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Morphological and molecular characterization of the rust fungus, *Phakopsora apoda* causing leaf rust on *Brachiaria* grass in Rwanda

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Abstract

Brachiaria grass is one of the major forages that plays a key role in push pull technology. Different diseases including leaf rust caused by rust fungus, *Phakopsora apoda* have recently become an important challenge resulting in yield loss of *Brachiaria* grass in different countries including Rwanda. The objective of this study was to provide morphological and molecular characteristics of *Phakopsora apoda* causing *Brachiaria* leaf rust. Spores isolated from rusted *Brachiaria* leaves collected in five districts of Rwanda were analyzed. Samples were put in paper bags and left at room temperature for two days. Thereafter, spores were removed using brushes and put in eppendorf tubes, stored at 4^{o}C in darkness for further characterization. For morphological characteristics, colour and measurements of spores were recorded using Optika B-350 microscope with installed camera and calibrated micrometer. Multigene analysis using rust specific primers and large subunit (LSU) of nuclear ribosomal RNA was performed for molecular characterization. The results indicated that the colour of spores was yellowish or brownish while the shape was ellipsoidal or circular with average measurements of 25.1µm and 16.8µm for length and width respectively. Primers amplified the size of DNA between 1291bp and 1381bp and the size between 874bp to 882bp for rust specific primers and LSU respectively. The identity of the sequence was 96% with e-value of 0, sequence coverage of 60% for rust primer and the sequence identity was 94.97% to 95.37%, sequence coverage of 99% to 100% and e-value of 0 for LSU primer. All sequences recovered from five isolates of leaf rust matched to the genebank accession number MG461668.1 for both rust specific and LSU sequences. The results of this study provide useful information to develop effective options for leaf rust disease management in Rwanda.

Key words: Internal transcribed spacer, large subunit, nuclear ribosomal RNA, *Phakopsora apoda*, sequence, spore.

Introduction

Brachiaria grass is one of the nutritious forages originating from Africa which is also its centre of biodiversity. It belongs to the *Poaceae* family and is appreciated by many farmers in Sub-Saharan region due to its different attributes such as high biomass production with high nutrient content, drought tolerance and adaptation to infertile soils. Improvement of this genus was done outside Africa, especially in America and Australia (Maass *et al*., 2015). Seven species originating from Africa including *Brachiaria ruziziensis; Brachiaria arrecta, Brachiaria humidicola; Brachiaria dictioneura, Brachiaria decumbens, Brachiaria brizantha* and *Brachiaria mutica* are used as forages. The development of improved cultivars outside Africa led to different challenges and little attention has been given to biotic stress including leaf rust. With the increased promotion of improved *Brachiaria* cultivation in East African countries, there have been reports of occurrence of various diseases including leaf rust (Nzioki *et al*., 2016; Uzayisenga *et al*., 2020, 2021).

The wide distribution of leaf rust on *Brachiaria* grass was reported in Rwanda (Uzayisenga *et al*., 2020) and other causal agents of leaf rust on *Brachiaria* grass species such as *Puccinia levis var. panici sanguinalis* and *Uromyces setariae-italicae* are well documented (Lenné, 1990; Lenné and Trutmann, 1994). Several *Brachiaria* cultivars including CIAT 6369, Llanero, CIAT 679 and CIAT 16126 showed susceptible reaction to leaf rust in Central and South America (Lenné and Trutmann, 1994). Rust disease symptoms are characterised by yellowish to blackish pustules on leaves. It is not easy to recognise rust disease at the beginning of infection (Lenné, 1990).

Leaf rust reduces the quantity and quality of *Brachiaria* biomass. The biomass reduction is up to 100% and the reduction of crude proteins of *Brachiaria* leaves was reduced to 49% - 53%. The availability of other nutrients was affected due to leaf rust infection (Lenné and Trutman, 1994). Leaf rust management options include establishment of hedges, acceleration of *Brachiaria* growth by application of nitrogen fertilizers; use of rust-free

planting materials, planting at appropriate time since leaf rust is favoured by rainfall, use of mixed *Brachiaria* genotypes and avoiding burning and early harvesting of *Brachiaria g*rass ranging between four and eight weeks (Alvarez *et al*., 2014). The objective of this study was to provide morphological and molecular characteristics of *Phakospora apoda*, the causal agent of *Brachiaria* leaf rust in Rwanda.

Materials and methods

Collection of samples and isolation of leaf rust spores

Brachiaria grass with leaf rust symptoms were sampled from five different *Brachiaria* fields located in different districts (Table 1). A total of five sample in each district was collected, put in separate paper bags and carried to Plant pathology laboratory of Rwanda Agriculture and Animal Resources Development Board (RAB) in Southern Province of Rwanda for processing and analysis. Leaf samples kept in paper bags were put on table to dry at normal room temperature for two days. Thereafter, brushes were gently used to remove the rust spores from *Brachiaria* leaves and the spores were collected on aluminium foil, put in eppendorf tubes and stored in darkness at – 4 ºC for further characterization (Guo *et al*., 2016).

Table 1. Sampling location and coordinates of collected leaf rust samples

Isolate name	Cultivar	District	Altitude (m.a.s.l)	GPS coordinates	
BRRWR1	Mulato II	Bugesera	1455	E030°01'58.6''	S02°15'33.8''
HYRWR2	Mulato II	Huye	1685	E29°46'.56.9″	S02°28'54.8″
NBRWR3	Cayman	Nyamagabe	1839	E29˚36'40.1″	S02˚26'06.7″
NRRWR4	Piata	Nyagatare	1346	E030°18.304'	S01° 18.936'
RN RWR5	Mulato II	Rwamagana	1520	E030 22.662'	S01 53.310'

Morphological characterization of the causal agent of *Brachiaria* leaf rust

Morphological characteristics including the colour, shape and measurements of leaf rust spores associated with *Brachiaria* grass were visualised using the microscope Optika B – 350 at x40 magnification and the digital image of leaf rust spore was recorded using a camera installed on the microscope. Measurements of the size of leaf rust spores were taken using a calibrated micrometre.

Molecular characterization of the causal agent of *Brachiaria* leaf rust

Genomic DNA (gDNA) was extracted from leaf rust spores obtained from five different samples representing five *Phakopsora apoda* isolates (BSRWR1, HYRWR2, NBRWR3, NRRWR4 and RNRWR5). Qiagen DNeasy Plant Mini Kit was used for DNA extraction. The manufacturer protocol was used and leaf rust spores were mixed with sterilized carborundum (0.01%) for easy breakage of cells. The crushing was done in liquid nitrogen using both cooled and sterile mortar and pestle. Further steps of DNA extraction used 20 mg of fine ground powder of each of the five samples. For confirmation of causal pathogen, multi-gene analysis was used where rust primers and 28S large subunit of nuclear ribosomal RNA (LSU) were used for identification of the causal agent of *Brachiaria* leaf rust. The PCR reaction volume was set at 25µl (3µl of diluted gDNA containing 20 to 40 ng DNA, 12.5µl premix, 0.5µl each primer (forward and reverse) and 8.5µl of water for molecular biology), along with the negative control reaction without DNA template. Rust specific primers ITS1rustF10d (5'-TGAACCTGCAGAAGGATCATTA-3') and rust1 (5'-GCTTACTGCCTTCCTCAATC-3') and 28S large subunit of nuclear ribosomal RNA LR5/LROR TCCTGAGGGAAACTTCG/ ACCCGCTGAACTTAAGC were used for DNA amplification (Barnes and Szabo, 2007).

The PCR conditions were 4 minutes of initial denaturation at 94°C, followed by 35 cycles of 94°C for 45 seconds, 59.5°C for 45 seconds and 72°C for 45 seconds, with final extension at 72°C for 10 minutes and hold at 4°C and initial denaturation step at 95 °C for 3 minutes followed by 34 cycles of 30 seconds of denaturation at 95°C, 30

seconds of annealing temperature at 52°C, 1 minute of elongation at 72°C, final extension of 10 min at 72°C and hold at 4◦C at the end for rust and LSU primers respectively. The presence of targeted products was verified by loading 3µl of PCR product on 1.5% agarose gel for one hour at 70 volts.

Thereafter, PCR products were purified using QIAquick PCR Purification Kit (QIAGEN) following instructions of the manufacturer. Purified PCR products were sanger-sequenced at BecA -ILRI, Nairobi, Kenya using the respective primer sets used for PCR amplification. Raw DNA sequences were cleaned and consensus sequences were determined by aligning nucleotide sequences generated by forward and reverse primers. Consensus sequences were submitted to the National Centre for Biotechnology Information (NCBI) for homology search and identification of species using Basic Local Alignment Search Tools (BLAST) programme (Altschul *et al*., 1990).

Pathogenicity test

A total of five isolates of *Phakopsora apoda* with one isolate from each district (BSRWR1, HYRWR2, NBRWR3, NRRWR4 and RNRWR5) were tested for pathogenicity using one-month old seedlings of *Brachiaria hybrid* cv. Mulato and leaf rust spores suspended in water and tween 20. The spore concentration was 10^6 ml^{-1}. To facilitate the penetration of fungus into host tissue, 1% carborandum was added into the inoculum. Three seedlings per isolate were inoculated by hand rubbing and the development of leaf rust symptom was checked on daily basis for 30 days. Symptoms on inoculated plants were compared with those which occur under natural conditions. Seedlings for negative control were inoculated with the inoculum prepared in the same way but leaf rust spores were not added.

To maintain high humidity, inoculated plants were covered in polyethylene bags for two days (Figure 1). The pathogen was re-isolated from artificially inoculated leaves of *Brachiaria* hybrid cv. Mulato using the same procedure as described before and morphological characteristics of re-isolated spores were compared with the original isolates.

Figure 1. *Brachiaria* hybrid cv. Mulato seedlings covered in plastic bags after leaf rust inoculation

Data analysis

The CLC Genomics Workbench Version 8.0.3 software (https://digitalinsights.qiagen.com) was used to process data of DNA sequences from Sanger sequencing. Trimming was performed using quality scores of 0.05. The analyses did not consider all sequences with scores rating below 50%. Nucleotide sequences generated by the forward and reverse primers were checked, edited and consensus sequences were also generated using CLC Workbench version 8.0.3 software. Sequences were aligned using the same software and gaps were considered as missing data. To allocate identities to the test isolates, identification of species was done using BLAST programme of NCBI sequence database and comparison of the GenBank database was based on high similarity, coverage and identity.

Results

Morphological characteristics of fungal species associated with *Brachiaria* leaf rust

The association of *Phakopsora apoda* with rust symptoms was identified through macroscopic and microscopic analysis. Observations indicated that pustules with yellowish or brownish colour were mostly found on adaxial surface of leaves. Spores were ellipsoidal or circle, yellowish and brownish with average length of 25.1µm and the average width of 16.8µm (Figure 2, Table 2).

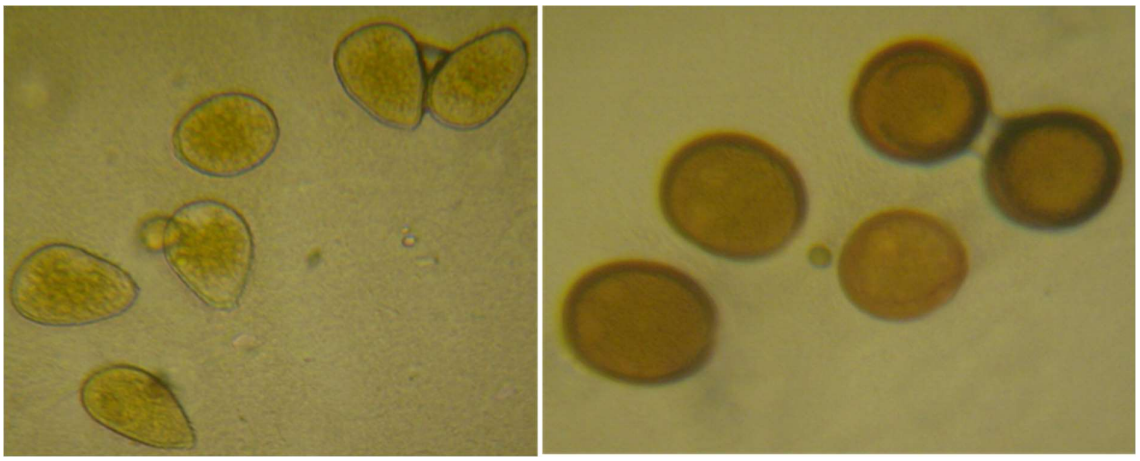

Figure 2. *Phakopsora apoda* spores isolated from rusted leaves of *Brachiaria* grass in Rwanda. (Photo taken using a camera installed on an OPTIKA B-350 microscope).

Table 2. Size of spores of *Phakopsora apoda* isolates

Isolate	Size of leaf rust spore (µm)	
	Length	Width
BRRWR1	23.8 ± 2.4^{ab}	16.7 ± 1.7^{a}
HYRWR2	23.3 ± 1.7^{ab}	15.8 ± 0.8^{a}
NBRWR3	29.2 ± 2.4^{a}	15.8 ± 2.0^{a}
NRRWR4	22.5 ± 1.7^{b}	18.3 ± 1.7^{a}
RN RWR5	26.7 ± 1.7^{ab}	17.5 ± 1.1^{a}
Mean	25.1 ± 0.9	16.8 ± 0.7

± is followed by standard error of the mean. Values with the same letters within the column are not statistically different at the probability level of 0.05.

Molecular identification of *Phakopsora apoda* isolates

For all five leaf rust isolates, rust specific primers amplified the size of DNA ranging between 1291bp and 1381bp while the DNA size varied between 874bp to 882bp when the amplification was done using LSU primers. The identity of the sequence was 96%, sequence coverage of 60% with e-value equal to zero for rust primers and the sequence identity was 94.97% to 95.37%, sequence coverage of 99% to 100% and e-value of 0 for LSU primers. All sequences recovered from five isolates of leaf rust matched the gene-bank accession number MG461668.1 for both rust specific and LSU sequences (Table 3).

Table 3. Five *Phakopsora apoda* isolates, rust primer and LSU sequence characteristics, homology search results

Primer	Isolate	Identified species	Sequence length (bp)	Sequence coverage (%)	e-value	Sequence identity (%)	Matching NCBI accession
Rust specific	BRRWR1	*P. apoda*	1370	60	0	96	MG461668.1
	HYRWR2	*P. apoda*	1381	60	0	96	MG461668.1
	NBRWR3	*P. apoda*	1374	60	0	96	MG461668.1
	NRRWR4	*P. apoda*	1291	60	0	96	MG461668.1
	RN RWR5	*P. apoda*	1378	60	0	96	MG461668.1
LSU	BRRWR1	*P. apoda*	882	100	0	95.37	MG461668.1
	HYRWR2	*P. apoda*	882	100	0	95.25	MG461668.1
	NBRWR3	*P. apoda*	868	100	0	94.72	MG461668.1
	NRRWR4	*P. apoda*	876	99	0	95.09	MG461668.1
	RN RWR5	*P. apoda*	874	99	0	94.97	MG461668.1

Pathogenicity of *Phakopsora apoda* isolates on susceptible *Brachiaria* seedlings

Results of the pathogenicity test revealed that all isolates produced a typical leaf rust symptom on leaves of *Brachiaria* hybrid cv. Mulato seedlings. Inoculated *Brachiaria* seedlings showed symptoms of leaf rust 10-18 days after inoculation for all isolates whereas leaves of seedlings used for negative controls did not show any disease symptoms (Figure 3). All five isolates were pathogenic to *Brachiaria* hybrid cv. Mulato and caused similar symptoms as the ones observed following natural infection. Symptomatic leaves from the inoculation experiment were used to re-isolate the causal agent.

Figure 3: Pathogenicity of *Phakopsora apoda* isolates from different regions of Rwanda on *Brachiaria* Hybrid cv. Mulato. 1: BSRWR1 (Bugesera isolate); 2: HYRWR2 (Huye isolate); 3: NBRWR3 (Nyamagabe isolate), 4: NBRWR3 (Nyagatare isolate), 5: RNRWR5 (Rwamagana isolate), 6: Negative control, 7: Leaf rust symptoms caused by *Phakopsora apoda* on naturally infected leaves of *Brachiaria* hybrid cv. Mulato.

Discussion

The isolation and identification of microorganism causing leaf rust of *Brachiaria* grass indicated that leaf rust was associated with *Phakopsora apoda*. Morphological characteristics of spores of *Phakopsora apoda* were ellipsoidal or circle, yellowish and brownish with average of 25.1µm and 16.8µm in length and width respectively. Morphological characteristics of the causal agent of *Brachiaria* leaf rust found in this study are in the range of the findings by other authors (Cherunya, *et.al.*, 2019)

Identification of leaf rust pathogen isolates using molecular techniques matched *Phakopsora apoda* sequence on top in the database of NCBI query cover of 60%. The low percent query cover found in this study might be attributed to rust fungi sequences from sequences that are unique and available in genebank. *Phakopsora apoda* has been proven to cause rust disease infecting Kikuyu grass (Gardner, 1984; Adendorff and Rijkenberg, 1995; Adendorff, 2014). Lenné (1990) indicated *Uromyces setariae-italicae* and *Puccinia levis* var. *panici-sanguinalis* as causal agents of rust disease affecting *Brachiaria* grass.

Likewise, analysis of sequences generated from rust and LSU primers confirmed *Phakopsora apoda* as the causal agent of *Brachiaria* grass leaf rust. The current study provides new etiological information about *Phakopsora apoda*, revealing these species for the first time as the causal agent of leaf rust on *Brachiaria* grass in Rwanda.

Conclusions and recommendations

Referring to the morphological features, rust and LSU sequence analysis and pathogenicity test of five isolates of leaf rust confirmed *Phakopsora apoda* as the causal agent of leaf rust of *Brachiaria* grass in Rwanda. All leaf rust isolates caused rust symptoms on *Brachiaria* hybrid cv. Mulato and had similar characteristics with re-isolates. Information generated in this study is highly important for development of leaf rust management strategies and will contribute to future studies on various aspects of *Phakopsora apoda* in order to protect *Brachiaria* grass.

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