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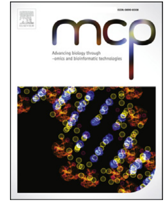

Development of field-applicable tests for rapid and sensitive detection of *Candidatus* Phytoplasma oryzae

Lillian Wambua [a, b, 1], Bernd Schneider [c, 1], Allan Okwaro [a, d], Joseph Odhiambo Wanga [a], Olive Imali [a], Peninah Nduku Wambua [a, b], Lavender Agutu [a], Cassandra Olds [e, f], Chris Stephen Jones [f], Daniel Masiga [a], Charles Midega [a], Zeyaur Khan [a], Joerg Jores [f, g], Anne Fischer [a, *, 2]

[a] International Centre of Insect Physiology and Ecology, P.O Box 30772, 00100 Nairobi, Kenya
[b] School of Biological Sciences, University of Nairobi, P.O Box 30197, 00100 Nairobi, Kenya
[c] Julius Kühn Institute, Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, 69221 Dossenheim, Germany
[d] Department of Biochemistry & Biotechnology, Kenyatta University, P.O. Box 43844, 00100 Nairobi, Kenya
[e] Washington State University, Pullman, WA, USA
[f] International Livestock Research Institute, P.O Box 30709, 00100 Nairobi, Kenya
[g] Institute of Veterinary Bacteriology, University of Bern, 3012 Bern, Switzerland

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ABSTRACT

Napier grass Stunt Disease (NSD) is a severe disease of Napier grass (*Pennisetum purpureum*) in Eastern Africa, caused by the leafhopper-transmitted bacterium *Candidatus* Phytoplasma oryzae. The pathogen severely impairs the growth of Napier grass, the major fodder for dairy cattle in Eastern Africa. NSD is associated with biomass losses of up to 70% of infected plants.

Diagnosis of NSD is done by nested PCR targeting the phytoplasma DNA, which is difficult to perform in developing countries with little infrastructure. We report the development of an easy to use, rapid, sensitive and specific molecular assay for field diagnosis of NSD. The procedure is based on recombinase polymerase amplification and targets the *imp* gene encoding a pathogen-specific immunodominant membrane protein. Therefore we followed a two-step process. First we developed an isothermal DNA amplification method for real time fluorescence application and then transferred this assay to a lateral flow format. The limit of detection for both procedures was estimated to be 10 organisms. We simplified the template preparation procedure by using freshly squeezed phloem sap from Napier grass. Additionally, we developed a laboratory serological assay with the potential to be converted to a lateral flow assay. Two murine monoclonal antibodies with high affinity and specificity to the immunodominant membrane protein IMP of *Candidatus* Phytoplasma oryzae were generated. Both antibodies specifically reacted with the denatured or native 17 kDa IMP protein. In dot blot experiments of extracts from infected plant, phytoplasmas were detected in as little as 12,5 μg of fresh plant material.

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1. Introduction

Phytoplasmas are insect transmitted phloem-limited bacterial plant pathogens parasitizing sieve tubes and causing profound disturbance of growth and productivity of numerous cereal, vegetable and fruit crops, as well as ornamental plants and trees worldwide [1]. In East Africa, Napier grass (*Pennisetum purpureum*), a fast-growing and high-yielding perennial grass native to Africa is affected by phytoplasmas in many regions. Infected grass exhibits severe yellowing and stunted growth prompting the disease name, Napier grass Stunt Disease (NSD). The disease causes up to 70% loss in biomass per infected plant, decimating the economic livelihoods of thousands of smallholder farmers who rely on Napier grass as feed for their dairy animals, which generate their income and provide their food. NSD was first reported in Kenya in 2004 [2], and

* Corresponding author.
E-mail address: jores.anne@gmail.com (A. Fischer).
[1] Equally contributed.
[2] Current address: SolidarMed, 6005 Luzern, Switzerland.

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later in Ethiopia [3], Uganda and Tanzania [4]. The phytoplasmas associated with NSD in Kenya, Uganda and Tanzania belong to the species *Candidatus* Phytoplasma oryzae (*Ca.* P. oryzae) [2,4] while the pathogen in Ethiopia is a member of the *Candidatus* Phytoplasma pruni species [3]. In Kenya *Maiestas banda* Kramer, a phloem-sucking leafhopper has been identified as a vector of Napier stunt phytoplasma [5], although other as yet undescribed vectors might exist. The common practice of sharing and trading cuttings and root splits of Napier grass by farmers as planting materials accelerates the spread of NSD. Wild grasses offer an alternative host for the Napier stunt phytoplasma [6] and the phytoplasma can potentially be transmitted to graminaceous cereals such as maize and sorghum, which are the most important staple food crops in Eastern Africa [7].

Phytoplasmas are unculturable *in vitro* and routine laboratory detection depends on molecular and serological methods. Molecular methods are mostly based on PCR assays using 16S ribosomal genes as the principal target for detection and characterization of phytoplasma species [1,8]. Varying primer combinations have been developed for universal [9], generic [10,11], and species-specific [12,13] detection of phytoplasmas in plant or insect samples. Routine detection of NSD phytoplasma is performed by amplification of the 16S rDNA in a nested PCR assay consisting of two subsequent PCRs [9]. This approach has been applied in very few laboratories across Eastern Africa to detect NSD phytoplasma in Napier grass [2–4], insect vectors [14] and wild grasses [6]. Nielsen et al. [4] used a real time Taqman PCR assay developed earlier [15] to simultaneously detect and quantify the NSD phytoplasma while using the plant 18S rDNA as internal control. Although PCR-based assays are sensitive, these methods are of restricted use in low and middle-income countries with poor laboratory infrastructure and limited resources as well as unreliable power supply. In addition, the current NSD phytoplasma diagnostics are based on the 16S rDNA gene, which is highly conserved and inadvertently raises the risk of false positives due to cross-amplification of related phytoplasmas and non-target bacteria with similar sequences, reducing the specificity of the tests [11]. Post-PCR procedures like RFLP analysis and DNA sequencing are required to verify the PCR amplicons unequivocally increasing the overall cost and expenditure of time for diagnosis.

Recent studies have demonstrated the feasibility of isothermal amplification technologies like loop-mediated amplification assay (LAMP) as viable alternatives for on-farm detection of phytoplasmas [16–18] but LAMP is highly susceptible to false-positive results. The lack of multiplexing capability and the complex primer design are additional disadvantages of the method. Although a LAMP assay for NSD detection based on 16S rDNA was previously developed [19], the specificity of the assay was low, data on the sensitivity of the assay were absent, and no field evaluation of the method was conducted. The recombinase polymerase amplification (RPA) technique is becoming a popular molecular tool for the rapid, sensitive and cost-effective detection of pathogens. It has been applied for the detection of plum pox virus among others and for the detection of methicillin-resistant *Staphylococcus aureus* [20], *Mycobacterium tuberculosis* [21], HIV-1 [22] and *Mycoplasma capricolum* subsp. *capripneumoniae* [23]. RPA is a versatile DNA amplification technique and can be combined with a range of detection procedures. Besides a real time assay based on fluorophore-labeled probes comparable to the Taqman real-time assays, amplification products can be visualized after incorporation of biotin-labeled primers with lateral flow devices. RPA and lateral flow assays (RPA-LF) have been developed for *Plasmodium falciparum* [24] and *Giardia duodenalis* [25]. Lateral flow assays are easy to use and therefore appropriate for resource-poor settings and nontechnical staff.

Serological detection methods are widely applied in plant pathology and belong to the standard repertoire of plant pathology laboratories and plant protection facilities. The diagnostic procedures are specific, easy to perform and cost efficient. Although a number of polyclonal antisera and monoclonal antibodies have been generated in the past against several phytoplasmas and varying epitopes, their employment for routine diagnosis is low and mostly restricted to the immunohistological localization of the pathogen [26]. The most widely used antigens for the generation of specific antibodies are the immunodominant membrane proteins (IMPs) [27–31]. IMPs are present in large numbers in the phytoplasma membrane and represent promising targets for the development of detection assays. Serological detection procedures are not available for NSD phytoplasma at present.

In this study, we developed an urgently needed field-applicable molecular diagnostic assay and a serological assay based on the immunodominant membrane protein gene (*imp*) and its respective product (IMP). Additionally, we compared the recombinase polymerase amplification (RPA) technology with the existing PCR-based assays. For the serological detection of IMP, high affinity monoclonal antibodies were generated and tested in immunoblot and enzyme-linked immunosorbent assay (ELISA) experiments for performance. Protocols for sample preparation from Napier grass and insects were also simplified for easy and rapid use in the field.

2. Materials and methods

2.1. Samples used in this study

About 200 leaf and stem cuttings of Napier grass (*Pennisetum purpureum*) cultivars were collected in February and March 2014 from farms in Kisumu, Vihiga, Butere, Busia, Teso, Bungoma, Siaya and Mbita districts of Western Kenya. They were screened for NSD infection and a subset of 96 samples representative of the different geographical origins were used for subsequent analyses (Table S1). A set of ten Napier grass plants maintained in *icipe*'s experimental plots at Thomas Odhiambo Campus in Mbita Point were used for the development of molecular and serological assays. Napier grass specimens free of any phytoplasma infection were accessed from healthy plants kept under insect-proof conditions in a greenhouse in *icipe*'s field station in Mbita Point and from the International Livestock Research Institute campus, an environment with no history of NSD.

Leaves of 17 periwinkle plants (*Catharanthus roseus*) infected with 15 different species of phytoplasmas available at the Julius Kühn Institute were also sampled for specificity testing (Table 1). In addition, two DNA extracts of *Candidatus* Phytoplasma cynodontis (CN, Kenya, 16Sr-XIV) were used for specificity testing.

Finally, 300 leafhoppers (*Maiestas banda*) fed on infected Napier grass in insectaries at *icipe* in Mbita Point were also included in the study. Upon collection, plant and insect samples were processed immediately or kept at −20 °C until DNA extraction.

2.2. DNA extraction

Genomic DNA was extracted from plants and insects using a cetyltrimethylammonium bromide (CTAB) method [32]. Briefly, 1 g of Napier grass, periwinkle leaves or two insects were placed in an extraction bag (Bioreba, Switzerland) and homogenized with a steel-ball roller in one ml of CTAB buffer. The homogenate was transferred to a microcentrifuge tube and extracted with an equal volume of chloroform:isoamyl alcohol (24:1). The aqueous and organic phases were separated by centrifugation. DNA was precipitated from the aqueous phase by adding an equal volume of isopropanol. The pellet was washed twice with 70% ethanol, air-

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