



Mycoflora and mycotoxins associated with stored cowpea (*Vigna unguiculata* L. Walp) seeds from smallholder farmers in South Africa

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Abstract Cowpea (*Vigna unguiculata* (L.) Walp) is a valuable legume crop for many people in tropical and subtropical countries worldwide and serves as an inexpensive source of proteins for humans and animals. Although it is not considered a major commercial crop in South Africa, cowpea plays an important part in subsistence and smallholder farming systems. Cowpea seeds when stored under unfavourable conditions are susceptible to fungal infestation and subsequent contamination of harmful mycotoxins. There is limited information regarding the natural occurrence of mycotoxigenic fungi and mycotoxin contamination of cowpea seed in South Africa. Therefore, this study was conducted to investigate which fungi and mycotoxins are naturally present in cowpea seed, and to determine whether there is a potential health risk for the consumers. Forty-eight seed samples were collected from South African subsistence and smallholder farmers from

the main growing areas. Surface sterilised and non-sterilised seeds were plated onto potato dextrose agar (PDA), fungi were isolated and identified morphologically, and molecularly using Sanger sequencing and phylogenetic analyses. Mycotoxins were analysed using ultra-performance liquid chromatography (UPLC) – electrospray ionization (ESI) tandem mass spectrometry (MS/MS). In total, sixteen different fungal genera were identified at various incidence levels. All samples showed high incidence of fungi (up to 70%), with *Fusarium* spp. being the most prominent. No mycotoxins were detected in the seed samples. Selected fungal isolates did however produce mycotoxins on maize cultures, including fumonisin B₁ (0.014–1092,402 mg/kg), B₂ (0,030–247,906 mg/kg) and B₃ (0,026–54,010 mg/kg), ochratoxin A (0,053 – 0,042 mg/kg), zearalenone (0,036–0,277 mg/kg), deoxynivalenol (0.190–0.658 mg/kg) and nivalenol (4.510 – 25,099 mg/kg). This study confirmed a high incidence of potentially harmful mycotoxigenic fungi isolated from stored cowpea seed samples across the cowpea-producing areas of South Africa. Mycotoxin production by these fungi could pose a severe health risk to both humans and animals since cowpea is produced as a nutritious food source.

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Introduction

Cowpea (*Vigna unguiculata* (L.) Walp) is one of the most important legume crops in farming systems in Africa as it is an inexpensive source of protein and minerals for both humans and animals (Dakora & Belane, 2019; Nkoma et al., 2021). Other than being a nutritious food source, cowpea is planted in crop rotation with crops such as maize (*Zea mays* L.) and cereals, directly increasing the production of these crops by enhancing nitrogen concentrations in the soil through biological nitrogen fixation (Da Silva et al., 2018). Additionally, consuming cowpea can be beneficial to one's health as it has anti-diabetic, anti-cancer, anti-hyperlipidaemic, anti-inflammatory and anti-hypertensive properties (Jayathilake et al., 2018). South Africa produces around for thousand six hundred tonnes per year (FAOSTAT, 2022) mainly in the Limpopo, Northwest, Mpumalanga and KwaZulu-Natal provinces of South Africa with some reports of production in the Eastern Cape (Dzemo et al., 2010; Directorate Plant Production, 2011).

Production of cowpea is influenced negatively by abiotic factors such as poor soil health, improper cultivation practises and drought (Nkoma et al., 2021). Biotic factors such as insect pests, competing weeds, fungal, bacterial and viral diseases can also cause a significant reduction in yield and seed quality, thus resulting in postharvest losses (Taiwo et al., 2009; Kareem et al., 2018; Durojaye et al., 2019). A major limitation to cowpea production is when the seed is naturally infected with seed-borne pathogens (Ushamalini et al., 1998), which often occurs during poor storage of the harvested grain (Gyasi et al., 2022). The decrease in yields can be attributed to the planting of fungal-infected seeds which influences seed germination, seedling mortality and an increased level of diseased plants (Biemond et al., 2013). Fawole et al., (2005) showed that cowpea seeds inoculated with fungi such as *Aspergillus flavus*, *Aspergillus niger*, *Cladosporium* sp., *Alternaria* sp., *Fusarium semitectum*, *Fusarium oxysporum*, *Fusarium solani* and *Penicillium* sp. had a detrimental effect on seedling germination. In addition to germination being affected, seed discoloration, rotting, shrinking and necrosis can also occur due to the seeds being infected with fungi such as *A. flavus*, *A. niger*, *Alternaria* sp., *F. oxysporum*, *Fusarium equiseti*, *Penicillium* spp., and *Rhizopus stolonifer* (Ameer Junaithal Begum et al., 2013).

Studies of fungi associated with cowpea seed in South Africa are limited. Kritzinger et al. (2003) isolated various species including seven *Fusarium* species (*F. equiseti*, *F. chlamydosporum*, *F. graminearum*, *F. proliferatum*, *F. sambucinum*, *F. semitectum*, and *F. subglutinans*) and three *Aspergillus* species (*A. flavus*, *A. glaucus* and *A. niger*) and *Penicillium* sp. from seed of four cowpea cultivars from South Africa. Certain fungi belonging to these genera are known producers of harmful mycotoxins (Ashiq et al., 2014), which when ingested can be detrimental to human and animal life and even be fatal. Mycotoxins produced by *Fusarium* species include primarily trichothecenes (deoxynivalenol, T-2 toxin), fumonisins and zearalenone (Cortinovis et al., 2013). Furthermore *Aspergillus* spp. are known to produce mycotoxins such as aflatoxin, ochratoxin and citrinin (produced by *A. flavus*, *A. ochraceus* and *A. niger*, respectively). These toxins are also produced by some *Penicillium* species such as *P. viridicatum* and *P. citrinum* (Fleurat-Lessard, 2017; Pitt, 2000). These metabolites can also be detrimental to plants. Fumonisin have been shown to have phytotoxic effects on crops such as maize and tomato (*Solanum lycopersicum* L.) (Lamprecht et al., 1994), as well as on cowpea (Kotze et al., 2017; Kritzinger et al., 2006), while aflatoxin has been found to reduce germination of common bean (*Phaseolus vulgaris* L.), pigeon pea (*Cajanus cajan* L. Millsp.), mung bean (*Vigna radiata* L.) and urad bean (*Vigna mungo* L.) (Janardhan et al., 2011).

The aim of this study was to determine the extent of mycoflora contamination of stored cowpea seeds produced by smallholder farmers in South Africa. Some isolates were selected to determine if they have the potential to produce mycotoxins. In addition, seeds were screened to determine if mycotoxins were naturally present in the stored samples. Results from this study can increase awareness on the potential health risk, as well as production constraints that improper storage of cowpea seeds can result in.

Materials and methods

Collection of samples

Smallholder and subsistence farmers located in the main cowpea-producing regions of the Gauteng,

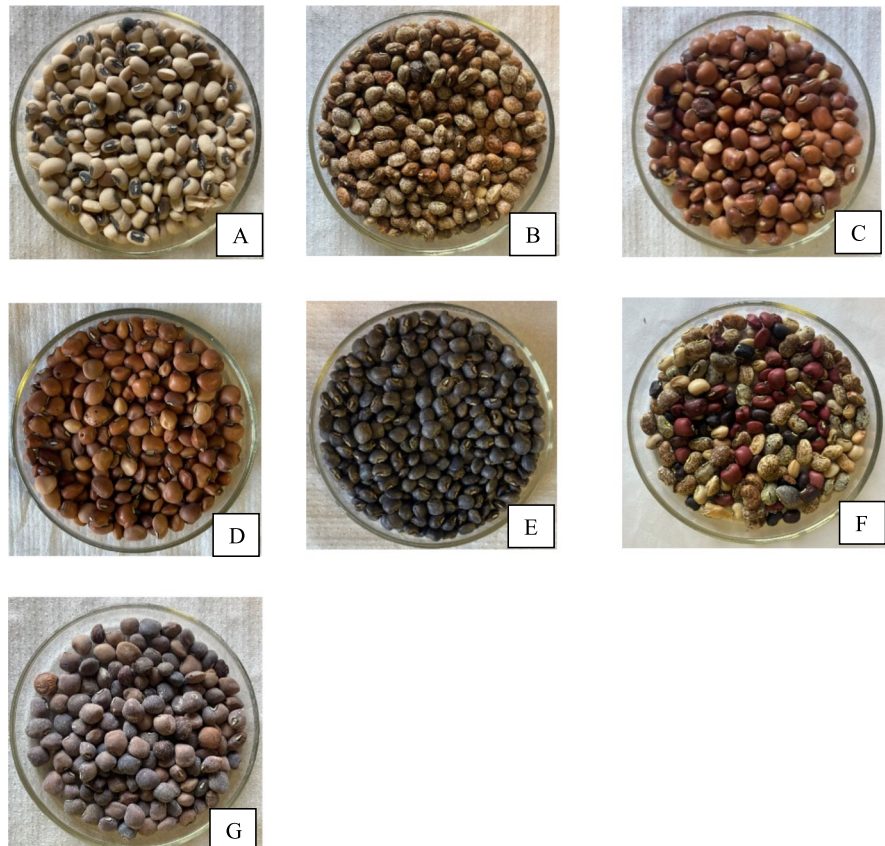
KwaZulu-Natal (KZN), Limpopo and Mpumalanga provinces of South Africa served as the collection source of seed. Identification of farmers and collection of seeds were done with the help of officials from the provincial agriculture departments and extension officers. In some cases, dry pods were collected, and the pods were dehulled to release the seeds. Samples were stored by the farmers under various conditions on farms, such as storing the seeds in cupboards, dried in a storeroom, placed in plastic bags, in plastic drums and laid bare under a shade tree. Forty-eight samples were collected and all repacked in labelled paper bags and stored at 4°C until use. Most of the samples were from Mpumalanga (44.6%) and Limpopo (42.5%), followed by KZN (10.6%) and Gauteng (2.1%). Samples were generally of good quality and represented a variety of colours (Fig. 1), with no single one being dominate.

Isolation and morphological identification of fungi from seed

Isolation of the fungi was done using the direct plating method (Makun et al., 2012; International Seed Testing Association (ISTA) 2016). Four hundred seeds were randomly selected from each sample. Of the 400 seeds, 200 seeds were plated directly onto the Petri dishes containing potato dextrose agar (PDA), amended with chloramphenicol, while the other 200 seeds were surface disinfected prior to plating. Surface disinfection was done by rinsing the seeds in 1% hypochlorite (NaOCl) for 3 min, then washing the seeds with sterilised distilled water thrice. Five seeds were aseptically placed on each agar plate (90 mm diameter). Plates were incubated at 22–25°C for 5 to 7 days. After the incubation period, infection incidence and frequency were calculated as follows (Bie-mond et al., 2013):

$$\text{Infection incidence (\%)} = \text{Number of seeds infected by a specific genus} / \text{total number of seeds} \times 100$$

Fig. 1 Colours of cowpea seed samples collected, marked as (A) white, (B) speckled, (C) red, (D) brown, (E) dark, (F) mixed, (G) purple



$$\text{Infection frequency (\%)} = \text{Number of samples in which a specific genus occurred} / \text{total number of samples} \times 100$$

Subcultures were made on PDA media and incubated at 22–25°C for 5 to 7 days. The different fungal isolates were identified to genus level based on their morphological characteristics using a light microscope (Von Arx, 1970; Watanabe 2002). All cultures were stored at 4°C on PDA plates, wrapped in Parafilm to avoid contamination. Fresh subcultures were made prior to molecular identification.

Molecular identification of fungal isolates

For the purpose of this study, isolates from major mycotoxin producing genera including *Fusarium*, *Aspergillus*, and *Penicillium* were identified to species level using molecular techniques. Using pure cultures of each isolate, DNA extraction was carried out by using a ZR Fungal/Bacterial DNA miniPrep™ extraction kit (Zymo Research) according to the manufacturers' instructions. The freshly extracted DNA were stored at –4°C in a freezer until use for polymerase chain reaction (PCR). For the PCR amplification, four different primer sets (Table 1) were used depending on the fungal genera. For *Aspergillus* species, the internal transcribed spacer (ITS) region and the calmodulin (cf) gene region was amplified. Identification of *Fusarium* species was done by amplifying the trans elongation factor-1 α (TEF-1 α) gene region, and the RNA polymerase II second largest subunit (Rpb2) gene region was amplified for *Penicillium* species.

The PCR reaction was conducted in 12.5 μ l solutions containing 1 μ l DNA (10 ng) added to 6.25 μ l Amplicon Taq DNA polymerase 2 $\times$ master mix red (1.5 mM), 0.4 μ l each of forward and reverse primer (10 μ M) and sterile distilled water (4.45 μ l). The Taq polymerase mix contained DNA polymerase, a NH₄⁺ buffer system, dNTP's (10 mM) and magnesium chloride (15 mM). The PCR was run on the different cycles for each specific primer (Table 1). After PCR amplification, an agarose gel electrophoresis was conducted to confirm if amplification was successful. PCR clean-up was done by adding 5 μ l of post-PCR product to 2 μ l of ExoSAP-IT™ reagent and incubating the mixture for 15 min at 37°C followed by incubation at 20 min at 80°C in the thermocycler.

Finally, a sequencing reaction was done according to the BigDye® sequencing protocol. A mixture containing 0.5 μ l BigDye, 0.3 μ l forward or reverse primer (10 μ M), 2.1 μ l sequencing buffer and 6.4 μ l double distilled water (ddH₂O) was prepared. Samples were heated at 96°C for 1 min, followed by a denaturing step at 96°C for 10 s, an annealing step at 50°C for 5 s and an elongation step at 60°C for 4 min. The cycle was repeated 25 times. Precipitation of the sequence reaction was done by adding 12 μ l of the sequence PCR product to 2 μ l of sodium acetate (3 M, pH=4.6) and 16 μ l 100% ethanol (EtOH). After centrifugation at 13 400 g for 30 min, the supernatant was discarded and 250 μ l of 70% EtOH was immediately added. The mixture was then centrifuged at 13 400 g for 5 min, the supernatant discarded, and the step was repeated. After drying overnight, samples were submitted to the DNA sequencing facility, University of Pretoria, where Sanger sequencing was performed. Submitted samples were prepared with ABI PRISM BigDye®, Terminator V3.1 cycle sequencing kit, sequencing 5X buffer and the HiDi™ formamide. Analysis was done on the ABI PRISM 3130xl and ABI PRISM 3500xl genetic analysers, using buffer 10X with EDTA (125 mM) on 50 cm long 16 capillary arrays for the 3130xl and anode buffer container and cathode buffer container on 50 cm long 24 capillary arrays for the 3500xl. All instruments, consumables, software and devices were supplied by Applied Biosystems™ / Life technologies, Carlsbad, US.

Phylogenetic analyses

Nucleotide sequences obtained from Sanger sequencing were aligned and manually edited using the CLC main workbench 8.0.1 (QIAGEN), where after they were entered in the National Centre for Biotechnology Information (NCBI) database (GenBank), and representative sequences with a similar identity of 97 to 100% were downloaded. For molecular identification of *Fusarium* spp., sequences were entered into the Fusarium-ID database (<http://isolate.fusariumdb.org/index.php>) and filtered to only compare sequences to known *Fusarium* isolates. Again only sequences with a similar identity of 97 to 100% were used.

To redefine the identities of the fungal isolates, phylogenetic analyses were performed. After editing,

Table 1 Primer sets, gene region amplified and cycling conditions used during polymerase chain reaction (PCR) amplification

Primers:	Gene region amplified:	PCR Conditions:	References:
ITS 1 F ITS 4	Internal Transcribed Spacer (ITS) CTT GGT CAT TTA GAG GAA GTA A TCC TCC GCT TAT TGA TAT GC	94° C for 1 min - Initial denaturation 94° C for 30 s - Denaturation 55° C for 30 s - Annealing 72° C for 1 min - Elongation 72° C for 8 min - Final elongation 4° C for infinity - Rest period x 35 cycles	White <i>et al.</i> 1990 Gardes and Bruns 1993
Rpb2-5f Rpb2-7cR	RNA polymerase II gene GAY GAY MGW CAT CAY TTY GG CCC ATR GCT TGY TTR CCC AT	94° C for 3 min - Initial denaturation 94° C for 20 s - Denaturation 55° C for 30 s - Annealing 72° C for 1 min - Elongation 72° C for 10 min - Final elongation 4° C for infinity - Rest period x 35 cycles	Liu <i>et al.</i> 1999
TEF 1 TEF 2	Translation Elongation factor 1-alpha (EF) ATG GGT AAG GAR GAC AAG AC GGA RGT ACC AGT AAT CAT CAT GTT	95° C for 3 min - Initial denaturation 95° C for 30 s - Denaturation 51° C for 30 s - Annealing 72° C for 1 min - Elongation 72° C for 7 min - Final elongation 4° C for infinity - Rest period x 35 cycles	O'Donnell <i>et al.</i> 1998
Cf1 Cf4	Calmodulin GCCGACTCTTTGACYGARGAR TTTTYTGATCATRAGYTGAC	95° C for 5 min - Initial denaturation 95° C for 45 s - Denaturation 55° C for 45 s - Annealing 72° C for 1 min - Elongation 72° C for 10 min - Final elongation 4° C for infinity - Rest period x 35 cycles	Yin <i>et al.</i> 2017

sequences were aligned using the MAFFT server (Katoh et al., 2019), and Maximum Likelihood (ML) phylogenetic analyses were performed using IQtree v. 2.2.2.6 (Minh et al., 2020).

Determining the presence of naturally occurring mycotoxins

To determine the natural occurrence of mycotoxins, present in the cowpea seed samples, 30 g of each seed sample were ground to a fine medium using a benchtop grinder. Five grams of each sample was then weighed off in duplicates and placed in sealed labelled plastic bags for analysis. Samples were sent to the Central Analytic Facilities (CAF) at Stellenbosch University for multi-mycotoxin analysis by ultra-performance liquid chromatography (UPLC) – electrospray ionization (ESI) tandem mass spectrometry (MS/MS). Mycotoxins screened for included aflatoxin B₁, deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEA), ochratoxin (OTA) and fumonisin B₁, B₂ and B₃ (FB₁, FB₂ and FB₃). Samples were analysed as described by Alberts et al., 2019 with minor adjustments. The UPLC-MS/MS was performed using a Waters UPLC coupled to a Waters Xevo TQS-Micro Mass Spectrometer (Waters, Milford, MA, USA). Using electrospray ionisation in positive mode, multi-mycotoxins were separated in multiple reaction monitoring (MRM). Operating parameters were as followed: capillary voltage 3.5 V, cone voltage range 15–50 V; collision energy range 10–40 eV; source temperature 140°C; desolvation temperature 400°C; desolvation gas 800 L/h and cone gas 50 L/h. Separation was achieved with a Waters UPLC BEH C18 column (2.1×100 mm, 1.7µ particle size) at 50°C and a flow rate of 0.35 ml/min. An injection volume of 2 µl was used and the mobile phase consisted of water acidified with 0.1% formic acid (A) and acetonitrile acidified with 0.1% formic acid (B). The following gradient was used: Initial conditions was 98% A, held for 0–0.5 min followed by a gradual gradient change of solvent A to 60% from 0.5–7 min. This was followed by a further gradient change of solvent A to 30% from 7–10 min. A quick gradient change followed for solvent A to 5% from 10–11 min. Another gradient change of solvent A followed to 0% from 11–12 min after which the gradient quickly changed to initial conditions of 98% solvent A from 12–12.1 min. Initial conditions of 98% solvent A were kept for 12.1–14 min. Table 2 summarizes the limit of detection (LOD) and limit of quantification (LOQ) for each analyte. Samples were

Table 2 Limit of detection (LOD in mg/kg) and limit of quantification (LOQ in mg/kg) for each analyte used with ultra-performance liquid chromatography (UPLC) – electrospray ionization (ESI) tandem mass spectrometry (MS/MS)

Analyte	LOD (mg/kg)	LOQ (mg/kg)
Aflatoxin B1	0.005	0.015
DON	0.01	0.03
Zearalenone	0.01	0.03
Nivalenol	0.01	0.03
Ochratoxin A	0.01	0.03
Fumonisin B1	0.001	0.003
Fumonisin B2	0.001	0.003
Fumonisin B3	0.001	0.003

processed using Waters Target Lynx software, with all calibration curves having a R² value above 0.99.

Mycotoxin producing potential of selected fungal isolates

Two isolates each of *Fusarium chlamydosporum*, *Fusarium incarnatum*, *Fusarium brachygibbosum*, *Fusarium verticillioides*, *Aspergillus niger* and *Aspergillus tubingensis* were cultured on PDA plates and incubated at 22–25°C for 5 – 7 days. Maize patty media was prepared by adding 25 g of ground maize kernels to 25 g of water. The mixture was then placed in Pyrex Petri dishes (100×18 mm) and autoclaved for 1 h at 121°C and 120 kPa on two consecutive days. The spore suspensions were prepared in 100 ml distilled water and 3 ml of each suspension was added to the sterile maize patty medium. The plates were incubated at 22–25°C for 21 days or until the entire plate was overgrown after which they were dried at 40°C for 4–5 days until completely dry (Alberts et al., 1993; Kritzing et al., 2006). The dried media was ground to a fine medium using a benchtop grinder and 5 g of each isolate was analysed for mycotoxins using UHPLC-ESI-MS/MS (Sect. "Phylogenetic analyses").

Results

Incidence and frequency of fungal genera isolated from cowpea seed.

Morphological identification indicated that a wide range of fungi were present in the samples. The

Table 3 Incidence and frequency of fungal isolates per genera isolated from 48 cowpea seed samples collected from smallholder farmers in various localities in South Africa

Fungal genera	Total isolates retrieved	Non-surface disinfected			Surface disinfected seeds		
		No. of isolates	Infection incidence* (%)	Infection frequency* (%)	No. of isolates	Infection incidence* (%)	Infection frequency* (%)
<i>Fusarium</i>	110	64	27.4	70.2	46	12.7	51.01
<i>Aspergillus</i>	29	19	8.4	34.0	10	3.4	17.0
<i>Penicillium</i>	22	14	40.4	25.5	8	16.7	14.9
<i>Rhizopus</i>	17	9	27.1	19.1	8	14.1	17.0
<i>Trichoderma</i>	10	4	4.0	8.5	6	6.8	10.6
<i>Macrophomina</i>	10	4	11.0	8.5	6	3.8	8.5
<i>Rhizoctonia</i>	4	2	1.0	2.1	2	3.0	4.3
<i>Chaetomium</i>	6	3	39.6	6.4	3	48.2	8.5
<i>Diaporthe</i>	3	1	10.0	2.1	2	1.5	4.3
<i>Epicoccum</i>	5	2	20.5	4.3	3	13.3	8.5
<i>Cladosporium</i>	28	21	39.7	31.9	7	6.7	12.8
<i>Phoma</i> like	38	28	36.1	36.2	10	9.3	21.3
<i>Colletotrichum</i>	1	1	3.0	2.1	0	0	0
<i>Alternaria</i>	47	16	37.8	29.8	31	21.9	53.2
<i>Botryosphaeria</i>	15	8	19.2	14.9	7	8.8	14.9
<i>Nigrospora</i>	2	0	0	0	2	11.0	4.3
Total (Average %)	347	196	(20.3)	(18.5)	151	(11.3)	(15.7)

*Infection incidence (%) = Number of seeds infected by a specific genus / total number of seeds $\times$ 100. Infection frequency (%) = Number of samples in which a specific genus occurred / total number of samples $\times$ 100

incidence and frequency of isolated fungi are presented in Table 3. All samples presented fungal contamination, with multiple genera isolated from the majority of the samples. In total, 16 different fungal genera were identified, representing a higher incidence of fungi in non-surface disinfected seeds (56.5%) compared to surface disinfected seeds (43.5%). Altogether 347 isolates were identified, with the majority belonging to the *Fusarium* genera (31.7%), followed by *Alternaria* (13.5%), *Phoma* like fungi (11.0%) and *Cladosporium* (8.1%). Other mycotoxin producing genera including *Aspergillus* and *Penicillium* had high occurrences making out 8.4% and 6.3%, respectively, of the total number of isolates.

The infection incidence of non-surface disinfected seeds (on average 20.3%) was double when compared to that of surface disinfected seed (11.3%). Infection frequency averages between surface disinfected compared to non-surface disinfected seed were 15.7% and 18.5%, respectively. Most genera were isolated from disinfected and non-disinfected seeds, except for *Colletotrichum* that was only found once on a non-surface

disinfected sample, and two isolates of *Nigrospora* that was isolated from a single surface disinfected sample.

Considering non-surface disinfected seeds, *Penicillium* had the highest infection incidence rate (40.4%), followed by *Cladosporium* (39.7%), *Chaetomium* (39.6%) and *Alternaria* (37.8%). *Rhizoctonia* had the lowest infection incidence and was only isolated twice. In terms of infection frequency, *Fusarium* was by far the most prevalent in all the samples (70.2%). *Aspergillus* (34.0%) and *Penicillium* (25.5%) were also found frequently in all the samples. When considering surface disinfected seeds, *Chaetomium* had the highest infection incidence (48.2%), while *Alternaria* (21.9%) had the second highest. Similar to non-surface disinfected seeds, *Fusarium* had a relative high infection frequency percentage (51.1%), with only *Alternaria* (53.2%) being more common in the samples.

Molecular identification and phylogenetics

All isolates used for molecular identification showed > 97% similarity when compared to reference

species in the GenBank and Fusarium-ID databases. In total, 38 isolates of *Fusarium* were obtained, and had consensus sequences of between 97 – 100%, which was used for phylogenetic analysis. Similarity, below 100% have been previously attributed to the possible existence of allelic variants, the lack of a sequence representative or the existence of a new species (Hsuan et al., 2011). Sequencing of the TEF-1 α gene region has shown to be quick and reliable when screening broad and unknown *Fusarium* species (Nitschke et al., 2009). Amplifying this region has also been confirmed to be ideal gene to be used as phylogenetic marker (Barik & Tayung 2012; Mkandawire et al., 2022). Comparison of the TEF-1 α sequences to those in Fusarium-ID database showed that the isolates represented four species complexes in *Fusarium*, including *F. chlamydosporum* species complex (FCSC), *F. incarnatum-equiseti* species complex (FIESC), *F. fujikuroi* species complex (FFSC), *F. sambucicum* species complex (FSAMSC) (Fig. 2).

Most isolates belonged to the FIESC (18 isolates), followed by FSAMSC (10 isolates), and FCSC (8 isolates), while there were only 2 isolates in FFSC. The isolates within the FIESC clade were grouped into five species, namely *F. incarnatum* (6 isolates), *F. caatingaense* (6 isolates), *F. lacertarum* (4 isolates), *F. compactum* (1 isolate) and *F. longidatum* (1 isolate). Within the FSAMSC clade, only one species was identified, that of *F. brachygibbosum* (10 isolates). Phylogenetic analysis of FCSC separated isolates into three species, *F. atrovinosum* (1 isolate), *F. chlamydosporum* (4 isolates) and *F. sporodochiale* (3 isolates). Similar to FSAMSC, the TEF-1 α based phylogeny only grouped FFSC under one species, that of *F. verticillioides* (2 isolates). In total, 10 different species representing different clades were identified using TEF-1 α based phylogeny.

Fifteen isolates of *Aspergillus* were molecularly identified based on their similarity to consensus sequences in the GenBank. All other isolates were shown to belong the *nigri* group of *Aspergillus* species for both CaM and ITS amplified regions. The concatenated ITS and CaM based phylogeny separated the isolates into three clades and four distinct known species groups (Fig. 3). These were *Aspergillus niger/welwitschiae* clade, *A. tubingensis* clade and the *Aspergillus aculeatus* clade.

Within the clades only four species were identified, *A. niger*, *A. neoniger*, *A. tubingensis* and *A. aculeatus*. The majority of the isolates (9) were identified as *A. niger*, with the remaining isolates equally represented as *A. neoniger*, *A. tubingensis* and *A. aculeatus* with two isolates each. Some *A. niger* isolates were thought to be *Aspergillus welwitschiae* based on the BLAST results of the ITS sequence.

When considering *Penicillium*, 12 isolates were identified based on the closest Rpb2 sequence match phylogenetic analysis was based on the amplification of this gene region. Similarity in the GenBank database were more than > 98.97%, and the Rpb2 phylogeny divided the isolates into four well defined clades (Fig. 4). Isolates examined represented *Fasciculata*, *Chrysogena*, *Brevicompacta* and *Citrina* clades. The phylogeny grouped the isolates into four species, *P. chrysogenum* (*Chrysogena*), *P. polonicum* (*Fasciculata*), *P. brevicompactum* (*Brevicompacta*) and *P. citrinum* (*Citrina*). When comparing incidence, five isolates were found to be *P. brevicompactum*, four isolates of *P. polonicum*, two isolates of *P. chrysogenum* with only one isolate belonging to *P. citrinum*.

Natural occurrence of mycotoxins and production of mycotoxins by isolates *in vitro*

None of the mycotoxins analysed for were found to be naturally present in any of the seed samples. Nevertheless, some isolates grown on maize patty media produced mycotoxins (Table 4). Deoxynivalenol (DON) was produced at low levels by all *Fusarium* isolates except *F. verticillioides*, ranging between 0.190 – 0.611 mg/kg. Zearalenone was produced at levels ranging between 0.222 and 1.119 mg/kg by the two *F. incarnatum* isolates, 0.035 and 0.046 mg/kg by the two *F. brachygibbosum* isolates, 0.109 and 0.237 mg/kg by the two *F. chlamydosporum* isolates, and 0.036 mg/kg by only one *F. verticillioides* isolate. Nivalenol (NIV) was produced by *F. incarnatum* (9.07–9.34 mg/kg), *F. brachygibbosum* isolates 1 and 2 (5.83–6.97 mg/kg), *F. caatingaense* isolates 1 and 2 (10.405–10.987 mg/kg) and *F. chlamydosporum* (4.51–25.01 mg/kg). Isolates 1 and 2 of *A. niger* were the only isolates to produce ochratoxin A (OTA), at relatively low concentrations (0.042–0.053 mg/kg).

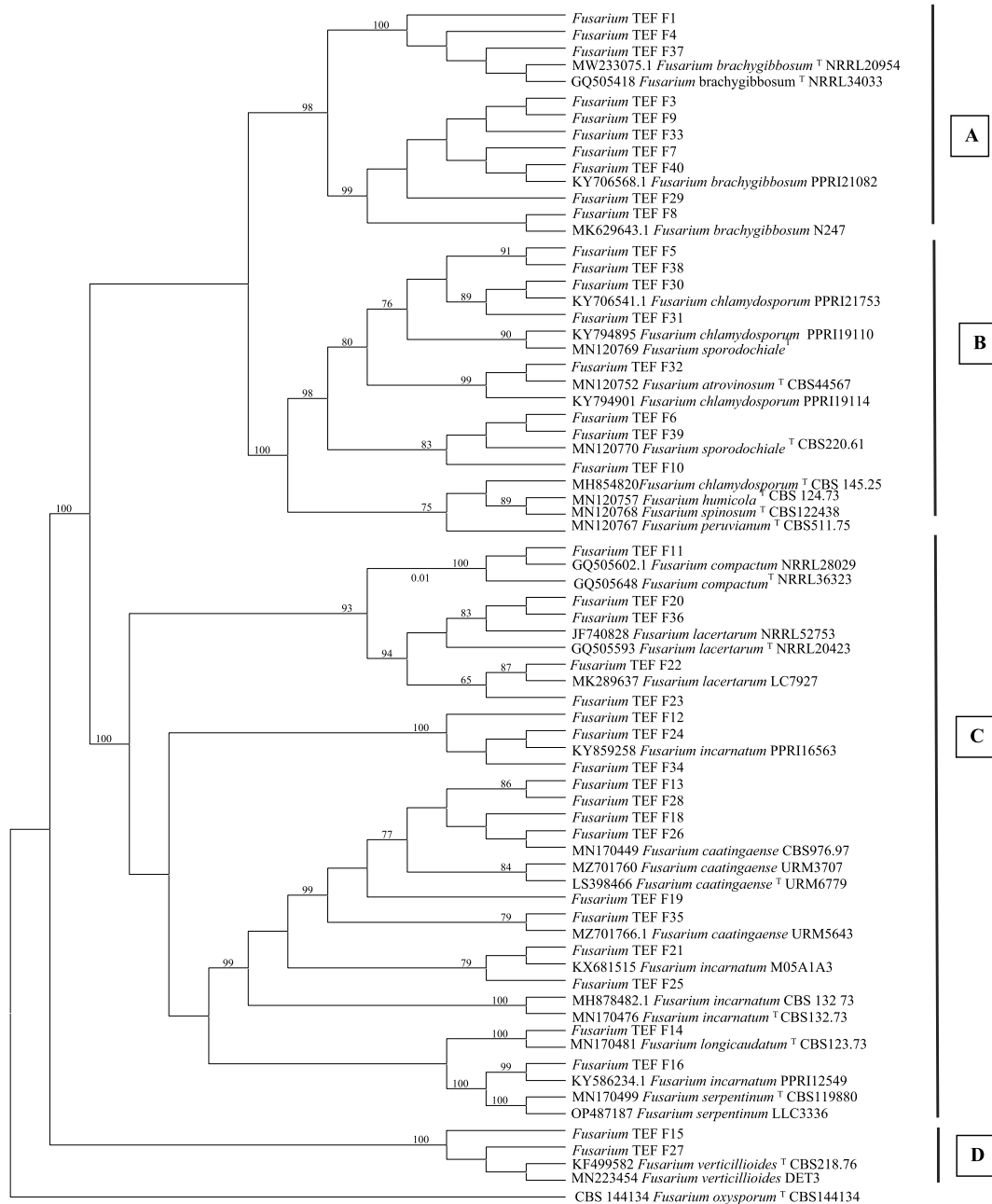


Fig. 2 Maximum likelihood tree based to the TEF-1α gene phylogeny. Percentage of replicate trees in which the taxa clustered together are shown next to branches based on the bootstrap test (1000 replicates). The tree is rooted in *Fusarium oxysporum* CBS 144134. Four clades are visible A: *F. sam-*

bucicum species complex (FSAMSC), B: *F. chlamydosporum* species complex (FCSC), C: *F. incarnatum-equiseti* species complex (FIESC), D: *F. fujikuroi* species complex (FFSC). [†] Denotes type material

Fig. 3 Maximum likelihood tree from the combined CaM and ITS sequence alignment. Percentage of replicate trees in which the taxa clustered together are shown next to branches based on the bootstrap test (1000 replicates). Three clades are visible **A**: *Aspergillus niger*/*welwitschiae* clade, **B**: *A. tubingensis* clade and the **C**: *Aspergillus aculeatus* clade. The tree is rooted with *Aspergillus flavus* NR1110441/FN594583. ^T Denotes type material

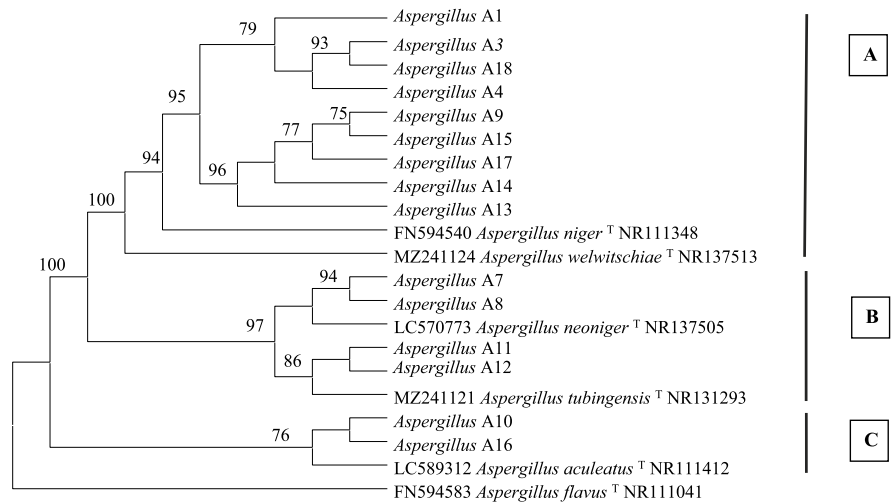
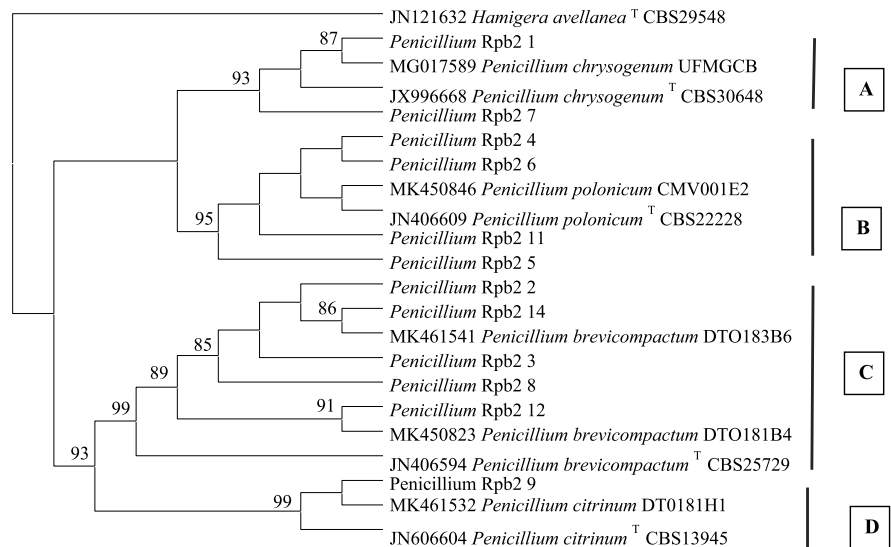


Fig. 4 Maximum likelihood tree based to the Rpb2 gene phylogeny. Percentage of replicate trees in which the taxa clustered together are shown next to branches based on the bootstrap test (1000 replicates). The tree is rooted in *Hamigera avellanea* CBS295 48. Four clades are observed, **(A)** Chrysogena, **(B)** Fasciculata, **(C)** Brevicompacta, **(D)** Citrina. ^T Denotes type material



Fumonisin B₁, B₂ and B₃ were produced predominantly by the *F. verticillioides* and *F. chlamydosporum* isolate 3. Fumonisin B₁ was produced at 1150,05 – 1182,87 mg/kg by *F. verticillioides*, while B₂ (227,37 – 247,91 mg/kg) and B₃ (50,68 – 54,01 mg/kg) production by the same isolates were also detected at higher concentrations than the other mycotoxins. Isolate 3 of *F. chlamydosporum* also produced fumonisins at similar concentrations that of the *F. verticillioides* isolate (B₁, B₂ and B₃ at 1092,40; 251,13 and 91,94 mg/kg, respectively). *Aspergillus niger* isolates produced relatively low concentrations of the fumonisin B₁,

B₂ and B₃, with the exception of isolates 3 and 4 that produced FB₂ at a much higher concentration (21,10 – 22,55 mg/kg).

Discussion

This study has shown that several fungal genera are naturally associated with cowpea seed in South African smallholder production systems. Non-surface disinfected seeds also had a higher incidence of fungi compare to surface disinfected seeds. What is of concern is the high incidence of potentially

Table 4 Molecularly identified fungal isolates from cowpea seed samples and the mycotoxins they produced when grown on maize patty cultures

Organism	Mycotoxin concentration in mg/kg*							
	AFB1	DON	ZEA	NIV	OTA	FB ₁	FB ₂	FB ₃
<i>F. incarnatum</i> —is1	-	0.475	1.119	9.065	-	0.014	-	-
<i>F. incarnatum</i> —is2	-	0.464	0.277	9.34	-	-	-	-
<i>F. brachygibbosum</i> – is1	-	0.283	0.035	6.972	-	-	-	-
<i>F. brachygibbosum</i> – is2	-	0.275	0.046	5.826	-	-	-	-
<i>F. caatingaense</i> – is1	-	0.611	-	10.405	-	0.034	0.092	-
<i>F. caatingaense</i> – is2	-	0.563	-	10.987	-	0.024	0.036	-
<i>F. chlamydosporum</i> – is1	-	0.305	0.109	-	-	0.197	0.024	-
<i>F. chlamydosporum</i> —is2	-	0.319	0.237	4.51	-	0.176	0.015	-
<i>F.chlamydosporum</i> —is3	-	0.261	-	25.099	-	1092.4	251.131	91.937
<i>F. verticillioides</i> – is1	-	-	-	-	-	1150.05	227.37	50.675
<i>A. niger</i> – is1	-	-	-	-	0.053	1.688	1.372	0.245
<i>A. niger</i> – is2	-	-	-	-	0.042	0.286	0.394	0.054
<i>A. niger</i> – is3	-	-	-	-	-	0.103	21.104	0.026
<i>A. niger</i> – is4	-	-	-	-	-	0.051	22.553	0.017
<i>A. tubingensis</i> – is1	-	-	-	-	-	-	-	-
<i>A. tubingensis</i> – is2	-	-	-	-	-	-	-	-

*AFB₁: Aflatoxin B₁,
 DON: Deoxynivalenol,
 ZEA: Zearalenone,
 NIV: Nivalenol, OTA:
 Ochratoxin A, FB₁:
 Fumonisin B₁, FB₂:
 Fumonisin B₂, FB₃:
 Fumonisin B₃

mycotoxigenic fungi belonging to the genera *Aspergillus*, *Fusarium* and *Penicillium* that were recovered. Isolates from the *Fusarium* genus were predominant. Classification of the clades based on TEF-1 α phylogeny found in this study aligned with previous findings (Laraba et al., 2021; Mkandawire et al., 2022). Molecular identification showed that species belonging to the FIESC was the most prominent in the current study. The complex is known to consist of over 30 phylogenetic species including *F. brevicaudatum*, *F. caatingaense*, *F. clavum*, *F. compactum*, *F. equiseti*, *F. guilinense*, *F. incarnatum*, *F. lacertarum*, *F. longicaudatum*, *F. scirpi* and *F. semitectum* (Xia et al., 2019). At least five of these species occur naturally on cowpea seed samples analysed in this study. Moreover, species not found in this study belonging to the FIESC have previously shown to be associated with cowpea seeds, including *F. semitectum* (Kritzing et al., 2003) and *F. equiseti* (Khare et al., 2016). This suggests some form of dominance of the species complex in the cowpea seeds. Although present in seed samples analyzed in this study, *F. caatingaense* has been shown to be non-pathogenic to cowpea in previous studies (Maciel et al., 2021).

One species, which forms part of the FSAMSC, were also found at a high incidence in the samples, namely, *F. brachygibbosum* classified under the *Brachygibbosum* sub-clade (Laraba et al., 2021).

Although research is limited on the presence of this species in cowpea seed, there have been some reports of *F. brachygibbosum* being associated with leguminous crops such as soybean (*Glycine max* (L.) Merr.) (Wang et al., 2021). Another species in the complex, *F. sambucinum*, has however been isolated from cowpea seed samples in the past (Kritzing et al., 2003). Isolates representing the FCSC formed a distinct clade in the phylogenetic analysis, and is represented by two species formally found on cowpea seeds, *F. chlamydosporum* (Kritzing et al., 2003) and *F. sporodochiale* (Rodrigues & Menezes, 2005). Furthermore, *F. atrovinosum*, which also resides in this clade was also identified in the present study. The species has been previously been shown to be associated with maize in South Africa (Visagie et al., 2024). Two isolates belonged in the FFSC, and were molecularly identified as *F. verticillioides*. Similarly to this study, the species have been associated with cowpea seeds in various previous studies (Rodrigues & Menezes, 2005; Zaidi, 2012; Biemond et al., 2013; Ghangaokar & Kshirasagar 2013), while another species in the complex, *F. proliferatum*, has been isolated from cowpea seeds collected from Benin (Kritzing et al., 2003). Results in the current study confirms that cowpea seed is a suitable host for a variety of *Fusarium* species to survive and proliferate. Most *Fusarium* species identified have also been associated

with maize production (Visagie et al., 2024), and given that maize is used for intercropping with cowpea in South Africa (Asiswe and Maimela 2021), the possibility of contamination cannot be excluded.

Infection of seed by *Fusarium* species can lead to diseases such as wilt, blight, root rot and canker which further reduces crop yield of legumes (Sampaio et al., 2020). The detrimental effect of *Fusarium* infection on cowpea seeds were previously confirmed where cowpea seed artificially inoculated with *F. verticillioides* and *F. proliferatum* had a significant reduction in germination (Kritzing et al., 2006). Similarly, *F. verticillioides* significantly decreased seedling emergence percentage, as well as the dry root and shoot seedling mass of cowpea seeds imbibed with the fungi (Kotze et al., 2017). It is also well known that *Fusarium* species produce various harmful mycotoxins. Fumonisin B₁, B₂ and B₃ were found to be produced by *F. chlamydosporum* at 0.007, 0.0015 and 0.0017 mg/L, respectively, in abalone feed (Greef-Laubser et al. 2020). The study also confirmed the production of these mycotoxins by *F. verticillioides* in feed (Greef-Laubser et al., 2020). Fumonisin analogs are produced by *F. verticillioides*, *F. proliferatum*, *F. subglutinans*, *F. oxysporum* and various other *Fusarium* species (Rheeder et al., 2002). Trichothecene mycotoxins such as NIV are mainly produced by species belonging to the *F. graminearum* clade (Desjardins & Proctor, 2011). However, NIV has been reported to be produced by fungi in FSAMSC, such as *F. brachygibbosum* isolated from soybean and wheat (*Triticum aestivum* L.) (Laraba et al., 2021). Additionally, it was shown that isolates belonging to the FIESC, such as *F. caatingaense* and *F. incarnatum*, can also produce NIV, ZEA and DON mycotoxins, similar to findings in this study (Avila et al., 2019), which is thought to be due to the presence of the *Tri5* gene in the trichothecene loci (Proctor et al., 2009). This is in accordance with Maciel et al. (2021) that found that *F. caatingaense* produced trichothecene mycotoxins such as DON.

Deoxynivalenol was detected in all the maize patty cultures inoculated with *Fusarium*, *F. brachygibbosum*, although this fungi is not known as a major producers of the toxin. Rabaaoui et al. (2021) however have previously found that *F. brachygibbosum* can produce trichothecene type mycotoxins. Although these isolates could produce the mycotoxins on maize patty cultures, none of these toxins were found

naturally in the cowpea seed samples. Fumonisin (B₁, B₂, B₃ and B₄), trichothecenes and ZEA all have been shown to be naturally associated with maize kernels, the crop being highly susceptible to infection by mycotoxigenic *Fusarium* spp. (Bryla et al., 2022). In contrast, cowpea has been suggested to be less vulnerable to *Fusarium* produced mycotoxin contamination compared to maize (Houssou et al., 2009), although fumonisin contamination have been reported in cowpea seed (Bankole et al., 2006; Kritzing et al., 2003). One explanation can be that cowpea contain relative high levels of ellagic acid (Sharifi-Rad et al., 2022), a phenolic compound which has shown to be able to decrease the mycotoxin production of *F. verticillioides* (Ferrigo et al., 2021). This could suggest why no toxins were found naturally in the collected seed.

Aspergillus isolates identified mainly belonged to the black aspergilli, section *Nigri*. Species of the group includes *A. niger*, *A. japonicus*, *A. tubingensis* (Nielsen et al., 2009), *A. neoniger* (Samson et al., 2014) and *A. welwitschiae* (Massi et al., 2020). The limitation of using the ITS region for sequence analysis is that species identification within a group is not always possible as the ITS region has limited sites which is not variable enough (Balajee et al., 2009). Distinguishing between *A. niger* and *A. welwitschiae* solely on ITS gene amplification is not possible as sequences have been shown to be near identical (Pařenicov et al., 2001). Previously it has been shown that by targeting the calmodulin gene, *Aspergillus* isolates showed better resolution compared to when ITS or β -tubulin regions were amplified (Alshehri & Manikandan, 2020). Clade classification based on the CaM gene phylogeny of the *Aspergillus* spp. are similar to the results previously obtained by Samson et al. (2014). Considering the phylogenetic nature of these species, Samson et al. (2014) found that *A. niger* and *A. welwitschiae* belong to the same clade when only considering the partial CaM gene sequences, while *A. tubingensis* and *A. neoniger* form part of the same clade, but with *A. aculeatus* and *A. japonicus* belonging to different clades. Studies using concatenated data sets consisting of gene amplifications with ITS, benA and CaM yielded similar results, grouping *A. niger* and *A. welwitschiae* together, but separating *A. tubingensis* and *A. aculeatus* into different clades (D'hooge et al., 2018). Phylogenetic analysis in this study yielded a similar distinction

between the groups. Species belonging to the section *Nigri* have been previously associated with cowpea. Early reports indicated *A. niger* being predominant on stored cowpea in Nigeria with infestations ranging from 31 to 100% for surface sterilised samples (Collison et al., 1992). More recently, *A. niger* was isolated from two different cultivars in Botswana but at much lower percentages, only 3 and 4%, respectively (Khare et al., 2016). The species is mostly associated with postharvest decay of vegetables, beans, cereals and fruit crops (Gautum et al., 2011). Also in the *Nigri* group, *A. japonicus* have been previously found in cowpea seed samples from Pakistan (Dawar et al. 2015).

Apart from species found in this study, other *Aspergillus* species have also been isolated from cowpea seed samples, including *A. flavus*, *A. flavipes*, *A. fumigatus* and *A. nidulans* (Jyoshna & Saxena, 2021). In terms of mycotoxin production, OTA is known to be produced by *A. niger* strains (Han et al., 2019), confirming results found in this study. Similarly, *A. tubingensis* has been shown to produce OTA in vitro ranging from 0.0053 – 0.1069 µg/g when grown on synthetic nutrient medium and Czapek yeast autolysate agar medium (Selouane et al., 2009). The species however is considered a weak producer of OTA, with limited isolates being able to produce the toxin (Storari et al., 2012). This could explain why the isolates tested in this study did not produce any OTA. In addition to the production of OTA, *A. niger* has also been shown to produce FBs such as B₂, B₄ and B₆, however not B₁ (Frisvad et al., 2011). Fumonisin production by *A. niger* could be due to a gene cluster which is also found in *F. verticillioides*, responsible for the production of these toxins (Onami et al., 2018). Susca et al. (2016) showed that *A. niger* produced fumonisin B₂ at levels ranging from 0.1–44 µg/g on a variety of crop substrates with significant production shown on cashew nuts (*Anacardium occidentale* L.) and raisins. In addition to these significantly important mycotoxins, *Aspergillus* sp. have also shown to produce other mycotoxins. For example, *A. aculeatus* is known produce secalonic acid D (Frisvad et al., 2006), a mycotoxin known to be toxic to chicken and mice embryos (Ren et al., 2006). Although mycotoxigenic *Aspergillus* spp. were isolated from cowpea seed samples, no mycotoxins were detected in these samples. It has been suggested that the presence of tannins in cowpea seeds, such as gallic acid has an influence on

the biosynthesis of some mycotoxins (Houssou et al., 2009). Romero et al. (2010) found that under in vitro conditions, production of OTA by *Aspergillus carbonarius* were significantly reduced in the presence of 100 mg/l gallic acid. Tannin content of cowpea seed coats have been found to increase as the seed colour intensity increases (Plahar et al., 1997). This could explain why no mycotoxins were found in the seed samples, given that samples varied in colour.

Penicillium species identified based on β-tubulin sequences were similar to the clades classified by Houbaken and Samson (2011). Amplifying gene regions such as Rpb1 and Rpb2 have been shown to be accurate in species level identification of *Penicillium* spp. (Raja et al., 2017). Various *Penicillium* species have been shown to be associated with cowpea seeds. Houssou et al. (2009) isolated *P. citrinum*, *P. corylophilum*, *P. chrysogenum*, *P. pinophilum* and *P. funiculosum* from seeds collected from farmer stock in Benin. Kahre et al., (2016) found *P. chrysogenum* in cowpea seeds samples collected from Botswana. Likewise, *Penicillium* species such as *P. chrysogenum*, *P. citrinum* and *P. notatum* have also been isolated from cowpea seed samples collected in India (Jyoshna & Saxena, 2021). Although there are no prior reports of *P. brevicompactum* associated with cowpea seeds, the fungus has been previously isolated from soil, vegetables, grains as well as textiles and dyes (Min et al., 2019). Additionally, the fungus has also occurred in legumes such as beans, soybeans as well as raw nuts such as pecans, pistachios and peanuts (Pitt & Hocking, 1997). Similarly, this study is the first indication of *P. polanicum* being associated with cowpea seeds. *Penicillium polanicum* is blue-green in colour when grown on PDA, consisting of two branched conidiophores, phialides that are bunched with smooth conidia (Al-Askar et al., 2021). This species is known to cause food spoilage on various crops including but not limited to peanuts (*Arachis hypogaea* L.), cereals, onions (*Allium cepa* L.) and green olives (*Olea europaea* L.) (Al-Askar et al., 2021). Some isolated *Penicillium* species from this study are known to produce mycotoxins. For example, *P. citrinum* produces citrinin, aflatoxin and OTA (Fleurat-Lessard, 2017), while citrinin was also produced by *P. chrysogenum* in bread (Reiss, 1977). Moreover *P. brevicompactum* is known to produce mycophenolic acid, a mycotoxin with low toxicity (Frisvad & Samson 2004),

Other fungi isolated and identified morphologically from cowpea include *Macrophomina* sp. which occur world wide and are generally found in the soil, affecting a variety of plant species (Marquez et al., 2021). Isolation of the fungus from cowpea seed was reported by Vasava et al. (2017). Cowpea seed decay and seedling damping can be the result of *Macrophomina* seed infection, while other diseases such as charcoal rot, leaf blight and stem blight is also caused by the pathogen (Oladimeji et al., 2012). Cowpea seeds inoculated with *M. phaseolina* also resulted in the reduction of reducing and non-reducing sugars compared to the control (Ushamalini et al., 1998). Although at a lower incidence, *Rhizopus* sp. was also isolated from the collected samples. Etaware and Ogungbemile (2019) similarly isolated *Rhizopus* when investigating the incidence of fungi on seed obtained from markets in Nigeria. Additionally *A. flavus*, *A. niger*, *A. fumigatus*, *A. parasiticus* and *F. oxysporum* were isolated from these samples. Inoculation of cowpea seed with *Rhizopus stolonifer* resulted in significant reduction of nutritional factors such as carbohydrate content, lipid content as well as fibre concentration when compared to a control, indicating the fungi's potential role in seed decay (Iyanyi & Ataga 2021). The current study also showed the presence of *Chaetomium*, although at a very low incidence. Several species of the fungus have shown to produce mycotoxins and is known to be the casual agent of onychomycosis, external skin infection and cerebral mycosis (Kedves et al., 2021). The presence of *Chaetomium* in this study correlates with previous findings by Bautista and Opina (1991) and Zohri et al., (1992). *R. solani* is a well known plant pathogen that causes severe yield losses in a variety of crops world wide (Sturrock et al., 2015). Dawar et al., (2015) isolated *R. solani* from cowpea seeds collected from various regions of Pakistan. Cowpea seed infection with *Rhizoctonia* have shown to cause significant reduction in seedling emergence accompanied with plant mortality (Pan & Das, 2011). The fungi is also frequently associated with cowpea seedlings and is known to cause damping off (Adandonon et al., 2004).

Another important seed borne pathogen associated with cowpea seed is *Diaporthe* sp., which is known to cause a variety of diseases in various crops including fruit rot, dieback, canker and blight (Dissanayake et al., 2015; Vasava et al., 2017). *Cladosporium*

which is a known plant pathogenic fungi (El-dawy et al., 2021), has been formerly isolated from cowpea seeds from samples collected in Pakistan (Dawar et al., 2015) and India (Jyoshna & Saxena, 2021). *Phoma* sp. have been previously isolated from cowpea seed samples in Ghana and India (Jyoshna & Saxena, 2021; Nutsugah et al., 2004). Although Jyoshna and Saxena (2021) did not find the fungus on sterilized seeds, the current study showed the converse with half the incidence rate compared to didinfected seeds. Damping off due to the infection of cowpea seedlings by *Phoma* has previously been reported (Adandonon et al., 2004).

An economically important disease of cowpea in South Africa is Colletotrichum stem disease caused by *Colletotrichum* sp. (Kinge et al., 2022). The fungus is also known to cause both cowpea anthracnose (*C. lindemuthianum*) and brown blotch (*C. truncatum*) in cowpea (Abebitan et al., 1992). The genus has been isolated from cowpea seed collected from markets in India, at an incidence of 7,0 – 16,5% per 100 seed of sample (Maske & Mogle, 2013). *Botryosphaeria* is known to cause disease on woody plants, such blight on pistachio (*Pistacia ver* L.), canker dieback and blight of walnut (*Juglans regia* L.), as well as band canker of almond (*Prunus dulcis* Mill.) (Moral et al., 2019). Although mainly a pathogen of tree plants, *Botryosphaeria* has been previously isolated from Italian common bean landraces (Dell'Olmo et al., 2023). Additional studies are needed to confirm the results. Found at a high incidence in this study, the plant pathogenic fungus *Alternaria alternata* has been associated with cowpea seeds in various studies (Vasava et al., 2017; Zanjare et al., 2020). The genus is known to produce multiple mycotoxins such as alternariol, alternariol monomethyl ether and tentoxin, which have shown to be carcinogenic and mutagenic (Escrivá et al., 2017). Fungi belonging to the *Nigrospora* genus are known plant pathogens causing stem and leaf blight on a wide variety of crops (Wang et al., 2017). *Nigrospora* were previously isolated from both surface sterilised and non-surface sterilised cowpea seed (Jyoshna & Saxena, 2021). It is clear that the variety of fungi isolates from the samples pose a threat to cowpea due to the pathogenicity of the various genera.

Considering the range of fungi isolated in this study, *Trichoderma* holds the least pathogenic threat for cowpea. Isolates from this genus have been used

extensively as biocontrol agents due to its antagonism against fungi such as *Rhizoctonia* and *Fusarium* (Mukhopadhyay & Kumar, 2020). Additionally, *Trichoderma* species also induces host plant resistance (Sood et al., 2020). Dawar et al., (2015) isolated *T. harzianum* from cowpea seed collected in Pakistan, and more recently seed samples collected in India confirmed the natural occurrence of *T. album* and *T. viridae* in the samples (Jyoshna & Saxena, 2021). Another potential beneficial fungal genera found in the study, is that of *Epicoccum*. Reports have shown the biological activity of species (especially *E. nigrum*) against pathogens such as *Sclerotinia sclerotiorum* on sunflower and *Pythium* on cotton (Fa'varo et al. 2011). The fungus has been previously associated with cowpea seeds when looking for biocontrol candidates of *Xanthomonas campestris* (Jindal & Thind, 1990).

The study confirms that various fungal genera are associated with cowpea seeds in rural production areas. The high incidence of mycotoxigenic fungi is of particular concern as they are known to produce harmful mycotoxins that can lead to serious health risks for humans and animals. Contamination of produce reduces smallholder farmers ability to consume or sell the seeds, which contributes to financial losses and reduces food security. In addition, other genera isolated from the samples are known to cause a variety of diseases of cowpea, which can ultimately lead to yield losses. Seed-borne fungi are known to proliferate under unfavourable storage conditions, which could be a starting point for farmers to protect their seeds by improving storage conditions. Properly drying of seed, use of clean storage containers and maintaining overall cool and dry storage conditions are some strategies smallholder farmers can use. Future work should focus on different storage conditions and the effect they have on the mycoflora spectrum of cowpea seeds. Also, many of the fungi identified in this study could be due to contamination of stored seeds. Smallholder farmers often have limited farming equipment, thus moving from one crop to another is inevitable. Most of the species identified here are associated with maize, a common crop used in rotation with cowpea or as an intercrop (Masvaya et al., 2017), which could explain their presence. No natural occurring mycotoxins were found in any of the samples. Literature suggests that the phenolic compounds present in cowpea could be

inhibit the production of mycotoxins. Investigating the effect of phenolic compounds on the biosynthesis of mycotoxins could provide valuable insight into reducing contamination of various foodstuff, reducing the risk of exposure of humans and animals.

Author contributions 1.Mr JJF Viljoen (first author; post-graduate student who conducted the research experiments and drafted the manuscript). 2.Dr Mariette Truter (assisted with the experiments; edited manuscript). 3.Prof Quenton Kritzing (supervisor of the study; edited manuscript).

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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