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IDENTIFICATION OF FUNGAL ORGANISMS ASSOCIATED WITH THE RHIZOSPHERE OF MAIZE (*Zea mays* L.): BASIC MOLECULAR TECHNIQUES

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Abstract
The rhizosphere of plants harbours diverse communities of microorganisms which play important roles to the plant and other living organisms. This study was aimed at isolating and characterizing fungi associated with the rhizosphere of *Zea mays* L. using both traditional cultural techniques and basic molecular methods. Fungi were isolated from the soil around the root of maize plant obtained from University of Port Harcourt, Rivers State. The fungal DNA was extracted using Zymo Fungal/Bacteria DNA Miniprep Kit and amplified using the primer pair: ITS4 and ITS5. Internal Trnascribed Spacer (ITS) sequences of the isolates were blasted on National Centre for Biotechnology Information (NCBI) database and identified as *Aspergillus pseudonomius* and *Penicillium* sp. Phylogenetic tree was constructed to access the relationship between the isolates obtained from this study and other isolates on GenBank. The sequences of the isolates have been deposited in GenBank under the accession numbers: MN187251 for *Aspergillus pseudonomius* and MT723948 for *Penicillium* sp. The molecular techniques used in this study were sufficient in classifying the fungal isolates. This study has given insight into some of the fungi that inhabit the rhizosphere of *Zea mays*.

Keywords: *Zea mays*, rhizospere, sequencing, phylogeny, and fungi

Introduction
Zea mays is one of the most important, popular and oldest cereal food crops worldwide, serving as a staple food and livestock feed (Huma *et al*., 2019). It is used in the human diet in both fresh and processed forms. The value added has been an economic driver in the specialty corn markets. Maize is a good source of carbohydrates. It also contains vitamin B-complex such as pantothenic acid, thiamine, riboflavin and niacin. It contains selenium, beta-carotene; and vitamins A, C and K (Kumar *et al*., 2013). It is a potential antioxidant that protects the body against harm by free radicals which are responsible for cellular damage, which may lead to cancer. It can reduce body pains and act as an analgesic (Owoloye *et al*., 2010). Maize grain is progressively used for the preparation of corn starch, corn syrup, corn oil dextrose, corn flakes, gluten, grain cake, lactic acid and acetone, which are used by various industries such as textile, fermentation and food industries. The oil from the embryo is used in cooking oils, margarine and salad dressings. Maize serves a replacement for wheat flour to make corn bread and other baked products. Starch extracted from maize can also be made into plastic, fabrics and can appear in many other household items such as syrup, ice cream, ink, batteries, mustard, glue, shoe polish, cosmetics aspirin and many other chemical products. Maize cob can also be used as biomass fuel source. Maize is extensively used as feedstock for the production of ethanol fuel (Torres, 2016).

Rhizosphere refers to the environment or habitat in the soil around the root of plants; usually accommodating high microbial activity. Rhizosphere and plant root play a major role in soil physical, chemical and biological process. The soil microbiome is made up of a diversity of organisms, with bacteria, fungi and archaea being the most researched organisms in rhizosphere microbiology studies (Spence and Bais, 2013). Fungal and bacterial organisms having a symbiotic or saprophytic relationship with plants, can either be beneficial or harmful to plants. The microorganisms in the rhizosphere establish interaction with plant roots by inhabiting the soil around the roots. They carry out several biogeochemical transformations in the soil which promote plant growth by increasing the amount of plant nutrients available to plants. They are also known to produce plant growth

hormones and protect plants against pathogens. The activity of soil microorganisms is an important determinant of the environmental quality necessary for a sustainable and improved food production. Some of these organisms remain in the rhizosphere while others referred to as endophytes are able to penetrate plant tissues where they continue their lives (Brader *et al*., 2014; Mercado-Blanco, 2015). Some of these endophytes circumvent the immune system of plants and colonize the plants without causing symptoms of disease. Some other endophytes produce important secondary metabolites; affect plant responses to pathogens and herbivores, plant growth and environmental changes.

Most traditional and phenotypic methods used in microbiological laboratories for identification of microorganisms are time and material consuming. These methods are not always suitable to accurately identify microorganism and distinguish all the species belonging to a specific complex (Criseo *et al*., 2015). Identifying microorganisms by media culturing is not reliable as the problem of misinterpretation may arise. The fact that some species cannot be cultured in the laboratory is also a shortfall of traditional cultural method. To reduce the short-falls of identification of microorganisms using traditional cultural techniques, the use of molecular biology techniques comes into play; this may also be combined with various molecular fingerprinting techniques (Castro-Escarpulli *et al*., 2016). Identification of fungal species using modern molecular techniques such as amplification by Polymerase Chain Reaction (PCR), and sequencing has proven to be a more reliable alternative to traditional cultural methods. This study was carried out to determine the species identity of fungal organisms associated with maize rhizosphere using basic molecular techniques. The traditional cultural techniques used in the study comprised of isolation of fungi and preparation of pure cultures of fungi using serial dilution method, and potato dextrose agar medium respectively.

Materials and Methods

Study Area and Sample Collection

The study was conducted at the Regional Centre for Biotechnology and Bioresources Research Laboratory, University of Port Harcourt, Choba, Rivers State, Nigeria. The PCR products were sequenced at the International Institute of Tropical Agriculture (IITA), Ibadan. Soil samples from the rhizosphere of *Zea mays* planted at the Botanical Garden, University of Port Harcourt, were taken at different points and bulked to form a composite sample in April 2019.

Isolation of Fungi from Rhizosphere Soil

Fungi were isolated from the maize rhizosphere soil samples following serial dilution method. One gram (1g) of soil was added to 10ml of sterile normal saline. The sample was serially diluted up to 10^{-5}. 0.1ml of each of 10^{-2} and 10^{-4} dilutions, were plated out on sterile Petri dish plates containing sterile Potato Dextrose Agar (PDA), and incubated at room temperature (27$\pm$2°C) for 7days. After incubation, the isolated fungi were sub-cultured on PDA to obtain pure cultures of fungi. Occurrence of fungi for each isolate was determined.

Fungal DNA Extraction

Deoxyribonucleic acid (DNA) extraction was carried out using Quick-DNA Fungal/Bacterial MiniPrepKit (Zymo Research Group, California, USA), according to the manufacturers' protocols with some modifications. The mycelium of each fungal isolate was scrapped off from the surface of the growth media, frozen with liquid nitrogen and homogenized using bashing bead buffer in a sterilized mortar. The homogenized mixture was centrifuged in a 1.5 ml microcentrifuge tube at 10,000 x *g* for 1 minute. Lysis, precipitation, pre-washing, washing and elution of DNA were carried out following the protocol of the above-mentioned kit. NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, USA) was used for the determination of DNA concentration and purity. DNA purity was indicated by the ratio of Ultraviolet Light (UV) absorbance by DNA at 260nm to absorbance at 280nm. DNA quality was determined using 1% agarose gel through gel electrophoresis.

PCR Amplification and Sequencing

The primer pair; ITS4: TCCTCCGCTTATTGATATGS, forward and ITS5: GGAAGTAAAAGTCGTAACAAGG, reverse were used for the PCR amplification. The PCR cocktail mix consist of 2.5µl of 10 x PCR buffer, 1µl of 25mM $MgCl_2$, 1 µl each of forward primer and reverse primer, 1µl of DMSO, 2µl of 2.5mMDNTPs, 0.1µl of 5µ/µl Taq DNA polymerase, and 3µl of 10ng/µl DNA. The total reaction volume was made up to 25µl using 13.4µl nuclease free water. The PCR reaction mix was subjected to the following conditions: initial denaturation was at 94˚C for 5 mins, followed by 36 cycles of denaturation at 94˚C for 30sec, annealing at 54˚C for 30secs, and elongation at 72˚C for 45sec. These were followed by a final elongation step at 72˚C for 7 mins, and hold temperature at 10 ˚C. Amplified fragments were visualized on Safe view- stained 1.5% agarose electrophoresis gel. Amplified products were sequenced on ABI 3500 Genetic Analyzer (Thermo Fisher Scientific, Massachusetts, United States).

Phylogenetic Analysis

Sequences were edited on Molecular Evolutionary Genetics Analysis (MEGA) software, version X (Kumar *et al*., 2018) and blasted on NCBI database for identification of species. The ITS1-2 gene sequences of the isolates were aligned with closely-related sequences in GenBank and a neighbor-joining phylogenetic tree was constructed using maximum composite likelihood method.

Results and Discussion

This study was carried out to isolate the fungal organisms associated with the rhizosphere of *Zea mays* using traditional cultural techniques and characterize these organisms using basic molecular techniques. Isolation was carried out using serial dilution method while pure cultures of fungi were obtained using Potato dextrose agar method. Basic molecular techniques employed in the

study were: DNA extraction, gel electrophoresis, PCR and Sanger sequencing.

Fungi associated with Rhizophere of Zea mays

Two fungi were isolated from the rhizosphere of *Zea mays*. The pure cultures of isolates R1 and R2 are shown in Plate 1. The colonial characteristics of the two isolates are presented in Table 1. The frequency of occurrence of sample 2 was higher than that of sample 1 as shown in Table 1.

Table 1: Frequency of occurrence of fungi isolated from rhizosphere of *Zea mays*

Sample ID	Morphological description	Frequency of occurrence
R1	Yellow to dark brown spores surrounded by white spores	2.1
R2	Green spores	2.3

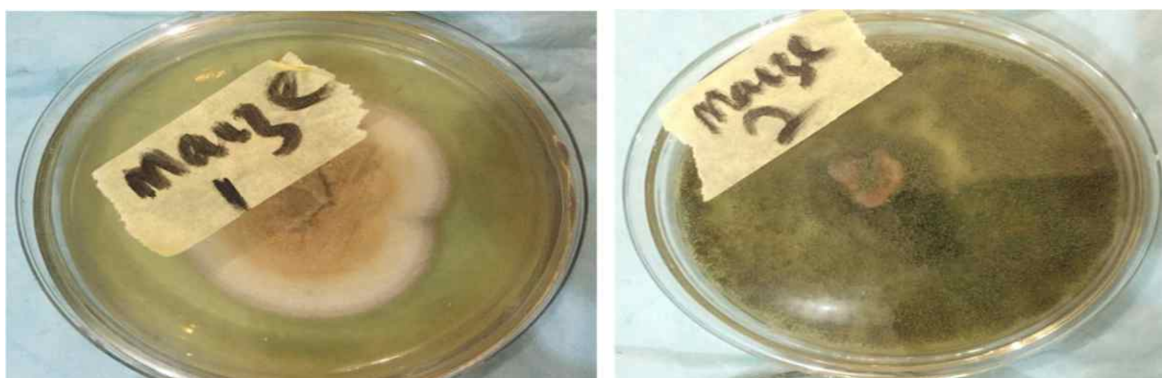

Plate 1: Pure cultures of fungal isolates from *Zea mays* rhizosphere

DNA Quantification and Gel Electrophoresis

The concentration of the DNA from the two isolates is presented in Table 2. The extracted DNA for isolates R1 and R2 showed clear bands on agarose gel when viewed under UV light as shown in Plate 2. This indicated that the DNA are of good quality.

Table 2: Concentration of genomic DNA obtained from fungal isolates of *Zea mays*

Sample ID	Nucleic acid conc. (ng/µl)	Absorbance at 260 nm/280 nm (Purity)
R1	45.6	1.85
R2	39.7	2.1

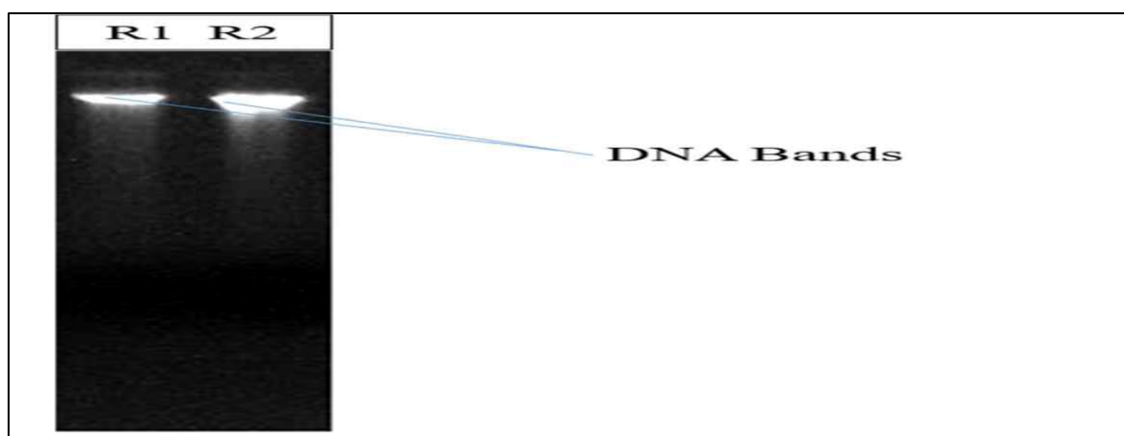

Plate 2: Gel electrophoresis of genomic DNA of fungal isolates

Polymerase Chain Reaction

PCR products viewed under UV light showed clear amplicons. The bands obtained from PCR amplification of the ITS gene are presented in Plate 3.

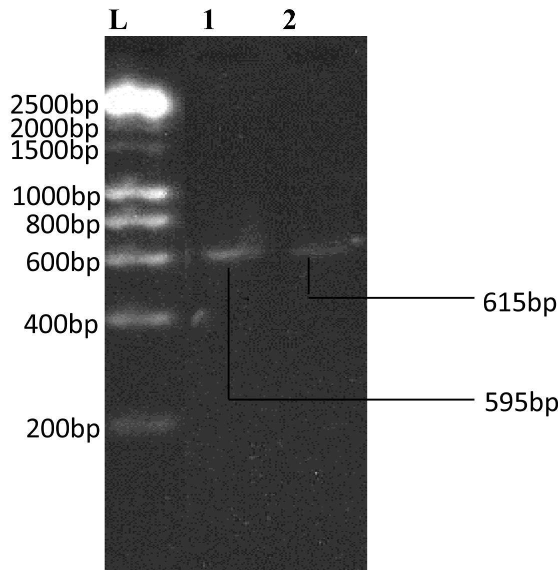

Plate 3: PCR products generated from amplification of ITS1-2 gene sequences of fungi M: 1 kb DNA Ladder; Lanes 2 and 3: PCR products for fungal samples R1 and R2

DNA Sequences and Phylogenetic Analysis of Isolates

After sequencing, the nucleotide lengths of the isolates were determined to be 595 and 615 base pairs for samples R1 and R2 respectively. The sequence alignments of the samples are shown in Plates 4 and 5 for samples 1 and 2 respectively. The DNA sequence of the ITS 1-2 gene obtained for each fungal isolate was more than 200 base pairs which is ideal for the identification of microorganisms.

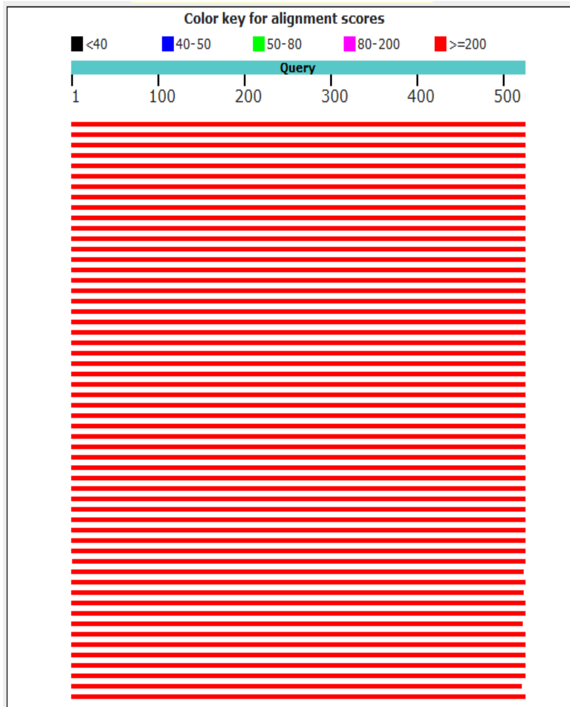

Plate 4: Sequence alignment of sample R1

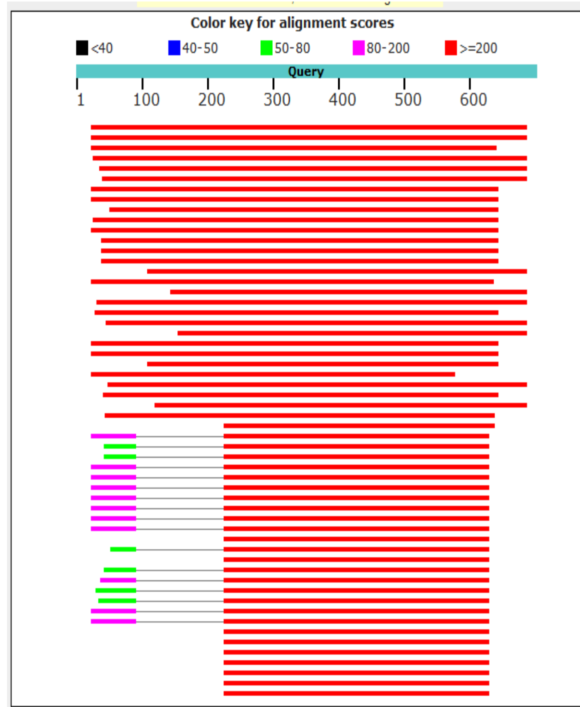

Plate 5: Sequence alignment of sample R2

The sequences were blasted on NCBI database and the identity of the isolates revealed to be *Aspergillus pseudoniums* and *Penicillium* sp. for samples R1 and R2 respectively. The percentage similarity and GenBank accession numbers of the hit search for each isolate are presented in Table 3.

Table 3: Putative taxonomic affinities of sequences types inferred from BLAST of ITS sequences of the isolates

Sample ID	Identity	GenBank accession No	Similarity (%)
R1	*Aspergillus pseudonomius*	MN187251.1	93.23
R2	*Penicillium* sp.	MN521825.1	80.91

The sequences were submitted on GenBank and accession numbers (in parenthesis) were assigned to the isolates as follows:

Sample 1: *Aspergillus pseudoniums* (MN626626) strain RCBBR_AEAPR5

Sample 2: *Penicillium* sp. (MT723948) strain RCBBR_AEAPR6

Phylogenetic Analysis

The neighbour-joining phylogenetic tree constructed showed the relationship between the isolates from this study and other fungal isolates on GenBank. The phylogenetic analysis showed that *Aspergillus nomius*, *Aspergillus zhaoquingensis*, *Penicillium sclerotiorum* and *Penicillium mallochii* were most closely related to the fungal isolates obtained from the rhizosphere of *Zea mays* as presented in Figure 1. The vertical lines on the trees indicate the difference between the branches. The greater the length of the vertical line, the more the difference between the branches.

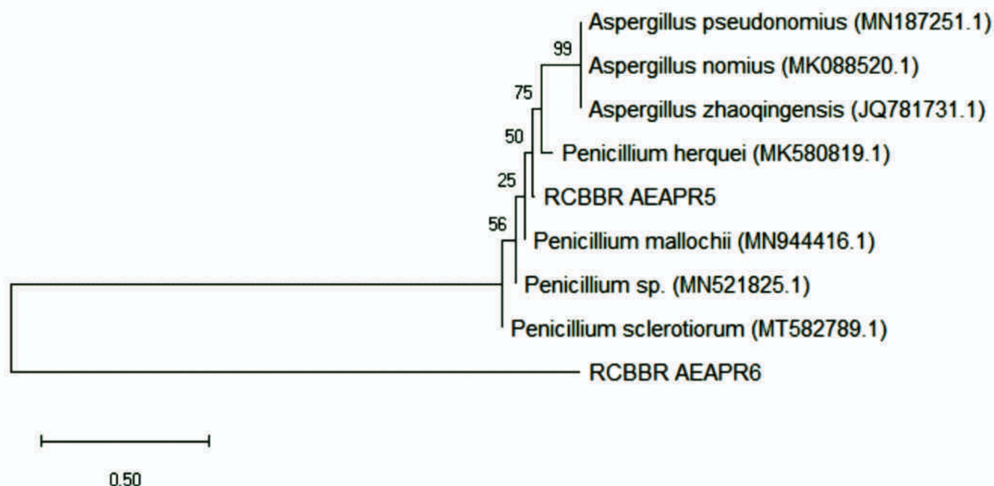

Figure 1: Neighbor-joining phylogenetic tree based on the sequenced ITS1-2 region of *Aspergillus pseudonomius* and *Penicillium* sp.

Polymerase chain reaction (PCR) of the internal transcribed spacer (ITS) region of the fungal genome and subsequent sequencing lead to the identification of the two isolates associated with the rhizosphere of *Zea mays* as *Aspergillus pseudonomius* and *Penicillium* sp. Both fungi obtained from this study belong to the division Ascomycota, class Eurotiomycetes, order Eurotiales and family Trichocomaceae. *Aspergillus* species are producers of mycotoxins. These mycotoxins are secondary metabolites which are harmful to animals and humans. The genus *Aspergillus* are used in oriental food fermentation, and as host for heterologous genes expression (Samson *et al*., 2014). They produce aflatoxins B_1, B_2, G_1, and G_2, ochratoxins and other mycotoxins (Frisvad *et al.*, 2019). *Aspergillus pseudonomius* was first isolated from insects and soil in the USA and has been reported to produce aflatoxin B_1, chrysogine and kojic acid (Varga *et al*., 2011). *A. pseudonomius* has also been reported to produce aflatoxin B_1, B_2, G_1, and G_2 (Palágyi *et al*., 2015). Brazil nut production is plagued by aflatoxins-producing fungi including *A. pseudonomius*. These nuts were infected by several toxigenic species than other nuts, and infections of Brazil nuts by *Aspergillus* species in the Flavi section can reach 100% (Calderari *et al*., 2013). *A. pseudonomius* has also been reported in a patient with fungal rhinosinusitis (Salah, *et al*., 2019). Agbetiameh *et al*. (2018) reported the incidence of aflatoxin contamination in maize and groundnut in Ghana caused by *Aspergillus flavus, A. parasiticus* and *A. tamarii* with *A. flavus* being the most prevalent. Other studies such as isolation of *Aspergillus flavus* from groundnut seed (Agbetiameh *et al*., 2019), reported that the species produced mycotoxins. *Aspergillus flavus* has also been reported on maize plants by Ortega-Beltran and Cotty (2018).

Several species of *Aspergillus* and *Penicilium* causing rot diseases was isolated from maize grains (Abe *et al.*, 2015).

Fusarium, Penicillium and *Aspergillus* species were the predominant organisms isolated from maize grains in South Africa (Ekwomadu *et al.*, 2018). Many *Penicillium* spp. have been recorded to cause postharvest fruit spoilage. *Penicillium expansum*, *P. crustosum* and *P. digitatum* have been reported to be pathogenic on apple fruits and; *P. brevicompactum* and. *P. solitum* on pear (Louw and Korsten. 2014). *Fusarium verticillioides*, *F.oxysporum*, *F. lateritium*, *F. flocciferum*, *Penicillium bilaiae*, *P. solitum*, *P. verrucosum*, *Aspergillus vesicolor*, *A. ustus*, *Cladosporium lunata*, *Trichoderma viride*, *T. harzianum*, *Curvularia lunata* and *Glioadium virens* have been reported to be associated with the rhizosphere of *Zea mays* in Turkey (Kucuk and Kyvanc, 2011).

The use of molecular techniques provides accurate identification of microorganisms unlike the cultural techniques which is based on the use of morphological and microscopic characteristics of the organisms. Many scientists in this part of the world still dwell on the use of cultural techniques in the identification of microorganisms and this can be misleading. The economic importance of the organisms isolated and identified in this study have been highlighted and this will give plant pathologists the insights required to proffer preventive and control measures towards reducing crop loss as a result of these pathogens.

Conclusion

Traditional method of identifying fungal organisms results in misinterpretation of the fungal community. Molecular characterization tools provide information concerning identification and characterization of unknown species; thereby, allowing the comparison of DNA sequences between known and unknown species. Molecular characterization is a reliable method because molecular data boost the explanation of phylogeny and provide relevant knowledge for understanding taxonomy and evolution of species. Accurate identification of microorganisms is pertinent in plant pathology as this determines the preventive or control measures to be employed. This study has given insight into some of the fungi that harbour the rhizosphere of *Zea mays*.

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