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CHARACTERIZATION AND DIVERSITY OF POTENTIAL MYCOTOXIN PRODUCING ENDOPHYTIC FUNGI ISOLATED FROM MAIZE HUSK LEAVES

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ABSTRACT

Maize (*Zea mays* L.) is an important staple food due to its good nutritional profile. Unfortunately, maize can be vulnerable to contamination by fungi, which are capable of producing mycotoxins that have an impact on human and animal health, crop productivity and lead to economic losses. In addition, the ubiquitous presence of mycotoxin-producing fungi in maize poses significant challenges to food safety and economic stability. Mycotoxins are toxic secondary metabolites produced by certain fungi, which, when ingested by humans or animals through contaminated maize products can lead to a variety of health issues. There is a shift of non-pathogenic microbes to pathogenic state due to unfavourable conditions arising. The aim of this study was to characterize and identify fungi that have the potential to produce mycotoxins isolated from maize husks. The study identified *Fusaria* sp., *Aspergillus* and *Penicillium* sp. as target species since they are often regarded as primary mycotoxin producing fungi, which often contaminates the maize plants. Ten (n=10) healthy (disease-free) and mature maize samples were obtained, and a total of 200 maize husk segments were utilized to isolate eighty-four (n=84) endophytic fungi. Preliminary identification was conducted using macroscopic and microscopic characteristics. Molecular processes using the Internal transcribed spacer region (ITS1 and ITS4) were used for PCR amplification as a confirmatory test. A total of 13 isolates were identified by macroscopic and microscopic characterization based on morphological features and their identification was confirmed using molecular techniques. *Fusaria* sp. were found to be predominant (46%), followed by *Aspergillus* sp. (39%) and *Penicillium* sp. (15%). The findings suggest a community of mycotoxin producing fungi inhibited within the maize husks. Under unfavourable conditions, these fungi might be the source of contamination and produce mycotoxins. In the face of changing environmental conditions and evolving fungal threats, our research serves as a valuable resource for understanding and addressing the challenges posed by mycotoxin contamination in maize. Effective strategies and interventions are essential to mitigate the impact of these fungi on food security, human health, and the global maize industry.

Key words: Endophytes; fungi; food safety; maize; maize husks; mycotoxin; mycotoxin-producing fungi

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INTRODUCTION

Maize (*Zea mays* L.) is regarded as one of the leading cereals used as staple food worldwide [1]. Maize, rice and wheat currently dominate human diets [2] and provide at least 30% of the food calories of more than 4.5 billion people in 94 developing countries. Maize alone contributes over 20% of the total calories in human diets in 21 low-income countries, and over 30% in 12 countries that are home to more than 310 million people. Due to diverse usage, maize is the world's most multi-purpose crop. Maize is important as a staple food for humans, animal feed (especially poultry) as well as a source of bio-fuel and other industrial products. The population growth, changing diets and a rapidly growing poultry sector, significantly contribute to a sharp increase in maize demand. Cultivation of maize worldwide is restricted by diseases, which cause grain loss of approximately 11% of the total production [3].

Pathogenic fungi are responsible for the deterioration and loss of maize grains. Fungi contribute to approximately 50-80% of damage on farmers' maize during the storage period if conditions are favourable for their development. During storage, numerous fungi might be associated with the maize seeds, either, causing their deterioration or simply remain viable to infect germinating seedlings. The fungi genera typically found in stored grains are *Aspergillus, Penicillium, Fusaria* and some xerophytic species, in which some are capable of producing mycotoxins, however in the present study the main focus was on *Aspergillus, Penicillium* and *Fusarium* species [4]. Fungi produce mycotoxin that might cause feed spoilage, reduced cattle productivity and impaired human and animal health. Mycotoxins are secondary fungal metabolites that are toxic to humans, animals and plants. These metabolites primarily affect the seed quality, germination viability, seedling vigour, root growth and coleoptile. In addition, since the fungi are responsible for the production of these mycotoxins, endophytes might react to unfavourable conditions by producing toxic metabolites, hence turning into parasites.

The fungus will infect and colonize living plant tissues, which leads to accumulation of mycotoxins in the plant tissues and ultimately the development of plant diseases. The presence of mycotoxins, even in the absence of disease symptoms, may still have subtle biological effects on the physiology of plants. Mycotoxin contamination in feed can cause serious health and productivity problems to livestock, which can lead to significant losses for farmers [5]. *Aspergillus* and *Penicillium* species might be responsible for numerous complaints in various plants including maize, and most importantly as a causative agent of mycotoxin contamination. One of the diseases caused by contaminated maize plants is ear rot. Ear rot is the most economically important maize disease in Europe [6]. It can be caused by a diverse group of fungi from *Aspergillus*, *Penicillium* and other

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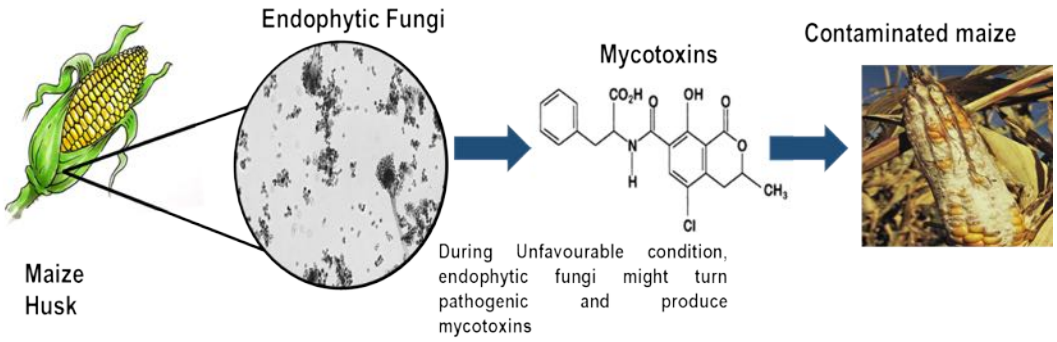
genera. However, the most important and harmful pathogens belong to *Fusaria* species [7]. The most important aspect of food and feed spoilage caused by these organisms is the formation of mycotoxins, which may have harmful effects on human and animal health. Several mycotoxins produced by *Aspergillus* and *Penicillium* have been identified in maize from previous studies [8, 9, 10].

Endophytes are microorganisms (mostly fungi and bacteria) that inhabit plant hosts for all or part of their life cycle. They colonize the internal plant tissues beneath the epidermal cell layers without causing any apparent harm or symptomatic infection to their host. They live within the intercellular spaces of the tissues and might penetrate the living cells [11]. Fungal endophytes provide protection to the host plant against herbivorous insects, plant pathogens and adverse environmental conditions such as heat, stress and prolonged periods of drought [12, 13]. Under stressful conditions, endophytes cause disease symptoms of the host plant [14]. This shift in balance might result in endophytes converting to parasites and eventually producing mycotoxins in maize. The aim of this study was to isolate and identify potential mycotoxin producing fungi, mainly *Fusaria*, *Aspergillus* and *Penicillium* from maize husks.



Graphical Abstract: Overview of potential mycotoxin producing endophytic fungi isolated from maize husk leaves

METHODOLOGY

Sample Collection

The maize plants used in this study were collected at Vrylof and Vriende farm in Vryburg, South Africa with geographical co-ordinates of 26.9566° South and 24.7284° East**.** Ten (n=10) healthy (disease-free) and mature maize samples were randomly collected from Vryburg in the North-West Province. The samples were aseptically collected and transported carefully to the North-West University (NWU), Mafikeng, Microbiology laboratory for further processing. The collected maize samples were processed within 48 hr and later subjected to surface sterilization.

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Isolation of Fungal Endophytes

Endophytic fungi were isolated according to the protocols described by Petrini [15], which were slightly modified based on preliminary tests. Prior to surface sterilization, the leaf samples were cut into 5 mm pieces. They were washed twice in distilled water (dH_2O), surface sterilized by immersion for 1 minute in 70% (v/v) ethanol, 5 minutes in sodium hypochlorite 2.5 % (v/v) (NaCIO) and 30 seconds in 70% (v/v) ethanol and then washed three times in sterilized distilled water for 1 minute. Finally, the leaf samples were aseptically transferred onto Potato dextrose agar plates (PDA; MERCK Biolab, Gauteng, South Africa), which were supplemented with 0.05 g of chloramphenicol (to suppress bacterial growth). Aliquots from the third wash were plated onto PDA to check that surface sterilization had been effective and all PDA plates were then incubated (Labcon 5018U, Labcon, South Africa) at 25ºC for 7-14 days.

Colonization Frequency (CF) was calculated as described by Sishuba *et al*. [16].

$$CF = \frac{Number\ of\ segments\ colonized\ by\ fungi}{Total\ number\ of\ segments\ observed} \times 100$$

Growth rate assay

The growth rate of fungal isolates was estimated by culturing them on Potato Dextrose Agar (PDA; MERCK Biolab, Gauteng, South Africa). The diameter of each isolate was measured (using a ruler, cm) after two days for 9 days (3, 5, 7, 9 days) and the results were recorded.

Morphological Characterization of Fungi

Macroscopic and microscopic characterization were based on morphological features of the fungi. The fungal isolates were mounted on sterile slides with a drop of distilled water and examined in 100X light microscopy (Reichert-Jung, 150, United States of America).

Deoxyribonucleic acid (DNA) Extraction

Deoxyribonucleic acid (DNA) extraction was performed for all fungal isolates using Zymo Research Quick-DNA TM Fungal/Bacterial Miniprep Kit, catalogue number D6005 purchased from Inqaba Biotec (Pretoria, South Africa). The extraction procedure was done according to the manufacturer's instructions.

Polymerase Chain Reaction (PCR)

Total genomic DNA was isolated using the miniprep kit method. For identification, ITS1 (5'TCCGTAGGTGAACCTGCGG) and ITS4 (5'TCCTCCGCTTATTGATATGC) primers were utilized to amplify the nuclear ribosomal internal transcribed spacer region (ITS) by using PCR. Final volume of PCR mixture (20 µl) consisted of 1 µl of fungal genomic DNA, 0.5 µl of each Primer, 10 µl of master mix and 8 µl of nuclease free water. The PCR program

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comprised initial denaturation for 3 min at 94 °C, 30 cycles of 0.30 sec at 94 °C, 0.45 sec at 52°C and 0.45 sec at 72 °C, and a final extension cycle of 7 minutes at 72 °C [17].

Gel Electrophoresis

Agarose gel electrophoresis is the separation of DNA or proteins in a matrix of stained agarose gel. The PCR products were separated on a 1% agarose gel in the electrophoresis machine containing 1X TAE buffer. The agarose gel was then stained with Ethidium bromide (1 µl) and visualized. The bands were exposed to ultraviolet light by using a UV gel documentation system/Chemi Doc with Universal hood III (Bio Rad Laboratories, South Africa) for visualization. A 1 kb DNA ladder was used as the size marker.

Sequencing

Amplified PCR amplicons were purified and sequenced by Inqaba Biotec (Pretoria, South Africa). Pair-wise evaluations, multiple alignment and consequence sequences were obtained using BioEdit Sequence Alignment Editor software version 7.2.5. A BLAST search on NCBI Web Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to confirm the fungal identities.

RESULTS AND DISCUSSION

Colonisation rate

From 200 maize leaf segments, eighty-four (n=84) endophytic fungi were successfully isolated. The colonization rate was therefore 42%. From all the isolates, only 13 fungal species were used for further investigation. Moreover, the colonization rate for our targeted fungi was 16%. All fungi isolates were cultivated on growth media and maintained as pure cultures.

Morphological identification

In this study, the morphological aspects such as colony colour, texture, specialized structures and nature of the hyphae were recorded. Morphological identification was based on macroscopic and microscopic features as documented in Table 1.

Fusaria isolates formed pink-white colonies, except one isolate (Sample ID: P7.1) that formed pink-yellow colonies when cultured on PDA. They also had a cotton texture, and the hyphae were septate when observed under the microscope. *Aspergillus* isolates formed green-blue colonies, with others having green-yellow and green-white colonies on PDA. *Aspergillus* isolates formed a powdery and cotton texture, and the hyphae were non-septate when observed under the microscope. Figure 1, illustrates the colour dimensions displayed by colonies, both front and back of *Fusarium*, *Aspergillus* and *Penicillium* species. The *Penicillium* isolates formed brown-green colonies with the exception of one isolate (Sample ID:

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P10) having green colonies when cultured on PDA. *Penicillium* isolates also had a smooth texture and the hyphae were septate.

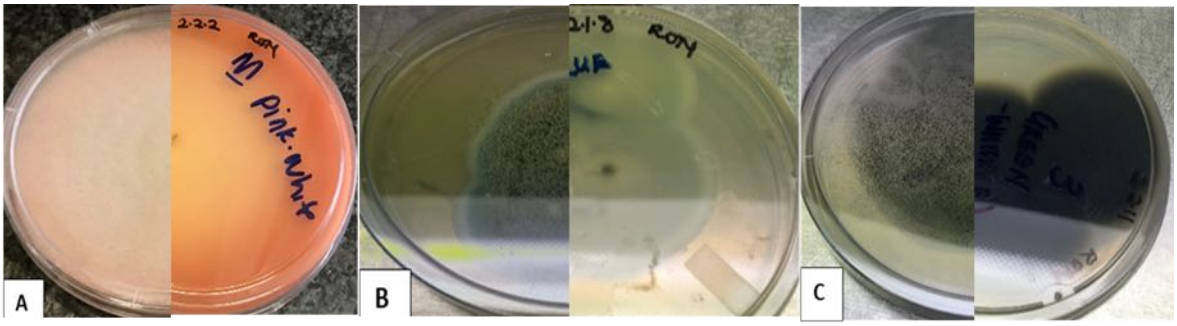

Figure 1: The colony colour on potato dextrose agar (PDA) Front and back (A) *Fusarium*, (B) *Aspergillus* and (C) *Penicillium*

Furthermore, with hyaline macroconidia which are septate, and generally have a foot-shaped or notched base to the basal cell, the identification process becomes more precise.

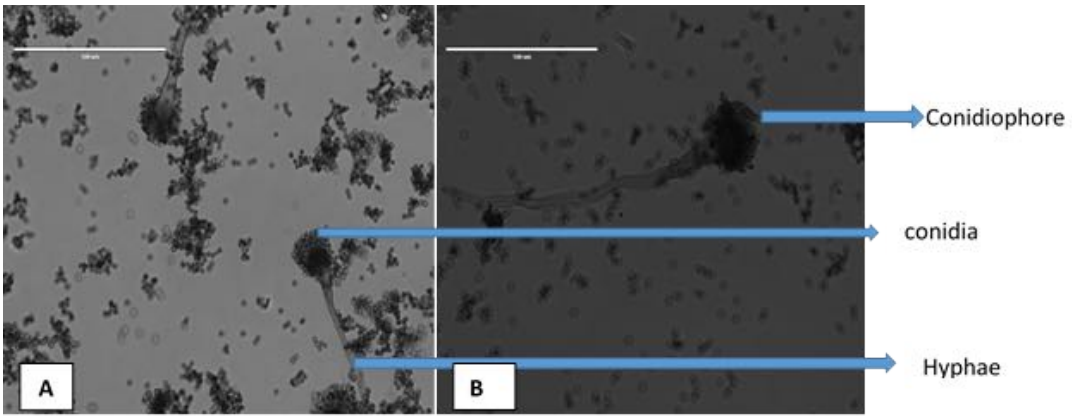

Figure 2: Microscopic structures of *Aspergillus* (A) Conidia and hyphae (B) Conidiophore

Based on morphological identification such as colony colour, shape, texture nature of hyphae and specialized structures (Figure 2), preliminary identification indicates that *Fusaria, Aspergillus* and *Penicillium* were identified.

Diversity of endophytic fungi

The fungal diversity in maize leaves were dominated by isolates belonging to the genus *Fusaria* (6 isolates, 46%), followed by *Aspergillus* (5 isolates, 39%) and lastly *Penicillium* (2 isolates, 15%), (Figure 3). Figure 3 represents the diversity of fungal endophytes in maize husk leaves.

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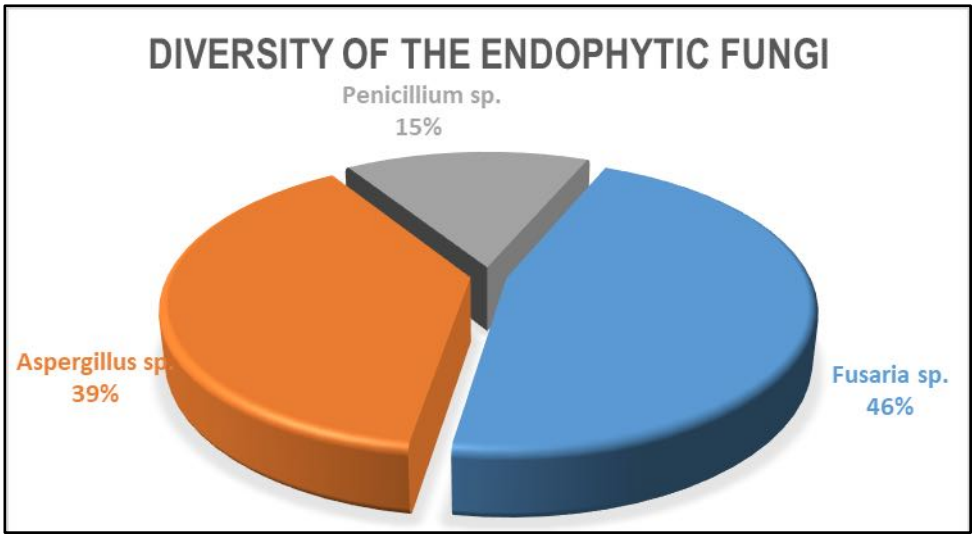

Figure 3: **Diversity of the isolated endophytic fungi**

Growth rate of endophytic fungi

The growth rate of 13 fungal isolates were measured for 9 days and recorded in Table 2. *Fusaria* species grew faster than *Aspergillus* and *Penicillium* (Figure 4). Two isolates (Sample ID: P1 and P8) of *Aspergillus* grew slower than other *Aspergillus* isolates. One isolate of *Penicillium* had a very slow growth rate (Sample ID: P10), while the isolate had a fast growth rate (Sample ID: P3). *Fusaria* species had a very good growth rate when compared to the other two species (*Penicillium* and *Aspergillus*). Table 2, represents the growth rate of fungal isolates for nine days which was measured in centimetres using a ruler; and Figure 4 shows the growth rate of fungal species in a bar graph. Within our target species *Fusaria* species had the highest growth rate.

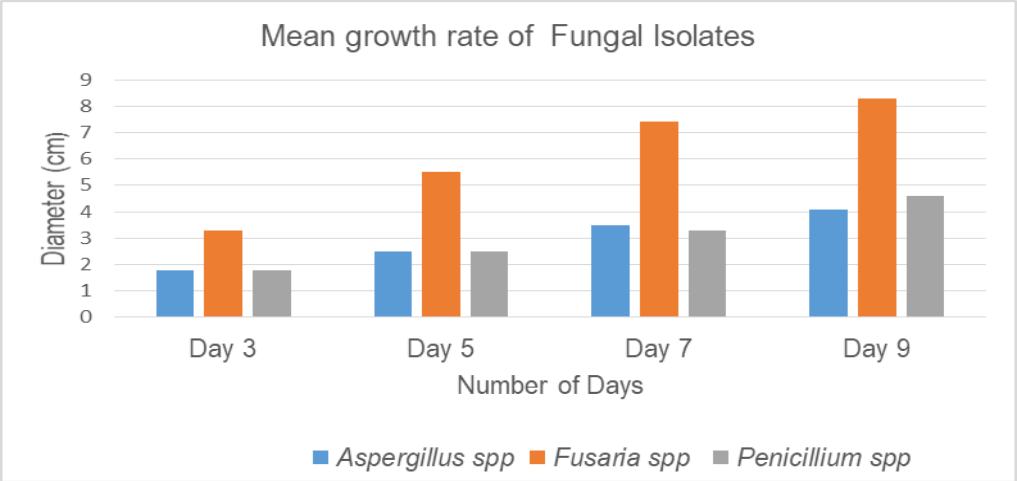

Figure 4: Growth rate of fungal species

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Molecular Identification

A total of 13 fungal isolates were subjected to DNA extraction and the genomic DNA was used for the amplification of the ITS region. To verify if the amplification of the DNA was successful, gel electrophoresis was carried out which separated the fragments. Agarose gel electrophoresis is the separation of DNA or proteins in a matrix of stained agarose gel. After running the gel electrophoresis, the isolates showed good quality DNA, which was indicated by a clear band in Figure 5 (A). PCR amplicons were amplified and indicated a band size of 625 bp on agarose gel electrophoresis (Figure 5 (B)).

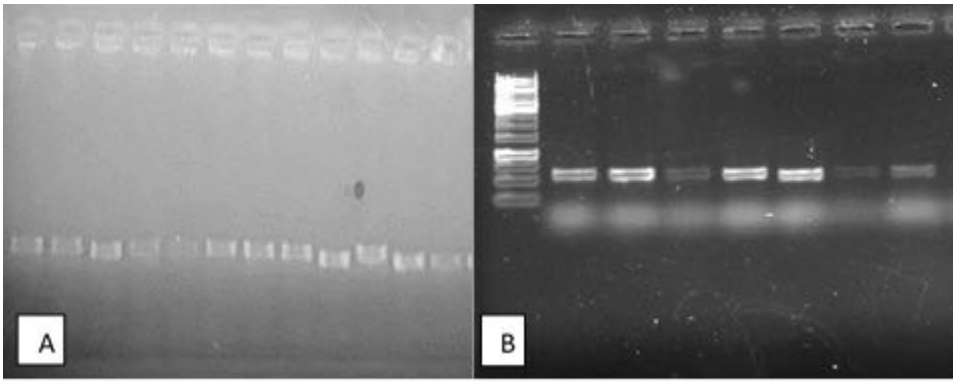

Figure 5: (A) Gel picture of DNA bands after extraction (B) Agarose gel electrophoresis of PCR amplicons

Table 3 represents a list of fungal isolates utilized in the current study, their GenBank accession numbers and identity percentages.

Microorganisms residing within the host plant may have various interactions, alternating from mutualistic symbiosis to latent pathogenicity [18]. These associations are characterized as plastic because, they are dependent on the genetic natures of both partners. Furthermore, the balance depends on microbes\plant development stage, availability of nutrients and environmental factors [19]. With the endophyte-host interaction, a balance must be maintained, if disturbed by a decrease in plant defence or an increase in fungal virulence, disease develops in the plant. Therefore, the endophyte will act as an opportunistic pathogen [20]. Some metabolites produced by endophytes regulate metabolism of the host, enforcing balance. To establish this, the endophyte must first overcome the epiphytes then compete with the pathogens to finally colonize the host [21]. Moreover, endophytic fungi understudied microbial community are capable of producing beneficial as well as detrimental bioactive secondary metabolites. Endophytic fungi have been reported to reside in the internal plant tissues without causing any harm. A number of studies have established that almost all higher plants co-exist with endophytes [22, 23]. In previous studies, various endophytic

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fungi had been isolated from maize leaves. Six genera of endophytic fungi were isolated [24]. Futhermore, colonies formed by Fusaria species had a white colour in the center and orange in the edge and the hyphae were hyaline. Aspergillus species produced a green colony colour at the core of growth and white in the margin, and their hyphae were non-septate [24]. Three of those genera, *Fusaria, Aspergillus* and *Penicillium* species were also found in the current study. In a previous study conducted by Renuka and Ramanujam [25], an overall colonization rate of fungi was 54.03% isolation from maize leaves. The isolation rate in the previous studies is higher than our isolation rate. Most *Fusaria* species are soil fungi and have a worldwide distribution in nature. Some are plant pathogens, causing root and stem rot, vascular wilt or fruit rot. Several species have emerged as important opportunistic pathogens in humans causing hyalohyphomycosis (especially in burn victims and bone marrow transplant patients), mycotic keratitis and onychomycosis [26]. In maize grains, a wider array of *Fusaria* species is found compared to other cereal grains. The colour colony may be white, cream, tan, salmon, cinnamon, yellow, red, violet, pink or purple and on the reverse, it may be colourless, tan, red, dark purple, or brown [27]. Penicillium is a very large and ubiquitous genus, which currently contains 354 accepted species [28]. Many species are common contaminants on various substrates, and are well-known as potential mycotoxin producers. Colonies are usually fast growing, in shades of green, sometimes white, mostly consisting of a dense felt of conidiophores. *Aspergillus* species are widely distributed and able to produce the most toxic mycotoxins called aflatoxins. Aflatoxins affect mainly the liver in both humans and animals [28]. Duan *et al.* [29] detected mycotoxins such as zearalenone and deoxynivalenol produced by pathogenic *Fusarium* spp., which were causing maize ear rot in China. The study also concluded that, *Fusarium* spp was the predominant pathogen responsible for producing mycotoxins. Negative plant-microbial interactions cause latent pathogenicity and might produce mycotoxins. There is a lack of research conducted in this field of study, hence this study forms a basis for the plant-microbial interaction affecting pathogenicity in maize plants.

CONCLUSION, AND RECOMMENDATIONS FOR DEVELOPMENT

This research was aimed to identify and isolate potential mycotoxins producing fungi from maize husk leaves. Based on microscopic, macroscopic and molecular identification, it can be concluded that, *Fusaria, Aspergillus and Penicillium species*, were successfully isolated and the genus *Fusaria* sp. was dominating followed by *Aspergillus*. Currently these species do not induce any diseases or associated symptoms. Moreover, these three genera have potential to produce mycotoxins, and in unfavourable conditions they might shift from endophytic to pathogenic, thus producing mycotoxins and causing diseases.

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DECLARATION OF COMPETING INTEREST

All authors declare that there is no conflict of interest.

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Table 1: Macroscopic and Microscopic characteristics of fungi

Sample ID	Probable ID	Macroscopic characteristics		Microscopic characteristics	Texture
		Colony colour		Nature of hyphae	
		Top	Bottom		
P1	*Aspergillus*	Light green-yellow	White	Non septate	Powdery
P2	*Fusarium*	Pink-white	Pink-reddish	Septate	Cotton
P3	*Penicillium*	Green-brown	White	Septate	Powdery
P4	*Aspergillus*	Blue-green	White	Non septate	Cotton
P5	*Aspergillus*	Brown-blue	White	Non septate	Cotton
P6	*Fusarium*	Pink-white	Orange	Septate	Cotton
P7.1	*Fusarium*	Pink-yellow	Yellow	Septate	Cotton
P7.2	*Fusarium*	White-pink	Pink-white	Septate	Cotton
P8	*Aspergillus*	Green	White	Non septate	Powdery
P9.1	*Fusarium*	White-pink	Reddish	Septate	Cotton
P9.2	*Fusarium*	White	Yellow	Septate	Cotton
P10	*Penicillium*	Green	White-yellow	Septate	Powdery
P11	*Aspergillus*	Green-white	Green	Non septate	Powdery

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Table 2: Growth rate of fungi

Sample ID	Probable ID	Diameter (cm)				
		Day 3	Day 5	Day 7	Day 9	Growth rate
P1	*Aspergillus*	1.5	1.5	2.0	2.1	Slow
P2	*Fusarium*	3.2	6.0	8.0	8.1	Fast
P3	*Penicillium*	1.9	2.9	3.8	6.0	Fast
P4	*Aspergillus*	2.9	3.6	4.8	5.0	Medium
P5	*Aspergillus*	1.7	2.3	3.5	4.0	Medium
P6	*Fusarium*	3.2	5.1	6.5	7.3	Fast
P7.1	*Fusarium*	3.5	6.1	7.9	8.8	Very fast
P7.2	*Fusarium*	2.9	5.1	6.8	8.3	Fast
P8	*Aspergillus*	1.4	2.3	2.9	3.4	Slow
P9.1	*Fusarium*	3.2	5.5	7.9	8.8	Very fast
P9.2	*Fusarium*	3.6	5.4	7.4	8.2	Fast
P10	*Penicillium*	1.6	2.1	2.8	3.1	Slow
P11	*Aspergillus*	1.9	3.0	4.1	5.9	Medium

Table 3: Sequence identities of potential mycotoxin producing microorganisms based on NCBI BLAST search

Sample ID	Closest related species	Identity %	Accession No.
P1	*Aspergillus niger*	99.67%	**MK256745.1**
P2	*Fusarium sp.*	100.00%	**DQ446211.2**
P3	*Penicillium chrysogenum*	98.98%	**MG250454.1**
P4	*Aspergillus niger*	97.73%	**MF078659.1**
P5	*Aspergillus sp.*	98.25%	**MG437322.1**
P6	*Fusarium oxysporum f. sp. lycopersici*	97.61%	**KY318499.1**
P7.1	*Fusarium equiseti*	99.64%	**MK168567.1**
P7.2	*Fusarium sp.*	99.53%	**KY318498.1**
P8	*Aspergillus terreus*	100.00%	**KY587312.1**
P9.1	*Fusarium sp.*	99.27%	**MK168565.1**
P9.2	*Fusarium oxysporum*	100.00%	**KY318483.1**
P10	*Penicillium rubens strain*	98.31%;	**MN413181.1**
P11	*Aspergillus fumigatus*	96.99%	**HE864321.1**

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