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Qualitative Distribution of Candidatus Phytoplasma Oryzae in Roots, Stems and Leave of Napier Grass (Pennisetum purpureum)

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Abstract
For sustainable production of Napier grass (*Pennisetum purpureum*), screening for the absence of phytoplasma on propagation material (stem) is carried by Nested-PCR in detection of 16S rDNA on leaves. However, the aim of this study was to investigate if there is uneven distribution of the pathogen in roots, stems and leaves of the infected plants that could influence screening results. A total of 294 Napier grasses infected with candidatus phytoplasma oryzae were sampled from western Kenya for detection of Napier Stunt Disease (Phytoplasma Oryzae) by nested PCR. There was significant difference on distribution of phytoplasma in roots, stems and leaves ($F=36.26$, $df=2$, $p<0.001$). The results show that high number of positive samples were detected from stem (94.1 %), leaves (77 %) and roots (44.1 %) samples, respectively. Conclusively, the study revealed uneven distribution thus inclusion of stem samples should be considered during screening procedures. Other factors like seasonal variation are also essential to be studied.
Keywords: Phytoplasma, Napier grass, nested PCR, Distribution of the pathogen, Propagation

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1.0 Introduction

Phytoplasmas constitute uncultivable cell wall-less prokaryotic pathogens classified as *Mollicutes* that colonize host-plant phloem and insect hosts (Music *et al*., 2008). Phytoplasmas have been identified to be Gram-positive bacteria that exist as small cells with smallest genome size among bacteria. The genus '*Candidatus* phytoplasma' has been considered on the basis of the phylogeny generated on 16S ribosomal DNA (Mehle *et al*., 2011). They are parasites that are majorly limited within the sieve elements of infected host plants, and are transmitted by phloem restricted vectors hemipterans, largely *Cicadellidae* including leafhoppers, plant hoppers and psyllids. Moreover, phytoplasmas are transmitted through cuttings, micro propagation, and grafting procedures (Mehle *et al*., 2011).

The colonization of host-plant species by phytoplasma is largely dependent on the host plant, plant organ, as well as season. They have been discovered in several unrelated plant species of economic significance. However, phytoplasmas are uncultivable in *axenic* media as their genome is tremendously reduced and lack genes that are vital for self-dividing organisms (Marzachi, 2006; Music *et al*., 2008). From their discovery, varied approaches have been used in detection of phytoplasmas in foliar, including electron microscopy and pathogenic nucleic acid staining techniques with little success. Nonetheless, the employment of molecular based procedures, including polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) evaluation provide sensitive, more accurate, and reasonably rapid determination of diseases linked to phytoplasmas (Marzachi *et al*., 2006).

Phytoplasmas infecting *Pennisetum purpureum* (Napier grass) have been determined by the use of molecular biology procedures including PCR (Adam *et al.*, 2015; Asudi *et al.*, 2016) and loop-mediated isothermal amplification (LAMP) (Obura *et al*., 2011) in western Kenya. Phytoplasmas observed to infect Napier grass in Kenya and Uganda have been identified to be of the subgroup 16SrXI (*Candidatus* phytoplasma oryzae) (Jones *et al*., 2004; Nielsen *et al*., 2007) and 16SrIII in Ethiopia (Jones *et al*., 2007) based on the conserved phytoplasma-specific 16S rDNA sequence. These phytoplasmas result in the development of Napier stunt disease (NSD) in Napier grass.

Napier stunt disease affects Napier grass production at rates between 30% and 90% in many small-holder farms in western Kenya (Mulaa *et al*., 2004). This extensive spread of NSD threatens to restrict the benefits obtained from this grass (Wambua *et al*., 2015). It elicits symptoms of reduced leaves, foliar yellowing, tillers proliferation, and shortening of internodes, hence stunting (Jones *et al*., 2004). Even so, accurate detection of phytoplasmas is notoriously problematic owing to their periodic fluctuating titres in host plants and, more importantly, their irregular distribution in infected plants (Mehle *et al*., 2011). This paper serves to provide an overview of phytoplasmas identified in Napier grass and their distribution within the Napier grass plants. Via

this, insight on Napier grass dissemination to the farmers will be essential as it will help in advising farmers on which part of Napier grass plant is suitable for propagation. Hence forming integral part of habitat management for Napier stunt disease.

2.0 Materials and methods

2.1.0 Plant samples

Symptomatic Napier grass stump samples were randomly collected between August 2015 and December 2015 from areas that are most prevalent with Napier Stunt Disease in western Kenya. Napier grass stumps uprooted then gently placed into a 2kg pots to avoid cross contamination. The stumps were washed with tap water to remove all the soils from the roots. From each stump; sub samples root, stems and leaves were aseptically collected (300 mg of each sample) into pre-labeled sampling tubes. The samples were stored in liquid nitrogen for transportation before being stored at -80°C in the laboratory prior to DNA extraction. Napier grass materials were collected during dry season.

2.1.1 Total DNA extraction

About 300 mgs of each fresh Napier grass sample was crushed to powder in liquid nitrogen in 1.5 milliliters eppendorf tubes. After which 600 microliters of 65°C pre-warmed Cetyltrimethylammonium bromide buffer (100mM Tris–HCl pH 8.0, 20mM EDTA, 2% CTAB, 1.4M NaCl, and 0.2% 2-mercaptoethanol) was dispensed to the powdered samples and vortexed to mix. The preparation was then incubated in hot water bath at 65°C for 1 hour. Thereafter 600 microliters of chloroform: isoamyl alcohol (24:1 v/v) was added to the preparation and vortexed. The preparation was centrifuged at 4000 revolutions per minute (rpm) for 10 minutes and the aqueous phase (supernatant) (about 600 microliters) layer cautiously transferred to sterile 1.5 milliliters eppendorf tubes. To each tube, 600 microliters of chilled isopropanol was added and incubated at -20°C overnight. The preparation was then centrifuged at 14000 rpm for 30 minutes and the supernatant carefully discarded. The sediment DNA pellet was washed with 1000 microliters of 70% ice-cold ethanol and the pellet left to air-dry. The dry DNA pellet was re-suspended in 50 microliters deionized, distilled, water (Doyle and Doyle 1990; Khan *et al.,* 2006; Stewart *et al*., 1993).

2.1.2 Polymerase chain reaction for DNA amplification

This reaction was aimed at amplifying the phytoplasma specific DNA fragments (16S rDNA) in the grass samples. Nested PCR was conducted using two pairs of oligonucleotides, phytoplasma universal primer pair P1 [5′-AAG AGT TTG ATC CTG GCT CAG GAT T-3′] and P6 [5′-CGG TAG GGA TAC CTT GTT ACG ACT TA-3′] (Inqaba Biotec^{TM}) in the first PCR reaction and NapF [5′-AGG AAA CTC TGA CCG AGC AAC-3′] and NapR [5′-ATT TTT CAT TGG CAG TCT CGT TA-3′] (Inqaba Biotec^{TM}) in the second PCR (Davis et al., 2003). The reaction mixture in the initial PCR contained 1.0µl of template DNA of each sample at 100ng/ µl, 1.0µl of both P1 and P6 primers for each sample, 2.5µl of dNTPs (deoxyribonucleotide triphosphates) for each sample, 0.25µl of Taq DNA polymerase (GenScript) for each sample and 2.5µl of 1x Taq Polymerase buffer (GenScript) for each sample used. The PCR reaction mixture was vortexed for 10 seconds to mix and 22.25µl of the mixture added to PCR tubes containing 1.0µl of each template. A 35 cycle PCR was conducted in a PTC-100® Thermal cycler (MJ Research - Incorporated, Lincoln Street, Massachusetts, USA) as follows; denaturation of DNA at 94ºC for 2 minutes for 1 cycle, annealing of the primers at 52ºC for 2 minutes for the first reaction and 372ºC for 3 minutes for the subsequent reactions and elongation reaction at 72ºC for 10 minutes for 1 cycle (Obura *et al*., 2011). The second amplification of the primary PCR products of the 16S rDNA fragment was carried out using a reaction mixture containing NapF/NapR primer pair (Inqaba BiotecTM). The second round reaction was performed using 0.5 µl of the first PCR amplicons. From each of the second PCR amplicons, 6.0 µl of the DNA was mixed with 4.0 µl of 6x loading dye (SIGMA - ALDRICH®) prior to loading into the gel wells. Electrophoresis was carried out at 70 volts for 45 minutes on 1.5% (w/v) agarose gel containing 0.3 µg/ mL ethidium bromide in 1x TAE (22.5mM Tris - acetate 1mM EDTA; pH 8.0) buffer. The gels were observed under UV transilluminator at 312nm wavelength to visualize the bands.

2.2 Data analysis

All data were analysed with R version 3.3.1. The numbers of positive samples were presented by frequency, while the mean of positive detections in roots, stem and leaves analysed with Analysis of Variance after angular transformed. The means were then compared using Turkey HSD at confidence interval of 95 %.

3.0 Results

There was more bands detected in the stems followed by leaves then in the roots (Figure 1)

3.1 Distribution of phytoplasma in Napier grass tissues

The PCR detection revealed significance difference in distribution of phytoplasma in the stem, leaves and roots

(F=36.26, df=2, p<0.001) (Table 1). The plant tissue with highest detection of phytoplasma was stem (94.1 %), leaves (77.0 %) and root (44 %), respectively (Table 1).

4.0 Discussion

Studies have showed that several wild grasses are reservoir for Phytoplasma affecting Napier grass (Adam *et al.,* 2015) making it difficult to control Napier stunt disease. In Bertaccini and Duduk (2009) review, they mentioned screening of phytoplasma-free and provision of resistant plant materials as the most feasible control and management strategies for phytoplasma. The available resistant materials to Napier stunt (Ouma II and South Africa varieties) are often screened before distribution to farmers. Standard method of leaves sampling for detection of 16s rDNA phytoplasma by nPCR is used. However, our studies showed that there is actually uneven distribution of phytoplasma in Napier grass tissues. Most samples from stems had the highest detection of phytoplasma followed by stem and roots. Our studies corroborated partly with several studies that have showed uneven phytoplasma distribution in host plants. In studies by Christensen *et al.*, (2004), phytoplasma levels were low in the roots and moderate in the stems with the highest titer found in mature leaves, with even a titer ≈40 times higher than that of the roots reported on leaves. Effect of season have been reported by Waterworth and Mock (1999) studies on deciduous woody plants suggesting that phytoplasmas disappear from the aerial parts of trees during the winter and survive in the root system to re-colonize the stem and branches in spring.

Pleomorphism exhibited by phytoplasma allows them to colonise many plant tissues. However, translocation studies have also showed that these pathogens are poor colonizers of the developing and differentiating tissues (meristem) (Wang and Valkonen, 2008). Factors that influence distribution and preference of phytoplasma in several tissues have not been investigated. However, Bertaccini and Duduk, (2009) in their review stated that phytoplasma lack of most biosynthetic pathways make them dependent on the nutrient-rich environment. So, this explains why most of stem and leaf samples in our studies tested positive for 16s rDNA Phytoplasma.

The increased emergence of phytoplasma diseases in recent years, has led to a continuous effort to improve the diagnostic procedures for quicker and more economic detection (Bertaccini and Duduk, 2009). For instance, to increase PCR efficiency, the low amount of phytoplasma DNA, often 1 % of the total DNA extracted from plant tissue (Bertaccini, 2007) has been improved by nested PCR, designed to increase specificity and sensitivity for low titre of DNA in sample. However, our studies revealed that apart from versatile detection technique, the knowledge on distribution of phytoplasma in plant tissue is necessary for screening. This was revealed by more positive samples of stems, the main propagation tissues than the leaves which are mostly the sources of injection of the pathogen by the vectors.

Knowledge of the movement of Phytoplasmas within the host plant and their final distribution in various organs is usually essential for understanding phytoplasma-host interactions and increasing the accuracy in sampling techniques. Moreover, sample collection for nPCR analysis would also have to be collected from stem tissues if conclusive reports are to be drawn on status of plants in question. Conclusively, these results could be used as a baseline in enhancing screening, multiplication and propagation systems of Napier grass. Additionally, it would also be important to study seasonal variation of Napier stunt disease since it is not well understood whether different season could affect the spread of Napier stunt disease. Based on our findings, it would also be very interesting to find out contribution of Napier grass propagation material towards the spread Napier stunt disease. Ideally, stem is the site of material cutting and propagation in the multiplication system. However, with such results at hand, methods of propagation will have to be revised to provide a safer method of material distribution. As such, disease spread could be easily controlled from the primary source. This experiment has promising results if further quantitatively analysed by qPCR (Real time PCR assay).

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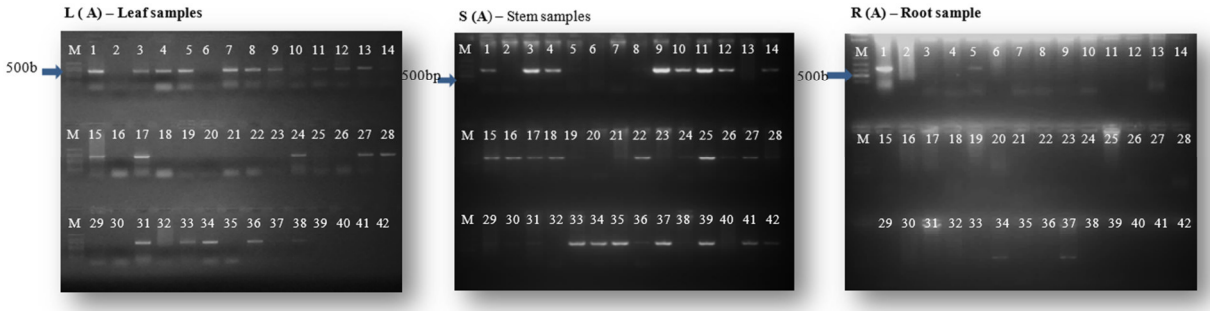

Figure 1: Detection of phytoplasma in different parts of Napier grass. **L**, leaf samples; **S**, stem samples; **R**, root samples. **A**, first batch of samples. Taq polymerase (Genscript) was used for nested polymerase chain reaction amplification. 500bp fragments were amplified with P1/P6 and NapF/NapR primers. Lane 1 in all the gel pictures is positive controls. 1000bp DNA molecular marker was used. **LA**, **SA**, **RA** means that they are all from the same Napier grass stump. For instance, **LA** (Lane2), **SA** (Lane 2) and **RA** (Lane 2) are samples from the same stalk of Napier grass and therefore comparison is made in **LA** (Lane 2 which is leaf sample 1), **SA** (Lane 2 which is stem sample 1) and **RA** (Lane 2 which is root sample 1) for the presence of phytoplasma through visible bright band. (Numbers in **L** correspond to numbers in **S** which also correspond to the ones in **R** thus comparison was observed among the corresponding numbers in leaves, stems and roots).

Table 1: Detection of *Phytoplasma* from different parts of phytoplasma-infected Napier grass trees by polymerase chain reaction assay

Plant tissue	Number of samples	% No. of Positive sample	Mean
Roots	294	44.0 %	10.6[a]
Stems	294	94.1 %	45.2[b]
Leaves	294	77.0 %	27.9[c]

Means followed by the different letters in each column are significantly different (P <0.05) by Tukey's HSD.