The fungal diversity and mycotoxins associated with South African soybeans

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by

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TABLE OF CONTENTS

DECLARATION OF ORIGINALITY

I, Shivan Bezuidenhout, declare that the thesis/dissertation, which I hereby submit for the degree Magister Scientiae at the University of Pretoria, contains my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature:

Date: April 2023

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PREFACE

Soybean is one of the most important oilseeds and is used for food, feed, and crop rotation. South Africa is the largest soybean producer in sub-Saharan Africa and mainly uses soybean in the feed industry. Soybean production is threatened by mycotoxin contamination and fungal disease that can impact the quality and quantity of seed production. Before this survey, there were limited studies on fungi and their mycotoxins associated with South African soybeans. This study thus provides important baseline knowledge to direct future research on this topic. The strains preserved in culture collections and their DNA sequences deposited in public databases will also serve as important reference materials.

The first chapter was written as a literature article focusing on the economic value and importance of soybeans, and discussing important mycotoxins associated with soybeans and soybean meal. A summary of each disease is provided with information on in-field symptoms, causal agents, and how to identify the species using both morphological and molecular data.

The second chapter explores the fungal diversity and mycotoxins associated with pre-stored South African soybean seeds. The aim was to monitor collected samples for common mycotoxins using LC/MS analysis and to then isolate and identify fungi to species based on morphology and DNA-sequence data and mycotoxin survey.

The third chapter focuses on the isolation and identification of fungal species associated with diseased soybean plant roots, stems, and leaves, collected across South Africa. The aim was to identify fungal species using DNA-sequence data. The overall of this project was to identify fungal diversity associated with soybeans and make the DNA sequence data available on Genbank as reference data for accurate and reliable identifications in the future.

CHAPTER 1

A Literature Review: An overview of mycotoxigenic fungi and fungal diseases affecting soybeans

INTRODUCTION

History of soybeans

Soybean (*Glycine max* (L.) Merr.) belongs to *Fabaceae* and is an important food legume. The origin of soybean domestication is ambiguous, but it is widely believed to be cultivated from wild soybean (*Glycine soja*) in East Asia around 1100 BC (Hymowitz, 2008; Sedivy *et al.*, 2017). Soybean was introduced to the western world around the 19th century (DAFF, 2010) and was first introduced to South Africa and other East African countries as an agricultural crop in 1903 by Chinese traders. Today, soybeans are one of the world's most important legumes and are widely commercialised.

Global soybean production and export

Soybean is a global commodity, with 170 countries participating in international trade as importers, exporters, or both (De Maria *et al.*, 2020). The largest soybean-producing and exporting countries during the 2020/21 season (USDA, 2022b) by value in million metric tons (MMT), were Brazil (±139,50 MMT), the United States (±114,75 MMT), and Argentina (±46,20 MMT). During the same season, China was the largest importer of soybeans, importing ±99.8 MMT mostly for its high feed demand in the swine and poultry sectors (USDA, 2021).

Soybean production and consumption in South Africa

Locally, 1 897 000 metric tons (MT) of soybeans were produced in the 2020/21 season, an increase from 1 245 000 MT in the 2019/20 season (SAGL, 2021). The season's soybean production is the highest recorded in South Africa for the last decade. Eight provinces contributed to production, including the Free State (766 500 MT), Mpumalanga (644 550 MT), North West (166 800 MT), KwaZulu-Natal (129 600 MT), Gauteng (105 000 MT), Limpopo (71 750 MT), Eastern Cape (9 300 MT), and Northern Cape (3 500 MT) provinces (SAGL, 2021). South Africa ranks 11th in the world for soybean production (USDA, 2022a).

SOYBEAN USAGE

The nutritional content of soybeans

Soybeans are desired for their relatively high protein content (Medic *et al.*, 2014; Natarajan S *et al.*, 2013). On average, seeds contain 38 % protein, 30 % carbohydrates, and 8 % fats (primarily unsaturated) (Kim *et al.*, 2021). They are known for their excellent amino acid profile because they contain the essential amino acids, such as isoleucine, histidine, leucine, lysine, methionine, phenylalanine, threonine, and tryptophan (Tessari *et al.*, 2016) The nutritional profile of soybeans makes them a desirable commodity for various uses in the food, animal feed, and farming sectors.

Soybeans in the food industry

The global soy food market was valued at US$ 44.7 billion in 2021 and projected to reach US$ 60.7 billion by 2027 (IMARC, 2022). Soybeans are an important protein source that has been consumed worldwide for centuries, especially in oriental cuisine. Popular soybean products include miso soup or paste, soy sauce, tempeh, and tofu (Chen K *et al.*, 2012). Today, these products are not only consumed in oriental countries but are favoured around the world, especially for individuals who are lactose intolerant or are shifting to a plant-based diet (Rizzo and Baroni, 2018). Veggie burgers, soymilk yogurt and cheese, soy flour, and tofu hot dogs are a few products that are marketed to substitute meat and traditional dairy products (Chen K *et al.*, 2012). Soy oil is also a versatile cooking oil in restaurants or used by families at home as margarine or salad dressing. South Africans consume approximately 1.3 million tons of vegetable and oilseed oils per year (USDA, 2020).

Soybeans in the livestock feed industry

Soybean meal is a by-product of the oil extraction process and an important component of the animal feed industry worldwide, including in South Africa. In the past, South Africa was a major producer and net exporter of fish meal (BFAP, 2021). Fish meal was used domestically in the feed industry (especially in broilers) because it is an inexpensive and readily available protein source (BFAP, 2021). Consequently, fish meal is less popular and available because of overfishing and increased global prices (BFAP, 2021). Approximately 80,000 tons of fishmeal was produced during 2020/21 for livestock feed in South Africa (DAF, 2021). Soybean meal (also known as oilcake) has become an alternative protein feed and plays a significant role in the soybean industry growth in South Africa (BFAP, 2021). An estimated 1.5 million tonnes of soybean meal was consumed per annum (DAF, 2021).

Benefits of soybean crop rotation

Food safety and security are complementary factors in a sustainable future (Vågsholm *et al.*, 2020). Thus, the agricultural sector must implement effective strategies to meet production demands. Many farmers use soybeans to rotate with crops like maize and improve soil fertility. Plant roots produce and require a wide variety of compounds, and have an interactive relationship with the surrounding environment. Soybeans have an advantageous relationship with bacteria inhabiting root nodules, which can fix atmospheric nitrogen and restore it to the soil (GrainSA, 2020). Crops like maize do not have the ability to return nitrogen to the soil and if grown continuously on the same land, the soil becomes depleted of nitrogen unless artificially added. Many farmers implement a crop rotation system in which they rotate legumes such as soybeans with grass crops. Crop rotation with soybeans can also help break the disease cycle of recurring pathogens and increase microbiome biodiversity in the soil (Shrestha, 2021). Rotating legumes with non-legume crops has the advantage of providing sufficient nitrogen and using less nitrogen fertiliser (GrainSA, 2020), which is economically and environmentally more friendly. High soil quality can improve biodiversity, increase water uptake, and improve yield tolerance to drought and other harsh growing conditions under various crop rotations (Garbeva *et al.*, 2004; Shah *et al.*, 2021; Shrestha, 2021).

SOYBEAN GROWTH AND DEVELOPMENT

Soybeans in South Africa are planted from early to mid-November and the optimum harvesting time ranges between 120–130 days after (March to April) depending on the climatic conditions or cultivar. The growth stages of soybeans are summarised below based on previous studies (Fig 1) (Baltazari *et al.*, 2020; Fehr *et al.*, 1971). Soybean growth stages are divided into vegetative (V) and reproductive (R) stages (Fig 1) (Bayer, 2021) In the latter phase, the plant begins to set flowers and produce soybean pods. The growth stages begin when the primary root or radicle emerges from the seed and pulls the cotyledon to the soil surface; this is known as the emergence stage (VE). The cotyledons supply plants with nutrients for 7–10 days after emergence. This is followed by the vegetative cotyledon (VC) stage, where the cotyledon becomes fully exposed and unifoliate leaves emerge at the second node and begin to photosynthesise to provide the plant with energy. The first trifoliate leaflets develop and extend at node 3 and lead the plant into the V1 (vegetive first trifoliate) stage of growth. Subsequently, the V2 (second trifoliate) stage is established through the development of second trifoliate leaves and root module development and initiates nitrogen fixation and the growth of lateral roots. In the third to sixth trifoliate (V3 to V6) phases, the axillary buds develop into flower clusters (racemes) at the top of the stem, and lateral roots grow.

The beginning bloom or first reproductive (R1) stage is initiated when one flower opens at any node. Flowering typically begins at nodes three to six of the main stem. The full-bloom stage (R2) occurs when an open flower develops at one of the top two nodes of the main stem. The beginning pod (R3) stage occurs when pods grow on at least one of the upper four nodes and are at least 5 mm long. This is followed by the “full pod” (R4) stage, where pods begin to grow and the seeds begin to develop. The beginner seed (R5) stage occurs when at least one (3 mm) long seed is present in a pod at one of the four upper nodes. At this stage, nitrogen fixation peaks, and plants attain the maximum height, node number, and leaf area. The full seed (R6) stage is commonly referred to as the ‘green bean’ stage because it marks the filling of the pod cavity. As the pod weight reaches the maximum, the leaves begin to turn yellow. As the plant begins the maturity (R7) phase, at least one normal pod on the main stem turns brown, and the other pods lose their green colour. Lastly, when most pods turn brown, the full maturity (R8) phase has been reached with 5 to 10 days of dry weather needed for good harvesting.

MYCOTOXINS: A THREAT TO FOOD AND FEED SECURITY

Safeguarding food and feed quality against fungi and their associated mycotoxins is critical for achieving sustainability. Mycotoxins are toxic compounds produced by certain fungi and affect global food safety. Mycotoxins are mainly produced by mycotoxigenic species that belong to the genera like *Aspergillus*, *Alternaria*, *Claviceps*, *Fusarium*, *Penicillium,* and *Stachybotrys* (Greeff-Laubscher *et al.*, 2020; Kumar *et al.*, 2020; Wambacq *et al.*, 2016). As technology improves, so do the analytical methods for detecting trace levels and identifying known and unknown mycotoxins. Even with the most well-studied mycotoxins, there remain many unanswered questions regarding mycotoxigenic fungi and mycotoxins. It is necessary to understand which commodities and geographic regions are more prone to mycotoxin contamination.

Mycotoxins

The potential health risks to animals and humans posed by mycotoxin consumption have been recognised internationally by the European Commission (EC) and its European Food Safety Authority (EFSA), the US Food and Drug Administration (FDA), the World Health Organisation (WHO) and the Food and Agricultural Organisation (FAO). Mycotoxins can be divided into three categories, regulated, emerging, and masked mycotoxins (Siri-anusornsak *et al.*, 2022). Regulated mycotoxins are those that have established regulatory limits or set maximum levels in animal feed and food commodities for humans. The most important regulated mycotoxins are aflatoxins (AFs), fumonisins (FUMs), ochratoxins (OTA), deoxynivalenols (DON), and zearalenones (ZEA) (Omotayo *et al.*, 2019; WHO, 2018). Emerging mycotoxins are a group of chemically diverse mycotoxins which are not routinely tested or don’t have regulatory limits. Some of the most important emerging mycotoxins are ergot alkaloids, enniatins, beauvericin, and moniliformin (Kovalsky *et al.*, 2016). The term masked mycotoxins refers to biologically modified mycotoxins that are conjugated by plants and undetected with conventional analytical techniques (Kovalsky *et al.*, 2016). Among the group of masked mycotoxins, ZEN-14-sulfate and DON-3-glucoside are most commonly observed in feed (Dall’Erta *et al.*, 2013). Mycotoxins threaten sustainable development goals by contaminating feed and food and causing health risks when consumed by animals and humans.

Mycotoxins and mycotoxigenic fungi associated with soybean seeds

Soybean seeds are susceptible to the growth of fungi and should be monitored for mycotoxin contamination to ensure safety for human and animal consumption. Some studies have found mycotoxigenic fungi as dominant communities, but no mycotoxins associated with soybean seeds. Soybean seeds harvested from the 2010/2011 crop season in Argentina identified *Alternaria, Aspergillus, Chaetomium, Cladosporium, Fusarium, Penicillium, Phomopsis, and Sclerotinia* as dominant genera (Garrido *et al.*, 2013). In particular, *Alternaria alternata* (100%), *Alternaria* *tenuissima* (95%), and *Aspergillus flavus* (57%) as the most frequently isolated species. Despite the presence of mycotoxigenic fungi, none of the mycotoxins tested for (AFs, ZEA, FBs, trichothecenes, and OTA) were detected in the samples. In South Africa, ten soybean samples representing different regions were collected during the 2011/2012 season and analysed for thirteen mycotoxins, including AFs (AFB_1, AFB_2, AFG_1, AFG_2), FUMs ($FUMB_1$, $FUMB_2$, $FUMB_3$), DON, OTA, ZEA, and T2 – toxin, but no mycotoxin residues were detected on any of the samples analysed (SAGL 2012).

Some studies have reported mycotoxin associated with soybeans, but are generally within regulatory limits. Egbuta *et al.* (2016) screened soybean seeds for fungi and mycotoxins contaminating collected from Nigerian markets. *Alternaria*, *Aspergillus*, *Fusarium*, and *Penicillium* were dominant genera found on soybean seed with dominant mycotoxigenic species including *A. flavus* (42.9 %), *A. parasiticus* (33.3 %), and *F. verticillioides* (23.8 %). Based on high-performance liquid chromatography (HPLC), seeds contained AFs (0.111 – 3.430 ug/g), $FUMB_1$ (0.033 – 2.270 ug/g), and OTA (0.000 – 0.051 ug/g). However, mycotoxins levels were not of concern since they were below the regulatory limits. The European and Nigerian regulatory limits of 4.0 µg/kg and 15 µg/kg respectively for aflatoxins, 5 µg/kg for OTA, and 4 mg/kg for FUMs (FDA mycotoxin regulatory guidance) (Egbuta *et al.*, 2016). In another study, soybean samples from Rwanda reported some AFs contamination (Niyibituronsa *et al.*, 2018). Most of the samples (n=291) were below the limits of detection (<1µg/kg). Only one positive sample (11.2µg/kg) exceeded the AFs regulatory limits and eight positive samples showed AFs levels ranging between 1–1.60 µg/kg which are below the maximum allowable limit (Niyibituronsa *et al.*, 2018). In 2012, twenty soybean samples from local Pakistan markets were tested for AFs. Two samples had a concentration ranging from 5.5 – 10.5 µg/kg above the maximum limit, and one sample of 3.2 µg/kg was below the suggested limits (Lutfullah and Hussain, 2012).

The presence of mycotoxigenic fungi, reported in these previous studies, may pose a threat later in downstream products, for example, soybean meal, if conditions are favourable for mycotoxin production and it is thus important to understand what fungi are commonly present in South African produced soybean. Despite the growing demand for this oilseed in South Africa, mycotoxins have not been monitored for soybeans since 2012.

Mycotoxins in soybean-based animal feed

Soybean meal is an important raw component of animal feed. Considering animal feed production involves several steps and processing, it is critically important to monitor raw materials and finalised products for mycotoxin contamination. Rodrigues and Naehrer (2012) investigated feedstuff worldwide over three years for the presence of mycotoxins AFs, DON, FUMs, OTA, and ZEA. Mycotoxins such as ZEA and DON were most frequently reported in soybean meals but mycotoxins were within regulatory limits except for one sample from the USA that contained DON (5500 ppb) and a sample from Turkey contaminated with FUM (5088 ppb)(Rodrigues and Naehrer, 2012). Li *et al.* (2014) investigated the occurrence of four major mycotoxins (AFB_1, DON, ZEA, and OTA) in feed ingredients (corn, wheat bran, soybean meal, and DDGS) and complete swine feeds from swine farms in China's Beijing region. Eleven soybean meal samples were tested and were contaminated with AFB_1 (36 %), DON (54 %), ZEA (54 %), and OTA (64 %), but mycotoxin levels were not of concern because they were below China's regulatory limits (30 µg/kg).

While studies have found that mycotoxin levels are generally low in soybean meals, there are surveys showing it can still be a problem. Iqbal *et al.* (2016) investigated mycotoxin contamination on important substrates used for the poultry industry. Soybean meal amongst other raw substrates was collected from farmers, industry, and general stores from large cities in Punjab, Pakistan. Soybean meal was contaminated with AFB_1 (range: 0.09–105.9 ug/kg) and ZEA (range: 0.15–120.89 ug/kg), with the levels of AFs above the EU legislative levels (20 µg/kg) (Iqbal *et al.*, 2016). International mycotoxin surveys show mycotoxin contamination below regulatory limits, but there are cases that exceed the limit showing soybean meal can present high contamination levels when conditions are favourable for mycotoxin production. There are no previous studies investigating the mycotoxins associated with soybean meal in South Africa.

Conclusion

Soybean seeds seem to be less susceptible to mycotoxin contamination in comparison to other commodities like maize. However, previous studies have shown the presence of mycotoxigenic fungi and mycotoxins. Mycotoxin levels in soybean have mostly been below the regulatory limits, in some cases exceeding them. It is also important to consider the mixtures of mycotoxins may have additive and/or synergistic effects. Soybean based-products like soybean meal are more commonly contaminated with mycotoxins compared to soybean seeds. It is important to maintain vigilance and regularly monitor for mycotoxins. Despite mycotoxin contamination being a major concern in South Africa and across the world, limited studies have been conducted on mycotoxins in food and feed. Therefore, regular screening of mycotoxins and their producers is important to ensure adequate human and animal nutrition and health and to achieve sustainable development goals.

FUNGAL AND OOMYCETE DISEASES OF SOYBEAN

Soybean is highly susceptible to diseases that cause substantial economic losses and contribute to food insecurity on a national and global level, threatening soybean production and yield (Savary *et al.*, 2019; UN, 2022). Fungi and oomycetes (commonly referred to as 'water moulds') are adapted to live in soil, water, and plant debris and typically live in close contact with other organisms such as plants. Some of these organisms can infect seeds, foliage, stems, and roots and cause disease.

Information on crop losses is limited, challenging to obtain, and difficult to compile and compare across soybean-producing regions. There is limited information on the contribution of disease-associated quantity loss due to a lack of information and consensus or a suitable loss estimation framework. The Yield Loss calculator of the crop protection network is used to share economically important information on the estimated crop loss from diseases (Crop Protection Network, 2022l). It is estimated that a $3.6 billion loss is caused by fungal diseases in the USA (2019–2021) (Crop Protection Network, 2022l).

It is important to know which diseases are prevalent in soybean fields, and it is impractical to simultaneously protect soybean from all diseases. In most cases, when a disease is observed, it is too late to implement effective management strategies for the season. Therefore, it is important to monitor diseases across regions annually to establish which diseases are of concern and establish effective management strategies. In the following section, we discuss the most common diseases reported for soybeans across the world (Table 1).

1. Anthracnose

Soybean anthracnose is caused by the fungus *Colletotrichum truncatum* (Hartman *et al.*, 2015b). This disease has been reported in China (Shi *et al.*, 2021), Argentina (Dias *et al.*, 2019), India (Bhatt *et al.*, 2021), and the USA (Crop Protection Network, 2022l). According to the Crop Protection Network (2022l), economic losses due to soybean anthracnose amounted to $36,6 million between 2019 and 2021 in the USA.

Disease symptoms: Infection symptoms include curling of the plant tissue in the petiole, known as shepherd's crook, and irregularly shaped red to dark brown blotches embedded on the stems and petioles (Fig 2A) (Malvick, 2018a). In severe infections, leaf rolling (Fig 2B), or premature defoliation is observed. Pods may be shrivelled, have blotches on their external surface (Fig 2C), and contain fewer or mouldy seeds. Plant tissues can show black fruiting bodies (acervuli) that produce black spine-like setae (Fig 2D) which can be seen with an unaided eye but are easier to observe with a hand lens (Hartman *et al.*, 2015a; Malvick, 2018a).

Causal organism: *Colletotrichum truncatum* (Schwein.) Andrus & W.D. Moore, Phytopathology 25: 121 (1935) [MB#280780] ≡ *Vermicularia truncata* Schwein., Transactions of the American Philosophical Society 4 (2): 230 (1832) [MB#144328] = *Colletotrichum dematium f. truncatum* (Schwein.) Arx, Phytopathologische Zeitschrift 29 (4): 459 (1957) [MB#352008].

Classification: *Ascomycota, Sordariomycetes, Hypocreomycetidae, Glomerellales, Glomerellaceae*

Morphological and molecular identification: *Colletotrichum truncatum* colonies are pale grey to dark grey, reverse dark brown (Liu *et al.*, 2016). For molecular identifications, the internal transcribed spacer region of rDNA (ITS) does not work well at the species level. The glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) gene region is suitable for initial identifications across most species complexes (Vieira *et al.*, 2020) but should in some cases be supplemented with calmodulin (*CaM*), translation elongation factor 1-alpha (*tef1-α*), and β-tubulin (*BenA)* (Bakhshi *et al.*, 2018) (Table 2).

2. Cercospora Leaf Blight and Purple seed stain

Cercospora kikuchii is the causal organism of two important soybean diseases: Cercospora Leaf Blight (CLB) and purple seed stain (PSS). Purple seed stain is named after the characteristic purple-stained spots on seeds (Matsumoto and Tomoyasu, 1925). It is referred to as CLB when *C. kikuchii* infects aerial parts of the plant, such as the leaves, stems, or petioles (Walters, 1980). These two diseases may occur simultaneously or individually, and the presence or severity of the disease does not influence the other. According to the Crop Protection Network (2022l), the estimated economic loss due to CLB and PSS was $308 million in the USA from 2019 to 2021.

Disease symptoms: Symptoms are observed on the upper leaves that are exposed to sunlight. The fungus produces cercosporin, a toxin stimulated by light, which causes irregularly shaped purple patches on the leaves. Later, the affected leaves may become leathery and dark purple with bronze highlights (Fig 3A). Cercospora seed stain develops noticeable purple patches on the seed and causes shrivelling and reduced size (Fig 3B) (Crop Protection Network, 2022h).

Causal organism: *Cercospora kikuchii* (T. Matsumoto & Tomoy.) M.W. Gardner, Proceedings of the Indiana Academy of Science 36: 242 (1927) [MB#252873] ≡ *Cercosporina kikuchii* Tak. Matsumoto & Tomoy., Ann. phytopath. Soc. Japan: 10 (1925) [MB#268620] = *Mycosphaerella phaseoli* Chona & Munjal, Indian Phytopathology 9 (1): 53 (1956) [MB#301600].

Classification: *Ascomycota*, *Dothideomycetes, Mycosphaerellales, Mycosphaerellaceae*

Morphological and molecular identification: Colonies are white on the edge and light greyish olive towards the center (Hartman *et al.*, 2015b). Visible red soluble pigmentation, due to cercosporin production, is usually seen in culture media. The ITS barcode can be used for initial identification to a genus level but *gapdh* is a promising gene for species identification. It is suggested to use additional gene regions *CaM*, *tef1-α*, and *BenA* with *gapdh* (Bakhshi *et al.*, 2018; Groenewald *et al.*, 2013) (Table 2).

3. Charcoal Rot

Macrophomina phaseolina is a generalist soil-borne fungus causing charcoal rot in soybean. The name refers to the microsclerotia produced by the fungus which gives the lower stem and taproots a discoloured light grey/black or charcoal-like appearance. The disease is also commonly referred to as dry-weather wilt or summer wilt (Hartman *et al.*, 2015b) because, unlike many soybean pathogens, *M. phaseolina* thrives in warm temperatures ranging between 28 – 40°C and low rainfall (Sarr *et al.*, 2014). According to the Crop Protection Network (2022l), economic losses due to charcoal rot were estimated at $112.9 million in the USA from 2019 to 2021.

Disease symptoms: Black microsclerotia form in the vascular tissue of the host and can be observed on and through the lower stems and roots of infected plants (Fig 4A) and can be observed in cracks in the seed coat (Fig 4B) (Marquez *et al.*, 2021b). The leaves remain attached to the infected plant but remain smaller than usual and turn yellow before wilting (Fig 4C) (Gupta *et al.*, 2012).

Causal organism: *Macrophomina phaseolina* (Tassi) Goid., Annali della Sperimentazione Agaria 1 (3): 457 (1947) [MB#300023] ≡ *Macrophoma phaseolina* Tassi, Bollettino del Laboratorio de Orto Botanico Reale Universita Siena 4: 9 (1901) [MB#236367] = *Tiarosporella phaseolina* (Tassi) Aa, The genera of fungi sporulating in pure culture: 208 (1981) [MB#116053] = *Macrophomina philippinensis* Petr., Annales Mycologici 21 (3-4): 314 (1923) [MB#274223].

Classification: *Ascomycota*, *Dothideomycetes, Botryosphaeriales*, *Botryosphaeriaceae*

Morphological and molecular identification: *Macrophomina phaseolina* colonies are generally grey to black (Nouri *et al.*, 2020). The fungus forms microsclerotia, a compact mass of hardened fungal mycelium that is spherical, oval, or oblong, with colours ranging from light brown in the early stage to dark brown/black that can be seen on potato dextrose agar (PDA) (Hartman *et al.*, 2015b; Marquez *et al.*, 2021a). However, the identification of *Macrophomina* to species with morphology is considered nearly impossible, mainly due to similar conidial features (Machado *et al*. 2019). It is not possible to identify *Macrophomina* species based on the ITS barcode. The *tef1-α* gene region presented sufficient for species discrimination and can possibly be used as a primary marker for *Macrophomina* species (Hyde *et al.*, 2014; Machado *et al.*, 2019). Additional markers including actin (*act)*, *BenA,* and *CaM* are used for species identification (Table 2) (Machado *et al.*, 2019; Sarr *et al.*, 2014). Alternatively, specific primers are designed by Santos *et al.* (2020) using a PCR approach for accurate, and rapid identification of *Macrophomina* species (Boonham *et al.*, 2016) (Table 2).

4. Downy mildew

Downy mildew is a soybean disease caused by *Peronospora manshurica*, an oomycete distributed across the world. According to the Crop Protection Network (2022l), the economic loss due to soybean downy mildew was $6.3 million from 2019 to 2021 in the USA.

Disease symptoms: Foliar symptoms appear on the upper surface of young leaves with pale green-to-yellow spots that later enlarge and turn into bright yellow spots (Fig 5A) (Crop Protection Network, 2022c). The centre of the spots turns brown, but the margins remain yellow (Fig 5B). On the lower leaf surface, lesions are covered with tufts of greyish to pale purple sporangiospores (Fig 5C) (Hartman *et al.*, 2015b). Soybean pods can be infected without external symptoms (Ryley, 2015) or be confused with white mould (caused by *Sclerotinia sclerotium*) because the seeds can be coated with white mycelia.

Causal organism: *Peronospora manshurica* (Naumov) Syd., Beiträge zur Kryptogamenflora der Schweiz 5 (4): 221 (1923) [MB#270522] ≡ *Peronospora trifoliorum* var. *manshurica* Naumov, Bull. Soc. mycol. Fr.: 73 (1914) [MB#157134].
Classification: *Chromista*, *Oomycota*, *Oomycetes, Peronosporales, Peronosporaceae*

Morphological and molecular identification: Sporangiophores emerge from the stomata on the lower surfaces of leaves. Sporangia are usually 19–24 um in diameter and oospores are light brown or yellow. Gene-specific primers, PM3 and PM4 are used to identify *P. manshurica* by PCR (Lai P *et al.*, 2022) (Table 2).

5. Frog-eye leaf spot

The fungus *Cercospora sojina* causes a foliar disease called Frogeye Leaf Spot (FLS), sometimes referred to as Cercospora leaf spot, which was reported in Argentina, the USA, and Canada (Crop protection network 2016; Soares *et al.*, 2015). According to the Crop Protection

Network (2022l), the estimated loss due to FLS was $227 million in the USA during 2019-2021.

Disease symptoms: Foliar symptoms include light grey centers with purple to red-brown margins (Fig 6A) and sometimes develop yellowing halos (Fig 6B) (Hartman *et al.*, 2015b). On the stem, long, narrow, dark lesions with flat centers. On soybean pods, lesions can be reddish-brown, and the seed coat may crack or flake. As the disease progresses, individual spots can merge to form larger lesions and leads to premature leaf drops (Crop Protection Network, 2022d).

Causal organisms: *Cercospora sojina* Hara, Agric. World (Nogyo Sekai), Tokyo: 28 (1915) [MB#119516] ≡ *Cercosporina sojina* (Hara) Hara, Jitsuyo-sakumotsu-byorigaku: 112. 1925 =*Cercosporidium sojinum* (Hara) X.J. Liu & Y.L. Guo, Acta Mycol. Sinica 1: 100. 1982 = *Passalora sojina* (Hara) Poonam Srivast., Journal of the Living World 1 (2): 118 (1994) [MB#492549] = *Passalora sojina* (Hara) H.D. Shin & U. Braun, Mycotaxon 58: 163 (1996) [MB#434535] = *Cercosporina daizu* Miura, Manchurian R.R. Agric. Exp. Sta. Bull.: 25 (1920) [MB#491072].

Classification: *Ascomycota*, *Dothideomycetes*, *Mycosphaerellales, Mycosphaerellaceae*

Morphological and molecular: These cercosporoid genera are mainly separated based on a combination of characters, of which the structure of the conidiogenous loci (scars) and hila, and the presence or absence of pigmentation (Bakhshi *et al.*, 2015). The genus *Cercospora* is recognised by having pigmented conidiophores with conspicuous (thickened and darkened) conidiogenous loci and hyaline conidia with conspicuous hila (Bakhshi *et al.*, 2015; Groenewald *et al.*, 2013). However, morphological characteristics are unreliable and should be supported with DNA sequences (Chupp, 1954). The ITS barcode can be used for initial identification to a genus level but *gapdh* is a promising gene for *C. kikuchii* (causing CLB and PSS). However, the *gapdh* cannot always identify *C. sojina* and therefore it is suggested to use *act* and histone (*his)* are used to identify *C. sojina* (Groenewald *et al.*, 2013) (Table 2).

6. Fusarium wilt or root rot

Fusarium wilt and root rot are soil-borne diseases mainly caused by *Fusarium* species belong to *Fusarium oxysporum* species complex (FOSC). According to the Crop Protection Network (2022l), Fusarium root rot caused an estimated $43,8 million in economic losses in the USA from 2019 to 2021.

Disease symptoms: When roots are infected, they will appear reddish-brown to dark brown and have poor nodulation (Crop Protection Network, 2022e) (Fig 7A). The fungus colonises the xylem tissue and prevents effective water transport in the plant. Foliar symptoms include scorching and yellowing on the upper canopy, and the lower leaves can turn chlorotic (Fig 7B) and later wither and drop from the plant (Fig 7C). When opened, the vascular tissue and stem will be brown and exhibit wilting (Crop Protection Network, 2022e)

Causal organism: *Fusarium oxysporum* sensu lato Schltdl., Flora Berolinensis, Pars secunda: Cryptogamia: 139 (1824) [MB#218372].
Classification: *Ascomycota*, *Sordariomycetes, Hypocreales, Nectriaceae, Fusarium oxysporum* species complex (FOSC)

Morphological and molecular identification: Colonies of *F. oxysporum are* white to pinkish, cottony, and dark-purple (reverse) on potato dextrose agar (Khan *et al.*, 2021). The *tef1-α* gene region can be used to identify *Fusarium oxysporum* and additionally gene regions used for species delimitation include DNA-directed RNA polymerase II subunit (*rpb1*) and the second largest subunit of RNA polymerase II (*rpb2*) (O'Donnell *et al.*, 2009) (Table 2).

7. Phomopsis Seed Decay/ Pod and Stem blight

Phomopsis seed decay is a destructive soybean seed disease caused by the fungus *Diaporthe sojae*. According to the (Crop Protection Network, 2022l), this disease cost an estimated $260 million in economic losses between 2019–2021 in the USA.
Disease symptoms: In the field, the fungal fruiting bodies (pycnidia), appear as black specks. The pycnidia appear linearly down the stem (Fig 8A) and the dark zone lines are observed on the stem (Fig 8B) (Crop Protection Network, 2022g). The soybean seeds appear wrinkled, white, and reduced in size (Fig 8C) (Hartman *et al.*, 2015b).

Causal organism: *Diaporthe sojae* Lehman, Annals of the Missouri Botanical Garden 10: 128 (1923) [MB#278338] = *Phomopsis longicolla* Hobbs, Mycologia 77 (3): 542 (1985) [MB#105685] = *Diaporthe longicolla* (Hobbs) J.M. Santos, Vrandecic & A.J.L. Phillips, Persoonia 27: 13 (2011) [MB#563213] = *Diaporthe melonis* var. brevistylospora Ts. Kobay. & Tak. Ohsawa, Annals of the Phytopathological Society of Japan 55 (4): 416 (1989) [MB#126352].
Classification: *Ascomycota, Sordariomycetes*, *Diaporthales, Diaporthaceae*

Morphological and molecular identification: Colonies of *D. sojae* on PDA have fluffy, dense, white, and sometimes greenish-yellow aerial mycelia (Manawasinghe *et al.*, 2019). The reverse side colour appears white with pigment-free but large, black stromata visible. *Diaporthe* species cannot be distinguished using the ITS gene region and is only useful for initial identification to a genus level. The *tef1-α* is used to identify *Diaporthe* species (Santos *et al.*, 2017; van Rensburg *et al.*, 2011) (Table 2).

8. Phytophthora root and stem rot

Phytophthora root and stem rot affects production in many countries throughout the world caused by the oomycete *Phytophthora sojae.* The disease was first identified in the USA around 1948, but *P. sojae* was only described in 1958 (Hartman *et al.*, 2015b; Kaufmann and JW, 1958). According to the Crop Protection Network (2022l), an estimated $489 million was lost due to this soybean disease in the USA between 2019 and 2021.

Disease symptoms: Continuous wet weather and soil saturation allow the zoospores to swim to and infect soybean roots (Douglas, 2020). Damping-off is a condition that kills or weakens seeds or seedlings before or after the seed can germinate. The most common symptom is a dark brown lesion on the lower stem that can extend to higher areas, in some cases as high as the nodes of the soybean plant (Fig 9A). The split stem shows brown discoloration (Fig 9B). Soybean plants are stunted or killed because of the restriction of water and nutrients (Hartman *et al.*, 2015b; Malvick, 2018b).

Causal organism: *Phytophthora sojae* Kaufm. & Gerd., Phytopathology 48: 207 (1958) [MB#303624] = *Phytophthora megasperma* var. *sojae* A.A. Hildebr., Canadian Journal of Botany 37 (5): 954 (1959) [MB#352395].
Classification: *Stramenopila, Oomycota, Peronosporomycetidae, Pythiales, Pythiaceae*

Morphological and molecular identification: Based on Dorrance A *et al.* (2012), *P. sojae* most commonly grows on Lima bean agar and has no colour. The oomycete doesn't grow on full-strength potato dextrose agar but reducing the concentration or opting for water agar can promote growth. On V8 juice agar, the hyphae of *P. sojae* are white (Dorrance A *et al.*, 2012). Previously, the PS1–PS2 (based on ITS gene region) primer set was developed for use with conventional PCR and real-time PCR to detect *P. sojae* (Wang *et al.*, 2006). However, it was challenging to distinguish *P. sojae* from other *Phytophthora* species. Species-specific primers (PSOJ primers) developed by Bienapfl *et al.* (2011) can identify *P. sojae* using PCR and distinguish it from other *Phytophthora* species (Table 2).

9. Powdery Mildew

Powdery mildew of soybean is caused by the fungus *Erysiphe diffusa* and has been recorded in Australia (McTaggart *et al.*, 2012), Brazil (Gonçalves *et al.*, 2002), India (Baiswar *et al.*, 2016), and Vietnam (Le *et al.*, 2017).

Disease symptoms: The most common symptom is the white, powdery coating on the upper surface of the leaves (Fig 10A). Small patches emerge and as the disease progresses, the whole leaf will be covered. Disease severity differs among soybean cultivars, but plants usually exhibit leaf yellowing, scorching, or rust-coloured patches (Malvick, 2018c). In more severe cases, necrotic lesions and defoliation occur (Fig 10B). Soybean pods are also affected by the disease, leading to reduced pod fill, and shrivelled, and underdeveloped seeds (Crop Protection Crop Protection Network, 2021b).

Causal organism: *Erysiphe diffusa* (Cooke & Peck) U. Braun & S. Takam., Schlechtendalia 4: 7 (2000) [MB#464293] ≡ *Microsphaera diffusa* Cooke & Peck, J. Bot. 10: 13 (1872) [MB#176571].
Classification: *Ascomycota, Leotiomycetes, Erysiphales, Erysiphaceae, Erysiphe*

Morphological and molecular identification: Morphological identification at a genus or species level is nearly impossible (Braun *et al.*, 2000; Heffer V *et al.*, 2006). The ITS DNA barcode can be used to identify *E. diffusa* (Bradshaw *et al.*, 2021; Le *et al.*, 2017) (Table 2).

10. Sclerotinia Stem Rot

Sclerotinia stem rot (SSR) also referred to as “white mold”, is caused by *Sclerotinia sclerotium* and is considered one of the most important soybean diseases. The disease was first reported in South Africa in 1979 and has become increasingly common over the years (DARD, 2021; Van der Hoven *et al.*, 2017). According to the Crop Protection Network (2022l), an estimated $677 million was lost due to SSR in the USA between 2019 and 2021.

Disease symptoms: Fluffy, white mycelia can be seen growing on the stem (Fig 10A). When the stem is cut open characteristic black sclerotia are visible (Fig 11B). The leaf tissue between prominent veins will turn greyish-green and die but remain attached to the stem (Giesler, 2022a). Soybean pods appear smaller, lighter, and cottony, and in some cases, the seeds are replaced by the sclerotia in the pod (Fig 11C) (Van der Hoven *et al.*, 2017). Sclerotia germinate to develop a structure called apothecia which can sometimes be seen in the soil (Fig 11D).

Causal organism: *Sclerotinia sclerotiorum* (Lib.) de Bary, Vergleichende Morphologie und Biologie der Pilze Mycetozoen und Bacterien: 56 (1884) [MB#212553] ≡ *Peziza sclerotiorum* Lib., Plantae Cryptogamae, quas in Arduenna collegit Fasc. 4: no. 326 (1837) [MB#168084] = *Sclerotinia libertiana* Fuckel, Jahrbücher des Nassauischen Vereins für Naturkunde 23-24: 331 (1870) [MB#191471].
Classification: *Ascomycota, Discomycetes, Helotiales, Sclerotiniaceae*

Morphological and molecular identification: *Sclerotinia sclerotiorum* can be variable in culture but generally is described as fluffy white (sometimes dirty white or brownish) aerial mycelia accompanied by black sclerotia production on PDA. The size, shape, and number, pattern of sclerotia formation varies between isolates (Md and Mme, 2022; Smolińska and Kowalska, 2018; Sousa Melo *et al.*, 2019). The ITS DNA barcode is used to identify *S. sclerotiorum* (Grabicoski *et al.*, 2015; Md and Mme, 2022) (Table 2).

11. Septoria brown spot

Septoria brown spot (SBS), commonly known as brown spot, is a soybean foliar disease that was first reported in the USA in 1922 (Hartman *et al.*, 2015b; Wolf, 1926). Today, the disease can be found in some of the top soybean-producing countries such as the United States (Allen *et al.*, 2017), Argentina, Brazil, and China (Hartman *et al.*, 2015a). Soybean SBS is one of the ten most destructive diseases (Allen *et al.*, 2017) and caused an estimated $155 million in losses in the USA from 2019 to 2021 Crop Protection Network (2022l).
Disease symptoms: Symptoms first appear on the lower leaves and progress to the mid-to-upper canopy. Small brown spots develop on the leaves (Fig 12A) and later enlarge, merge, and develop into irregular brown, yellow blotches and the leaves die and drop prematurely (Fig 12B) (Hartman *et al.*, 2015b; Malvick, 2018d).

Causal organism: *Septoria glycines* Hemmi, Transactions of the Sapporo Natural History Society 6: 15 (1915) [MB#215541].
Classification: *Ascomycota, Dothideomycetes, Capnodiales*, *Mycosphaerellaceae*

Morphological and molecular identification: White, circular slightly appressed mycelium growing on reddish brown hyphae. Dark brown pycnidia are produced in concentric circles around the white mycelia (Mirza, 2002). The ITS barcode can be used to identify to a genus level but *BenA* is informative to identify species of *Septoria* (An Y *et al.*, 2021) (Table 2).

12. Soybean Rust

Soybean rust is caused by two closely related fungi, *Phakopsora pachyrhizi,* and *Phakopsora meibomiae*. *Phakopsora pachyrhizi* is known to cause Asian soybean rust and is widely distributed across Asia, Africa, South America, and the U.S. (CABI, 2009; Giesler, 2023). *Phakopsora meibomiae* is native to the tropical and subtropical regions of the Americas and is considered less aggressive than *P. pachyrhizi* (Rupe and Sconyers, 2008). *Phakopsora pachyrhizi* is considered the most important of the two rust pathogens for serval reasons. *Phakopsora pachyrhizi* populations from different countries are composed of different races or pathotypes, with pathotypes often having multiple virulence factors (Hartman *et al.*, 2015b; Rupe and Sconyers, 2008). Additionally, it has a wider geographic distribution, is known to overcome sources of resistance, and causes more damage throughout its range of occurrence (Hartman *et al.*, 2015b). The Crop Protection Network (2022l) estimated a $36 million loss in the USA from 2019 to 2021 due to soybean rust.

Disease symptoms: Diseases symptoms from the two rust species cannot be distinguished by observing foliar symptoms in the field. Symptoms are small, tan-reddish lesions (spots) that develop on the underside of the leaves as described by Malvick (2018e) (Fig 13A). Lesions will enlarge within a few days, and rust pustules (blister-like) will become visible (Fig 13B). It is challenging to distinguish between soybean rust and other common leaf diseases before the pustules develop.

Causal organism: *Phakopsora pachyrhizi* Syd. & P. Syd., Annales Mycologici 12 (2): 108 (1914) [MB#121037].

Causal organism: *Phakopsora meibomiae* (Arthur) Arthur, Bulletin of the Torrey Botanical Club 44: 509 (1917) [MB#102469] ≡ *Physopella meibomiae* Arthur, Mycologia 9 (2): 59 (1917) [MB#202229] = *Malupa meibomiae (*Arthur) Y. Ono, Buriticá & J.F. Hennen, Mycological Research 96: 828 (1992) [MB#357586]

Classification: *Basidiomycota, Pucciniomycotina, Pucciniomycetes, Pucciniales, Raveneliineae*, *Phakopsoraceae*

Morphological and molecular identification: The ITS barcode and species-specific primers are the most useful ways to identify *P. pachyrhizi* and *P. meibomiae* (Tapiero *et al*., 2021) (Table 2). The species-specific include Ppm1 and Pme2 for *P. meibomiae*, and Ppm1, Ppa2, Ppa3, and Ppa4 for *P. pachyrhizi* (Frederick *et al*., 2001; Tapiero *et al*., 2021).

13. Soybean stem canker

Stem cankers are classified as either northern stem canker (caused by *Diaporthe caulivora*) or southern stem canker (caused by *Diaporthe aspalathi*). The names are based on their occurrence in the northern and southern regions of the U.S. and are caused by two different species. *Diaporthe* species typically live as endophytes within a wide range of plant hosts (Gomes *et al*., 2013). Certain members of the genus *Diaporthe* are well-studied phytopathogenic fungal species and are commonly referred to as Diaporthe/Phomopsis Disease Complex (DPC) because they cause serious destructive diseases on soybeans such as seed decay, pod & stem blight, and stem canker (Hosseini *et al*., 2020; Santos *et al*., 2011). According to the Crop Protection Network (2022l), it is estimated loss of stem canker is $163 million.

Disease symptoms: Initially small, reddish-brown lesions develop on the main stem at the base of the lower nodes (Fig 14A) and grey streaking can be seen when splitting the stem or taproots (Fig 14B) (Malvick, 2018f). Lesions tend to elongate and eventually become sunken with dark brown to black margins expanding down below the soil line (Hartman *et al*., 2015b). Small black spots, called the stroma, are compact masses of fungal hyphae in which perithecia (the sexual fruiting structures of the fungus), will be present in infected tissue. Foliar symptoms include interveinal chlorosis which leads to necrotic lesions (Giesler, 2022b). Eventually, the leaves die and generally remain attached to the stem. There is no difference documented for the disease symptoms between the two stem cankers.

Causal organism (Northern stem canker): *Diaporthe caulivora* (Athow & Caldwell) J.M. Santos, Vrandecic & A.J.L. Phillips, Persoonia 27: 13 (2011) [MB#518520] ≡ *Diaporthe phaseolorum* var. *caulivora* Athow & Caldwell, Phytopathology 44: 323 (1954) [MB#346720] = *Diaporthe phaseolorum f.sp*. *caulivora* Kulik, Mycologia 76: 288 (1984) [MB#118262].

Causal organism (Southern stem canker): *Diaporthe aspalathi* E. Jansen, Castl. & Crous, Studies in Mycology 55: 71 (2006) [MB#500803] ≡ *Diaporthe phaseolorum* var. *meridionalis* F.A. Fern., Mycologia 88 (3): 438 (1996) [MB#416161].
Classification: *Ascomycota, Sordariomycetes*, *Diaporthales*, *Diaporthaceae*

Morphological and molecular identification: *Diaporthe caulivora* cultured on PDA and Sabouraud Dextrose Agar (SDA) show white aerial mycelia with zones of white-brownish pigmentation. On the reverse side, colonies have yellow pigmentation with cream to pale brown in the center with clustered perithecia on PDA (Mena *et al.*, 2019). *Diaporthe aspalathi* produces white colonies with interspersed cottony tufts. Some isolates produce large stromata in which perithecia form. Morphologically it is challenging to identify *Diaporthe* species, with DNA sequencing needed to confirm observations. The ITS barcode is only useful for genus identification but insufficient for species identification. The t*ef1-α* gene region is typically used as an identification marker (Santos *et al.*, 2017; van Rensburg *et al.*, 2011) (Table 2).

14. Sudden Death Syndrome

Soybean sudden death syndrome (SDS) is an emerging disease of great economic importance worldwide. The disease was first detected in Arkansas (USA) in 1971 and has since been reported in South Africa (Tewoldemedhin YT *et al.*, 2014; Tewoldemedhin *et al.*, 2017). According to Crop Protection Network (2022l), it is estimated that economic losses due to SDS amounted to $652 million in the USA between 2019 and 2021. SDS is caused by the fungus *Neocosmospora phaseoli*. *Neocosmospora* (previously the *Fusarium solani* species complex) differs from other fusarioid fungi based on their ecology, the morphology of sexual and asexual characteristics, and extrolite production (Crous *et al.*, 2021b; Sandoval-Denis *et al.*, 2019).

Disease symptoms: Scattered, yellow spots begin to form on soybean leaves and grow to form large chlorotic and necrotic blotches between the leaf veins, while the midvein and major lateral veins remain green (Hartman *et al.*, 2015b; Westphal *et al.*, 2018) (Fig 15A). Severely infected leaves will fall off, but the petioles remain on the stem. Roots are easily removed from the soil with reduced mass and discoloration (Fig 15B)(Westphal *et al.*, 2018). Longitudinal cuts along the stem will show grey or brown discoloration, but the pith remains white. The fungus can be isolated from the roots or lower stem near the soil lines (Hartman *et al.*, 2015b).

Causal organism: *Neocosmospora phaseoli* (Burkh.) L. Lombard & Crous, Studies in Mycology 80: 227 (2015) [MB#810962] ≡ *Fusarium martii f. phaseoli* Burkh., Memoirs of the Cornell University Agricultural Experimental Station 26: 1007 (1919) [MB#489076] = *Fusarium solani f.sp. phaseoli* (Burkh.) W.C. Snyder & H.N. Hansen, American Journal of Botany 28: 740 (1941) [MB#445914] = *Fusarium phaseoli* (Burkh.) T. Aoki & O'Donnell, Mycologia 95 (4): 671 (2003) [MB#488914] = *Fusarium martii* var. *minus* Sherb., Memoirs of the Cornell University Agricultural Experimental Station 6: 249 (1915) [MB#139254] = *Fusarium solani* f. 3 W.C. Snyder, Zentralblatt für Bakteriologie und Parasitenkunde, Abteilung 2 91: 179 (1934) [MB#494338] = *Fusarium tucumaniae* T. Aoki, O'Donnell, Yosh. Homma & Lattanzi, Mycologia 95 (4): 664 (2003) [MB#489463] = *Fusarium virguliforme* O'Donnell & T. Aoki, Mycologia 95 (4): 667 (2003) [MB#489315] = *Neocosmospora virguliformis* (O'Donnell & T. Aoki) L. Lombard & Crous, Studies in Mycology 80: 228 (2015) [MB#810967] = *Neocosmospora tucumaniae* (T. Aoki, O'Donnell, Yosh. Homma & Lattanzi) L' lombard & Crous, Studies in Mycology 80: 228 (2015) [MB#810966] = *Fusarium brasiliense* T. Aoki & O'Donnell, Mycoscience 46 (3): 166 (2005) [MB#338753] = *Fusarium cuneirostrum* O'Donnell & T. Aoki, Mycoscience 46 (3): 170 (2005) [MB#341392] = *Fusarium crassistipitatum* Scandiani, T. Aoki & O'Donnell, Mycoscience 53 (3): 171 (2012) [MB#561257] = *Fusarium azukiicola* T. Aoki, H. Suga, F. Tanaka, Scandiani & O'Donnell, Mycologia 104 (5): 1075 (2012) [MB#565190].

Classification: *Ascomycota, Sordariomycetes, Hypocreales, Nectriaceae*

Morphological and molecular identification: The macroconidia are usually thick-walled, with blunt, rounded apical cells, and they usually have inconspicuous foot-shaped basal cells (Crous *et al.*, 2021a). Microconidia are produced on very long, narrow phialides. However, it can be challenging to distinguish *Neocosmospora* from other fusarioid genera based on morphological characteristics. Molecular identification using ITS barcode is only useful to genus level, with *tef1-α* suggested for species identification (Crous *et al.*, 2021b) (Table 2).

15. Soybean red crown rot

Red crown rot (RCR) is caused by the fungus *Calonectria ilicicola* and causes the deterioration of soybean roots and stems. *Calonectria ilicicola* is a soilborne plant-pathogenic fungus and has become an emerging threat to crop production in the USA (Kleczewski *et al.*, 2019), and Taiwan (Liu *et al.*, 2020) in recent years. According to Crop Protection Network (2022l), it is estimated that $ 465 000 was lost in the USA (2019–2021) due to RCR.

Disease symptoms: Foliar symptoms initially appear as small light green to yellow blotches in between leaf veins (Hartman *et al.*, 2015b). Later the symptoms developed into interveinal necrosis (Fig 16A) and leaves can die prematurely. Reddish discoloration begins on the lower stem (Fig 16B) and can become flush with white hyphae and red/rusty brown spore-bearing structures (Fig 16C and 16D) if prolonged moisture occurs late in the growing season (Crop Protection Network, 2022j).

Causal organism: *Calonectria ilicicola* Boedijn & Reitsma, Reinwardtia 1: 58 (1950) [MB#293933] = *Cylindrocladium parasiticum* Crous, M.J. Wingf. & Alfenas, Mycological Research 97 (7): 892 (1993) [MB#360237].
Classification: *Ascomycota*, *Pezizomycotina*, *Sordariomycetes, Hypocreomycetidae, Hypocreales, Nectriaceae*

Morphological and molecular identification: Orange to red perithecia-like structures can be observed on the basal stem and crown (Kleczewski *et al.*, 2019). Colonies grown on MEA and V8 medium are initially white and dense and later exhibit brown to black pigments on the reverse (Kleczewski *et al.*, 2019; Liu *et al.*, 2020). The ITS region is not sufficient for distinguishing between species of *Calonectria*. The *tub2* locus is needed to identify *C. illicicola* (Crous *et al.*, 2000; Crous *et al.*, 2006; Kleczewski *et al.*, 2019) (Table 2).

16. Red Leaf Blotch

Red leaf blotch (RLB) is endemic to African countries of soybean (*Glycine max*) and caused by the fungus *Coniothyrium glycines*. The disease is also commonly referred to as “Dactuliophora leaf spot”, “Pyrenochaeta leaf blotch” and “Pyrennochaeta leaf spot” (Hartman *et al.*, 2015b). Red leaf blotch can potentially become a major foliar disease if it spreads to other soybean-producing countries like the U.S. (Hartman *et al.*, 2011). *Coniothyrium glycines* are currently listed as one (position 62nd) of the United States Department of Agriculture (USDA) Plant Protection and Quarantine Select Agents and Toxins (Federal Select Agent Program, 2021).

Disease symptoms: Lesions (1–3 mm in diameter) can be observed on leaves, petioles, pods, and stems. As the disease progresses, lesions appear as dark red spots on upper leaf surfaces with reddish-brown borders (Fig 17A) (Hartman *et al.*, 2015b). The uniqueness of the sclerotia may provide a characteristic that can be used for field diagnosis as they can be seen clearly with the aid of a hand lens. Orange to red perithecia-like structures can be observed on the basal stem and crown region.

Causal organism: *Coniothyrium glycines* (R.B. Stewart) Verkley & Gruyter, Studies in Mycology 75: 23 (2012) [MB#564777] ≡ *Pyrenochaeta glycines* R.B. Stewart, Mycologia 49: 115 (1957) [MB#304859]= *Dactuliochaeta glycines* (R.B. Stewart) G.L. Hartman. & J.B. Sinclair, Mycologia 80 (5): 699 (1988) [MB#135398]= *Phoma glycinicola* Gruyter & Boerema, Persoonia 17 (4): 554 (2002) [MB#478437]
Classification: *Ascomycota, Dothideomycetes, Pleosporales, Leptosphaeriaceae*

Morphological and molecular identification: Hartman and Sinclair (1988) described *Coniothyrium glycines* (previously *Dactuliochaeta glycines*) was described as woolly, pink to orange-red to brown colonies on malt extract agar (MEA). Reverse brown to black in the center red to pink with white to tan margins. Colonies on oatmeal agar (OA) are red, turning green to blue-green with NaOH. Pycnidia (subhyaline to brown) are immersed to semi-immersed in host tissue or agar media, growing out from the surface of sclerotia which is dark brown, scattered, and spherical. For molecular identification, the ITS, *BenA*, *RPB2* and *tef-α* are used for the identification (De Gruyter *et al.*, 2013; de Gruyter *et al.*, 2010; Quaedvlieg *et al.*, 2013).

17. Other soybean diseases

The above-mentioned soybean diseases are generally found worldwide in soybean-producing areas and cause economic or yield losses. There are other diseases including Alternaria leaf spot, Aristastoma leaf spot (*Aristastoma camarograghioides* & *A. guttulosum*), Black leaf blight (*Arkoola nigra*), Choarephora leaf blight (*Choanephora infundibulifera* & *C. cucurbitarum*), Drechslera Blight *(Drechslera glycini*) and soybean scab (*Elsinoe glycines)* (Hartman *et al.*, 2015b) have either been identified long ago or have limited information about causal agents, recent outbreaks, or geographical distribution.

Conclusion

Soybean is a protein-rich oilseed used as animal feed, food, and for crop rotation in South Africa. Many fungal species associated with soybeans can cause diseases, reduce seed quality, or produce mycotoxins. No information is currently available on fungal diversity associated with stored soybean seeds in South Africa and the last mycotoxin survey was completed more than a decade ago. Certain mycotoxins have serious health implications when consumed by humans and animals and are regulated in many countries. It is important to know which diseases are prevalent in soybean fields to help implement necessary management strategies or to generate knowledge of diseases in subsequent seasons. Once the baseline information is established, the next goal might be to tie this into environmental conditions to predict which diseases may become problematic. Previous studies have used morphology or molecular methods, targeting the ITS gene region, for fungal identification on soybean seed or as causal agents of soybean disease. It is critically important to identify causal agents at a species level using DNA markers that can identify to a species level.

The aim of this project was, therefore, to 1) identify the fungal diversity and mycotoxins associated with pre-stored soybean seeds, and 2) identify the fungal diversity associated with diseased soybeans from South Africa. The knowledge generated from this study will form baseline knowledge on the fungi and mycotoxins associated with soybeans in South Africa.

FIGURES

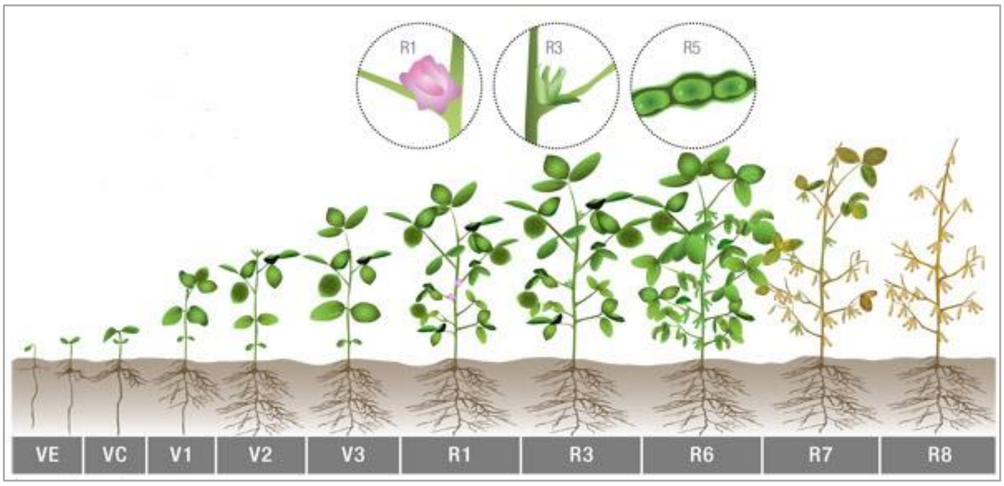

Figure 1. The development vegetative (V) reproductive (R) stages of soybean (Dekalb, 2021).

Figure 2. Soybean Anthracnose symptoms (A) irregularly shaped red to dark brown blotches embedded on the stems and petioles (B) leaf rolling before premature defoliation (C) pods may be shriveled and have blotches on the external surface (D) black acervuli (Crop Protection Network, 2022a).

Figure 3. Cercospora purple seed stain of soybean (A) foliar symptoms (B) purple seed stain symptoms on seeds (Crop Protection Network, 2022i).

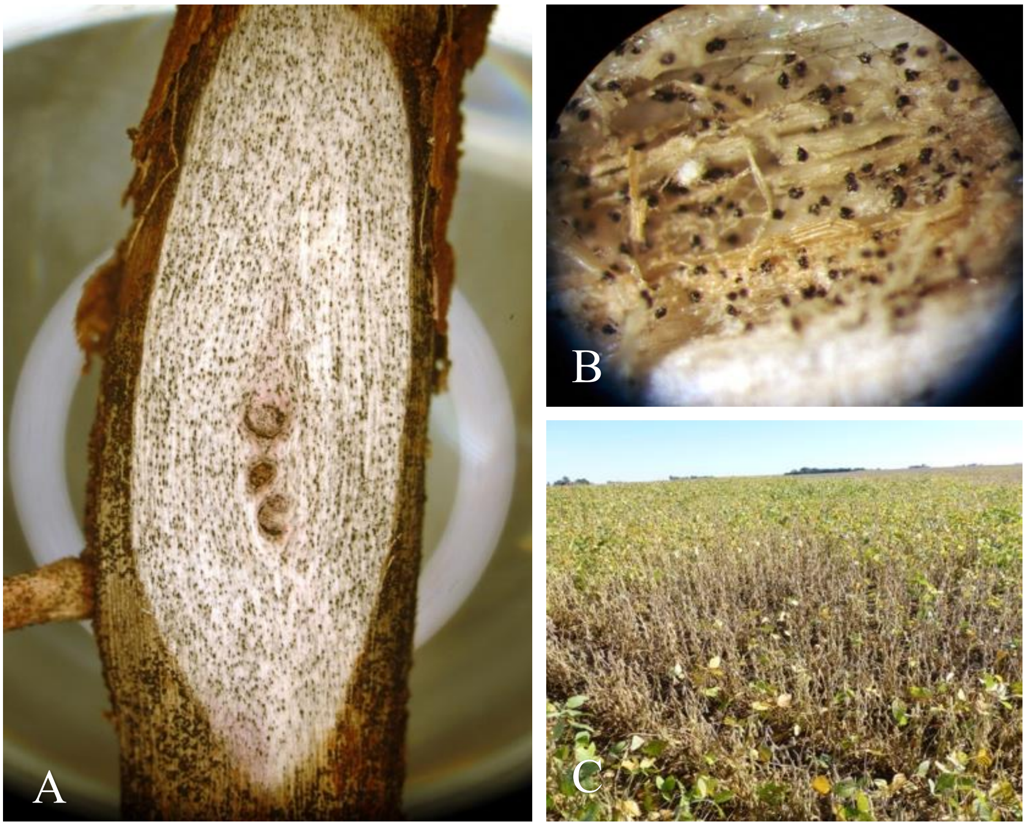

Figure 4. Soybean charcoal rot symptoms (A) charcoal-like, grey discoloration from microsclerotia on the internal and external parts of soybean stem (B) microsclerotia in stem and (C) stunted or wilted patches of soybean plants (Crop Protection Network, 2022b).

Figure 5. Soybean downy mildew (A–B) lesions beginning turning from yellow to brown on upper leaf side (C) grey mycelia growth on the leaf underside (Crop Protection Network, 2021a).

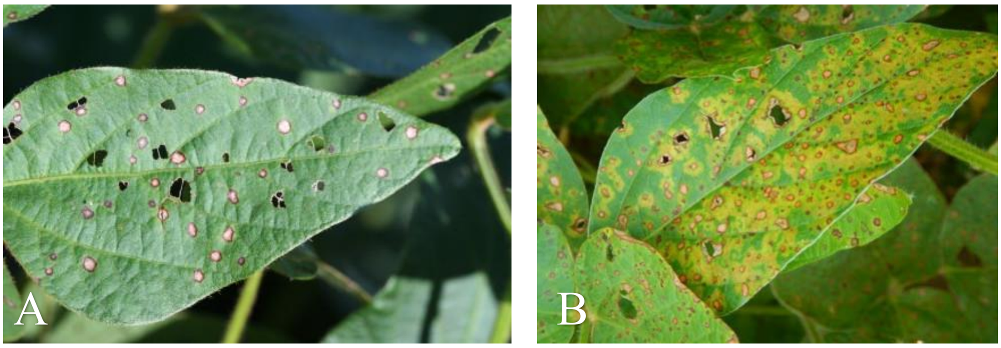

Figure 6. Soybean Frog-eye leaf Spot (A) reddish purple margins surround the grey centers on mature lesions (B) light green to yellow halos around lesions (Crop Protection Network, 2022d).

Figure 7. Soybean Fusarium wilt or root rot (A) reduced root mass and nodulation (B) foliar symptoms include scorching and yellowing on the upper canopy (C) leaves wither and drop (Crop Protection Network, 2022e).

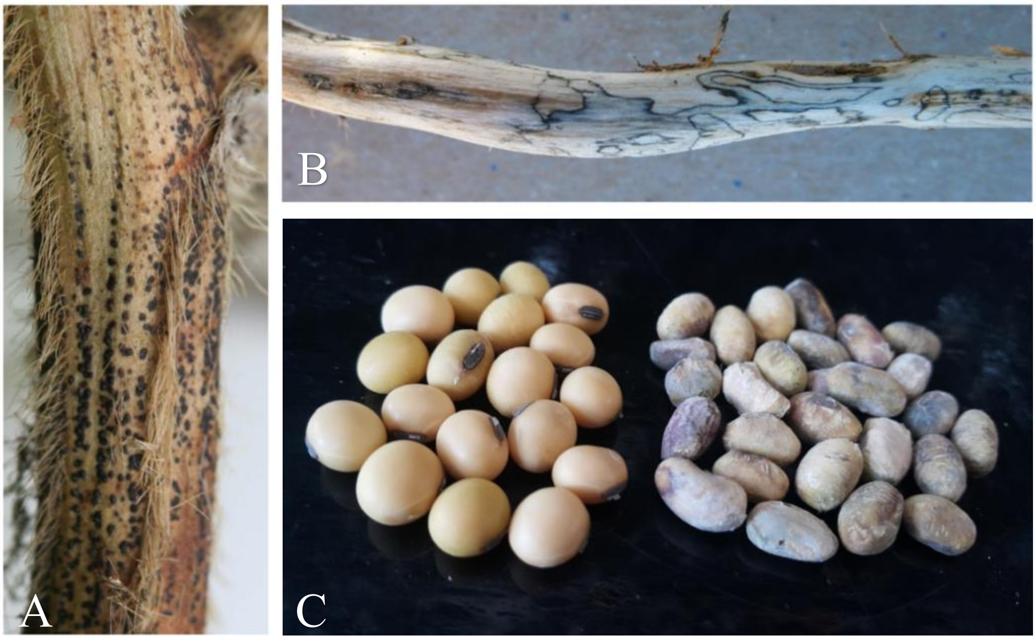

Figure 8. Soybean Phomopsis Seed Decay/ Pod and Stem blight (A) linear black pycnidia along the stem (B) dark zone lines in the longitudinal section of the lower stem (C) seeds showing Phomopsis decay (right) compared to healthy seeds (left) (Crop Protection Network, 2022g).

Figure 9. Soybean Phytophthora root and stem rot (A) brown discoloration of soybean stem (B) split stem showing brown discoloration due to Phytophthora infection (upper) compared to a healthy stem (lower) (Crop Protection Network, 2022f).

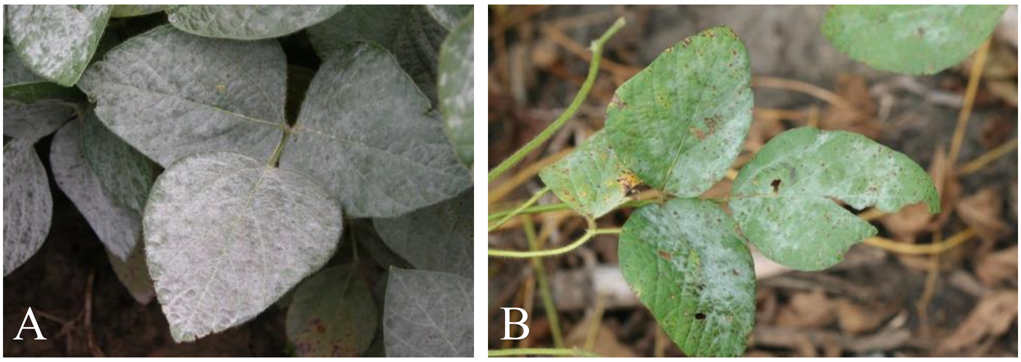

Figure 10. Soybean Powdery Mildew (A) white, powdery symptoms on foliar tissue and (B) with necrotic lesions shown (Crop Protection Network, 2021b).

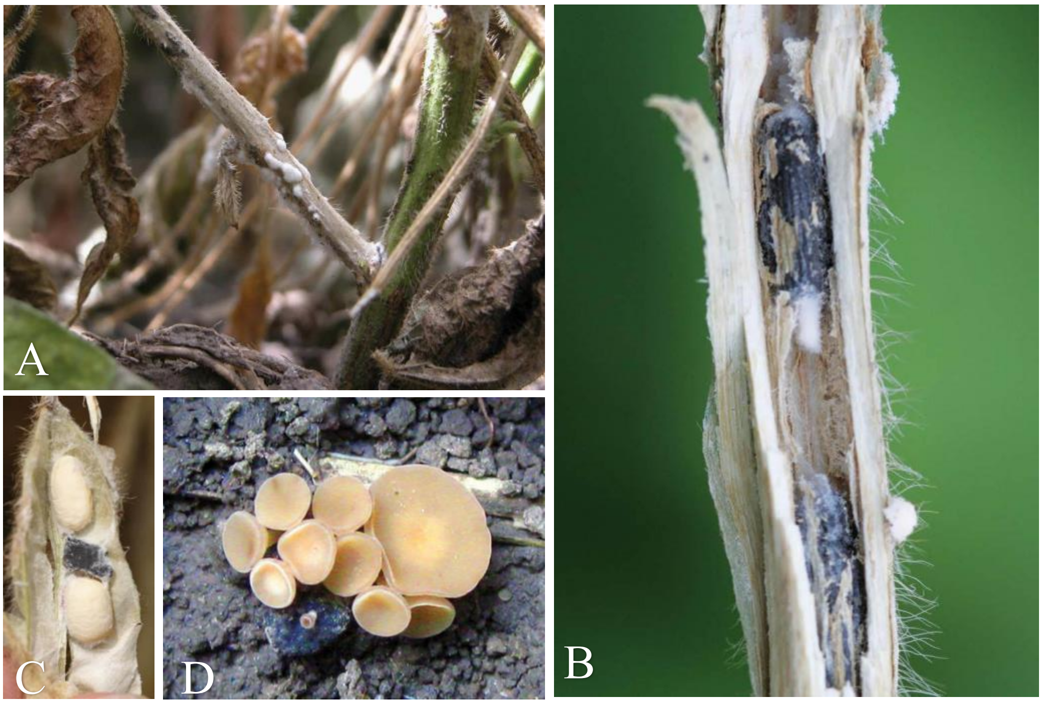

Figure 11. Soybean Sclerotinia Stem Rot (A) *S. sclerotiorum* white mycelium and sclerotia produced on the outside stem tissue (B) sclerotiorum inside a soybean stem (C) sclerotiorum inside a soybean pod (D) apothecia which produce ascospores (Crop Protection Network, 2022o).

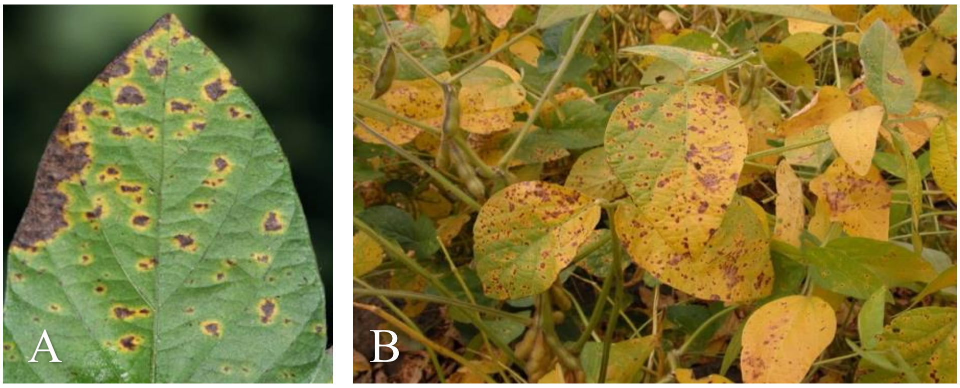

Figure 12. Soybean Septoria brown spot (A) small, irregular, dark brown spots on leaf (B) infected leaves turn yellow and drop prematurely (Crop Protection Network, 2022k).

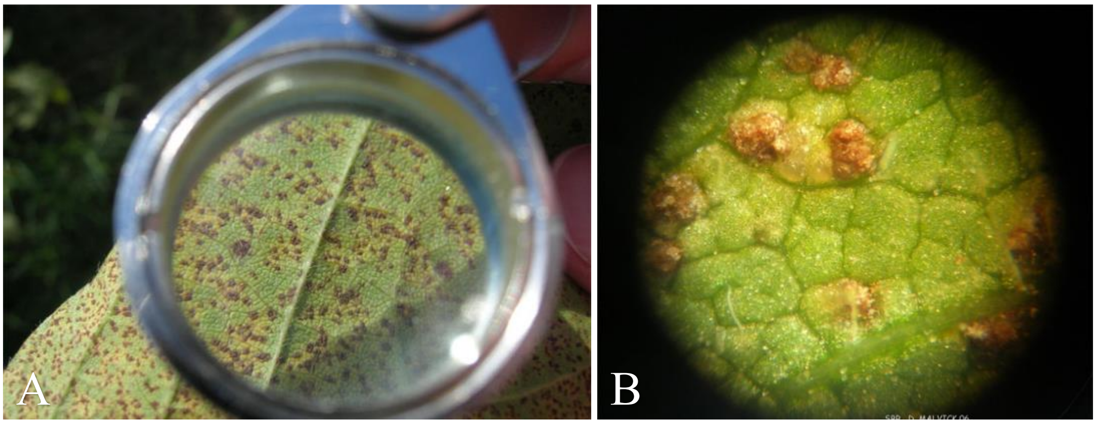

Figure 13. Soybean rust (A) small, tan-reddish lesions (spots) develop on the underside of the leaves (B) enlarged lesions develop into rust pustules (Malvick, 2018e).

Figure 14. Soybean stem canker (A) small, reddish-brown lesions on stem tissue (B) grey zone lines inside the stem (Crop Protection Network, 2022m).

Figure 15. Soybean sudden death syndrome (A) scattered, yellow spots to large chlorotic and necrotic blotches between the leaf veins (B) roots with reduced mass and discoloration (Crop Protection Network, 2022n; Wakefield, 1924).

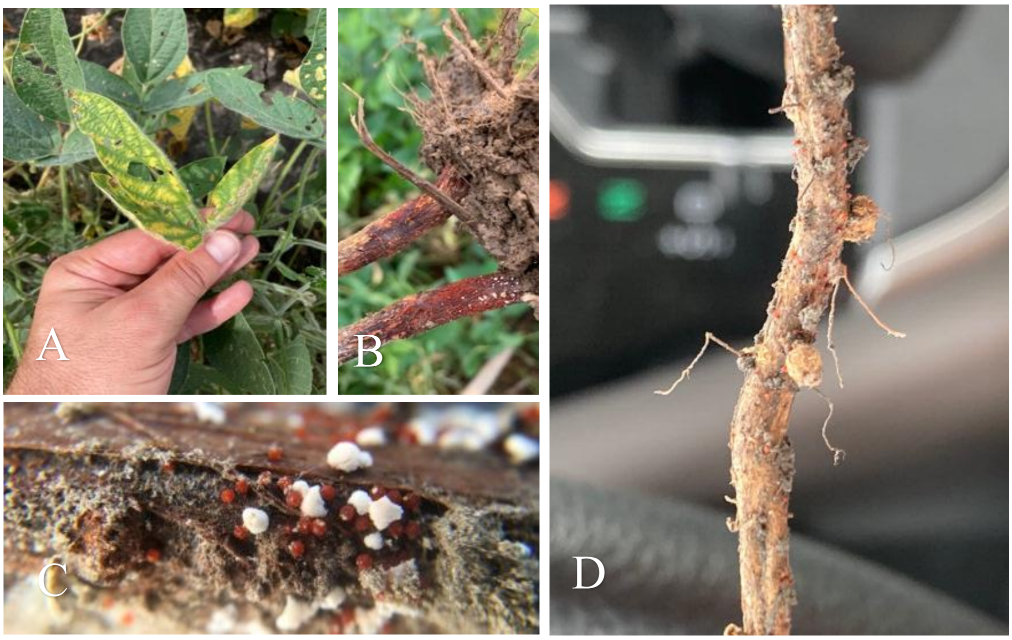

Figure 16 Soybean red crown rot (A) interveinal chlorosis and necrotic lesion on leaves (B) reddish discoloration on stem tissue above the soil line (C) reddish spore-bearing perithecia and white mycelia on lower stem (D) perithecia, appears as bright, red orange on stem tissue (Crop Protection Network, 2022j; Welker, 2022).

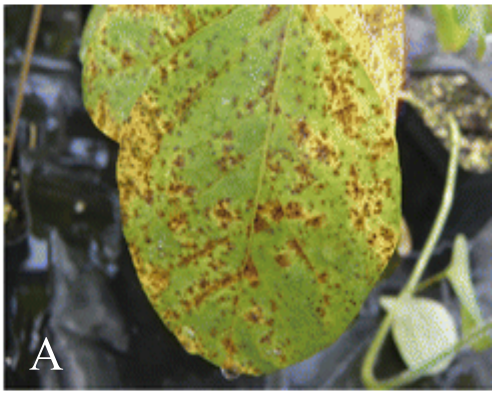

Figure 17. Soybean red leaf blotch (A) *C. glycines* causing dark red spots on upper leaf surface (Tooley, 2017).

TABLES

Table 1 Summary of important soybean diseases, causal agents, and the estimated loss for USA between 2019 to 2021 from the Crop Protection Network

Disease symptoms	Disease	Causal organism	Estimated loss ($ million) between 2019 to 2021 in the USA	Reference
Fig 2	Anthracnose	*Colletotrichum truncatum*	36.6	(Crop Protection Network, 2022a)
Fig 3	Cercospora purple seed stain & Cercospora Leaf Blight	*Cercospora kikuchii*	308	(Crop Protection Network, 2022h)
Fig 4	Charcoal rot	*Macrophomina phaseolina*	112.9	(Crop Protection Network, 2022d)
Fig 5	Downy mildew	*Peronospora manshurica*	6.3	(Crop Protection Network, 2021a)
Fig 6	Frogeye leaf spot	*Cercospora sojina*	227	(Crop Protection Network, 2022d)
Fig 7	Fusarium wilt	*Fusarium oxysporum* s.l.	43.8	(Crop Protection Network, 2022e)
Fig 8	Phomopsis seed decay/ pod and stem blight	*Diaporthe sojae*	260	(Crop Protection Network, 2022g)
Fig 9	Phytophthora root and stem rot	*Phytophthora sojae*	489	(Crop Protection Network, 2022f)
Fig 10	Powdery mildew	*Erysiphe diffusa*	N. A	N. A

Fig 11	Sclerotinia stem rot	*Sclerotinia sclerotium*	677	(Crop Protection Network, 2022o)
Fig 12	Septoria brown spot	*Septoria glycines*	155	(Crop Protection Network, 2022k)
Fig 13	Soybean rust	*Phakopsora pachyrhizi* & *P. meibomiae*	36	(Crop Protection Network, 2022l)
Fig 14	Soybean stem canker	*Diaporthe aspalathi* & *D. caulivora*	163	(Crop Protection Network, 2022m)
Fig 15	Sudden death syndrome	*Neocosmospora phaseoli*	652	(Crop Protection Network, 2022n)
Fig 16	Soybean Red Crown Rot	*Calonectria ilicicola*	0.465	(Crop Protection Network, 2022j)
Fig 17	Red leaf Blotch	*Coniothyrium glycines*	N. A	N. A

N.A: no information available on the economic loss in the USA.

Table 2 Primers and amplification protocols to identify causal agents of soybean disease.

Disease and causal agent	Locus	Primer	Primer sequence 5'-3'	Amplification profile	Reference
Soybean Anthracnose *Colletotrichum truncatum*	*act*	ACT-512F	ATGTGCAAGGCCGGTTTCGC	95°C 3 min, 34 cycles at 95°C 1 min, 52°C 30s, 72°C 1 min, final 72°C 10 min	(Carbone and Kohn, 1999)
		ACT-784R	TACGAGTCCTTCTGGCCCAT		(Carbone and Kohn, 1999)
	BenA	T1	AACATGCGTGAGATTGTAAGT		(Glass and Donaldson, 1995; O'Donnell and Cigelnik, 1997)
		Bt2b	ACCCTCAGTGTAGTGACCCTTGGC		(Glass and Donaldson, 1995; O'Donnell and Cigelnik, 1997)
	gapdh	GDF1	GCCGTCAACGACCCCTTCATTGA	94°C 4 min, 34 cycles at 94°C 45 s, 60°C 45 s, 72°C 1 min, final 72°C 10 min.	(Berbee *et al.*, 1999)
		GDR1	GGGTGGAGTCGTACTTG AGCATGT		(Berbee *et al.*, 1999)
Purple seed stains/leaf blight, and Frog-eye leaf spot *Cercospora* species	*act*	ACT-512F	ATGTGCAAGGCCGGTTTCGC	95°C 3 min, 34 cycles at 95°C 1 min, 52°C 30 s, 72°C 1 min, final 10 min 72 °C	(Carbone and Kohn, 1999)
		ACT-783R	TACGAGTCCTTCTGGCCCAT		(Carbone and Kohn, 1999)
	tef-1α	EF1-728F	CATCGAGAAGTTCGAGAAGG		(Carbone and Kohn, 1999)
		EF1-986R	TACTTGAAGGAACCCTTACC		(Carbone and Kohn, 1999)
	CaM	CAL-228F	GAGTTCAAGGAGGCCTTCTCCC		(Carbone and Kohn, 1999)

		CAL-737R	CATCTTCTGGCCATCATGG		(Carbone and Kohn, 1999)
	his	CylH3F	AGGTCC ACTGGTGGCAAG		(Crous *et al*. 2004c)
		CylH3R	AGC TGG ATG TCC TTG GAC TG		(Crous *et al*. 2004c)
	gapdh	Gpd1-LM	ATT GGC CGC ATC GTC TTC CGC AA	94°C 45 s, 60°C 45 s, 72°C 90 s, 5 cycles of 94 °C, 45 s, 58 °C, 45 s, 72 °C, 90 s, 30 cycles of 94 °C, 45 s, 53 °C, final 72°C 5 min.	(Myllys *et al*., 2002)
		Gpd2-LM	CCC ACT CGT TGT CGT ACC A		(Myllys *et al*., 2002)
	BenA	T1	AAC ATG CGT GAG ATT GTAAGT	94°C 3 min, 40 cycles of 94°C 30 s, 48°C or 30 s, 72°C 45 s , final 72°C 5 min.	(O’Donnell and Cigelnik, 1997)
		β-Sandy-R	GCR CGN GGV ACR TAC TTG TT		(Stukenbrock *et al*., 2012)
		BT-1F	GTC CWC ACC GCC CCT GAT		
		BT-1R	CTT GTT RCC RGAAGC CTR TGS		
Charcoal rot *Macrophomina phaseolina*	*BenA*	T1	AACATGCGTGAGATTGTAAGT	94°C 2 min , 35 cycles 94°C 1 min, 52-55°C 30 s, 72°C 1 min, final 72°C 10 min . Annealing conditions (52°C for *tef-1α*, 55°C for *CaM*, *act* and *BenA*).	(O’Donnell and Cigelnik, 1997)
		CYLTUB1R	AGT TGT CGG GAC GGA AGA G		(Crous *et al*., 2004b);
	act	ACT-512F	ATGTGCAAGGCCGGTTTCGC		(Carbone and Kohn, 1999)
		ACT-783R	TACGAGTCCTTCTGGCCCAT		(Carbone and Kohn, 1999)
		ACT-2RD	ARR TCR CGD CCR GCC ATG TC		(Groenewald *et al*., 2013)
	tef-1α	EF1-728F	CATCGAGAAGTTCGAGAAGG		(Carbone and Kohn, 1999)
		EF2	GGA RGT ACC AGT SAT CAT GTT		(O’Donnell *et al*., 1998)
		EF 986R	TACTTGAAGGAACCCTTACC		(O’Donnell and Cigelnik, 1997)
	CaM	CAL-228F	TACTTGAAGGAACCCTTACC		(Carbone and Kohn, 1999)

		CAL-737R	CATCTTCTGGCCATCATGG		(Carbone and Kohn, 1999)
Downy mildew *Peronospora manshurica*	Not specified	PM3	GGCTGGCTGCTACTGGGCA	92°C 1 min , 30 cycles 55°C 45s, final 30s at 72°C	(Lai P *et al.*, 2022)
		PM4	GCCGACTGGCCACGCGGA		(Lai P *et al.*, 2022)
Fusarium Wilt and root rot Sudden Death syndrome *Fusarium oxysporum* & *Neocosmospora phaseoli*	*tef-1α*	EF1	ATGGGTAAGGARGACAAGAC	95°C 5 min, 35 cycles of 95°C 45 s, 52°C 45 s, 72°C 90 s, final 72°C 8 min	(O'Donnell *et al.*, 1998)
		EF2	GGARGTACCAGTSATCATG		(O'Donnell *et al.*, 1998)
	CaM	CL1	GARTWCAAGGAGGCCTTCTC	94°C 90 s, 35 cycles of 94°C 45 s, 50°C 45 s, 72°C 1 min, final 72°C 10 min	(O'Donnell *et al.* 2000)
		CL2A	TTTTTGCATCATGAGTTGGAC		(O'Donnell *et al.* 2000)
	rpb1	FA	CAYAARGARTCYATGATGGGWC	94°C 90 s, 5 cycles of 94°C 45 s, 54°C 45 s, 72°C 2 min, 5 cycles of 94°C 45 s, 53°C 45 s, 72°C 2 min, 35 cycles of 94°C 45 s, 52°C 45s, 72°C 2 min, final 72°C 10 min	(Hofstetter *et al.* 2007)
		R8	CAATGAGACCTTCTCGACCAGC		O'Donnell *et al.* 2010
		FA	TTCTTCCACGCCATGGCTGGTCG	94°C 90 s, 5 cycles of 94°C 45 s, 56°C 45 s, 72°C 2 min, 5 cycles of 94°C 45 s, 55°C 45 s, 72°C 2 min, 35 cycles of 94°C 45 s, 54°C 45s, 72°C 2 min, 72°C 10 min, 10°C soak	(O'Donnell *et al.* 2010)
		G2R	GTCATYTGDGTDGCDGGYTCDCC		(O'Donnell *et al.* 2010)
	rpb2	5F2	GGTGTCAGTCARGTGYTGAA	95°C 5 min, 40 cycles of 94°C 30 s, 51°C 90 s, 68°C 2 min, 68°C 5 min	(Reeb *et al.* 2004)
		7Cr	CCCATRGCTTGYTTRCCCAT		(Liu *et al.*, 1999)

		7Cf	ATGGGYAARCAAGCYATGGG	95°C 5 min, 40 cycles of 94°C 30 s, 51°C 90 s, 68°C 2 min, 68°C 5 min	(Liu *et al.*, 1999)
		11ar	GCRTGGATCTTRTCRTCSACC		(Liu *et al.*, 1999)
	BenA	T1	AACATGCGTGAGATTGTAAGT	95°C 5 min, 35 cycles of 95°C 45 s, 52°C 45 s, 72°C 90 s, 72°C 8 min	(O’Donnell and Cigelnik, 1997)
		T2	TAGTGACCCTTGGCCCAGTTG		(O’Donnell and Cigelnik, 1997)
Phomopsis seed decay and Stem canker in soybeans *Diaporthe* species	*tef-1α*	EF1-728F	CATCGAGAAGTTCGAGAAGG	95 ºC 5 min, 34 cycles 95 ºC 30 s, 58 ºC 30 s, 72 ºC 1 min, final 72 ºC 10 min	(Carbone and Kohn, 1999)
		EF1-986R	TACTTGAAGGAACCCTTACC		(Carbone and Kohn, 1999)
Phytophthora root and stem rot *Phytophthora sojae*	Species-specific primers	PSOJF1	GCCTGC TCTGTGTGGCTGT	94°C 5 min, 35 cycles of 94°C 30 s, 66°C 30 s, and 72°C 30 s, 72°C 10 min.	(Bienapfl *et al.*, 2011)
		PSOJR1	GGTTTAAAAAGTGGGCTCATGATC		
Powdery mildew *Erysiphe diffusa*	ITS	ITS1/PM6	TCCTCCGCTTATTGATATGC	94°C 5 min, 94°C 30s, (54–52 °C, stepdown approach −1°C in each cycle) 30s ,72°C 40s, final 72°C 5 min	(White *et al.*, 1990)
		ITS4/PM5	GGA AGT AAA AGT CGT AAC AAG G		(White *et al.*, 1990)
Sclerotinia Stem Rot *Sclerotinia sclerotium*	ITS	ITS-1	TCCGTAGGTGAACCTGCGG	94°C 3 min, 35 cycles 94°C 60 s, 56°C 60 s, 72°C 2 min, final 72°C 7 min	(White *et al.*, 1990)
		ITS-4	TCCTCCGCTTATTGATATGC		(White *et al.*, 1990)
Brown spot *Septoria glycines*	*BenA*	Bt1	GGTCGAGAACTCCGATGAGA	94 °C 2 min, 35 cycles of 94 °C 15 s, 60 °C 60 s, 72 °C 30 s, final 72 °C 1 min	(Glass and Donaldson, 1995)

		Bt2	TACGAGGGGTTGTTGAGCTT		(Glass and Donaldson, 1995)
Soybean rust *Phakopsora pachyrhizi*, or *P. meibomiae*	ITS	ITS4	TCCTCCGCTTATTGATATGC	96°C 2 min, 35 cycles of 96°C 1 min, 55°C 1 min, 72°C 2 min, final 72°C 10 min.	(White *et al.*, 1990)
		ITS 5	GGAAGTAAAAGTCGTAACAAGG		(White *et al.*, 1990)
	Species-specific primers	Ppa1	TAAGATCTTTGGGCAATGGT	94°C 1 min, 25 cycles of 94°C 15 s, 65°C 15 s, and 72°C 15 s, final 72°C 6 min	(Frederick *et al.*, 2001)
		Ppa2	GCAACACTCAAAATCCAACAAT		
		Ppa3	CCCATTTAATTGGCTCATTG		
		Ppa4	TCAAAATCCAACAATTTCCC		
Red Crown Rot *Calonectria ilicicola*	*BenA*	T1	AACATGCGTGAGATTGTAAGT	96°C 10 min, 30 cycles of 96°C 15 s, 55°C 35 s, and 75°C 35 s, final 75°C 2 min	(O'Donnell and Cigelnik, 1997)
		Bt2b	ACCCTCAGTGTAGTGACCCTTGGC		(Glass and Donaldson, 1995)
Leaf Blotch *Coniothyrium glycines*	*tef-1α*	EF1-728F	CATCGAGAAGTTCGAGAAGG	95ºC 5 min, 34 cycles 95ºC 30 s, 58ºC 30 s, 72ºC 1 min, final 72ºC 10 min	(Carbone and Kohn, 1999)

		EF-2	GGARGTACCAGTSATCATGTT		(O'Donnell *et al.*, 1998)
	BenA	T1	AACATGCGTGAGATTGTAAGT	96°C 10 min, 30 cycles of 96°C 15 s, 55°C 35 s, and 75°C 35 s, final 75°C 2 min	(O’Donnell and Cigelnik, 1997)
		β-Sandy-R	GCRCGNGGVACRTACTTGTT		(Stukenbrock *et al.*, 2012)

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CHAPTER 2

The fungal diversity and mycotoxins associated with South African pre-stored soybean seeds

ABSTRACT

Soybean (*Glycine max*) is a significant oilseed crop and contributes to the South African food and feed industry. However, pre-stored soybean seeds may be susceptible to various fungi that can cause disease or sometimes lead to elevated levels of mycotoxins when stored under suboptimal conditions. Currently, there is limited information on the fungal diversity and mycotoxins associated with South African soybeans. This study thus aimed to complete a survey of fungi and mycotoxins associated with pre-stored soybean seeds collected across South Africa. A multi-mycotoxin analysis of 150 seed samples was performed using liquid chromatography-mass spectrometry (LC-MS/MS) that tested for 13 commonly occurring mycotoxins including aflatoxins, ochratoxins, fumonisins, and trichothecenes. Surface-disinfested soybean seeds were plated onto potato dextrose agar (PDA), Dichloran 18% glycerol agar (DG18), and water agar (WA), and isolations were subsequently made into pure culture. Strains were identified to species level using DNA sequence data that included the nuclear ribosomal internal transcribed spacer regions (ITS), partial sequences of beta-tubulin (*BenA*), calmodulin (*CaM*), translation elongation factor 1-α (*tef-1*α), glyceraldehyde 3-phosphate dehydrogenase (*gapdh*), and/or DNA-directed RNA polymerase II subunit (*rpb2*). We isolated 411 fungal strains that belonged to 24 genera and 81 species. Most notably, the most commonly isolated genera were *Aspergillus* (n=139), *Cladosporium* (n=78), *Penicillium* (n=55), *Didymella* (n=31), *Alternaria* (n=29), and *Fusarium* (n=28). No mycotoxins were detected in any of the samples analysed, even though several mycotoxigenic species like *Aspergillus flavus*, *A. parasiticus, A. westerdijkiae*, *Fusarium verticillioides,* and *F. boothii* were detected. This project generated baseline knowledge on the fungal diversity associated with South African soybean seeds, with strains and their DNA sequences that will serve as important reference data for future studies.

INTRODUCTION

Soybean (*Glycine max*) is one of the most important oilseeds in the world and a growing component of Africa's agricultural economy (Engelbrecht *et al.*, 2020) but are susceptible to fungal contamination in the field and post-harvest during storage. Mycotoxigenic genera, such as *Aspergillus*, *Penicillium*, and *Fusarium* can contaminate agricultural commodities and under suboptimum storage conditions produce mycotoxins. Consuming mycotoxins in contaminated food and feed can have harmful health and economic effects. Fungal diseases and mycotoxigenic fungi can threaten soybean seed quality and thus impact on the second United Nations Sustainable Development Goal (SDG 2) of 'Zero Hunger' to improve nutrition, achieve food security, and attain healthy agro-economic growth (WHO, 2022). Therefore, it is critical to monitor fungal diversity and mycotoxins in stored soybean seeds to provide baseline information for South Africa and understand the potential risks associated with it.

South Africa produced 1 897 000 tons of soybeans in 2020/21 (SAGL, 2021). Soybeans are a significant source of high-quality edible oils and proteins for humans and animals (Liu, 1997; Sudarić, 2020) but their production is mostly driven by the demand from the animal-feed industry (SAGL, 2021). Soybeans are used to produce food products such as soy sauce, tofu, and soy oil for human consumption. Farmers use soybeans for crop rotation with non-legume commodities such as maize and wheat, to restore nitrogen back into the soil or break disease cycles (Shrestha, 2021). Safe and good-quality food and feed are key pillars for improving food security and livestock productivity (DPME, 2017; Islam *et al.*, 2022; SAGL, 2019; STATS SA, 2019).

Some of the most common fungal diseases affecting soybean include phomopsis seed decay (caused by *Diaporthe longicolla*, cercospora purple seed stain (caused by *Cercospora kikuchii*), charcoal rot (*Macrophomina phasaeolina*), and sclerotinia stem rot (caused by *Sclerotinia sclerotiorum*)(Hartman and Murithi, 2020; Hartman *et al.*, 2015; Hobbs *et al.*, 1985; Shovan *et al.*, 2008; Sun *et al.*, 2013; Turner *et al.*, 2020). Other genera commonly isolated from seeds include *Alternaria, Aspergillus, Cladosporium, Fusarium,* and *Penicillium* (Balendres *et al.*, 2019; Greeff-Laubscher *et al.*, 2020; Marasas *et al.*, 2008). Some fungal species can produce mycotoxins, and consumption of foods contaminated with high levels of these toxic compounds can have serious health implications. For example, aflatoxins have been shown to be genotoxic carcinogens, and have been linked to cancer in both humans and animals (NCI, 2022). In particular, aflatoxin B1 is regarded as a class 1 carcinogen by the WHO (2018). The impact of mycotoxins includes the loss of human and animal life, increased healthcare costs, reduced livestock production, loss of crops, and contribution to the failure of exports and food waste (Xu *et al.*, 2022; Zain, 2011).

Knowledge of the fungal diversity and diseases associated with South African soybeans remains limited, while seeds are typically not subjected to mycotoxin screening. The aim of this study was thus to determine mycotoxin levels and to isolate, preserve and identify fungi associated with soybean seeds collected from commercial soybean production areas across South Africa.

MATERIALS AND METHODS

Sampling

One hundred and fifty pre-storage soybean seed samples from all South African production regions were collected during the 2018/19 season by the South African Grain Laboratory (SAGL). All samples were analysed for mycotoxins by the South African Grain Laboratory using the methodology described by Meyer *et al.* (2019). Twenty samples were randomly selected from the Free State (4), Gauteng (7), Mpumalanga (8), and North-West (1) for fungal isolations.

Mycotoxin analysis

Mycotoxins levels were analysed using the methodology of Meyer *et al.* (2019). Soybean samples were milled with a 1 mm sieve (Retsch ZM 200 mill) and mechanically mixed for 90 min. Subsamples (10.00 g ± 0.05 g) were weighed and added to 40 mL extraction solvent, comprising 50:25:25 ultra-pure water(<18.2 MΩ.cm): methanol (MeOH, for high-performance liquid chromatography (HPLC), >99.9%): acetonitrile (AcCN, ACS/HPLC grade, Burdick and Jackson). The samples were blended using an overhead stirrer for 1 min. The samples were transferred to a 50-mL polypropylene centrifuge tube and placed on a mechanical shaker in a horizontal position at 260 rpm for 15 min. The samples were centrifuged at 3000 rpm for 10 min. A 5-mL aliquot was pipetted into a volumetric flask and diluted by adding 5 mL of the dilution solution (25% MeOH in H2O (v/v)). The final sample extracts were filtered through 13-mm, 0.22-µm syringe filters (Nylon, Membrane Solutions) into HPLC amber vials LC-MS/MS.

Thirteen mycotoxin levels were tested, including aflatoxins (AFs including B_1, B_2, G_1, G_2), fumonisins (FUMs; including B_1, B_2, B_3), deoxynivalenol (DON), 15-acetyldeoxynivalenol (15-ADON), HT-2 toxin (HT2), ochratoxin A (OTA), T-2 toxin (T2), and zearalenone (ZEA). A standard (10 µg/mL) was prepared for AFs, DON, 15-ADON, FUM(B_1), OTA, T2, and ZEA; the HT2 toxin calibrant solution (100 µg/mL)(Romner Labs Diagnostic GmbH; Tulln, Austria); and FUMs (B_2 and B_3; Cape Peninsula University of Technology, Cape Town, South Africa). Additionally, negative controls were prepared using only the extraction solvent, and positive controls (spiked samples) were prepared at concentrations 5 and 50 µg/kg, respectively, in 40 mL of the extraction solvent.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed using an ultra-performance liquid chromatograph (Waters Acquity UPLC) with a C18 column (Acquity BEH) at 30°C in a Waters tandem quadrupole mass spectrometer (Acquity TQD). A programmed gradient elution buffer comprising of mobile phase A (0.5 mM ammonium acetate (Purity ≥ 98% Sigma-Aldrich/Merck) and 0.1% formic acid (98–100% Suprapur, Merck)), and mobile phase B (AcCN with 0.1% formic acid) was used for the separation of the thirteen mycotoxins.

Fungal isolations

Three isolation media were used to target a broad range of fungi based on their growth requirements. These included Dichloran 18% Glycerol Agar (DG18), which selects for xerophilic or osmotolerant fungi, and the generalist media ½ Potato Dextrose Agar (½ PDA) and Water agar (WA). Chloramphenicol (50mg/mL) was added to ½ PDA and WA to prevent bacterial growth. The direct plating method was performed for each sample by plating five surface-disinfested seeds, five non-disinfected seeds, and milled seeds in triplicate onto isolation media. To surface-disinfest the seeds, they were soaked in 2% bleach for 3 min, then in 70% ethanol for 30 s, and finally in distilled H_2O for 10 s. Milled seeds were sprinkled onto isolation media using a sterile spatula. Plates were incubated at 25°C for 3–7 d and regularly inspected for fungal growth.

Pure fungal cultures were obtained by observing growth with a stereomicroscope and transferring spores or mycelia onto ¼ PDA or DG18 using a sterile needle and incubated at 25°C for 7 d in order to obtain pure colonies. For *Fusarium*, single spore cultures were performed following the recommendations of (Leslie and Summerell, 2006). Isolates were identified to the genus level based on their macromorphological characters and microscopic characters. Isolates were subsequently preserved in vials containing 10% glycerol and stored at -80°C. The strains were accessioned into a working collection (CN) of the Applied Mycology group housed at FABI (Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa). Representative strains were also deposited into the CMW culture collection also housed at FABI).

DNA extraction, amplification (PCR), sequencing, and identification

For fungi with pigmented conidia, like *Alternaria*, *Aspergillus*, *Cladosporium*, and *Penicillium*, genomic DNA was extracted using the Quick-DNA Fungal/Bacterial Miniprep kit™ (Zymo Research, California, USA) following the manufacturer’s instructions. For hyaline fungi, like *Fusarium*, DNA was extracted using Prepman™ Ultra Sample Preparation reagent (Thermo Fisher Scientific, Waltham, Massachusetts USA) following the manufacturer’s instructions. DNA extracts were accessioned into the CN-DNA collection of the Applied Mycology group housed at FABI and stored at -20°C.

PCR amplifications were prepared in 25 µL reactions and consisted of 17.85 µL MilliQ H_2O, 5 µL BioLine 5X MyTaq™ Reaction Buffer (Meridian BioScience, USA), 0.50 µL of each primer (10 µM), and 0.15 µL BioLine MyTaq™ DNA Polymerase (Meridian BioScience, USA) and 1 uL of template DNA. For *Fusarium*, a reaction mixture of 25 µL consisted of 17.3 µL MilliQ H_2O, 2.5 µL (10×) FastStart PCR reaction buffer (Roche, Basel, Switzerland), 0.5 µL (2.5 mM) $MgCl_2$, 200 µM of each dNTP, 0.5 µL (0.8 µM) of each primer (forward and reverse), 0.2 µL (1U) FastStart Taq DNA Polymerase (Roche, Basel, Switzerland) and 1 uL of DNA as described in Yilmaz *et al.* (2021). PCR amplification was done using primers and amplification profiles as shown in Table 1.

Loci targeted for each genus include the partial beta-tubulin (*BenA*) for *Penicillium* (Glass and Donaldson, 1995; Visagie *et al.*, 2014), calmodulin (*CaM*) for *Aspergillus* (Hong *et al.*, 2006; Samson *et al.*, 2014), translation elongation factor 1–α (*tef-1*α) for *Fusarium* (O'Donnell *et al.*, 1998), and *Cladosporium* (Carbone and Kohn, 1999), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) for *Alternaria* and other *Pleosporales* (Berbee *et al.*, 1999) and DNA-directed RNA polymerase II second largest subunit for (*rpb2*) for *Didymellaceae* (Hou *et al.*, 2020). The nuclear ribosomal internal transcribed spacer (ITS) rDNA region was sequenced strains could not be identified to genus using morphology (de Hoog and Gerrits van den Ende, 1998; Schoch *et al.*, 2012). The following primers were used for identification: T10 (O'Donnell and Cigelnik, 1997) and Bt2b (Glass and Donaldson, 1995), and Bt2b (Glass and Donaldson, 1995) for *BenA*; Cmd5 (Hong *et al.*, 2006) and Cmd6 (Hong *et al.*, 2006) for the *CaM* gene region; V9G (de Hoog and Gerrits van den Ende, 1998) and LS266 (Masclaux *et al.*, 1995) for ITS. For *tef-1*α, the primers used were EF-1 (O'Donnell *et al.*, 1998) and EF-2 (O'Donnell *et al.*, 1998) for *Fusarium* or EF1-728F (Carbone and Kohn, 1999), and EF-983 (Rehner and Buckley, 2005) for *Cladosporium*. For part of the *rpb2* primers F1 (Sung *et al*., 2007) and 7CR (Liu *et al.*, 1999), and *gapdh* primers, gpd1 and gpd2 (Berbee *et al.*, 1999) were used.

PCR products were stained with GelRed (Biotium, Inc., California, USA), electrophoresed on a 1% agarose gel, and examined under UV light to confirm successful amplification reactions and amplicon sizes. Amplicons were purified using the ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific, Massachusetts, USA) and sequenced in both directions using the BigDye terminator sequencing kit v. 3.1 (Applied Biosystems, Forster City, California USA) using the same primers used for PCR amplification followed by sodium acetate precipitation. Sequence analyses were performed at the DNA Sanger Sequencing facility, Faculty of Natural and Agricultural Sciences, the University of Pretoria on an ABI3500xL Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA). The contigs were assembled and base calls were made using Geneious Prime v. 2019.0.4 (BioMatters Ltd., Auckland, New Zealand).

In general, sequence-based identifications were made by comparing sequence data to the National Centre for Biotechnology Information (NCBI's) nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). *Aspergillus*, *Penicillium*, and *Talaromyces* strains were identified by comparing their sequences with curated reference databases (Houbraken *et al.*, 2020; Samson *et al.*, 2014; Visagie *et al.*, 2014; Yilmaz *et al.*, 2014). *Fusarium* strains were identified using FUSARIOID-ID (https://www.fusarium.org/) which is based on the dataset published by Crous *et al.* (2022). Newly generated sequences were submitted to GenBank with accession numbers listed in Table 2.

RESULTS

Mycotoxin analysis

The limit of detection (LOD) and limit of quantification (LOQ) for each mycotoxin are shown in Table 3. No mycotoxins were detected in any collected soybean samples.

Fungal identifications

Most of the fungal growth was observed on non-disinfected seeds plated onto PDA. Isolations from 20 seed samples resulted in 411 strains recovered that were identified as 24 genera (Fig 3) and 81 species (Table 2). The dominant genera isolated were *Aspergillus* (n=139), *Cladosporium* (n=78), *Penicillium* (n=59), *Didymella*, (n=31), *Alternaria* (n=29), *Fusarium* (n=28), and *Talaromyces* (n=23). Other less dominant genera include *Boeremia* (n=6), *Epicoccum* (n=3), *Neodidymelliopsis* (n=3), *Lectera* (n=2), *Nigrospora* (n=2), *Allophoma* (n=1), *Aureobasidium* (n=1), *Chaetocapnodium* (n=1), *Clonostachys* (n=1), *Coniochaeta* (n=1), *Diaporthe* (n=1), *Neurospora* (n=1), *Periconia* (n=1), *Pestalotiopsis* (n=1), *Sarocladium* (n=1), *Scopulariopsis* (n=1), and *Wallemia* (n=1) (Fig 1).

Aspergillus was the dominant genus with strains belonging to nine sections and 18 species including section *Aspergillus* (*A. chevalieri* (n=22), *A. montevidensis* (n=12), *A. proliferans* (n=12), *A. pseudoglaucus* (n=16), *A. ruber* (n=13)), sect *Clavati* (*A. clavatus* (n=5)), sect *Circumdati* (*A. ostianus* (n=2)), sect *Cremei* (*A. wentii* (n=1)), sect *Flavi* (*A. flavus* (n=24), *A. parasiticus* (n=1), *A. tamarii* (n=2)), sect *Fumigati* (*A. fumigatus* (n=3)), sect *Nidulantes* (*A. creber* (n=14), *A. sydowii* (n=6), *A. penicillioides* (n=2)), sect *Nigri* (*A. welwitschiae* (n=2), *A. tubingensi* (n=1)), and sect *Terrei (A. terreus* (n=1)). (Table 2 and Fig 2). Potential mycotoxigenic species included *A. flavus* (AFs), *A. parasiticus* (AFs), and *A. welwitschiae* (OTA).

Nine sections and 18 *Penicillium* species were identified including section *Aspergilloides* (*P. glabrum* (n=1), *P. rudallense* (n=1)), sect *Brevicompacta* (*P. brevicompactum* (n=5)), sect *Charlesia* (*P.

charlesii (n=1)), sect *Chrysogena* (*P. chrysogenum* (n=6), *P. lanosocoeruleum* (n=1), *P. vanluykii* (n=12)), sect *Cinnamopurpurea* (*P.cinnamopurpureum* (n=1)), sect *Citrina* (*P. citrinum* (n=5), *P. sizovae* (n=1)), sect *Fasciculata* (*P.aurantiogriseum* (n=10), *P.crustosum* (n=1), *P. melanoconidium* (n=1), *P. polonicum* (n=2), *P. viridicatum* (n=4)), sect *Ramigena* (*P. hispanicum* (n=1)), sect *Ramosum* (*P. scabrosum* (n=1)), and sect *Robsamsonia* (*P. griseofulvum* (n=1)) (Table 2 and Fig 3).

Seven *Fusarium* species were identified (Table 2 and Fig 4), belonging to four species complexes, namely, the *Fusarium fujikuroi* species complex (FFSC) (*F. andiyazi* (n=1), *F. temperatum* (n=5), and *F. verticillioides* (n=16)), *Fusarium incarnatum-equiseti* species complex (FIESC) (*F. clavus* (n=3), and *F. coffeatum* (n=1)), *Fusarium oxysporum* species complex (FOSC) (*F. callistephi* (n=1)), and *Fusarium sambucinum* species complex (FSAMSC) (*F. boothii* (n=1)). *Fusarium verticillioides* was the most frequently isolated species. Potential mycotoxigenic species included *F. boothii* (15-ADON, DON, and ZEA), and *F. verticillioides* (FUMs).

Cladosporium was a commonly isolated genus, representing four species, including *C. anthropophilum* (n=2), *C. cladosporioides* (n=27), *C. myrtacearum* (n=1), *C. pseudocladosporioides* (n=16), followed by *Cladosporium* species (n=32). Other isolated species include *Allophoma* sp. (n=1), *Alternaria alternata* (n=26), *Al. burnsii* (n=1), *Alternaria* sp. (n=2), *Aureobasidium namibiae* (n=1), *Boeremia linicola* (n=2), *Boeremia* sp. (n=4). *Chaetocapnodium* sp. (n=1), *Clonostachys eriocamporesiana* (n=1), *Coniochaeta canina* (n=1), *Diaporthe miriciae* (n=1), *Didymella americana* (n=2), *Di. calidophila* (n=2), *Di. glomerata* (n=2), *Di. pomorum* (n=22), *Di. subglomerata* (n=2), *Di. subherbarum* (n=1), *Epicoccum dendrobii* (n=1), *E. keratinophilum* (n=1), *Epicoccum* sp. (n=1), *Lectera nordwiniana* (n=2), *Neodidymelliopsis ranunculi* (n=3), *Neurospora* sp. (n=1), *Nigrospora oryzae* (n=1), *Ni. sphaerica* (n=1), *Periconia byssoides* (n=1), *Pestalotiopsis* sp. (n=1), *Sarocladium zeae* (n=1), *Scopulariopsis* sp. (n=1), *Talaromyces adpressus* (n=2), *T. amestolkiae* (n=7), *T. diversus* (n=1), *T. pseudofuniculosus* (n=4), *T. purpureogenus* (n=2), *T. stollii* (n=2), *T. tiftonensis* (n=1), *T. veerkampii* (n=1), *T. verruculosus* (n=1), *T. wortmannii* (n=2), and *Wallemia mellicola* (n=1) (Table 2).

DISCUSSION

Soybeans are a valuable crop in South Africa due to their diverse uses, including crop rotation, food, and feed production, as well as their significant contribution to the country's trade and export industry (BFAP, 2021; SAGL, 2021). Soybean production is, however, threatened by fungal diseases and mycotoxigenic species that pose a potential risk of mycotoxin contamination if seeds are not stored correctly. The aim of this study was to determine the fungal diversity and mycotoxins associated with soybeans produced in South Africa. No mycotoxins were detected in the 150 collected samples, even though several mycotoxigenic species were identified amongst the relatively diverse communities in the 20 soybean samples from which isolations were made.

During this survey, 411 fungal strains were obtained with *Aspergillus*, *Cladosporium Penicillium*, *Didymella, Alternaria,* and *Fusarium* the most frequently isolated genera. Communities largely reflect those previously reported from soybean. The dominant communities associated with soybean seeds in Turkey included *Cladosporium*, *Fusarium*, *Penicillium*, and *Aspergillus* (Üstün *et al*., 2021). Ahmed *et al.* (2016) reported *Aspergillus*, *Curvularia*, *Fusarium*, *Penicillium*, and *Phomopsis* as dominant genera associated with soybean in Nigeria. A study from the USA reported *Alternaria*, *Diaporthe*, *Fusarium*, *Phoma*, *Penicillium*, and *Cladosporium* as common (Escamilla *et al*., 2019). Previous studies frequently reported *Fusarium* from soybean (Egbuta *et al.*, 2016; Escamilla *et al.*, 2019; Naeem *et al.*, 2019). *Aspergillus*, *Penicillium*, and *Fusarium* are important mycotoxigenic genera with certain species known to produce mycotoxins relevant to the agricultural industry.

The presence of these xerophilic species can be attributed to the nature of soybean seed harvesting practice or storage. Soybean seeds are harvested when the plant is dry and can be easily mixed with dust, soil, and other plant debris. After harvest, soybean seeds can be exposed to airborne or indoor fungi. Many of the species identified in this study are known as post-harvests pathogens and have been isolated from these environments. Oilseed storage is designed to be as dry as possible, especially in temperate countries to keep seeds in the best condition possible. However, these conditions can also be ideal for xerophilic or osmotolerant *Aspergillus* and *Penicillium* species that tend to grow in storage facilities with low water activity. It is thus important to keep soybean seeds as dry as possible after harvest and impossible to eliminate mycotoxigenic and pathogenic fungi from agricultural commodities. Therefore, it is essential to regularly monitor for the presence of mycotoxigenic fungi and screen for mycotoxins

Aspergillus was the most commonly isolated genus during this survey. The genus is taxonomically diverse with 452 accepted species that occur worldwide in various habitats but are typically considered storage fungi (Houbraken *et al.*, 2020). Many species identified in this study are xerophilic like *A. clavatus*, *A. chevalieri*, *A. fumigatus A. flavus*, *A. ruber*, *A. sydowii*, *A. tamarii*, *A. terreus*, *A. montevidensis*, *A. pseudoglaucus*, *A. parasiticus*, *A. penicillioides*, *A. wentii*, and *A. westerdijkiae* (Samson *et al.*, 2019) which can explain their presence on dried soybean seeds.

Important species identified in this survey include *A. flavus* and *A. parasiticus* (section *Flavi*) which are known to produce AFs (Frisvad *et al.*, 2006; Frisvad *et al.*, 2018; Frisvad *et al.*, 2019). *Aspergillus flavus* was the most frequently isolated species during this study (Fig 2) and was found to occur in 11 of 20 samples processed. *Aspergillus parasiticus* was isolated from only one sample during this study. The presence of aflatoxigenic *A. flavus* and *A. parasiticus* is significant to the agriculture industry (Frisvad *et al.*, 2018). The wide range of growth temperature makes it challenging to control storage conditions to reduce *A. flavus* growth, therefore it's important to constantly monitor for aflatoxins. In this study, two strains were identified as *A. westerdijkiae* (sect *Circumdati*) which has been reported as an important OTA producer under warm conditions (Visagie *et al.*, 2014).

Although species such as *A. flavus* were commonly isolated, the presence of mycotoxigenic species does not guarantee mycotoxin production. For example, aflatoxin-producing potential varies widely between *A. flavus* strains, some having genotypes unable to produce AFs. Aflatoxin gene clusters are tightly clustered and gene deletions and the presence of single nucleotide polymorphisms (SNPs) in aflatoxin biosynthesis genes are often associated with *A. flavus* inability to produce AFs (Adhikari *et al.*, 2016; Chang *et al.*, 2005). It is possible the strains collected during this study are non-aflatoxigenic and can explain the absence of AF in the soybean samples. If the strains have the AFs gene clusters, there are many factors like relative humidity, temperature, and oxidative stress (Beukes *et al.*, 2018; Caceres *et al.*, 2020; Mshelia *et al.*, 2020) that may determine AF production.

Aspergillus flavus can produce mycotoxins at a relative humidity ranging between 85 to 95% (Ding *et al.*, 2015). The fungus grows at a wide range of temperatures from 12°C to 48°C, but 28°C to 37°C is the optimum temperature (Hawkins *et al.*, 2005; Siciliano *et al.*, 2017). Normally at high temperatures, AFB production is higher than AFG, but at low temperatures, there is no significant difference between AFB and AFG production (Matumba *et al.*, 2015). The availability of O_2 and CO_2 also influences aflatoxin production. Aflatoxins production and fungal growth are inhibited at a higher level of CO_2 and a lower level of O_2 (Mahbobinejhad *et al.*, 2019). The factors influencing *A. flavus* aflatoxin production have not been determined on soybean seeds or on soy-based media, therefore further studies are needed to determine the factors influencing aflatoxin production on soybean in vitro. No mycotoxins were detected in this study, but should be monitored as aflatoxins have been previously detected in downstream products like soybean meal (Iqbal *et al.*, 2016; Li *et al.*, 2014).

Penicillium species were commonly isolated from soybean seeds during this study. The genus contains 535 accepted species, that also inhabit various environments such as soil, plants, food, and indoor environments (Houbraken *et al.*, 2020; Samson *et al.*, 2019; Visagie *et al.*, 2014). *Penicillium* species are considered storage or post-harvest pathogens due to their psychrophilic, xerophilic, or osmotolerant nature (Visagie *et al.*, 2017). Species identified in this study like *P. aurantiogriseum*, *P. brevicompactum*, *P. charlesii*, *P. chrysogenum*, *P. citrinum,* and *P. griseofulvum* prefer environments with low water activity as reported by Samson *et al.* (2019). *Penicillium* is an important genus because some species can produce mycotoxins (Frisvad *et al.*, 2004). However, the *Penicillium* species identified in this study have not been reported to produce regulated mycotoxins.

Fusarium was frequently isolated during this study. The genus is widely distributed in plants and soils and commonly begins invading the roots. Some species can also overwinter in the soil as chlamydospores (Summerell, 2019). *Fusarium* will commonly occur when the conditions are cool temperature and wet soil during soybean growth or when there is injury e.g. caused by nematodes (Hartman *et al.*, 2015). Previous studies frequently reported *Fusarium* from soybean (Chang *et al.*, 2020; Egbuta *et al.*, 2016; Escamilla *et al.*, 2019; Naeem *et al.*, 2019). Chang *et al.* (2020) identified five *Fusarium* species based on *tef-1*α and *RBP2* secondary markers. *Fusarium fujikuroi* was found as the most frequently encountered species, followed by *F. incarnatum* and *F. proliferatum*, *F. asiaticum*, and *F. verticillioides*. In contrast, this survey found *F. verticillioides* as the most commonly isolated species followed by *F. temperatum, F. clavus*, *F. andiyazi*, *F. boothii, F. callistephi,* and *F. coffeatum.*

Some *Fusarium* species are infamously known for their ability to cause diseases or produce mycotoxins like fumonisins, zearalenones, deoxynivalenol, and trichothecenes (Ekwomadu *et al.*, 2021). During this study, *F. verticillioides*, and *F. boothii* are known to be mycotoxin producers. *Fusarium verticillioides* is a major pathogen in the FFSC and is a common contaminant of soybean, and can be an important producer of FUMs under certain environmental factors (Lee and Ryu, 2017; Mshelia *et al.*, 2020; Schoeman *et al.*, 2018; Yilmaz *et al.*, 2021). *Fusarium boothii,* belonging to the FSAMSC, has been reported on soybean by Chiotta *et al.* (2015) and has the potential to produce 15-ADON, DON, and ZEA (Beukes *et al.*, 2018; Gryzenhout *et al.*, 2016). *Fusarium temperatum* has previously been thought to be an important FUM producer (Munkvold *et al.*, 2021; Scauflaire *et al.*, 2011). However, studies by Fumero *et al.* (2020) have shown that the FUM biosynthetic gene clusters lacking in these species and that the status of toxin production by these species needs further work. If the biosynthetic gene clusters are present in the genome, then mycotoxins are the product of complex pathways involving clustered genes and many enzymatic reactions (Caceres *et al.*, 2020). Additionally, factors like temperature, a_w, and CO_2 levels many studies have described environmental factors influencing mycotoxin production. For example, Garcia *et al.* (2012) studied the influence of temperature on FUM production in *F. verticillioides* on soy cultures and soybean. The results showed *F. verticillioides* grew optimally at 25°C but only produced $FUMB_1$ at 30°C after 15 days of growth. However, at 30°C the strains were unable to grow on soybean seeds and only grew on culture media. Thus, mycotoxin production depends on many different factors and there can be many explanations for the absence of the mycotoxins tested on soybean seeds.

Many of the previous fungal diversity studies on soybean have based identifications on morphology or DNA sequences from the ITS barcode (Ahmed *et al.*, 2016; Escamilla *et al.*, 2019; Krsmanović *et al.*, 2020; Naeem *et al.*, 2019; Russo *et al.*, 2016). ITS is sufficient for genus-level identification but unreliable for species-level identification, especially in common genera like *Alternaria, Aspergillus*, *Cladosporium*, *Fusarium,* and *Penicillium* species (Houbraken *et al.*, 2020). In contrast, this study focused on species identification using morphology and molecular sequencing using the recognised identification markers for the genera detected during this study species identification. DNA sequences generated during this study were deposited to Genbank with accession numbers available to aid future fungal identifications. Strains were also deposited into culture collections and is available for future studies. Culture collections are a valuable resource for preserving fungal diversity. Preserved strains can be revived for morphological descriptions, novel secondary metabolite analysis, or for in vitro mycotoxin production studies.

CONCLUSION

Fungal and mycotoxin surveys are essential for ensuring safe food and feed in South Africa, but very few have studied this for South African soybeans. This survey is the first to determine the mycotoxin levels and fungal species present on soybeans and it thus provides important baseline knowledge for an increasingly important crop. During this survey, no mycotoxins were detected in any of the samples analysed, even though several mycotoxigenic species like *A. flavus*, *A. parasiticus, A. westerdijkiae*, *F. boothii,* and *F. verticillioides* were isolated. The reasons for this result are not clear but indicate that downstream processing should be aware of the mycotoxins risk these fungi pose.

FIGURES

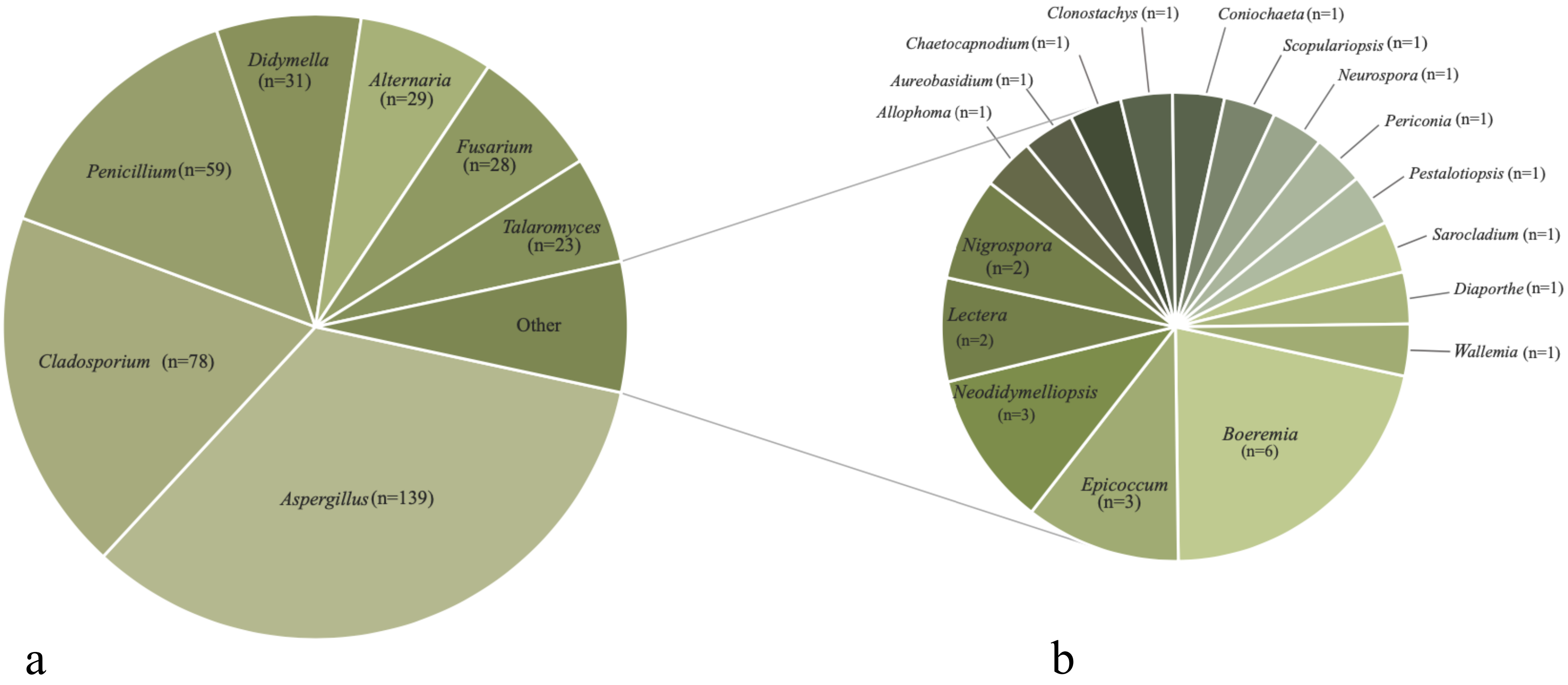

Figure 1. The fungal genera isolated from stored soybean seeds in South Africa (a) genera isolated frequently and (b) genera isolated less frequently. The numbers of strains (n=) are shown for each genus.

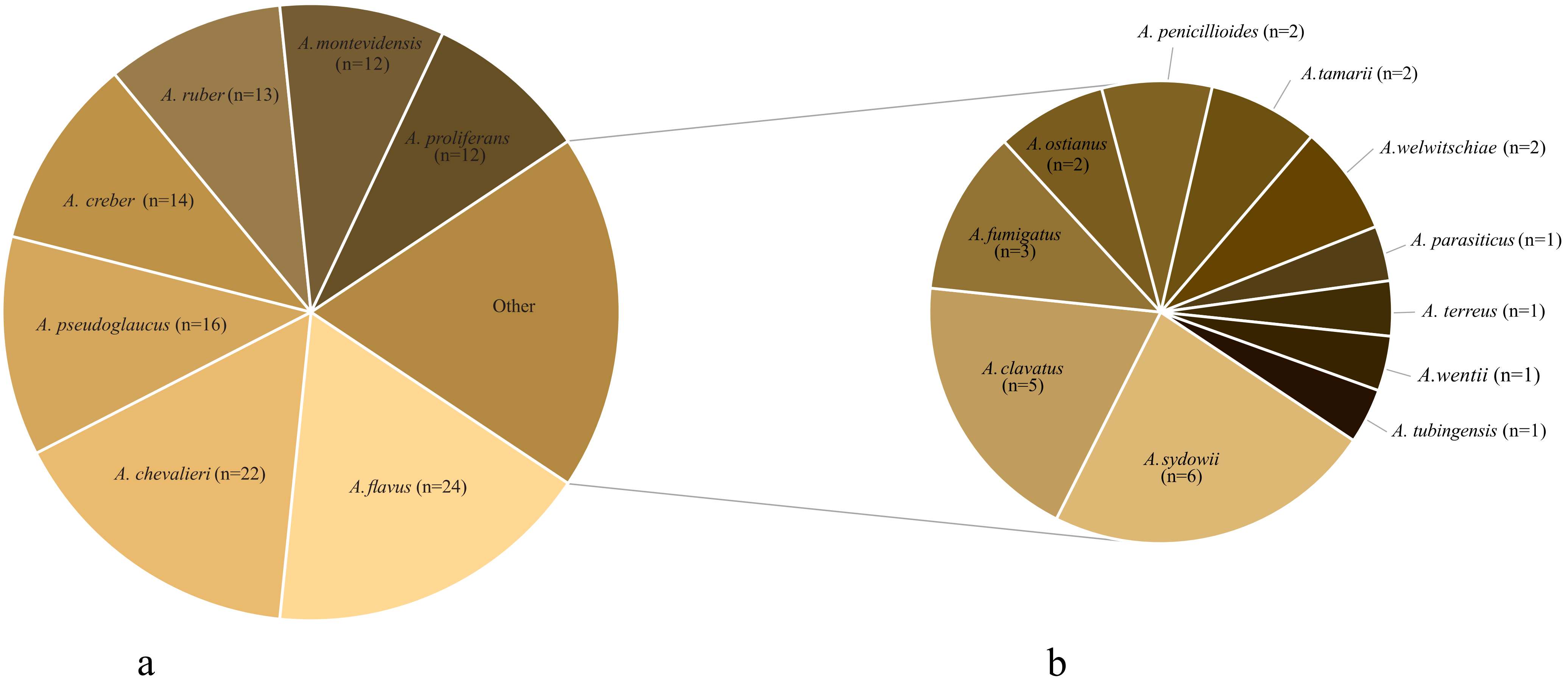

Figure 2. The *Aspergillus* species isolated from stored soybean seeds in South Africa (a) species isolated frequently and (b) species isolated less frequently. The numbers of strains (n=) are shown for each species.

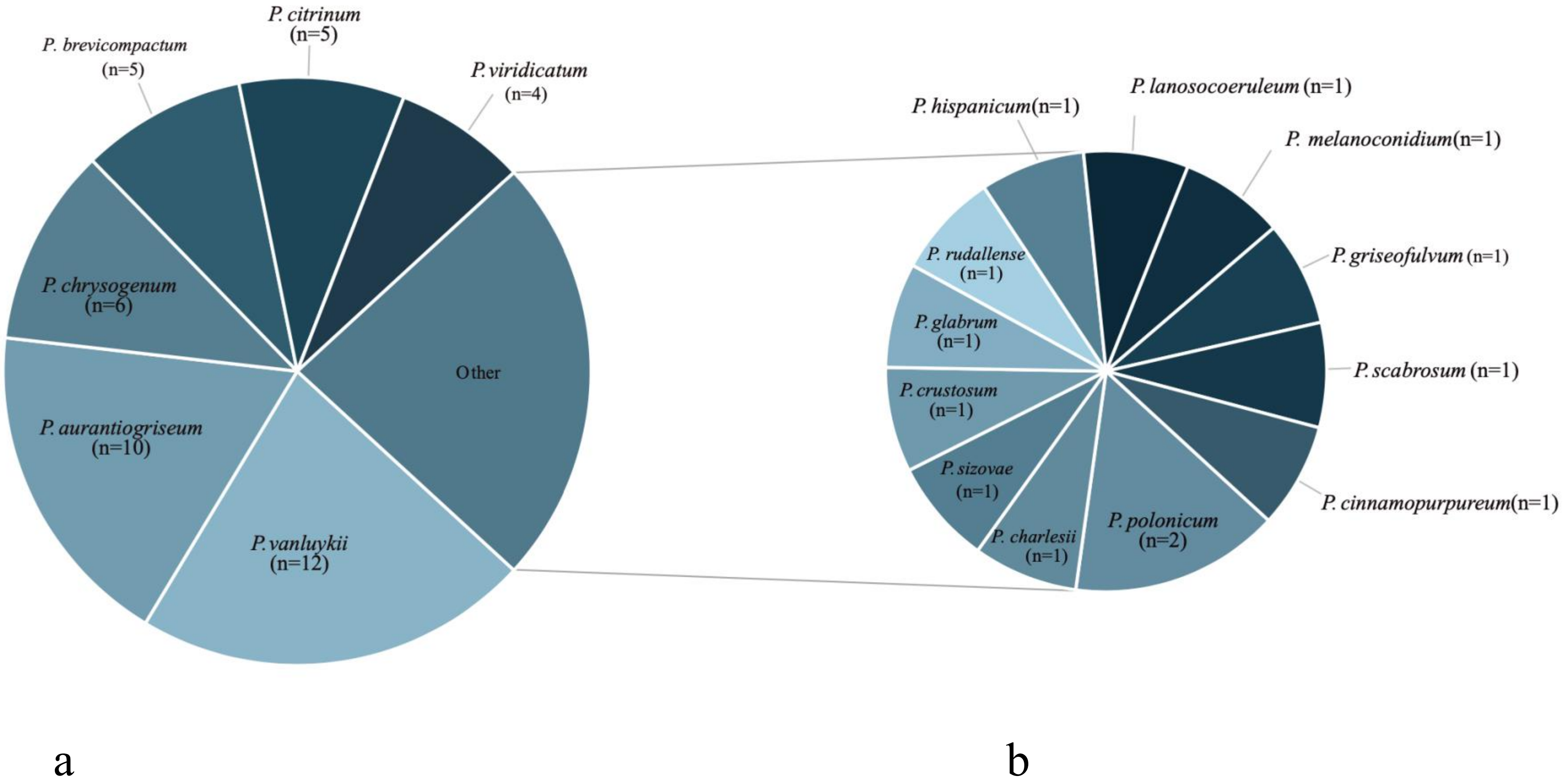

Figure 3. The *Penicillium* species isolated from stored soybean seeds in South Africa (a) species isolated frequently and (b) species isolated less frequently. The numbers of strains (n=) are shown for each species.

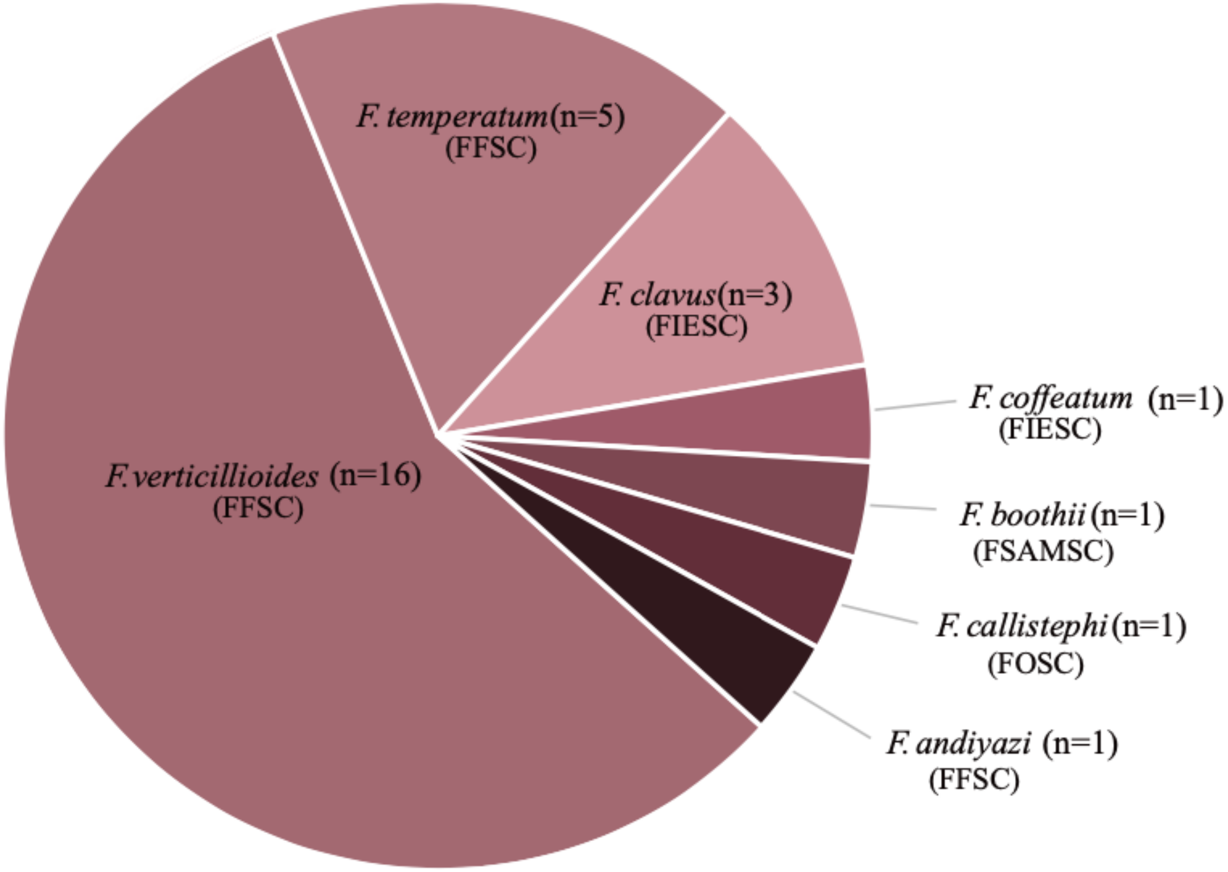

Figure 4. The *Fusarium* species isolated from stored soybean seeds in South Africa. The number of strains (n=) are shown for each species.

TABLES

Table 1. Primers pairs, amplification profile, and references used in this study

Locus	**Primer**	**Sequence (5' to 3')**	**Amplification profile**	**Reference**
Beta-tubulin (*BenA*)	T10	ACGATAGGTTCACCTCCAGAC	94°C 3 min; 35 cycles of 94°C 45s; 52°C 45s, 72°C , 1 min 30s; final extension of 72°C 8 min.	(Glass and Donaldson, 1995)
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC		(Glass and Donaldson, 1995)
Calmodulin (*CaM*)	Cmd5	CCGAGTACAAGGARGCCTTC	94°C 5 min; 35 cycles of 94°C 45s; 55°C 45s; 72°C 1 min; final extension of 72°C 7 min	(Hong *et al.*, 2006)
	Cmd6	CCGATRGAGGTCATRACGTGG		(Hong *et al.*, 2006)
Glyceraldehyde-3 phosphate dehydrogenase (*gapdh*)	GDP1	CAACGGCTTCGGTCGCATTG	94°C 5 min; 30 cycles of 94°C 45s 52°C 1 min 72°C 1 min; final extension of 72°C 7 min	(Berbee *et al.*, 1999)
	GDP2	GCCAAGCAGTTGGTTGTGC		(Berbee *et al.*, 1999)

Internal transcribed spacer (ITS)	V9G	TTACGTCCCTGCCCTTTGTA	94°C 3 min; 35 cycles of 94°C 45s; 52°C 45s; 72°C 1 min 30s; final extension of 72°C 8 min	(de Hoog and Gerrits van den Ende, 1998)
	LS266	GCATTCCCAAACAACTCGACTC		(Masclaux *et al.*, 1995)
DNA-directed RNA polymerase II (*rpb2*)	5F	GGGGWGAYCAGAAGAAGGC	94°C 5 min; 35 cycles of 95°C 45s; 54°C 1 min 20s; 72°C 2 min; final extension of 72°C 10 min	(Sung *et al.*, 2007)
	7CR	CCCATRGCTTGYTTRCCCAT		(Liu *et al.*, 1999)
Translation elongation factor (*tef-1*a)	EF1-728F	CATCGAGAAGTTCGAGAAGG	94°C 3 min; 35 cycles of 94°C 45s; 52°C 45s; 72°C 1 min 20s; final extension of 72°C 8 min	(Carbone and Kohn, 1999)
	986R	TACTTGAAGGAACCCTTACC		(O'Donnell *et al.*, 1998)
	EF1	ATGGGTAAGGARGACAAGAC	94°C 5 min; 35 cycles of 94°C 45s; 53°C 45s; 72°C 60s; final extension of 72°C 7min	(O'Donnell *et al.*, 1998)
	EF2	ATGGGTAAGGARGACAAGAC		(O'Donnell *et al.*, 1998)

Table 2. Genbank accession number for strains identified in this study

	Strain	**Genbank Accession numbers**					
		ITS	***CaM***	***BenA***	***gapdh***	***rpb2***	***tef-1α***
Allophoma species	CN095F6	OP596036	-	-	-	-	-
Alternaria alternata	CN088G7	OP595970	-	-	OP723110	-	-
	CN088G8	OP595971	-	-	OP723111	-	-
	CN088G9	OP595972	-	-	OP723112	-	-
	CN089C9	OP595979	-	-	OP723113	-	-
	CN090D7	-	-	-	OP723114	-	-
	CN090E1	OP595991	-	-	OP893543	-	-
	CN091E5=CMW59032	OP596009	-	-	OP723115	-	-
	CN091F1=CMW59033	OP596011	-	-	OP893544	-	-
	CN095E6	-	-	-	OP893545	-	-
	CN095E7	-	-	-	OP723117	-	-
	CN095E8	-	-	-	OP723118	-	-
	CN095E9	-	-	-	OP723119	-	-
	CN096E1	-	-	-	OP723120	-	-
	CN096E4	OP596037	-	-	OP723121	-	-
	CN096E5	OP596038	-	-	OP723122	-	-

	Strain	**Genbank Accession numbers**					
		ITS	***CaM***	***BenA***	***gapdh***	***rpb2***	***tef-1α***
	CN096E7	OP596040	-	-	OP893546	-	-
	CN097A1	-	-	-	OP723123	-	-
	CN097A2	-	-	-	OP893547	-	-
	CN097A3	-	-	-	OP723124	-	-
	CN097A9	OP596043	-	-	OP723125	-	-
	CN100G8	-	-	-	OP723126	-	-
	CN100G9	-	-	-	OP723127	-	-
	CN101A7	-	-	-	OP723128	-	-
	CN101A8	-	-	-	OP723129	-	-
	CN101B1	OP596059	-	-	OP723130	-	-
	CN101D6	-	-	-	OP723131	-	-
Alternaria burnsii	CN092B2=CMW59034	OP596028	-	-	OP723116	-	-
Alternaria species	CN091I2	OP596019	-	-	-	-	-
	CN088E9	OP595963	-	-	-	-	-
Aspergillus chevalieri	CN089A5	-	OP720056	-	-	-	-
	CN089B9	-	OP720057	-	-	-	-
	CN090F7	-	OP720058	-	-	-	-

	Strain	**Genbank Accession numbers**					
		ITS	***CaM***	***BenA***	***gapdh***	***rpb2***	***tef-1α***
	CN090F9	-	OP720059	-	-	-	-
	CN090G2	-	OP720060	-	-	-	-
	CN091D6	-	OP720061	-	-	-	-
	CN091D7	-	OP720062	-	-	-	-
	CN091D8	-	OP720063	-	-	-	-
	CN091D9	-	OP720064	-	-	-	-
	CN091E1	-	OP720065	-	-	-	-
	CN091E2=CMW59041	-	OP720066	-	-	-	-
	CN095C2=CMW59042	-	OP720067	OP660572	-	-	-
	CN095D2	-	OP720068	-	-	-	-
	CN095F4	OP596035	OP893557	-	-	-	-
	CN095F5	-	OP720069	-	-	-	-
	CN096G4	-	OP720070	-	-	-	-
	CN096G9	-	OP720071	-	-	-	-
	CN096H8	OP596041	OP720072	-	-	-	-
	CN100D9	-	OP720073	-	-	-	-
	CN100E1	-	OP720074	-	-	-	-
	CN100E2	-	OP720075	-	-	-	-
	CN100H8	-	OP720076	-	-	-	-

	Strain	Genbank Accession numbers					
		ITS	***CaM***	***BenA***	***gapdh***	***rpb2***	***tef-1α***
Aspergillus clavatus	CN090H1=CMW59040	OP595999	OP720190	-	-	-	-
	CN095H2=CMW59044	-	OP720206	OP660631	-	-	-
	CN095H7	-	OP720207	OP660634	-	-	-
	CN095I9	-	OP720208	OP660633	-	-	-
	CN101C2	-	OP720236	OP660632	-	-	-
Aspergillus creber	CN088I5	-	OP720077	-	-	-	-
	CN088I6	-	OP720078	-	-	-	-
	CN088I7	-	OP720079	-	-	-	-
	CN088I9	-	OP720080	-	-	-	-
	CN089A1=CMW59037	-	OP720081	-	-	-	-
	CN089A2=CMW59038	-	OP720082	-	-	-	-
	CN089A3	-	OP720083	-	-	-	-
	CN089A4	-	OP720084	-	-	-	-
	CN090F5	-	OP720085	-	-	-	-
	CN090G3	OP595993	OP720086	-	-	-	-
	CN091B1	OP596005	OP720087	-	-	-	-
	CN091E3	OP596007	OP720088	-	-	-	-
	CN095F3	OP596034	OP720089	-	-	-	-
	CN096A1	-	OP893553	-	-	-	-

	Strain	Genbank Accession numbers					
		ITS	***CaM***	***BenA***	***gapdh***	***rpb2***	***tef-1α***
Aspergillus flavus	CN088D9	-	OP720090	-	-	-	-
	CN088E1	-	OP720091	-	-	-	-
	CN088E2	-	OP720092	-	-	-	-
	CN088H3	-	OP720093	-	-	-	-
	CN088H4	-	OP720094	-	-	-	-
	CN088H5	-	OP720095	-	-	-	-
	CN090G8	OP595997	-	-	-	-	-
	CN091B4	-	OP720096	-	-	-	-
	CN091B5	-	OP720097	-	-	-	-
	CN091C3	-	OP720098	-	-	-	-
	CN091G4	-	OP720099	-	-	-	-
	CN096A3	-	OP720100	OP660569	-	-	-
	CN096A7	-	OP720101	-	-	-	-
	CN096A8	-	OP720102	-	-	-	-
	CN096F2	-	-	OP660570	-	-	-
	CN100C2	-	OP720103	-	-	-	-
	CN100C3	-	OP720104	-	-	-	-
	CN100C4	-	OP720105	-	-	-	-
	CN100F8	-	OP720106	-	-	-	-

	Strain	**Genbank Accession numbers**					
		ITS	***CaM***	***BenA***	***gapdh***	***rpb2***	***tef-1α***
	CN100I4	-	OP720107	-	-	-	-
	CN100I5	-	OP720108	-	-	-	-
	CN101C3	-	OP720109	-	-	-	-
	CN101C4=CMW59065	-	OP720110	-	-	-	-
	CN101C5=CMW59066	-	OP720111	-	-	-	-
Aspergillus fumigatus	CN096G2=CMW59049	-	OP720215	-	-	-	-
	CN100D7=CMW59055	-	OP720226	OP660630	-	-	-
	CN088E7	OP595962	-	-	-	-	-
Aspergillus montevidensis	CN089F6	OP595985	-	-	-	-	-
	CN089F7	OP595986	-	-	-	-	-
	CN089F9	OP595988	OP720181	-	-	-	-
	CN090G7	OP595996	OP720188	-	-	-	-
	CN090G9	OP595998	OP720189	-	-	-	-
	CN091G2	-	-	OP660571	-	-	-
	CN096A9	-	OP720210	-	-	-	-
	CN096H1	-	OP720218	-	-	-	-
	CN096H9	OP596042	OP720220	-	-	-	-
	CN096I8	-	OP720222	-	-	-	-
	CN096I9=CMW59052	-	OP720223	-	-	-	-

	Strain	**Genbank Accession numbers**					
		ITS	***CaM***	***BenA***	***gapdh***	***rpb2***	***tef-1α***
	CN100C5=CMW59054	-	OP720225	-	-	-	-
Aspergillus ostianus	CN096B3=CMW59046	-	OP893558	-	-	-	-
	CN096B4=CMW59047	-	OP893559	-	-	-	-
Aspergillus parasiticus	CN100D8=CMW59056	-	OP720227	-	-	-	-
Aspergillus penicillioides	CN096A2=CMW59045	-	OP720209	OP660635	-	-	-
	CN090F8	-	OP720185	-	-	-	-
Aspergillus proliferans	CN090G1	-	OP720186	-	-	-	-
	CN091B8	-	OP720191	-	-	-	-
	CN091B9	-	OP720192	-	-	-	-
	CN091C1	-	OP720193	-	-	-	-
	CN092A3	-	OP720199	-	-	-	-
	CN095D3	-	OP720202	-	-	-	-
	CN095D5	-	OP720203	-	-	-	-
	CN095D7	-	OP720204	-	-	-	-
	CN096C3	-	OP720212	-	-	-	-
	CN096D3	-	OP720213	-	-	-	-
	CN096D4	-	OP720214	-	-	-	-
	CN096H2=CMW59050	-	OP720219	-	-	-	-
Aspergillus pseudoglaucus	CN088I2	OP595973	-	-	-	-	-

	Strain	**Genbank Accession numbers**					
		ITS	***CaM***	***BenA***	***gapdh***	***rpb2***	***tef-1α***
	CN088I3	OP595974	OP720175	-	-	-	-
	CN089C1	OP595976	OP720179	-	-	-	-
	CN089F8	OP595987	OP720180	-	-	-	-
	CN089G1	OP595989	OP720182	-	-	-	-
	CN090G5	OP595994	OP720187	-	-	-	-
	CN091C2	-	OP720194	-	-	-	-
	CN091E4	OP596008	OP720197	-	-	-	-
	CN095D1	-	OP720201	-	-	-	-
	CN096B6	-	OP720211	-	-	-	-
	CN096G5	-	OP720216	-	-	-	-
	CN100F7	-	OP720230	-	-	-	-
	CN100I6	-	OP720231	-	-	-	-
	CN100I7	-	OP720232	-	-	-	-
	CN100I8=CMW59062	-	OP720233	-	-	-	-
	CN100I9=CMW59063	-	OP720234	-	-	-	-
Aspergillus ruber	CN088I4	-	OP720176	-	-	-	-
	CN089A6	-	OP720177	-	-	-	-
	CN089G2	-	OP720183	-	-	-	-
	CN089G3	-	OP720184	-	-	-	-

	Strain	**Genbank Accession numbers**					
		ITS	***CaM***	***BenA***	***gapdh***	***rpb2***	***tef-1α***
	CN090F6	-	OP893556	-	-	-	-
	CN091C4	-	OP720195	-	-	-	-
	CN091C5	-	OP720196	-	-	-	-
	CN091G3	-	OP720198	-	-	-	-
	CN092A4	-	OP720200	-	-	-	-
	CN095E2	-	OP720205	-	-	-	-
	CN096G6	-	OP720217	-	-	-	-
	CN096I4	-	OP720221	-	-	-	-
	CN101A1=CMW59064	-	OP720235	-	-	-	-
Aspergillus sydowii	CN088D8	-	OP720112	-	-	-	-
	CN088E6	-	OP893552	-	-	-	-
	CN089B6	-	OP720113	-	-	-	-
	CN091B7	-	OP720114	-	-	-	-
	CN096F3=CMW59048	-	OP720115	-	-	-	-
	CN096I3=CMW59051	-	OP720116	-	-	-	-
Aspergillus tamarii	CN088H2=CMW59035	-	OP720173	-	-	-	-
	CN088H6=CMW59036	-	OP720174	-	-	-	-
Aspergillus terreus	CN100E4=CMW59058	-	OP720228	-	-	-	-
Aspergillus tubingensis	CN089B8=CMW59039	-	OP720178	-	-	-	-

	Strain	Genbank Accession numbers					
		ITS	***CaM***	***BenA***	***gapdh***	***rpb2***	***tef-1α***
Aspergillus welwitschiae	CN100E5=CMW59059	-	OP893555	-	-	-	-
	CN100E6=CMW59060	-	OP720229	-	-	-	-
Aspergillus wentii	CN097B2=CMW59053	OP596044	OP720224	-	-	-	-
Aureobasidium namibiae	CN100H3=CMW59067	OP596054	-	-	-	-	-
Boeremia linicola	CN092B4=CMW59069	OP596030	-	-	-	OP729382	-
	CN092B6=CMW59070	OP596032	-	-	-	OP729383	-
Boeremia species	CN091A8	OP596004	-	-	-	-	-
	CN092B1	OP596027	-	-	-	-	-
	CN092B3	OP596029	-	-	-	-	-
	CN092B5	OP596031	-	-	-	-	-
Chaetocapnodium species	CN090E6	OP595992	-	-	-	-	-
Cladosporium anthropophilum	CN090H9=CMW59073	OP590104	-	-	-	-	OP893580
	CN100G3=CMW59074	OP590144	-	-	-	-	OP893589
Cladosporium cladosporioides	CN088E3	OP590076	-	-	-	-	OP893560
	CN088E4	OP590077	-	-	-	-	OP893561
	CN090E7	OP590094	-	-	-	-	OP893576
	CN090H2	OP590098	-	-	-	-	-
	CN090H3	OP590099	-	-	-	-	OP893578
	CN090H5	OP590100	-	-	-	-	OP893579

	Strain	**Genbank Accession numbers**					
		ITS	***CaM***	***BenA***	***gapdh***	***rpb2***	***tef-1α***
	CN090H6	OP590101	-	-	-	-	OP893562
	CN090H7	OP590102	-	-	-	-	-
	CN090H8	OP590103	-	-	-	-	OP893563
	CN090I2	OP590106	-	-	-	-	OP893581
	CN090I3	OP590107	-	-	-	-	OP893564
	CN090I4	OP590108	-	-	-	-	OP893582
	CN090I6	OP590109	-	-	-	-	OP893583
	CN090I7	OP590110	-	-	-	-	OP893565
	CN091A2	OP590113	-	-	-	-	OP893584
	CN091A3	OP590114	-	-	-	-	OP893585
	CN091E7	OP590117	-	-	-	-	OP893566
	CN091E8	OP590118	-	-	-	-	OP893586
	CN091H1	OP590120	-	-	-	-	OP893567
	CN091H5	OP590124	-	-	-	-	OP893568
	CN091I8	OP590127	-	-	-	-	OP893569
	CN092A2	OP590129	-	-	-	-	OP893570
	CN095E3	OP590130	-	-	-	-	OP893571
	CN096D7	OP590131	-	-	-	-	OP893587
	CN097A6	OP590135	-	-	-	-	OP893588

	Strain	**Genbank Accession numbers**					
		ITS	***CaM***	***BenA***	***gapdh***	***rpb2***	***tef-1α***
	CN101A4	OP590146	-	-	-	-	OP893572
	CN101C6	-	-	-	-	-	OP893573
Cladosporium myrtacearum	CN090E9=CMW59072	OP590096	-	-	-	-	OP893577
Cladosporium pseudocladosporioides	CN089E8	OP590086	-	-	-	-	OP893600
	CN090E8	OP590095	-	-	-	-	-
	CN090G4	OP590097	-	-	-	-	OP893601
	CN090I1	OP590105	-	-	-	-	-
	CN090I8	OP590111	-	-	-	-	-
	CN090I9	OP590112	-	-	-	-	-
	CN091A7	OP590115	-	-	-	-	OP893602
	CN091F2	OP590119	-	-	-	-	OP893603
	CN091H6	OP590125	-	-	-	-	-
	CN092A1	OP590128	-	-	-	-	OP893604
	CN095E4	-	-	-	-	-	OP893605
	CN096D8	OP590132	-	-	-	-	OP893606
	CN097B1	OP590136	-	-	-	-	-
	CN100C6	OP590137	-	-	-	-	-
	CN100C7	OP590138	-	-	-	-	-

	Strain	**Genbank Accession numbers**					
		ITS	***CaM***	***BenA***	***gapdh***	***rpb2***	***tef-1α***
	CN101C7	-	-	-	-	-	OP893607
Cladosporium species	CN089E7	OP590085	-	-	-	-	-
	CN089F3	OP590087	-	-	-	-	-
	CN090D5	OP590088	-	-	-	-	-
	CN090D6	OP590089	-	-	-	-	-
	CN090D8	-	-	-	-	-	-
	CN090D9	OP590090	-	-	-	-	-
	CN090E2	OP590091	-	-	-	-	-
	CN090E4	OP590092	-	-	-	-	-
	CN090E5	OP590093	-	-	-	-	-
	CN091A9	OP590116	-	-	-	-	-
	CN091C6	-	-	-	-	-	-
	CN091C7	-	-	-	-	-	-
	CN091H3	OP590122	-	-	-	-	-
	CN091H4	OP590123	-	-	-	-	-
	CN091I6	OP590126	-	-	-	-	-
	CN096H7	-	-	-	-	-	-
	CN097A4	OP590133	-	-	-	-	-

	Strain	**Genbank Accession numbers**					
		ITS	***CaM***	***BenA***	***gapdh***	***rpb2***	***tef-1α***
	CN097A5	OP590134	-	-	-	-	-
	CN100C8	OP590139	-	-	-	-	-
	CN100E7	OP590140	-	-	-	-	-
	CN100E8	OP590141	-	-	-	-	-
	CN100G1	OP590142	-	-	-	-	-
	CN100G2	OP590143	-	-	-	-	-
	CN100G4	-	-	-	-	-	
	CN101A3	OP590145	-	-	-	-	
	CN088E5	OP590078	-	-	-	-	-
	CN088F1	OP590079	-	-	-	-	-
	CN088F7	OP590080	-	-	-	-	-
	CN089B5	OP590081	-	-	-	-	-
	CN089C5	OP590082	-	-	-	-	-
	CN089C6	OP590083	-	-	-	-	-
	CN089E5	OP590084	-	-	-	-	-
Clonostachys eriocamporesiana	CN097D9=CMW59079	OP596046	-	-	-	-	-
Coniochaeta canina	CN100H2=CMW59080	OP596053	-	-	-	-	-
Diaporthe miriciae	CN092A6=CMW59083	OP596024	-	-	-	-	-
Didymella americana	CN092A7=CMW59091	OP596025	-	-	-	OP720130	-

	Strain	**Genbank Accession numbers**					
		ITS	***CaM***	***BenA***	***gapdh***	***rpb2***	***tef-1α***
	CN092A8=CMW59092	OP596026	-	-	-	-	-
Didymella calidophila	CN096E6=CMW59093	OP596039	-	-	-	-	-
	CN101D4=CMW59095	OP596063	-	-	-	OP720135	-
Didymella glomerata	CN091I1=CMW59088	OP596018	-	-	-	OP720127	-
	CN091I4=CMW59089	OP596020	-	-	-	-	-
Didymella pomorum	CN089E9	OP595980	-	-	-	-	-
	CN089F2	OP595982	-	-	-	OP720117	-
	CN090D4	OP595990	-	-	-	OP720118	-
	CN091A1	OP596000	-	-	-	OP720119	-
	CN091A4	OP596001	-	-	-	OP720120	-
	CN091A5	OP596002	-	-	-	-	-
	CN091A6	OP596003	-	-	-	-	-
	CN091E9	OP596010	-	-	-	OP720121	-
	CN091F5	OP596013	-	-	-	OP720123	-
	CN091H7	OP596015	-	-	-	OP720124	-
	CN091H8	OP596016	-	-	-	OP720125	-
	CN091H9	OP596017	-	-	-	OP720126	-
	CN091I7	OP596022	-	-	-	OP720129	-
	CN092A5	OP596023	-	-	-	-	-

	Strain	**Genbank Accession numbers**					
		ITS	***CaM***	***BenA***	***gapdh***	***rpb2***	***tef-1α***
	CN100D2	OP596047	-	-	-	-	-
	CN100E9	OP596048	-	-	-	-	-
	CN100F2	-	-	-	OP729315	OP720131	-
	CN100H1	-	-	-	OP729316	OP720133	-
	CN101A5	OP596056	-	-	-	-	-
	CN101A6	OP596057	-	-	-	-	-
	CN101A9	OP596058	-	-	-	OP720134	-
	CN101D7	-	-	-	OP729317	-	-
Didymella subglomerata	CN091I5=CMW59090	OP596021	-	-	-	OP720128	-
	CN100F3=CMW59094	OP596049	-	-	-	OP720132	-
Didymella subherbarum	CN091F4	OP596012	-	-	-	OP720122	-
Epicoccum dendrobii	CN100H4	OP596055	-	-	-	OP720154	-
Epicoccum keratinophilum	CN088F4=CMW59102	OP595966	-	-	-	-	-
Epicoccum species	CN100G5	OP596051	-	-	-	-	-
Fusarium andiyazi	CN097D1=CMW59118	-	-	-	-	-	OP729331
Fusarium boothii	CN089C2	-	-	-	-	-	OP729322
Fusarium callistephi	CN097E9	-	-	-	-	-	OP729342
Fusarium clavus	CN097F3	-	-	-	-	-	OP729345
	CN100F1	-	-	-	-	-	OP729349

	Strain	Genbank Accession numbers					
		ITS	*CaM*	*BenA*	*gapdh*	*rpb2*	*tef-1α*
	CN090G6	OP595995	-	-	-	-	OP729324
Fusarium coffeatum	CN097C4	-	-	-	-	-	OP729328
Fusarium temperatum	CN097D2	-	-	-	-	-	OP729332
	CN097D7	-	-	-	-	-	OP729335
	CN097E5=CMW59121	-	-	-	-	-	OP729341
	CN097F4	-	-	-	-	-	OP729346
	CN097F6	-	-	-	-	-	OP729348
Fusarium verticillioides	CN097B7	-	-	-	-	-	OP729325
	CN097B8	-	-	-	-	-	OP729326
	CN097C2	-	-	-	-	-	OP729327
	CN097C6	-	-	-	-	-	OP729329
	CN097C9	-	-	-	-	-	OP729330
	CN097D3	-	-	-	-	-	OP729333
	CN097D5	-	-	-	-	-	OP729334
	CN097D8	-	-	-	-	-	OP729336
	CN097E1	-	-	-	-	-	OP729337

	Strain	**Genbank Accession numbers**					
		ITS	***CaM***	***BenA***	***gapdh***	***rpb2***	***tef-1α***
	CN097E2	-	-	-	-	-	OP729338
	CN097E3	-	-	-	-	-	OP729339
	CN097E4=CMW59120	-	-	-	-	-	OP729340
	CN097F1	-	-	-	-	-	OP729343
	CN097F2	-	-	-	-	-	OP729344
	CN097F5=CMW59123	-	-	-	-	-	OP729347
	CN089D4	-	-	-	-	-	OP729323
Lectera nordwiniana	CN091F6=CMW59124	OP596014	-	-	-	-	-
	CN101B5=CMW59125	OP596061	-	-	-	-	-
Neodidymelliopsis ranunculi	CN089F1=CMW59129	OP595981	-	-	-	-	-
	CN089F4=CMW59130	OP595983	-	-	-	-	-
	CN089F5	OP595984	-	-	-	-	-
Neurospora species	CN088F9	OP595969	-	-	-	-	-
Nigrospora sphaerica	CN095E5	OP596033	-	-	-	-	-
Nigrospora oryzae	CN101D1	-	-	-	-	-	-
Penicillium aurantiogriseum	CN088D7= CMW59133	-	-	OP660574	-	-	-
	CN088H9=CMW59136	-	-	OP660577	-	-	-
	CN089D7	-	-	OP660575	-	-	-
	CN089D8	-	-	OP660610	-	-	-

	Strain	**Genbank Accession numbers**					
		ITS	***CaM***	***BenA***	***gapdh***	***rpb2***	***tef-1α***
	CN089E4	-	-	OP660586	-	-	-
	CN089G6	-	-	OP660636	-	-	-
	CN089G9	-	-	OP660618	-	-	-
	CN089H1	-	-	OP660623	-	-	-
	CN095C4	-	-	OP660624	-	-	-
	CN101B8	-	-	OP660584	-	-	-
Penicillium brevicompactum	CN089E3=CMW59141	-	-	OP660583	-	-	-
	CN091B2=CMW59146	OP596006	-	OP660598	-	-	-
	CN100B9	-	-	OP660611	-	-	-
	CN100C1	-	-	OP660578	-	-	-
	CN101B6	-	-	OP660579	-	-	-
Penicillium charlesii	CN090F3=CMW59145	-	-	OP660580	-	-	-
Penicillium chrysogenum	CN091C9=CMW59149	-	-	OP660581	-	-	-
	CN091D1=CMW59150	-	-	OP660608	-	-	-
	CN091D3	-	-	OP660599	-	-	-
	CN095C7	-	-	OP660625	-	-	-
	CN095C8	-	-	OP660626	-	-	-
	CN095C9	-	-	OP660612	-	-	-
Penicillium cinnamopurpureum	CN089B7=CMW59140	-	-	OP660590	-	-	-

	Strain	**Genbank Accession numbers**					
		ITS	***CaM***	***BenA***	***gapdh***	***rpb2***	***tef-1α***
Penicillium citrinum	CN089A9=CMW59138	-	-	OP660597	-	-	-
	CN100D5=CMW59154	-	-	OP660609	-	-	-
	CN100H5	-	-	OP660604	-	-	-
	CN100H7	-	-	OP660606	-	-	-
	CN101C1	-	-	OP660587	-	-	-
Penicillium crustosum	CN091B6=CMW59147	-	-	OP660605	-	-	-
Penicillium glabrum	CN101B2=CMW59160	OP596060	-	OP660588	-	-	-
Penicillium griseofulvum	CN091C8=CMW59148	-	-	OP660589	-	-	-
Penicillium hispanicum	CN088I1=CMW59137	-	-	OP660585	-	-	-
Penicillium lanosocoeruleum	CN089H2=CMW59143	-	-	OP660603	-	-	-
Penicillium melanoconidium	CN091D4=CMW59151	-	-	OP660607	-	-	-
Penicillium polonicum	CN095F7	-	-	OP660591	-	-	-
	CN095H6	-	-	OP660596	-	-	-
Penicillium rudallense	CN100I3=CMW59159	-	-	OP660627	-	-	-
Penicillium scabrosum	CN088H8=CMW59135	-	-	OP660610	-	-	-
Penicillium sizovae	CN089B1=CMW59139	-	-	OP660623	-	-	-

	Strain	**Genbank Accession numbers**					
		ITS	***CaM***	***BenA***	***gapdh***	***rpb2***	***tef-1α***
Penicillium vanluykii	CN089E1	-	-	OP660619	-	-	-
	CN090F1	-	-	OP660614	-	-	-
	CN095C5	-	-	OP660594	-	-	-
	CN095C6	-	-	OP660620	-	-	-
	CN095F9	-	-	OP660600	-	-	-
	CN095G2	-	-	OP660622	-	-	-
	CN095G5	-	-	OP660602	-	-	-
	CN095G7	-	-	OP660592	-	-	-
	CN095H8	-	-	OP660595	-	-	-
	CN100B8	-	-	OP660627	-	-	-
	CN100H6=CMW59155	-	-	OP660628	-	-	-
	CN100H9=CMW59156	-	-	OP660615	-	-	-
Penicillium viridicatum	CN088D6	-	-	OP660617	-	-	-
	CN088H7=CMW59134	-	-	OP660582	-	-	-
	CN089G5=CMW59142	-	-	OP660576	-	-	-
	CN101B9=CMW59161	-	-	OP660621	-	-	-
Periconia byssoides	CN089C8=CMW59163	OP595978	-	-	-	-	-
Pestalotiopsis species	CN100G6	OP596052	-	-	-	-	-
Sarocladium zeae	CN097C5=CMW59166	OP596045	-	-	-	-	-

	Strain	**Genbank Accession numbers**					
		ITS	***CaM***	***BenA***	***gapdh***	***rpb2***	***tef-1α***
Scopulariopsis species	CN089A7	OP595975	-	-	-	-	-
Talaromyces adpressus	CN088D5= CMW59170	-	-	OP660551	-	-	-
	CN088E8=CMW59171	-	-	OP660552	-	-	-
Talaromyces amestolkiae	CN089A8=CMW59176	-	-	OP660553	-	-	-
	CN089D5=CMW59179	-	-	OP660556	-	-	-
	CN089D6	-	-	OP660557	-	-	-
	CN089E2	-	-	OP660555	-	-	-
	CN089G4	-	-	OP660554	-	-	-
	CN091F9	-	-	OP660558	-	-	-
	CN100D4	-	-	OP660559	-	-	-
Talaromyces diversus	CN089C7=CMW59177	OP595977	-		-	-	-
Talaromyces pseudofuniculosus	CN089D1	-	-	OP660562	-	-	-
	CN089D2	-	-	OP660564	-	-	-
	CN100F4=CMW59184	-	-	OP660563	-	-	-
	CN100F5=CMW59185	OP596050	-	-	-	-	-
Talaromyces purpureogenus	CN088F2=CMW59172	OP595964	-	-	-	-	-
	CN089D3=CMW59178	-	-	OP660565	-	-	-

	Strain	**Genbank Accession numbers**					
		ITS	***CaM***	***BenA***	***gapdh***	***rpb2***	***tef-1α***
Talaromyces stollii	CN089D9=CMW59180	-	-	OP660560	-	-	-
	CN091F3=CMW59182	-	-	OP660561	-	-	-
Talaromyces tiftonensis	CN088F5=CMW59173	OP595967	-	-	-	-	-
Talaromyces veerkampii	CN088F6=CMW59174	OP595968	-	-	-	-	-
Talaromyces verruculosus	CN088I8=CMW59175	-	-	-	-	-	-
Talaromyces wortmannii	CN090F4=CMW59181	-	-	OP660566	-	-	-
	CN091G1=CMW59183	-	-	OP660567	-	-	-
Wallemia mellicola	CN101D2=CMW59186	OP596062	-	-	-	-	-

Table 3. Limit of detection (LOD) and limit of quantification (LQD) for the 13 mycotoxins tested during the study.

Mycotoxin	**Limit of quantitation (LOQ), µg/kg**	**Limit of detection (LOD), µg/kg**
Aflatoxin B1	5	2.5
Aflatoxin B2	5	2.5
Aflatoxin G1	5	2.5
Aflatoxin G2	5	2.5
Deoxynivalenol	100	50
15-Acetyl-Deoxynivalenol	100	50
Fumonisin B_1	20	10
Fumonisin B_2	20	10
Fumonisin B_3	20	10
Ochratoxin A	5	2.5
T2-Toxin	20	10
HT2-Toxin	20	10
Zearalenone	20	10

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CHAPTER 3

Fungi associated with soybean leaves, stems, and roots in South Africa

ABSTRACT

Soybeans (*Glycine max*) are a significant source of oil and protein and a growing component of Africa's agricultural economy. Knowledge of fungal diversity, including those species that can cause soybean diseases, is to some degree documented worldwide but is lacking in South Africa. This study aimed to characterise the fungal diversity associated with South African soybean plants. Diseased materials were collected in 2021 from various farms across South Africa and included symptomatic roots, stems, leaves, and seeds from which fungi were cultured. A total of 200 strains were isolated, preserved, and identified based on their morphological characteristics and DNA sequences of the nuclear ribosomal internal transcribed spacers (ITS), DNA-directed RNA polymerase II subunit (*rpb2*), glyceraldehyde 3-phosphate dehydrogenase (*gapdh*), and/or partial sequences of translation elongation factor 1 (*tef-α*). Strains were identified in 22 genera and 38 species. The most dominant genera and the number of strains isolated during this study are *Sclerotinia* (n=46), *Epicoccum* (n=32), *Alternaria* (n=27), *Didymella* (19), *Diaporthe* (n=19), and *Fusarium* (n=19). Certain species identified from these genera like *Sclerotinia sclerotiorum, Alternaria alternata, Diaporthe longicolla,* and *Macrophomina phaseolina* have been reported as the causal agents of soybean diseases. The results of this study provide information on the fungal diversity and show which fungi may pose a threat to South African soybeans. Providing local farmers and seed breeding companies with knowledge of important fungi specific to their geographic location can allow for more targeted prevention of prevalent diseases.

INTRODUCTION

Soybean (*Glycine max* (L.) Merr.) is a major source of protein and extracted oil, and is widely consumed worldwide. South Africa is one of the largest producers in Sub-Saharan Africa and produced a record 1.89 tons in 2021/22 (Engelbrecht *et al.*, 2020; SAGL, 2021). The demand for soybeans and dedicated land for production is increasing (SAGL, 2021). Soybeans play a crucial role in South Africa's agriculture and food industry (Engelbrecht *et al.*, 2020), supporting both livestock and human nutrition, while also contributing to the country's economy through export markets (DAF, 2021; Engelbrecht *et al.*, 2020).

Studying fungal diversity can help identify beneficial and harmful fungal species, leading to more sustainable and efficient agricultural practices. One major constraint to soybean production is losses due to fungal diseases (Engelbrecht *et al.*, 2020; Hartman *et al.*, 2015). The most relevant fungal diseases affecting soybean production include alternaria leaf spot, anthracnose, brown stem rot, cercospora leaf blight, charcoal rot, fusarium wilt/root rot, sclerotinia stem rot, stem canker, soybean sudden death syndrome, soybean rust, red leaf blotch, red crown rot, purple seed stain, and phomopsis seed decay (Hartman *et al.*, 2015). In South Africa, fungal diseases affecting soybean production include soybean sudden death syndrome (caused by *Neocosmospora phaseoli* (previously *Fusarium virguliforme*, and *F. brasiliense*), soybean rust (caused by *Phakopsora pachyrhizi,* or/and *Phakopsora meibomiae*), and sclerotinia stem rot (caused by *Sclerotinia sclerotiorum*) (Caldwell and McLaren, 2004; Rothmann and McLaren, 2022; Tewoldemedhin YT *et al.*, 2014; Tewoldemedhin *et al.*, 2017; Thompson GC and AH, 1979; Van der Hoven *et al.*, 2017). The yield and economic loss depend on factors like type and severity of disease, climate, disease control practices, genetic diversity of the pathogens, and soybean cultivars (Lin *et al.*, 2022).

Seed breeding companies require knowledge of fungal diversity to develop resistant soybean cultivars. One way fungal diseases can be spread or remain prevalent in subsequent seasons is by farmers who plant soybean seeds saved from the previous seasons. These farm-saved seeds can negatively impact yields and reduce the funding to seed and breeding companies (BFAP, 2022). In an attempt to solve this problem, a breeding and technology levy is a statutory levy that has been implemented to promote the development of new cultivars adapted to local growing conditions and diseases, increase yield, and reduce imports to South African companies (BFAP, 2022; SANSOR, 2019). The levy falls under the Marketing of Agricultural Products Act of 1996, developed by the South African Cultivar & Technology Agency (SANSOR, 2019). It is also important to consider that pathogens have the ability to evolve and can overcome resistance (Anderson *et al.*, 2004; McDonald and Stukenbrock, 2016; Ristaino *et al.*, 2021) and it’s unattainable to develop soybean cultivars resistant to all diseases.

Despite the impact of fungal diseases on soybean production, very little data exists on the fungal diversity associated with South African soybeans. Seed breeding companies and producers are reliant on the knowledge of fungal diversity to develop and select the appropriate local cultivars needed for subsequent seasons. Therefore, the aim of this study was to generate baseline information on the fungal diversity associated with South African soybean plants and to make fungal strains and data available in culture collections.

MATERIALS AND METHODS

Sampling

One hundred and thirty-six soybean samples were collected from 17 collection sites from soybean-producing farms located in the Free State (n=21), Gauteng (n=11), Kwazulu-Natal (n=10), Mpumalanga (n=86), and the North-West (n=8) in South Africa (Fig 1 and Table 2). Symptomatic soybean stems, roots, and foliar samples were collected in 2021 (Fig 2 and Fig 3).

Fungal isolations

Two isolation media were used to target a broad range of fungi based on their varying growth requirements. These included the generalist media ½ Potato Dextrose Agar (½ PDA) and Water agar (WA). Chloramphenicol (50mg/mL) was added to ½ PDA and WA to prevent bacterial growth. Symptomatic soybean material was surface disinfested and directly plated onto each type of medium and on filter paper in 90 mm Petri dishes. All plates were incubated at 25°C for 3–7 d and regularly inspected for fungal growth. Pure fungal cultures were obtained by observing growth with a stereomicroscope and transferring spores or mycelia onto fresh growth media using a sterile needle. For *Fusarium*, single spore cultures were prepared following the recommendations by Leslie and Summerell (2006). Media used for pure cultures included ¼ PDA, WA, or DG18, and cultures were incubated at 25°C for 7d.

Isolates were identified to genus level based on their colony and microscopic characters. Isolates were subsequently preserved by collecting fungal material from pure cultures using a sterile scalpel blade into vials containing 10% glycerol and stored at -80°C. The strains were accessioned into the working collection (CN) of the Applied Mycology group housed at FABI (Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa). Additionally, representative strains of each species were deposited into the CMW culture collection also housed at FABI.

DNA extraction, amplification (PCR), and sequencing

The Quick-DNA Fungal/Bacterial Miniprep kit™ (ZYMO RESEARCH, California, USA) was used to extract the DNA from heavily pigmented fungi, such as *Alternaria*, *Epicoccum*, and *Didymella*. The

Prepman™ Ultra Sample Preparation reagent (Thermo Fisher Scientific, Waltham, Massachusetts) was used to extract DNA from fungi lacking dark pigments, like *Fusarium*, according to manufacturing protocol. The gene regions and primer sequences used during the study are shown in Table 1.

For PCR amplification, the reaction mixture of 25 µL consisted of 17.85 µL MilliQ H_2O, 5 µL BioLine 5X MyTaq™ Reaction Buffer (Meridian BioScience, USA), 0.50 µL of each primer (10 µM), and 0.15 µL BioLine MyTaq™ DNA Polymerase (Meridian BioScience, USA) and 1 µL of DNA. For *Fusarium*, a reaction mixture of 25 µL consisted of 17.3 µL MilliQ H_2O, 2.5 µL (10x) PCR reaction buffer, 0.5 µL (2.5 mM) $MgCl_2$, 200 µM of each dNTP, 0.5 µL (0.8 µM) of each primer (forward and reverse), 0.2 µL (1U) FastStart Taq DNA Polymerase (Roche, Basel, Switzerland) and 1 µL of DNA.

Loci targeted for each genus include translation elongation factor 1–α (*tef-1α*) (O'Donnell *et al.*, 1998) for *Cladosporium*, *Diaporthe*, *Fusarium*, *Macrophomina* and *Neocosmospora*, glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) for *Alternaria* and other *Pleosporales* (Berbee *et al.*, 1999) and DNA-directed RNA polymerase II second largest subunit for (*rpb2*) for *Didymellaceae* (Hou *et al.*, 2020). The nuclear ribosomal internal transcribed spacer (ITS) rDNA region for strains was used to identify strains that could not be identified to genus using morphology (de Hoog and Gerrits van den Ende, 1998; Schoch *et al.*, 2012). The following primers were used for identification: V9G (de Hoog and Gerrits van den Ende, 1998) and LS266 (Masclaux *et al.*, 1995) for the internal transcribed spacer regions (ITS), including 5.8S nrRNA gene region. For *tef-1α* primers used were EF-1 and EF-2 (O'Donnell *et al.*, 1998) for *Fusarium* and *Neocosmospora* or EF1-728F (Carbone and Kohn, 1999) and EF-983 (Rehner and Buckley, 2005) for *Cladosporium*, *Diaporthe,* and *Macrophomina*. For part of the *rpb2,* primers F1 (Sung *et al.*, 2007) and 7CR (Liu *et al.*, 1999), and *gapdh* primers, gpd1, and gpd2 (Berbee *et al.*, 1999) were used.

PCR products were stained with GelRed (Biotium, Inc., California, USA), electrophoresed on 1% agarose gel, and examined under UV light to confirm correct amplicon sizes. Amplicons were purified using the ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific, Massachusetts, USA) and sequenced in both directions using the BigDye terminator sequencing kit v.3.1 (Applied Biosystems, Forster City, California USA) using the same primers used for PCR amplification (Table 1). Sequence analyses were performed at the DNA Sanger Sequencing Facility, Faculty of Natural and Agricultural Sciences, the University of Pretoria on an ABI3500xL Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA). The contigs were assembled and base calls were made using Geneious Prime v. 2019.0.4 (BioMatters Ltd., Auckland, New Zealand).

Species identifications

Sequence-based identifications were made by comparing sequence data to the National Centre for Biotechnology Information (NCBI's) nucleotide database using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). *Fusarium* strains were identified using FUSARIOID-ID (https://www.fusarium.org/) and the dataset published by Crous *et al.* (2022). Newly generated sequences were submitted to GenBank with accession numbers listed in (Table 2).

RESULTS

Fungal identifications

Isolations resulted in 200 fungal strains representing 22 genera, and 38 species. Genera identified in this study were *Alternaria* (n=27), *Aureobasidium* (n=1), *Boeremia* (n=3), *Cladosporium* (n=7), *Colletotrichum* (n=1), *Cumuliphoma* (n=2), *Diaporthe* (n=18), *Didymella* (n=19), *Epicoccum* (n=32), *Exserohilum* (n=1), *Fusariella* (n=1), *Fusarium* (n=16), *Macrophomina* (n=6), *Neoascochyta* (n=1), *Neocosmospora* (n=1), *Nigrospora* (n=13), *Penicillium* (n=1), *Periconia* (n=1), *Phaeosphaeria* (n=1), *Phialophora* (n=1), *Sclerotinia* (n=46), and *Setophaeosphaeria* (n=1) (Fig 4). The most frequently isolated genera were *Sclerotinia*, *Epicoccum*, *Alternaria*, and *Fusarium*. Most of the fungal growth was observed on PDA and most genera were isolated from soybean leaves, stems, seeds/pods and roots, respectively (Fig 5).

Species were identified in this study including *Alternaria alternata* (n=25), *Alternaria* sp. (n=2), *Aureobasidium melanogenum* (n=1), *Boeremia coffeae* (n=1), *Boeremia* sp.(n=2), *Cladosporium cladosporioides* (n=2), *Cladosporium pseudocladosporioides* (n=4), *Cladosporium* sp. (n=1), *Colletotrichum* sp. (n=1), *Cumuliphoma pneumoniae* (n=2), *Diaporthe longicolla* (n=16), *Diaporthe miriciae (*n=1), *D. sojae* (n=1), *Didymella americana* (n=7), *Di. glomerata* (n=1), *Di. pomorum* (n=6), *Di. sinensis* (n=2), *Di. subherbarum* (n=3), *Epicoccum dendrobii* (n=4), *E. draconis* (n=9), *E. italicum* (n=5), *E. mezzettii* (n=4), *E. nigrum* (n=1), *E. ovisporum* (n=1), *E. sorghinum* (n=8), *Exserohilum rostratum* (n=1), *Fusariella hughesii* (n=1), *Fusarium acuminatum* (n=1), *F. boothii* (n=7), *F. clavus* (n=4), *F. fabacearum* (n=1), *F. glycines* (n=1), *F. verticillioides* (n=2), *Macrophomina phaseolina* (n=6), *Neoascochyta argentina* (n=1), *Neocosmospora solani* (n=1), *Nigrospora sphaerica* (n=13), *Penicillium glabrum* (n=1), *Periconia byssoides* (n=1), *Phaeosphaeria* sp. (n=1), *Phialophora cyclaminis* (n=1), *Sclerotinia sclerotiorum* (n=46), and *Setophaeosphaeria badalingensis* (n=1) (Table 2). The most isolated species in this study were *Sclerotinia sclerotiorum* (n=46), *Alternaria alternata* (n=25), *Diaporthe longicolla* (n=16), and *Nigrospora sphaerica* (n=13) (Fig 3).

DISCUSSION

Soybeans play an important role in South Africa's agriculture and food industry, supporting both livestock and human nutrition while also contributing to the country's economy through export markets (DAF, 2021; Engelbrecht *et al.*, 2020). Soybean production can be threatened by several fungal diseases. The aim of this study was to determine the fungal diversity associated with symptomatic soybeans in South Africa.

During this survey, 206 fungal strains were identified as 22 genera and 38 species. The most frequently isolated genera were *Sclerotinia*, *Epicoccum*, *Alternaria*, *Fusarium,* and *Diaporthe*, respectively. Similar genera have been found on soybean stems in Minnesota (USA) including *Cladosporium*, *Alternaria, Diaporthe*, and *Epicoccum*, respectively, based on the ITS barcode (Impullitti and Malvick, 2013). Chang *et al.* (2020) found *Fusarium*, *Colletotrichum*, *Alternaria,* and *Diaporthe* (previously *Phomopsis*) commonly isolated from soybean seeds based on the ITS, *tef-1α* and *RBB2* barcodes. Many of the species identified have been previously isolated from soybeans or are known as the causal agents of soybean diseases. The most commonly encountered species in this study were *Sclerotinia sclerotiorum*, *Alternaria alternata*, and *Diaporthe longicolla*. *Macrophomina phaseolina* and *Fusarium oxysporum* were less dominant but have significance to the soybean industry.

Sclerotinia sclerotiorum is soil-borne and is known as the causal agent of stem rot or white mold in soybean (Hartman *et al.*, 2015). *Sclerotinia sclerotiorum* has been reported from soybean by Pawlowski *et al.* (2019) who used the ITS barcode for identification. The disease can be characterized by the production of hard, melanized mycelial masses called sclerotia that can remain in the soil for up to 10 years and can serve as a source of inoculum for new infections (Rothmann and McLaren, 2018). These structures can reside in the stem (Fig 2) or pods of soybean. Most of the strains in this study were isolated from soybean stems, including some recovered from foliar samples. Soybean seeds can drastically degrade in value if sclerotia are present, and thus the South African Grain Laboratory (SAGL) grades seeds from commercial soybean-producing farms annually. Similar to this study, *S. sclerotiorum* has been previously associated with South African soybeans. Rothmann and McLaren (2022) recovered sclerotia from soybean seeds in South African silos in 2018 (63.2% incidence from 19 samples), 2019 (61.1% incidence in 18 samples), and 202 (78.3% incidence from 23 samples) in soybean production regions. Similar to the results of this study, strains of *S. sclerotiorum* (and/or sclerotia) have been previously collected and identified from soybeans in South Africa

(Mbedzi *et al.*, 2019; Thompson GC and AH, 1979). Most of the *S. sclerotiorum* strains collected during this study were isolated from the soybean seeds, pods, and stem (Fig 5).

Alternaria alternata is mainly necrotrophic causing leaf blight in soybeans, and will usually be a secondary invader following mechanical injury or if infected with other diseases. *Alternaria alternata* has been identified as the causal agent of Alternaria leaf spot in soybean (Hartman *et al.*, 2015). Alternaria leaf blight on soybeans has been reported in Argentina (Rothen *et al.*, 2017; Russo *et al.*, 2016), Turkey (Ustun *et al.*, 2019), India (Fagodiya *et al.*, 2021; Zade and Ingle, 2022), and China (Li *et al.*, 2023). Some studies have used *gapdh* to identify *A. alternaria* (Li *et al.*, 2023), while some studies based identification only on the ITS barcode (Rothen *et al.*, 2017; Russo *et al.*, 2016; Ustun *et al.*, 2019) or morphology (Fagodiya *et al.*, 2022; Zade and Ingle, 2022). *Alternaria alternata* has been frequently isolated from soybean seeds in the U.S.A (Escamilla *et al.*, 2019). Similarly, the results of this study show *A. alternaria* as a dominant species associated with soybeans. Most of the *A. alternata* strains collected during this study were from soybean leaves (Fig 5). In South Africa, there are no reports of Alternaria leaf spot or of *A. alternata* associated with soybean. *Alternaria alternata* has only been reported as a causal agent of sunflower leaf blight (Kgatle *et al.*, 2018). Sunflowers can be used for crop rotation with soybeans and potentially infect the latter (Kgatle *et al.*, 2018). However, further studies are needed to determine if this fungus can cause soybean leaf blight in South Africa.

Previous studies have reported *Diaporthe* species on soybean stem in the USA (Impullitti and Malvick, 2013; Pimentel *et al.*, 2022) based on the ITS barcode. *Diaporthe longicolla* was a frequently isolated species in this study and has been reported associated with soybean stems *Diaporthe longicolla* (syn. *Phomopsis longicolla*) is known as the major cause of Phomopsis seed decay, pods, and stem blight in soybean (Hartman *et al.*, 2015; Petrović *et al.*, 2021). *Diaporthe longicolla* has been previously reported on soybean stem (Santos *et al.*, 2011) and seeds (Hosseini *et al.*, 2020) based on the *tef-1α*. *Diaporthe longicolla* has been associated with soybean in countries like Canada (Abdelmagid *et al.*, 2022), China (Shan *et al.*, 2012), and Colombia (López-Cardona *et al.*, 2022) based on the *tef-1α* barcode. Strains from this study were collected from dark zone lines in the longitudinal section of the lower stem can be an indicator of *Diaporthe* infection (Fig 3). Most of the *Diaporthe* strains collected in this study were isolated from the stem base and roots (Fig 5). Infected seeds can appear shriveled, cracked, or covered in white mold (Crop Protection Network, 2015; Hartman *et al.*, 2015). Phomopsis seed decay is an important disease because it can reduce the seed weight and oil content (Hartman *et al.*, 2015).

Macrophomina phaseolina was less dominant during this study but is an important causal agent of soybean charcoal rot. Similar to the results of this study, *M. phaseolina* has been reported on soybeans in the USA (Bradley and Río, 2003) based on morphology and Brazil (Machado *et al.*, 2019) and

Taiwan (Shih *et al.*, 2022) based on the *tef-1α* and in Argentina (Rothen *et al.*, 2017) and Brazil (Rothen *et al.*, 2017) based on the ITS barcode. The fungus is soil-borne and survives in regions with temperatures ranging between 30–35°C and moisture stress levels below 60% (Arora and Pareek, 2013; Gupta *et al.*, 2012; Kaur *et al.*, 2012; Marquez *et al.*, 2021; Meena *et al.*, 2018; Saleh *et al.*, 2010). Microsclerotia is the primary inoculum that can reside in the soil for up to 15 years and can sometimes be observed inside the stem and roots (Gupta *et al.*, 2012). Most strains isolated during this study were collected from soybean roots and stems (Fig 5). Yield loss due to charcoal rot can vary over years but has been ranked as one of the most yield-reducing soybean diseases in the U.S.A (Allen *et al.*, 2017; Romero Luna *et al.*, 2017). Charcoal rot is usually triggered by drought stress, which can be partially problematic in South Africa given its droughts and warm weather (Hartman *et al.*, 2015).

Fusarium oxysporum (FOSC) was a less dominant species in this study but is important to the soybean industry. The fungus is widespread and can overwinter in the soil and plant debris for long periods of time (Leslie and Summerell, 2008). *Fusarium oxysporum* has been reported to cause Fusarium wilt and root rot in soybean (Arias *et al.*, 2013; Duvnjak *et al.*, 2017; Hartman *et al.*, 2015). *Fusarium oxysporum* has been previously reported on soybean in Canada (Zhou *et al.*, 2018), China (Chang *et al.*, 2018), Croatia (Duvnjak *et al.*, 2017), and the U.S.A (Pimentel *et al.*, 2022). *Fusarium oxysporum* was isolated from symptomatic soybean roots and identified using the *tef-1α* (Chang *et al.*, 2018; Pimentel *et al.*, 2022; Zhou *et al.*, 2018), or using morphology and the ITS barcode (Fernandes *et al.*, 2015). Similarly, *Fusarium oxysporum* was identified in this study. Species *F. fabacearum* and *F. glycines* were isolated in this study and are similar to the results of Lombard *et al.* (2019), who initially described these species from soybean in South Africa. Soybean material with SDS symptoms, i.e. root necrosis and foliar chlorosis and necrosis, were collected during this study. *Neocosmospora solani* (previously *Fusarium solani*) was isolated from the roots during this study (Fig 5). Soybean SDS has been previously reported in South Africa (Tewoldemedhin YT *et al.*, 2014; Tewoldemedhin *et al.*, 2017), but the causal agent was not found in this study.

In this study, valuable information was gained on the fungal diversity associated with soybeans in South Africa. This knowledge combined with future surveys can help seed breeding companies select or breed appropriate cultivars for subsequent seasons, and rotate soybeans with other crops. Most past studies identified fungal species based on morphology or the ITS barcode. It is essential to identify species using DNA sequences that can identify and distinguish between closely related species. This study generated DNA sequences that will expand biological resources like Genbank and serve as important reference material for future studies (Table 2). Generated DNA sequences can help capture infra-species variation and will make future identifications easier. This study is one of the first surveys to characterise the fungal diversity associated with soybeans in South Africa.

CONCLUSION

There is a growing demand for soybeans in South Africa. Fungal diseases can have devasting effects on soybean production, which will limit the expansion of the industry. Seed companies and soybean producers need baseline information to make important decisions on which cultivars are needed for soybean production. For this study, important fungi like *Diaporthe longicolla*, *Sclerotinia sclerotiorum*, and *Alternaria alternata* were dominant. Less dominant species like *Fusarium oxysporum* and *Macrophomina phaseolina* were identified but are important since they have been identified as the causal agents of soybean diseases. The pathogenicity of these species on South African soybeans needs to be confirmed by Koch's postulates. DNA sequences obtained during this study are available on Genbank and cultures are deposited into culture collections for future studies.

FIGURES

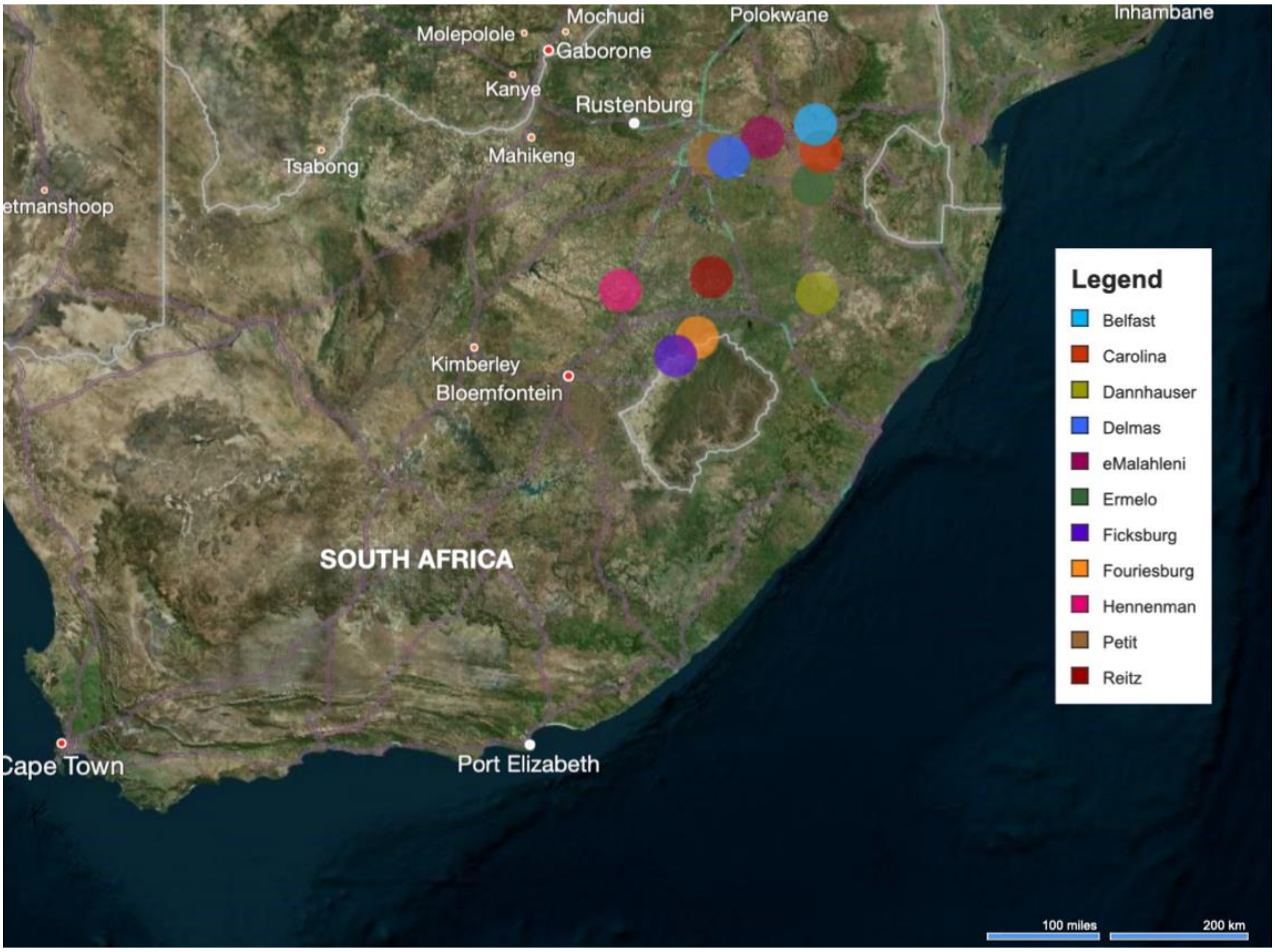

Figure 1. Diseased soybean sampling sites in South Africa.

Figure 2. Sclerotia of *Sclerotinia sclerotiorum* embedded inside soybean stems.

Figure 3. Dark zone lines in the longitudinal section of the lower stem are an indicator of *Diaporthe longicolla* collected from stems in South Africa.

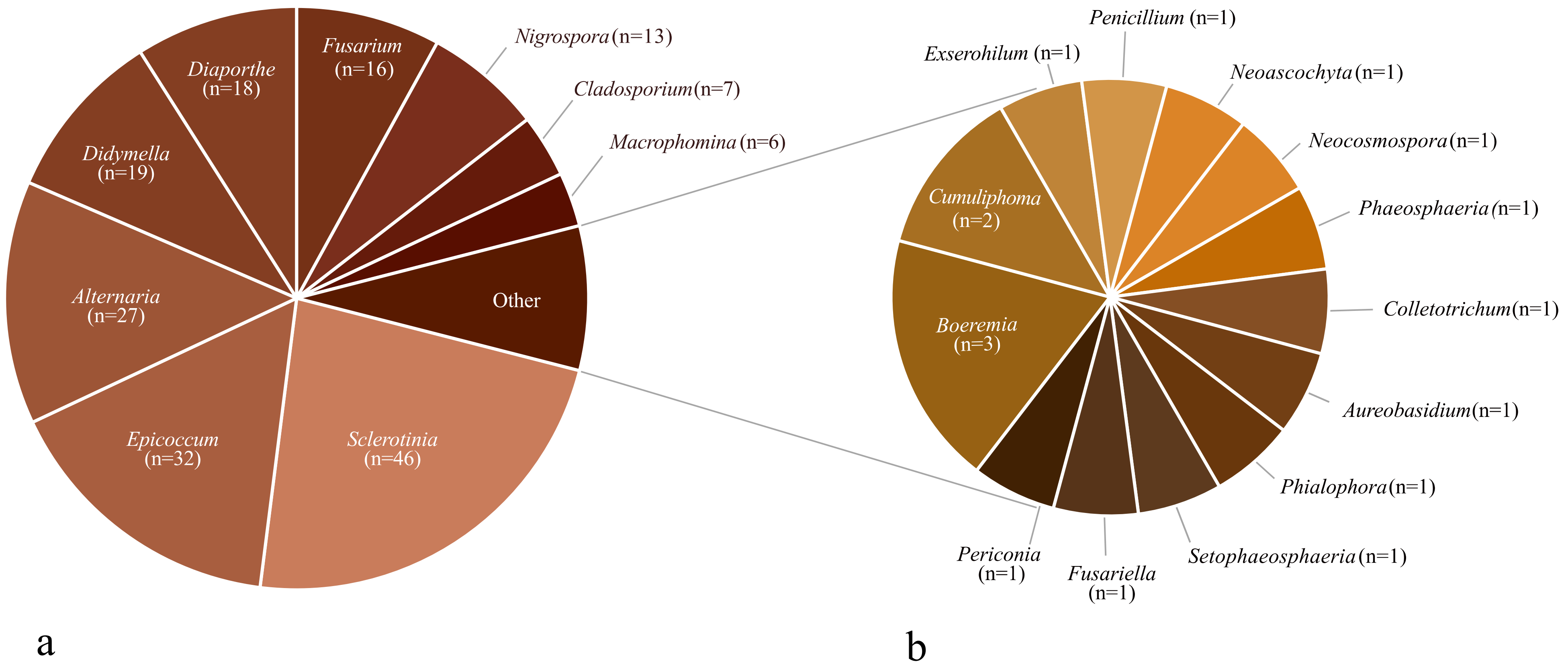

Figure 4. Genera isolated from symptomatic soybean material in South Africa (a) Genera isolated frequently and (b) genera isolated less frequently. The number of strains (n) isolated are shown for each genus.

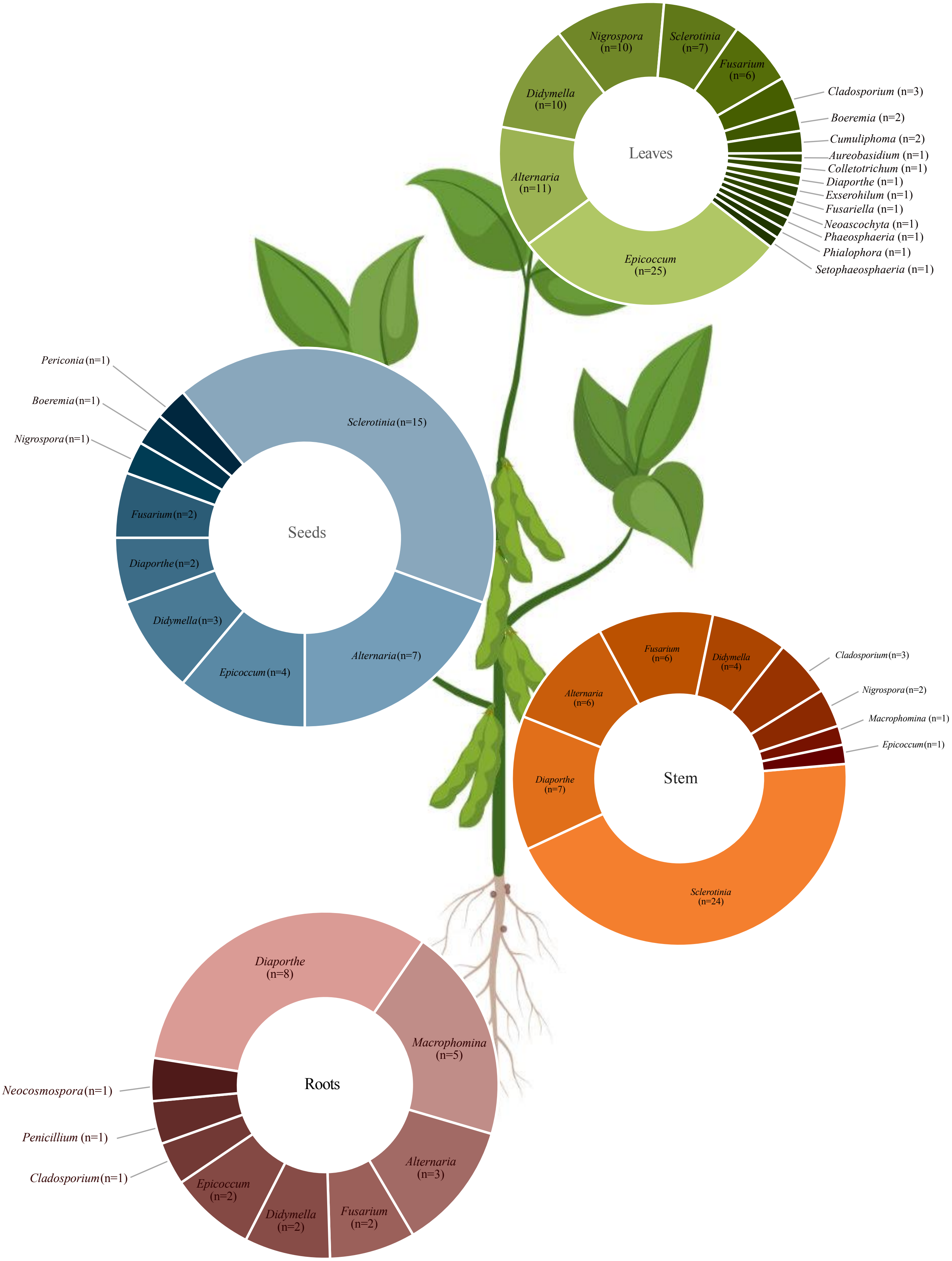

Figure 5. The genera isolated from symptomatic soybean material in South Africa. The number of strains (n) are shown for each genus.

TABLES

Table 1. The primer pairs, amplification profile, and references used in this study.

Locus	Primers	Sequence (5’ to 3’)	Amplification profile	Reference
Beta-tubulin (*BenA*)	T10	ACGATAGGTTCACCTCCAGAC	94°C 3 min; 35 cycles of 94°C 45s, 52°C 45s, 72°C 1 min 30s, final extension 72°C 8 min	(Glass and Donaldson, 1995)
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC		(Glass and Donaldson, 1995)
Glyceraldehyde-3phosphate dehydrogenase (*gapdh*)	gdp1	CAACGGCTTCGGTCGCATTG	94°C 5 min; 30 cycles of 94°C 45s, 52°C 1 min, 72°C 1 min, and final extension 72°C 7 min	(Berbee *et al.*, 1999)
	gdp2	GCCAAGCAGTTGGTTGTGC		(Berbee *et al.*, 1999)
Internal transcribed spacer (ITS)	V9G	TTACGTCCCTGCCCTTTGTA	94°C 3 min, 35 cycles of 94°C 45s, 52°C 45s, 72°C 90s, and final extension 72°C 8 min	(de Hoog and Gerrits van den Ende, 1998)
	LS266	GCATTCCCAAACAACTCGACTC		(Masclaux *et al.*, 1995)

DNA-directed RNA polymerase II (*rpb2*)	5F	GGGGWGAYCAGAAGAAGGC	94°C 5 min, 35 cycles of 95°C 45s, 54°C 80s, 72°C 2 min, and final extension of 72°C 10 min	(Sung *et al.*, 2007)
	7CR	CCCATRGCTTGYTTRCCCAT		(Liu *et al.*, 1999)
Translation elongation factor (*tef-1α*)	EF1-728F	CATCGAGAAGTTCGAGAAGG	94°C 3 min; 35 cycles of 94°C 45s, 52°C 45s, 72°C 80s, final extension of 72°C 8 min	(Carbone and Kohn, 1999)
	986R	TACTTGAAGGAACCCTTACC		(O'Donnell *et al.*, 1998)
	EF1	ATGGGTAAGGARGACAAGAC	94°C 5 min, 35 cycles of 94°C 45s, 53°C 45s, 72°C 60s, final extension of 72°C 7min	(O'Donnell *et al.*, 1998)
	EF2	ATGGGTAAGGARGACAAGAC		(O'Donnell *et al.*, 1998)

Table 2. The fungal strains isolated and identified for this study.

	Strain	Genbank accession number					Substrate	Town
		ITS	*tef-1a*	*gapdh*	*rpb2*	*BenA*		
Alternaria alternata	CN119F9	OP596066	-	OP723132	-	-	Stem	Petit
	CN119G1	OP596067	-	OP723133	-	-	Roots	Petit
	CN119G3	OP596068	-	OP893548	-	-	Stem	Petit
	CN119I1	OP596076	-	OP723134	-	-	Seed	Petit
	CN119I2	OP596077	-	OP723135	-	-	Seed	Petit
	CN119I3	OP596078	-	OP723136	-	-	Stem	Petit
	CN119I4	OP596079	-	OP723137	-	-	Roots	Petit
	CN119I5	OP596080	-	OP893549	-	-	Roots	Petit
	CN134I6	-	-	OP723138		-	Stem	Hennenman
	CN135A2	-	-	OP723139	-	-	Leaf	Hennenman
	CN135B2	-	-	OP723140	-	-	Leaf	Dannhauser
	CN135B5	-	-	OP893550	-	-	Leaf	Ficksburg
	CN135I1	-	-	OP723141	-	-	Leaf	Carolina
	CN136B4	OP596124	-	OP723142	-	-	Leaf	Carolina
	CN136B5	OP596125	-	OP723143	-	-	Leaf	Carolina
	CN136B6	-	-	OP723144	-	-	Leaf	Carolina
	CN136C2	-	-	OP723145	-	-	Stem	Belfast
	CN136C3	-	-	OP723146	-	-	Stem	Belfast
	CN136D6	-	-	OP893551	-	-	Leaf	eMalahleni
	CN136D7	-	-	OP723147	-	-	Leaf	eMalahleni
	CN136F5	-	-	OP723148	-	-	Leaf	Delmas

	CN136F6	-	-	OP723149	-	-	Leaf	Delmas
	CN145B4	-	-	OP723150	-	-	Seed	Delmas
	CN145B5	-	-	OP723151	-	-	Seed	Delmas
	CN145B6	-	-	OP723152	-	-	Seed	Delmas
Alternaria species	CN145C4	OP596149	-	-	-	-	Seed	Delmas
	CN136F8	OP596143	-	-	-	-	Seed	Delmas
Aureobasidium melanogenum	CN135A6=CMW59068	OP596095	-	-	-	-	Leaf	Hennenman
Boeremia species	CN136F1	OP596138	-	-	-	-	Leaf	Carolina
	CN136F2	OP596139	-	-	-	-	Leaf	Carolina
Boeremia coffeae	CN145D3=CMW59071	OP596156	-	-	OP729384	-	Seed	Belfast
Cladosporium cladosporioides	CN119I7	-	OP893574	-	-	-	Stem	Petit
	CN136D5=CMW59078	-	OP893575	-	-	-	Leaf	Petit
Cladosporium pseudocladosporioides	CN119G2	-	OP893608	-	-	-	Stem	Petit
	CN119H2=CMW59075	OP596073	OP893609	-	-	-	Roots	Petit
	CN119H4	-	OP893610	-	-	-	Stem	Petit
	CN136D4=CMW59077	-	OP893611	-	-	-	Leaf	Belfast
Cladosporium species	CN136B7	OP596126	-	-	-	-	Leaf	Carolina
Colletotrichum species	CN136F4	OP596141	-	-	-	-	Leaf	eMalahleni
Cumuliphoma pneumoniae	CN135C3=CMW59081	OP596104	-	-	OP893598	-	Leaf	Dannhauser
	CN135C4=CMW59082	OP596105	-	-	OP893599	-	Leaf	Dannhauser
Diaporthe longicolla	CN119H3	OP596074	-	-	-	-	Stem	Petit
	CN126E4	OP596081	-	-	-	-	Stem	Petit
	CN126E5	OP596082	OP893590	-	-	-	Root	Petit
	CN126F6	OP596084	-	-	-	-	Root	Petit

	CN126F7	OP596085	OP893591	-	-	-	Stem	Petit
	CN126F8	OP596086	-	-	-	-	Root	Petit
	CN126F9	-	OP893592	-	-	-	Root	Petit
	CN126G1	OP596087	OP893593	-	-	-	Stem	Petit
	CN126G6	OP596088	OP893594	-	-	-	Root	Petit
	CN126G7	OP596089	-	-	-	-	Root	Petit
	CN126G8	OP596090	-	-	-	-	Stem	Petit
	CN126G9	OP596091	OP893595	-	-	-	Root	Petit
	CN126H2	OP596092	OP893596	-	-	-	Stem	Petit
	CN126H5	OP596093	-	-	-	-	Root	Petit
	CN136F7=CMW59084	OP596142	-	-	-	-	Leaf	Delmas
	CN145D1=CMW59087	OP596154	-	-	-	-	Seed	Schweizer-Reneke
Diaporthe miriciae	CN145C6=CMW59085	OP596151	-	-	-	-	Stem	Belfast
Diaporthe sojae	CN145C7=CMW59086	OP596152	OP893597	-	-	-	Seed	Belfast
Didymella americana	CN119F3	OP596064	-	-	OP720136	-	Stem	Petit
	CN119F4	OP596065	-	-	OP720137	-	Roots	Petit
	CN119G7	OP596070	-	-	OP720138	-	Roots	Petit
	CN119G9	OP596072	-	-	OP720140	-	Stem	Petit
	CN145B7	-	-	-	OP720151	-	Pod	Ermelo
	CN145C3	OP596148	-	-	OP720152	-	Seed	Delmas
	CN145C5	OP596150	-	-	OP720153	-	Seed	Delmas
Didymella glomerata	CN136A4	-	-	-	OP720147	-	Leaf	Carolina
Didymella pomorum	CN119G8	OP596071	-	-	OP720139	-	Stem	Petit
	CN135B8	OP596100	-	-	OP720143	-	Leaf	Dannhauser

	CN135I4	OP596112	-	-	OP720145	-	Leaf	Carolina
	CN136A3=CMW59098	-	-	-	OP720146	-	Leaf	Carolina
	CN136A5=CMW59099	-	-	-	OP720148	-	Leaf	Carolina
	CN136E8	-	-	-	OP720149	-	Leaf	eMalahleni
Didymella sinensis	CN135C7=CMW59097	OP596108	-	-	OP720144	-	Leaf	Dannhauser
	CN136B3=CMW59100	OP596123	-	-	-	-	Leaf	Carolina
Didymella subherbarum	CN135B3	OP596098	-	-	OP720141	-	Stem	Ficksburg
	CN135B4=CMW59096	OP596099	-	-	OP720142	-	Leaf	Ficksburg
	CN136E9=CMW59101	OP596137	-	-	OP720150	-	Leaf	Delmas
Epicoccum dendrobii	CN134I9	OP596094	-	-	OP720155	-	Leaf	Hennenman
	CN136A9	OP596120	-	-	OP720165	-	Leaf	Carolina
	CN136B2=CMW59108	OP596122	-	-	OP720167	-	Leaf	Carolina
	CN145D4=CMW59112	-	-	-	OP720170	-	Seed	Delmas
Epicoccum draconis	CN135A7	OP596096	-	-	-	-	Leaf	Dannhauser
	CN135C1	OP596102	-	-	OP720157	-	Leaf	Dannhauser
	CN135C8	OP596109	-	-	-	-	Leaf	Dannhauser
	CN135C9	OP596110	-	-	OP720159	-	Leaf	Dannhauser
	CN135I8	OP596114	-	-	OP720161	-	Leaf	Carolina
	CN136A1	OP596116	-	-	OP720163	-	Leaf	Carolina
	CN136A2	OP596117	-	-	OP720164	-	Leaf	eMalahleni
	CN136B1=CMW59107	OP596121	-	-	OP720166	-	Leaf	Carolina
	CN145D6=CMW59114	OP596158	-	-	OP720172	-	Seed	Belfast
Epicoccum italicum	CN119F1	-	-	-	OP729377	-	Roots	Petit
	CN119F2	-	-	-	OP729378	-	Seed	Petit
	CN119G5	OP596069	-	-	OP729379	-	Roots	Petit

	CN135I2=CMW59106	-	-	-	OP729380	-	Leaf	Carolina
	CN136C5=CMW59110	-	-	-	OP729381	-	Leaf	Belfast
Epicoccum mezzettii	CN134I7	-	-	-	OP729373	-	Leaf	Hennenman
	CN134I8	-	-	-	OP729374	-	Leaf	Hennenman
	CN135A8=CMW59103	OP596097	-	-	OP729375	-	Leaf	Dannhauser
	CN136C4=CMW59109	-	-	-	OP729376	-	Leaf	Belfast
Epicoccum nigrum	CN135A9	-	-	-	OP720156	-	Leaf	Dannhauser
Epicoccum ovisporum	CN135C5=CMW59105	OP596106	-	-	OP720158	-	Leaf	Dannhauser
Epicoccum sorghinum	CN119H5	OP596075	-	-	-	-	Seed	Petit
	CN135I3	OP596111	-	-	-	-	Leaf	Carolina
	CN135I5	OP596113	-	-	OP720160	-	Leaf	Carolina
	CN135I9	OP596115	-	-	OP720162	-	Leaf	Carolina
	CN136A8	OP596119	-	-	-	-	Leaf	Carolina
	CN136B9	OP596127	-	-	OP720168	-	Leaf	Carolina
	CN136F3=CMW59111	OP596140	-	-	OP720169	-	Leaf	eMalahleni
	CN145D5=CMW59113	OP596157	-	-	OP720171	-	Stem	Delmas
Exserohilum rostratum	CN135B9=CMW59115	OP596101	-	OP729318	-	-	Leaf	Dannhauser
Fusariella hughesii	CN136F9=CMW59116	OP596144	-	-	-	-	Leaf	Delmas
Fusarium acuminatum	CN145D9	-	OP729366	-	-	-	Leaf	Fouriesburg
Fusarium boothii	CN126D7	-	OP729351	-	-	-	Stem	Petit
	CN137C2	-	OP729361	-	-	-	Leaf	Dannhauser
	CN137C3	-	OP729362	-	-	-	Leaf	Dannhauser
	CN137C5	-	OP729363	-	-	-	Leaf	Dannhauser
	CN145D7	-	OP729364	-	-	-	Seed	Belfast
	CN145D8	-	OP729365	-	-	-	Seed	Belfast

	CN119F6	-	OP729350	-	-	-	Stem	Petit
Fusarium clavus	CN126E6	-	OP729354	-	-	-	Root	Petit
	CN126E8	-	OP729355	-	-	-	Stem	Petit
	CN126E9	-	OP729356	-	-	-	Stem	Petit
	CN126F2	-	OP729358	-	-	-	Stem	Petit
Fusarium fabacearum	CN126E2	-	OP729353	-	-	-	Stem	Petit
Fusarium glycines	CN126F1	-	OP729357	-	-	-	Roots	Petit
Fusarium verticillioides	CN137B9	-	OP729359	-	-	-	Leaf	Dannhauser
	CN137C1	-	OP729360	-	-	-	Leaf	Dannhauser
Macrophomina phaseolina	CN119F7	OP596161	OP729367	-	-	-	Stem	Petit
	CN119F8	OP596162	OP729368	-	-	-	Roots	Petit
	CN119H6	OP596163	OP729369	-	-	-	Roots	Petit
	CN119H7	OP596164	OP729370	-	-	-	Roots	Petit
	CN119H8=CMW59126	OP596165	OP729371	-	-	-	Roots	Petit
	CN119H9=CMW59127	OP596166	OP729372	-	-	-	Root	Petit
Neoascochyta argentina	CN135C6=CMW59128	OP596107	-	-	-	-	Leaf	Dannhauser
Neocosmospora solani	CN126E1	-	OP729352	-	-	-	Roots	Petit
Nigrospora sphaerica	CN126E7	OP596083	-	-	-	-	Stem	Petit
	CN136A7	OP596118	-	-	-	-	Leaf	Carolina
	CN136C1	OP596128	-	-	-	-	Leaf	Carolina
	CN136D1	OP596130	-	-	-	-	Leaf	Belfast
	CN136D2	OP596131	-	-	-	-	Leaf	Belfast
	CN136D3	OP596132	-	-	-	-	Leaf	Belfast
	CN136D9	OP596133	-	-	-	-	Leaf	eMalahleni
	CN136E1	OP596134	-	-	-	-	Leaf	eMalahleni

	CN136E2	OP596135	-	-	-	-	Leaf	eMalahleni
	CN136E3	OP596136	-	-	-	-	Leaf	eMalahleni
	CN136G2	OP596146	-	-	-	-	Leaf	Delmas
	CN145C9=CMW59131	OP596153	-	-	-	-	Pod	Reitz
	CN145E1=CMW59132	OP596159	-	-	-	-	Stem	Delmas
Penicillium glabrum	CN119G6=CMW59162	-	-	-	-	OP660629	Roots	Petit
Periconia byssoides	CN145D2=CMW59164	OP596155	-	-	-	-	Seed	Belfast
Phaeosphaeria species	CN136G3	OP596147	-	-	-	-	Leaf	Delmas
Phialophora cyclaminis	CN136C7=CMW59165	OP596129	-	-	-	-	Leaf	Belfast
Sclerotinia sclerotiorum	CN144F4	OP596167	-	-	-	-	Seed	Belfast
	CN144F5	OP596168	-	-	-	-	Seed	Belfast
	CN144F6	OP596169	-	-	-	-	Seed	Belfast
	CN144F7	OP596170	-	-	-	-	Seed	Belfast
	CN144F8	OP596171	-	-	-	-	Pod	Reitz
	CN144F9	OP596172	-	-	-	-	Seed	Belfast
	CN144G1	OP596173	-	-	-	-	Stem	Ermelo
	CN144G2	OP596174	-	-	-	-	Stem	Schweizer-Reneke
	CN144G3	OP596175	-	-	-	-	Stem	Ficksburg
	CN144G4	OP596176	-	-	-	-	Stem	Ficksburg
	CN144G5	OP596177	-	-	-	-	Stem	Fouriesburg
	CN144G6	OP596178	-	-	-	-	Pod	Fouriesburg
	CN144G7	OP596179	-	-	-	-	Leaf	Ermelo
	CN144G8	OP596180	-	-	-	-	Stem	Reitz

	CN144G9	OP596181	-	-	-	-	Seed	Reitz
	CN144H1	OP210214	-	-	-	-	Leaf	Ermelo
	CN144H2	OP210215	-	-	-	-	Leaf	Delmas
	CN144H3	OP210216	-	-	-	-	Stem	Delmas
	CN144H4	OP210217	-	-	-	-	Stem	Delmas
	CN144H5	OP210218	-	-	-	-	Seed	Belfast
	CN144H6	OP210219	-	-	-	-	Stem	Delmas
	CN144H7	OP210220	-	-	-	-	Stem	Delmas
	CN144H8	OP210221	-	-	-	-	Stem	Delmas
	CN144H9	OP210222	-	-	-	-	Pod	Fouriesburg
	CN144I1	OP210223	-	-	-	-	Leaf	Ermelo
	CN144I2	OP210224	-	-	-	-	Seed	Belfast
	CN144I3	OP210225	-	-	-	-	Stem	Ermelo
	CN144I4	OP210226	-	-	-	-	Stem	Ermelo
	CN144I5	OP210227	-	-	-	-	Stem	Ermelo
	CN144I6	OP210228	-	-	-	-	Stem	Ermelo
	CN144I7	OP210229	-	-	-	-	Stem	Ermelo
	CN144I8	OP210230	-	-	-	-	Leaf	Ermelo
	CN144I9	OP210231	-	-	-	-	Stem	Delmas
	CN145A1	OP210232	-	-	-	-	Seed	Belfast
	CN145A2	OP210233	-	-	-	-	Stem	Schweizer-Reneke
	CN145A3	OP210234	-	-	-	-	Leaf	Ermelo
	CN145A4	OP210235	-	-	-	-	Seed	Belfast

	CN145A5	OP210236	-	-	-	-	Stem	Schweizer-Reneke
	CN145A6	OP210237	-	-	-	-	Stem	Schweizer-Reneke
	CN145A7	OP210238	-	-	-	-	Stem	Ermelo
	CN145A8	OP210239	-	-	-	-	Stem	Ermelo
	CN145A9	OP210240	-	-	-	-	Stem	Ermelo
	CN145B1	OP210241	-	-	-	-	Leaf	Ermelo
	CN145B2=CMW59167	OP210242	-	-	-	-	Seed	Belfast
	CN145B3=CMW59168	OP210243	-	-	-	-	Seed	Belfast
	CN145E2	OP596182	-	-	-	-	Stem	Ermelo
Setophaeosphaeria badalingensis	CN136G1=CMW59169	OP596145	-	-	-	-	Leaf	Delmas

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SUMMARY

Soybeans (*Glycine max*) are a significant source of oil and protein and a growing component of Africa's agricultural economy. Knowledge of fungal diversity, including those that can cause soybean diseases, is to some degree documented worldwide but is lacking in South Africa. Fungal and mycotoxin surveys are essential for ensuring safe food and feed in South Africa, but very few have studied this for South African soybeans.

The first chapter of the dissertation provides a literature overview of the fungal and oomycete diseases that affect soybeans. The chapter emphasizes the significance of soybeans in the global economy and highlights the crucial role of soybeans as a source of essential nutrients for both humans and animals. Moreover, the chapter elucidates the different mycotoxins that are commonly associated with soybean seeds and soy meal. In addition, the chapter provides a detailed summary of each disease, outlining the characteristic in-field symptoms, causal agents, and techniques used for identifying the species based on both morphological and molecular data.

The second chapter aimed to identify fungal diversity and mycotoxins associated with pre-stored soybean seeds in South Africa. This study resulted in 411 fungal strains recovered that were identified as 24 genera and 81 species. The dominant genera isolated were *Aspergillus*, *Cladosporium*, *Penicillium*, *Fusarium*, *Alternaria Didymella*, and *Talaromyces*. No mycotoxins were detected in any of the samples analysed, even though several mycotoxigenic species like *A. flavus*, *A. parasiticus*, *A. westerdijkiae*, *F. boothii*, and *F. verticillioides* were isolated. This survey is the first to determine the mycotoxin levels and fungal species present in soybeans and it thus provides important baseline knowledge for an increasingly important oilseed crop.

The third chapter aimed to identify the fungal diversity associated with symptomatic soybean leaves, roots, stems, and seeds in South Africa. This study resulted in 200 fungal strains representing 22 genera and 38 species. Important genera and the number of strains isolated during this study are *Sclerotinia*, *Epicoccum*, *Alternaria*, *Fusarium*, *Diaporthe*, and *Macrophomina*, respectively. Certain species identified from these genera have been previously reported as the causal agents of soybean diseases alternaria leaf spot (caused by *Alternaria alternata*), charcoal rot (caused by *Macrophomina phaseolina*), sclerotinia stem rot (caused by *Sclerotinia sclerotiorum*), and phomopsis seed decay (mainly caused by *Diaporthe longicolla*). There are limited studies on fungal diversity associated with soybeans in South Africa. Koch's postulates are required to determine if these species can cause soybean diseases in South Africa.

The results of this study provide information on fungal diversity and show which fungi may potentially pose a threat to South African soybeans This study provides important knowledge on fungal diversity associated with South African soybeans which are currently very limited. Previous international fungal diversity studies have used the ITS barcode or morphology to identify fungal species. The aim of this study was to identify fungal species using morphology and molecular sequencing. DNA sequences were generated based on markers that accurately identify and distinguish between closely related species. This study has generated important baseline knowledge on fungal diversity in South Africa. The DNA sequences generated during this study were deposited to Genbank with accession numbers available to aid future fungal identifications. Strains have been preserved in culture collection and are readily available for future studies such as novel secondary metabolite analysis, in vitro mycotoxin production, or Koch’s postulates to determine causal agents of soybean diseases in South Africa. This project provides baseline knowledge and contributes to the aim of safe food and feed in South Africa.