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Original Research Article

Molecular Characterization of the Mycorrhizae and Other Root Associated Fungi Abundance of Some Crop Plants Grown in Kano, Nigeria

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ABSTRACT

This work looked into the molecular characterization of crop plants grown in Kano, North-Western Nigeria. This was aimed at finding what class of mycorrhizal fungi is associated with these crops for better understanding of the plant ecosystem. This was done using DNA extraction and Polymerase chain reaction techniques. Sequence results were associated to the Ascomycota and Mucuromycota Division. Fungal isolates identities were above 85% and were recognized to be *Rhizopus microsporus, Fusarium oxysporum* and *Sodaria* spp.

Keywords: Climate change, Mycorrhizae fungi, Molecular characterization, Nigeria, Roots.

INTRODUCTION

The mycorrhizal symbiosis is arguably the most important symbiosis on earth. Fossil records indicate that arbuscular mycorrhizal interactions evolved 400 to 450 million years ago (Smith and Read, 2008) and that they played a critical role in the colonization of land by plants. The arbuscular mycorrhiza (AM) is the most commonly found mycorrhizal symbiosis, formed between a wide range of plant taxa including a large number of species, and the obligate symbiotic soil fungi belonging to the monophyletic Phylum Glomeromycota. The Phylum comprises only about 250 described species (Vita *et al*., 2018). The majority of these mycorrhizal interactions is mutually beneficial for both partners and is characterized by a bidirectional exchange of resources across the mycorrhizal interface. The mycorrhizal fungus provides the host plant with nutrients, such as phosphate and nitrogen, and increases the abiotic (drought, salinity, heavy metals) and biotic (root pathogens) stress resistance of the host. In return for their beneficial effect on nutrient uptake, the host plant transfers between 4 and 20% of its photosynthetically fixed carbon to the mycorrhizal fungus (Wrigh *et al*., 1998). These legumes and grains are staples of high nutritional value to almost every household in this region and limited information exist on the type of mycorrhizae species colonizing these crops in the study area. The determination and identification of local fungal species will help determine more compatible fungi for eventual sustainable agriculture, leading to boast in food production, improve soil conditions against stressful cues and directly help combat climate change. The aim of this work was molecular characterization of the mycorrhizal and other root associated fungi abundance of some crop plants grown in Kano, North-Western Nigeria.

MATERIALS AND METHODS

Study Area

This work was carried out at Centre for Dryland Agriculture (CDA), and the Old Campus of Bayero University Kano, Nigeria. It was conducted within the months of August 2019 to January 2020. Crop plants were grown in the field at the CDA farms located at Bayero University Kano New campus and the Old Campus of Bayero University Kano. The coordinates for the sites are 11°58'58"N 8°24'53"E and 11°58'42"N 8°28'40"E, respectively.

Media Preparation and Inoculation of Fungi

Thirty nine (39) grams of commercialized Potato Dextrose Agar (PDA) powder was added to 1 liter of distilled water. The PDA contains Potato infusion 200 gm, Dextrose 20gm, and Agar 20 gm. This was boiled while mixing

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until completely dissolved. The media was sterilized by autoclaving at 121℃ for 15 minutes. When cooled to 50℃, tetracycline was used to aseptically dispense into sterile Petri dishes, in a sterilized safety cabinet.

For the inoculation, a clamp was sterilized using a spirit lamp, and the representative root sample transferred into the Petri dishes and allowed for 24 – 48 hours to observe the growth. Some colonies were sub-cultured for a more finely fungal sample.

Microscopic and Molecular Identification of Arbuscular Mycorrhizal Fungi

For microscopic identification, fungal samples were collected from the already growing samples of inoculated root of crops. Fungal specimens were collected using wire loop and carefully mounted on a slide, stained using methylene blue. This was observed under a compound microscope at x10 and x40 magnification, with a camera connected for capturing the identified fungi. To perform molecular characterization of the AM fungi, the nested PCR protocol as described by Lee *et al*. (2008) was adopted. The AML sequences obtained were further analyzed using BLAST search program from the National Center of Biotechnology Information (NCBI) website to find identical species in the GenBank.

LIVAK DNA Extraction Protocol

LIVAK Homogenising Buffer: 1.6ml 5M NaCl, 5.48g Sucrose, 1.57g Tris, 10.16 ml 0.5M EDTA, 2.5ml 20% SDS Bring volume to 100ml, filter sterilize. Store 5ml aliquots at -20℃. Heat in Water bath and mix before each use to re-dissolve precipitate.

About 1.5ml of the broth culture was pipetted in new 1.5 ml Eppendorf tube and centrifuge at 10,000rpm for 5min (at 4℃). The supernatant was discardedand pellet washed with distilled sterile and deionized water three times. 100 µL preheated homogenising buffer was Pipette into the supernatant and homogenized, and transfer immediately to 65℃.It was incubated at 65℃approx 30 min. Microfuged briefly to collect condensation.14µL 8M K-acetate was added (to final concentration of 1M) and mixed.Incubated on ice for approximately 30 minutes, centrifuged for 20 min at 4 ℃. Supernatant transferred to new 1.5 ml eppendorf, carefully not to transfer any debris. Add 200µL 100 % ethanol and was mixed, spinned for 15 min at 4℃. Remove and discard supernatant, rinse pellet in approx 100µL ice-cold 70% ethanol, being careful not to dislodge pellet. Dry pellet were left open in tubes on bench top for approximately 1 hour. Pellet were suspended in 100µL dH_2O, incubate at 65℃ for 10 minutes. The overall concentration of the DNA was checked using nanodrop spectrophotometry.

Polymerase Chain Reaction (PCR) For Fungal Species

The PCR procedure was carried out using KAPATaq DNA polymerase. The Total reaction volume was 25µL. Reaction mix comprised of 2µL each of the genomic DNA, 2.5 µL of 10 TaqA Buffer, ~0.4M (0.85 µL) of each of forward and reverse primers, 1.25 mM (1.5 µL) of $MgCl_2$, 0.25 mM (0.2 µL) of dNTP mixes and 0.2 µL of Taq DNA polymerase, in ddH_2O. Amplification was carried out using the following conditions: initial denaturation of 5 min at 95℃, followed by 35 cycles each of 30s at 94℃ (denaturation), 47℃ (primer annealing) and 1 min at 72℃ (extension). This was followed with 10mins final extension at 72℃. PCR products were separated in a 1.5% agarose gel stained with ethidium bromide.

The primers should are18srRNA fung ITS-FATATGCTTAAGTTCAGCGGGT (5^1-3^1), 18srRNAfung-ITS-RGTTCCGTAGGTGAACCTGC (5^1-3^1) 550-600bp at 47℃.

Sequence Analysis

Sequences were edited with the open access program BioEdit (Buck *et al*., 2019) and aligned in MEGA 6 (Lee *et al*., 2006). BLAST searches for AM fungal species was performed against the NCBI Reference Sequence Database (Buck *et al*., 2019). The bootstrap values will be estimated with 1000 replicates.

An Arbuscular mycorrhizal operational taxonomic unit (OTUs) will be defined on the basis of sequence similarities as surrogates for species. A threshold of 97% similarity will be selected as the minimum value to assign a sequence to the same OTU since this value has been commonly used by various authors (Buck *et al*., 2019).

RESULTS AND DISCUSSION

Microscopic Characterization of Mycorrhizal Fungi

The results of the microscopic fungi actively colonizing the plant root using microscopic analyses showed different fungal forms. Microscopic images of the selected plants roots colonized by arbuscular mycorrhizal fungi are presented below. Figures 1, 2, 3 and 4 showed representative microscopic images of Groundnut, Cowpea, Millet and Sorghum roots which were colonized by arbuscular mycorrhizae in Bayero University Kano Old Site and Bayero University Kano New Site during the three (3) collection phases (Pre-flowering, Flowering, and Budding).

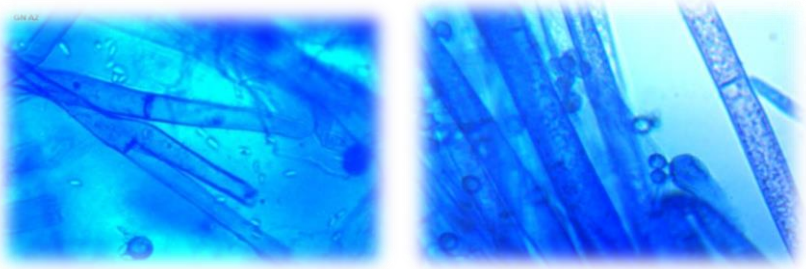

Figure 1. Representative fungi from Groundnut roots of Sites A and B. X40 (*Rhizopus* and *Acremonium*).

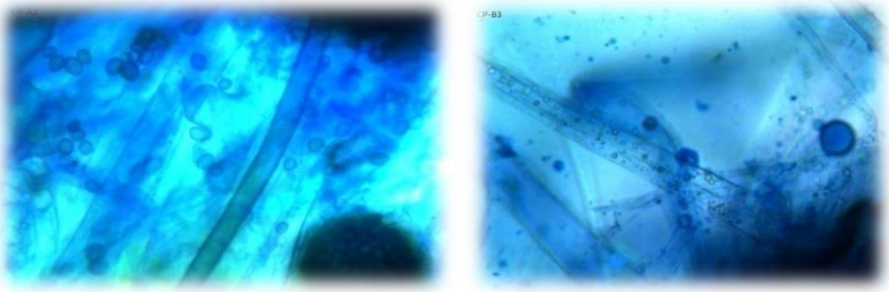

Figure 2. Representative fungi from Cowpea roots of Sites A and B. X40 (*Rhizopus* and *Aureabasidium*).

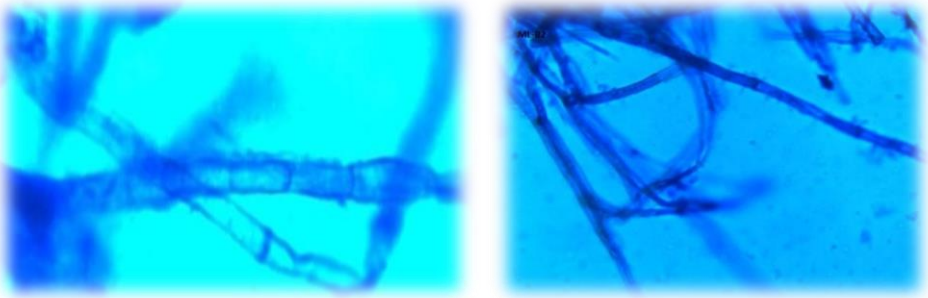

Figure 3. Representative fungi from Millet roots of Sites A and B. X40 (*Cireinella* and *Aureabasidium*).

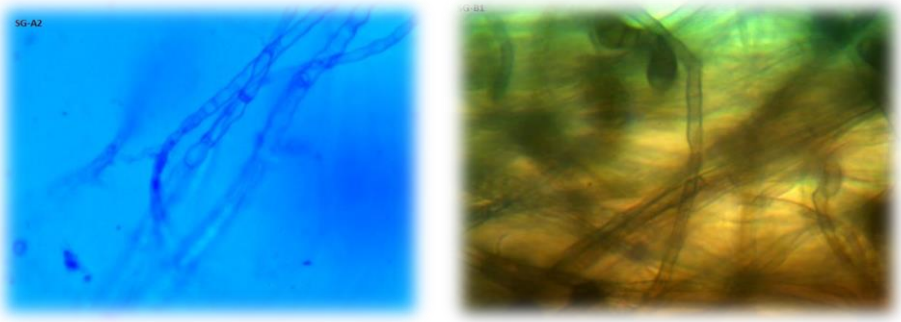

Figure 4. Representative fungi from Sorghum roots of Sites A and B. X40 (*Streptomyces* and *Talaromyces*)

Molecular Analyses

The ITS region, targeted by the universal primers ITS1 and ITS4 was amplified in all three isolates. PCR products varied in size from 504 to 655 bp.The hyper ladder 1kb, Bioline USA, calibrated in two parts. The lower five has an increase of 200bps while the upper has an increase of 1000bps (200bp, 400bp, 600bp, 800bp, 1000bp, 1500bp, 2000bp, 2500bp, 3000bp, 4000bp, 5000bp, 6000bp, 8000bp and 10037bp). But from the Gel image below we only have five (5) visible ladder bands, the remaining nine (10) did not separate. The longest fragment (655) was from cowpea isolate and the shortest (504) from millet fungal isolate. This is similar to the findings of Yildirim *et al.* (2007) who observed amplicon of approximately 601bps in electrophoretic gel bands.The procedure was carried out at 110v for 35 minutes.

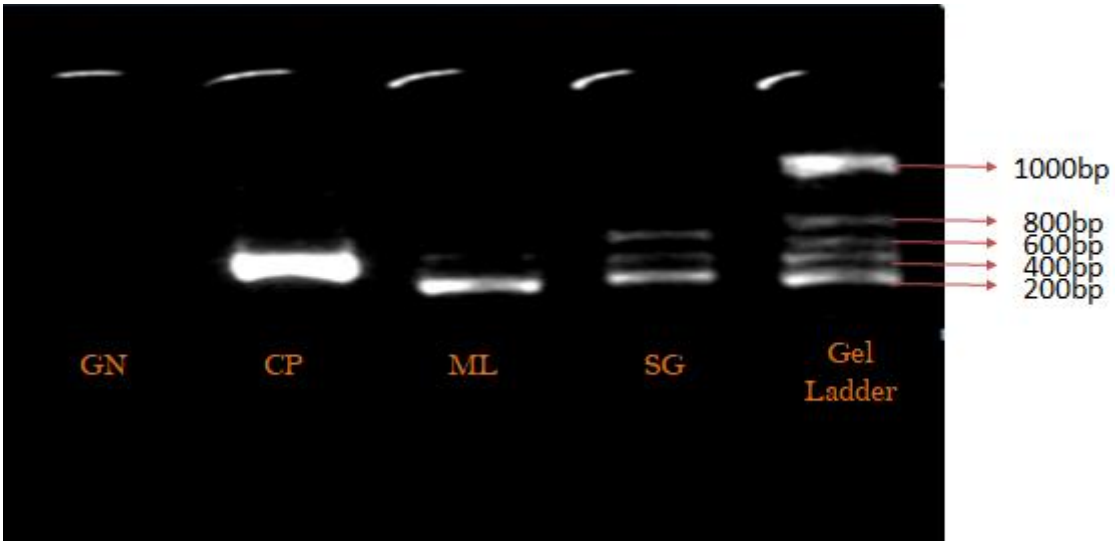

Figure 5. Electrophoretic gels of arbuscular mycorrhizal ITS regions from fungal isolates of groundnut (GN), cowpea (CP), millet (ML) and sorghum (SG).

Sequence Results

The sequence is a product of PCR by Inqaba Biotech Laboratories, South Africa. The Sequence of the three fungal representative samples is given in their graphic format form NCBI Blast for the first 10 results of each sequence and the identity organisms. All sequence results were found to have high identity match (over 85-100%) to AMF associated with the Glomeromycota phylum. This is in agreement with the work of Mahdhi *et al*., (2018), form which their Blast searches in the GenBank database showed that all of the sequences had a high identity (85–100%) to AMF and affiliated to the Glomeromycota phylum. The absence in this study of AMF belonging to the *Glomus* and *Scutellospora* which were detected in other studies might be due to the high specificity of the primers FLR3 and FLR4 to Glomeromycota which was not used for this study. The FLR4 primer has a perfect match only

with glomalean LSU rDNA sequences and although the Primer FLR3 primer could recognize DNA from some Basidiomycetes as well as from Glomales.

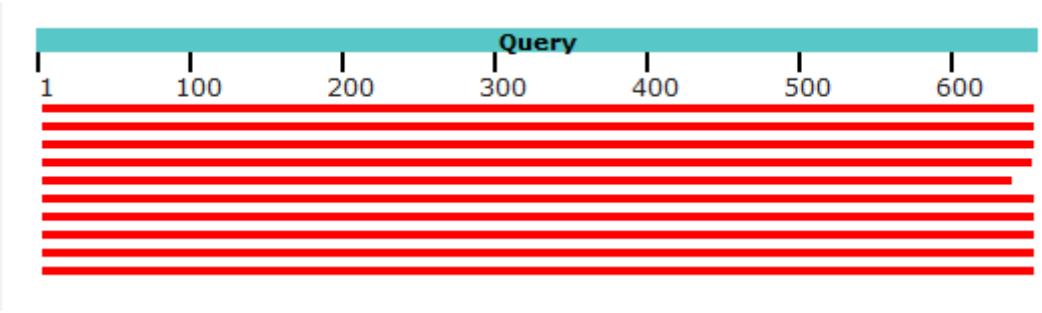

Figure 6. Graphic representation of the first 10 results from cowpea sequence.

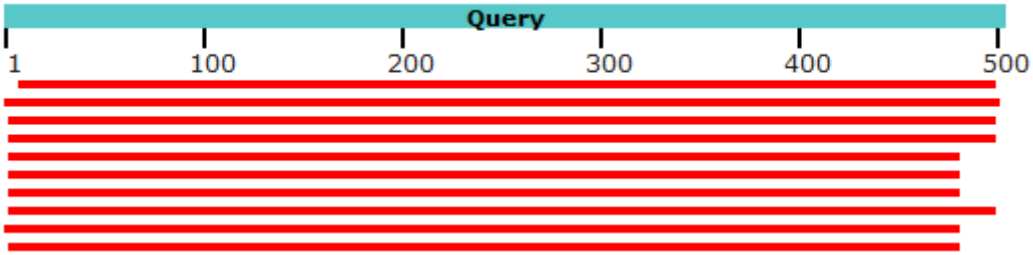

Figure 7. Graphic representation of the first 10 results from millet sequence.

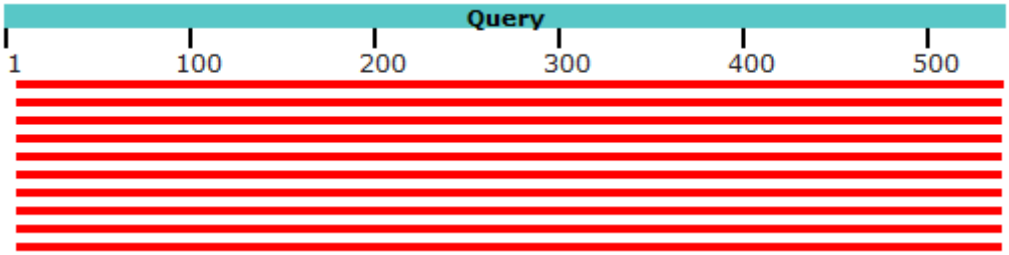

Figure 8. Graphic representation of the first 10 results from sorghum sequence.

Table 1. Pairwise alignment of ITS1 and ITS2 amplicons of cowpea, millet and sorghum.

Organism	Score (bits)	Identities	Gaps	Isolate Crop
Rhizopus microsporus strain AY899 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	1116 bits(604)	638/653(98%)	7/653(1%)	Cowpea
Fusarium oxysporum isolate FusCic45B 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene,	542 bits(293)	429/496(86%)	5/496(1%)	Millet

and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence				
Sordaria sp. P10E4 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	948 bits(513)	529/536(99%)	3/536(0%)	Sorghum

Phylogenetic Tree

Molecular phylogeny is commonly used to infer taxonomic relationships and patterns of genetic diversity among species. The phylogenetic tree above was drawn to show the distance and or similarities between the representative isolates, this was not in reference to any known phylogeny of other phylum from the fungi kingdom. From the Figure 9, we can conclude the representative fungal isolates of sorghum (*Sordaria species* Z4) and millet (*Fusarium oxysporum* Z3) are from the same node, making them more closely related as sister taxa compared to cowpea (*Rhyzopus microsporus* Z2) which is far away from the root starting point.

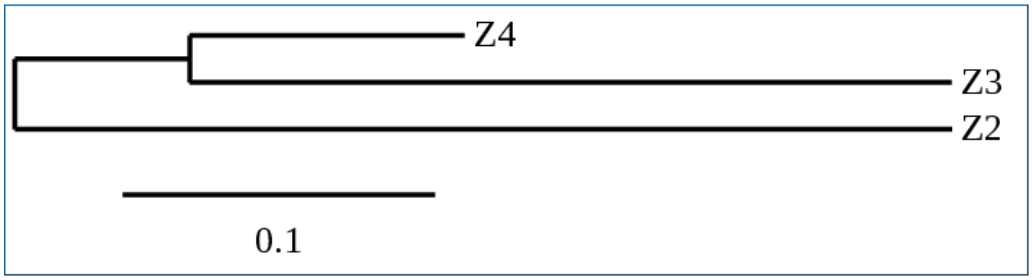

Figure 9. Phylogenetic Tree of the representative organisms from crop plant roots.

CONCLUSIONS

Our study is the first to report the mycorrhizal characterization of these plants in the North-Western part of Nigeria. Little is currently known about the molecular aspect of these crops and thereby needs more investigation. This study concludes that two phylum of Ascomycota and Mucoromycota were recovered from the findings with Acomycota been the dominant of the two classes. This work is in line about the ongoing debate to classify ascomycota and mucuromycota as mycorrhizae fungi and not just endophytes as all distinct features of mycorrhizae has been observed.

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