Tropical Plant Pathology
https://doi.org/10.1007/s40858-021-00422-8

ORIGINAL ARTICLE

Root and crown rot pathogens found on dry beans grown in Mozambique

Suzanna Fernandes[1,2] · Graciela Godoy-Lutz[1,3] · Celestina Jochua[1,2] · Carlos Urrea[4] · Kent Eskridge[5] · James R. Steadman[1] · Joshua R. Herr[1,6,7]

Received: 16 March 2019 / Accepted: 16 February 2021

Abstract

Dry edible beans are a vital food source in Mozambique, East Africa—one that alleviates hunger and malnutrition and adds value to the economy. In recent years, root/crown rot (RCR) pathogens have emerged as limiting constraints in dry bean production. Not much has been characterized concerning the causal agents of RCR in Mozambique. The purpose of this study was to identify the primary pathogen(s) associated with RCR dry bean samples collected at breeder nursery sites and farmer fields in Mozambique using molecular sequencing and culture-based methods. Sequencing revealed, not surprisingly, an increased diversity of fungal/oomycete operational taxonomic units when compared to culture-based methods oof diversity. Species of *Fusarium*, mainly *F. oxysporum*, were the dominant taxa detected in RCR dry beans through sequencing the ITS rDNA region and partial EF-1α gene. Collectively, 333 fungi and/or Oomycetes were isolated in culture during the 2014–2015 growing seasons and tested for pathogenicity on healthy bean seedlings. *Fusarium* species were identified by both morphological and molecular characters. At least 60% of the isolates inoculated on common bean were recognized as potentially pathogenic. From both isolation frequency and pathogenicity testing, *F. oxysporum* and related species play an important role in the bean RCR complex. We found similar results from dry beans grown in the two main bean-growing regions of Mozambique. These findings will allow breeders to screen for resistance to *F. oxysporum* in greenhouse grown bean plants as well as within field grown bean cultivars.

Keywords Soil-borne fungal pathogens · *Fusarium* · FTA® Cards · High-throughput sequencing · Bean root and crown rot · *Macrophomina* · *Fusarium oxysporum*

Introduction

Dry edible beans (*Phaseolus vulgaris L.*), with a yearly production harvest area of 100,000 ha, are one of the most important food sources in Mozambique (Wortmann et al. 1998). Beans provide an important source of dietary protein, micro-nutrients, and caloric intake for the people of Mozambique, as well as a crucial source of income for the

Suzanna Fernandes and Graciela Godoy-Lutz contributed equally to this work.

✉ Joshua R. Herr
jherr@unl.edu

[1] Department of Plant Pathology, University of Nebraska, Lincoln, NE 68583, USA

[2] Present address: Directorate of Agronomy and Natural Resources (DARN), The Agricultural Research Institute of Mozambique (IIAM), Maputo, Mozambique

[3] Present address: Instituto Dominicano de Investigaciones Agropecurarias y Forestales (IDIAF), Santo Domingo, Dominican Republic

[4] Department of Agronomy & Horticulture, University of Nebraska, Lincoln, NE 68583, USA

[5] Department of Statistics, University of Nebraska, Lincoln, NE 68583, USA

[6] Center for Plant Science Innovation, University of Nebraska, Lincoln, NE 68588, USA

[7] Genetics, Cell, and Molecular Biology Graduate Program, School of Biological Sciences, University of Nebraska, Lincoln, NE 68588, USA

Published online: 25 March 2021

small-scale farmers (Wortmann et al. 1998). Mozambique, which is located on the southeastern coast of Africa, has many challenges for bean production, due in large part to climate variation through cyclical drought and excessive rainfall and plant diseases are exacerbated these changes in climate (Walker et al. 2006). Consequently, diseases such as root/crown rot (RCR) are emerging as limiting factors in bean production enhanced by changing environmental conditions (Farrow et al. 2011; Bodah 2017).

Bean RCR comprises a syndrome attributed to a suite of soil-borne pathogens. These pathogens may be reported either as individual strains or synergistic infections whose occurrence and severity varies by location, site-specific incidences, soil properties and nutrients, and/or other variable environmental conditions that contribute to disease propagation and spread (Rusuku et al. 1997; Wortmann et al. 1998; Farrow et al. 2011). Most species of soil-borne pathogens have been identified as causal agents using traditional culture methods and pathogenicity tests. The most relevant species reported in Mozambique are *Fusarium solani* (Mart.) Appel, Wollenv. *F. sp. phaseoli* (Burk.) Synd. & Hans, *F. oxysporum* Schlecht. *F. sp. phaseoli* Kendrick & Synder, *Pythium spp*., *Rhizoctonia solani* Kühn (teleomorph: *Thanatephorus cucumeris* (Tassi) Goid), *Athelia rolfsii* (Curzi) C.C. Tu & Kimbr., and *Macrophomina phaseolina* (Tassi) Goid (Abawi 1989; Rusuku et al. 1997; Paparu et al. 2018). Recently, in eastern and southern Africa, species of *Pythium* have received more attention due to their higher prevalence over other pathogens in disease surveys (Nzungize et al. 2011a, Nzungize et al. 2011b). As a result, breeding programs in some countries have focused on only developing RCR resistance for *Pythium* species.

In Mozambique, there is limited information on the pathogenic species causing, or associated with, bean RCR symptoms. This is despite widespread disease occurrence in the Gurue and Chokwe provinces where beans are most consistently grown. The proper identification of the primary causal pathogen(s) can lead to better disease management strategies and may improve bean production due to the fact that yield loss has not been accurately calculated for each pathogen. Morphological features of pathogens cultured from infected plants provide important information for identification in the laboratory (Narayanasamy 2011), and live pathogenic specimens are vital for disease-resistance inoculations and greenhouse evaluations in breeding programs. In addition to traditional morphological characters, molecular methods, such as high-throughput sequencing, have become commonplace in pathogen detection and species identification (Herr et al. 2015; Hibbett et al. 2016). In the present study, we employed a suite of morphological and molecular identification techniques to identify the diversity of the predominant pathogen (or pathogens) in Mozambique that are associated with bean RCR. We additionally focused on the population structure of *Fusarium oxysporum* isolates recovered from the same bean RCR samples.

Materials and methods

Sample collection and processing

We took advantage of collecting plants from a field test of four Andean Diversity Panels for Bean (Cichy et al. 2015). These panels were situated where root rot screening has already been in effect for at least a decade. The survey locations in the present study included the R10 (North West) region, which has the greatest levels of bean production, and is fed by rainfall; the R3 (South West) region where production is under dry winter irrigation nurseries; and lastly, from two farmer fields: R3 (Chokwe) and R10 (Gurue), respectively, from the Gaza and Zambesia provinces in well-studied agro-ecological zones (Wortmann et al. 1998). The sampling locations in 2014 and 2015 represented contrasting climatic and soil conditions (Fig. 1 and Table S-3). The essential differences of these conditions consisted the sampling locations of Chokwe, where the site is close to sea level, had organic matter–rich Salic Fluvisol soils and recognized as a semi-arid climate, and the sampling locations of Gurue, which is approximately 700 m above sea level, and consist of Ferrossols with less organic matter, and a slightly cooler climate due to the elevation gain of slightly more than 100 m.

Within 30 days after bean plant emergence, we identified and sampled 88 bean plants with RCR symptoms (Fig. 1) along with a healthy control plant in close proximity, typically no more than 1 m away. Plants were harvested in the field, placed in a cooler with ice, and brought to the laboratory for processing. Soil and associated debris were removed from the plants and lesions (approximately 2–4 mm in size) at both the diseased and healthy interface (Fig. 2a) were extracted according to published protocols (Mukuma et al. 2020). Lesions of infected tissue were isolated, flash-frozen, and ground with a micro-pestle (Fig. 2b). Liquid extracts of the lesions were spotted onto the absorbent matrix of Whatman FTA® Cards and matching tissue samples were placed in a coin envelope and labeled with the sample number, bean cultivar name, description of phenotypic symptoms, the location and dates of sampling, and any other field-related data taken during collection, such as unique climate and precipitation notes.

Fungal/Oomycete cultural isolation

Small segments of 2–4 mm of the RCR diseased plant tissue were surface sterilized by immersing in 10% v/v NaOCI/water for 15–30 s, then transferring to 70% alcohol for 15–30 s, and finally to a distilled water wash for 1–2 min. The infected segments of plant tissue were then blotted on sterile

Fig. 1 (a) Bean field affected by root/crown rot (RCR) in Chokwe, Mozambique. (b) Inset photo of row of RCR and wilt of bean plants. (c) Topographical map of Mozambique with specific location on the continent of Africa and the general locations that were sampled in this study

Whatman™ filter paper to air dry, and then transferred using sterile forceps onto 2% water agar where segments reached about 4 mm in length. After this growth period, the resulting isolate hyphae was transferred onto potato dextrose agar (PDA) to allow further growth. Within 2–4 days, 4-mm mycelial plugs were transferred to four PDA plates and one water

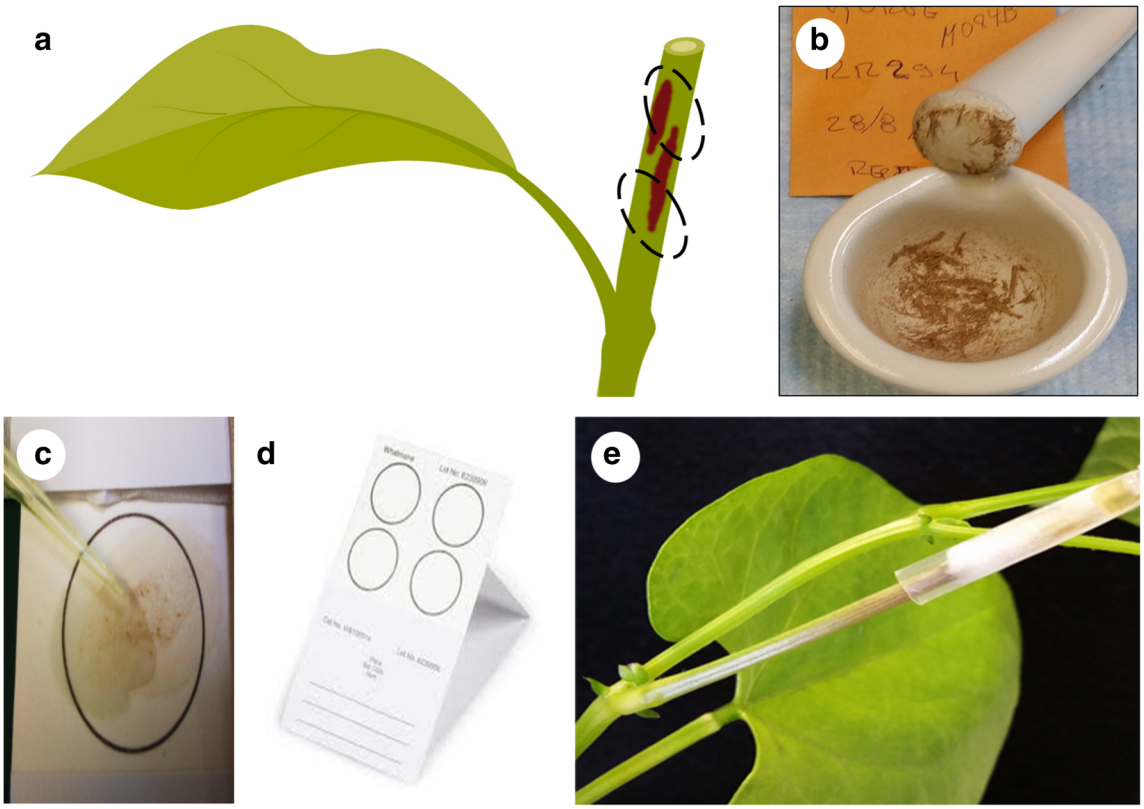


Fig. 2 (a) Schematic representation of typical bean plant showing infection site and location of where tissues were sampled. (b) Ground RCR tissue for DNA extraction. (c) Tissue extracts blotted onto FTA Card. (d) Entire FTA ® Card shipped to laboratory. (e) Photo of the pathogenicity test by the "straw method" conducted in the greenhouse

agar (WA) plate for each isolate. These plates were used for morphological observations, future pathogenicity tests, and mycelial DNA extraction for subsequent nucleotide sequencing. Culture characteristics were examined across three different culture media: WA, PDA, and carnation leaf agar (CLA) (Fisher et al. 1982: Leslie and Summerell, 2006). The characteristics observed were growth pattern, colony texture and pigmentation, spore size and shape, and growth rate of the mycelial edge (Fig. 3). Mycelial plugs of a 6-mm diameter were taken from the WA colony edge of each isolate and transferred to PDA plates and incubated under continuous darkness for 9 days at 22–25 °C. Colony diameter was measured on the bottom of the plate. Colony texture and color on PDA were evaluated for each isolate. For fungi and Oomycetes, the genus and species names were assigned using several identification keys (Dhingra and Sinclair 1978; Dugan 2006; Watanabe 2010). Species of *Fusarium* were identified based on the color and growth pattern of colonies grown on PDA, then transferred to CLA for identification using dichotomous keys (Burgess et al. 1994; Leslie and Summerell 2006) focusing on characters such as the size and morphology of phialides, macro- and micro-conidia, and chlamydospores. *Fusarium* isolates grown on CLA media at 20–24 °C with a 12-h light/12-h dark regime for 10 days sporulated on CLA, and spore masses growing on the leaf surface were transferred to a blue drop of lactophenol cotton blue on a glass slide to observe spore size and shape. Diagnostic characters were then photographed using a compound light microscope mounted with a Motic camera (Motic North America, British Columbia, Canada) at × 40 magnification.

DNA extraction from FTA® Cards and plant tissue

In order to extract DNA from field collected samples, the Whatman FTA® Cards and matching tissue samples were sent to the laboratory located at the Department of Plant Pathology at the University of Nebraska-Lincoln, NE, USA, under the USDA-APHIS Permit P526P-17-02138 issued to J.R. Steadman. Samples waiting to be processed were kept in a desiccant chamber at room temperature. Due to a small number of samples from the Whatman FTA® Cards that we were not able to acquire enough DNA for sequencing, as well as the overall proximity and similarities of some of the sampling locations, we evaluated all the data from the closely located geographic locations Chato and Chissano (analyzed further as the Gurue (R10) growing region) along with the larger number of samples from Chokwe (R3) growing region (Fig. 1).

Genomic DNA from the FTA® Cards was recovered from an excised 1-cm^2 section of the Card by incubation in 200–300 μl of TRIS-EDTA buffer solution (Fluka Analytical, Sigma-Aldrich Co., St. Louis, MO, USA) at 4 °C overnight. After this incubation, 100 μl of the buffer solution was used for DNA extraction with the PowerClean Pro DNA Cleanup Kit (Catalog No.12997-50 MO BIO Laboratories, Inc., Carlsbad, CA, USA). Genomic DNA was extracted from 100 mg of cryogenic ground RCR diseased plant tissue or isolate mycelia using the PowerPlant Pro DNA isolation kit (Catalog No. 13400-50, MO BIO Laboratories Inc., Carlsbad, CA, USA). Concentration and purity of total DNA from each sample was determined using a DeNovix DS-11 Nanodrop

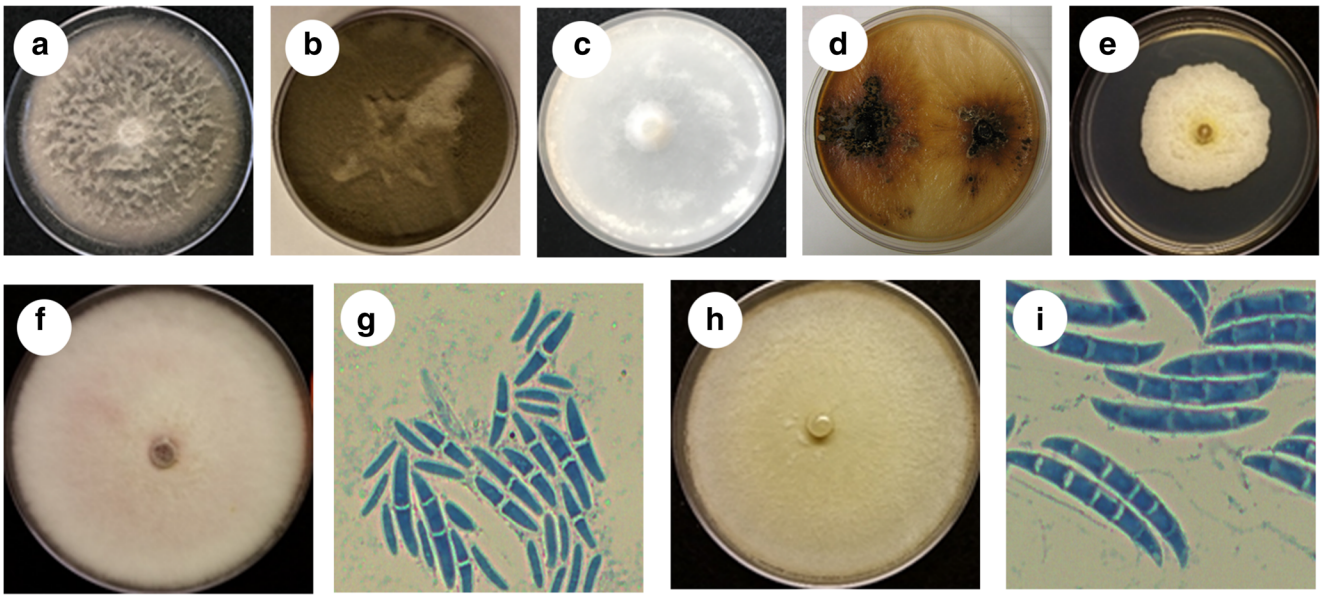


Fig. 3 Photos of Fungi and Oomycetes isolated in this study: (a) *Macrophomina phaseolina*; (b) *Alternaria alternate*; (c) *Pythium ultimum*; (d) *Rhizoctonia solani*; (e) *Fusarium equiseti*; (f) *Fusarium oxysporum*; (g) spores of *Fusarium oxysporum* stained with lactophenol cotton blue; (h) *Fusarium solani*; (i) spores of *Fusarium solani* stained with lactophenol cotton blue

Spectrophotometer (DeNovix Inc., Wilmington, DE). Genomic DNA from the extraction process was stored at −20 °C until sequencing.

PCR amplification and DNA sequencing

Taxonomic identification of the fungal/Oomycete isolates from cultural methods was initially based on morphology, but we subsequently used DNA from each isolate amplified by polymerase chain reaction (PCR) with primer pairs ITS4/ITS5 (White et al. 1990) to verify our identification. To aid in the identification of *Fusarium* species, we sequenced a second marker region, the partial EF-1α gene using primer pairs EF1/EF2 (O'Donnell et al. 1998). We identified four main pathogen groups associated with diseased RCR plant tissue from sequencing of both the DNA from the FTA® Cards and/or plant tissue extracts. To verify these species complexes, we utilized isolate-specific markers for these pathogens using the following specific primer pairs (Table S-4): FM66/58 COX II for *Pythium* species (Martin 2000); ITSFu F/ITSFu R for *Fusariu*m species (Abd-Elsalam et al. 2003); RS4 primers for/RS4 primers for *Rhizoctonia solani* (Guillemaut et al. 2003) and MpkF1/MpkR1 primers for *Macrophomina phaseolina* (Babu et al. 2007). Isolates of *Macrophomina phaseolina*, *Pythium ultimum*, *Rhizoctonia solani* strain AG-4, and *F. oxysporum* collected from field grown dry beans from Scottsbluff, NE, USA, were used throughout our tests for PCR quality control. For each amplification, 25 μl of PCR reaction mixture was prepared by adding 1 μl of genomic DNA to 24 μl of a master mix/tube containing 9.5 μl PCR grade sterile ddH_2O, 12.5 μl Econotaq® PLUS GREEN 2× Master mix (Lucigen, Madison, WI, USA), and using 1 μl of 0.2 mM/μl of each forward and reverse primers. Reactions were performed in a PTC-100 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). Amplification fragments were separated by electrophoresis in a 1.5% Ultra-Pure® and Quick Dissolve agarose (Invitrogen, Carlsbad, CA, USA) in TBE buffer and stained with ethidium bromide for visualization under a UV light using a ChemiDoc EQ System with the software package Quantity One (Bio-Rad Laboratories, CA, USA). Amplified fragments for Sanger sequencing were purified with Ultra-Clean PCR Clean-Up Kit (Cat. No. 12500-50 MO BIO Laboratories, Inc., Carlsbad, CA, USA), and sequenced on an ABI 3730XL Sanger Sequencer by ACGT, Inc. (Wheeling, IL, USA).

DNA extracted from the FTA® Cards of field infected RCR plants was isolated with the goal of using both sequence identification methods and phylogenetic analysis of the fungal/Oomycete composition in the plant lesions (Herr et al. 2015; Hibbett et al. 2016). Sequencing was conducted at Molecular Research LP (www.mrdnalab.com—Shallowater, TX, USA) on an Illumina MiSeq (Ilumina, Inc., San Diego, CA, USA) using PCR primers Euk SSU euk7F/euk570R amplifying the 18S rRNA gene V4 variable region. Sequence data was processed using a taxonomic analysis pipeline (Chiodini et al. 2016) where the raw data set was demultiplexed and barcodes and primers were trimmed from the sequences. The sequence data was subsequently processed by removing sequences <200 bp, as well as reads with homopolymer runs exceeding 10 bp and ambiguous base calls. Sequencing chimeras were detected and removed, and operational taxonomic units (OTUs) were generated by centroid clustering at 3% divergence (using vsearch at 97% similarity; Rognes et al. 2016) followed by removal of true singleton sequences for downstream analysis (Chiodini et al. 2016). Final OTUs were identified using BLASTn against the curated GreenGenes (DeSantis et al. 2006, McDonald et al. 2012), RDPII (Cole et al. 2005), and Silva (Quast et al. 2012) databases, respectively.

Pathogenicity assay

Pathogenicity assays were conducted using 333 fungal and/or Oomycete isolates recovered from the RCR diseased plant tissue. Two-week-old susceptible PINTO 114 bean seedlings, grown in the greenhouse using 6 × 4 cm plastic pots containing steam pasteurized sterilized soil mix, were inoculated using a modified straw test method (Mukuma et al. 2020). Each inoculation was replicated five times and plants were kept in a mist chamber set for humidity ≥ 80% and temperatures approximating 23 °C. Control plants were inoculated with clean PDA plugs containing no visible organismal growth. After 48 h, all plants were moved to greenhouse benches and arranged in a complete randomized block design. Lesion length for each plant was measured and recorded 48 h after removal from the mist chamber (Fig. 2e). Lesion data were analyzed using Statistical Analysis System (SAS) v 9.2 (SAS Institute, Inc. Cary, NY, USA). Mean comparisons were made using Fisher's protected least significance difference at $P = 0.05$. To fulfill Koch's postulates, the fungi were re-isolated from the infected petiole, grown on WA, and subsequently transferred to PDA (as previously described) to re-validate cultural and morphological features.

PCR fingerprinting of *Fusarium oxysporum* with microsatellite markers

The genetic diversity of *F. oxysporum* isolates recovered from RCR diseased plant tissue was determined using PCR-fingerprinting analysis with four types of microsatellites. Previously utilized for *Fusarium* taxa (Kang et al. 2002), the universal rice primer (URP) (5′ CCCAGCAACTGATC GCACAC 3′) was used to amplify *Fusarium* isolates and microsatellites with the repeated motifs $(GTG)_5$ (Lieckfeldt et al. 1993; Brasileiro et al. 2004), $(ACG)_5$, and $(AGG)_5$ (Bahkali et al. 2012). Two primers, the (ACG)s and (AGG)s

motifs, were chosen from a set of 3 URPs, because they yielded the most consistent and unambiguous fragments and amplified most of the *Fusarium* isolates we obtained. DNA samples from 93 *F. oxysporum* isolates (72 from Gurue and 21 from Chokwe) were amplified with the primers following published protocols (Table S-4). The PCR amplification reactions were prepared for each of the primers and band visualization was done as previously described. Band size and number in the gel were determined in the Band Analysis tools of ImageLab software, version 4.1 (Bio-Rad, Carlsbad, CA, USA). After being scored, the resulting data set was analyzed using the nei.dist() function within Poppr (Nei 1973, Kamvar et al. 2014) using R (R Core Team 2018).

Clear and unambiguous bands, amplified by the microsatellite markers, were selected and scored for presence (1) or absence (0) of the corresponding band, which ranged from 300 to 1000 bp. A nonclone-corrected binary data matrix was computed with Genalex v. 6.502 (Peakall and Smouse 2006) and SAS v. 9.2 (SAS Institute, Inc. Cary, NY, USA) utilizing morphological characteristics from the original isolate used in the inoculation assay.

Data visualization and analysis

Sequence chromatograms of ITS rDNA and the partial EF-1α genes were visualized with Chromat version 2.6.4 (Technelysium Pty., Australia) and after inspection, the resulting FASTA files were exported for further analysis. To infer the identification of the isolates at putative species level, the ITS rDNA sequences were identified using the NCBI BLAST web interface (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against the NCBI GenBank nt database. Identification of the partial EF-1α gene to the closest matching *Fusarium* species was conducted by BLAST analysis against the *Fusarium* EF blast cyber-Infrastructure website (http://fusariumdb.org/index.php) available through the Pennsylvania State University, University Park, PA, USA (Park, et al. 2010). For phylogenetic analysis and placement, the FASTA files of the partial EF-1α gene sequences were subjected to multiple sequence alignment (MSA) using MUSCLE (Edgar 2004) and the resulting alignment was used for the phylogenetic tree construction. Phylogenic analysis was conducted using Bayesian inference with the BEAST pipeline (Drummond and Rambaut 2007). Sequences of *F. oxysporum* from various *F. oxysporum forma specialis phaseoli* accessions from NCBI GenBank showing 99 to 95% synteny were selected as outgroups and to add resolution beyond those sequences collected for this study. The resultant tree was visualized using iTOL (Interactive Tree of Life—http://itol.embl.de/; Letunic and Bork 2016).

The overall abundance of taxa among the samples from the different sampling locations and years were estimated from the identification of OTUs from the Illumina MiSeq sequencing. Heat maps were constructed in R (R Core Team 2018) using the ggplot2 (Wickham 2016) and RColorBrewer packages (Neuwirth, 2014), based on the percent of relative abundance obtained from Illumina MiSeq 18S sequencing for FTA® Cards and RCR diseased plant tissue. Sequences with reads depth less than 10, which we considered putative sequencing artifacts, were removed from further analysis. The overall sample diversity, which was measured as Shannon (Shannon and Wiener, 1963) and Simpson (Simpson 1949) indices, was calculated with the PAST (PAleontological STastics) v 3.12 program (Hammer et al. 2001). Venn diagrams, constructed with Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/), were drawn to display similarities (shared reads) and distinctiveness (unique reads) among *F. oxysporum* for the Gurue and Chokwe locations based on Illumina MiSeq 18S and EF-1α sequence data. Sequencing data from the MiSeq run was used to validate the culture-based and PCR amplified methods for detecting the four main RCR pathogens we identified in this field study. This analysis was conducted by Spearman's rank and rho correlation which was also calculated within the PAST program.

The frequency of the four main fungal/Oomycete genera and species (*Fusarium* spp., *Macrophomina phaseolina*, *Rhizoctonia solani* and P*ythium* spp.) was generated by PCR amplification with specific primers. A binary matrix was constructed with the color-coded data set for each as presence (1) and absence (0) of the expected fragment. Tables were constructed for each location per year.

Results

Cultural and morphological characterization of isolates obtained from RCE tissue

We obtained 333 fungal and Oomycete isolates in culture, as examined by macro- and microscopic characteristics, from 79% of RCR tissue samples collected in Chokwe and Gurue in 2014 and 2015. Most of the isolates were initially assigned at the genus or species level, based on their characteristic mycelial and spore features, although some isolates did not sporulate or had no diagnostic cultural characteristics. Isolates of *Alternaria* spp., *Fusarium* spp., *Pythium* spp., *M. phaseolina*, and *R. solani* were significantly more abundant than other taxa in the samples. Among the identified isolates, *Pythium* spp. had the fastest growth rate and *M. phaseolina* the slowest. The morphology and size of both phialides and spores, based on growth characters from CLA plates, were used to tentatively identify the *Fusarium* species into 3 morphotypes and/or species complexes: *F. solani*, *F. oxysporum*, and *F. equiseti* (Fig. 3), prior to DNA sequencing analysis.

Identification of cultured isolates

We identified the taxa of cultured isolates of fungi and Oomycetes through DNA sequencing and subsequently compared the recovery of the isolates from Whatman FTA® Cards and RCR tissue. Nucleotide sequence data were identified to closest match using BLAST queries with a cut-off value >98% match and a resultant 42 distinct fungal taxa were identified (Table S-5). *Fusarium oxysporum* was by far the most common genus and species (Figs. 4, 5 and 6). In comparison, the most frequently isolated genus and species in both locations were *F. oxysporum* (34%) *F. equiseti* (13%), *F. solani* (10%), *A. alternata* (9%), *M. phaseolina* (4%), and *R. solani* (2%) (Fig. 4).

Pathogenicity assay

The 333 isolates recovered from RCR plant tissues were tested for pathogenicity and 204 were identified as inducing pathogenic-like responses in bean assays (Table S-5) in the greenhouse. We used the criteria of water-soaking followed by necrosis or wilting of the stem of 14-day-old Pinto 114 bean plants observed 3 days after inoculation as exhibiting traits of pathogen damage. Mean lesions ranged from 0.4 to 8 cm in length. The most common isolate recovered from our pathogenicity tests was *Fusarium oxysporum*, followed by *F. equiseti* and *F. solani* from both locations of farm fields, with the exception of samples from Chokwe 2015, where *Macrophomina phaseolina* was the most virulent isolate we encountered.

Detection of four primary pathogenic isolates associated with RCR

Microsatellite amplicon sequences from the infected samples and positive controls were consistent with the expected fragment sizes for *Fusarium* spp., *Pythium* spp., *Rhizoctonia solani*, and *Macrophomina phaseolina*. With the primers we utilized, the presence or absence of each genus and/or species per sample was detected (Fig. 7). The frequency of detection varied with both location and year; however, *Fusarium* spp. were detected in highest frequencies and in RCR tissue more than in Whatman FTA® Cards (Fig. 7).

When Illumina sequencing was used for DNA analysis from both FTA® Cards and RCR tissue, not surprisingly, we identified a greater depth of species of fungi/Oomycetes

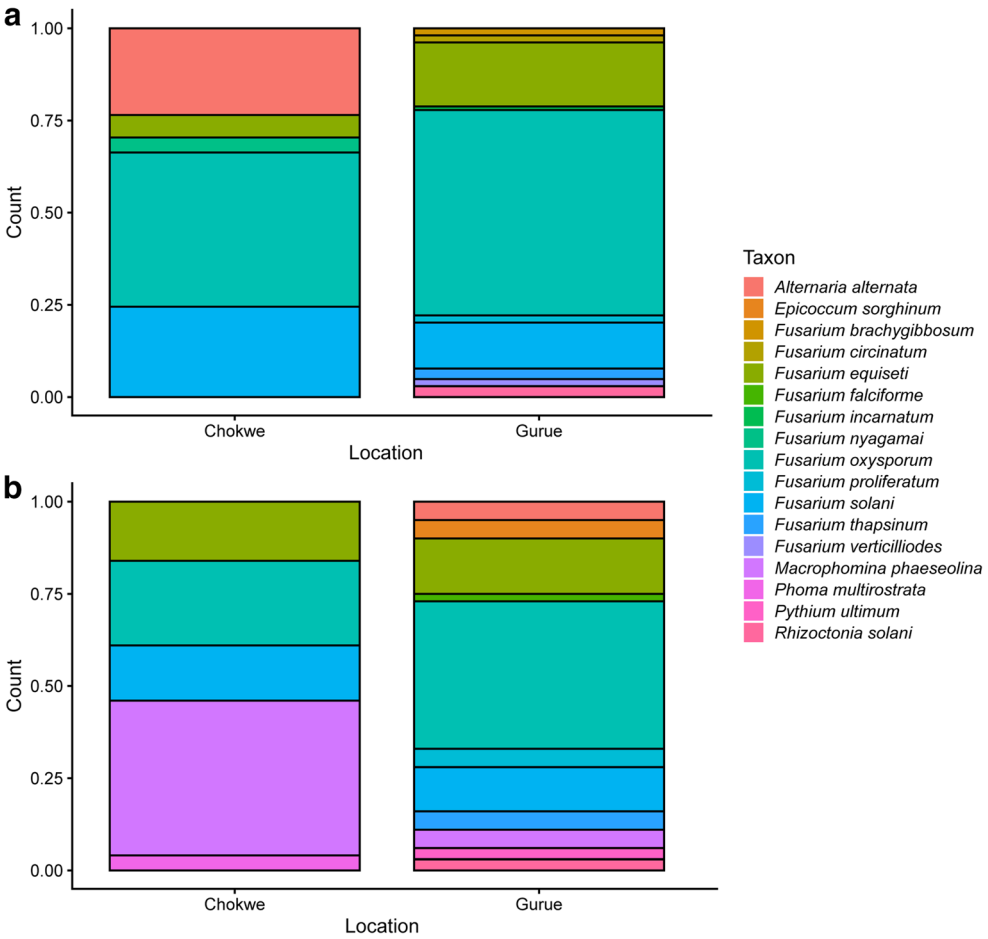
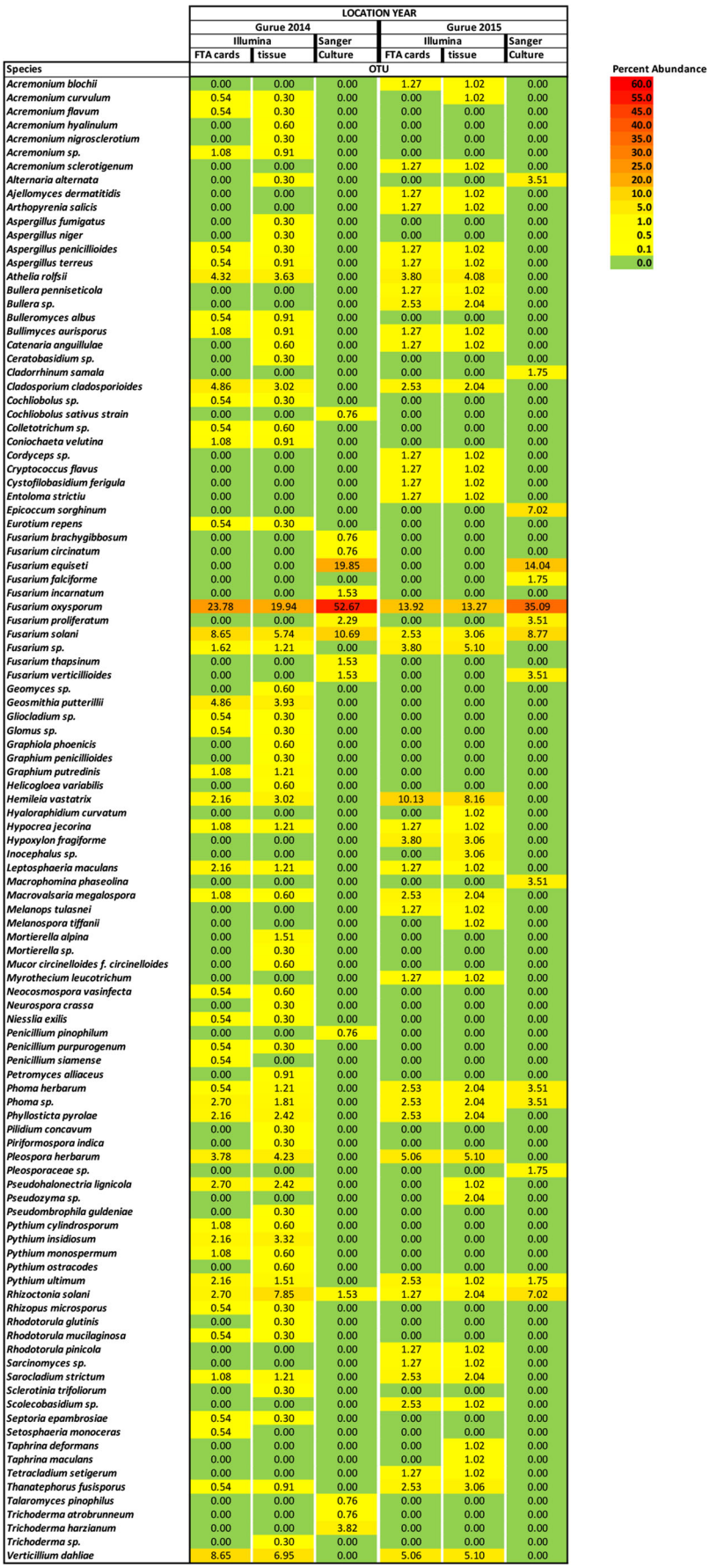


Fig. 4 Relative abundance of the 17 most prevalent identified pathogenic fungi and Oomycetes isolated from isolated RCR bean tissue through 18S rDNA Illumina sequencing for years (a) 2014 and (b) 2015, by location of pooled Chokwe and pooled Gurue growing regions

Fig. 5 Heat map of normalized relative abundance of the top 92 fungal operational taxonomic units associated to RCR-symptomatic beans based on 18S rDNA region Illumina sequencing from the pooled Chokwe growing regions in 2014 and 2015

	LOCATION YEAR					
	Chokwe 2014			Chokwe 2015		
	Illumina		Sanger	Illumina		Sanger
	FTA cards	tissue	Culture	FTA cards	tissue	Culture
Species	OTU					
Acremonium curvulum	0.53	0.31	0.00	0.00	0.51	0.00
Acremonium flavum	0.53	0.31	0.00	0.66	0.51	0.00
Acremonium hyalinulum	0.00	0.62	0.00	0.66	0.51	0.00
Acremonium radiatum	0.53	0.31	0.00	0.00	0.00	0.00
Acremonium sp.	1.60	0.93	0.00	0.00	0.00	0.00
Alternaria alternata	0.00	0.31	26.14	0.00	0.00	0.00
Alternaria arborescens	0.00	0.00	3.41	0.00	0.00	0.00
Alternaria macrospora	0.00	0.00	1.14	0.00	0.00	0.00
Alternaria sp.	0.00	0.00	1.14	0.00	0.00	0.00
Amylomyces rouxii	0.00	0.00	0.00	1.99	2.55	7.84
Aspergillus calidoustus	0.00	0.00	0.00	0.00	0.00	1.96
Aspergillus fumigatus	0.53	0.31	0.00	0.00	0.51	0.00
Aspergillus niger	0.53	0.00	0.00	0.00	0.00	0.00
Aspergillus penicillioides	0.53	0.31	0.00	0.66	0.00	0.00
Aspergillus terreus	0.00	0.62	0.00	0.00	0.00	0.00
Athelia rolfsii	4.26	2.17	0.00	7.28	6.12	0.00
Bulleromyces albus	1.06	0.93	0.00	0.66	1.02	0.00
Bullimyces aurisporus	0.53	0.31	0.00	0.66	0.51	0.00
Candida ishiwadae	0.53	0.00	0.00	0.00	0.00	0.00
Catenaria anguillulae	0.00	0.62	0.00	0.00	0.00	0.00
Ceratobasidium sp.	0.00	0.31	0.00	0.00	0.00	0.00
Chaetomium fanicola	0.00	0.00	1.14	0.00	0.00	0.00
Chaetomium globosum	0.00	0.00	2.27	0.00	0.00	0.00
Chaetomium sp.	0.00	0.00	0.00	0.00	0.00	3.92
Cladosporium halotolerans	0.00	0.00	1.14	0.00	0.00	0.00
Cladosporium sp.	0.00	0.00	2.27	0.00	0.00	0.00
Cladosporium ternuissimun	0.00	0.00	1.14	0.00	0.00	0.00
Cladosporium cladosporioides	4.79	3.10	1.14	3.97	3.57	0.00
Cochliobolus sp.	0.53	0.31	0.00	0.00	0.51	0.00
Colletotrichum sp.	0.00	0.31	0.00	0.00	0.00	0.00
Coniochaeta velutina	1.60	0.93	0.00	0.66	1.02	0.00
Curvularia hawaiiensis	0.00	0.00	0.00	0.00	0.00	1.96
Curvularia lunata	0.00	0.00	3.41	0.00	0.00	0.00
Epicocum nigrum	0.00	0.00	6.82	0.00	0.00	0.00
Eurotium repens	0.53	0.31	0.00	0.66	0.51	0.00
Fusarium equiseti	0.00	0.00	3.41	0.00	0.00	9.80
Fusarium nyagamai	0.00	0.00	1.14	0.00	0.00	0.00
Fusarium oxysporum	20.21	19.50	21.59	16.56	18.37	11.76
Fusarium solani	6.91	5.26	10.23	6.62	6.12	7.84
Fusarium sp.	1.06	0.93	0.00	0.00	1.02	0.00
Fusarium thapsinum	0.00	0.00	4.55	0.00	0.00	0.00
Fusarium verticillioides	0.00	0.00	1.14	0.00	0.00	0.00
Geomyces sp.	0.00	0.62	0.00	0.00	0.00	0.00
Geosmithia putterillii	3.19	3.41	0.00	2.65	2.55	0.00
Gliocladium sp.	0.53	0.31	0.00	0.66	0.51	0.00
Glomus sp.	0.53	0.31	0.00	0.00	0.00	0.00
Graphiola phoenicis	0.00	0.31	0.00	0.00	0.00	0.00
Graphium penicillioides	0.00	0.31	0.00	0.00	0.00	0.00
Graphium putredinis	1.60	0.62	0.00	2.65	1.53	0.00
Helicogloea variabilis	0.53	0.62	0.00	0.66	0.51	0.00
Hemileia vastatrix	3.19	4.02	0.00	7.28	4.08	0.00
Hypocrea jecorina	0.53	1.24	0.00	0.00	0.00	0.00
Leptosphaeria maculans	1.06	1.55	0.00	1.32	2.55	0.00
Macrophomina phaseolina	0.00	0.00	0.00	0.00	0.00	21.57
Macrovalsaria megalospora	1.06	0.93	0.00	1.32	2.04	0.00
Mortierella alpina	0.00	2.48	0.00	1.99	0.51	0.00
Mucor circinelloides f. circinelloides	1.06	0.93	1.14	0.00	0.00	0.00
Neocosmospora vasinfecta	0.53	0.31	0.00	0.66	0.51	0.00
Neurospora crassa	0.00	0.31	0.00	0.66	0.00	0.00
Niesslia exilis	0.53	0.31	0.00	0.66	0.51	0.00
Penicillium purpurogenum	0.53	0.31	0.00	0.00	0.51	0.00
Penicillium siamense	0.53	0.00	0.00	0.00	0.51	0.00
Petromyces alliaceus	0.00	0.93	0.00	0.00	0.00	0.00
Peyronellaea glomerata	0.00	0.00	0.00	0.00	0.00	1.96
Phaeosphaeriopsis sp.	0.00	0.00	0.00	0.00	0.00	1.96
Phoma herbarum	1.06	1.24	0.00	0.66	1.02	0.00
Phoma multirostrata	0.00	0.00	0.00	0.00	0.00	17.65
Phoma sp.	2.13	2.17	0.00	2.65	2.55	3.92
Phyllosticta pyrolae	4.26	2.79	0.00	4.64	5.10	0.00
Pilidium concavum	0.00	0.31	0.00	0.00	0.51	0.00
Pleospora herbarum	5.32	5.26	0.00	2.65	4.08	0.00
Pseudohalonectria lignicola	2.13	2.48	0.00	1.99	2.55	0.00
Pseudombrophila guldeniae	0.00	0.31	0.00	0.00	0.00	0.00
Pythium cylindrosporum	1.60	0.93	0.00	1.32	1.53	0.00
Pythium insidiosum	2.13	2.79	0.00	5.96	5.61	0.00
Pythium monospermum	1.06	0.62	0.00	1.32	1.02	0.00
Pythium ostracodes	0.53	0.31	0.00	0.00	0.51	0.00
Pythium ultimum	1.60	2.17	0.00	3.31	3.06	0.00
Rhizoctonia solani	5.85	7.74	0.00	5.30	5.10	0.00
Rhizoctonia zeae	0.00	0.00	3.41	0.00	0.00	0.00
Rhizopus microsporus	0.00	0.31	0.00	0.00	0.00	0.00
Rhodotorula glutinis	0.53	0.31	0.00	0.66	0.51	0.00
Rhodotorula mucilaginosa	1.06	0.93	0.00	0.66	0.00	0.00
Saccobolus dilutellus	0.00	0.31	0.00	0.00	0.00	0.00
Sarocladium strictum	1.60	0.93	0.00	1.32	1.02	0.00
Sclerotinia trifoliorum	0.53	0.31	0.00	0.00	0.51	0.00
Septoria epambrosiae	0.53	0.00	0.00	0.66	0.51	0.00
Setosphaeria monoceras	0.00	0.62	0.00	0.66	0.51	0.00
Setosphaeria rostrata	0.00	0.00	0.00	0.00	0.00	7.84
Thanatephorus fusisporus	0.53	1.24	0.00	1.32	1.02	0.00
Thielavia terricola	0.00	0.00	2.27	0.00	0.00	0.00
Trichoderma sp.	0.00	0.31	0.00	0.00	0.00	0.00
Verticillium dahliae	5.32	6.81	0.00	3.31	3.57	0.00

Percent Abundance: 30.0, 25.0, 20.0, 15.0, 10.0, 5.0, 1.0, 0.5, 0.1, 0.0



	LOCATION YEAR					
	Gurue 2014			Gurue 2015		
	Illumina		Sanger	Illumina		Sanger
	FTA cards	tissue	Culture	FTA cards	tissue	Culture
Species	OTU					
Acremonium blochii	0.00	0.00	0.00	1.27	1.02	0.00
Acremonium curvulum	0.54	0.30	0.00	0.00	1.02	0.00
Acremonium flavum	0.54	0.30	0.00	0.00	0.00	0.00
Acremonium hyalinulum	0.00	0.60	0.00	0.00	0.00	0.00
Acremonium nigrosclerotium	0.00	0.30	0.00	0.00	0.00	0.00
Acremonium sp.	1.08	0.91	0.00	0.00	0.00	0.00
Acremonium sclerotigenum	0.00	0.00	0.00	1.27	1.02	0.00
Alternaria alternata	0.00	0.30	0.00	0.00	0.00	3.51
Ajellomyces dermatitidis	0.00	0.00	0.00	1.27	1.02	0.00
Arthopyrenia salicis	0.00	0.00	0.00	1.27	1.02	0.00
Aspergillus fumigatus	0.00	0.30	0.00	0.00	0.00	0.00
Aspergillus niger	0.00	0.30	0.00	0.00	0.00	0.00
Aspergillus penicillioides	0.54	0.30	0.00	1.27	1.02	0.00
Aspergillus terreus	0.54	0.91	0.00	1.27	1.02	0.00
Athelia rolfsii	4.32	3.63	0.00	3.80	4.08	0.00
Bullera penniseticola	0.00	0.00	0.00	1.27	1.02	0.00
Bullera sp.	0.00	0.00	0.00	2.53	2.04	0.00
Bulleromyces albus	0.54	0.91	0.00	0.00	0.00	0.00
Bullimyces aurisporus	1.08	0.91	0.00	1.27	1.02	0.00
Catenaria anguillulae	0.00	0.60	0.00	1.27	1.02	0.00
Ceratobasidium sp.	0.00	0.30	0.00	0.00	0.00	0.00
Cladorrhinum samala	0.00	0.00	0.00	0.00	0.00	1.75
Cladosporium cladosporioides	4.86	3.02	0.00	2.53	2.04	0.00
Cochliobolus sp.	0.54	0.30	0.00	0.00	0.00	0.00
Cochliobolus sativus strain	0.00	0.00	0.76	0.00	0.00	0.00
Colletotrichum sp.	0.54	0.60	0.00	0.00	0.00	0.00
Coniochaeta velutina	1.08	0.91	0.00	0.00	0.00	0.00
Cordyceps sp.	0.00	0.00	0.00	1.27	1.02	0.00
Cryptococcus flavus	0.00	0.00	0.00	1.27	1.02	0.00
Cystofilobasidium ferigula	0.00	0.00	0.00	1.27	1.02	0.00
Entoloma strictiu	0.00	0.00	0.00	1.27	1.02	0.00
Epicoccum sorghinum	0.00	0.00	0.00	0.00	0.00	7.02
Eurotium repens	0.54	0.30	0.00	0.00	0.00	0.00
Fusarium brachygibbosum	0.00	0.00	0.76	0.00	0.00	0.00
Fusarium circinatum	0.00	0.00	0.76	0.00	0.00	0.00
Fusarium equiseti	0.00	0.00	19.85	0.00	0.00	14.04
Fusarium falciforme	0.00	0.00	0.00	0.00	0.00	1.75
Fusarium incarnatum	0.00	0.00	1.53	0.00	0.00	0.00
Fusarium oxysporum	23.78	19.94	52.67	13.92	13.27	35.09
Fusarium proliferatum	0.00	0.00	2.29	0.00	0.00	3.51
Fusarium solani	8.65	5.74	10.69	2.53	3.06	8.77
Fusarium sp.	1.62	1.21	0.00	3.80	5.10	0.00
Fusarium thapsinum	0.00	0.00	1.53	0.00	0.00	0.00
Fusarium verticillioides	0.00	0.00	1.53	0.00	0.00	3.51
Geomyces sp.	0.00	0.60	0.00	0.00	0.00	0.00
Geosmithia putterillii	4.86	3.93	0.00	0.00	0.00	0.00
Gliocladium sp.	0.54	0.30	0.00	0.00	0.00	0.00
Glomus sp.	0.54	0.30	0.00	0.00	0.00	0.00
Graphiola phoenicis	0.00	0.60	0.00	0.00	0.00	0.00
Graphium penicillioides	0.00	0.30	0.00	0.00	0.00	0.00
Graphium putredinis	1.08	1.21	0.00	0.00	0.00	0.00
Helicogloea variabilis	0.00	0.60	0.00	0.00	0.00	0.00
Hemileia vastatrix	2.16	3.02	0.00	10.13	8.16	0.00
Hyaloraphidium curvatum	0.00	0.00	0.00	0.00	1.02	0.00
Hypocrea jecorina	1.08	1.21	0.00	1.27	1.02	0.00
Hypoxylon fragiforme	0.00	0.00	0.00	3.80	3.06	0.00
Inocephalus sp.	0.00	0.00	0.00	0.00	3.06	0.00
Leptosphaeria maculans	2.16	1.21	0.00	1.27	1.02	0.00
Macrophomina phaseolina	0.00	0.00	0.00	0.00	0.00	3.51
Macrovalsaria megalospora	1.08	0.60	0.00	2.53	2.04	0.00
Melanops tulasnei	0.00	0.00	0.00	1.27	1.02	0.00
Melanospora tiffanii	0.00	0.00	0.00	0.00	1.02	0.00
Mortierella alpina	0.00	1.51	0.00	0.00	0.00	0.00
Mortierella sp.	0.00	0.30	0.00	0.00	0.00	0.00
Mucor circinelloides f. circinelloides	0.00	0.60	0.00	0.00	0.00	0.00
Myrothecium leucotrichum	0.00	0.00	0.00	1.27	1.02	0.00
Neocosmospora vasinfecta	0.54	0.60	0.00	0.00	0.00	0.00
Neurospora crassa	0.00	0.30	0.00	0.00	0.00	0.00
Niesslia exilis	0.54	0.30	0.00	0.00	0.00	0.00
Penicillium pinophilum	0.00	0.00	0.76	0.00	0.00	0.00
Penicillium purpurogenum	0.54	0.30	0.00	0.00	0.00	0.00
Penicillium siamense	0.54	0.00	0.00	0.00	0.00	0.00
Petromyces alliaceus	0.00	0.91	0.00	0.00	0.00	0.00
Phoma herbarum	0.54	1.21	0.00	2.53	2.04	3.51
Phoma sp.	2.70	1.81	0.00	2.53	2.04	3.51
Phyllosticta pyrolae	2.16	2.42	0.00	2.53	2.04	0.00
Pilidium concavum	0.00	0.30	0.00	0.00	0.00	0.00
Piriformospora indica	0.00	0.30	0.00	0.00	0.00	0.00
Pleospora herbarum	3.78	4.23	0.00	5.06	5.10	0.00
Pleosporaceae sp.	0.00	0.00	0.00	0.00	0.00	1.75
Pseudohalonectria lignicola	2.70	2.42	0.00	0.00	1.02	0.00
Pseudozyma sp.	0.00	0.00	0.00	0.00	2.04	0.00
Pseudombrophila guldeniae	0.00	0.30	0.00	0.00	0.00	0.00
Pythium cylindrosporum	1.08	0.60	0.00	0.00	0.00	0.00
Pythium insidiosum	2.16	3.32	0.00	0.00	0.00	0.00
Pythium monospermum	1.08	0.60	0.00	0.00	0.00	0.00
Pythium ostracodes	0.00	0.60	0.00	0.00	0.00	0.00
Pythium ultimum	2.16	1.51	0.00	2.53	1.02	1.75
Rhizoctonia solani	2.70	7.85	1.53	1.27	2.04	7.02
Rhizopus microsporus	0.54	0.30	0.00	0.00	0.00	0.00
Rhodotorula glutinis	0.00	0.30	0.00	0.00	0.00	0.00
Rhodotorula mucilaginosa	0.54	0.30	0.00	0.00	0.00	0.00
Rhodotorula pinicola	0.00	0.00	0.00	1.27	1.02	0.00
Sarcinomyces sp.	0.00	0.00	0.00	1.27	1.02	0.00
Saroccladium strictum	1.08	1.21	0.00	2.53	2.04	0.00
Sclerotinia trifoliorum	0.00	0.30	0.00	0.00	0.00	0.00
Scolecobasidium sp.	0.00	0.00	0.00	2.53	1.02	0.00
Septoria epambrosiae	0.54	0.30	0.00	0.00	0.00	0.00
Setosphaeria monoceras	0.54	0.00	0.00	0.00	0.00	0.00
Taphrina deformans	0.00	0.00	0.00	0.00	1.02	0.00
Taphrina maculans	0.00	0.00	0.00	0.00	1.02	0.00
Tetracladium setigerum	0.00	0.00	0.00	1.27	1.02	0.00
Thanatephorus fusisporus	0.54	0.91	0.00	2.53	3.06	0.00
Talaromyces pinophilus	0.00	0.00	0.76	0.00	0.00	0.00
Trichoderma atrobrunneum	0.00	0.00	0.76	0.00	0.00	0.00
Trichoderma harzianum	0.00	0.00	3.82	0.00	0.00	0.00
Trichoderma sp.	0.00	0.30	0.00	0.00	0.00	0.00
Verticillium dahliae	8.65	6.95	0.00	5.06	5.10	0.00

Fig. 6 Heat map of normalized relative abundance of the top 107 fungal operational taxonomic units associated to RCR-symptomatic beans based on 18S rDNA region Illumina sequencing from the pooled Gurue growing regions in 2014 and 2015

than from the culture-based method (Fig. 7). By location and year, overall, *F. oxysporum* was the most frequently identified isolate in both FTA® Cards and RCR tissue, followed by *F. solani* and other *Fusarium* species, as well as *Rhizoctonia solani* (Figs. 4, 5, and 7). Other fungal/Oomycete species that have been associated with RCR symptoms such as *Pythium ultimum* were less frequently observed. We found it notable that a fungal taxon identified in culture from RCR infected legumes, *M. phaseolina*, was not detected by Illumina amplicon sequencing in either location or year with the Whatman FTA® Cards. Finally, there was a significant correlation between information obtained by FTA® Cards and RCR tissue values based on the Spearman correlation coefficient for Gurue (0.759 and 9.86516, $P < 0.001$) and Chokwe (0.70288 and 0.865, $P < 0.001$) for 2014 and 2015, respectively.

Phylogenetic affinities of *Fusarium oxysporum* and genetic variation for *Fusarium oxysporum* assessed with microsatellite primers

To detect the four major pathogen genera and species (*Fusarium* spp., *R. solani*, *P. ultimum*, *M. phaseolina*), we employed Illumina amplicon sequencing, PCR-based microsatellites, and culture-based methods. The Illumina amplicon sequencing identified the most fungi and Oomycetes from FTA® Cards. However, PCR-based methods from RCR tissue were best at detecting *M. phaseolina* (Fig. 7). Every method that we employed identified *Fusarium* spp. as the most abundant pathogen group in RCR tissues. Other *Pythium* species, as well as *R. solani*, were also detected by amplicon sequencing at higher frequencies than PCR-based or culture-based methods. The frequency of detection of the

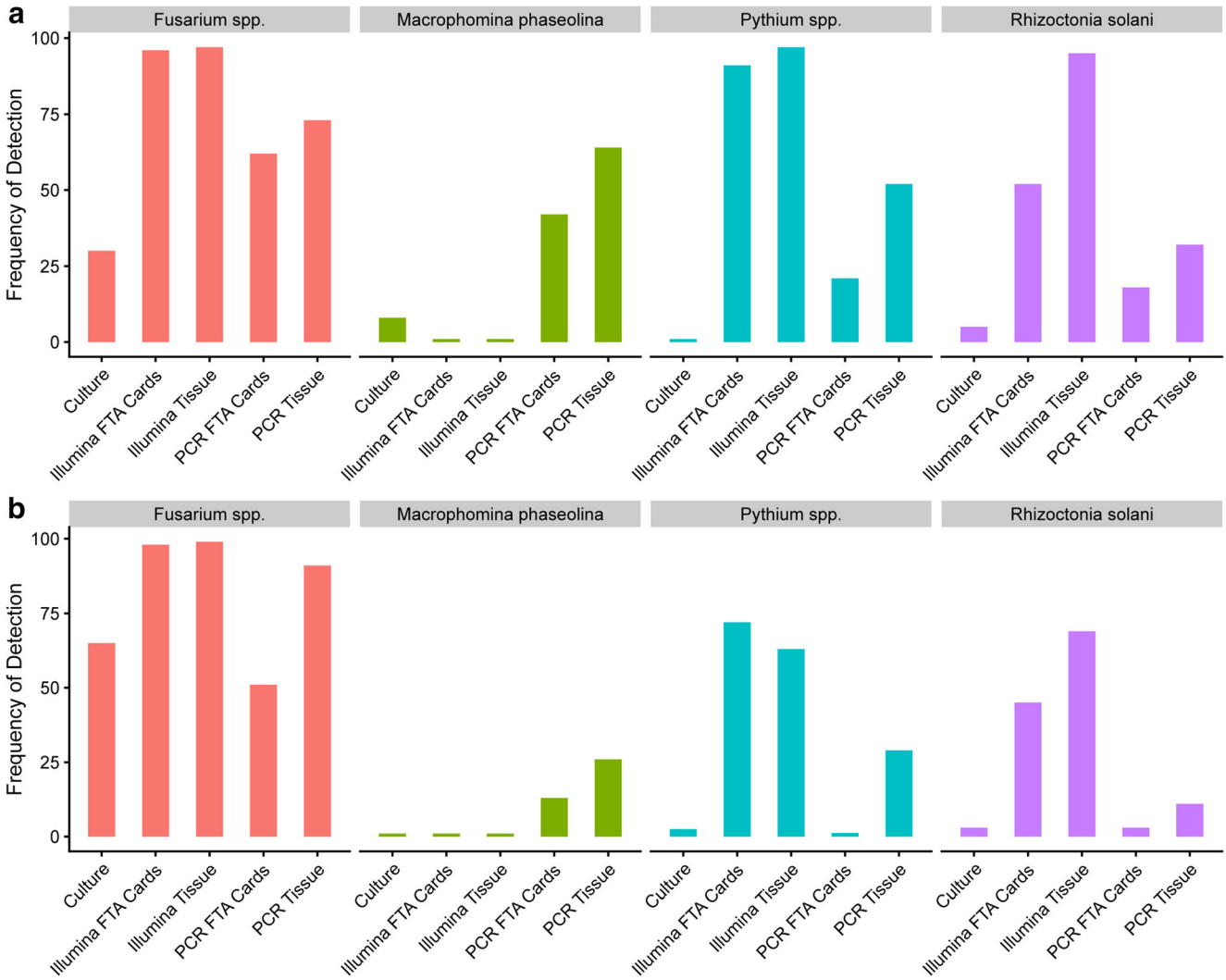


Fig. 7 Detection frequency across the years of 2014 and 2015 for the four main pathogens we identified (all *Fusarium* species, *Macrophonima phaseolina*, all *Pythium* species, and *Rhizoctonia solani*) associated with RCR of beans from sampling sites of (a) Chokwe and (b) Gurue in Mozambique. The methods used for detection in this study were (1) culture-based, (2) Illumina MiSeq FTA Card, (3) Illumina MiSeq tissue sample, (4) primer-specific polymerase chain reaction amplification FTA Card, and (5) primer-specific polymerase chain reaction amplification of tissue sample

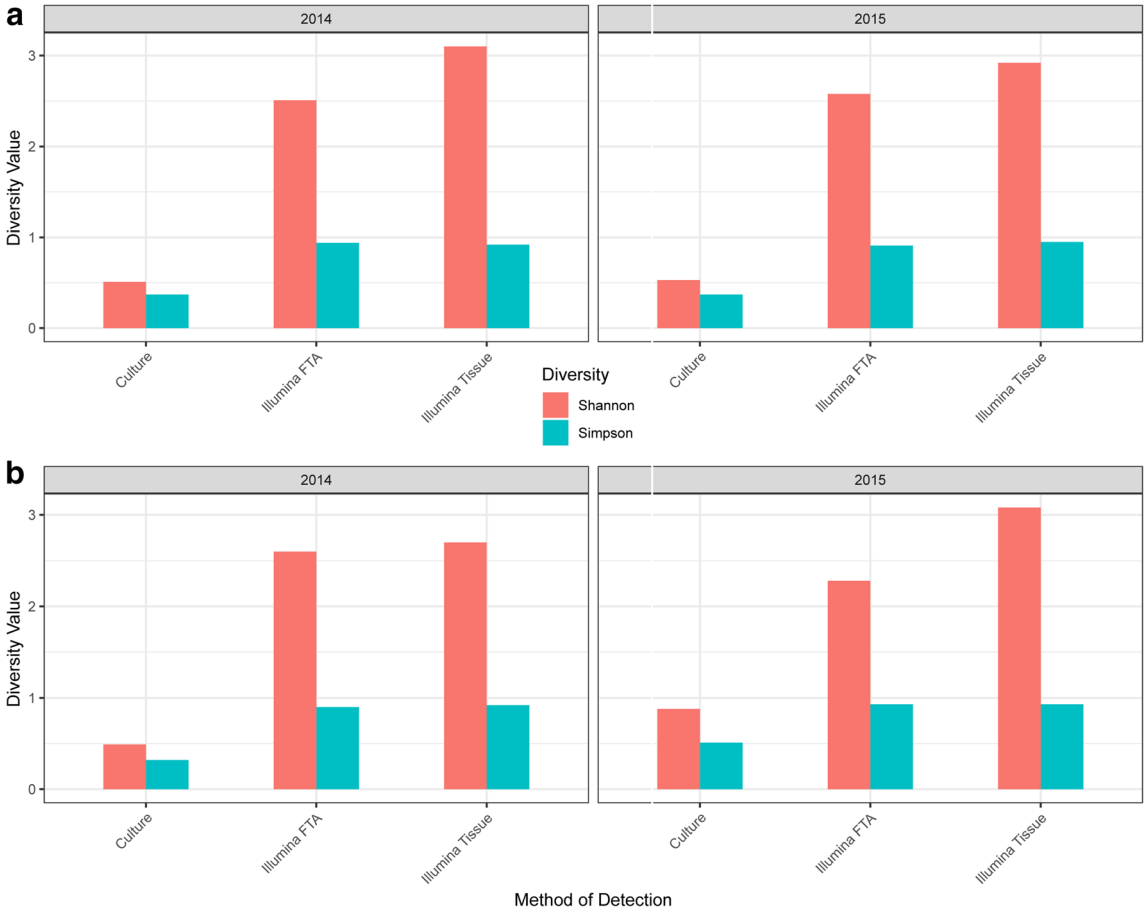


Fig. 8 Diversity metrics (Simpson and Shannon indices) for the (a) pooled Chokwe growing region RCR bean samples and the (b) pooled Gurue growing region RCR bean samples in 2014 and 2015 using the different diagnostic analysis methods we employed: (1) culture-based methods, (2) Illumina sequencing of FTA Card DNA extractions, and (3) Illumina sequencing of directly sampled RCR tissue DNA extractions directly sampled tissue samples

four most abundant pathogens was higher in the direct assay of RCR diseased plant tissue than in FTA® Cards for samples from both farm field locations in Mozambique. The culture-based methods had the lowest detection frequency for species of *Pythium*, *R. solani*, and *M. phaseolina*, but they isolated and identified numerous species of *Fusarium*. The two locations sampled showed differences in community diversity (based on the value of $H' > 2.5$) which revealed the presence of diverse array of isolates associated with RCR disease, whereas Simpson values close to 1 show the predominance of one species or group, which was the case for *Fusarium*, where these taxa were found in the same plant niche for most locations and year tested (Fig. 8). The exception was for the Chokwe location in 2015, where *M. phaseolina* was the predominant fungal species in culture. Values of Shannon and Simpson diversity indices were similar for both locations and years where *F. oxysporum* had the highest percentage of relative abundance. Of the most predominant species, *F. oxysporum*, was further examined for better taxonomic resolution by sequencing the partial EF-alpha gene and the sequences were used to construct a phylogenetic tree (Fig. 9). The phylogenetic tree revealed at least seven well-defined clusters within *F. oxysporum* species complex indicating intraspecific variability of the partial EF-1 gene. Additionally, most of the isolate sequences were separated from those of published *F. oxysporum* accessions from the NCBI GenBank database indicating the new isolates from Mozambique are not found in databases. There was no evidence that geographical origins of the isolates influenced the clustering pattern because sequences from both locations were found in species complex clusters. Phylogenetically informative nucleotide positions in the 18S rRNA sequences (generated by the Illumina amplicon sequencing) were used to compare common or shared OTUs in different combinations between the Chokwe and Gurue locations for both years and presented in Venn diagrams (Fig. 10). These common, or shared, amplicon counts are displayed in the overlapping panels and unique OTUs in the non-overlapping areas.

There were 91 isolates of *F. oxysporum* from Chokwe and Gurue characterized with single microsatellite primers (URP2R, $(GTG)_5$, $(ACG)_5$, and $(AGG)_5$) which generated 16 reproducible fragments, as well as 27 scorable fragments. The fragments (or allele) sizes ranged from 100 to >2000 bp,

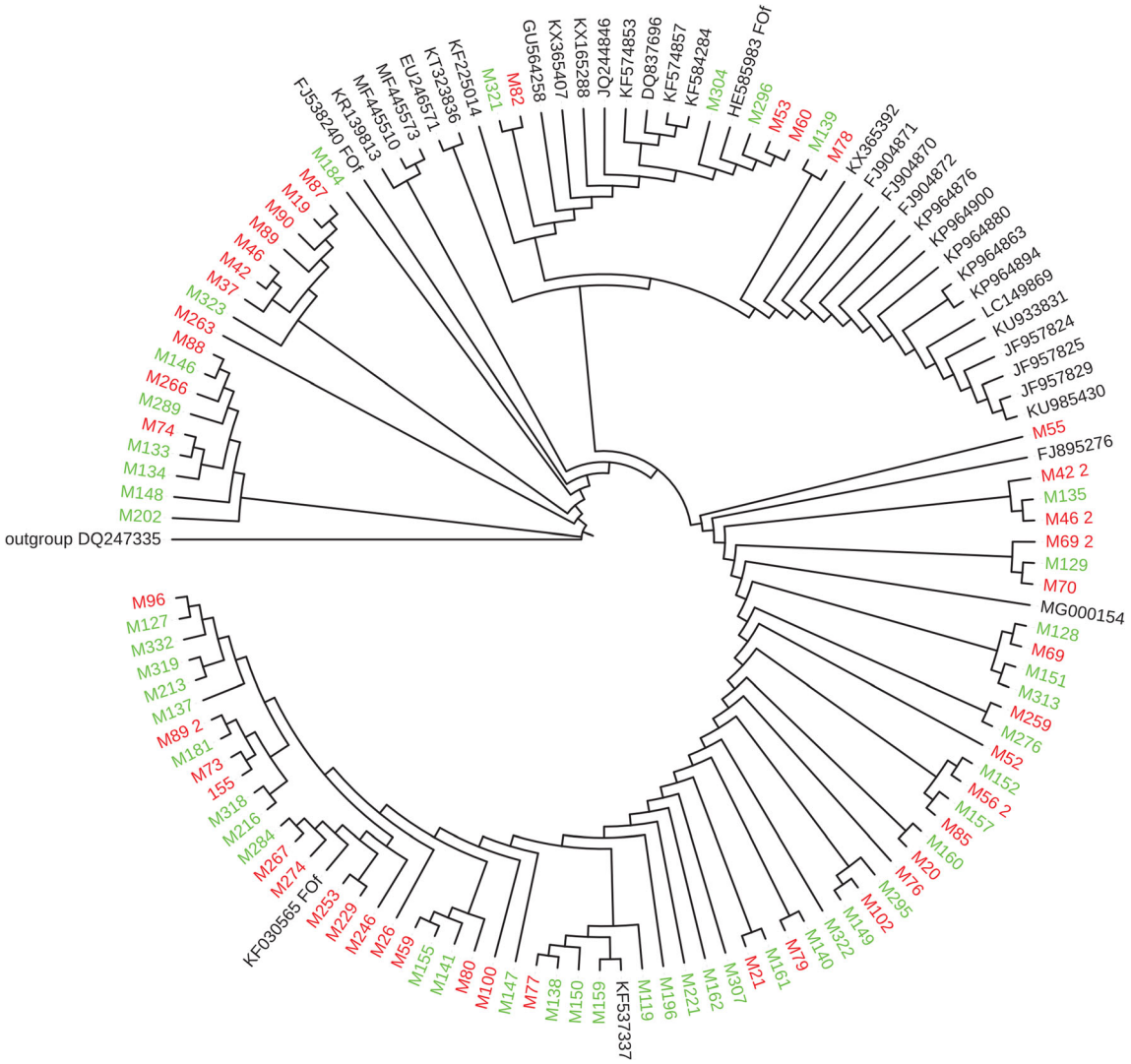


Fig. 9 Bayesian likelihood phylogenetic tree derived from partial EF-alpha gene region sequences of *Fusarium oxysporum* cultured isolates from Mozambique. Leaf names in green were isolated in from RCR-symptomatic samples from the Gurue region and leaf names in red were isolated from RCR-symptomatic samples from the Chokwe region. Leaf names in black represent 36 *Fusarium oxysporum* partial EF-alpha gene accessions from database of NCBI GenBank

although for ease of interpretation we only scored those with the range of 200 to 1000 bp. Not all of the isolates amplified using all four primers with the same efficiency. We identified diversity indicators for the Chokwe and Gurue group of isolates utilizing the microsatellite primers URP2R, $(GTG)_5$, $(ACG)_5$, and $(AGG)_5$ (Table S-1), and for example, the percentage of the polymorphisms was highest when we utilized the $(AGG)_5$ primer set when compared to the rest of the primers. Nei's gene diversity (for heterozygosity estimates), unbiased Nei's gene diversity (a correction for small number of samples), and Shannon's Information Index (extent of genetic variation) (Table S-2) all displayed low values indicating a small genetic distance between the Chokwe and Gurue *F. oxysporum* isolate groups. Analysis of molecular variance revealed that the percentage of total variation within the locations was higher than the variation found between locations. In conclusion, we detected no structural population differences between *F. oxysporum* isolate groups for each location based on the number of isolates and primers tested (Table S-1).

Discussion

The identification of plant pathogens is an important first step to develop preventive management strategies and identify resistant dry edible bean cultivars to root and crown rot (RCR) diseases. In this study we employed three methods to identify the primary causal agent of RCR in dry edible beans in Mozambique: amplicon sequencing of the 18S rRNA and EF1 marker regions, genus and species-specific microsatellite primer amplification paired with morphological identification

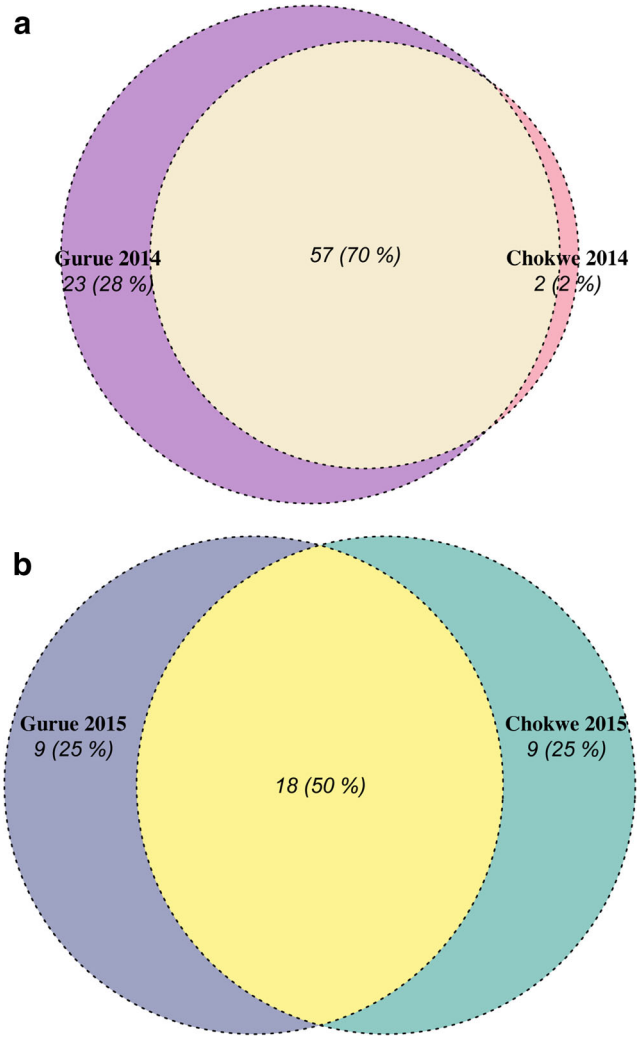


Fig. 10 Euler diagrams depicting shared (intersecting) and unique (not intersecting) operational taxonomic units of Fusarium oxysporum identified with Illumina MiSeq analysis of the 18S rDNA region sequences from RCR-symptomatic beans plants in (A) 2014 and (B) 2015 from the pooled samples of the Chokwe and Gurue growing regions in Mozambique

of cultural material, and DNA sequencing of the ITS rDNA region of the cultured fungi and Oomycetes.

Amplicon sequencing methods revealed the presence of a greater diversity of fungal and Oomycete species than culture-based methods. In all of the methods we employed, species of *Fusarium*, mainly *F. oxysporum*, were the dominant fungal isolates detected either on DNA extracted from FTA® Cards and RCR diseased plant tissue, or from isolates recovered through conventional culture methods. Other RCR disease–associated fungi, such as *R. solani*, *Pythium* spp., *A. rolsfii*, and *M. phaseolina*, were detected or isolated in cultured samples but were lower in abundance and typically inconsistent in their presence and absence. The exception to this was found in the samples from Chokwe in 2015 where we identified *M. phaseolina* from both the sequencing and culture methods. Other fungal species we detected by amplicon sequencing, widely distributed across the Ascomycota and Basidiomycota, are not known to be associated with RCR and could be considered plant endophytes. The role of these fungi as endophytes in healthy or diseased dry bean plant tissue is yet to be well established (Nair and Padmavathy 2014). Despite the high frequency of isolation of *M. phaseolina* from the 2015 samples collected in Chokwe, the symptoms observed in the collected root/crown samples did not match symptoms typically caused by *M. phaseolina*, which is known to infect the upper stem of more mature plants. We hypothesize that increased rainfall in the Chokwe region during our study resulted in this finding, as *M. phaseolina* has been reported to increase abundance in regions of the tropics and subtropics where crops might be exposed to water stress (Songa and Hillocks 1996). The high isolation frequency of *M. phaseolina* is likely due to climate variability in the 2014 and 2015 growing seasons, which were characterized by low rainfall from October to December of 2014, and the resulting drought in the southern region from January to March of 2015. Later, an El Niño phenomenon was reported in Mozambique in the 2015 growing season which a rainy season in the south of the country was followed by additional pronounced and excessive rain or floods in the northern region (C. Jochua, personal observations). Most of the symptomatic bean samples, which exhibited signs of wilting and necrosis of the stem even before the flowering stage, were associated with infection by species of *Fusarium*. These symptoms were typically associated with recurrent cyclical weather patterns in the country, such as flooding and drought, and have caused environmental conditions which favor the abundance of *Fusarium* species in all locations of Mozambique.

The results of this study are in agreement with previous work (Mukuma et al. 2020) that found *F. oxysporum* was the predominant pathogen associated with RCR of bean in Zambia. However, Rusuku et al. (1997) also studied soil-borne pathogens causing the RCR disease from culture isolation and found species of *Pythium* to be the most frequently isolated pathogens, over *F. oxysporum*, *M. phaseolina*, and *R. solani* in the RCR complex in Rwanda. To complement the methods used in the previous studies conducted in Zambia and Rwanda, we implemented both amplicon sequencing and microsatellite-based methods as they reveal a finer scale genetic profile of pathogens rather than phenotypic profiles obtained *via* only using cultural methods (Hilton et al. 2016; Mukuma et al. 2020).

In spite of the limitations of not being able to culture all possible fungal isolates, the fungi that we did isolate could be directly tested for pathogenicity to screen dry bean germplasm for disease resistance. Collectively, we identified 333 fungi and/or Oomycetes isolates from Chokwe and Gurue regions in the 2014 and 2015 growing seasons and tested these for pathogenicity against a diversity panel of beans. At least 60% of the isolates were pathogenic to bean, and species of *Fusarium*, primarily *F. oxysporum*, collected from all locations and years

produced the longest mean lesion lengths in our pathogenicity assays. Based on the isolation frequency from the field collections and overall pathogenicity observed across the bean diversity panel, we conclude that *F. oxysporum* plays the most predominant role in the bean RCR complex in Mozambique.

More than one pathogen causing RCR of bean was additionally reported by Rusuku et al. (1997) and Mukuma et al. (2020), suggesting a complex of pathogens such as *Pythium* spp., *R. solani*, *M. phaseolina*, *F. oxysporum* f.sp. *phaseoli*, and *A. rolfsii* may be interacting to cause bean RCR. The hypothesis that more than one pathogen causes RCR on beans may have implications in the identification and subsequent breeding in response to broad pathogen-specific RCR disease resistance (Abawi and Pastor-Corrales 1990; Wortmann et al. 1998; Chaudhary et al. 2006; Clare et al. 2010).

With regard to the *Fusarium* species we identified, *F. oxysporum* was the most frequently isolated in our study and identified among the most pathogenic fungi and/or Oomycetes isolated within Mozambique. These results support other studies conducted in Latin America, Spain, and the USA, where *F. oxysporum* has caused RCR outbreaks (Pastor-Corrales and Abawi, 1987; Alves-Santos et al. 2002). In addition, *F. oxysporum* has also been identified as a major pathogen in Central Africa where serious losses on improved climbing bean varieties were reported (Buruchara and Camacho 2000). This study found that pathogenic isolates of *Fusarium*, with *F. oxysporum* being most notable, were co-existing with other individual *Fusarium* taxa that were not observed to be pathogenic within the same plant. The finding that some of the *Fusarium* species were pathogenic and others were not detrimental to a plant host may be explained by their ubiquitous nature in habitats such as soil, water, and as plant endophytes (Lofgren et al. 2018). Therefore, it is not unusual to have pathogenic and non-pathogenic isolates together in a healthy plant, which contribute to the early development and severity of disease when conditions are unfavorable for bean growth (Leslie and Summerell 2006; Estevez de Jensen et al. 2004; Harveson et al. 2005). It is important to note that *Fusarium* taxa that are typically recognized as opportunistic pathogens and will infect plants under the induction of stressful conditions such as those common in changing climate scenarios (Valverde-Bogantes et al. 2019). We note that, along with *F. oxysporum*, a large portion of the *Fusarium* species complexes identified here were identified as *F. solani* and *F. equiseti* and that we did not investigate in more detail here. We expect these species complexes will be the focus of additional studies inspecting morphotypes and genetic diversity of all RCR bean–associated *Fusarium* taxa.

Based on the amplicon sequencing portion of our study, we identified a diverse community of fungal and Oomycete taxa associated with RCR diseases of bean in two geographically distant regions in Mozambique. A relatively high fungal diversity in different environments has been reported in other studies of the dry bean microbiomes in tropical regions (Pastor-Corrales and Abawi 1987; Alves-Santos et al 2002, Buruchara and Camacho 2000), supporting high-throughput sequencing methods which provide better estimates of the overall fungal diversity of a sample when compared to other identification methods. High-throughput sequencing methods, such as the amplicon sequencing methods we employed in this study, are sensitive methods to identify pathogens associated with RCR when compared to culture-based techniques using morphological approaches that are better suited to only detect individual taxa or those that are easier to culture. Culture-based assessments of diversity can be problematic as some particular species can be isolated with ease in a laboratory setting as they rapidly respond to simple media formulations and are quick to outgrow other organisms. It has been estimated that many fungal taxa are difficult or are unable to be cultured with our current methodology which hinders the process of identification and experimentation (Hilton et al. 2016). We emphasize that the importance of culturing is vital for establishing cultural material of both fungal and/or Oomycete pathogens which are needed for screening bean lines for RCR resistance.

As previously mentioned, the DNA sequencing methods we used as identification methods were not always in agreement with all the isolated cultures we collected. In general, the combination of molecular- and morphological-based methods, such as the microsatellite and cultural isolation methods we employed here, was better for identifying *Macrophomina phaseolina*, which was not detected by amplicon sequencing from either the FTA® Cards or direct RCR lesions. DNA-based methods, such as high-throughput Illumina amplicon sequencing and PCR-based microsatellite amplification we used in this study, are the most sensitive technique available for the detection of plant pathogens, due in part to the overall depth of sequencing and the specificity of the oligonucleotide primers designed for *Fusarium* taxa (Capote et al. 2012). We hypothesize that the poor amplification of numerous *Macrophomina phaseolina* isolates can be explained by a lack of specificity regarding the oligonucleotide primers and probes, but we have yet to test this hypothesis. Sanger-based sequencing analyses were based on the direct analysis of the genomic DNA of the ITS-2 region which may be variable in the case of *M. phaseolina*. In addition, the high recovery of *M. phaseolina* isolates in samples from the Chokwe region in 2015 may be attributed to environmental conditions in the field, such as high humidity and temperature, where *M. phaseolina* has a high optimal temperature for growth and infection (Songa and Hillocks 1996).

Amplicon sequencing using the Illumina high-throughput platform was used to compare the efficacy of the FTA® Card method for collecting fungi and/or Oomycetes with direct isolation from RCR tissue. The taxonomic diversity assessed by both methods were highly correlated, indicating that FTA® Cards are an acceptable alternative for collection and storage

of DNA, particularly in locations where DNA collection *via* direct placement in liquid buffers may be prohibitive, such as field studies. These results are in agreement with the findings obtained by Ndunguru et al. (2005) who used FTA® Cards for sampling and retrieval of DNA and RNA viruses from plant tissues and conducted molecular analysis on viral diversity. Additionally, the proper use of FTA® Cards for DNA collection and storage largely circumvents the security issues related to the import and export of infected plant tissues. These findings demonstrate the importance of using a combination of diagnostic methods to address identification and phylogenetic placement of primary pathogens associated with host-plants. In this study we used specific primers for the ITS-2 and 18S rDNA regions from RCR fungal pathogen isolates, in addition to species-specific oligonucleotide hybridization, which led to the sequencing and analysis of target DNA suited for both taxonomic and phylogenetic assessment of diversity (Herr et al. 2015; Hibbett et al. 2016). However, we stress the importance of culture-based methods for establishing fungal isolate collections that can be used to test for pathogenicity in field and greenhouse experiments with dry beans and assist in breeding efforts to select for pathogen resistance.

A greater amount of genetic variance within fungal populations from a specific location, rather than between the two regions, was most notable in our microsatellite analysis. Despite using the relatively small suite of microsatellites we tested, our results suggest that either gene flow of the fungi exist between the two regions we surveyed or the fungal isolates are broad in their geographic ranges. Even though the Chokwe and Gurue regions are 1200 km apart, this finding may be explained by a number of factors. For example, many fungi may be seed derived, and an active bean seed exchange between farming locations in Mozambique, as well as with neighboring countries such as Zambia, could be contributing to broad geographic ranges of these fungi. *Fusarium oxysporum* is known to be seed transmitted (Gargouri et al. 2000; Garibaldi et al. 2004; Pires da Silva et al. 2014) and this species complex consists of morphotypes consisting of similar genotypes with diverse lineages (Pires da Silva et al. 2014; Gordon 2017). Additionally, the *F. oxysporum* species complex is not well understood and has no identified sexual reproduction system, and, as a result, the diversity among the isolates may be typical resulting in a limited number of clonal lineages on a large geographic scale (Gordon and Martyn 1997; Valverde-Bogantes et al. 2019).

To our knowledge, this is the first study which utilized both molecular and culture methods to determine the primary pathogens associated with RCR disease of bean in Mozambique. The fact that there are similar pathogen profiles in the two main bean-growing regions of Mozambique allows breeders to broadly screen for resistance to *Fusarium* species, mainly *F. oxysporum*, and cultural methods can provide the pathogen isolates needed for screening across the country as a whole. Currently, the breeding programs in East Africa are predominantly evaluating *Pythium* spp.; however, the results from this study should provide relevant pathogen information and we hope that it will inform breeding for bean and root rot disease resistance in Mozambique. Lastly, we hope that the findings presented here will provide a basis for future studies of agroecosystem microbiomes (Dundore-Arias et al. 2020) at a finer sampling scale for RCR diseases of dry bean in Mozambique, as well as other tropical and temperate growing regions.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s40858-021-00422-8.

Acknowledgements We recognize the help of many regional farmers, most notably R. Joseph, J. Tenga, and E. Langa, as well as many whom remain anonymous, in the field identification and collection of beans with RCR symptoms. We acknowledge the help of Edgar Nieto who provided recommendations on heatmap visualization tools using the R statistical framework. We also want to thank the anonymous reviewers who provided feedback and comments on the initial submission of this manuscript.

Author contribution GG-L, CJ, CU, KE, JRS, and JRH were involved in planning and supervised the work; SF, CJ, and GG-L performed the experiments; GG-L and JRH processed the experimental data and designed the figures; SF, GG-L, JRS, and JRH drafted the manuscript. All authors discussed the results and commented on the manuscript.

Funding This work was completed using the Holland Computing Center of the University of Nebraska, which receives support from the Nebraska Research Initiative. JRS recognizes funding from USDA-NIFA (Grant #11172136) which directly supported this research. JRH acknowledges funding from the US National Air & Space Administration (Grant #80NSSC17K0737), the US National Science Foundation (EPSCoR Grant #1557417), and US National Institute of Justice (Grant #2017-IJ-CX-0025), as well as start-up funding from the University of Nebraska Agricultural Research Division and the University of Nebraska Office of Research and Economic Development, which indirectly supported this research through the support of research facilities. The funding agencies had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Data availability The data sets generated during and/or analyzed during the current study are available in the following data repository: https://github.com/HerrLab/Fernandes_et_al_2019.

Declarations

Conflict of interest The authors declare no competing interests.

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