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Characterization of the Endophytic Mycobiome in Cowpea (*Vigna unguiculata*) from a Single Location Using Illumina Sequencing

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Abstract: Cowpea is an important crop for small-scale farmers in poor areas but is also being developed for commercial agriculture as a possible substitute for commercial legumes. Endophytic fungi are omnipresent and play crucial but diverse roles in plants. This study characterized the endophyte component of the cowpea mycobiome from leaves, main and crown stems and roots using Illumina MiSeq of the ITS2 region of the ribosomal operon. Ascomycetes exhibited the highest diversity, with Molecular Operational Taxonomic Units (MOTUs) assigned as *Macrophomina*, *Cladosporium*, *Phoma*, *Fusarium* and *Cryptococcus*, among the most dominant genera. Certain MOTUS showed preferential colonization patterns for above or below ground tissues. Several MOTU generic groups known to include phytopathogenic species were found, with relative abundances ranging from high to very low. Phylogenetic analyses of reads for some MOTUs showed that a level of identification could be obtained to species level. It also confirmed the absences of other species, including phytopathogens. This is the first study that adopted a holistic metagenomic typing approach to study the fungal endophytes of cowpea from a single location, a crop that is so integral for low-income households of the world.

Keywords: cowpea; endophyte; fungal diversity; environmental sequencing; phytobiome

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1. Introduction

Cowpea (*Vigna unguiculata*) is one of the most economically important indigenous African legume crops [1] and is cultivated in more than 60 countries of Asia, Oceania, the Middle East, southern Europe, Africa, southern United States of America and Central and South America [2]. Cowpeas are grown mostly for their edible beans, but the leaves, green seeds, dry seeds and pods can also be consumed. Cowpeas are usually cooked to make them edible, usually by boiling [3–5]. They are widely used as an inexpensive protein source in most rural and semi-urban areas and as animal feed and a cash crop [6,7]. Africa is a major area of production where the crop is crucial for low input agriculture that is the basic characteristic of most parts of the continent [8]. In South Africa, small-scale farmers form a large producer group of cowpea under dryland farming conditions [9], although there are no records with regard to the size of the areas under production and the quantities produced. Cowpea crop fixes 80% nitrogen for its growth demand from the atmosphere [10], thereby reducing nitrogen fertilizer demand and costs of its production. It is also an important companion crop in most cereal-legume cropping systems because of the benefit from the residual nitrogen originating from the decay of its leaf litter, roots and root nodules [11].

Cowpea yield can be low due to diseases such as damping-off [12] and stem rot which have been reported in many countries [13]. Damping-off and stem rot diseases are caused by many different species of fungi, including *Pythium aphanidermatum*, *Rhizoctonia solani*,

Phytophthora sp., *Fusarium solani* and *Sclerotium rolfsii* [14,15]. Another important disease is Fusarium wilt caused by *F. oxysporum* f. sp. *tracheiphilum* [16–18]. In Brazil, this disease is responsible for heavy losses in cowpea production, while in Nigeria and the United States of America, plant mortality can reach levels above 50% [18]. In South Africa *F. equiseti*, *F. graminearum*, *F. chlamydosporum*, *F. sambucinum* and *F. subglutinans* [19] have been isolated from cowpea. These *Fusarium* species were mycotoxin producers. Other diseases of cowpea in South Africa are Colletotrichum stem disease caused by *Colletotrichum dematium* [20]. *P. ultimum* [15] and *R. solani* [12] also have been consistently isolated from cowpea seedlings with symptoms of damping-off. Cercospora leaf spot is caused by *Mycosphaerella cruenta* [21] and *Alternaria cassiae* also affects leaves [22].

Cowpea seed have been reported to be susceptible to *Aspergillus* infection and to aflatoxin production [23]. *Fusarium* toxins such as fumonisin B1, B2 and B3 have a major effect on the health of farm animals and humans [24]. Indeed, the same toxins produced by *F. proliferatum*, *F. nygamaia* and *F. verticillioides* isolates in South Africa have a major toxicological significance in animal and human health [19]. Fumonisin B1 causes equine leukoencephalomalacia in horses and pulmonary edema in pigs. High incidence of human esophageal cancer in the Transkei region, Southern Africa has been associated with incidence of *F. verticillioides* infection on home grown maize [19].

Culture-based and environmental sequencing studies of surface sterilized stems and leaves have revealed an astounding diversity of fungal species existing sub-cuticularly or deeper within the tissues of healthy plants [25,26]. Such fungi are designated as endophytes, a term long used to indicate fungal residence within plant tissues rather than on plant surface asymptomatically without causing visible disease symptoms [27]. They can be obligatory or facultative bio-trophic and have a continuum of ecological functions from mutualists to saprophytes to latent pathogens [28]. In fact, a number of known pathogens have cryptic endophytic life stages, thus evading detection and complicating disease management programmes [28]. Others have been shown to improve plant health [29,30].

Phytobiome research of agricultural plants aims to maximize sustainable food production by generating, optimizing and translating knowledge of all factors influencing the plant into practice [31–36]. The last two decades of research have increasingly highlighted mechanisms of microbial facilitation of plant nutrient use. For example, nutrients that are not readily mineralized in the absence of the plant can be available to plant-microbe interactions, including widespread priming effects on C and N mineralization and solubilization of phosphate in the rhizosphere [32–34]. The first step in phytobiome research is often to characterize the microbes associated with the particular crop [35,36].

With the appearance of next generation sequencing (NGS), metagenomic surveillance approaches have revealed greater microbial diversity than culture-based methods and promoted phytobiome research [31,37]. Furthermore, these environmental sequencing approaches can detect microorganisms that could be missed in culture-dependent approaches and rare taxa [38]. When focusing on endophytes in cowpea plants, their identification and quantification can provide a foundation towards understanding the interactions between cowpea plants and endophytes, including pathogenic and beneficial species. Furthermore, the ability to detect pathogens and their relative abundance will aid studies in the epidemiology of pathogens and could benefit disease management and monitoring. Characterizing the endophytic communities in the entire plant, including above and below ground tissues, will lead to a more holistic approach to improve plant health.

Cowpea is one of the most important crops for livelihood in Africa, including South Africa. The present study aims to characterize the composition of the fungal endophyte communities associated with different above and below ground cowpea tissues with a NGS approach, in this case using an Illumina MiSeq platform. We specifically focused on three main questions: (i) What is the composition of the fungal endophytic communities found in cowpea from a single location in South Africa at a single time? (ii) What is the fungal endophyte composition in the different plant tissues of cowpea at a single time? (iii) Do the plant parts harbour any latent pathogens or potentially beneficial fungi? The study represents the first high throughput

sequencing based phytobiome characterization of cowpea not only in Africa, but also in the world, and will generate the first set of baseline knowledge of the fungal communities of cowpea plants in the field from this particular location. It also served to establish the technique and to illustrate its usefulness for the industry and growers in South Africa.

2. Materials and Methods

2.1. Field Sampling

Asymptomatic cowpea plants (6-weeks-old) were collected from a trial plot at the Small Grain Institute (Agricultural Research Council) in Potchefstroom, South Africa, in February 2016 (late summer). The plants were planted in three replicated blocks following standard cultivation processes, with the blocks randomly distributed between fallow plots and plots containing other crops, namely sorghum, soybean, dry bean and Bambara groundnut. The adjacent fields to the trial plot were sowed with sunflower and maize plants. From each block, five plants were randomly chosen and transported in a cool box to the laboratory of the Department of Genetics, University of the Free State, for further processing. The plants were not treated with fungicides at the time of sampling. All necessary permissions were obtained to sample and transport the plants in accordance with national and institutional regulations.

The roots, main stem, crown stem and leaves were the focus of this study. These plant parts were separated from the plants. Ten leaves from each plant were collected, cut into 1-cm-diam squares and 10 squares per leaf were randomly picked. The stems and roots per plant were cut into 1-cm-long pieces, and 10 random pieces were then selected. The plant material was placed in separate falcon tubes and surface sterilized in 3% sodium hypochlorite for 3 min, followed by rinsing in sterile distilled H_2O for 1 min, immersion in 70% ethanol for 2 min and a final rinse with sterile distilled water for 1 min. The plant material was freeze dried and pulverized in a Qiagen Tissue Lyser II cell disrupter (Whitehead Scientific, Cape Town, South Africa) for the environmental DNA extraction.

2.2. Illumina Sequencing of the Metagenomic DNA

The ribosomal RNA Internal Transcribed Spacer 2 (ITS2) region has been targeted because its minimum length invariability lacks the problem of co-amplification with the SSU intron, and it is better represented in the environmental sequence databases in comparison to ITS1 gene sequences [39]. Metagenomic DNA from 0.1 g of each pulverized plant sample was extracted using the Nucleospin® Plant II mini Kit (Macherey Nagel, Düren, Germany). DNA concentrations were determined using a Nanodrop LITE spectrophotometer (Thermo Scientific, Waltham, MA, USA) and diluted to a standard 10 ng/µL for Polymerase Chain Reactions (PCR). The Internal Transcribed Spacer 2 region of the ribosomal operon was amplified using the primers ITS3F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAG ACAGGCATCGATGAAGAACGCAGC3′) and ITS4R (5′-GTCTCGTGGGCTCGGAGATGT GTATAAGAGACAGTCCTCCGCTTATTGATATG-3′), with Illumina MiSeq (Sigma Aldrich, St. Louis, MO, USA) specific adaptor sequences in bold [40]. Reactions (25 µL) consisted of 20 ng of template DNA, 0.6 µM of each of the primers and 2U of HiFi Ready Mix DNA Polymerase (KAPA Biosystems, Lasec, SA, Cape Town, South Africa). The PCRs were performed in a G-Storm GS04822 thermal cycler (Somerton Biotechnology Centre, Bristol, UK) with parameters set to an initial denaturation at 95 °C for 3 min, followed by 25 cycles of denaturation at 98 °C for 20 s, annealing at 65 °C for 30 s and extension at 72 °C for 30 s. A final extension was performed at 72 °C for 5 min. The PCR products were visualized on 2% agarose gel electrophoresis supplemented with GelRed (Biotium, Inc., Separations, Johannesberg, South Africa) fluorescent nucleic acid dye.

The PCR amplicons were sent for sequencing library construction and subsequent sequencing at the Next Generation Sequencing Facility, Department of Health Sciences, University of the Free State, South Africa. The quality and quantity assessment of the PCR products were performed using a 2100 Bioanalyzer using a DNA 12,000 Chip (Agilent Technologies, Santa Clara, CA, USA). The amplified PCR products were gel purified using the Agencourt AMPure XP Bead Clean-up kit (Beckman Coulter, Atlanta, Georgia, United

States), normalized, pooled and denatured before being submitted to the MiSeq platform with paired 300 bp reads and MiSeq v3 reagents (Illumina Inc., San Diego, CA, USA).

2.3. Cluster Analysis

The obtained DNA sequence data were analysed using QIIME 1 as has been applied in a previous publication [41]. Briefly, before running the QIIME 1 pipeline, the quality of the sequencing was assessed and quality control performed using PrinSeq-lite v0.20.4 [42]. All datasets were pre-processed and trimmed to obtain an average quality score of ≥20 using a 5 nt window with a 3 nt step. All sequences shorter than 200 bp were filtered out and paired end reads merged using PEAR 0.9.6 [43]. The demultiplex and quality filtering script in QIIME 2 was run without any additional inputs to obtain a FASTA output file that could be analysed in the QIIME 2 pipeline. Chimeric sequences were identified, using usearch 6.1.544 as the chimera detection method [44], and filtered out of the quality trimmed reads by using identify_chimeric_seqs.py and filter_fasta.py commands, respectively, in QIIME 1. Fungal Principal Coordinate analysis (PCoA) was performed based on unweighted UniFrac distances.

Molecular Operational Taxonomic Unit (MOTU) was defined and tentative taxonomic names were assigned to representative MOTUs using the pick_open_reference_otus.py script. This was done at 99% sequence identity against the UNITE ITS reference database (alpha version 12_11) [45]. Fungal alpha diversities were analysed in QIIME 1 with alpha_rarefaction.py using the Shannon diversity metric. Beta-diversities were analysed using Principal Coordinates Analysis (PCoA) plots in R (www.r-project.org, accessed on 25 July 2021) using "plot_ordination" in the "Phyloseq" package using Bray–Curtis distance [46]. Prior to beta diversity analysis, the OTU-table was normalized using normalize_table.py in QIIME with the CSS normalization option [47]. The results for the PCoA and relative abundances were compared using 99% identity level. Sequence data were submitted to Genbank as BioProject ID PRJNA738463.

2.4. Phylogenetic Analysis

Phylogenetic analyses were carried out on sequence reads in order to refine the identities of selected representatives of genera that could be of possible importance as phytopathogens or possible biocontrol agents. Sequences obtained were queried using nucleotide BLAST searches against the National Center for Biotechnology Information (NCBI) database (Genbank). At least five representative sequences with a 99 to 100% level of similar identity and a high maximum coverage (higher than 95%) to respective query sequences were downloaded and aligned with the query sequence using the MAFFT server [48] and default parameters. In other cases, the query sequences were included in more comprehensive datasets for a genus, such as that of *Fusarium* [41]. The alignments were edited manually where needed. Maximum Likelihood (ML) phylogenetic analyses were performed with MEGA 7.2.2 [49] with a 1000 bootstrap replication performed to determine the support of branches [50]. Specific evolutionary models for the ML analyses were determined prior for each dataset using MEGA. Phylogenies were not produced for certain important genera, such as *Alternaria*, *Phoma* and *Cladosporium*, since it was previously shown that the ITS2 region, which is often used for Illumina deep sequencing, is not even adequate to distinguish the genera in these groups [41].

3. Results

3.1. Illumina Sequencing of the Metagenomic DNA

After quality checking and data filtering, a minimum of 11,417 to a maximum of 21,680 sequences were retained for the different plant parts (Table 1). Read lengths ranged from 200 to 251 bp. The sequences represented a minimum of 51 MOTUs to a maximum of 135 MOTUs for the different plant parts (Table 1), while there were 175 unique MOTUs. Richness and diversity were significantly greater in the roots while they were lowest in crown stem (Figure 1). Rarefaction curves showed that the sampling depth and sequencing coverage were adequate for all four plant parts (Supplementary Figure S1).

Table 1. Statistical summary of reads and Molecular Operational Taxonomic Units (MOTs) for the four cowpea plant tissues investigated.

Substrates	Total Number of Reads after QC	Total Number of MOTUs
Main stem	11,843	61
Crown stem	13,596	51
Leaves	11,417	77
Roots	21,680	135

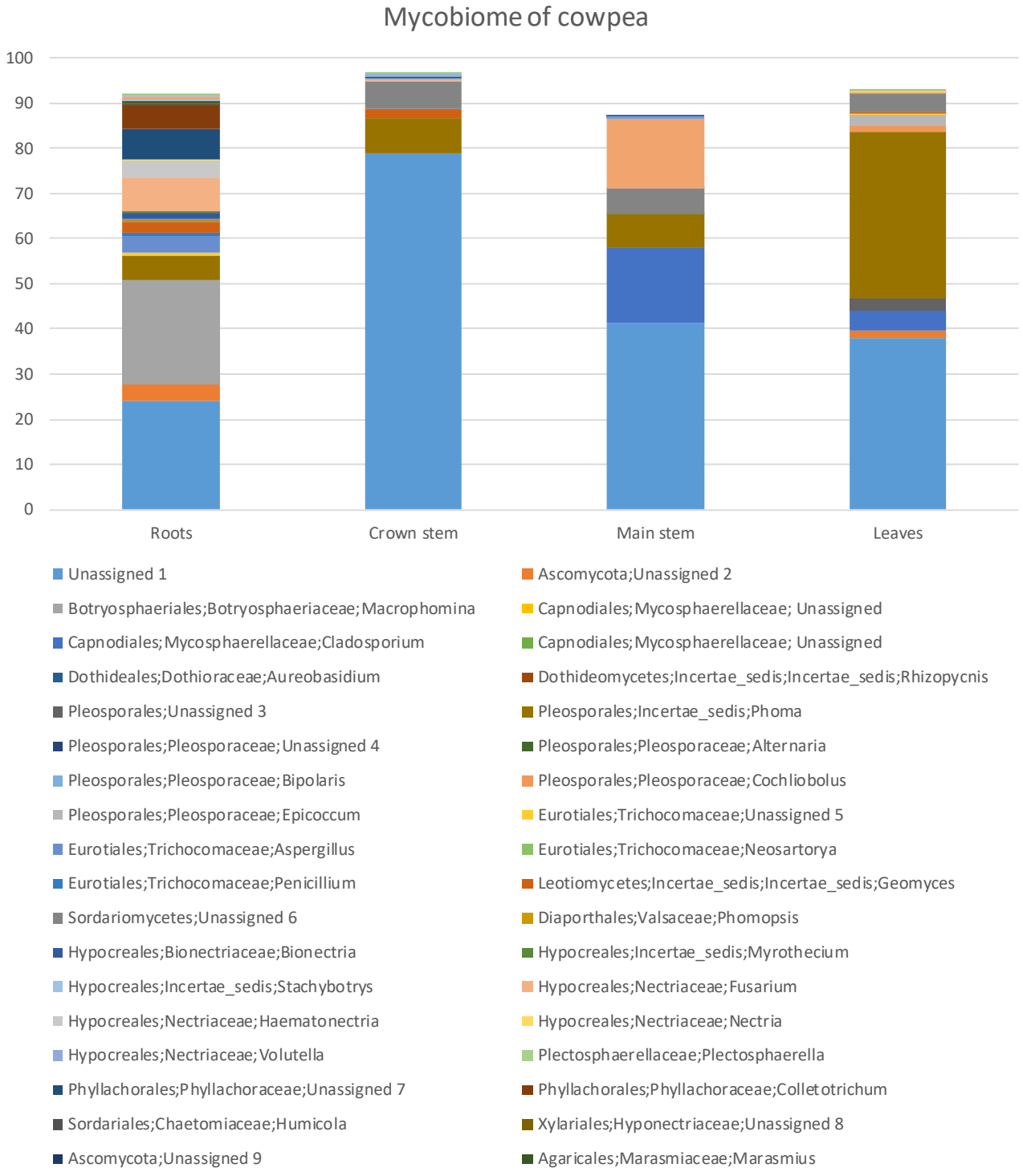

Figure 1. Percentage fungal relative abundance of Molecular Operational Taxonomic Units (MOTUs) up to genus level from roots, crown and main stem and leaves of cowpea at 99% level of sequence similarity.

3.2. Cluster Analysis

The percentage relative abundances (Supplementary Table S1) showed that the Ascomycota was highest in the roots with 58.74% and least in the crown stem with 13.45%. The Basidiomycota had the highest diversity in the main stem with 15.65% and the least diversity in the leaves where this phylum was absent (Supplementary Table S1). Overall, fungal diversity was highest in the roots followed by the leaves, main stem and the least was the crown stem (Figure 1). Some of the MOTUs were unidentified or unassigned.

In the Ascomycota (Supplementary Table S1), the Dothideomycetes were by far the most abundant with 45.14% in the leaves, followed with 29.14% in the roots, 23.92% in the main stem and 7.79% in the crown stem. The Dothideomycetes was represented by five genera across three orders and four families. The Eurotiomycetes was highest in the roots (4.32%) and least in the leaves (0.21%). The Eurotiomycetes was represented with three genera across one order and family. The Leotiomycetes was highest in the roots with 2.55% and least in the leaves with 0.21%, represented by one genus of uncertain position (Geomyces). The Sordariomycetes (22.73%) resided mostly in the roots and was made up of 11 genera, six orders and nine families.

MOTUs in the Basidiomycota (Supplementary Table S1) resided in the Agaricomycetes with two genera, two orders and two families. Tremellomycetes relative abundance was highest in the main stem (15.42%) and lowest in roots (0.92%) and was represented by one genus, order and family, namely *Cryptococcus*. The Ustilaginomycetes occurred in main stems (0.23%) and in roots (0.21%). They were represented by two genera from two orders and families. The Mucoromycota was highest in the roots with 0.35% and least in the leaves with 0.10% consisting of one genus, order and species, namely *Rhizopus*.

In total there were 43 genera (Supplementary Table S1). Roots had the highest number of MOTUs (22 MOTUs) and relative abundances (67.64%), followed by leaves (12, 51.06%). The most abundant genera (Figure 1, Supplementary Table S1) in the roots were MOTUs assigned as *Macrophomina* (22.86%), *Fusarium* (7.38%) and *Phoma* (5.68%) while in the crown stem, the most abundant MOTUs were assigned as *Phoma* (7.79%) and *Geomyces* (2.04%). In the main stem, the most abundant MOTUs were *Cladosporium* (16.45%), *Cryptococcus* (15.42%) and *Phoma* (7.47%). *Phoma* (37.02%), *Cladosporium* (4.43%) and *Epicoccum* (2.32%) were prominent in the leaves. A large proportion of MOTUs could not be satisfactorily assigned at order level or higher (Figure 1).

For the plant tissues (roots, main stem, crown stem, leaves) (Supplementary Table S1), eight genera assigned by the pipeline were dominant (having relative abundances higher than 2%). However, these genera showed varying patterns of colonization, especially with regards to presence below and above ground. *Macrophomina* (22.86%) was only found in the roots. *Aspergillus* was more present in the roots (3.83%) and less so in leaves (0.21%), similarly to *Colletotrichum* (4.90%) but with the difference that the latter co-occurred in the crown. *Cladosporium* was only found in above soil parts in this study, being dominant in the main stem at 16.45% but also present in leaves at 4.43%. A MOTU assigned as *Epicoccum* only occurred in leaves (2.32%).

MOTUs found in all of the tissues included *Phoma*, which were highly dominant in the cowpea plants, with relative abundances higher than 5% in all tissues but being most prominent in leaves (37.02%). *Geomyces* occurred below ground in roots (2.55%) and the crown (2.04%) but to a lesser degree in the main stem (0.23%) and leaves (0.21%). Similarly, *Fusarium* was more prominent in the roots (7.38% and 3.62% as *Haematonectria*) but present in the other plant parts to a lesser degree (0.37% in the crown, 0.11% in stems, 0.10% in leaves). Interestingly, the remaining MOTU assigned as *Cryptococcus* with relative abundances higher than 2% were dominant in the main stem (15.42%) and then only again found in the roots (0.92%).

MOTUs that represented possible phytopathogen groups were detected in the plant parts (Figure 1, Supplementary Table S1). MOTUs assigned as *Macrophomina*, *Cladosporium*, *Phoma*, *Epicoccum* and *Fusarium* that occurred relatively dominantly represented genera known to include plant pathogens [51,52]. Other potential pathogenic MOTUs occurring in

low relative abundances were *Cochliobolus*, *Thecaphora* and *Ustilago*. Some of these MOTUS are also known to include taxa known to cause diseases or mycotoxin issues of cowpea, such as *Rhizopus* [12], *Colletotrichum* [53] and *Fusarium* [19].

The PCoA analysis based on pairwise unweighted UniFrac distances (Figure 2) showed two principal coordinates explained at 86.4% of the variations (68.1% for PC1 and 18.3% for PC2, respectively). The analysis showed that the four plant parts orientated into two distinct groups. The first group was comprised of the above ground leaf and main stem samples, as well as the crown area just below soil level. Although these three groups did not cluster closely together, they were all positioned to the left of the plot. The second group was positioned to the right and represented the root samples.

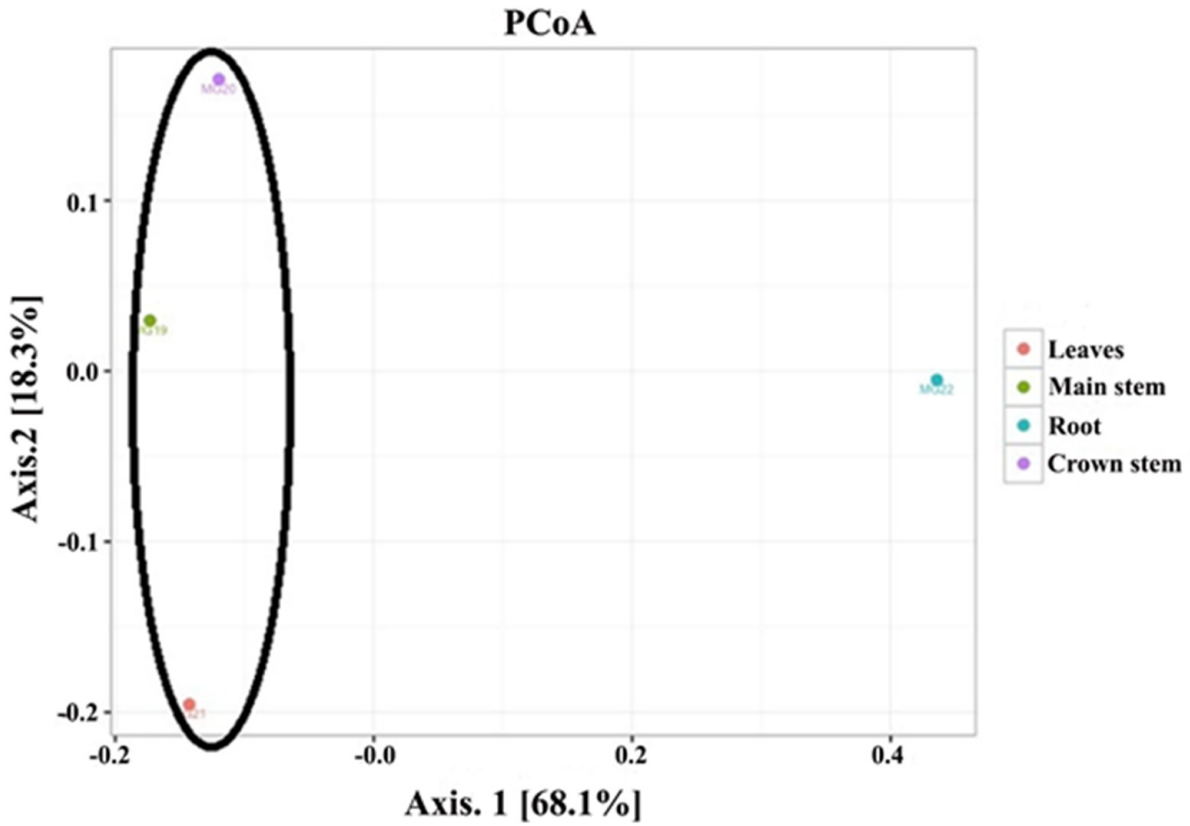

Figure 2. Principal coordinate analysis for the four plant tissues (roots, main stem, crown stem, leaves) of cowpea.

3.3. Phylogenetic Analysis

Phylogenetic analyses were quite informative in providing a more robust indication of the possible species or species complexes that selected MOTUs could represent. Eight representatives of the MOTUs assigned as *Fusarium* grouped in five species complexes in the dataset of *Fusarium* and other closely related genera (Figure 3). These included a MOTU from roots (MG22 5414) in the *F. chlamydosporum* species complex (FCSC), another (MG22 137) from roots in the *F. solani* species complex (FSSC), two MOTUs from roots (MG22 17908) and stems (MG19 2895), respectively, in the *F. oxysporum* species complex (FOSC) and another set of two from roots (MG22 2563) and stems (MG19 2852), respectively, in the *F. incarnatum-equiseti* species complex (FIESC). A MOTU from the crown (MG20 20923) grouped in *Bisifusarium*, while another from roots (MG22 20923) grouped separately from sequences of any *Fusarium* or closely related genus (Figure 3).

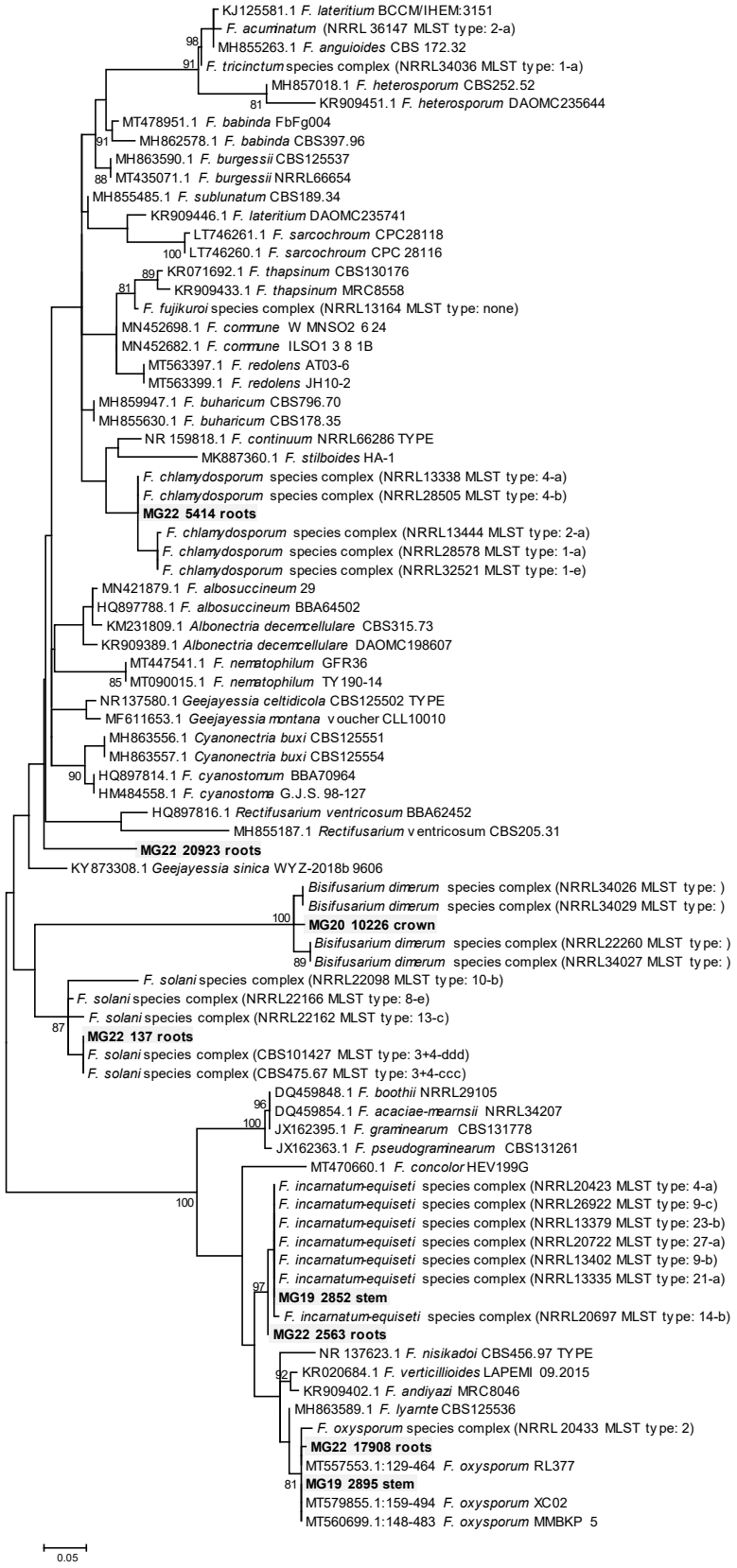

Figure 3. Phylogram of Molecular Operational Taxonomic Units assigned as *Fusarium*. The phylogram was generated with Maximum Likelihood analyses of the Internal Transcribed Spacer 2 region (used evolutionary model indicated). Confidence levels $\geq$ 80% (1000 replicate bootstrap analysis) are indicated on the branches. Sequence reads generated in this study are indicated with MG codes.

A MOTU from the main stem that was assigned in the Ustilaginomycetes (MG19 3597) grouped with *U. trichophora* with a 100% Bootstrap support (Figure 4A). A MOTU from the crown (MG20 5929) grouped with sequences of *R. solani*, with the previously used teleomorph name of *Thanetophorus cucumeris* still used for some of the deposited sequences (Figure 4B). Comparisons of a crown MOTU, namely MG20 2659, showed it to possibly represent *Plectosphaerella cucumerina* (Figure 5), while MG22 6502 from roots grouped in *Clonostachys* (synonym *Bionectria*) but its position could not be resolved. A root MOTU (MG22 17361) grouped together with *Colletotrichum coccodes* and *C. nigrum*, separate from the known cowpea pathogens *C. demiatum* and *C. truncatum* (Figure 5).

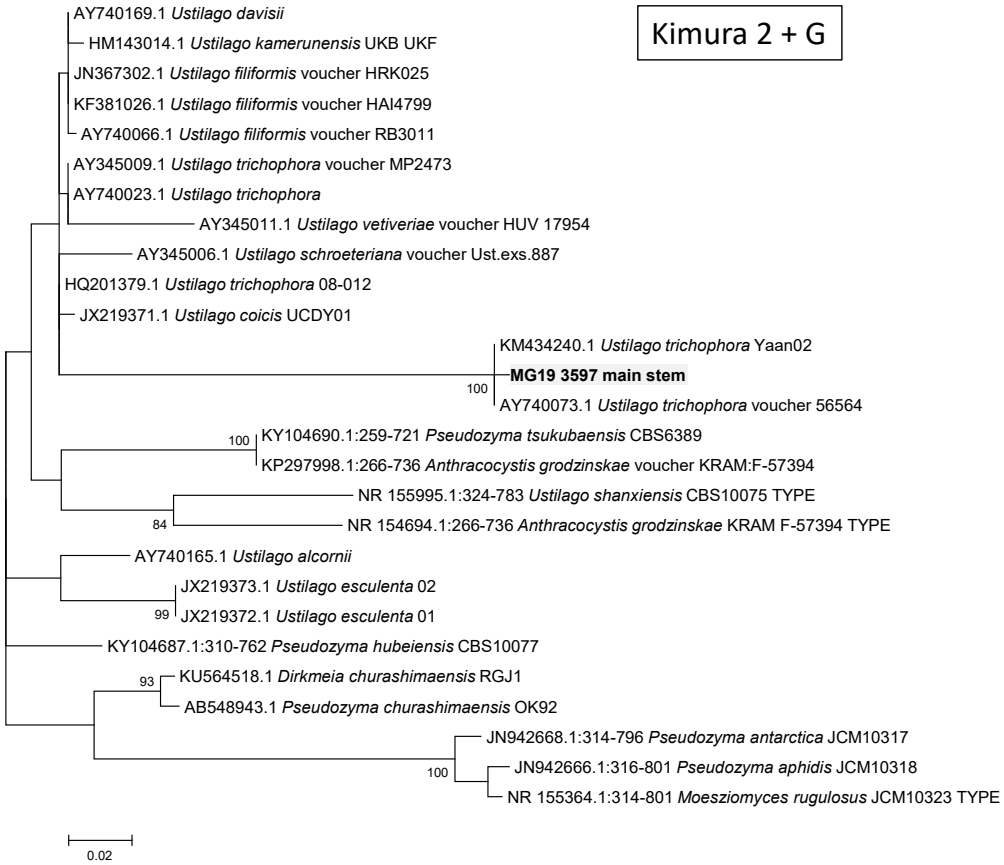

Figure 4. Phylograms of Molecular Operational Taxonomic Units. (**A**) *Ustilago*. (**B**) *Rhizoctonia*. The phylograms were generated with Maximum Likelihood analyses of the Internal Transcribed Spacer 2 region (used evolutionary model indicated). Confidence levels $\geq$ 80% (1000 replicate bootstrap analysis) are indicated on the branches. Sequence reads generated in this study are indicated with MG codes.

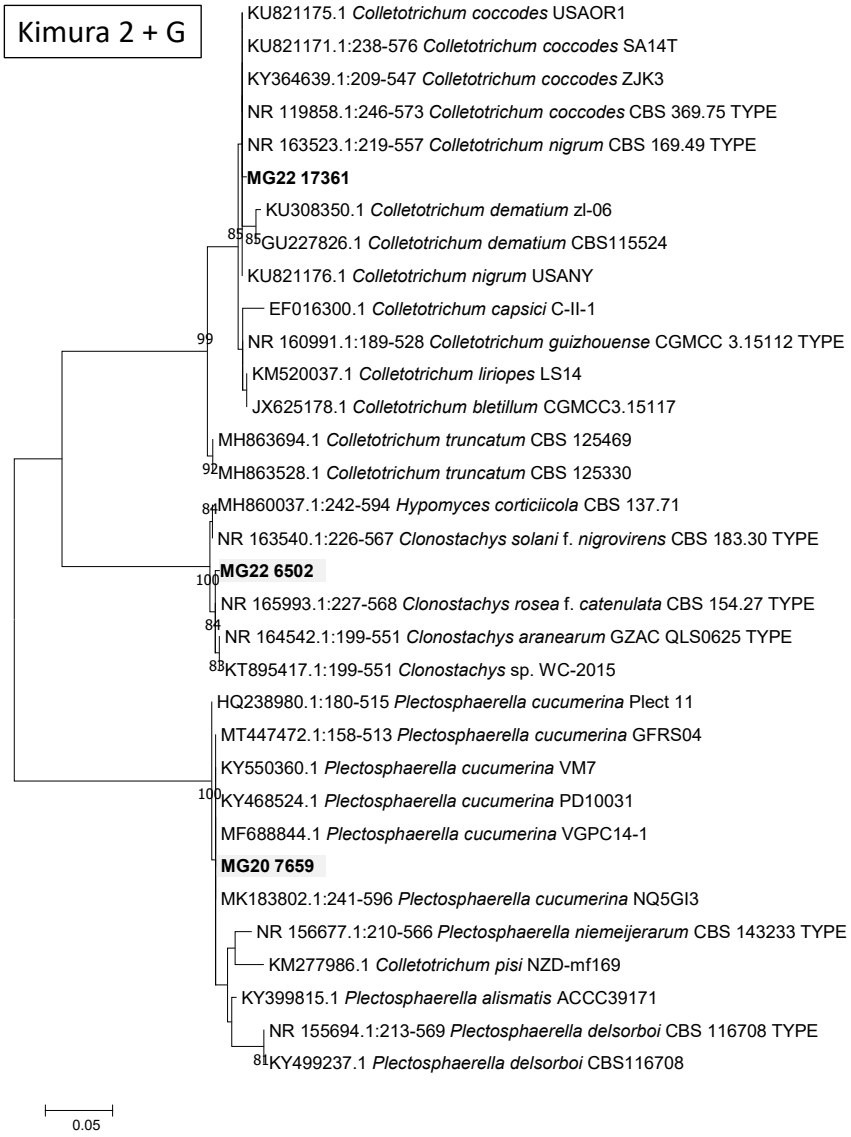

Figure 5. Phylograms of Molecular Operational Taxonomic Units. A *Ustilago* (A). B *Rhizoctonia*. The phylograms were generated with Maximum Likelihood analyses of the Internal Transcribed Spacer 2 region (used evolutionary model indicated). Confidence levels $\geq$ 80% (1000 replicate bootstrap analysis) are indicated on the branches. Sequence reads generated in this study are indicated with MG codes.

4. Discussion

Research and production of cowpea have largely been neglected in South Africa [9]. Although not as widely planted and commercialized as dry bean (*Phaseolus vulgaris*) or soybean (*Glycine max*), this crop has the potential to become an important substitute for other legumes or crops to improve food security, ensure production resilience and maintain the health of consumers. A number of prominent to rare MOTUs have been detected that are associated with cowpea at the trial site. Some of these showed possible colonization trends in specific plant tissues, while genera known to include phytopathogens were detected. This study represents the first of its kind on cowpea and is an important first step for such future studies towards crop improvement.

Results detected 43 genera from one location. Genera such as *Fusarium*, *Cladosporium*, *Macrophomina*, *Cryptococcus* and MOTUs assigned as *Phoma* in the Didymellaceae were among the dominant groups in this study. All of these prominent groups contain plant pathogens, except for *Cryptococcus* where some species have been shown to exhibit potential

biocontrol [54–58]. *Clonostachys* represented another genus known to include species used for biocontrol [59–62]. Other potential pathogen genera included *Plectosphaerella*, *Colletotrichum*, *Cochliobolus*, *Thecaphora*, *Ustilago* and *Rhizoctonia*.

Previous cultivation-based studies revealed that the fungal community associated with cowpea was dominated by three genera, namely *Fusarium*, *Cladosporium* and *Phoma* [12]. Other fungi, such as *S. rolfsii*, *P. ultimum*, *R. solani*, a *Rhizopus* sp. and *Trichoderma harzianum*, were present more rarely. The prevalence of *Fusarium*, *Cladosporium* and members of the Didymellaceae such as *Phoma* was confirmed in this study. *Cladosporium*, *Phoma* and *Fusarium* were also shared among the four plant parts. NGS based studies on other crops such as rice, sugarcane, wheat and *Arabidopsis thaliana* found more or less the same groups to occur as those found in this study [63–67].

In this study the fungal community from below ground roots grouped quite separately from those of the other tissues above ground including the crown area that is at the soil–air interface. Some MOTUs were only detected in the roots, such as *Macrophomina* and *Colletotrichum*. On the other hand, the *Cladosporium* and *Cryptococcus* only occurred above ground. Others were very dominant in roots and only present in above ground tissues at low relative abundances, such as *Fusarium*. *Phoma* had an interesting occurrence where it was dominantly present in all plant tissues (>5%) but had an exceedingly high relative abundance of 37% in the leaves.

A sense of colonization patterns of tissues and variation in prominence could be obtained with the Illumina sequencing. In this study results showed that only a small number of genera were prominently associated with cowpeas. Interestingly, the absence of others was also detected, such as the commonly occurring and cosmopolitan phytopathogen genus *Alternaria*. The approach could thus be useful to study community structure changes from a baseline when various agronomical effects are applied and management options against plant pathogens of cowpea are tested. However, more extensive environmental sequencing studies, as well as confirmation based on isolates, are needed to confirm observed patterns.

Some of the groups detected are known to include phytopathogen species of cowpea, as well as other plants. This is despite the fact that only healthy cowpea plants were used, indicating that these fungi could be potential pathogens [68,69]. *Fusarium* species have previously been associated with disease symptoms on cowpea, including South Africa [12]. Species in the *F. oxysporum*, *F. chlamydosporum*, *F. incarnatum-equiseti* and *F. solani* species complexes were possibly detected in this study, which include known pathogens and mycotoxin producers [70]. Similarly, members of the *Phoma* and *Epicoccum* groups in the Didymellaceae [71,72], and *Cladosporium* (Cladosporiaceae) [73] include known pathogens, including on legumes [74]. Other interesting pathogens detected include smuts possibly representing *Thecaphora* that include pathogens of potato [75], peanut [76], rhubarbs [77] and *U. trichophora*, which is a pathogen of rice, and *Echinochloa crus-galli* [78]. *Plec. cucumerina* is a pathogen of horticultural crops [79] and leafy vegetable crops [80]. *Colletotrichum* is possibly related to *C. nigrum* and *C. coccodes*, which are both phytopathogens of peppers and tomato [54]. The important cowpea pathogens *C. dematium* [81] and *C. truncatum* [82] were not detected.

The NGS approach detected interesting MOTUs from the healthy cowpea plants, some at low relative abundances. For example, *U. trichophora* was detected in cowpea stem, the first report in cowpea from South Africa. However, the biological significance of this finding is unclear and in vivo assays may be required to validate it. Even though other approaches would be needed to confirm the presence of this species, phylogenetic placing of these MOTUs derived from partial ITS data was used, which also represents the first report from South Africa.

Although phylogenies generated in this study from the ITS2 region generated by Illumina sequencing are limited because they only represent a portion of the ITS region and the ITS region does not always distinguish between species of certain genera [83], valuable information could in some cases be gained. It was shown that MOTUs assigned as *Fusarium*

or initial names consisted of more than one species complex. The identities of others could be ascertained to some degree, for example, those of *R. solani* and *Plec. cucumerina*. The presence of known pathogens of cowpea could be ruled out, even if other members of the genus were present. For example, although the identity of the *Colletotrichum* could not be confirmed with certainty, it was clear that it did not represent the previously reported pathogens *C. truncatum* and *C. demiatum*. This approach has also been used in a previous study to determine species identities or absences more accurately [41].

Our description of the mycobiome associated with parts of cowpea provides an interesting baseline for cowpea grown in the Potchefstroom area that can be used for further monitoring to improve risk assessments and crop improvement for this crop. Future studies with adequate samples and results for robust statistical analyses can build further on this study. Sound knowledge on the pathogens that threaten this crop is still largely lacking, even though these pathogens threaten cowpea production, which forms such an integral part of the livelihoods of many. Knowing how to improve growth and yield or how this crop improves growth of other crops and assessing the potential threat of mycotoxins will aid numerous human communities, especially those that are poor and heavily depend on the products of this crop. It is essential to gain knowledge of the cowpea core mycobiome to proceed to studies on the functions of these fungi [35,36,66,84] and how these fungal communities may change in the plants due to various biotic and abiotic changes [85]. Such an approach can be used to develop targeted control strategies that are focused on managing the most prevalent phytopathogens in a given region. Future analyses with additional biogeographical datasets of cowpea mycoflora will help to identify whether or not the core mycobiome ascribed to cowpea in this study will be similar elsewhere and what the sources are. The occurrence of important mycotoxins can also be studied. Future work on expanded biogeographical regions will help to provide such answers and build a more complete baseline on the fungi associated with cowpea.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12030333/s1, Figure S1: Rarefaction curves for the four cowpea plant tissues (roots, main stem, crown stem, leaves); Table S1: Relative abundances of MOTUs (differentiated at 99% similarity) from the different plant parts of cowpea, excluding unassigned or unknown taxa that could not be placed in a family. Values in bold are higher than 2%, while values of 0% were omitted. Values at phylum, class, order and family level represented totals of the relative abundances of the generic names.

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