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Screening Napier grass accessions for resistance to Napier grass stunt disease using the loop-mediated isothermal amplification of DNA (LAMP)

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ABSTRACT

Napier grass stunt (NGS) disease is a major threat to Napier grass cultivation and the smallholder dairy industry in East and Central Africa. The disease is caused by a phytoplasma, which is transmitted by the leafhopper *Maiestas banda* (Kramer) (Hemiptera: Cicadellidae). The current study was conducted to identify among 65 Napier grass accessions that could be resistant to NGS disease using the loop-mediated isothermal amplification of DNA (LAMP). The accessions were caged with NGS phytoplasma-infected Napier grass as inoculum source and *M. banda* as the vector. All Napier grass accessions were subjected to phytoplasma testing thereafter 18 phytoplasma negative and five asymptomatic accessions were selected and used in further screening by subjecting the extracted DNA to LAMP. Plant response to the NGS phytoplasma by symptom expression, impact on yield-related parameters and phytoplasma infection was used to evaluate tolerance or resistance over a period of three months. Most Napier grass accessions were susceptible to the disease except plants belonging to accession 16789 which were negative by LAMP. Napier grass accession 16807 was found to be tolerant with 60% plants positive by LAMP and 90% plants symptomless. Accessions 16822 and 16817 had moderate tolerance with one and two plants positive by LAMP, respectively. Accession 16812 was slightly tolerant with 58.3% plants positive by LAMP and 33.3% of the plants showing symptom remission in the second re-growth. This study indicates there could be resistance and tolerance to NGS disease which could be exploited in the development of an integrated management strategy for this disease.

1. Introduction

Napier grass (*Pennisetum purpureum* Schumach) (Poaceae) is the most important fodder crop grown for intensive and semi-intensive dairy production systems in East and Central Africa. The fodder crop is preferred to other grasses because of its high yielding capacity, ease of propagation and management within a wide ecological range (Muyekho et al., 2003; Orodho, 2006). In East Africa, the grass is used as a trap plant to control cereal stem borers, which are the most injurious grain pests in the push-pull strategy. Additionally, it serves as a windbreak in maize fields and is used to stabilize soil by holding particles together thereby preventing soil erosion (Orodho, 2006; Cook et al., 2007; Pickett et al., 2014). Surplus Napier grass is a source of income and employment to smallholder farmers and proceeds from its sales are used to cater for school fees and other household needs (Khan et al., 2014). Despite these advantages, the performance of Napier grass is threatened by the emergence of Napier grass stunt (NGS) disease in

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the region.

NGS disease is caused by a phytoplasma, a cell wall-less bacterium parasitizing the plants' sieve elements. Phytoplasmas are responsible for numerous crop diseases worldwide. They are non-culturable and a provisional taxon '*Candidatus* (*Ca.*) Phytoplasma' has been established. Together with other cell wall-less bacteria they are members of the class Mollicutes (Lee et al., 2000; IRPCM, 2004). Phytoplasmas were mostly identified and classified based on symptoms, vector specificity and host range and recently on molecular tools such as polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the conserved 16S ribosomal gene (16S rDNA). PCR provided a more rapid and reliable method of classifying phytoplasmas (Lee et al., 1998, 2000). However, the method is often laborious and sometimes requires two rounds of PCR. Therefore, loop-mediated isothermal amplification of DNA (LAMP) has been developed for NGS phytoplasma classification. The method is simpler, efficient and allows for detection of phytoplasma titers below the detection threshold of nested PCR (Obura et al., 2011).

Based on 16S rDNA sequences, phytoplasmas associated with NGS in Kenya, Tanzania and Uganda belong to the 16SrXI group, represented by the type strain '*Ca.* Phytoplasma oryzae' whereas NGS in Ethiopia is caused by the African sugarcane yellow leaf phytoplasma, a member of the 16SrIII group, represented by '*Ca.* P. pruni' (Jones et al., 2004, 2007; Nielsen et al., 2007). In Kenya NGS disease was first described in Bungoma district in 1997, in Uganda in Masaka district in 2001 (Alicai et al., 2004) and in Ethiopia in 2004, and has also been reported in Tanzania recently (Alicai et al., 2004; Orodho, 2006; Jones et al., 2004, 2007; Nielsen et al., 2007; Asudi et al., 2015, 2016a). Symptoms of phytoplasma-infected plants include small chlorotic leaves, proliferation of tillers, and shortening of internodes to the extent that clumps appear very stunted, ultimately resulting in death of the plant (Ajanga, 2005; Orodho, 2006; Kabirizi et al., 2007; Asudi et al., 2015). In Kenya, *Maiestas banda* (Kramer) (Hemiptera: Cicadellidae), a leafhopper in the tribe Deltocephalini was identified as the vector of NGS-disease (Obura et al., 2009). In Ethiopia, the leafhopper *Exitianus* spp. (Hemiptera: Cicadellidae) and the planthopper *Leptodelphax dymas,* (Fennah) (Hemiptera: Delphacidae) were suggested as potential vectors of the NGS phytoplasma (Arocha et al., 2009). The vectors passively ingest the phytoplasmas with the phloem sap from infected plants. During a latency period, the phytoplasmas cross the gut wall and establish in the salivary gland through the hematocele. They are then are ejected with saliva to healthy plants during feeding (Weintraub and Beanland, 2006). A further way of phytoplasma spread is by vegetative propagation of infected plant material (Orodho, 2006; Koji et al., 2012; Asudi et al., 2015). In western Kenya, the disease affects up to 90% of Napier grass plants in smallholder farms and causes forage yield loss of 40–90% (Orodho, 2006; Mulaa and Ajanga, 2005). In some areas, many smallholder farmers have lost their entire Napier grass crop and are forced to reduce the number of animals or purchase fodder from the local market (Arocha and Jones, 2010). The disease is spreading very fast in East Africa and has reached the central and rift valley provinces of Kenya resulting in a serious threat to the dairy sector in other parts of the region (Kabirizi et al., 2007; Khan et al., 2014; Asudi et al., 2015; Kawube et al., 2015). A significant reduction in milk output has been reported in areas ravaged by the disease, and has led to decline in household incomes (Khan et al., 2014). The current mitigation strategies that include use of fertilizer, roguing and careful visual selection of planting material are not effective in controlling this disease (Kabirizi et al., 2007; Asudi et al., 2015). In addition, cultivars and accessions selected in the past have lost the ability to resist the disease (Mulaa et al., 2010; Kawube et al., 2014). Therefore, the objective of the study was to select Napier grass plants with resistance or tolerance to the NGS phytoplasma among 65 Napier grass accessions using the loop-mediated isothermal amplification of DNA (LAMP).

2. Materials and methods

2.1. Study area and plant materials

The study was conducted at the International Centre of Insect Physiology and Ecology, Thomas Odhiambo Campus at Mbita Point (0°25′ S, 34°12′ E) located at the shores of Lake Victoria in Homabay county, Kenya. Napier grass cultivars and accessions used in the study included accessions grown by farmers and collections made by the International Livestock Research Institute and Kenya Agricultural and Livestock Research Organization (KALRO) (Table 1). Three plants from each accession were grown in a screen house in pots with sterile black cotton soil and irrigated every morning. All Napier grass accessions were subjected to phytoplasma testing using nested PCR thereafter phytoplasma-negative and asymptomatic accessions were selected and used in further screening by subjecting the extracted DNA to LAMP using the protocol described by Obura et al. (2011).

2.2. Rearing of the healthy insect vector

Maiestas banda were obtained from the vector-rearing screen house at the Campus where they were reared on disease-free pearl millet in wooden framed cages measuring 45 × 45 × 60 cm and surrounded with a 0.25 mm netting material. Phytoplasma-free insects that hatched from eggs in these cages (F1 generation offspring) were used in the transmission experiments.

2.3. Transmission tests

Eighteen Napier grass accessions without phytoplasma and five phytoplasma positive accessions selected following initial screening of 65 Napier grass accessions were sown in 500 ml cups in sterilized black cotton soil and maintained in a screen house for 50 days. The insect vector *M. banda* was used as inoculum carrier to infect the test plants as described by Obura et al. (2009). The diseased Napier grass plant was placed at the centre of the cage surrounded by five healthy phytoplasma-free potted plants and Bana grass as control with 50 gravid female *M. banda* recognized by their abdomen (Fig. 1). The experiment consisted of 12 plants from each accession. Occasionally the insects were disturbed in the inoculation cages to redistribute the population. After 30 days, the inoculation setup was terminated and the exposed plants transferred to a separate screen house for phytoplasma testing and disease symptoms expression. Exposed Bana plants were used as positive controls while healthy screen house-propagated Bana plants as negative control.

2.4. Phytoplasma DNA isolation and amplification

DNA was extracted from 0.3 g of fresh leaves from each test plant as described by Doyle and Doyle (1990). The DNA pellet was then dried and reconstituted in 50 μL of sterile water. Phytoplasma was amplified in Napier grass accessions according to published protocols (Lee et al., 1998) using nested PCR based on the 16S rDNA gene with primer pair P1/P6 (Deng and Hiruki, 1991) in the first round PCR followed by primer pair NapF/NapR (Obura, 2012) in a PTC-100 Thermal Cycler (MJ Research Inc.). LAMP reaction based on the 16S rDNA gene was conducted on the Napier grass accessions using three pairs of primers including NGS-BIP, NGS-FIP, NGS-B3, NGS-F3, NGS-FL and NGS-BL (Obura et al., 2011). In all

Table 1
Napier grass accessions screened against Napier grass stunt phytoplasma, country of origin and their potential yield.

Accession/cultivar	% positive	% negative	Symptoms	Country of origin	Yield (ton/ha)	Source of information
Bgm 20	0.00	100	None	Kenya	–	Mulaa et al., 2012
Clone 13	0.00	100	None	Kenya	21.9	Muyekho et al., 2006
Ex-Malawi	0.00	100	None	Malawi	15.8	Muyekho et al., 2006
Ex-Mariakani	0.00	100	None	Kenya	9.5	Muyekho et al., 2006
Nigeria 14	0.00	100	None	Humid WCA	17.1	Muyekho et al., 2006
15743 cv. Mott	0.00	100	None	USA	–	Lowe et al., 2003
16621	0.00	100	None	Namibia	–	Lowe et al., 2003
16789	0.00	100	None	Swaziland	–	Lowe et al., 2003
16791(Kk 1)	0.00	100	None	Swaziland	23.8	Lowe et al., 2003; Muyekho et al., 2006
16794	0.00	100	None	Mozambique	–	Lowe et al., 2003
16808	0.00	100	None	USA	–	Lowe et al., 2003
16809	0.00	100	None	USA	–	Lowe et al., 2003
16812	0.00	100	None	USA	–	Lowe et al., 2003
16817	0.00	100	None	USA	–	Lowe et al., 2003
16822	0.00	100	None	Malawi	–	Lowe et al., 2003
16840	0.00	100	None	Unknown	–	Lowe et al., 2003
Uganda hairless	0.00	100	None	Uganda	11.6	Muyekho et al., 2006
Uganda L11	0.00	100	None	Uganda	17.8	Muyekho et al., 2006
Ex-Bokole	100	0.00	Asymptomatic	Unknown	16.3	Muyekho et al., 2006
16798 (Kk 2)	100	0.00	Asymptomatic	Zimbabwe	20.8	Lowe et al., 2003; Muyekho et al., 2006
16807	66.7	33.3	Asymptomatic	USA	–	Lowe et al., 2003
16815	66.7	33.3	Asymptomatic	USA	–	Lowe et al., 2003
18438	66.7	33.3	Asymptomatic	Unknown	–	–
Btr 86	33.3	66.7	Symptomatic	Kenya	–	Mulaa et al., 2012
14984	33.3	66.7	Symptomatic	Unknown	–	Lowe et al., 2003
16803	33.3	66.7	Symptomatic	Zimbabwe	–	Lowe et al., 2003
16804	33.3	66.7	Symptomatic	USA	–	Lowe et al., 2003
16814	33.3	66.7	Symptomatic	USA	–	Lowe et al., 2003
Nairobi L8	33.3	66.7	Symptomatic	Kenya	19.5	Muyekho et al., 2006
BSA 105	66.7	33.3	Symptomatic	Kenya	–	Mulaa et al., 2012
BSA 112	66.7	33.3	Symptomatic	Kenya	–	Mulaa et al., 2012
BSA 60	66.7	33.3	Symptomatic	Kenya	–	Mulaa et al., 2012
Cameroon 4E	66.7	33.3	Symptomatic	Cameroon	18.0	Muyekho et al., 2006
Ex-Matuga	66.7	33.3	Symptomatic	Unknown	19.0	Muyekho et al., 2006
L13	66.7	33.3	Symptomatic	South Africa	22.3	Muyekho et al., 2006
L4	66.7	33.3	Symptomatic	South Africa	11.5	Muyekho et al., 2006
16484	66.7	33.3	Symptomatic	Unknown	–	Lowe et al., 2003
16702	66.7	33.3	Symptomatic	Unknown	–	Lowe et al., 2003
16787	66.7	33.3	Symptomatic	Swaziland	–	Lowe et al., 2003
16838	66.7	33.3	Symptomatic	Unknown	6.8	Muyekho et al., 2006
L16	66.7	33.3	Symptomatic	Unknown	17.5	Muyekho et al., 2006
Muguga bana	66.7	33.3	Symptomatic	Kenya	–	Muyekho et al., 2006
Nairobi L9	66.7	33.3	Symptomatic	Kenya	12.5	Muyekho et al., 2006
Pakistan hybrid	66.7	33.3	Symptomatic	Unknown	12.3	Muyekho et al., 2006
Bgm 1 (A) 10	100	0.00	Symptomatic	Kenya	8.6	Mulaa et al., 2012
Bgm 3 (A) 16	100	0.00	Symptomatic	Kenya	–	Mulaa et al., 2012
Bgm 3 (B) 28	100	0.00	Symptomatic	Kenya	–	Mulaa et al., 2012
Bgm 3 (B) 31	100	0.00	Symptomatic	Kenya	–	Mulaa et al., 2012
Bgm 76	100	0.00	Symptomatic	Kenya	–	Mulaa et al., 2012
BSA 31	100	0.00	Symptomatic	Kenya	–	Mulaa et al., 2012
Btr 23	100	0.00	Symptomatic	Kenya	–	Mulaa et al., 2012
Btr 89	100	0.00	Symptomatic	Kenya	–	Mulaa et al., 2012
Congo Kinshasa	100	0.00	Symptomatic	DRC	13.5	Muyekho et al., 2006
French Cameroon	100	0.00	Symptomatic	Cameroon	21.7	Muyekho et al., 2006
Gold coast	100	0.00	Symptomatic	Ghana	19.8	Muyekho et al., 2006
16785	100	0.00	Symptomatic	Tanzania	–	Lowe et al., 2003
16786 (Kk 3)	100	0.00	Symptomatic	Swaziland	22.8	Muyekho et al., 2006
16792	100	0.00	Symptomatic	Mozambique	–	Lowe et al., 2003
16805	100	0.00	Symptomatic	USA	–	Lowe et al., 2003
16811	100	0.00	Symptomatic	USA	–	Lowe et al., 2003
16836	100	0.00	Symptomatic	Unknown	–	Lowe et al., 2003
16837	100	0.00	Symptomatic	Unknown	14.0	Lowe et al., 2003; Muyekho et al., 2006
52503	100	0.00	Symptomatic	Gold Coast	14.4	Lowe et al., 2003; Muyekho et al., 2006
Mott Napier	100	0.00	Symptomatic	USA	–	Mulaa et al., 2012
Uganda border	100	0.00	Symptomatic	Uganda	19.2	Muyekho et al., 2006

Not all individuals in symptomatic accessions had observable Napier grass stunt disease symptoms. DRC = Democratic Republic of Congo; WCA = West-Central Africa; Kk = Kakamega.

experiments, the DNA extract form healthy NGS negative Bana variety of Napier controls were included as the source of plant nucleic acid added to the PCR/LAMP reaction mix as negative controls while DNA extracted from diseased Napier grass was used as a positive control. Amplified products were visualized by gel electrophoresis in a 2% (w/v) agarose gel stained with ethidium bromide using 1xTAE (40 mM Tris acetate, 1 mM EDTA pH8.0) as running buffer and photographed.

Fig. 1. Arrangement of potted Napier grass plants in experimental cages during inoculation. The plant in the center is the inoculum source surrounded by six healthy Napier grass plants. The cage contained 50 gravid *Maiestas banda* to act as phytoplasma vectors.

2.5. Evaluation of the effect of NGS disease on plant growth parameters

After the transmission trials, the plants were tested monthly for three months for the presence of NGS phytoplasma using LAMP procedure. Individual plants were monitored for leaf symptom development, severity and mortality rates 30 days after the first cut back. Disease was scored on the first, second and third re-growth at intervals of 30 days each using the disease response rate by indicating presence or absence of disease symptoms on plants to show levels of tolerance or resistance. These symptoms were foliar yellow leaves, stunted growth or necrosis of leaves (Orodho, 2006). The period taken for the plants to express symptoms compared to presence/absence of NGS phytoplasma in the plant DNA was used to evaluate the ability of the test plant to tolerate or resist the disease. Seven of the 23 Napier grass accessions namely Nigeria 14, Bgm 20, 15743, 16808, 16807, 16789 and 16812 were assessed for the effect of the disease on their yields before death. Individual plants were assessed for plant height, leaf length and width. Plant height was measured using a tape measure from the soil surface to the tip of the youngest growing leaf. Leaf length represented an average of two of the longest leaves measured from the petiole end to the tip. Leaf width represented an average of two longest leaves measured out from one end to the other using a ruler meter (Asudi et al., 2016b). The feeding behavior of the insect vector on the Napier grass accession 16789 was also evaluated using a Safranine dye technique (Khan and Saxena, 1984).

2.6. Data analysis

The number of plants that were positive for NGS phytoplasma was expressed as a percentage, calculated as the proportion of exposed plants to phytoplasma-positive plants using the Statistical Package for Social Sciences (SPSS) version 18 (SPSS Inc. Chicago, IL, USA). The percentages of plants that developed NGS disease symptoms and mortality were calculated the same way. The scores for LAMP results, symptom development and the death of plants were analyzed using correlation analysis in proc corr using SAS software (v9.1). Single tailed t-tests using the SPSS v18 were conducted on plant growth parameters (leaf length, leaf width and plant height) to compare performance of plants before and after exposure to NGS phytoplasma and statistical significance determined at 95% confidence level. The data on plant growth parameters was subjected to one-way analysis of variance and means having significant differences separated using Tukey's studentized range tests.

3. Results

3.1. Vector transmission experiments

At the pre-screening stage, a 778-bp DNA nested PCR fragment (Fig. 2) was amplified from 47 accessions and the positive controls, indicating that the plants acquired NGS phytoplasma. The percentage of plants infected in each accession ranged from 33.3 to 100% (Table 1). However, 18 Napier grass accessions including 15743, 16621, 16789, 16791, 16794, 16808, 16809, 16812, 16817, 16822, 16840, Bgm 20, Clone 13, Malawi, Ex-Mariakani, Nigeria 14, Uganda hairless and Uganda L11 were uninfected by NGS phytoplasma. Five of the infected accessions were asymptomatic for NGS disease and included 16798 and Ex-Bokole with 100% phytoplasma-infected plants, and 16815, 18438 and 16807 each with 66.7% infection (Table 1).

A LAMP product of 240-bp of the 16Sr DNA sequence of the NGS phytoplasma (Fig. 3) was obtained from the DNA of first re-growing leaves. Phytoplasma-positive plants were identified in four accessions including Ex-Malawi with 9.1%, Ex-Mariakani with 16.7%, Nigeria 14 with 20% and 15743 with 33.3% infected plants (Table 2). At the second month of screening, another seven accessions were infected. Nigeria14 had the highest proportion of infected plants (100%), followed by Ex-Malawi with 45.5%, 16791 with 27.3%, 16840 with 25%, 18438 with 18.2%, Ex-Mariakani, Uganda L11, 16621 and 16798 with 16.7%, 16807 with 10% and 16812 with 8.3% infection (Table 2). All plants in accessions Bgm 20 and 15743 and 40% of the uninfected plants belonging to accession Ex-Bokole died at this stage and therefore were excluded from further screening. At the third month of screening, only plants in 16789, Uganda hairless and Clone 13 accessions were NGS phytoplasma negative by LAMP. The proportions of plants within acessions bearing the pathogen ranged from 8.3% in 16822 and 16808, to 91.7% in 16794 while all plants belonging to Nigeria 14 died before the third screening stage (Table 2).

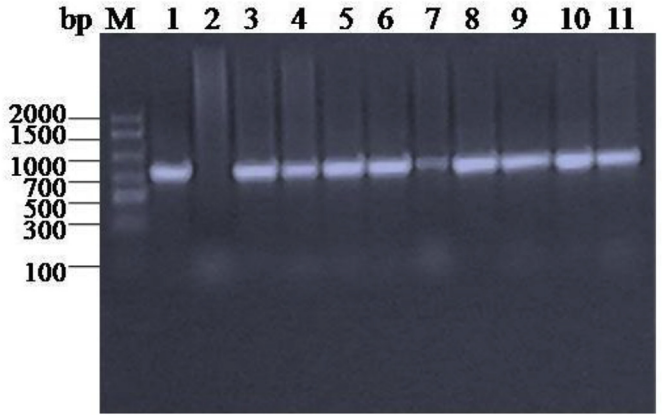

Fig. 2. Electropherogram of nested PCR products amplified with P1/P6 followed by NapF/NapR primers. M: 1 kb DNA marker (GenScript Inc.); lane 1: reference Napier grass stunt phytoplasma; 2: negative control (water); 3–11: diseased Napier grass plants.

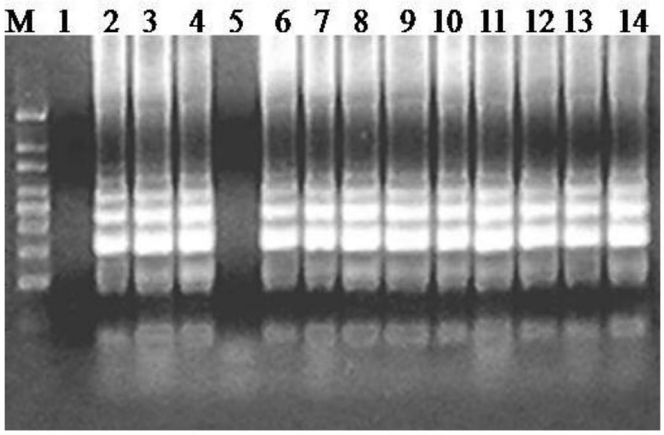

Fig. 3. Electropherogram of LAMP products. Lane M-100 bp marker (GenScript, USA Inc.); lane 1, negative control (water); lane 2 reference Napier grass stunt phytoplasma; lanes 3, 4, 6, 7, 8, 9, 10, 11, 12,13 and 14 representative plants positive for phytoplasma; 5 phytoplasma negative plants.

3.2. Symptom development in Napier grass accessions

Ten out of the 23 Napier grass accessions screened showed symptoms of NGS disease in the first re-growth. High proportions of symptomatic plants were recorded in 16812 with 33.3% plants showing symptoms followed by 16808 with 27.3%, Ex-Mariakani and 16840 with 25%, Ex-Bokole with 20%, 16794 and Clone 13 with 16.7%, 16791 with 9.1%, and 15743 and Uganda hairless with 8.3%. In the second re-growth, 12 accessions exhibited symptoms of NGS disease with five new accessions becoming symptomatic including 16798 with 16.7%, Ex-Malawi with 18.2%, Nigeria 14 with 20%, 16621 with 25% and 18438 with 27.3% symptomatic plants. Accessions 16791, Clone 13 and 16794 maintained the proportions of symptomatic plants in the second re-growth while the proportions of plants with symptoms in Uganda hairless, 16840, 16808 and Ex-Mariakani increased to 25%, 33.3%, 36.4% and 50% respectively (Table 2). During this stage, two symptomatic plants in Uganda hairless died before the re-growth while plants in accession 16812 became asymptomatic for the disease. The number of symptomatic Napier grass accessions increased to 14 in the third re-growth with the number of plants displaying symptoms ranging from 10 to 63.6%. The highest number of plants with symptoms was recorded in 16808 with 63.6% symptomatic plants, followed by Ex-Mariakani with 50%, 18438 with 45.5%, 16840 with 41.7%, 16791 with 27.3%, 16621, 16815 and 16798 with 25%, 16809 and Ex-Malawi with 18.2%, Uganda hairless, clone 13 and 16794 with 16.7%, and Ex-Bokole with 10%. By the end of the third re-growth 50% of 16,822, 88.9% of 16817 and 90% of 16807 plants remained alive and did not develop symptoms of NGS disease (Fig. 4). However, accession 16807 had the highest proportions of infected plants (60%) (Table 2; Fig. 3) followed by accessions 16,822 and 16817 with one and two plants infected respectively. 83.3% of plants within Napier grass accession 16789 did show observable NGS disease symptoms throughout the screening, while 16.7% died yet all the test plants were phytoplasma negative by LAMP. The plants within the accession also had less tillers compared to other accessions 16807 and 16840 (Table 2; Fig. 4).

3.3. Correlation between symptom development, LAMP scores and death of plants

The general correlation analysis between symptoms, LAMP scores and death of plants for all the 23 accessions screened indicated a positive correlation between accessions and symptom expression ($R = 0.15$; $P < 0.05$) and a negative correlation between symptom expression and death ($R = -0.15$; $P < 0.05$). The correlation analysis per specific Napier grass plant accessions indicated a positive correlation between symptom expression and LAMP results for two Napier grass accessions (18743 with $R = 1.00$; $P < 0.05$ and 16621 with $R = 0.817$; $P < 0.05$). A negative correlation between LAMP results and the death of plants was found in accessions 16794 and Uganda L11 ($R = -1.00$; $P < 0.05$ and $R = -0.67$; $P < 0.05$ respectively). Napier grass accessions 16798 and 16621 had a

Table 2
Proportions of Napier grass accessions symptomatic to or positive to Napier grass stunt phytoplasma at first, second and third re-growth of the plants.

Napier grass accession	First re-growth			Second re-growth			Third re-growth		
	% symptomatic	% positive	% dead	% symptomatic	% positive	% dead	% symptomatic	% positive	% dead
Bgm 20	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0
Clone 13	16.7	0.0	0.0	16.7	0.0	0.0	16.7	0.0	16.7
Ex-Bokole	20.0	0.0	0.0	0.0	0.0	40.0	10.0	0.0	0.0
Ex-Malawi	0.0	9.1	0.0	18.2	45.5	9.1	18.2	36.4	36.4
Ex-Mariakani	25.0	16.7	0.0	50.0	16.7	0.0	50.0	41.7	16.7
15743	8.3	33.3	0.0	0.0	0.0	100.0	0.0	0.0	0.0
16621	0.0	0.0	0.0	25.0	16.7	33.3	25.0	33.3	66.7
16789	0.0	0.0	0.0	0.0	0.0	16.7	0.0	0.0	16.7
16791	9.1	0.0	0.0	9.1	27.3	0.0	27.3	45.5	27.3
16794	16.7	0.0	0.0	16.7	0.0	0.0	16.7	91.7	0.0
16798	0.0	0.0	0.0	16.7	16.7	0.0	25.0	33.3	8.3
16807	0.0	0.0	0.0	0.0	10.0	0.0	0.0	60.0	0.0
16808	27.3	0.0	0.0	36.4	0.0	0.0	63.6	9.1	0.0
16809	0.0	0.0	0.0	0.0	0.0	0.0	18.2	45.5	9.1
16812	33.3	0.0	0.0	0.0	8.3	8.3	0.0	58.3	8.3
16815	0.0	0.0	0.0	0.0	0.0	0.0	25.0	33.3	0.0
16817	0.0	0.0	0.0	0.0	0.0	11.1	0.0	22.2	22.2
16822	0.0	0.0	16.7	0.0	0.0	16.7	0.0	8.3	16.7
16840	25.0	0.0	0.0	33.3	25.0	0.0	41.7	33.3	16.7
18438	0.0	0.0	0.0	27.3	18.2	0.0	45.5	45.5	0.0
Nigeria14	0.0	20.0	0.0	20.0	100.0	0.0	0.0	0.0	100.0
Uganda hairless	8.3	0.0	0.0	25.0	0.0	0.0	16.7	0.0	8.3
Uganda L11	0.0	0.0	0.0	0.0	16.7	25.0	0.0	0.0	75.0

% +ve: represents the number of Napier grass plants that tested positive by LAMP following transmission of Napier grass stunt phytoplasma using *Maiestas banda* as inoculum carrier.

Fig. 4. Morphological comparison of the Napier grass stunt phytoplasma-infected (16840) with typical diseased symptoms, uninfected (16789), and asymptomatic (16807) accessions after four months of incubation with the phytoplasma.

correlation between symptom expression and the death of plants with $R = 0.63$; $P < 0.05$ and $R = -0.82$; $P < 0.05$ respectively. There was no correlation of symptom expression, LAMP results and death of the plants found in the rest of the Napier accessions.

3.4. Effects of NGS phytoplasma on plant yield parameters

The analysis of variance showed that there were significant differences in plant height between accessions before exposure and at the end of the experiment ($P < 0.05$) (Table 3). However, plant height within accessions Bgm 20, Ex-Bokole, 15743, 16789, 16807, 16808 and 16812 did not differ significantly before exposure. At the end of the experiment, the plant height decreased in accessions Bgm 20, Ex-Bokole, 15743, 16807, 16808 and Nigeria 14 but increased significantly in accessions 16789 ($P < 0.05$) and 16812 ($P < 0.05$). Accession Nigeria 14 had the highest reduced height followed by Ex-Bokole, 15743, Bgm 20, 16808 and 16807 (Table 3). The analysis of variance before exposure and at the end of the experiment showed significant differences in mean leaf lengths among Napier grass accessions ($F = 3.52$; $P < 0.05$ and $F = 9.89$; $P < 0.05$) respectively. Accession Nigeria 14 had the highest leaf length compared to the other accessions tested (Table 3). Similarly, at the end of the experiment, Nigeria 14 was the most infested accession with the highest reduced leaf length followed by accessions Bgm 20, 15743, 16808 and 16807. However, the leaf length increased slightly in accessions Ex-Bokole and 16789, and significantly in 16812 ($P < 0.05$). Data on the leaf width differed significantly within the accessions before ($F = 11.45$; $P < 0.05$) and after exposure ($F = 11.49$; $P < 0.05$). Similarly, Nigeria14 had the highest reduced leaf width followed by 16808, 15743, 16807 and 16812 while Bgm 20 had the highest increase in leaf width followed by Ex-Bokole and 16789 (Table 3).

The treatment of the filter paper discs with 0.1% ninhydrin-acetone solution resulted in Bluish amino acid spots, indicating that *M. banda* fed on the phloem of both the resistant Napier grass accession 16789 and the susceptible (control) Bana variety.

4. Discussion

In order to identify tolerance or resistance to the NGS disease, 65 Napier grass accessions were screened against NGS phytoplasma using LAMP. The study showed that all Napier grass accessions were susceptible to the disease with an exception of accession 16789, which was not infected. However, the infection stage varied greatly with 47 accessions being infected in the initial screening. Subsequently, the 23 Napier grass accessions tested differed in the time taken to express symptoms with the number of symptomatic plants increasing in the second and the third re-growths indicating a possible build up of the phytoplasma titre or further break down in the defense of plants with time (Agrios, 2004).

Napier grass accession 16791 had the lowest number of symptomatic plants in the second re-growth, confirming the findings of Muyekho et al. (2006) while two accessions Nigeria 14 and Ex-Malawi had the highest number of plant population infected and could be considered highly susceptible to the NGS phytoplasma. At the same period, all the plants in 15743 and Bgm 20, and symptomatic plants in Ex-Bokole plants died possibly due to other factors such as plant culture, fungal or bacterial infection. However, symptomatic plants in accession 16812 reverted to asymptomatic state. Although there is scarcity of information on recovery responses of gramineous plants from phytoplasma infections, this effect has been observed in woody plants infected with other phytoplasmas where infected plants showed reduced symptom expression up to complete recovery (Musetti et al., 2007; Romanazzi et al., 2007). Hence, presence and titre of phytoplasma in different parts of the plants including roots and stems need to be considered before recovery is used as a potential strategy to manage phytoplasma diseases (Morone et al., 2007).

Uganda hairless and Clone 13 had plants with foliar yellow leaves in the third re-growth but were phytoplasma negative by LAMP. This could have been possible due to change in environmental conditions, water shortage or due to presence of other disease-causing organisms (Sinclair et al. 1994; Bertaccini et al., 1996; Lockhart et al., 1996). The absence of phytoplasma in such

Table 3
Effect of Napier grass stunt phytoplasma infection on growth parameters of eight Napier grass accessions.

Accession	Plant height (inches)				*t*	P
	Before exposure	End	Mean difference	Mean proportional difference		
Bgm 20	23.3 ± 2.3a	18.8 ± 2.5b	−4.5	−16.7 ± 10.6bc	1.65	0.159
Ex-Bokole	22.7 ± 1.3a	16.2 ± 2.2a	−6.5	−35.3 ± 7.9ab	4.39	0.007
15743	26.1 ± 2.0a	24.0 ± 1.0c	−2.1	−19.6 ± 2.9abc	6.88	0.092
16789	25.1 ± 1.9a	28.7 ± 0.8c	3.6	17.9 ± 6.3d	−2.35	0.043
16807	26.7 ± 1.4a	24.8 ± 1.2bc	−1.9	−6.7 ± 2.4cd	2.77	0.02
16808	22.1 ± 0.6a	20.2 ± 1.2ab	−1.9	−7.7 ± 4.7bcd	1.61	0.167
16812	26.3 ± 1.2a	38.2 ± 1.1d	11.9	48.7 ± 8.9e	−7.21	<0.001
Nigeria 14	39.3 ± 1.0b	18.1 ± 3.0ab	−21.2	−53.9 ± 7.8a	7.17	0.002
F	8.9	23.4		19.0		
P	<0.001	<0.001		<0.001		
Accession	Leaf length (inches)				*t*	P
	Before exposure	End	Mean difference	Mean proportional difference		
Bgm 20	17.1 ± 1.7a	16.1 ± 0.0ab	−0.9	−5.0 ± 5.7a	0.83	0.445
Ex-Bokole	15.2 ± 0.7a	15.5 ± 0.8a	0.3	−3.9 ± 3.0a	1.47	0.203
15743	17.5 ± 0.5 ab	16.8 ± 0.3ab	−0.8	−7.2 ± 0.9a	6.50	0.097
16789	18.9 ± 1.7 ab	21.2 ± 0.7b	2.2	16.7 ± 7.7 ab	−1.57	0.15
16807	18.9 ± 1.3 ab	18.7 ± 1.2ab	−0.3	−1.3 ± 0.7a	1.75	0.11
16808	16.3 ± 1.1a	15.9 ± 1.8a	−0.4	1.6 ± 4.9a	−0.27	0.8
16812	19.9 ± 1.7 ab	26.7 ± 1.2c	6.8	−1.3 ± 0.7b	−4.04	0.002
Nigeria 14	25.2 ± 0.9b	19.2 ± 1.3ab	−6.0	−23.2 ± 6.4a	3.32	0.03
F	3.52	9.89		5.3		
P	0.003	<0.001		<0.001		
Accession	Leaf width (inches)				*t*	P
	Before exposure	End	Mean difference	Mean proportional difference		
BGM20	0.9 ± 0.13 ab	1.1 ± 0.1 ab	0.2	22.3 ± 9.9ac	−2.99	0.031
Ex-Bokole	0.9 ± 0.13 ab	1.0 ± 0.1a	0.1	0.2 ± 4.4abc	0.00	1
N15743	1.3 ± 0.1abc	1.1 ± 0.1 ab	−0.2	4.6 ± 4.6abc	−1.00	0.5
N16789	1.8 ± 0.17d	1.9 ± 0.1d	0.1	7.7 ± 9.4abc	−0.12	0.91
N16807	1.9 ± 0.14d	1.8 ± 0.1cd	−0.1	−4.3 ± 1.4abc	2.89	0.016
N16808	1.0 ± 0.1 ab	0.9 ± 0.1a	−0.2	−12.4 ± 4.6 ab	2.91	0.034
N16812	1.5 ± 0.1cd	1.5 ± 0.1bcd	−0.1	0.1 ± 8.4abc	0.41	0.693
Nigeria14	1.6 ± 0.1bcd	1.3 ± 0.1abc	−0.3	−17.1 ± 4.9a	3.50	0.025
F	11.45	11.49		2.10		
P	<0.001	<0.001		<0.001		

symptomatic plants could also be due to uneven distribution of phytoplasmas in the phloem of infected plants or low concentrations and variations in phytoplasma titre with season and plant organ (Firrao et al., 2007) as the pathogen was not detected in the leaves. Also phytoplasma infection has been reported to cause symptoms that are induced because of the stress placed on the plant by infection rather than specific pathogenicity of the phytoplasma (Bertamini et al., 2004). It could also be a result of poor correlation between phytoplasma presence and phloem aberrations or external symptoms occurring in some parts of infected plants, where by a long-distance effect of phytoplasmal infections is hypothesized (Marcone, 2010).

Four Napier grass accessions namely 16812, 16822, 16817 and 16807 remained asymptomatic throughout the study, an indication of tolerance to the NGS disease while accession 16789 was uninfected by the NGS phytoplasma throughout the screening period and could be due to the ability to resist the disease. The proportions of asymptomatic plants in 16822 decreased in the third re-growth due to plant death while accessions 16817, 16789 and 16807 maintained constant proportions of asymptomatic plants throughout the screening period. Phytoplasmas are obligate parasites inducing characteristic symptoms in host plants including slow growth rate, yellowing of the leaves, reduced leaf size, stunting, dieback, lack of fruits, virescence and phyllody. These symptoms indicate interference in the normal balance of plant hormones and vary with the phytoplasma strain, host plant, stage of the disease, age of the plant at the time of infection and environmental conditions (Lee et al., 2000; Jones et al., 2004, 2007; Nielsen et al., 2007; Asudi et al., 2015). In Napier grass, phytoplasma-infected plants usually manifest symptoms following several cuttings or grazing by animals (Orodho, 2006; Muyekho et al., 2006). Thus, the current study corroborates previous research as most plants exhibited yellow leaf symptoms and stunted growth on the third re-growth with fewer plants showing symptoms on the first and second re-growths. In rare cases, phytoplasma-infected plants are fully non-symptomatic over their lifetime and a temporary or permanent remission of symptoms may occur. Such associations between phytoplasmas and their hosts may indicate that those plants are tolerant to the phytoplasma infection (Lee et al., 2000). However, this also presents challenges to the NGS disease management as insect vectors can feed and acquire phytoplasmas from asymptomatic plants and disseminate them to new host plants thereby continuing its spread (Asudi et al., 2016a). Besides, Napier grass is propagated vegetatively, hence exchange of cuttings could contribute to the spread of the disease within and out of the farm when such asymptomatic cuttings are used (Orodho, 2006; Koji et al., 2012; Asudi et al., 2016b).

The NGS phytoplasma also greatly reduced the plant heights and leaf lengths of the plants in some accessions assessed including Nigeria 14, 15743 and Bgm 20 confirming their high susceptibility to the NGS disease. However, the effect was least in 16789 and 16812 supporting their ability to resist or tolerate the NGS disease respectively. There was also a greater effect of the phytoplasma on

the leaf width in Nigeria 14 and 16808 and less effect on accessions 16789 and Bgm 20. Phytoplasmas cause diseases to a number plant species worldwide leading to reduced yields, general decline or death of the plants. They also severely affect the phloem function in susceptible plants impairing transfer of soluble organic material to the roots. However, the symptoms can be mild or absent in resistant plants (Lee et al., 2000; Asudi et al., 2015, 2016b; Seemüller and Harries, 2010).

The Napier grass accessions used in the study were collected from different countries with most being infected with the NGS phytoplasma indicating the importance of the disease and its threat to the dairy sector in many countries. This also means that many regions are likely to be affected calling for an urgent need of a quarantine measure to control the movement of Napier grass cuttings and spread of the insect vectors to regions currently not infested with the disease. Unlike random screening of Napier grass accessions in the field in the past (Muyekho et al., 2006; Mulaa et al., 2010; Kawube et al., 2014), the method used in the current study was rigorous and the applied detection method of LAMP very sensitive (Obura et al., 2011). Thus, tolerant or resistant Napier grass accessions identified here are likely to stand for even longer period in the farmers' fields. In addition, we demonstrated that the vector (*M. banda*) fed on accession 16789, hence absence of NGS phytoplasma in the accession's DNA could not be alluded to plant escape from the vector during inoculation confirming the accession's ability to resist the effects of the phytoplasma. The accession 16789 is therefore a good source of resistance for future development of Napier grass varieties with good agronomic traits for release to farmers. However, it is important to establish the mechanism and duration for this resistance, and identify the genes responsible for this resistance and use them in breeding for resistance in Napier grass against the NGS disease. There is also need to test for phytoplasmas in other parts of the plants including the roots and stems and to quantify the phytoplasma titre in these accessions to validate tolerance and resistance. Field trials in different agro-climatic regions could also help to establish the effect of climate on both tolerant and resistant Napier grass accessions.

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