes: (1, 3 & 5), Negative hybridizations; (2 & 4), Positive hybridizations.

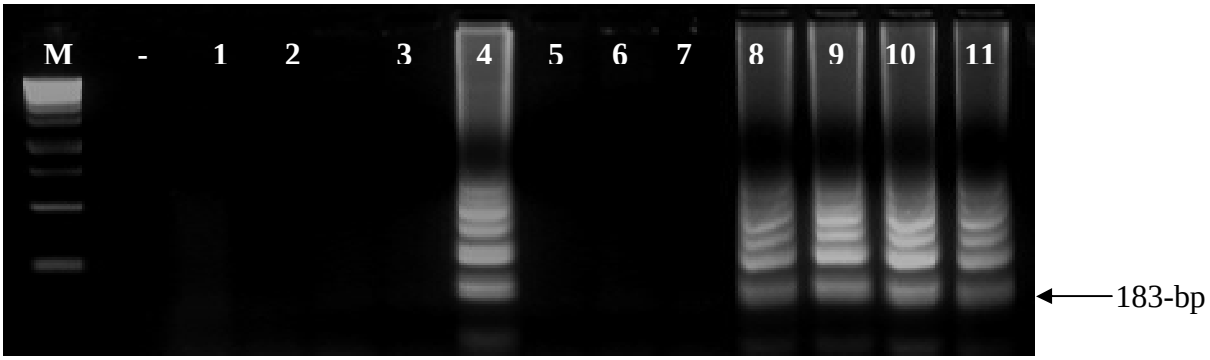

Figure 22: 2% Agarose gel electrophoresis of 11 phytoplasma groups tested for specific amplification by Loop mediated isothermal amplification of DNA (LAMP). Lanes: M, 1 kb DNA ladder (Promega); 1, Western X; 2, Cape St. Paul wilt of Coconut; 3, Tomato big bud; 4, Napier stunting disease; 5, Strawberry green petal; 6, Soybean phyllody; 7, Hydrangea MLO; 8, Pear decline; 9, Bermuda grass white leaf; 10, Stolbur; 11, Brinjal little leaf.

Figure 22 summarizes isothermal diagnosis of 16S DNA from 11 phytoplasma groups. Positive diagnosis were observed with 5 groups: Brinjal little leaf (BLL, 16SrVI), Bermuda grass white leaf (BGWL, 16SrXIV), Pear decline (PD, 16SrX), Napier stunting disease (NSD, 16SrXI), and Stolbur (Stol, 16SrXII). The other 6 phytoplasma groups tested were negative. The positive samples belonged to phytoplasma groups (16Sr X, XI, XII and XIV) with higher sequence homology to 16SrXI phytoplasma.

4.3.3 LAMP diagnosis using ITS primers

The primers based on the ITS gene for specific diagnosis of Ns-disease are presented in Table 9. The ITS gene sequence and the primer annealing sites are also presented in Figure 25. The FIP primer consisted of complementary sequence of F1 (25 nt), a TTTT linker and F2 (25 nt). The BIP for same DNA consisted of B1 (26 nt), a TTTT linker and the complementary sequence of B2 (20 nt). The outer primers were F3 (25 nt) and B3 (20 nt). The loop primers were LF (20 nt) and LB (19 nt). Isothermal diagnosis amplifies copies of DNA within 1 h under isothermal conditions. It uses strand displacement DNA polymerase and a set of four specially designed primers that recognize six distinct regions on the target sequence. Addition of loop primers prompted faster and more specific diagnosis. The position of the loop primer is the region between F2 and F1 (or B1 and B2) in the direction of F1 to F2 (or B1 to B2) (Fig. 23). The Isothermal diagnosis was optimized at ideal settings of 65°C for 60 minutes.

Table 9: Detail of primers for isothermal diagnosis based on the ITS gene.

Primer	Type	Sequence (5'-3')
NgsFIP	Forward inner ($F1_C$+TTTT+F_2)	AGAAAGATGACCTTTTTCAGTTGGT-TTTT-GTGGTTA GAGCACACGCCTGATAAG
NgsFIP	Backward inner (B1c+TTTT+B_2)	CAAAGTAAAATAATAAAATCAAAGGA-TTTT-CATCGGC TCTTAGTGCCAAG
NgsF3	Forward outer	AAGAAGGAGGGCCTATAGCTCAGT
NgsB3	Backward outer	ATATCGCTGTTAATTACGTC
NgsFL	Forward Loop	ATGGACTTGAACCATCGACC
NgsBL	Backward Loop	AAGGGCGTACAGTGGATGC

GGATGGATCACCTCCTTTCTAAGGAAAAGTTTTTAAATTTTCATCTTCAGTTTTGAAAGACTTA

GTTCTTATAAGTTTTTCCTTTTTTAAGAAAAAAAAGAAGGAGGGCCTATAGCTCAGTTGGTTAG

F3

AGCACACGCCTGATAAGCGTGAGGTCGATGGTTCAAGTCCATTTAGGCCCACCAACTGAAAAAG

F2 FL

GTCATCTTTCTTATTCTTAACAAAAGTTCTTTGAAAAGTAGATAAACAAAGTAAAATAATAAAA

F1 B1

TCAAAGGAATCAAGGGCGTACAGTGGATGCCTTGGCACTAAGAGCCGATGAAGGACGTAATTAA

F2 FL

CAGCGATATGCCACGGTTAGTTGTAAATAAATGAAGATCCGTGGATTTCCGAATAGGGCAACCT

B3

ACTACATTAAAATTGTAGTTTTTTGATTTTTTTTGTCAAAAAGGGAAACGCAGTGAACTGAAAT

ATCTAAGTAACTGCAGGAAAAGAAAGTAACAACGATTCTGGTAGTAGTGACGAGCGAATCCGGA

ACAGCCTGATACTATTCAAACTAGTTACAAAATTTTAATTAACTTGAATTTTTTGGAAAGAAAA

ACCAAAGGAGGTGATAGTCCTGTAGAGGAATAATTAAAATCTAGAGTATCAAAAAGTACGGCGA

GACACGAGAAATCTTGTCGGAAGACGGGAGGACCATCTCCCAAGGCTAAATACTCCTTAGTGAC

CGATAGTGAACTAGTACCGTGAGGGAAAGGCGAA

Figure 23: ITS gene (GenBank acc. EU168784). LAMP primer annealing sites are underlined.

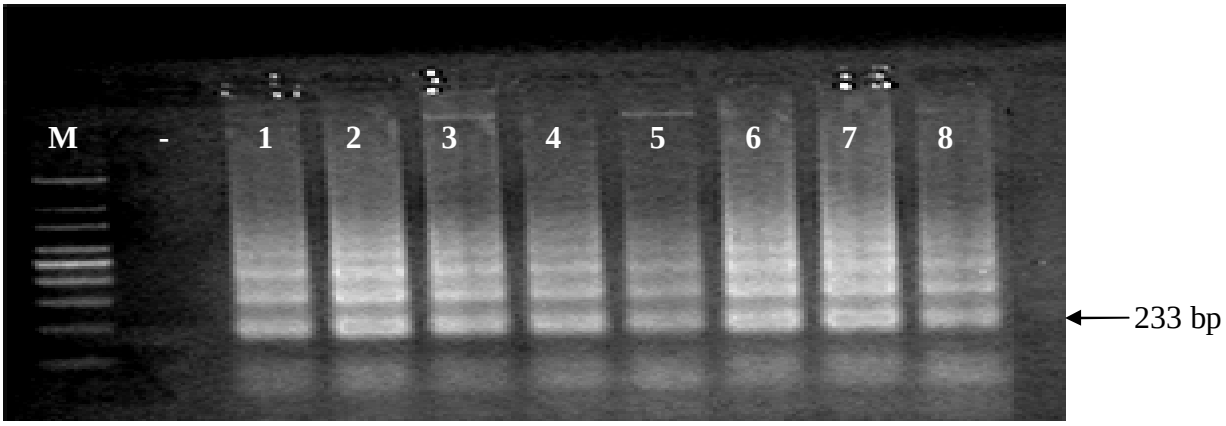

Figure 24: 2% Agarose gel electrophoresis of 8 symptomatic grasses tested for phytoplasma by isothermal diagnosis. M, PCR DNA ladder (Genscript); (-), Negative control; (1 –8), Napier grass samples.

LAMP diagnosis based on the ITS sequence amplified a 233 bp fragment of the target gene sequence from symptomatic grasses (Fig. 24). The band size was similar to predicted size (Fig. 23). The products were characteristic ladder-like or elongated gene sequences caused by repeats of the target sequence. Specificity of isothermal diagnosis based on ITS gene is presented in Figure 25. The isothermal primers were reactive to phytoplasmas in lanes 1 and 2 (RYD phytoplasma), and lane 12 (BGWL phytoplasma). All other phytoplasma groups were negative.

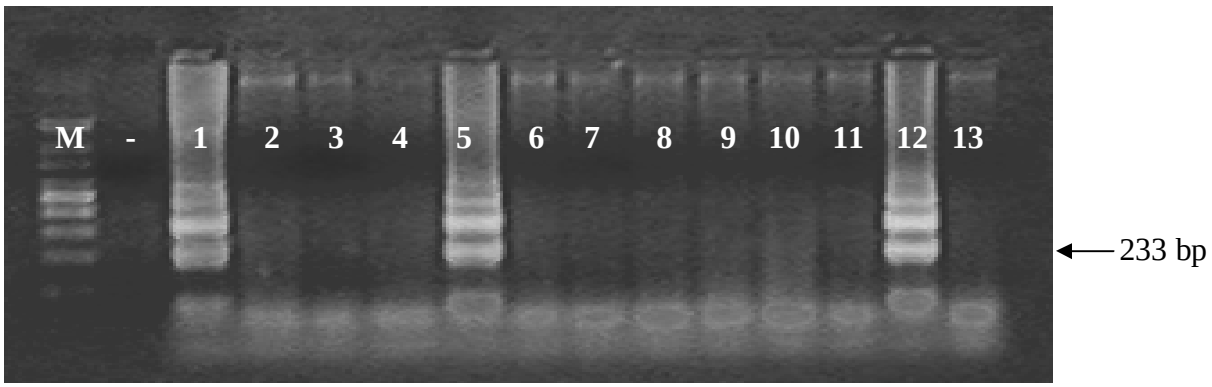

Figure 25: 2% Agarose gel electrophoresis of 11 phytoplasma groups tested for specific isothermal diagnosis of phytoplasma.Lanes: M, PCR DNA ladder (Genscript); (-), Negative control-water; 1, Hyparrhenia grass white leaf; 2, Western X; 3, Cape St. Paul wilt of Coconut; 4, Tomato big bud; 5, Napier stunting disease; 6, Strawberry green petal; 7, Soybean phyllody; 8, Hydrangea MLO; 9, Pear decline; 10, Stolbur; 11, Brinjal little leaf; 12, Bermuda grass white leaf; 13, (Negative control-healthy Napier grass).

4.4 Discussion

Accurate diagnosis of Ns-disease is required for successful management of the disease, through disease monitoring, and to identification of insect vectors. According to Maliyakal (1992), phytoplasma pathogen is difficult to diagnose, due to their uncultivable nature, low copies, and uneven distribution in the host plant. The distinct phytoplasma symptoms on Napier grass were sufficient for preliminary diagnosis of Ns-disease (Fig. 3). Nested polymerase Chain Reaction (PCR) was standard for confirmative phytoplasma diagnosis.

The oligonucleotode probes developed in this study were based on phytoplasma 16S rRNA gene. The 16S nucleotide sequence is standard for characterization of prokaryotes (IRPCM, 2004). The nested PCR using the newly developed NapF/NapR primers, based on the 16S were robust and reliable for diagnosis of Ns-disease. This was demonstrated by the thicker DNA bands on the gel and positive amplification in all the 8 diseased plants. According to Lee et al. (1994), nested-

PCR assay is highly sensitive in the detection of target DNA even in cases of unusually low titres of DNA present. The performance of NapF/NapR primer pair was excellent in PCR because they were specifically designed from Ns-disease phytoplasma 16S gene sequence. The primer hybridization to the target sequence was optimal. The primer parameters were also optimal for robust performance in PCR. Nevertheless, the target gene sequence was shorter (778 bp) compared to sequence amplified by R16F2n/R16R2 (1200 bp). This enhanced maximum sequence elongation in nested PCR.

The new primers (NapF/NapR) were non-specific, positively diagnosed 8 different phytoplasma groups. This agrees with sequence analysis predictions from database search. The new primers had the advantage of high annealing temperatures and the lack of significant sequence homology with chloroplast sequences. Non-specificity was attributed to the conserved nature of 16S rRNA gene among phytoplasmas. A broad-based diagnosis of phytoplasma is excellent in disease management since a host plant can be infected by more than one phytoplasma strain (Lee et al. 1994, 1995). Since it is not possible to exclude a latent infection with Ns-phytoplasma, a broad-based primer pair is preferable for Ns-disease diagnosis. The nested PCR based on the new primers was advantageous since it allowed further genetic characterization of the detected pathogen.

LAMP diagnostic tool was developed to replace standard PCR in routine testing, due to the high sensitivity, the characteristics of speed, sensitivity, and robustness. Copies of DNA were obtained within 1 h under isothermal condition of 65^0C. The specificity and reliability of LAMP reaction was accelerated by the addition of two primers, termed loop primers. The mechanism and expected reaction steps of LAMP using loop primers are detailed in Nagamine et al. (2002). The addition of loop primers increased target selectivity from 6 regions to 8 regions. LAMP was performed by incubating a mixture of the target gene, four different primers, Bst DNA polymerase, nucleotides and a suitable buffer, at an isothermal temperature of 65°C for 1 h. The technique was essentially an auto cycling process, since there was no denaturization, annealing and extension temperature. The isothermal temperature at 65 °C was optimal for the enzyme used for LAMP, Bst DNA polymerase. In LAMP, four primers hybridized to six distinct regions in the targeted DNA, to initiate DNA synthesis from the original un-amplified DNA. Therefore, target selectivity and specificity was expected to be high. This accounted for the 20-fold higher

analytical sensitivity of LAMP than nested PCR observed in this investigation. It is difficult to design specific PCR primers, based on 16S gene, which could separate closely related phytoplasma groups. The ITS region was useful for development of group-specific isothermal diagnosis. The 16s/23s ribosomal spacer sequences of bacteria are under minimal selective pressure during evolution and therefore should vary more extensively than sequences within genes that have functional roles (Barry et al. 1991; Saiki et al. 1988).

Predictive values were used to evaluate the usefulness of isothermal diagnosis. Compared to diagnosis based on symptoms, isothermal diagnosis was sensitive with 100% chance of true positive diagnosis, and zero chance of making a false negative diagnosis. The predictive values are important in disease management as only those seed canes negative by the assay should be certified for planting. The 20-fold higher sensitivity of isothermal diagnosis was predicted. Isothermal diagnosis uses 4 primers which hybridize to 6 distinct regions of the target gene, while nested PCR uses 2 primer primers which hybridize to 2 regions of the target gene. Three asymptomatic samples were positive by LAMP but negative by nested PCR. On the basis of higher analytical sensitivity, the 3 asymptomatic plants were not false positives, but had phytoplasma titers below the detection threshold of nested PCR.

The 16S region targeted for LAMP diagnosis is conserved among phytoplasmas. This explains why the isothermal primers targeting 16S gene sequence were reactive to 5 out of 11 phytoplasma groups. However, nested PCR primers also developed for the same gene, reacted to all the 11 phytoplasma groups. This is explained by the higher specificity of isothermal diagnosis. The assay uses up to 6 probes to target a single gene fragment, while nested PCR uses only 2 probes, therefore target selectivity is higher in LAMP compared to nested PCR. BsrI endonuclease was expected to cut F3 region of the targeted DNA into 66 and 113 bp. The two predicted fragments were restricted by BsrI endonuclease. The positive hybridizations of the probe DNA to the BsrI profiles served as molecular evidence that the DNA products produced by isothermal amplification had a similar structure to the one in Fig. 17.

New diagnostic tools were developed for robust detection of Ns-disease, however, it is nevertheless interesting to develop future assay methods with a higher multiplexing potential, thus improving the efficiency or ability to detect multiple phytoplasmas in a single step. In the

last few years, several other procedures have been proposed for the analysis of the PCR amplification products from phytoplasma infected plants, including PCR-ELISA (Poggi Pollini et al. 1997), PCR-dot blot (Bertin et al. 2004), heteroduplex mobility assay (Wand and Hiruki, 2000, 2001), 16S-23S spacer length polymorphism (Palmano and Firrao, 2000), microarray (Frosini et al. 2002) and nanobiotransducer hybridization (Firrao et al. 2005). Although these techniques may not have the characteristics of speed, sensitivity, and robustness of real time PCR, they are nevertheless interesting for developing future assay methods with a higher multiplexing potential, thus improving the efficiency or ability to detect multiple phytoplasmas in asingle step. It should be noted, however, that the major limitation to the development of high throughput, robust diagnostic assays for phytoplasmas remains the difficulty in developing a rapid and cost/labour effective preparation of representative nucleic acids extracts. It is well known that the phytoplasmas may be distributed very irregularly in infected plants. The most reliable diagnostic protocols, therefore, include the collection of samples as pools of subsamples taken from different parts of the individual plant to be tested. In order to reduce the amount of material to be processed usually the samples are enriched for phytoplasma and/or phytoplasma containing tissues before proceeding with nucleic acid extraction. Although this is a lengthy step, its suppression would lead to the occurrence of an unacceptable number of false negatives. Due to the intrinsic characteristics of phytoplasma diseases, i.e. the low concentration and irregular distribution of the pathogens, it is foreseen that the problem of sample representativeness of the sample is the major obstacle to further boost diagnosis of these plant pathogens.

4.5 Conclusion

Accurate diagnosis of plant pathogens is a prerequisite for its characterization and successful management of associated diseases. This study developed nested PCR and isothermal Ns-disease diagnostic tools. The putative phytoplasmas in Napier grass, insect vectors and other host plants can be accurately confirmed and defined on the basis of molecular phylogenetics. The newly developed protocols has advanced the diagnosis of Ns-disease. The nested PCR NapF/NapR primers targeted a 778 bp fragment of 16S rRNA gene, a smaller gene fragment from template DNA previously amplified by P1/P6 primers. The newly developed nested PCR NapF/NapR primers were reactive to 8 phytoplasma groups. The new primers were more sensitive than

conventional R16F2n/R16R2 nested PCR primers. Nested PCR was excellent because it allowed for genetic characterization of the target pathogen through sequencing and phylogenetic analysis.

This study also developed molecular primers for isothermal diagnosis of Ns-disease. This tool is set to replace nested PCR in phytoplasma diagnosis, due to its high sensitivity, the characteristics of speed, sensitivity, and robustness. The tool uses two sets of isothermal primers to target 16S, and the 16S/23S intergenic spacer region or the ITS gene. Like nested PCR, the isothermal primers targeting 16S gene fragment were reactive to 5 of 11 phytoplasma groups tested. This is due to the conserved nature of this gene across many phytoplasmas. Isothermal primers based on the ITS gene fragment were specific to closely related phytoplasmas; Ns-disease, HGWL, and BGWL phytoplasmas. The ITS gene has a higher sequence variability between phytoplasmas. This accounted for high its high specificity. Isothermal diagnosis based on the ITS gene also targeted 8 regions within the target DNA. This also increased target selectivity.

Compared to nested PCR, isothermal diagnosis was equipment-free, requiring only a device such as a water bath to maintain a constant isothermal temperature. It demanded less technical instrumentation than nested PCR. Detectable copies of DNA were obtained after only 1 h of incubation under isothermal condition of 65°C. When compared to the nested PCR which required up to 9 h, isothermal diagnosis produced results within a short time. The assay was 20-fold more sensitive than nested PCR. However, the higher analytical sensitivity increased the possibility of false positive reactions. Therefore, users of this technique should take every precaution to avoid contamination, for example by frequent change of gloves and separating areas for reagents, sample handing and analysis.

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CHAPTER 5

ALTERNATIVE HOST GRASSES OF NAPIER STUNTING DISEASE

5.1 Introduction

The pathosystem of Ns-disease is already known to consist of three basic components: the Rice Yellow Dwarf phytoplasma as the causative pathogen, Napier grass as the susceptible host plant, and *Maiestas banda* as a competent vector feeding on the host plant (Chapter 3). However, many phytoplasmas are maintained by natural disease cycles that include wild host plants, therefore, the pathosystem of Ns-disease may involve additional wild plant hosts which are significant as alternative sources of inoculum. According to Weintraub and Beanland (2006), the contemporaneous presence of a phytoplasma, weed reservoir and a competent vector has often been the cause of severe epidemics on food crops. For Ns-disease, the wild weed reservoir hosts were largely unknown. The inclusivity of Cereals in Ns-disease pathosystem was also unknown. This study identified the alternative host grasses of Ns-disease phytoplasmas. In this study, two phytoplasma surveys were conducted in western Kenya to determine active phytoplasma infections on the common grasses in the region, while through natural transmission experiments, the study determined inoculation, retention and responses to phytoplasma disease by Cereals grown in western Kenya. The present study therefore reports the wild and cultivated grasses which act as natural hosts of phytoplasma. The phytoplasma infections were also classified and their phylogenetic relationship with Ns-phytoplasma determined. The surveys mainly addressed the symptomatic plants, with the purpose being to monitor the presence of Ns-disease phytoplasma, its pathogenicity on other grass species, and to assess the presence and distribution of additional phytoplasmas in the Napier grass agro-ecology in western Kenya. The aim of the work was to improve knowledge of epidemiology of the phytoplasma disease in the region.

5.2 Materials and Methods

5.2.1 Infection of cultivated grasses

Five cultivated grasses were investigated for phytoplasma inoculation, retention and pathogenicity. The grasses were: finger millet, pearl millet, maize, sorghum, and rice. Each grass species was replicated in 12 pots and exposed to phytoplasma at 4 leaf stage. The grasses were then inoculated with phytoplasma in cages, containing six plants, using the leafhopper vector *Maiestas banda.* The inoculation procedure followed the protocol described in 3.2.2. A setup of unexposed plants served as control. The exposed grasses were incubated for symptoms appearance and phytoplasma detection by nested polymerase chain reaction.

5.2.2 Surveys

Field surveys were conducted at several locations in Napier grass agro-ecology in Bungoma, Busia, Vihiga, and Kakamega Districts (western Province) in 2009, principally areas of intensive Napier grass cultivation. A similar survey was conducted in 2010 at several locations across Ruma National Park, an area of little human activity. These surveys were conducted to determine active phytoplasma infections. In the first survey, five Napier grass plots in each locality were visited (Fig. 26), and each plot was divided into 2 m wide transects. Each transect was surveyed for active phytoplasma infections, and grasses showing phytoplasma-like symptoms were collected. In the second survey, sampling was done on grasses, 2 metres on each side of the road through the Ruma National park (Fig. 26). In both surveys, grasses showing the following symptoms were sampled for phytoplasma analysis: Bushy appearance, yellowing or whitening of leaves, and stunting. Leaves of symptomatic grasses were collected and surface sterilized in 70% Ethanol. The leaves were then chopped finely, dessicated over filter paper and on completion of the surveys, stored at -20^0C prior to testing. The symptomatic grasses were photographed. A herbarium of grasses showing phytoplasma symptoms from the field were also prepared for taxonomic identification. The identification of grasses was done using the grass identification manual of Barrion et al. (2007).

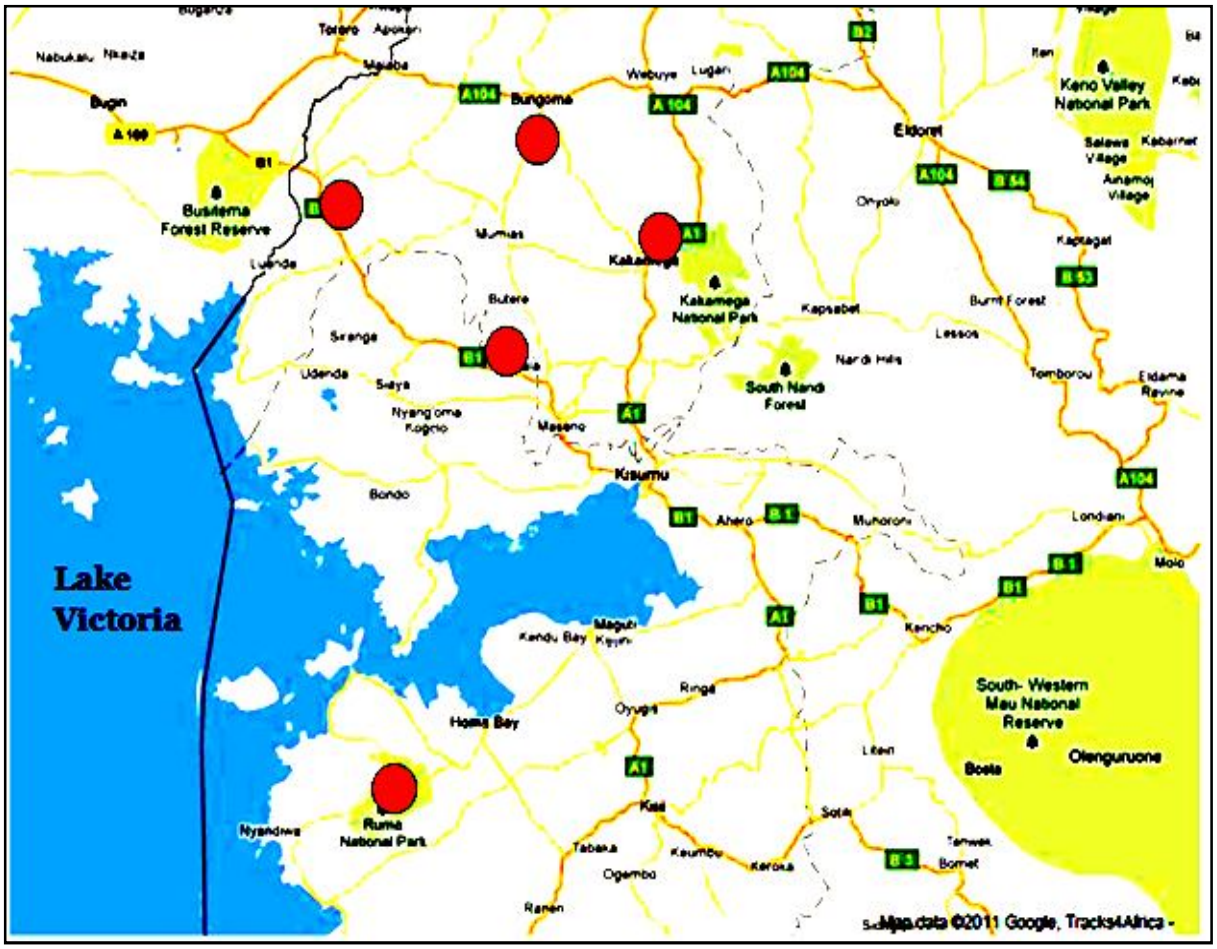

Figure 26: Google map of western Kenya showing the areas (indicated in red dots) where phytoplasma field surveys were conducted.

5.2.3 DNA extraction and nested polymerase chain reaction

Nucleic acids were extracted from the plant materials using the phytoplasma enrichment procedure of Doyle and Doyle (1990). The extracted DNA was diluted in water (dilutions were 1:10) to reduce the concentration of PCR inhibitory substances which might be present in the grasses. The enriched DNA was used as template in phytoplasma nested PCR using the 16S rDNA universal primers P1/P6 (Deng and Hiruki, 1991) for the first round PCR, followed by a nested PCR with R16F2n/R16R2 phytoplasma specific primers (Gundersen and Lee, 1996). For screening Food crops for phytoplasma, Ns-specific NapF/NapR nested PCR oligonucleotide probes were used. PCR and nested PCR were conducted as described in method 4.2.5. The PCR products were then electrophoresed on ethidium bromide stained 1% agarose gel. The presence of phytoplasma specific bands on the agarose gel was confirmed by visualizing the gel under UV-transillumination and photographed.

5.2.4 Sequence analysis

The plants that gave phytoplasma specific bands on the agarose gel were noted. The DNA bands were characterized further by sequencing the 16SrRNA gene to compare with data from other phytoplasma types. The PCR products were excised from the agarose, purified (GeneJET™ Gel Extraction Kit, Fermentas) according to manufacturer's protocol and sequenced directly using the ABI prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems, Scoresby, Victoria) with the phytoplasma specific primers R16F2n and R16R2 (Lee et al. 1993). Gel separation of the sequencing reactions was done at Segolilab (BecA, ILRI). Consensus sequences were generated using Genedoc software version 2.7 (Nicholas et al. 1997). The consensus nucleotide sequence was then deposited in the GenBank, and used to query the non-redundant database using BLAST (Altschul et al. 1990). Once the most similar sequence within the GenBank was identified, that sequence and several others representing the major groups of phytoplasmas (Table 10) were submitted for phylogenetic analysis using Mega version 4 (Koichiro et al. 2007) which utilizes the programs ClustalW (fast) (Thompson et al. 1994) and DNAdist, Neighbour and DrawGram (Felsentein 1989).

Table 10: Phytoplasmas used for phylogenetic analysis.

16Sr group	Group name	Species	GenBank Acc. No.
16SrI	Aster yellows	*Ca.* P. asteris	AY265211
16SrII	Peanut witch's-broom	*Ca.* P. aurantifolia	JN582265.1
16SrIII	X-disease	*Ca.* P. pruni	HQ589204.1
16SrIV	Coconut lethal yellowing	*Ca.* P. palmae	DQ384857.1
16SrV	Elm yellows	*Ca.* P. vitis	HQ199312
16SrVI	Clover proliferation	*Ca.* P. trifolii	HQ609490.1
16SrVII	Ash yellows	*Ca.* P. fraxini	HQ589190.1
16SrIX	Pigeon pea witch's-broom	*Ca.* P. phoenicium	HM988986
16SrX	Apple proliferation	*Ca.* P. mali	EF193361
16SrXI	Rice yellow dwarf	*Ca.* P. oryzae	JN223446
16SrXII	Stobur	*Ca.* P. solani	EF153634
16SrXIII	Mexican periwinkle virescence	Undefined	AF248960
16SrIV	Bermuda grass white leaf	*Ca.* P. cynodontis	EF444486

5.3 Results

5.3.1 Infection of food crops

Maize plants exposed to phtyoplasma remained negative after 3 months, showing that the vector was unable to inoculate maize. However, other cereals rice, finger millet, pearl millet and sorghum were able to acquire and retain phytoplasma through the activity of a leafhopper vector *M. banda*. PCR results showed 778 bp phytoplasma specific bands to confirm the presence of phytoplasma in these crops (Fig. 27). Transmission efficiency was high in the millets (90%) than other crops: 54% in rice and 33% in sorghum. After 3 months, all the potted crops attained maturity. Analysis of the plants, compared with the control setup, revealed no symptoms associated to the infected cereals under laboratory conditions.

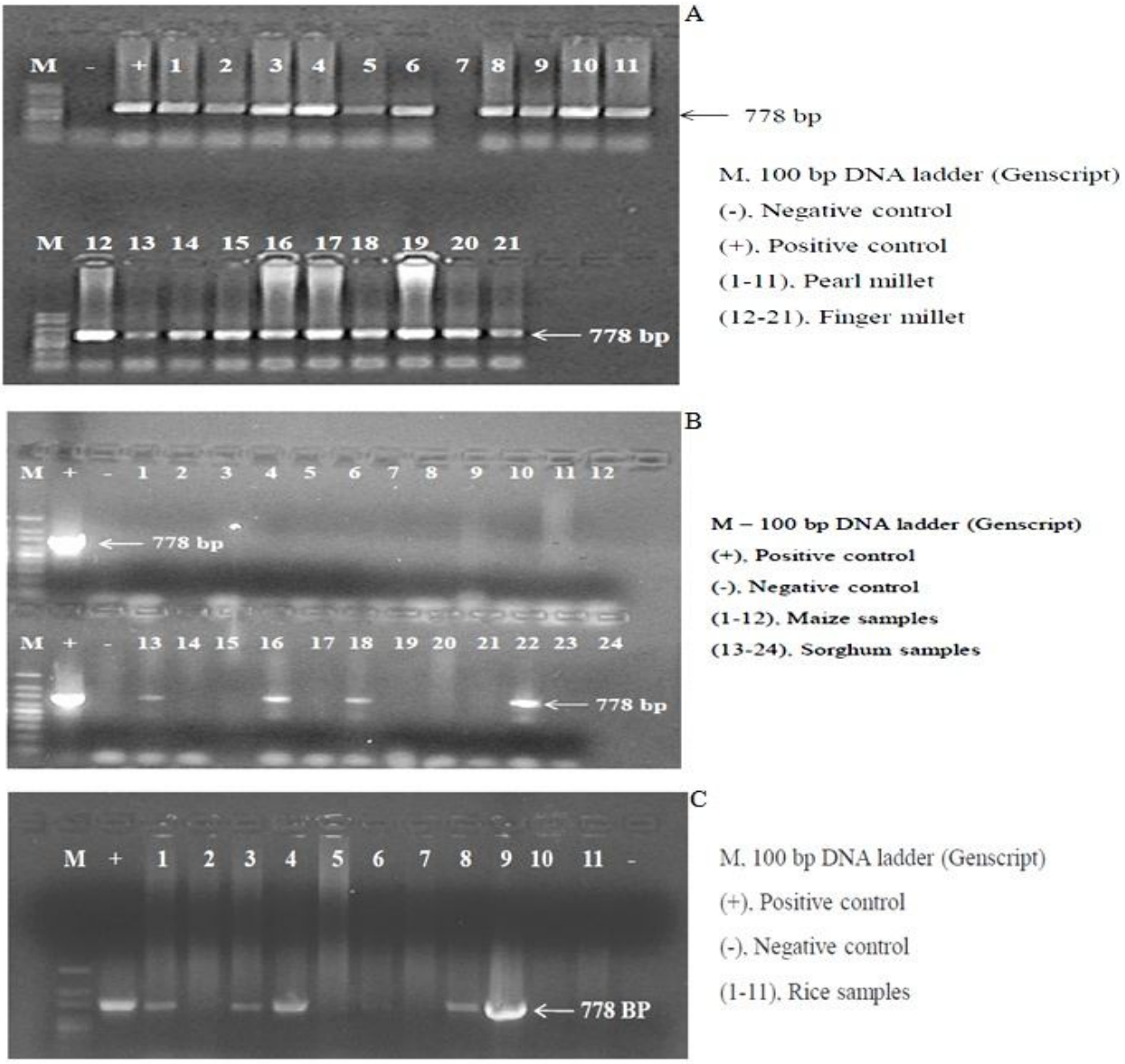

Figure 27: Agarose gel electrophoresis of PCR products from exposed pearl millet, finger millet (Gel A), maize, sorghum (Gel B) and rice (Gel C).

5.3.2 Field surveys

The phytoplasma-like symptoms observed were little leaves (reduced foliar size), leaf chlorosis (whitening of leaves), and witches broom (bushy growing habit, plus small leaves). (chlorosis), yellowing symptoms as decribed by Jones et al. (2004) were observed in Napier grass, mainly in Bungoma, Busia, Kakamega and Vihiga areas. A total of 27 phytoplasma records were confirmed by PCR from 3 different species (Table 11). Four records were from Busia, two were from Vihiga, five were from Bungoma, and the remainder were from Kakamega District (Table 11). In addition, a new phytoplasma disease was found in Bermuda grass *Cynodon dactylon* (Fig. 28) and Thatching grass *Hyparrhenia rufa* (Fig. 30). These plants were showing typical phytoplasma-like symptoms. Bermuda grass *Cynodon dactylon* plants observed were showing symptoms similar to Bermuda grass white leaf (BGWL) disease, caused by a phytoplasma (Marcone et al. 1997). The affected plants exhibited whitening of leaves, bushy growing habit, small leaves, shortened stolons/rhizomes, stunting, proliferation of auxiliary shoots and death. For the typical whitening of leaves, the disease observed in Bermuda grass in western Kenya was named Bermuda grass white leaf disease. Phytoplasma infection was confirmed by the amplification of a 1200 bp 16S rDNA nPCR fragment from all symptom-bearing *C. dactylon* plants tested (6/6, Fig. 29). No amplification was recorded in the symptomless plants.

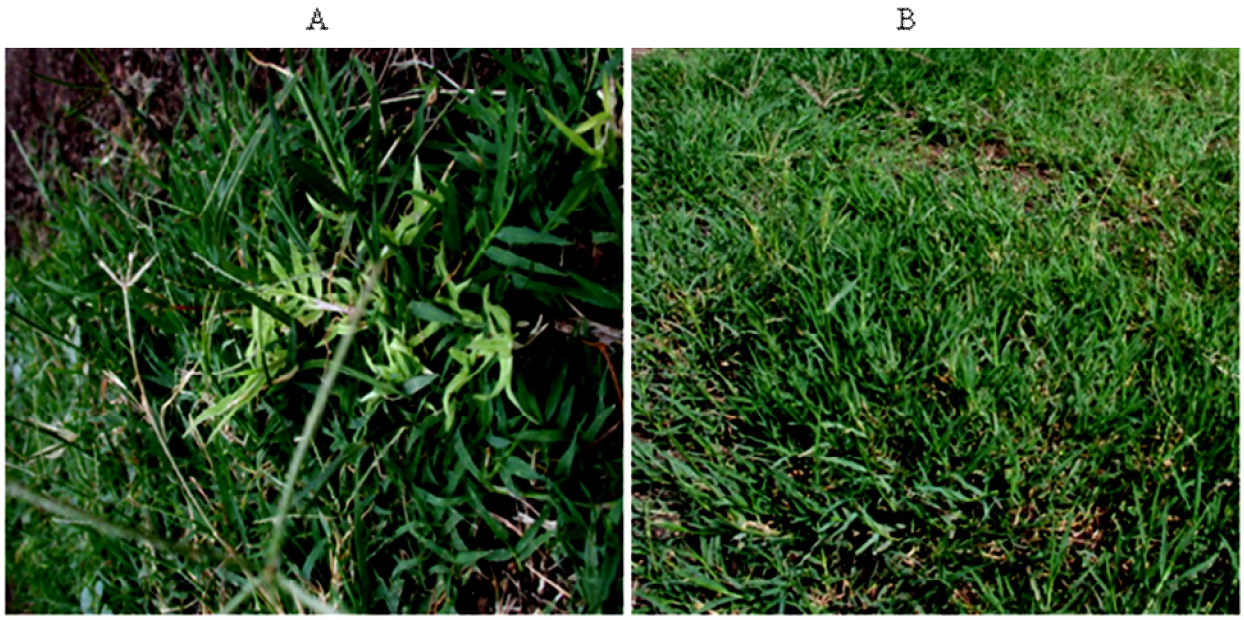

Figure 28: Photograph of Bermuda grass *Cynodon dactylon* in the field at Bungoma area in western Kenya. A: Symptomatic grasses (center) showing Bermuda grass white leaf (BGWL) symptoms; B: Asymptomatic grasses. Notice the flowering in asymptomatic grasses.

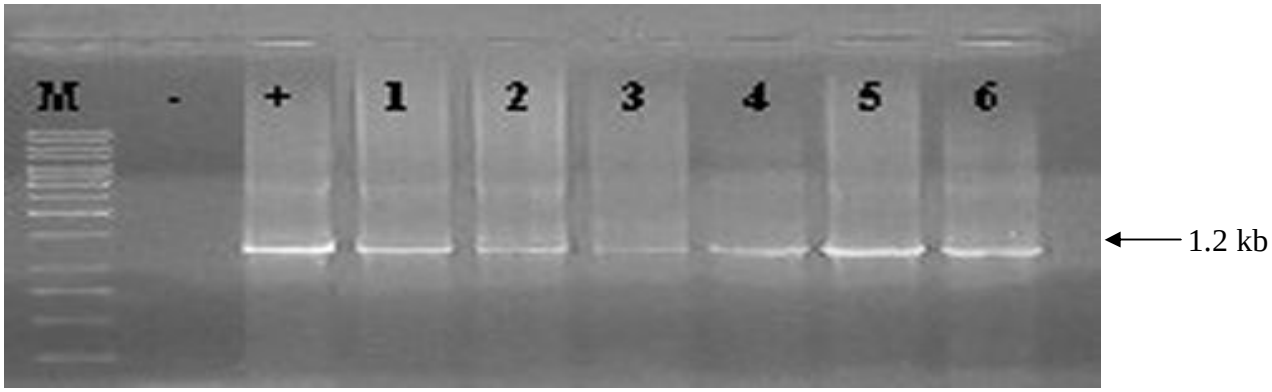

Figure 29: 1% agarose gel electrophoresis of *C. dactylon* infected with the BGWL phytoplasma. M: 1 Kb DNA ladder (Fermentas); (-): negative control, ddH2O; (+): positive control, Napier stunting phytoplasma DNA (16SrXI group); (1 to 6): C. dactylon showing BGWL symptoms and infected with the BGWL phytoplasma.

Table 11: Phytoplasma disease surveys in western Kenya (2009 and 2010).

Grass species	Collection no.	Approximate location[A]	PCR[B]	Strain cluster[C]
Cynodon dactylon	cd1,	Busia	+	BGWL
	cd2	Vihiga	+	BGWL
	cd3	Bungoma	+	BGWL
	Cd4	Kakamega	+	BGWL
Hyparrhenia rufa	Hyr1-Hyr11	Ruma Nat. Park	+	RYD
	cd5, cd6	Ruma Nat. Park	+	BGWL
Pennisetum purpureum	pp1-pp3	Busia	+	RYD
	pp4-pp7	Bungoma	+	RYD
	pp8, pp	Vihiga	+	RYD
	pp9, pp10	Kakamega	+	RYD
Digitaria scalarum	ds1-ds4	Busia	-	-
	ds3	Vihiga	-	-
	ds4-ds7	Bungoma	-	-
	ds8-ds10	Kakamega	-	-

[A]Locations: Busia, Bungoma, Vihiga and Kakamega (western Province) and Ruma National Park where the symptoms were observed.
[B]Phytoplasma infection confirmed by PCR test (+) or phytoplasma infection could not be confirmed by PCR (-).
[C]Determined by sequence analysis.

The symptomatic *H. rufa* were stunted and appeared bushy, with small white leaves (Fig. 30), symptoms resembling those associated with Napier stunting disease in Kenya (Jones et al. 2004). Unlike the adjacent healthy plants that were flowering, plants with these symptoms did not produce flowers. For the typical whitening of leaves symptoms, the disease observed in *H. rufa* was named Hyparrhenia grass white leaf (HGWL) disease. Phytoplasma infection was confirmed by amplification of 1200 bp rDNA fragments from all the samples showing symptoms (11/11, Fig. 31), while no amplification was recorded in the symptomless samples (0/11).

Figure 30: Photographs of *Hyparrhenia rufa* in the field at Lambwe valley in western Kenya. A: Symptomatic grasses; B: Asymptomatic grasses. Notice the savere chlorosis, absence of flowers, and general stunting of *H. rufa* plant in photograph A.

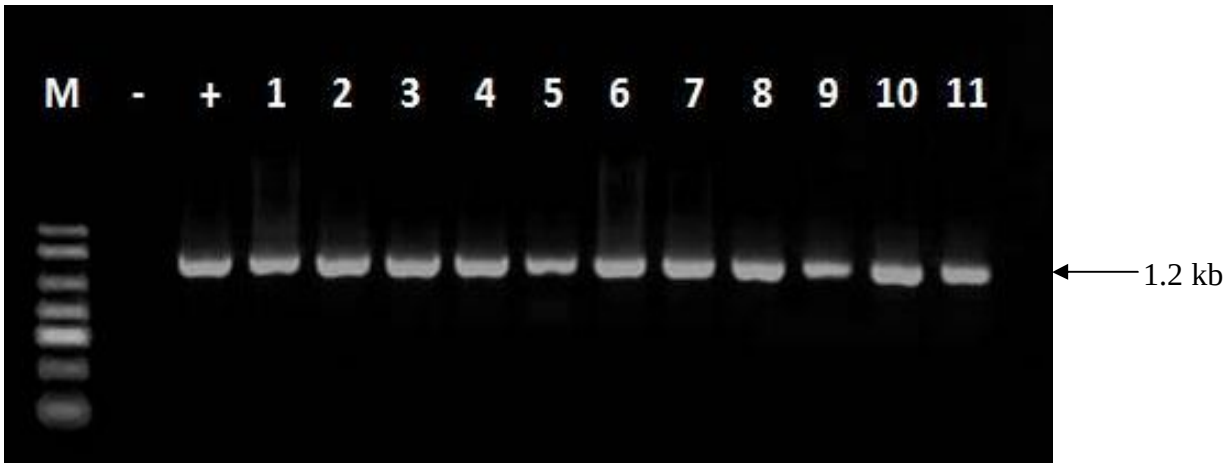

Figure 31: 1% agarose gel electrophoresis of DNA from *Hyparhenni rufa* infected with the HGWL phytoplasma. M: 1 Kb DNA ladder (Genescript); (-): negative control, healthy *H. rufa*; (+): positive control, Napier stunting phytoplasma DNA template; (lanes 1 to 11): Phytoplasma-infected *H. rufa* samples.

Figure 32: Photographs of Napier grass *Pennisetum purpureum* taken in the field at ICIPE's Mbita point field station in western Kenya. Notice the plant yellowing, general stunting and bunchy appearance of diseased plant. A: Symptomatic grass; B: Asymptomatic grass.

Figure 33: Specimens of *Digitaria scalarum.* A: Symptomatic grass; B: Asymptomatic grass. In photograph A (diseased), notice the massive proliferation of auxillary shoots giving the grass a bunchy appearance.

Figure 32 shows healthy and diseased Napier grass plants observed in the field during phytoplasma surveys. The grasses exhibited short internodes, bushy appearance, and lethal yellowing symptoms. PCR analysis on these plants revealed phytoplasma infection. In addition to the phytoplasmas characterized by molecular methods, suspected phytoplasma infection was documented in *Digitaria scalarum* (Fig. 33). This was a grass showing typical phytoplasma-like symptoms, but infection could not be confirmed by PCR even after dilutions were made to reduce PCR inhibition.

5.3.3 Sequence analysis

The partial 16S rDNA sequence of phytoplasma isolated from Bermuda grass was submitted to GenBank (Accession No. GU944766). The BGWL phytoplasma 16S rDNA sequence exhibited 100% of identity with that of '*Candidatus* Phytoplasma cynodontis' strain LY-C1 (EU409293), which belongs to the BGWL group (16SrXIV), and 99% of sequence identity with other BGWL phytoplasma members. The consensus partial nucleotide sequence of phytoplasma isolated from *H. rufa* was submitted to GenBank (Accession No. JN112372). Sequence analysis revealed highest 16S rDNA sequence identity (99%) of this phytoplasma with that of the Napier grass stunting phytoplasma (GenBank Accession No. AY377876), confirming the HGWL phytoplasma as a member of the 16SrXI phytoplasma group (‘*Candidatus* Phytoplasma oryzae’). The phytoplasma isolated from Napier grass was deposited in the GenBank (Acc. No. FM999728). The sequence of the 16S rRNA region of Napier stunting phytoplasma was 100% similar to other Napier stunting phytoplasma sequences in the database.The BLAST analysis therefore showed a clear relationship between phytoplasmas discovered in these surveys and other phytoplasmas. The distinct relationships were further revealed by constructing a phylogenetic tree of these new phytoplasmas and selected phytoplasmas (Fig. 34).

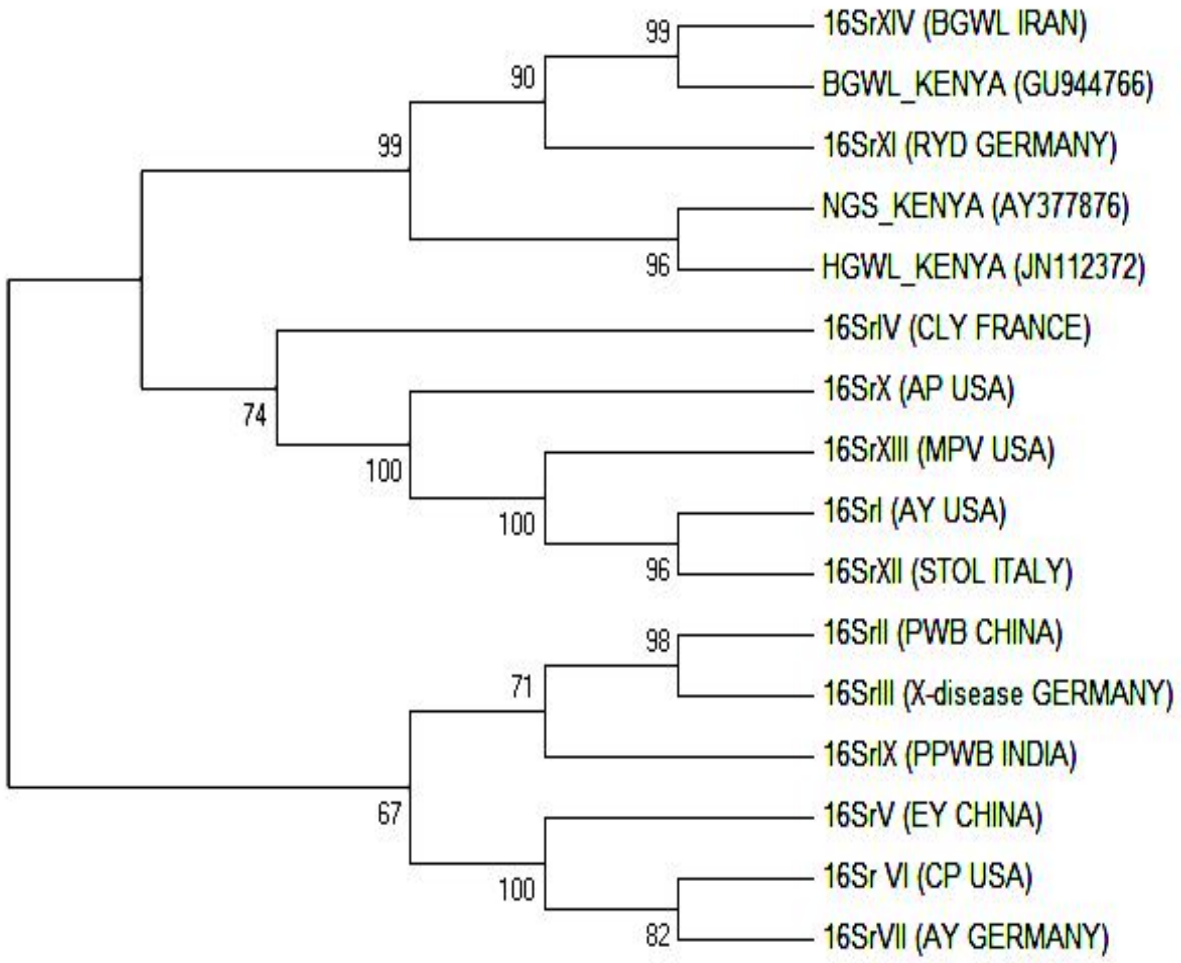

Figure 34: Phylogenetic tree using 16S sequences showing the relationship between phytoplasma isolated from Napier grass stunting (NGS-Kenya, AY377876), Bermuda grass white leaf (BGWL-Kenya, GU944766), Bermuda grass white leaf (BGWL-Iran, EF444486), *Hyparrhenia rufa* (HGWL, JN112372) with STOL (Stolbur, EF153634), MPV (Mexican periwinkle virescence, AF248960), AY (Aster yellows, AY265211), AP (Apple proliferation, EF193361), PWB (Peanut witch's broom, JN582265), X-disease (HQ589204), PPWB (Pigeon pea witch's-broom, HM988986), CLY (Coconut lethal yellowing, DQ384857), AY (Ash yellows, HQ589190), CP (Clover proliferation, HQ609490), EY (Elm yellows, HQ199312), RYD (Rice yellow dwarf, JN223446). The bar represents a phylogenetic distance of 1%.

5.4 Discussion

Under laboratory conditions, it has been demonstrated that *M. banda* can transmit phytoplasma to food crops. There was distinct hierarchy in transmission efficiency which ranged 0% in Maize to 90 in the millets. This hierarchy is similar to the survivorship of *M. banda* on various grasses, where the survivorship rate was lowest in Sorghum (0.13) and highest in Pearl millet (0.74). Feeding preference, duration of feeding and successful phytoplasma inoculation are correlated. The insect vector requires optimal feeding time to successfully inoculate enough titer to initiate infection. This is called Inoculation Access Period or IAA (Weintraub and Beanland, 2006). In the experimental setup, a choice was presented in the transmission cages, when disease Napier grass (inoculums source), a preferable host plant sat in the same cage with the crops. It is likely that all/most of the insects moved to Napier grass when a less suitable host plant was in the vicinity. This reduced phytoplasma transmission efficiency in the less preferred host plants. These results demonstrate that *M. banda* is also able to probe and transmit phytoplasma to crops in the absence of Napier grass.

The two independent surveys carried out in this study, have demonstrated the presence of active phytoplasma infections in wild grasslands in western Kenya, in addition to Napier stunting disease. Two different groups of phytoplasmas, 16SrIV, and 16SrXI were identified, and of the 6 regions visited, all were demonstrated to harbour phytoplasmas. At Ruma National park, only a single plant species was tested and phytoplasmas were detected. The 16SrIV phytoplasmas were found to be most widespread, and were present at all the six sites where phytoplasma infections were found. The study reports two new phytoplasma host records for Kenya (*C. dactylon* and *Hyparrhenia rufa)*. These diseases were named Bermuda grass white leaf (BGWL) and Hyparrhenia grass white leaf (HGWL) diseases. This is because the infections led to characteristic whitening of foliage. In plant pathology, as in human and animal medicine, not only pathogens but also the diseases they cause historically have been named largely on the basis of symptoms exhibited by infected hosts. Plant diseases attributed to phytoplasmas are no exception to this practice. For example, many phytoplasmal diseases have been classified in general terms, either as decline or virescence diseases. Disease names, such as aster yellows,

strawberry multiplier, pear decline, palm lethal yellows, and others, indicate the importance of perceived symptoms in the assignment of common names to diseases.

Surprisingly, only two new phytoplasma records were found in western Kenya. This might be because field surveys were conducted in intensively cultivated plots where hand removal of abnormal plants was a common practice. The most notable phytoplasma found in these surveys was BGWL, associated with white leaf disease in *C. dactylon.* The fact that it was found in many localities, suggests that it may be indigenous phytoplasma. Phylogenetic analysis of BGWL, HGWL and Ns-disease phytoplasmas (Fig. 34) shows that they are closely related. According to Moran and Plague (2004), Phytoplasmas, like other prokaryotes, undergo genomic changes following host restriction. It can be hypothesized that the three phytoplasmas are similar, but diverged slightly to colonize new plant niches. In chapter 4, the highly specific isothermal probes developed based on the highly conserved ITS gene of Ns-phytoplasma reacted with the DNA template of BGWL, Ns-phytoplasma and HGWL phytoplasma. This is a strong evidence of genetic similarity. The leafhopper *M. banda* is the putative vector of the three phytoplasmas.

Bermuda grass (=Star grass), *Cynodon dactylon*, is a perennial plant of the family Poaceae that is native to the African Savannah but is now widely distributed over many warm countries of the world (Marcone et al. 1997). *C. dactylon* or Bermuda grass, has been well documented to harbour phytoplasmas in other countries throughout the world, and the symptoms found in western Kenya were consistent with those caused by phytoplasma infection in this grass worldwide. BGWL is a lethal disease especially in warmer areas. It was first reported in Taiwan (Chen et al. 1972), but is now known to occur in other Asian countries (Zahoor et al. 1995; Lee et al. 2000; Rao et al. 2007; Jung et al. 2003), Africa (Daffala and Cousin, 1988), Australia (Padovan *et al*. 1999; Tran-Nguyen et al. 2000), Europe (Marcone *et al*. 1997) and Cuba (Arocha et al. 2005). To our knowledge, this is the first report of the BGWL disease and its associated phytoplasma in Kenya. The disease is significant since *C. dactylon* is widely used for forage and turf in the region. As the disease progresses, rangeland productivity is bound to decline. This will compromise the provision of pasture for livestock and wildlife. BGWL disease will also affect turf production and will dramatically reduce the role of turf in preventing soil erosion. It was evident that Ns-phytoplasma and BGWL phytoplasma were closely related. Marcone et al. (1997) showed that the BGWL phytoplasma has the smallest genome of all known

phytoplasmas. The BGWL phytoplasma evolved from RYD phytoplasmasby evolutionary changes after changing ecological niche to *C dactylon* by vector feeding. Typical changes included a large increase in the frequency of mobile elements in the genome, chromosomal rearrangements mediated by recombination among these elements, pseudogene formation, and deletions of varying size (Moran and Plague, 2004). These changes represent a general syndrome of genome evolution, which is observed repeatedly in host-restricted lineages from numerous phylogenetic groups. Considerable variation also exists, however, in part reflecting unstudied aspects of the population structure and ecology of host-restricted bacterial lineages.

Thatching grass *Hyparrhenia rufa* is commonly found throughout the tropics where it also serves as valuable cattle fodder and border grass to prevent soil erosion (Skerman and Riveros, 1990). *H. rufa* have never been documented to harbor phytoplasma, and this is the first account of this grass species reported to host phytoplasma. To our knowledge, this is the first record of the group 16SrXI, '*Ca.* Phytoplasma oryzae' associated with the white leaf disease of *H. rufa,* and the first record of the thatching grass as a host for a phytoplasma. This report also shows that *H. rufa* may be an alternative host plant for the Napier grass stunting phytoplasma and might play a role in the epidemiology of Napier grass stunting disease in East Africa. As the disease spreads, it will affect the continued use of *H. rufa* as cattle fodder and thatching grass. The disease will negatively impact soil conservation efforts in parts of East Africa where *H. rufa* is the main border grass. The study of Obura et al. (2009) implicated the leafhopper *M. banda* as vectors of Ns-phytoplasma in western Kenya. The vector is present in western Kenya and nearby regions. If indeed vectors are available and the conditions in western Kenya are suitable for successful transmission, these observations could be explained by pathogenic variation within Ns-phytoplasma and HGWL that has not yet been identified.

It was evident that HGWL phytoplasma had 100% similarity to Ns-disease phytoplasma (Fig. 34), indicative of a natural phytoplasma reservoir. In chapter 3, the survivorship rate of the Ns-disease vector *M. banda* was very low in *H. rufa.* Being able to colonize both *H. rufa* and *P. purpureum*, Ns-phytoplasma has multiple host plants, and the host range might include a number of fodder grasses and cereals in western Kenya. Even though phytoplasma specificity is known to exist for plant hosts (Lee *et al*, 1998), it is evident that Ns-phytoplasma is not plant host specific. It is evident that Ns-phytoplasma is maintained by natural disease cycle that includes *H.*

rufa as a wild host plant. However, additional vectors are probably involved. It can be hypothesized that vector feeding on infected *H. rufa* occurred in the same environment with Napier grass resulted in a phytoplasma infection to Napier grass. However, the fact that HGWL disease was only sampled in the wild grasslands away from Napier grass, and the fact that HGWL infections from the road side localities were aggregated together suggests possible presence of additional vector apart from *M. banda*, and an additional pathosystem probably exists. Since *M. banda* shows a narrow feeding preference on two closely related pennisetum grasses (Napier grass and Pearl millet), the newly emerged disease was then transmitted independently from the original pathosystem system involving *H. rufa*. The management of Ns-disease, where the affected crop (Napier grass) is involved in the pathosystem, is relatively easy because both the phytoplasma and its vector are endemic and available in Napier grass vegetation. Eradication of infected Napier grass will therefore hold up disease progress and the application of insecticide in Napier grass fields will affect the vector. *H. rufa*, the alternative host plant for Ns-disease plays a key role in Ns-disease pathosystem as the grass provides alternative food sources that would increase infection pressure to Napier grass.

5.5 Conclusion

It has been demonstrated that Ns-disease is transmissible to cultivated grasses under laboratory conditions. In the field, the high vector-host fidelity may largely restrict phytoplasma to Napier grass. The RYD phytoplasma was discovered in wild Thatching grass, where it is pathogenic. In disease management, eradication of Napier grass alone will not lead to direct eradication of Ns-disease, as a significant reserve will still remain in Thatching grass. A second phytoplasma, belonging to 16SrXIV-group was discovered Bermuda grass. Phylogenetic analysis revealed that Ns-phytoplasma, HGWL, BGWL phytoplasmas share a common ancestry with the RYD strain cluster (Fig. 34). This finding demonstrates a smaller genetic diversity in Kenyan phytoplasmas, and raises further questions about the validity of separating BGWL from Ns-disease phytoplasma based on ecology of the two phytoplasmas. In order to get clearer view of Ns-incidence, an extensive epidemiological study should be done monitoring the active role of *H. rufa* in western Kenya, searching other alternative host plants and performing vector transmission trials. The fact that new phytoplasmas

(BGWL and HGWL) have been found in common wild grasses in western Kenya implies that surveys focusing on native grasses might uncover more phytoplasma types.

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CHAPTER 6

GENETIC DIVERSITY AND RESPONSE TO PHYTOPLASMA DISEASE AMONG THE LOCAL NAPIER GRASS VARIETIES IN WESTERN KENYA.

6.1 Introduction

Phytoplasmas differentially colonize plants depending on the pathogen type, and properties of the host plant. In chapter 6, Napier grass was identified as the main host plant of Ns-disease phytoplasma, and the abundance of susceptible Napier grass varieties increases disease incidence. The pathogen induces yellowing of Napier grass folia, caused by Phytoplasma presence in the phloem, affecting its function and changing the transport of carbohydrates (Muast et al. 2003). The infected plants also appear bushy and are stunted due to changes in normal growth patterns caused by the infection. Napier grass germplasm in the region comprises predominantly the elite varieties, of excellent agronomic characteristics, which were introduced to farmers, through Kenya Agricultural Research Institute (KARI), by African Feeds Resources Network (AFRNET) in 1991. Remnants of these elite collections are available at Kenya Agricultural Research Institute (KARI, Kitale). Since the outbreak of Ns-disease in 2004, farmers have responded by selecting potentially resistant varieties.

The AFRNET collection available at KARI, plus the farmer selected varieties form a significant representative sample of Napier grass germplasm in western Kenya. However, the varieties are phenotypically indistinguishable (Orodho, 2006), unless through the use of Molecular fingerprinting tools. These tools are becoming essential in plant genetics (Gupta et al. 1999) and have several advantages over the traditional phenotypic markers that are difficult or time-consuming to select. These DNA type markers are not influenced by environmental conditions and are detectable at all plant growth stages. There are several different DNA analysis procedures that have been used to develop molecular markers. Each procedure has its own requirements, sensitivity, and reliability.

AFLP is one of the most reliable DNA fingerprinting procedure. It combines assay flexibility with high degree of sensitivity and reproducibility (Vos et al. 1995). The degree of polymorphism detected per reaction is much higher than other DNA fingerprinting techniques (Lin et al. 1996). AFLP has been extensively used for developing polymorphic markers

associated with disease resistance in different grasses like *Hordeum* (Altinkut et al. 2003), *Zea mays* (Agrama et al. 2002), and *Oryzae* (Jain et al. 2004). The objective of this study was to use AFLP procedure to assess the diversity of Napier grass germplasm in western Kenya and determine its response to phytoplasma disease under laboratory conditions. Polymorphism analysis as well as development of molecular markers could be helpful in identification of the genome segments associated with phytoplasma disease resistance in Napier grass, which may in turn help in selecting disease resistant genotypes.

6.2 Materials and Methods

6.2.1 Plant varieties

The varieties of Napier grass analyzed in this study are presented in the Table 14. Fifteen varieties were collected from farmers in Bungoma, Busia, Mumias, Siaya and Suba districts of western Kenya, areas with high phytoplasma disease pressure. Twenty one varieties were obtained from KARI at their station in Kitale, western Kenya. After the collections, the varieties were maintained in the screen house, and their phytoplasma status determined by nested PCR testing. Diseased Napier grasses, or sources of phytoplasma inoculum, were also collected from the field at ICIPE and maintained in a separate screen house.

6.2.2 Insects

Phytoplasma vector *Maiestas banda* (Obura et al. 2009) were collected by suction pump from Napier grass field at Mbita (0°25′ S, 34°12′ E) in western Kenya. *M. banda* were identified and selected from the sampled insects before being transferred to the screen house for mass rearing. Rearing was done on potted Napier grass diet in the screen house, at 20–28°C and 65–70% RH, in cages (25×25×60 cm) made of wooden frame. Periodically, these colonies were infused with insects collected from the field to minimize inbreeding.

6.2.3 Phytoplasma inoculation and disease monitoring

The genotypes were replicated in twelve pots each then naturally inoculated with phytoplasma using the *M. banda* vector. Inoculation was done in cages containing six plants according to the standard protocol (Weintraub & Beanland, 2006). Inoculation setup was as follows: a diseased Napier grass (inoculum) was surrounded by 6 healthy test plants in a cage, 50 infected female

insects were introduced into the same cage, then the setup incubated for one month. Un-exposed plants served as control. After exposure, the plants were cut every two months, and the re-growth tested for phytoplasma by nested polymerase chain reaction (PCR), and observed for phytoplasma infection and response. Response of each cultivar to phytoplasma infection was measured by 3 parameters: a) disease expression, b) time taken to express disease, and c) number of plants which expressed disease. Disease expression was determined using a 0-2 rating scale where 0=no symptom/PCR negative (resistant), 1=no symptom/PCR positive (Tolerant), 2=symptomatic/PCR positive (Susceptible). Time taken to express disease was determined in a 1-3 rating scale, where after 1 cutting =highly susceptible, 2 cuttings = very susceptible, 3 cuttings= susceptible, 4 cuttings=moderately susceptible. For the proportion of plants expressing symptoms, the higher the proportion, the more susceptible a cultivar was to phytoplasma.

6.2.4 Detection of Phytoplasma in test plants

Test plants before and after exposure were assayed for phytoplasma by nested PCR. To obtain DNA for molecular analysis, leaf samples were taken from the test plants and stored in Liquid Nitrogen. Total DNA was extracted from the leaf samples using the CTAB extraction method of Doyle and Doyle (1990). Leaf tissue (300mg) was powdered in liquid Nitrogen, and incubated in preheated buffer (CTAB buffer: 2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris–HCl, pH8.0, 0.2% 2-mercaptoethanol). DNA was extracted with 1 volume of Chloroform:Isoamyl Alcohol (24:1) and precipitated overnight in a -20 freezer using isopropanol. Following centrifugation at 14000 rpm (Centrifuge 5415C, Eppendorf), the DNA pellet was rinsed with 800 µl of 70% Ethanol, dried and dissolved in 50µl Tris-EDTA (20mM Tris, 1mM EDTA pH 8) buffer. The extracted DNA was used as template in a nested PCR assay. Nested PCR mix and cycling conditions followed the published protocols (Lee et al. 1998), using primer pair P1/P6 (Deng and Hiruki 1991) and nested PCR with NapF/NapR (Chapter 6) primers, in a PTC 100 programmable thermocycler (MJ Research). DNA amplified in PCR primed by P1/P6 was diluted 1:50 with sterile water and vortexed to mix, and 1µl used as a template in an nPCR. PCR products were detected in a 1% ethidium-bromide stained Agarose gel using 1× TAE (40 mM Tris acetate, 1 mM EDTA pH8.0) as running buffer, and photographed.

6.2.5 DNA Extraction and AFLP analysis

DNA was extracted using the hot CTAB method of Doyle and Doyle (1990) with a few modifications. 1g of Napier grass leaf tissue was powdered in liquid Nitrogen, 3.5ml of warm CTAB buffer was added, and then the tissue-CTAB mixture was incubated at 65^0 C for 1 hour. Napier grass genomic DNA was then extracted twice with Chloroform: Isoamyl alcohol (24:1), then precipitated with Isopropanol in a -20^0C freezer. The DNA precipitate was then washed twice with 800 µl of 70% Ethanol, air dried for 30 mins, then eluted in 100 µl of Tris–EDTA buffer (40 mM Tris acetate, 1 mM EDTA pH8.0). To remove RNA, 5µl of RNase (10 mg/ml) was added and the mixture incubated at 37 for 30 mins. RNase was then deactivated by incubating the tubes at 65^0 C for 30 mins. DNA purity and concentration was determined using a NanoDrop ND-1000 spectrophotometer while the DNA quality was confirmed by electrophoresis on a 1% Agarose gel. The concentration of Napier grass genomic DNA was standardized at 200 ng/µl and stored in -20°C pending AFLP analysis. Amplified fragment length polymorphism (AFLP, Vos et al. 1995) analysis was done using the "Plant Mapping Protocol" and AFLP Kit Module [Applied Biosystems (ABI), USA]. Selective amplification was conducted using five MseI and *EcorI* primer combinations (Table 12) [*EcorI*-ACC/MseI-CAA, *EcorI*-AGG/MseI-CTC, *EcorI*-AGG/MseI-CAT, *EcorI*-ACT/MseI-CAT and *EcorI*-ACA/MseI-CTC] selected from an earlier study (Table 12, Wanjala et al. draft thesis). The capillary system (ABI PRISM 3730TM) was used to resolve selective amplification products. From the ABI PRISM 3730, the sample data were directed to the GeneMapper^{TM} software for tabulation. The results were displayed as electrograms and allele frequency data of product presence /absence.

Table 12: Oligonucleotide adapters and primer names and sequences for the 5 selective amplified fragment length polymorphism (AFLP) primers.

Name	Sequence (5'-3')
MseI Adapter	CTACTCAGGACTC/AT
	GATGAGTCCTGAG
EcorI Adapter	GACTGCGTACC
	CTGACGCATGG/TTAA
EcorI + 1	GACTGCGTACC-AATT/N
MseI + 1	CATCTGACGCATGG-TTAA/N
EcorI + 3-ACC	GACTGCGTACC-AATT/ACC
EcorI + 3-AGG	GACTGCGTACC-AATT/AGG
EcorI + 3-ACT	GACTGCGTACC-AATT/ACT
EcorI + 3-ACA	GACTGCGTACC-AATT/ACA
MseI + 3-CAA	GATGAGTCCTGAG-TA/CAA
MseI + 3-CTC	GATGAGTCCTGAG-TA/CTC
MseI + 3-CAT	GATGAGTCCTGAG-TA/CAT

6.2.6 Data analysis

AFLP data was subjected to cluster analysis using PopGen32 population genetic analysis software (Yeh et al. 1997). Outputs of PopGen32 included genetic distance, dendrogram, gene diversity over loci, polymorphic loci and gene frequency (Nei 1987; McDermott and McDonald 1993). A distance matrix and Principal Co-ordinates Analysis (PCoA) was generated using Darwin software (Perrier, 2006).

6.3 Results

A total of 36 samples were analyzed for genetic diversity and response to phytoplasma disease infection. Farmers had selected 15 varieties to be resistant to be Ns-disease. These varieties were obtained from the farmers and included in the analysis. The remnants of AFRNET varieties available at KARI station (Kitale) were 21. These varieties were also included in the analysis. AFLP analysis identified the varieties from each other and established the genetic relationship between them. Closely related varieties clustered together while and varieties that were more

distantly related were identified (Fig. 35 and 36). The selected 5 primer- pair combinations (Table 12) generated clear-cut AFLP profiles from the 36 varieties. These are the best primer combinations that generated maximum number of fragments (Bramwel et al. MSc Draft thesis). Fingerprinting revealed a total number of 133 polymorphic gene loci, with an average of 26.6 loci per primer combination. The AFLP fragments ranged from approximately 50 to 500 base pairs (bp). The number of polymorphic alleles were 94 representing a level of polymorphism of 71%. A summary of the genetic distance among the varieties is presented in Table (13). The genetic distance estimates ranged from 1 to 94 gene loci. The highest genetic distance (94 alleles) was between varieties Bana grass and Ex-Mariakani. While, the lowest genetic distance (7 alleles) was observed between the farmer varieties RT-A and Ex-Teso.

The genetic relationships were also expressed as dendrogram (Fig. 35) which illustrates the graphical representation of the genetic distances between the 36 Napier grass varieties. Principal Coordinate (PCo) plots (Fig. 36) based on genetic distance (Table 13) also showed the relationships between the varieties in two dimensional space. The x and y coordinates accounted for 68.56% of the total percentage variation. Clustering pattern was based on the geographical origin of the cultivars, herbage yield and resistance to fungal diseases. Cluster one, the largest cluster, formed 59% of the varieties analyzed. All the farmer varieties clustered together in this group except OP, OU-C and OU-D. Cluster two, the second largest cluster, formed 16% of the varieties analyzed. Cluster three and four formed 8% and 13% of the varieties analyzed respectively.

Table 13: Distance matrix shows the distance index between the western Kenya Napier grass varieties (numbers indicate the serial number of genotypes as in Table 14.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	ID
0																																				1
7	0																																			2
24	23	0																																		3
64	67	56	0																																	4
52	47	50	74	0																																5
56	51	56	88	26	0																															6
31	28	31	69	47	45	0																														7
50	47	48	50	64	70	57	0																													8
34	35	32	54	38	56	43	42	0																												9
51	56	43	41	57	67	60	73	47	0																											10
29	34	31	65	47	51	48	51	35	44	0																										11
34	35	38	62	46	56	43	50	32	61	35	0																									12
38	37	26	50	42	52	41	54	24	37	41	46	0																								13
51	50	51	59	71	65	50	29	51	76	68	51	65	0																							14
53	56	41	33	61	75	46	71	49	30	52	57	47	76	0																						15
53	58	51	37	69	79	54	31	51	60	58	53	49	44	58	0																					16
39	38	43	75	45	43	44	55	35	70	40	31	53	52	66	62	0																				17
59	64	49	31	61	75	62	75	41	22	56	59	37	76	20	58	70	0																			18
59	54	57	59	69	61	56	25	55	82	74	61	65	20	84	44	56	84	0																		19
54	59	44	32	68	80	65	68	44	17	55	58	38	71	25	55	71	15	77	0																	20
54	57	38	32	66	76	53	74	50	29	51	52	44	77	15	59	71	23	85	24	0																21
70	73	62	34	76	88	71	56	66	55	77	68	62	61	43	49	75	43	61	48	48	0															22
44	43	38	56	60	62	53	58	38	43	37	52	28	71	51	59	47	49	71	48	50	58	0														23
36	35	32	60	38	58	45	52	18	51	35	30	32	59	53	61	39	55	63	54	50	70	32	0													24
31	30	33	61	39	51	36	49	19	52	34	33	33	52	50	60	34	52	52	55	51	69	41	21	0												25
44	37	44	76	42	34	33	64	48	73	53	54	54	55	61	71	35	75	55	80	68	80	62	50	39	0											26
62	61	52	30	60	74	57	40	48	55	57	56	48	51	43	35	61	45	53	48	48	40	56	56	53	70	0										27
35	36	35	57	47	55	34	61	33	48	40	45	37	54	48	58	46	52	60	51	45	67	45	35	34	43	55	0									28
43	38	45	73	45	37	44	67	43	64	54	51	43	50	72	74	42	70	42	67	71	77	43	35	36	35	67	36	0								29
48	47	46	48	62	74	53	14	34	69	57	54	48	29	69	29	57	71	25	66	72	56	58	44	41	60	42	59	63	0							30
61	62	55	45	55	67	66	83	55	28	40	57	53	92	30	70	64	30	94	37	31	55	45	53	54	69	59	52	72	85	0						31
57	56	51	43	69	81	60	23	47	64	60	61	49	38	62	24	62	66	38	61	65	51	57	57	56	69	33	62	70	21	76	0					32
26	25	20	56	44	50	35	52	32	43	33	32	30	51	49	57	31	53	57	48	48	64	34	26	29	46	52	33	37	50	47	57	0				33
38	33	34	68	46	52	47	46	30	53	25	34	42	59	55	65	33	65	65	62	54	74	40	32	33	48	54	39	49	54	53	57	34	0			34
37	30	35	69	49	47	42	51	37	62	36	31	49	48	58	70	34	66	56	67	57	75	45	31	28	45	55	44	44	55	58	58	31	25	0		35
35	36	35	59	39	53	44	59	33	48	34	41	33	70	52	54	42	52	74	55	53	67	33	27	34	47	49	34	38	57	50	54	29	37	44	0	36

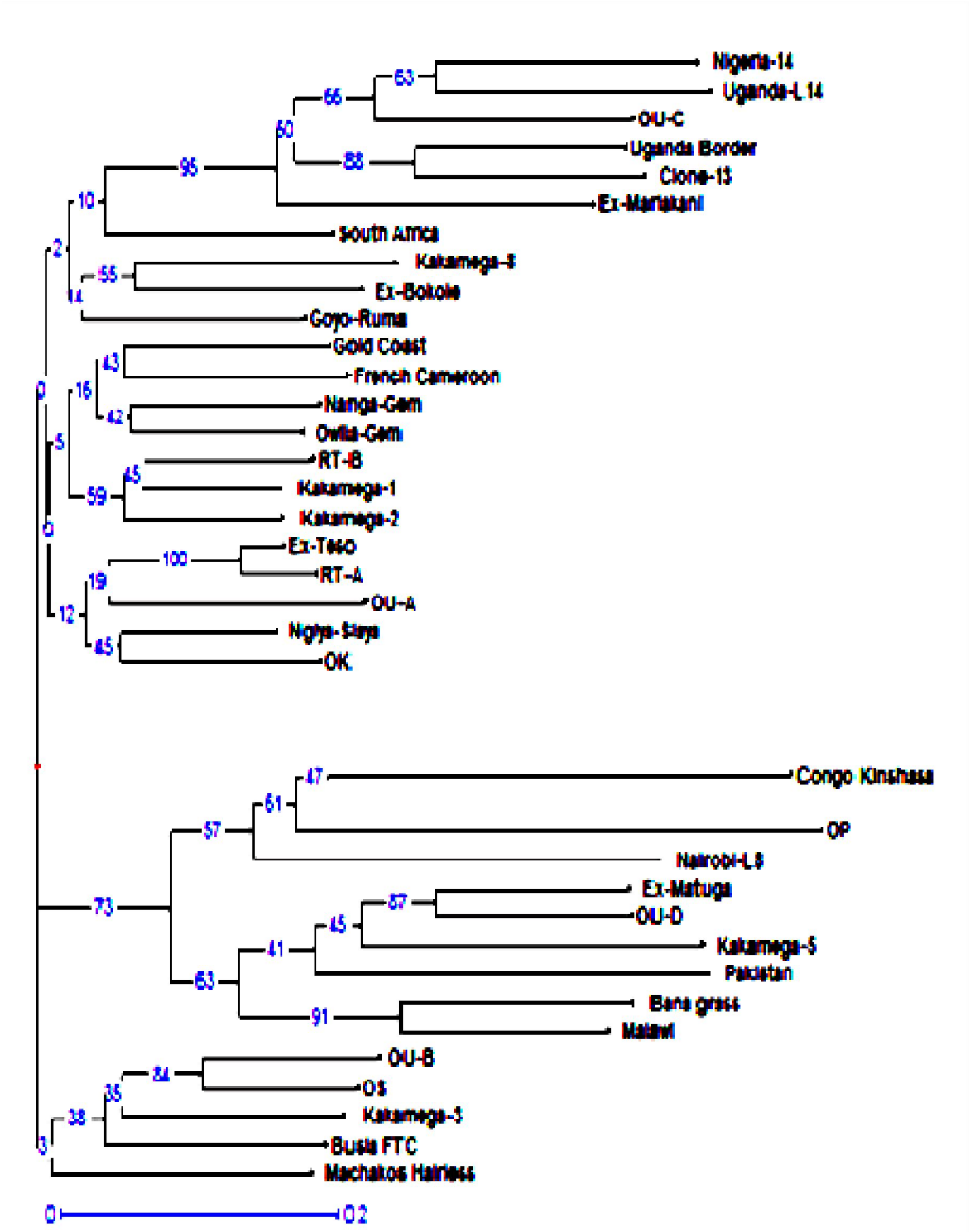

Figure 35: UPGMA dendrogram of 36 Napier grass varieties revealed by AFLP data based on Dice coefficient.

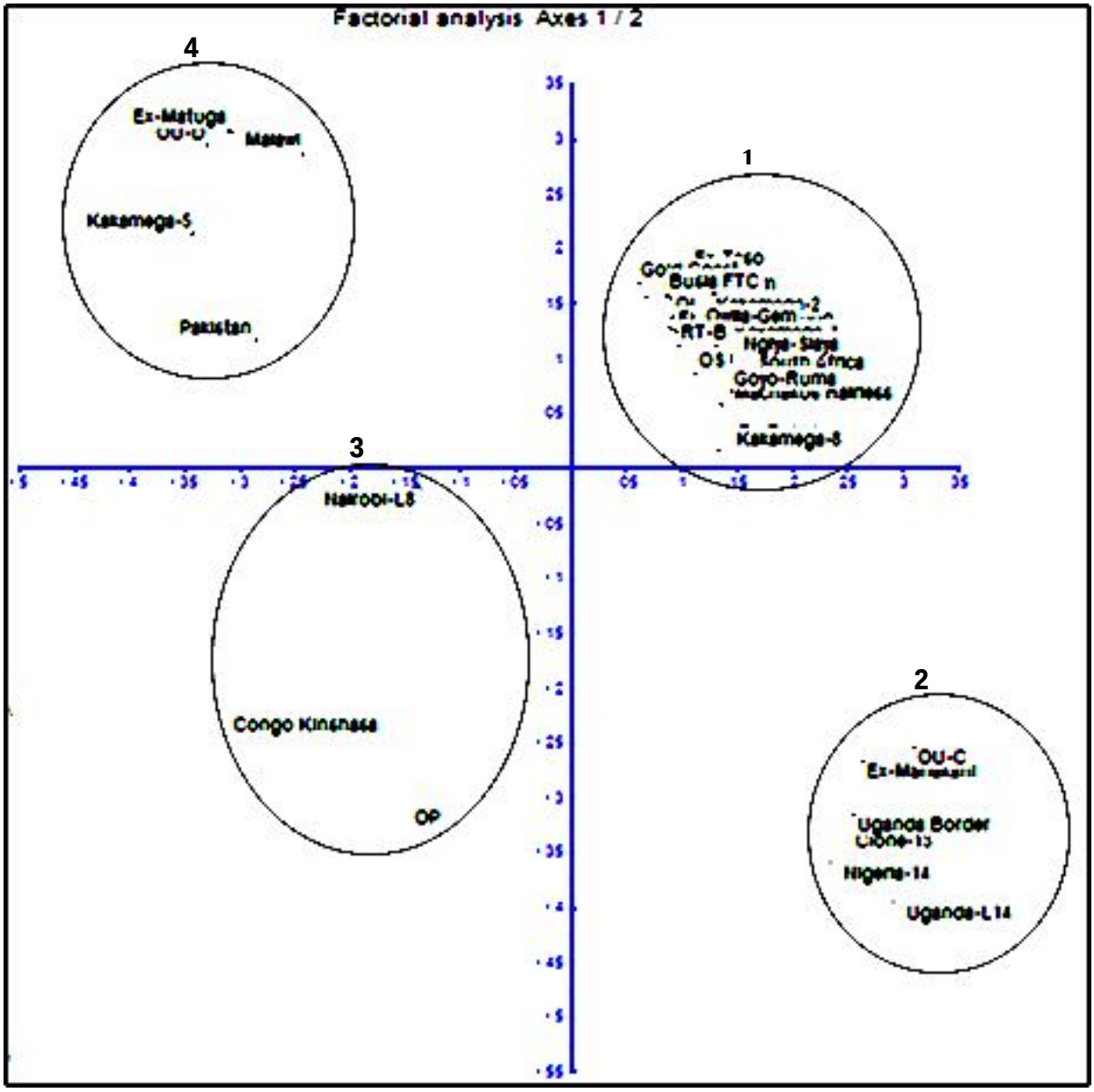

Figure 36: PCo plot showing the diversity of Napier grass varieties in the western Kenya gene pool.

All the 36 varieties investigated for disease response were susceptible to phytoplasma (Table 14). However, the varieties differed in time taken to express disease symptoms, and the proportion of plants expressing the symptoms. Some accessions succumbed to the disease quickly while others showed tolerance up to the 4th cutting (Table 14). On the basis of time taken to express symptoms, and the proportion of plants expressing symptoms, the farmer varieties; O-Gem, N-Ngiya and Busia FTC, and KARI varieties; Nairobi-8, South Africa, Malawi, Machakos hairless and Kakamega 5, expressed moderate susceptibility to phytoplasma. The results tally with earlier analysis by Muyekho et al. (2008) where Nairobi L8 genotype also did not progress to severe phytoplasma symptoms in his analysis, while Machakos hairless and South Africa genotypes only developed severe symptoms after the 4th harvest/cutting.

Table 14: Napier grass varieties, their origin and response to Ns-disease.

ID	Cultivar[A]	Origin[B]	Response[C]	Time[D]	PCR[E]	Cluster[F]
1	RT-A	Mumias, Kenya	2	1	0.4	1
2	Ex-Teso	Teso, Kenya	2	2	0.4	1
3	Ok	Mumias, Kenya	2	1	0.7	1
5	OS	Busia, Kenya	2	2	0.7	1
6	AO-B	Busia, Kenya	2	2	0.5	1
11	L 13	South Africa	2	3	0.2	1
7	AO-A	Busia, Kenya	2	1	0.7	1
12	French Cameroon	Cameroon	2	1	0.4	1
13	Ex-Bokole	Bokole – Kenyan Coast	2	1	0.8	1
23	Kakamega 8	ILRI Accession	2	3	0.5	1
24	Kakamega 1	ILRI Accession	2	2	0.6	1
25	Kakamega 2	ILRI Accession	2	2	0.5	1
26	Kakamega 3	ILRI Accession	2	2	0.4	1
17	L10	Gold coast, Ghana	2	2	0.7	1
28	Machakos Hairless	Machakos, Kenya	2	4	0.8	1
33	N-Siaya	Siaya District, Kenya	2	4	0.1	1
34	O-Gem	Gem, Kenya	2	4	0.1	1
35	N-Gem	Gem, Kenya	2	4	0.3	1
36	G-Ruma	Lambwe, Kenya	2	2	0.3	1
9	RT-B	Mumias, Kenya	2	3	0.8	1
29	B-FTC	Busia, Kenya	2	4	1	1
15	Clone 13	KARI, Kenya	2	1	0.8	2
31	Ex-Mariakani	Mariakani, Kenyan Coast	2	2	0.5	2
18	L 14	Uganda	2	2	0.5	2
20	R14	Nigeria	2	2	0.6	2
10	AO-C	Busia, Kenya	2	2	0.5	2
21	L 1	Uganda Boarder	2	1	0.7	2
4	OP	Busia, Kenya	2	2	0.5	3
27	L 8	Nairobi, Kenya	2	4	0.5	3
22	L 12	Congo Kinshasha	2	1	0.7	3
14	R 6	Malawi	2	4	0.5	4
16	Pakistant Hybrid	Pakistan	2	2	0.5	4
19	Bana grass	KARI, Kenya	2	3	0.3	4
8	AO-D	Busia, Kenya	2	1	0.5	4
30	Ex-Matuga	Matuga, Kenyan Coast	2	2	0.4	4
32	Kakamega 5	ILRI Accession	2	4	0.3	4

[ID]Sample identity in the distance matrix table
[A]Material/Cultivar Name
[B]Locality where the material was collected
[C]Presence or absence of phytoplasma-like symptoms (0=Resistant, 1=Tolerant, 2=Susceptible)
[D]Number of harvests before symptoms were expressed (1=highly susceptible, 2=very susceptible, 3=susceptible, 4=moderately susceptible)
[E]Proportion of infected plants (Ranges from 0-1, the higher the proportion, the more susceptible the cultivar)
[F]Clustering group in the PCo plot

6.4 Discussion

The AFLP technique permitted evaluation of polymorphism at a large number of loci distributed throughout a plant genome, within a very short period of time and required very small quantities of DNA. It provided an excellent opportunity for mapping the Napier grass genome to reveal diversity among the varieties investigated. The AFLP technique was specifically chosen in this study because there was no prior sequence information available for Napier grass. In the 36 Napier grass varieties screened, a high number of polymorphic loci (133) were found in western Kenya. Diversity was further confirmed by the genetic distance coefficients based on Dice statistic which diversely varied from 0 to 94 among the 36 varieties. A greater genetic diversity at the DNA molecular level implies a longer evolutionary history of the species (Li et al. 2005). The high number of polymorphic bands suggest that AFLPs are highly discriminatory and powerful markers for classification, fingerprinting and diversity analysis in Napier grass and most likely in wild relatives and populations as well. Furthermore, the high polymorphism noted makes AFLP markers a powerful tool for genotyping a large number of accessions and suitable for the evaluation of genetic diversity in large Napier grass gene banks.

According to Xavier et al. (1995), the intraspecific diversity in Napier grass is bound to be high. The diversity is preserved through the occurrence of protogyny, which favors cross fertilization, and through the predominance of vegetative propagation. The diversity also expanded due to heavy breading programs which imported genes from other species such as Pearl millet into Napier grass germplasm. The germplasm is also highly heterozygous and heterogeneous, suggesting the existence of a large amount of genetic variability within the species (Hanna, 1999). Earlier, Harris (2009), using 4 primer pairs in an AFLP analysis, divided Kenyan Napier grass genotypes into three groups. However, the sample composition and the number of selective primers used differed between that used in this study and Harris AFLP analysis. A more recent AFLP analysis (Bramwel et al. Draft thesis) using 5 selective primers on Napier grass collections from East Africa, divided the population into 4 district groups based on UPGMA clustering and principal coordinate analysis. The high genetic diversity in the western Kenya germplasm, may be explained in part by the fact that most of the varieties were introduced from elsewhere (Table 14).

Study showed that there was measurable genetic diversity between the varieties grown by farmers. Clustering of the varieties was according to geographical origin of the varieties, herbage yield and resistance to fungus disease. The bulk of the varieties (59%) belonged to the Kakamega 1 group, resistant to Napier head smut disease, caused by the fungus *Ustilago kamerunensis* (Orodho, 2006). The dry matter yield for Kakamega 1 is the highest at 23tha^{-1}(Muyekho et al. 2008). Apart from their resistance to smut fungus, the Kakamega 1 group is preferable because of their high biomass yield, accounting for the higher numbers of this group in the Napier grass gene pool in western Kenya. Cluster two or the Clone 13 group made up 16% of the genotypes. The group included the Clone 13 genotype which has been promoted for its resistance to snow-mold fungus *Biniowskia sphaeroides* (Orodho, 2006). Varieties of this group are high yielding with the dry matter yield of clone 13 being 21tha^{-1} (Muyekho et al. 2008). The lower representation of members of this group in the western Kenya gene pool is due to the less prevalence of snow mold fungus in the region. Cluster three and four or the Nairobi L8 and Malawi groups respectively formed 25% of the varieties investigated. The varieties in these groups are low yielding as the average dry matter yield for Nairobi L8 and Malawi are 13tha^{-1} and 15tha^{-1} respectively. This accounts for the fewer number of these groups in the Napier grass gene pool in western Kenya.

All the varieties were susceptible to phytoplasma, and developed symptoms after the 4th cutting. However, on the basis of time taken to express symptoms, certain varieties showed the promise of delayed responses, which could relate to the slower replication rate by the phytoplasma or higher inoculum dosage required by these plants to initiate phytoplasma infection (Weintraub and Beanland, 2006). Under field conditions where inoculum dose is much lower, the plants may take a longer time to succumb. The susceptibility of these varieties accounts for the rapid spread of Ns-disease in western Kenya, as all the farmer selected varieties were susceptible. However, as the biological interactions between Napier grass *P. purpureum* and Ns-phytoplasma is low in specificity (Chapter 5, *Hyparrhenia rufa* is alternative host plant), hence the widespread use of resistant varieties may not exert a strong selection pressure on the phytoplasma to favour new virulent biotypes (strains) capable of surviving and reproducing on resistant Napier grass. To aid in the control of phytoplasma based on resistant Napier grass, the plant breeders will have to continually identify new sources of resistance genes to phytoplasma.

6.5 Conclusion

There was measurable genetic diversity between the Napier grass varieties grown by farmers in western Kenya. The PCA plot showing the dimensional association of the varieties divided them into 4 distinct clusters. Many (59%) of the varieties were closely related to Kakamega 1 Napier grass, which has been promoted for its high Biomass yield and resistance to Napier head smut fungus. In spite of the high genetic diversity, the germplasm quality was poor as all the varieties were susceptible to Ns-disease. The poor germplasm quality contributed to the high Ns-disease incidence in the region. The small geographical region in this study, however, restricts the relevance of the analysis for more generalized conclusions. Further studies should therefore be carried out, using larger cultivar samples derived from more extended geographical regions to clarify the general level of Napier grass genetic variation and define valuable germplasms for improvement of this important grass.

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CHAPTER 7

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7.1 Introduction.

Napier stunting (Ns) disease, caused by Rice Yellow Dwarf (RYD) phytoplasma, is a threat to Napier grass production and food security in Kenya (Jones et al., 2004). The disease leads to reduction in fodder yield by up to 90% in affected farms (Lusweti et al. 2004; Mulaa et al. 2004). Like other phytoplasma diseases, the disease is maintained by a natural disease cycle consisting of the susceptible Napier grass, an insect vector and a wild grass reservoir host. For Ns-disease, the insect vector, and wild reservoir grasses were unknown. The threat of Ns-disease to cereals was also unknown. The genetic diversity of local Napier grass germplasm, and its potential as source of Ns-disease resistance was also unknown. There was also need to develop robust diagnostic tools to monitor active and latent phytoplasma infections in host plants and insect vector. This study therefore described the four components of the disease pathosystem. The study also developed quicker, more economic and robust methods for diagnosis of Ns-disease.

7.2 The insect vector of Napier stunting disease.

Transmission of Ns-disease in western Kenya is through the activity of a leafhopper vector *Maiestas (=Recilia) banda.* The vector has been barcoded for future taxonomy. Reference vector specimens are available at ICIPE (BSU, Nairobi). The vector had distinct preference hierarchy in its food choice with Pearl millet becoming the most preferred host plant species followed by Napier grass. In western Kenya, where Pearl millet is rare, Napier grass acts as the main vector reservoir and the insect completes its life cycle on the infected crop. Intensification of Napier grass farming will directly increase vector population. Infective females did not transmit phytoplasma to their nymphs, therefore all nymphs are born free of phytoplasma, and acquire the pathogen by feeding on diseased plants. The pathosystem of Ns-disease is simple as it only involves a single phytoplasma strain and a single vector species. The vector has limited host preference to two closely related pennisetum species: Pearl millet and Napier grass. The vector feeding habits and mobility, as well as the synchrony of Napier grass and vector phenology will affect the epidemiology of Ns-disease in western Kenya. Other members of the genus *Maiestas,*

M. dorsalis and *M. mica* have been associated with the transmission of plant viruses and phytoplasmas in Asia and West Africa (Desmier de Chenon 1979; Rivera et al. 1963; Takata 1985). The genus *Maiestas* has long history of association with plant pathogens. The Ns-disease in Kenya and Uganda are similar (Chapter 3), therefore *M. banda* surveys should be performed in Uganda to identify its active role in Ns-disease epidemiology. Meanwhile, Ns-disease in Ethiopia is genetically different from Uganda and Kenyan isolates. The pathosystem of Ns-disease in Ethiopia may exclude *M. banda.* Fresh vector studies should therefore be conducted in Ethiopia.

7.3 Molecular diagnostics.

Studies of the aetiology and epidemiology of phytoplasma diseases depend on sensitive and reliable techniques for the detection of the pathogens in plant and insect hosts. This study was devoted to the development of quicker, more economic and robust methods for diagnosis of Ns-disease, through the development and optimization of LAMP and PCR based oligonucleotide probes (Chapter 4). These probes replaced the traditional nested PCR in efforts to increase the speed and sensitivity of detection and to improve techniques for mass screening, and to identify phytoplasma vectors. The diagnostics is also a prerequisite for successful management of Ns-disease. The probes were also reactive to other phytoplasma in addition to Ns-disease and were essentially broad based. This is attributed to the highly conserved nature of 16S rRNA operon used to design the probes. However, the use of broad based oligonucleotide probes for Ns-disease diagnosis is recommended because a host plant can be infected by more than one phytoplasma strain (Lee et al. 1994, 1995), and the probes permitted positive diagnosis across a wide spectrum of phytoplasma strains. For a more specific diagnostics of Ns-disease, additional LAMP oligonucleotides set were developed from the variable intergenic spacer region (SR) of Ns-phytoplasma. Apart from Ns-disease, the specific LAMP probes were only reactive to BGWL, a molecular evidence that the two phytoplasmas are closely related. In general, the assay time, cost and simplicity were significantly reduced by the LAMP based probes, and the assay is a candidate to replace nested PCR in routine phytoplasma diagnostics.

7.4 Wild grass reservoirs.

The host range of RYD phytoplasma, causing Ns-disease, was found to be wide, and the phytoplasma is not wholly restricted to Napier grass. RYD infection was discovered in thatching grass *Hyparrhenia rufa* (Obura et al. 2011), probably, latent infections exist in other grasses. Hypothesis for the emergence of Ns-disease is the branching, through vector feeding, of a natural pathosystem from wild Thatching grass, to Napier grass, enabling the phytoplasma to exploit a new ecological niche. The RYD infection was pathogenic, causing Hyparrhenia grass white leaf (HGWL) disease. The HGWL phytoplasma may be exposed to a new group of insect vectors in a new pathosystem and serve as alternative source of phytoplasma for other different grass species or ecological niche. In western Kenya, additional symptomatic infection was found in Bermuda grass *Cynodon dactylon* (Obura et al. 2010). The Bermuda grass phytoplasma 16S sequence exhibited 100% of identity with that of '*Candidatus* Phytoplasma cynodontis', which belongs to the BGWL group (16SrXIV). However, the Ns-disease specific PCR and LAMP probes (Chapter 4) were reactive to BGWL phytoplasma DNA. The probes served as molecular evidence that Ns-disease and BGWL phytoplasmas, in 2 different ecological niches, are closely related. The BGWL phytoplasma may operate under a different pathosystem.

Under laboratory conditions, Ns-disease is transmissible to cultivated Cereals. Hence, the plant host range of a *Maiestas banda*, rather than lack of phytoplasma-specific cell membrane receptors, will limit the spread of Ns-disease to Cereals. This explains the reasons why there is no field report of Ns-disease outbreak in cultivated grasses. This study predicts that phytoplasma transmission will occur to Cereals through opportunistic feeding when Napier grass is cut and the insects move to nearby Cereal plots. Even in this case, the long incubation period of phytoplasmas (Weintraub and Beanland, 2006) will ensure that Cereals matures before disease development.

7.5 Napier grass varieties.

Napier grass germplasm in western Kenya comprise the elite varieties, of excellent agronomic characteristics, which were introduced to farmers, through KARI, by African Feeds Resources Network (AFRNET) in 1991. AFLP analysis divided the varieties into 4 genetically distinct groups. Many of the varieties (59%) grouped together with Kakamega 1 variety, an AFRNET

variety originally promoted by KARI for high yield and resistance to Napier head smut disease, caused by the fungus (Muyekho et al. 2008). Other varieties (16%) clustered together with Clone 13, an AFRNET variety promoted by KARI for high herbage yield and resistance to snow-mold fungus. Cluster 3 was least represented at 3 varieties, while cluster 4 formed 17% of varieties in western Kenya. The clusters 3 and 4 are low yielding Napier grass varieties, and this accounted for their less prevalence in the region. In spite of this measurable germplasm diversity, it is poor as potential source of resistance to Ns-disease. Results showed that, there was minimal variation towards phytoplasma disease reaction among all the 36 Napier grass varieties investigated, and a resistant variety was absent in the germplasm. There is need to extensify resistance screening outside this germplasm.

7.6 Conclusions and Recommendations

Ns-disease pathogen circulates between Napier grass and wild thatching grass, through vector feeding, corresponding to polycyclic epidemic. A closely related phytoplasma (BGWL) was isolated from pasture grass *C. dactylon*. The epidemiology cycle of BGWL disease should be extensively studied. The role of thatching grass in the Ns-disease pathosystem should be considered in future studies. These studies should focus on the insect vector, and disease host range. To control Ns-disease, the following prophylactic management strategies have been recommended: elimination of infected plants (Napier grass and thatching grass), certification of planting material and chemical control of the insect vector. Chemical application against the vector is possible, but this approach is not sustainable. Chemical residues pollute ground water systems and few insecticides will discriminate between the vector and beneficial insects. Other methods such as physical barriers or fine-mesh fabric (Bextine et al. 2001), although not economically viable, will exclude the vector insect from Napier grass and consequently reduce the impact of Ns-disease. Physical barriers will also reduce insect damage to Napier grass as a result of direct feeding. In general, other than on experimental scale, literature is scanty on successful control of phytoplasma diseases through vector management. Investigation of migration patterns of *M. banda* may be useful to predict new disease outbreaks and disease spread, thus contributing to effective management of Ns-disease.

Breeding of phytoplasma-resistant or tolerant Napier grass varieties may be the most cost effective way to protect Napier grass from phytoplasma infection. However, there are only a few success stories so far, such as coconut resistant to lethal yellowing (Cardena et al. 2003) and paulownia resistant to paulownia witches' broom (Du et al. 2005). Traditional plant breeding approaches to develop disease resistant/tolerant varieties are time consuming and sometimes difficult. Genetic engineering has made it possible to introduce certain pathogen/pest resistant genes into plants (Chen and Chen 1998) and the method is time efficient. These approaches may be preferred alternatives to manage Ns-disease in the future. Control of Ns-disease should involve monitoring active and latent phytoplasma infections in natural plant hosts and insect vectors. The monitoring can be done using the newly developed LAMP and nested PCR diagnostic tools.

A novel approach to reduce vector competence is through the manipulation of its symbiotic bacteria. Many arthropods carry a diverse assembly of symbiotic microorganisms that are maternally inherited and which have major effects on their hosts. The microbial flora in *M. banda* can be genetically modified to express anti-phytoplasma molecules and reduce its competence to host and transmit phytoplasma. Since the vector feeds specifically in phloem cells, obtaining nutrition from free amino acids and sugars, the activity of carbohydrate binding plant lectins would also affect *M. banda* nutrition and/or be toxic, and therefore is a potential means of controlling the insect. These lectins are usually inserted into the target plant by genetic engineering (Saha et al. 2006). These plant lectins includes the snowdrop lectin (*Galanthus nivalis* agglutinin, GNA) (Nagadhara et al. 2004) and a 25-kDa homodimeric lectin, *Allium sativum* leaf lectin (ASAL) (Dutta et al. 2005). Resistance to *M. banda* in Napier grass can also be induced through systemic acquired resistance (SAR). SAR can be elicited by a number of chemicals (Sticher et al. 1997) such as treatment with benzothiadiazole (BTH) to protect plants from insect vectors of phytoplasma. The mechanism for this effect is not clear, but the BTH is thought to elicit the production of a substance inhibiting vector feeding, hence inhibiting transmission

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APPENDICES

1. Papers from this research

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2. Manuscripts in preparation

Obura E, Midega D, Khan ZR, 2012. Hoppers (Hemiptera: Auchenorrhyncha) associated to Napier grass in western Kenya (Draft Manuscript)

Obura E, Masiga D, Wanjala BW, Harvey J, Wachira FN, Midega C, and Khan ZR. 2012. Genetic diversity and response to phytoplasma disease among the local Napier grass genotypes in western Kenya (Draft Manuscript)

Obura E, Midega C, Khan ZR, Masiga D, 2012. Survivorship and growth of *Maiestas banda* (Hemiptera: Cicadellidae) on Grasses (Draft Manuscript)

3. Conference and oral presentation

Obura E, Midega C, masiga D, Khan ZR, 2010. Napier stunting disease is transmitted by a leafhopper vector *Maiestas banda* in Western Kenya. *A conference titled: Mitigating the impact of Napier grass smut and stunting diseases for the smallholder dairy sector: Sharing results*. ILRI, Addis Ababa, Ethiopia from 4th June 2010 to 5th June 2010.

4. Sequences Submitted to the GenBank

FJ862999 16S rRNA gene partial sequence of phytoplasma isolated from *Maiestas (=Maiestas) banda* (Auchenorrhyncha: Cicadellidae), a vector of Napier stunting phytoplasma. GI|237654733|GB|FJ862999.2|[237654733]

FJ862998 16S rRNA gene partial sequence of phytoplasma isolated from Napier grass exposed to *Maiestas (=Maiestas) banda* (Auchenorrhyncha: Cicadellidae), a vector of Napier stunting phytoplasma in Kenya. GI|237654732|GB|FJ862998.2|[237654732]

FJ862997 16S rRNA gene partial sequence isolated from Napier grass GI|237654731|GB|FJ862997|[237654731]

JN112372 Hyparrhenia grass white leaf phytoplasma 16S ribosomal RNA gene, partial sequence. GI|339899491|GB|JN112372.1|[339899491]

GU944766 Star grass white leaf phytoplasma 16S ribosomal RNA gene, partial sequence. GI|294346666|GB|GU944766.1|[294346666]

5. COI Sequences of insects

>Thriambus strennus

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>Cicadullina mbila

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>Sogatella manetho

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