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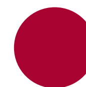

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DNA barcoding and isolation of vertically transmitted ascomycetes in sorghum from Burkina Faso: *Epicoccum sorghinum* is dominant in seedlings and appears as a common root pathogen

Michaela S. Stokholm[a], Ednar G. Wulff[b], Elisabeth P. Zida[c], Ibié G. Thio[c], James B. Néya[c], Romain W. Soalla[c], Sylwia E. Głazowska[a], Marianne Andresen[a], Henrik B. Topbjerg[d], Birte Boelt[d], Ole S. Lund[a,*]

[a] Institute of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen, Hoejbakkegaard Allé 13, DK-2630 Taastrup, Denmark
[b] Division of Plant Diagnostics, Danish Veterinary and Food Administration, Ministry of Food, Agriculture and Fisheries, Soendervang 4, DK-4100 Ringsted, Denmark
[c] Kamboinsé Research Station, INERA (Institut de l'Environnement et de Recherches Agricoles), 01 P.O. Box 476 Ouagadougou 01, Burkina Faso
[d] Department of Agroecology, Science and Technology, Aarhus University, Forsoegsvej 1, DK-4200 Slagelse, Denmark

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ABSTRACT

Molecular identification of fungal taxa commonly transmitted through seeds of sorghum in Western Africa is lacking. In the present study, farm-saved seeds, collected from four villages in Northern Burkina Faso, were surface sterilized and the distribution of fungal DNA in seeds and seven-day-old seedlings was analyzed by *18S* ribosomal DNA (rDNA) amplicon sequencing. More than 99% of the fungal rDNA was found to originate from ascomycetes. The distribution of ascomycetes at species level was subsequently analyzed by barcoding of *ITS2* rDNA. Eighteen Operational Taxonomic Units (OTUs) were identified from seedlings, compared to 29 OTUs from seeds. The top-eight most abundant ascomycete OTUs from seedlings were annotated as: *Epicoccum sorghinum*, *Fusarium thapsinum*, four different *Curvularia* spp., *Exserohilum rostratum* and *Alternaria longissima*. These OTUs were also present in amplicons from seed samples collected in Central Burkina Faso confirming a common occurrence. *E. sorghinum* was highly predominant in seedlings both measured by DNA analysis and by isolation. The dominance of *E. sorghinum* was particularly strong in roots from poorly growing seedlings. Pathogenicity of *E. sorghinum* isolates was compared to *F. thapsinum* by inoculation to seeds *in vitro*. Both fungal species caused significant inhibition of seedling growth ($P < 0.001$) and Koch's postulates were fulfilled. Extensive, dark necrosis in roots was a typical symptom of *E. sorghinum*, whereas wilting of leaves was caused primarily by *F. thapsinum*. This study provides the first molecular approach to characterize the seedling mycoflora of sorghum in Western Africa and suggests *E. sorghinum* as a common root pathogen.

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1. Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is the fifth most important grain crop in the world and is one of the major staple food crops of the semi-arid tropics of Africa, Asia and Latin America (Rooney, 1996; Dicko et al., 2006). In terms of annual production, sorghum is the most important crop in Burkina Faso, accounting for 1.7 million tonnes in 2014 with an average yield of 1.1 t/ha (FAO stat, 2015). Antifungal seed treatment of sorghum has previously shown a 25% yield increase in field trials in Burkina Faso (Zida et al., 2012). However, precise molecular information about the actual species composition of the seed transmitted mycobiome *in planta* is lacking. Fungal species associated with sorghum seeds and field plants in Burkina Faso have hitherto been identified on a morphological level in two country-wide studies (Zida et al., 2008; Zida et al., 2014). In both studies, a large number of ascomycetes were found to dominate the mycoflora. One of the most common taxon's encountered was *Fusarium moniliforme* (teleomorph: *Gibberella fujikuroi*), which is part of a morphologically defined complex, the *Gibberella fujikuroi* species complex that contains several distinct *Fusarium* species (Leslie et al., 2005). Due to the morphologically based taxonomy applied in these studies, it was not determined which species

* Corresponding author.
E-mail address: osl@plen.ku.dk (O.S. Lund).

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of the *G. fujikuroi* complex were actually represented. In the study of sorghum seed mycoflora (Zida et al., 2008), isolates of *F. moniliforme* were found to be highly pathogenic in seedlings, whereas in the study by Zida et al. (2014), *F. moniliforme* was found to be three times more frequently isolated from well performing (big) plants, compared to less well performing (small) plants, indicating a possible beneficial interaction. In addition to *Fusarium*, several abundantly occurring genera were incompletely resolved at the species level in these previous studies. *Phoma* spp. was for example found on 33% of seeds (Zida et al., 2008) and *Curvularia* spp. was found endophytically in 24% of young field plants (Zida et al., 2014). In order to challenge and further analyze these findings, it is necessary to apply a culture-independent molecularly based fungal taxonomy. The aim of the present study was therefore, to apply DNA barcoding of fungal taxa to investigate the mycobiome transmitted vertically *in planta* from seeds to seedlings of sorghum in Burkina Faso. Seeds from the Northern region were selected as the primary target for analysis. In order to eliminate horizontally transmitted fungi and superficially associated saprotrophs from the analysis, we used surface sterilized seeds to study the fungal transmission. The diversity of fungal species that could be isolated from roots and stems was also evaluated, and the most frequently isolated species were determined. The pathogenic nature of isolates, from the predominantly transmitted fungal species, was assessed *in vitro* on seedlings grown from inoculated seeds.

2. Materials and methods

2.1. Plant material

Eight farmer-saved seed samples of sorghum (*Sorghum bicolor* L.) were collected (200 g/sample) from four villages (Bani, Pobé, You and Ouahigouya) in Northern Burkina Faso, also known as the Sahelian agroecological zone. In addition three farmer-saved seed samples were collected, one from Northern Burkina Faso (Pobé-Mengao) and two from the villages Kindi and Diapangou in Central Burkina Faso (Northern Sudanian agroecological zone). The seed sample 49.071, used for pathogenicity testing, was obtained from the INERA Research Centre in Burkina Faso. Root material from 3-month-old sorghum plants was collected from field grown plants in the villages Kindi and Diapangou.

2.2. Surface sterilization of seeds

Seeds used for testing of vertical transmission of fungi were surface disinfected by immersion in 70% ethanol for 1 min and then transferred to 1.5-2% sodium hypochlorite for 3 min. Seeds were subsequently washed three times in sterile distilled H_2O. To assure that seeds were surface disinfected, sterility was tested after seed washing by plating aliquots of the last wash on potato dextrose agar (PDA) supplemented with streptomycin sulphate (0.3 g/L PDA) (Samson et al., 2002). In case fungal growth was detected on the water-control PDA plates after incubation at 20 °C for 1 week, the seed samples were discarded and the disinfection procedure repeated. After disinfection, seeds were left to dry in the laminar flow cabinet in Petri dishes containing sterile filter paper and subsequently stored at 4 °C until use.

2.3. DNA-extraction of plant material

For each of the eight seed samples collected in Northern Burkina Faso, 200 surface sterilized seeds were germinated between two layers of sterile, moistened filter-paper at 24 °C, as described by Mathur et al. (2003). For each sample, 30 viable and normal appearing seedlings were selected on day 7 and were subsequently surface sterilized and washed, as described above. Each group of 30 surface sterilized whole seedlings were ground into a fine powder in liquid nitrogen, followed by DNA extraction using DNeasy Plant Mini Kit (Qiagen Group, CA, USA). DNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer, according to the manufacturer's specifications (NanoDrop Technologies Inc., Wilmington, Delaware, USA). For each of the corresponding seed samples, DNA was similarly extracted from 30 seeds (surface sterilized and washed). Two DNA pools, consisting of one pool of seedling DNA and one pool of seed DNA, were generated by mixing equimolar amounts of DNA from each seed or seedling sample, respectively. DNA from non-surface sterilized plant material was extracted using the same method. Non-surface sterilized plant material included roots from sorghum field plants, as well as the three individual seed samples (400 seeds/sample) collected in Pobé-Mengao, Kindi and Diapangou.

2.4. Amplification of 18S from eukaryotes and of ITS2 ribosomal DNA from ascomycetes

One forward and four reverse primers for multiplex PCR targeting the small *18S* ribosomal subunit of plant associated microbial eukaryotes (basidiomycetes, ascomycetes, glomeromycetes, zygomycetes, oomycetes, plasmodiophorids and nematodes) were designed (Table 1) and obtained from Eurofins Genomics, Ebersberg, Germany. All primers contained at least 1 mismatch relative to the corresponding *18S* sequence of higher plants (angiosperms). The *18S* forward primer was fused with different multiplex identifier (MID) tags for barcoding of different samples, as well as 454-pyrosequencing adapters in both forward and reverse primers, according to Eurofins Genomics pyrosequencing manual (Table 1). The *18S* multiplex PCR (1 ×) consisted of 10 ng DNA, 1.0 μl forward primer Pyro-18-F2 extended with MID tag and pyrosequencing adapter (100 pmol/μl), 0.25 μl of each of four reverse primers: Pyro-18-R2a/e/g/h (100 pmol/μl), extended with pyrosequencing adapters, 3.2 μl $MgCl_2$ (25 mM), 8.3 μl 5 × colorless GoTaq Reaction buffer, 0.2 μl GoTaq DNA Polymerase (5 U/μl) (Promega Corporation, WI, USA) and 6.7 μl dNTP (1.25 mM), 2.5% DMSO and H_2O to a final volume of 40.8 μl. PCR amplification was conducted in a thermal cycler (MJ mini^{TM} Personal Thermal Cycler, Bio-Rad Laboratories Inc.) with the following program: 3 min initial DNA denaturation at 95 °C, followed by 35 cycles of 20 s at 94 °C, 15 s at 56 °C, 25 s at 72 °C and 7 min final extension at 72 °C. Ascomycete specific amplification of the ribosomal internal transcribed spacer region 2 (*ITS2*), was done using primers modified from Louarn et al. (2013), which were fused to MID tags and adaptors required for pyrosequencing, as above (Table 1). The *ITS2* multiplex PCR was performed with two technical replicates, as described above using 10 ng DNA as template, 0.8 μl of the forward and 0.4 μl of each of two reverse primers, excluding DMSO. The *ITS2* rDNA was amplified as follows: 3 min initial DNA denaturation at 95 °C, followed by 32 cycles of 20 s at 94 °C, 15 s primer annealing at 56 °C, 15 s extension at 72 °C and 3 min final extension at 72 °C. PCR products were assessed by gel electrophoresis in a 1.5% agarose gel. Amplicons (400–500 bp for *18S* rDNA and 180–250 bp for *ITS2*) were excised from gels and purified using MinElute Gel Extraction Kit (Qiagen Group, CA, USA). DNA concentrations of the purified amplicons were determined spectrophotometrically, as described above.

2.5. Pyrosequencing and cluster analysis

Equimolar concentrations of the seed and seedling amplicons were mixed and 454-pyrosequenced at the Roche GS FLX Titanium platform, followed by initial sorting of sequence data at Eurofins Genomics, according to sample specific MID tags. The pyrosequencing datasets were processed through a series of sequence trimming and quality control (QC) steps to extract high quality regions (HQR),

followed by single linkage clustering, using the open source SCATA pipeline (Sequence Clustering and Analysis of Tagged Amplicons (http://scata.mykopat.slu.se); Durling et al., 2015). QC for *18S* rDNA amplicons: Sequences containing a >90% match to the forward primer sequence were accepted, followed by removal of the forward primer. Sequences shorter than 250 bp, or sequences of low quality with a mean quality score <20 or bases with a score <10 were discarded. In addition, sequences of the *18S* amplicons were truncated to a maximum of 350 bp, which also ensured removal of the various combinations of reverse primer sequences. QC for *ITS2* rDNA amplicons: Prior to primer sequence removal, only sequences containing a >90% match to the forward and a >70% match to the reverse primer sequences were accepted. In addition, sequences shorter than 125 bp, or sequences of low quality with a mean quality score <20 or bases with a score <10 were discarded. Sequences passing QC were clustered into operational taxonomic units (OTUs) using a 99.5% similarity cut-off for *18S* and 99% cut-off for *ITS2* amplicons and 95% coverage for aligned sequences. The chosen cut-offs were found appropriate to separate sequences from the *18S* amplicons into OTUs, with genera as the finest taxonomic resolution, while *ITS2* amplicon sequences allowed a separation of most OTUs at the species level resolution, due to a higher degree of interspecies variation in this target. A slightly lower similarity cut-off = 98.5% is commonly applied to *ITS2* rDNA data subjected to single linkage clustering in the SCATA pipeline (Ihrmark et al., 2012; Karlsson et al., 2014). Additional settings were as follows: Mismatch penalty = 1, gap-open penalty = 10 for *18S* amplicons and 0 for *ITS2* amplicons, gap-extension = 1, end-gap weight = 0, homopolymer collapse to 3 bp for *ITS2* and 5 bp for *18S*. Usearch was used as cluster engine and singletons were removed. In addition to removal of singletons, an operational limit of detection for OTUs was set to 0.1% of the total clustered rDNA reads.

2.6. Taxonomic assignment of individual OTUs

Representative OTUs (most abundant sequence from each OTU) from all *18S* amplicons were taxonomically assigned to phylum level (Table 2). The OTUs from the *18S* amplicon from seedlings were further assigned to genus level, or to the finest possible taxonomic level, by comparison to sequence accessions in GenBank (http://www.ncbi.nlm.nih.gov/) with ≥99% sequence identity to the representative sequences. Taxonomic assignment was also made according to the SILVA SSU database for *18S* sequences (http://www.arb-silva.de/browser/) (Yilmaz et al., 2014) (Table 3). Representative OTUs from *ITS2* rDNA amplicons were taxonomically assigned to the finest possible taxonomic level by sequence identity ≥98% to accessions from type culture isolates (Table 5). In case sequence accessions from type strains were not available, representative OTUs were compared to sequence accessions from isolates described in peer-reviewed journals (Table 5). Annotation of *ITS2* sequences by type and reference isolates in GenBank was further consolidated by additional designation to a '*species hypothesis*' (SH) accession number, generated through the UNITE fungal *ITS* database (https://unite.ut.ee/index.php; Kõljalg et al., 2013), (Table 5).

2.7. Biological isolation and molecular identification of vertically transmitted ascomycetes

More than 100 sorghum seeds from a pool of the eight seed samples collected in Northern Burkina Faso, were surface sterilized as described above, dried and sown on top of agar in test tubes: 1 seed per tube, each containing 20 ml solidified sterile bacteriological grade agar (6 g/L, SCHARLAU) including 1 × Hoagland's plant growth medium no. 2. basal salt mixture (SIGMA-ALDRICH H2395). Seeds were then incubated for germination in a climate chamber at

Table 1
Applied PCR primers. Primers used for pyrosequencing were fused with MID tags and sequencing adaptors.

Barcoding target	Forward fusion primer	Sequencing primer A	MID tag[a]	Specific primer sequence	References
18S	Pyro-18-F2	5′CGTATCGCCTCCCTCGCGCCATCAG	07,08, 40, 44	AGGATTGACAGATTGA′3	This study
ITS2 - ASCO	ASCO-F1	5′CGTATCGCCTCCCTCGCGCCATCAG	22,23, 26, 28	GCCTGTTCGAGCGTCATT′3	Louarn et al. (2013)
Barcoding target	**Reverse fusion primer**	**Sequencing primer B**		**Specific primer sequence**	
18S	Pyro-18-R2a	5′CTATGCGCCTTGCCAGCCCGCTCAG		GCAGGGACGTAATC′3	This study
18S	Pyro-18-R2e	5′CTATGCGCCTTGCCAGCCCGCTCAG		GTCAGGGACGTAATC′3	This study
18S	Pyro-18-R2g	5′CTATGCGCCTTGCCAGCCCGCTCAG		GTAGTCAACGCAAG′3	This study
18S	Pyro-18-R2h	5′CTATGCGCCTTGCCAGCCCGCTCAG		GCAGGGACGTATTC′3	This study
ITS2 - ASCO	FITS-R1c	5′CTATGCGCCTTGCCAGCCCGCTCAG		CCTACCTGATYCGAGGTCAA′3	Modified after Louarn et al. (2013)
ITS2 - ASCO	FITS-R1d	5′CTATGCGCCTTGCCAGCCCGCTCAG		CCTACCTGATCYGAGGTCAA′3	Modified after Louarn et al. (2013)
Single spore amplicon	**Forward primer**	**Primer sequence**			
ITS1-5.8S-ITS2	ITS1-F	5′CTTGGTCATTTAGAGGAAGTAA′3			Gardes and Bruns (1993)
TEF-1α	EF1	5′ATGGGTAAGGARGACAAGAC′3			Geiser et al. (2004)
Single spore amplicon	**Reverse primer**	**Primer sequence**			
ITS1-5.8S-ITS2	ITS4	5′GCATATCAATAAGCGGAGGA′3			White et al. (1990)
TEF-1α	EF2	5′GGARGTACCAGTSATCATGTT′3			Geiser et al. (2004)

[a] MID-07: CGTGTCTCTA; MID-08: CTCGCGTGTC; MID-22: TACGAGTATG; MID-23: TACTCTCGTG; MID-26: ACATACGCGT; MID-28: ACTACTATGT; MID-40: TACGCTGTCT; MID-44: TCTAGCGACT.

Table 2
18S rDNA from pools of DNA extracted from seeds and seedlings.

	Seeds	Seedlings	Roots (control)
	Surface sterilized	7-day old, grown *in vitro* from surface sterilized seeds	From 3 month old field plants
Total rDNA reads	2053	2319	2508
Reads [excluding plant reads and OTUs <0.1% of total reads]	1738	1965	1947
Phylum distribution%			
Ascomycota	99.4	100	71
Basidiomycota	0	0	27.8
Nematoda	0	0	1.2
Arthropoda	0.6	0	0
Total%	100	100	100

Table 3
Taxonomic assignment of representative *18S* OTUs from the community of ascomycetes in seedlings of sorghum, according to SILVA taxonomy and GenBank reference accessions.

Taxonomic assignment of *18S* OTUs	OTU accession	SILVA SSU r121 taxonomy	GenBank reference	Identity to OTU sequence (%)	Reference paper
Epicoccum	KJ584944	*Phoma*	EU167568	100	Simon et al. (2009)
Fusarium	KJ584946	*Hypocreales*	JF807401	100	Gao et al. (2011)
Curvularia	KJ584945	*Pleosporales*	DQ677995	100	Schoch et al. (2006)
Cladosporium	KJ584952	*Cladosporium*	AY251091	100	Braun et al. (2003)
Gloeocercospora	KR024567	*Microdochium*	AF548077	100	Wu et al. (2003)
Aureobasidium	KJ584947	*Aureobasidium*	EU682922	100	Loncaric et al. (2009)
Lasiodiplodia	KU856660	*Botryosphaeriaceae*	JX646832	100	Liu et al. (2012)
Colletotrichum	KR024568	*Colletotrichum*	AJ301942	99	Nirenberg et al. (2002)
Nigrospora	KJ584948	*Trichosphaeriales*	AB220234	99	Ogawa et al. (2007)[a]

[a] Available in public databases, but not in a peer-reviewed journal.

25 °C with light/dark ratio of 14/10 h using a continuous photon flux density of 58–65 μmol s^{-1} m^{-1} photosynthetically active radiation (PAR) for 7 days. Stems and primary roots were surface sterilized, washed and plated on PDA (including streptomycin), with each stem and root cut in 2 pieces and each plate containing stem or root pieces from four plants. Plates with stems and roots were incubated under near UV light for 5–7 days to allow vertically transmitted filamentous fungi to grow out from the plant tissue. Single spore isolates were sub-cultured and provisionally identified morphologically. For molecular species identification, selected isolates were subjected to DNA purification and PCR amplification of the entire *ITS1-5.8S-ITS2* (*ITS*) rDNA region by application of the ITS1-F (Gardes and Bruns, 1993) and ITS4 primers (White et al., 1990; Table 1). Since not all *Fusarium* spp. are equally well-separated in the *ITS* locus, all isolates of *Fusarium* were additionally subjected to sequencing of the translation elongation factor 1-α (*TEF-1α*), using the EF1 and EF2 primers (Geiser et al., 2004; Table 1). PCR for amplification of both *ITS* and *TEF-1α* was conducted essentially as above with 2 μM of each forward and reverse primer. The PCR amplification program was as follows: 3 min initial DNA denaturation at 95 °C, followed by 35 cycles of 30 s at 94 °C, 30 s primer annealing at 52 °C for *ITS* and 53 °C for *TEF-1α*, 40 s extension at 72 °C and 3 min final extension at 72 °C. PCR products were subjected to gel electrophoresis and amplicons of ~600 bp (*ITS*) and ~700 bp (*TEF-1α*) were excised and purified, as described above. The DNA concentration of the purified PCR products was adjusted to 5 ng/μl and Value read sequenced at Eurofins, using either the forward primer of *ITS* or *TEF-1α*.

2.8. Distribution of Epicoccum sorghinum in seedlings

An *in vitro* test tube assay was set up, as described above: At least 100 sorghum seeds from each of four seed samples, including a pool of the eight seed samples from Northern Burkina Faso, as well as the three individual seed samples from Pobé-Mengao, Kindi and Diapangou, were surface sterilized, dried and sown on top of agar in test tubes, followed by incubation in a climate chamber for seven days, as described above. At harvest, fresh weight of shoots was registered. Plants were then divided into four different pools, based on shoot fresh-weight category: 1) <20 mg, 2) 20–60 mg, 3) 60–90 mg, 4) >90 mg. Stems and primary roots from category 2–4 were surface sterilized, washed and plated on PDA (including streptomycin), with each stem and root cut into two pieces and each plate containing stem or root pieces from four plants. Plates with stems and roots were incubated under near UV light for 5–7 days prior to morphological identification. *Epicoccum sorghinum* isolates were readily identified as the most commonly occurring species and quantified in relation to presence or absence on each stem or root. Other filamentous fungi were quantified as one group. However, to verify the distribution of different species among these other filamentous fungi, representative isolates were molecularly identified, as described above.

An *in soil* assay was set up as follows: 2 × 96 seeds, from a pool of the eight seed samples from Northern Burkina Faso, were surface sterilized and sown in 2 × 8 pots with sterilized soil (12 seeds/pot). Plants were harvested after 3 weeks, washed to remove soil from roots, and divided into three shoot-weight categories: 1) <150 mg, 2) 150–190 mg, 3) >190 mg. Stems and roots were surface sterilized, as described above with 1.5% hypochlorite and plated on PDA to determine the proportion of stems and roots harboring *Epicoccum* sp. and other filamentous fungi. This procedure was repeated with 2 × 96 seeds from Diapangou (Central Burkina Faso), with the only difference being the shoot-weight categories, which were: 1) <200 mg, 2) 200–300 mg, 3) >300 mg, in order to get an approximate equal distribution of plants in each weight category. The differential distribution of *Epicoccum* in stems and roots from different shoot-weight categories was tested by a X^2-test (Chi-square test) for contingency tables, using the software PAST (Hammer et al., 2001).

Table 4
ITS2 rDNA of ascomycete species vertically transmitted in sorghum from seeds to seedlings, with taxonomic assignment of OTUs and distribution shown in % of total reads. 454-pyrosequencing data from *ITS2* amplicons was clustered in SCATA with a 99% similarity cut-off.

Representative *ITS2* sequences[a,b]			Ascomycete DNA in % of total reads	
OTU ID	OTU accessions	Taxonomic assignment	Seeds	Seedlings
			n[c] = 4253	n[c] = 4220
Epicoccum				
Epi-01	KF218620	*Epicoccum sorghinum*[d]	14.48	48.96
Fusarium				
Fus-01	KF218624	*Fusarium thapsinum*[d]	4.21	20.92
Fus-03	KF218629	*Fusarium equiseti*[d]	0.45	0.66
Fus-02	KF218625	*Fusarium pseudonygamai*[d]	4.09	0.05
Fus-05	KR024557	*Fusarium proliferatum*	0.75	0
Curvularia				
Curv-Bi-02	KF218632	*Curvularia lunata var. aeria*[d]	9.48	5.71
Curv-Bi-04	KF218639	*Curvularia alcornii*[d]	3.97	5.26
Curv-Bi-01	KF218640	*Curvularia lunata*[d]	14.01	5.02
Curv-Bi-03	KF218633	*Curvularia intermedia*[d]	6.61	1.52
Curv-Bi-10	KF218644	*Curvularia asianensis*	0	0.24
Curv-Bi-09	KF218643	*Curvularia heteropogonis*	0	0.21
Curv-Bi-16	KR024558	*Curvularia specifera*	1.25	0.02
Curv-Bi-05	KF218635	*Curvularia eragrostidis*[d]	1.69	0
Curv-Bi-17	KR024559	*Curvularia alcornii*	1.62	0
Curv-Bi-06	KF218636	*Curvularia kusanoi*	1.55	0
Exserohilum				
Exs-01	KF218607	*Exserohilum rostratum*[d]	9.24	5.26
Alternaria				
Alt-01	KR024560	*Alternaria longissima*	0.87	2.44
Peyronellaea				
Pey-01	KF218621	*Peyronellaea zeae-maydis*	0.12	0.9
Cladosporium				
Cla-01	KF218608	*Cladosporium cladosporioides*	1.67	0.36
Cla-02	KR024561	*Cladosporium herbarum*[d]	1.67	0.12
Gloeocercospora				
Glo-01	KF218609	*Gloeocercospora sorghi*	0.33	0.4
Aureobasidium				
Aur-01	KF218617	*Aureobasidium pullulans*	0	0.36
Lasiodiplodia				
Las-01	KR024562	*Lasiodiplodia crassispora*	0	0.24
Colletotrichum				
Col-01	KF218610	*Colletotrichum sublineola*	1.95	0.12
Aspergillus				
Asp-01	KJ584937	*Aspergillus flavus*	1.67	0.05
Asp-02	KF218611	*Aspergillus cibarius*	4.16	0
Asp-03	KR024563	*Aspergillus aurantiobrunneus*	2.96	0
Phomopsis				
Phom-01	KF218612	*Phomopsis vexans*	3.74	0.02
Nigrospora				
Nig-01	KF218613	*Nigrospora oryzae*[d]	0.12	0
Pseudofusicoccum				
Pseu-01	KR024564	*Pseudofusicoccum olivaceum*	0.28	0
Trichothecium				
Tri-01	KR024565	*Trichothecium roseum*	1.67	0
Eremothecium				
Ere-01	KJ584936	*Eremothecium coryli*	3.36	0
Saccharomyces				
Sac-01	KR024566	*Saccharomyces cerevisiae*	1.01	0
Ascomycetes				
Other Ascomycetes			1.01	1.16
Sum			100	100

[a] OTUs ordered according to abundance of genera in seedlings.
[b] Presence of OTU recorded if constituting >0.1% of total reads from seed and seedling pools.
[c] Total reads after quality trimming and subtraction of singletons.
[d] Also identified by *ITS* rDNA sequencing of single-spore isolates from surface sterilized seedlings.

2.9. *Pathogenicity testing of* Epicoccum sorghinum *and* Fusarium thapsinum *in sorghum*

An *in vitro* pathogenicity assay was performed testing two representative vertically transmitted isolates from *E. sorghinum* (EpSo 2 and EpSo 3) and *F. thapsinum* (Fus 11 and Fus 12). The pathogenicity assay, previously described by Leslie et al. (2005), was used with the following modifications: Sorghum seeds from a seed sample (49.071) from the INERA Research Centre in Burkina Faso were heat treated in a water bath at 55 °C for 40 min, in order to reduce the natural inoculum of seed-borne fungi. Seeds were dried overnight before sowing on top of agar in test tubes, as described above. One day after sowing, each seed was inoculated with 250 μl of sterilized water as control or 250 μl of 2×10^4 spores/ml from either of the four fungal isolates. Plants were incubated in a climate chamber at 25 °C with light/dark ratio of 14/10 h using a continuous photon flux density of 58–65 μmol s^{-1} m^{-1} photosynthetically active radiation (PAR) for 15 days. Plant growth was visually assessed 11 days

Table 5
Taxonomic assignment of representative *ITS2* OTUs according to GenBank reference sequences from type cultures, where possible, as well as to UNITE SH clusters.

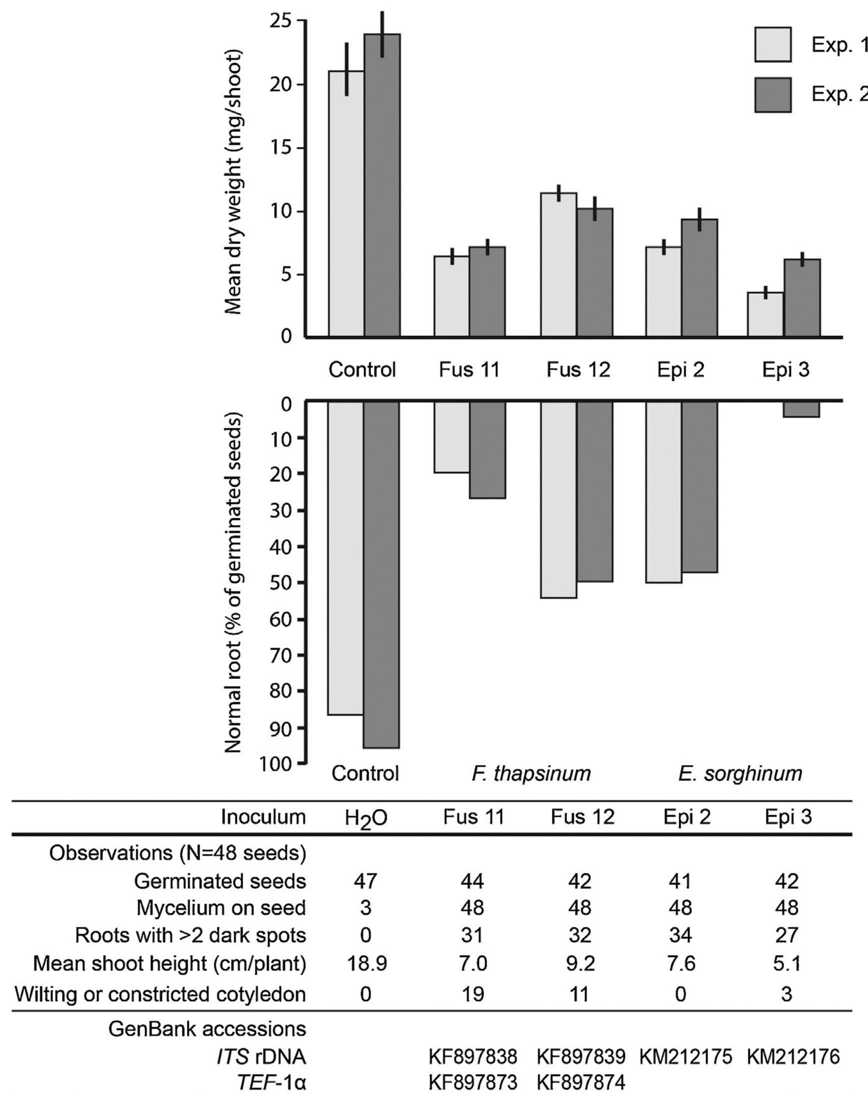
Representative *ITS2* sequences							
OTU ID	OTU accession	Reference species	Reference isolate	GenBank reference	Identity to OTU sequence (%)	UNITE SH cluster	Reference paper
Epicoccum							
Epi-01	KF218620	*Epicoccum sorghinum*	SA05_01	EU626192	100	SH202146.06FU	Pažoutová (2009)
Fusarium							
Fus-01	KF218624	*Fusarium thapsinum*	NRRL 22045	U34560	99	SH063008.06FU	O'Donnell and Cigelnik (1997)
Fus-03	KF218629	*Fusarium equiseti*	NRRL 26419	GQ505688[a]	99	SH004348.06FU	O'Donnell et al. (2009)
Fus-02	KF218625	*Fusarium pseudonygamai*	NRRL 13592	U34563[a]	99	SH289467.06FU	Nirenberg and O'Donnell (1998)
Fus-05	KR024557	*Fusarium proliferatum*	CBS 216.76	AB587006[a]	99	SH219673.07FU	Watanabe et al. (2011)
Curvularia							
Curv-Bi-02	KF218632	*Curvularia lunata var. aeria*	CBS 294.61	HE861850[a]	98	SH224799.06FU	da Cunha et al. (2013)
Curv-Bi-04	KF218639	*Curvularia alcornii*	MFLUCC 10-0703	JX256420[a]	98	SH187563.07FU	Manamgoda et al. (2012)
Curv-Bi-01	KF218640	*Curvularia lunata*	CBS 730.96	JX256429[a]	99	SH224802.06FU	Manamgoda et al. (2012)
Curv-Bi-03	KF218633	*Curvularia intermedia*	CBS 334.64	HE861853	99	SH224819.06FU	da Cunha et al. (2013)
Curv-Bi-10	KF218644	*Curvularia asianensis*	MFLUCC 10-0711	JX256424[a]	100	SH215499.06FU	Manamgoda et al. (2012)
Curv-Bi-09	KF218643	*Curvularia heteropogonis*	CBS 284.91	JN192379[a]	98	SH187563.07FU	Manamgoda et al. (2011)
Curv-Bi-16	KR024558	*Curvularia specifera*	CBS 274.52	JN192387	95	SH187563.07FU	Manamgoda et al. (2012)
Curv-Bi-05	KF218635	*Curvularia eragrostidis*	NBRC 32566	JN943448	99	SH224806.06FU	Schoch et al. (2006)
Curv-Bi-17	KR024559	*Curvularia alcornii*	MFLUCC 10-0703	JX256420[a]	100	SH187563.07FU	Manamgoda et al. (2012)
Curv-Bi-06	KF218636	*Curvularia kusanoi*	GRMP-25	JQ818178	99	SH224836.06FU	Gokulraj et al. (2014)
Exserohilum							
Exs-01	KF218607	*Exserohilum rostratum*	CBS 467.75	HE664034[a]	99	SH025405.06FU	da Cunha et al. (2012)
Alternaria							
Alt-01	KR024560	*Alternaria longissima*	ATCC 18552	AF229489	100	SH192117.07FU	Pryor and Gilbertson (2000)
Peyronellaea							
Pey-01	KF218621	*Peyronellaea zeae-maydis*	CBS 588.69	FJ427086[a]	100	SH100149.06FU	Aveskamp et al. (2010)
Cladosporium							
Cla-01	KF218608	*Cladosporium cladosporioides*	CBS 112.388	HM148003[a]	99	SH093668.06FU	Bensch et al. (2010)
Cla-02	KR024561	*Cladosporium herbarum*	CBS 121.621	EF679363[a]	100	SH253764.06FU	Schubert et al. (2007)
Gloeocercospora							
Glo-01	KF218609	*Gloeocercospora sorghi*	NBRC 7266	LC063851	100	SH026820.07FU	Ban et al. (2015)[b]
Aureobasidium							
Aur-01	KF218617	*Aureobasidium pullulans*	CBS 105.22	FJ150886[a]	100	SH270544.06FU	Zalar et al. (2008)
Lasiodiplodia							
Las-01	KR024562	*Lasiodiplodia crassispora*	CBS 118741	DQ103550[a]	99	SH272199.06FU	Burgess et al. (2006)
Colletotrichum							
Col-01	KF218610	*Colletotrichum sublineola*	CBS 131301	JQ005771[a]	100	SH310052.06FU	O'Connell et al. (2012)
Aspergillus							
Asp-01	KJ584937	*Aspergillus flavus*	ATCC 16883	AF138287[a]	99	SH216041.07FU	Henry et al. (2000)
Asp-02	KF218611	*Aspergillus cibarius*	KACC 46346	JQ918177[a]	99	SH179237.07FU	Hong et al. (2012)
Asp-03	KR024563	*Aspergillus aurantiobrunneus*	NRRL 4545	EF652465[a]	99	SH206406.06FU	Peterson (2008)
Phomopsis							
Phom-01	KF218612	*Phomopsis vexans*	CBS 127.14	KC343229	100	SH016945.06FU	Gomes et al. (2013)
Nigrospora							
Nig-01	KF218613	*Nigrospora oryzae*	NRRL 54030	GQ328855	95	SH177637.07FU	Sarkar et al. (2013)
Pseudofusicoccum							
Pseu-01	KR024564	*Pseudofusicoccum olivaceum*	CBS 124939	FJ888459[a]	99	SH320713.06FU	Mehl et al. (2011)
Trichothecium							
Tri-01	KR024565	*Trichothecium roseum*	CBS 113.334	EU552162	99	SH223989.06FU	Marincowitz et al. (2008)
Eremothecium							
Ere-01	KJ584936	*Eremothecium coryli*	CID1339	EF682106	98	SH235559.06FU	Shipunov et al. (2008)
Saccharomyces							
Sac-01	KR024566	*Saccharomyces cerevisiae*	CBS 1171	AB018043	99	SH241331.06FU	Sugita et al. (1999)

[a] Accession from type culture.
[b] Available in public databases, but not in a peer-reviewed journal.

Fig. 1. Distribution of OTUs in DNA pools from seeds and seedlings from Northern Burkina Faso, divided into A: the eight largest OTUs identified from seedlings and B: the remaining OTUs constituting <1% read abundance in seedlings. Values (% rDNA reads) represent OTU size in% of total reads for each pool. " + " indicate occurrence of species in individual and independent seed samples from Northern Burkina Faso: Pobé-Mengao (x) and from Central Burkina Faso: Kindi (y) and Diapangou (z). "●" indicate sequenced single spore isolates of fungal species transmitted to seedlings.

Fig. 2. Three-week-old sorghum seedlings grown in soil from surface sterilized seeds, from a pool of eight seed accessions from Northern Burkina Faso. Representative whole seedlings were divided into small, medium and large plants, according to estimated shoot weight.

post inoculation (dpi) by recording of germination, shoot height and root growth. Normal or abnormal root development was determined for each plant, where a normal root was identified by the presence of a primary root >4 cm with lateral roots >1 cm. In addition, the presence or absence of disease symptoms on each plant was assessed 11 dpi, including mycelium on seeds, dark necrotic spots on roots as well as wilting and/or constriction of cotyledons. At 15 dpi, plants were harvested and shoot dry weight was measured after drying at 70 °C for three days. The assay consisted of two biological repetitions with n = 24 seeds per treatment. Two-way



Inoculum	H_2O	Fus 11	Fus 12	Epi 2	Epi 3
Observations (N=48 seeds)					
Germinated seeds	47	44	42	41	42
Mycelium on seed	3	48	48	48	48
Roots with >2 dark spots	0	31	32	34	27
Mean shoot height (cm/plant)	18.9	7.0	9.2	7.6	5.1
Wilting or constricted cotyledon	0	19	11	0	3
GenBank accessions					
ITS rDNA		KF897838	KF897839	KM212175	KM212176
TEF-1α		KF897873	KF897874		

Fig. 3. Pathogenicity of *Epicoccum sorghinum* compared to *Fusarium thapsinum* in sorghum seedlings. Mean dry weight of sorghum shoots (mg/shoot) 15 days post inoculation (dpi) with vertically transmitted *F. thapsinum* and *E. sorghinum* isolates (Fus 11, Fus 12 and Epi 2, Epi 3). Normal root development (% of germinated seeds) was recorded 11 dpi, together with observations of germination, shoot length, and disease symptoms. Data from two independent experiments with n = 24 seeds/repeat is shown with means of dry weight ± SEM. There was no difference in shoot dry weight between repetitions of the same treatment. Multiple comparison of dry weight means showed a significant difference between treatments and water control ($P < 0.001$), and normal root development was significantly inhibited in inoculated plants compared to control ($P < 0.001$; X^2-test).

analysis of variance (ANOVA) was performed on the resulting dry weights. The analysis was done using the lm() function in the statistical software R version 3.2.1 (R Core Team, 2015). Further, multiple comparisons were done using the multicomp package applying the Tukey method (Hothorn et al., 2008). Similarity of binomial root development (normal/abnormal) between groups was tested by a X^2-test, using the software PAST (Hammer et al., 2001). Koch's postulates were tested by inoculation of the two different isolates of *F. thapsinum* and *E. sorghinum* to seeds, as described above, followed by re-isolation from necrotic root tissues in seedlings. Re-isolated fungi were tested by re-inoculation to sorghum seeds and verified by *ITS*-sequencing, as described above.

3. Results

3.1. 18S *rDNA analysis of seeds and seedlings from pooled DNA samples*

Surface sterilized seeds harvested from eight different villages in Northern Burkina Faso, and 7-day-old, healthy appearing seedlings thereof, were subjected to *18S* rDNA PCR amplification and 454-pyrosequencing. Root material from field-grown sorghum was included as a control containing both ascomycetes and basidiomycetes. The seed and seedling pools yielded 2053 and 2319 reads, respectively, after sequence trimming, quality control and subtraction of singletons. Operational taxonomic units (OTUs) of non-plant origin, constituting >0.1% of total reads, were after cluster analysis assigned to genus level and categorized into four major phyla: *Ascomycota*, *Basidiomycota*, *Nematoda* and *Arthropoda* (Table 2). The phylum *Ascomycota* was found to constitute more than 99% of the amplified rDNA from both seeds and seedlings. Representative sequences of *18S* OTUs from the phylum of ascomycetes in seedlings are shown in Table 3.

3.2. ITS2 *rDNA profiling of ascomycetes in seeds and seedlings*

To increase the taxonomic resolution of ascomycetes present in sorghum, the DNA pools from seeds and seedlings were subsequently subjected to ascomycete specific amplification of *ITS2*

rDNA (Table 4). From seeds and seedlings, a total of 33 OTUs each constituting >0.1% of total reads were identified: 29 OTUs from seeds and 18 OTUs from seedlings (Table 4). The eight largest OTUs identified from seedlings accounted as a group for 95% of the total seedling reads. The corresponding representative OTU sequences were annotated, as described in materials and methods, to the following species (Table 5): *Epicoccum sorghinum*, *Fusarium thapsinum*, *Curvularia lunata* var. *aeria*, *Exserohilum rostratum*, *Curvularia alcornii*, *Curvularia lunata*, *Alternaria longissima* and *Curvularia intermedia*. Of these, *E. sorghinum* and *F. thapsinum* were the most dominating OTUs, accounting for 48 and 20% of the total number of reads, respectively (Fig. 1a). A large group of OTUs, including several saprotrophic species (e.g. *Aspergillus* spp. and *Eremothecium* sp. *coryli*) were easily detected in seeds, but were each found to constitute <0.1% in seedlings (Fig. 1b). The technical replicates of the *ITS2* rDNA amplicons confirmed the profiles of the ascomycetous community composition in the sorghum seed and seedling pools, as described above (data not shown). At the genus level, the distribution of *ITS2* rDNA OTUs observed in seedlings corresponded well with the OTU distribution of the *18S* rDNA amplicon (Table 6).

3.3. ITS2 *rDNA profiling of ascomycetes in individual seed samples*

ITS2 rDNA cluster analysis was performed as above on DNA extracted directly from three individual seed samples, one collected from Northern Burkina Faso and two from the Central region. The seed rDNA from Popé-Mengao, Kindi and Diapangou yielded 14086, 11572 and 10095 reads, respectively, after sequence trimming, quality control and subtraction of singletons. All eight dominant OTUs identified above, were also detected in each of these three seed samples, confirming their common distribution in the country (Fig. 1a). In addition, seven other OTUs found in the seedling pool above, were also represented in at least two of three independent seed samples, including: *Fusarium equiseti*, *F. pseudonygamai*, *F. proliferatum*, *Cladosporium cladosporioides, Cladosporium herbarum*, *Colletotrichum sublineola* and *Gloeocercospora sorghi* (Fig. 1b).

3.4. *Isolation and molecular identification of vertically transmitted ascomycetes*

From the seed samples collected in Northern Burkina Faso, a total of 58 vertically transmitted single spore fungal isolates were obtained from 7-day-old seedlings grown from surface sterilized seeds. Isolates were picked and sub-cultured from explants of roots and stems plated on PDA. Isolates were sequenced (*ITS* rDNA) and annotation of the resulting sequences was essentially done as described for the representative OTU sequences above. A total of 12 different ascomycetous species were identified, including seven out of eight taxa corresponding to the largest OTUs described above (Fig. 1A): *Epicoccum sorghinum* (KM212175, KM212176), *Fusarium thapsinum* (KF897838, KF897838), *Curvularia lunata* var. *aeria* (genotype A-B-AC: KU856647, KU856633, KU856632), *Exserohilum rostratum* (genotype 1–2: KU856642, KU856643), *Curvularia alcornii* (KU856619), *Curvularia lunata* (genotype 1–2: KU856628, KU856629) and *Curvularia intermedia* (genotype 1–2-3: KU856621, KU856622, KU856624). In addition, isolates belonging to the following species were obtained: *Fusarium equiseti* (KU856645), *Fusarium pseudonygamai* (KU856650), *Curvularia eragrostidis* (KU856617), *Cladosporium herbarum* (KU856614) and *Nigrospora sphaerica* (KU856654) (Fig. 1B). Specifically for isolates of *Fusarium*, the annotation of *ITS* rDNA sequences was confirmed by annotation of their corresponding *TEF-1α* sequences: *Fusarium thapsinum* (KF897873, KF897874), *Fusarium equiseti* (KU856656), and *Fusarium pseudonygamai* (KU856658).

3.5. *Natural distribution of* Epicoccum sorghinum *in seedlings*

From stems and primary roots of 444 sorghum seedlings, grown *in vitro* for 7 days, *E. sorghinum* was the most common single species isolated, in particular from roots ($P < 5 \times 10^{-7}$) and stems ($P < 8 \times 10^{-4}$) of poorly growing seedlings (shoot-weight 20–60 mg; (Table 7a). Isolates of other fungal species were not individually enumerated, but 11 different species were identified by sequencing of single-spore isolates, as described above. A different pattern was observed when sorghum was grown in sterile soil for 3 weeks (Table 7b), after which *E. sorghinum* could be isolated from 20% of roots, but only from 1% of stems ($P < 3 \times 10^{-8}$). Still, *E. sorghinum* was strongly associated with roots of poorly growing seedlings (shoot-weight <150 mg), of which 38% were infected with this fungus, while only 7% of roots from the largest plants (shoot-weight >190 mg) were infected with *E. sorghinum* ($P < 4 \times 10^{-5}$) (Fig. 2). The infection percentage in roots by other filamentous fungi as a group did not decrease with increasing shoot size of sorghum grown in soil during 3 weeks (Table 7b). A similar pattern was observed in seedlings grown from Diapangou seeds, in which 30% of roots from the smallest seedlings were infected with *E. sorghinum*, whereas only 10% of roots from the largest plants were infected with this pathogen ($P < 6.2 \times 10^{-2}$) (Table 7c; Fig. 2).

3.6. *Pathogenicity test of* Epicoccum sorghinum *and* Fusarium thapsinum

The apparent association of *E. sorghinum* with poorly growing plants was further investigated in an *in vitro* pathogenicity assay, by comparison to the well-known sorghum pathogen *F. thapsinum* (second largest OTU in the seedling *ITS2* rDNA amplicon). Two isolates of each species were obtained from seedlings and the species identity was confirmed by *ITS* rDNA sequencing of each isolate and additional sequencing of *TEF-1α* from the isolates of *F. thapsinum* (Fig. 3). Pathogenicity in seedlings was tested by inoculation of fungal spores to heat treated seeds (reduced natural inoculum) and subsequent analysis of seedlings grown in agar after 11 and 15 days (Fig. 3). The isolates of both species turned out to be highly pathogenic. A highly significant ($P < 0.001$) difference in shoot dry weight was found between control treatment (inoculation with pure water) and any of the groups of plants inoculated with fungal spores, which showed at least 40% reduction in shoot dry weight. Likewise, in all groups of plants inoculated with fungal spores, development of a normal root (primary root >4 cm with presence of lateral roots) was prevented in more than 45% of the plants, compared to the water treated control ($P < 0.001$; X^2-test). In addition, specific disease symptoms, such as dark spots on roots, were observed in more than 55% of plants germinating from inoculated seeds (Figs. 3 and 4). In particular, *E. sorghinum* caused extensive dark necrosis in roots as a typical symptom, whereas *Fusarium* isolates typically resulted in wilting or constriction of the cotyledon leaf in up to 63% of plants after reaching a shoot length above 5 cm (Figs. 3 and 4). Koch's postulates were fulfilled, since re-isolations of the two different isolates of *Fusarium* and *Epicoccum* from symptomatic tissue, followed by re-inoculation, resulted in identical symptoms as described above for the primary isolates. Sequencing of the *ITS* region of the secondary isolates confirmed their identity to the primary isolates (KX171658-KX171661). Furthermore, no filamentous fungi could be isolated from roots of mock-inoculated plants. Symptoms on roots from re-inoculated plants are shown in (Fig. 4c).

4. Discussion

In the present study, the vertically transmitted mycobiome of sorghum from Burkina Faso was for the first time investigated

by both culture dependent and culture-independent methods and by use of a fungal taxonomy solely based on DNA-barcodes. Ascomycetes, and in particular, *Epicoccum sorghinum* (previously *Phoma sorghinum*, Aveskamp et al., 2010), were found to dominate the vertically transmitted mycoflora, irrespective of the methodology applied. New and statistically robust evidence of *E. sorghinum* occurring as a root pathogen in seedlings was further found, considering both a natural seed-borne inoculum and artificial inoculation under controlled conditions. The scientific context and perspectives of the findings are discussed below.

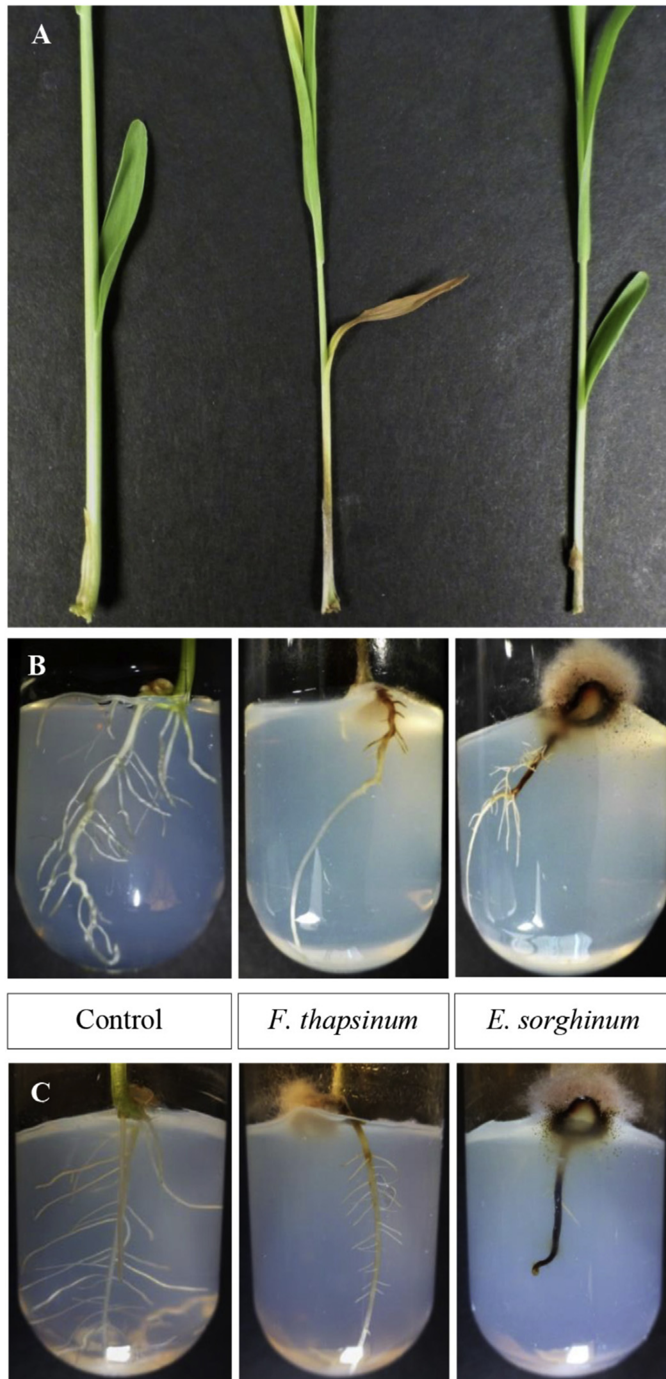


Fig. 4. Symptoms in sorghum shoots and roots after inoculation with *F. thapsinum* (Fus 12) and *E. sorghinum* (Epi 2) compared to water control (A–B), as well as symptoms in roots caused by corresponding re-isolates (C).

Table 6
Comparison of ascomycete profiles in sorghum seedlings, measured by 454-pyrosequencing of *ITS2* and *18S*[a] amplicons.

Ranked after *ITS2* rDNA sequence abundance	OTUs in % of total reads	
	DNA in seedlings	
	n[b] = 4220	n[b] = 1965
Taxonomic assignment to genus	*ITS2*[c]	*18S*[d]
Epicoccum + *Alternaria*[e]	51.4	53.0
Fusarium	22.0	22.7
Curvularia + *Exserohilum*[e]	23.2	22.7
Cladosporium	0.5	0.3
Gloeocercospora	0.4	0.4
Aureobasidium	0.4	0.2
Lasiodiplodia	0.2	0.2
Colletotrichum	0.1	0.5
Nigrospora	0.0	0.1
Other ascomycetes	1.8	0.0
Total% of ascomycetes	100	100

[a] Clustered in SCATA v. 2015 with 99% and 99.5% similarity threshold for *ITS2* and *18S* amplicons, respectively.
[b] Total reads after quality trimming and subtraction of singletons.
[c] *ITS2* rDNA represent sum of OTUs/genera.
[d] *18S* amplicon = only reads belonging to ascomycetous DNA.
[e] These genera cannot be reliably separated in *18S*, due to low sequence variability.

4.1. *DNA barcoding of* Sorghum mycoflora

A majority of the total of 33 species identified here in seeds and/or in seedlings by DNA barcoding has previously been reported from morphologically based surveys of the sorghum mycoflora in Burkina Faso (Zida et al., 2008, 2014). In comparison to the previous studies, the *ITS2* rDNA barcoding applied in this study allowed a dissection of the *Gibberella fujikuroi* species complex (Leslie et al., 2005) into *F. thapsinum*, *F. pseudonygamai* and *F. proliferatum*. Of these three species, only *F. thapsinum* has previously been identified in sorghum material from Burkina Faso (Andresen et al., 2015). *F. thapsinum* was first described by Klittich et al. (1997) and is globally regarded as a common seed-borne pathogen of sorghum (Frederiksen 2000; Bandyopadhyay et al., 2002). *F. equiseti* was detected with low sequence abundance as the only *Fusarium* species outside of the *Gibberella fujikuroi* species complex. However, *F. equiseti* was recently found to be a major species among ascomycetes detected in roots of mature field-grown sorghum plants (Zida et al., 2016). A high diversity of the genus *Curvularia* was reported both in the present and in previous studies, including *C. lunata* and *C. eragrostidis* (Zida et al., 2008). Three *Curvularia* species, *C. lunata* var *aeria*, *C. alcornii* and *C. intermedia*, found in the present study are new to Burkina Faso and were all found by both amplicon sequencing (including individual seed samples) and by isolation of single spore isolates. A high diversity of *Curvularia* species in the seed mycoflora of sorghum has also been reported from India (Girish et al., 2011). Species belonging to the closely related genus *Bipolaris* have previously been found in several studies of the sorghum seed mycoflora (Zida et al., 2008; Girish et al., 2011). However, many *Bipolaris* species have taxonomically been re-assigned to the genus *Curvularia* (teleomorph: *Cochliobulus*) following a re-organization of the *Curvularia-Bipolaris* complex, based on molecular phylogeny (Manamgoda et al., 2012). This might explain why DNA barcodes corresponding to species of *Bipolaris* were not encountered in the present study.

4.2. *Vertically transmitted species*

Both the *18S* and *ITS2* barcoding targets resulted in almost the same distribution of fungal OTUs at the genus level in seedlings (Table 6). However, for the analysis at species level, only *ITS2* bar-

Table 7a
Evaluation of fungi from 7-day-old seedlings (root and stem) grown *in vitro*.

	Sorghum grown 7 days *in vitro*[a]							
Shoot weight (mg)	20-60	60-90	>90	20-60	60-90	>90	Total	
Transmission of fungi from seeds		Root			Stem		Root	Stem
Plants[b]	95	177	172	95	177	172	444	444
Plants infected with *E. sorghinum* (%)	17.9	6.8	1.2	15.8	5.1	4.1	7.0	7.0
P-value for *E. sorghinum*[c]		5×10^{-7}			8×10^{-4}		ns	
Plants infected with other fungi (%)	13.7	7.9	0.6	24.2	15.8	4.7	6.3	13.3

[a] Results from a pool of eight seed accessions and individual seed samples from Pobé-Mengao, Kindi and Diapangou.
[b] Seedlings surface sterilized with 2% hypochlorite.
[c] X^2-value for contingency test of the *E. sorghinum* distribution.

Table 7b
Evaluation of fungi from 3-week-old seedlings (root and stem) grown in soil from a pool of eight seed accessions from Northern Burkina Faso.

	Sorghum grown 3 weeks in soil							
Shoot weight (mg)	<150	150-190	>190	<150	150-190	>190	Total	
Transmission of fungi from seeds		Root			Stem		Root	Stem
Plants[a]	60	49	56	60	49	56	165	165
Plants infected with *E. sorghinum* (%)	38.3	12.2	7.1	1.7	0.0	1.8	20.0	1.2
P-value for *E. sorghinum*[b]		4×10^{-5}			ns		3×10^{-8}	
Plants infected with other fungi (%)	30.0	20.4	41.1	11.7	4.1	1.8	30.9	6.1

[a] Seedlings surface sterilized with 1.5% hypochlorite.
[b] X^2-value for contingency test of the *E. sorghinum* distribution.

Table 7c
Evaluation of fungi from 3-week-old seedlings (root and stem) grown in soil from seeds collected in Diapangou (Central Burkina Faso).

Shoot weight (mg)	<200	200-300	>300	<200	200-300	>300	Total	
Transmission of fungi from seeds		Root			Stem		Root	Stem
Plants[a]	60	58	38	60	58	38	156	156
Plants infected with *E. sorghinum* (%)	30.0	19.0	10.5	0.0	1.7	2.6	21.2	1.3
P-value for *E. sorghinum*[b]		6.2×10^{-2}			ns		7×10^{-9}	
Plants infected with other fungi (%)	21.7	24.1	10.5	5.0	10.3	2.6	19.9	6.4

[a] Seedlings surface sterilized with 1.5% hypochlorite.
[b] X^2-value for contingency test of the *E. sorghinum* distribution.

coding was used, due to a higher inter-species sequence diversity within this amplicon.

Taking the differences between molecular and morphological taxonomy described above for the genera *Fusarium* and *Curvularia* into account, a high degree of correspondence exists between the most common, vertically transmitted species identified in the present study by molecular methods, compared to the species morphologically identified as common, seed-borne fungi in the previous study by Zida et al. (2008). Nevertheless, a comparative quantification of the seedling mycoflora, transmitted from a natural inoculum in seeds, has to our knowledge not previously been conducted, and the present finding of *E. sorghinum* as a predominant, naturally transmitted species is therefore new. This finding, obtained by both isolation of fungi and amplification of DNA, is on the other hand likely to depend on the geographical origin of the analyzed plant material, since *E. sorghinum* is known to be highly common on seeds of sorghum particularly in Burkina Faso and other countries of Western Africa: Ghana and Cameroon (Mathur and Manandhar, 2003).

4.3. Pathogenicity of E. sorghinum

Of the two species tested in the present work, *F. thapsinum* and *E. sorghinum*, the first has already been described as a seedling pathogen of sorghum using the same *in vitro* assay as in Leslie et al. (2005). In addition, our results fully confirm the previous observations of root pathogenicity (crown rot) and early wilting associated with *F. thapsinum* (Andresen et al., 2015). *Epicoccum* or *Phoma* spp. have been known to infect sorghum for almost 100 years (Koch and Rumbold, 1921) and in particular *E. sorghinum* has been frequently associated with the sorghum grain mold complex (Forbes et al., 1992; Bandyopadhyay et al., 2000; Frederiksen, 2000). However, to our knowledge no specific symptoms of *E. sorghinum* have been shown before in seeds or seedlings of sorghum, except for the production of black pycnidia on the surface of infected seeds (Williams and Rao, 1980). Our present findings, supported by fulfillment of Koch's postulates, clearly demonstrate that *E. sorghinum* is the causal agent of the development of necrotic root lesions in sorghum seedlings (Fig. 3 and 4). It has previously been shown that *E. sorghinum* can be found both in the seed pericarb, the endosperm and in the embryo of naturally infected sorghum seeds (Bonzi et al., 2012), which explains both the capability of the fungus to survive surface sterilization of seeds and its ability to be efficiently transmitted to seedlings. The finding that a natural inoculum of *E. sorghinum* was more frequently isolated from roots of poorly growing sorghum plants in soil (Tables 7b and 7c, Fig. 2) indicates that a natural inoculum of *E. sorghinum* can exert a substantial pathogenic pressure in roots of young seedlings. Somewhat contrasting is that *E. sorghinum* (named as *Leptosphaeria sacchari*) recently was found to be the most frequently isolated endophytic fungal species from leaf sheath tissue of individual sorghum field plants that performed above average at the 3–5 leaf stage (Zida et al., 2014). In other plant species, *E. sorghinum* has been reported to occur either as a true endophyte (Borges and Pupo, 2006) or as a facultative pathogen (Fisher and Petrini, 1992). In the tropical grass crop, sugarcane, a growth promoting effect was recently demonstrated by

an endophytic interaction with another species of the genus *Epicoccum*: *E. nigrum* (Fávaro et al., 2012). Thus, despite the apparent pathogenic nature of *E. sorghinum* revealed in the present work, the plant-microbe interaction under field conditions remains to be investigated, including to which extent natural infection of *E. sorghinum* affects the growth of sorghum during the entire growing season.

4.4. Conclusions and perspectives

The molecular species identification applied in the present study has provided a more precise dissection of the diversity and composition of the seed transmitted mycobiome in sorghum in Western Africa, compared to previous morphologically based studies. The two most prominent taxa identified, *E. sorghinum* and *F. thapsinum* were both highly pathogenic when inoculated to seeds *in vitro* and it was demonstrated that *E. sorghinum* is a root pathogen of sorghum seedlings. Seed-borne fungal pathogens play a significant, yield-limiting role in sorghum production world-wide. The application of an antifungal seed treatment (thiram) has been found to increase both yield and emergence (Hasan, 2001; Girish et al., 2004) and specifically in Burkina Faso, the application of a binary pesticide, Calthio C, containing thiram was found to cause a yield increase of 25% (Zida et al., 2012). In comparison, an aqueous extract of the plant species *Eclipta alba* applied as seed treatment in the same study, resulted in an average yield increase of 17%. Interestingly, the extract of *Eclipta alba* was also found to confer antifungal activity particularly against *E. sorghinum*. The present findings of *E. sorghinum* as a potentially pathogenic, predominant, and vertically transmitted species in sorghum in Burkina Faso, together with the previous findings of the effects of the *Eclipta alba* seed treatment, generate a simple hypothesis suggesting *E. sorghinum* as a seed-borne, yield limiting pathogen in Burkina Faso. A rigorous testing hereof seems therefore highly justified. Another interesting aspect from this study that merits further investigation is: to verify whether some of the potentially non-pathogenic fungal isolates that were obtained, have biocontrol or plant growth promoting properties in sorghum, such as *Cladosporium herbarum*, which previously has been shown to increase yields in wheat (Singh and Kapoor, 1999).

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