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PREVALENCE OF MYCOTOXIN-PRODUCING FUNGI IN *BRASSICA OLERACEA* SOLD IN PORT HARCOURT METROPOLIS, NIGERIA

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ABSTRACT: *This study investigated the prevalence of mycotoxin-producing fungi from Brassica oleracea sold in Port Harcourt metropolis, Nigeria using molecular techniques. Brassica oleracea showing signs of fungal infestations were collected from markets across Port Harcourt Metropolis, Nigeria. The samples were first observed for signs and symptoms. Fungi were isolated from the vegetable following standard methods. Capacity to produce mycotoxin by the fungal isolates was investigated by polymerase chain reaction (PCR)-based screening of the isolates. The presence of five mycotoxigenic genes: nor1, tri6, otanps, fum13 and Zea encoding for the following mycotoxins: aflatoxin, trichothecene, Ochratoxin A, fumonisin, and zearalenone, respectively were evaluated. Fourteen fungi were isolated from the vegetable, out of which four isolates were positive for mycotoxigenic genes. This represents a 28.6% prevalence rate. The mycotoxigenic genes found in the isolates included Ochratoxin A, Trichothecene and Aflatoxin. Trichothecene was produced by Pithomyces chartarum RCBBR_Sf5 and Fusarium longifundum RCBBR_Mf4 while aflatoxin and ochratoxin A were produced by Trametes duplexa RCBBR_Mf1 and Trametes versicolor RCBBR_Sf2a, respectively. The ITS gene sequences of the four fungi have been deposited in GenBank under the accession numbers OR816040; OR816043-OR816045. This study has demonstrated that mycotoxin-producing fungi are prevalent in Brassica oleracea sold in Port Harcourt Metropolis. This observation raises both public health and food security concerns.*

KEYWORDS: Mycotoxin; *Brassica oleracea; Prevalence; mycotoxigenic genes; polymerase chain reaction; ITS* gene.

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INTRODUCTION

Cabbage (*Brassica oleracea var. Capitata*) belongs to the exotic vegetable group consumed widely in Nigeria and commonly grown in some states such as Plateau because of the near temperate climate. Cabbage is commercially cultivated for its large, leafy head that is rich in vitamin C. It is generally believed to have originated from the wild, leafy, non-heading types of vegetables which are found growing in Europe (Grubben & Denton, 2004). *Brassica oleracea var. capitata* is regarded as the most important member of the Cruciferaeor mustard family and has remained one of the world's leading vegetable crops.

Because of its economic importance, its production and cultivation have been widely studied (Mondédji *et al*., 2021). Cabbage is one of the most popular vegetables in the world because of its adaptability to a wide range of climatic conditions and soil types, ease of production and storage, and its food value. In Nigeria, production of this important vegetable is mostly carried out in the north, especially Plateau State (Ogedegbe & Law-Ogbomo, 2013).

Šamec et al. (2017) noted that cabbage is consumed fresh in the form of salad, stir-fried, boiled or fermented. It is an essential component of most diets because it is affordable and available in local markets. The nutritional value of cabbage is high, being rich in fibre, vitamins (C, K, A and folate), and minerals (Ca, P and K). Many studies have reported the presence of beneficial secondary metabolites in cabbage. Some of the frequently reported secondary metabolites include glucosinolates containing Sulphur and s-methyl cysteine sulfoxide, alkaloids, anthocyanins, coumarins, carotenoids, flavonoids, tannins, saponins, phenolic compounds, tannins, terpenes, phytosterols, and chlorophylls (Nawaz et al., 2018).

Mycotoxins infestation of *Brassica oleracea var. Capitata* have been previously reported by Gruber-Dorninger *et al*. (2017); however, reports on the types and quantity of mycotoxins in *Brassica oleracea var. capitata* sold within Rivers is still scanty. Moreover, there is dearth in literature of the prevalence of mycotoxin in the vegetable. This study was therefore designed to investigate the prevalence of mycotoxin-producing fungi in *Brassica oleracea* sold in Port Harcourt Metropolis, Nigeria.

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MATERIALS AND METHODS

Study Area

The study was carried out in Rivers State. The map of the study area is given in Figure 1.

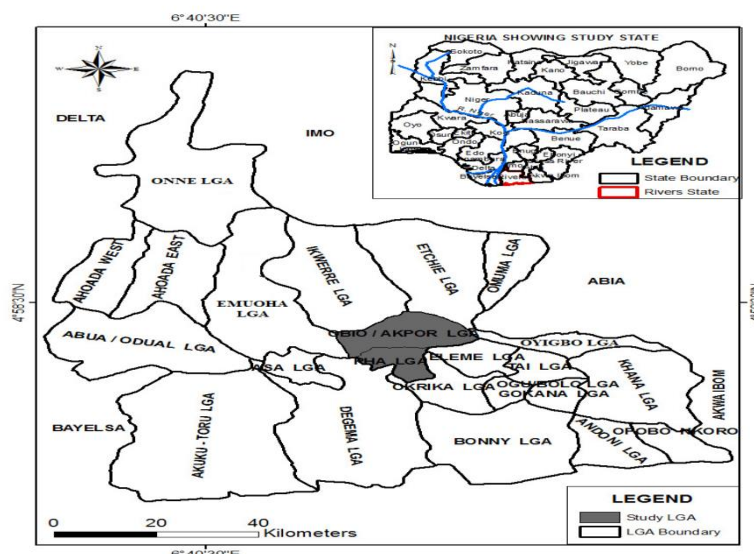

Figure 1: Map of the study area

Sample Collection

Brassica oleracea showing signs of infection were randomly collected from four different markets (Mile 3 and Choba markets for Obio Akpor; Mile 1 and fruit markets for Port Harcourt) in Obio Akpor and Port Harcourt L.G.As in Rivers State, Nigeria. A total of 12 cabbage samples collected from three sellers in each of the four separate markets were used in the study. A sample of the vegetable used in the study is presented in Figure 2. The samples were processed as described by Kłapeć *et al*. (2021). Briefly, the vegetables were separately packed into clean foil bags, and then transported to the laboratory. Subsequently, the mean samples were cut into smaller pieces using a sterile scalpel. Twenty grams (20g) of the fragmented vegetables was homogenized for 5 min in 180 ml of a diluent (normal saline), with the addition of 10% Tween 80. From the homogenates prepared in this way, ten-fold serial dilutions were performed and used for fungal isolation and cultivation.

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Figure 2: Image of *Brassica oleracea* with signs of fungal infestation

Isolation of Pathogenic Fungi

Filamentous fungi were isolated from the processed vegetables following the method of plate dilutions on malt agar previously reported by Kłapeć *et al*. (2021) with the addition of chloramphenicol to inhibit bacterial growth.

For identification, fungal colonies were checked for purity by microscopic and cultural methods and eventually subcultured onto malt agar slants. The fungal isolates were identified based on macroscopic and microscopic methods (Color Atlas) and molecular techniques.

Screening of Fungal Isolates for Mycotoxigenic Genes

DNA Extraction

Fungal DNA was extracted using Zymo Quick DNA Fungal/Bacterial Kit following the manufacturer's instruction. After the DNA extraction, NanoDrop Spectrophotometer 2000c was used to check for concentration and purity of samples.

Primer Designing for Mycotoxigenic Gene Amplification

The primers used in the study were custom-synthesized by Inqaba Biotec, West Africa. Specific genes to detect mycotoxigenic fungal species were identified after a thorough literature survey. Four metabolic pathway genes specific to major mycotoxigenic fungal species, namely *nor1* for aflatoxigenic Aspergilli, Tri6 and *FUM13* for trichothecene- and fumonisin-producing *Fusarium* species, respectively and *otanps* for ochratoxigenic *Penicillium* species were employed. The sequences of all primers (Table 1) were evaluated using PRIMER-BLAST and BLASTN tools (http:// www.ncbi.nhn.nih.gov/tools/primer-blast/) to identify any non-specific targets and to determine the specificity of the PCR assay.

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Polymerase Chain Reaction (PCR)

Amplification reaction was performed in 12.5 µl of reaction volume that contained 2 ng/µL of template DNA, 6.25µL of OneTaq® 2X Master Mix with Standard Buffer (comprising 1.5 mM $MgCl_2$, 20 mM Tris–HCl pH 8.4, 50 mM KCl, 0.2 mM dNTP mix, 0.1 U Fast-Start DNA Polymerase, 1×Reaction Buffer) and 0.25 µL of each of the five primers (0.5mM). A SafeView dye (New England Biolabs, England) was used for all reactions. The following amplification programme was applied: 95 °C, 10 min; 45 cycles of 15 s at 95 °C, 15 s at 53 °C and 30 s at 72 °C. Thereafter, the PCR products were separated on a 1.5 % agarose gel. One hundred base pair (100 bp) DNA ladders (Solis Biodyne) were used as DNA molecular weight markers. Electrophoresis was performed at 100 V for 40 min, and the gel was viewed under UV light after staining with EZ Vision Dye Blue Light (New England Biolabs, England).

Table 1: Primer name, primer sequences, targeted genes and amplicon sizes for the multiplex PCR assay

Primer name	Primer sequence (5-3')	Gene targeted	Amplicon size (bp)	References
nor1 F	ACCGCTACGCCGGCACTCTCGG	*nor1*	396	Rashimi *et al.*, 2012
nor1 R	GGCCGCCAGCTTCGACACTCCG			
tri6 F	GATCTAAACGACTATGAATCACC	*Tri6*	541	Ramana *et al.* 2011
tri6 R	GCCTATAGTGATCTCGCATGT			
otanps F	AGTCTTCGCTGGGTGCTTCC	*otanps*	750	Bogs *et al.* 2006
otanps R	CAGCACTTTTCCCTCCATCTATC C			
fum 13 F	GAGCTTGTCCTTCTCACTGG	*FUM13*	982	Rashimi *et al.*, 2012
fum 13 R	GAGCCGACATCATAATCAGT			
ZEA-F	CTGAGAAATATCGCTACACTAC CGAC	Zea	192	Atoui et al., 2011
ZEA-R	CCACTCAGGTTGATTTTCGTC			

Sequence Analysis (BLAST/Phylogenetic Tree Construction)

Sanger sequencing was applied to determine the order of nucleotides in the fungi. The sequences generated by the sequencer were visualized using Bioinformatic Algorithms such as Chromas Lite for base calling. BioEdit was used for sequence editing, before performing a Basic Local Alignment Search Tool (BLAST) using NCBI (National Centre for Biotechnology Information) database (https://blast.ncbi.nlm.nih.gov/ Blast.cgi). Similar sequences were downloaded and aligned with Clustal W.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nej, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1500 replicates) were shown next to the branches (Felsentein, 1985). The trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances

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used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method and were in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA11 (Tamura *et al*., 2021).

Statistical Analysis

The prevalence rate of each of the mycotoxigenic gene was calculated using the formula:

$$Prevalence\ rate\ (\%) = \frac{Total\ number\ of\ positive\ cases}{Total\ number\ of\ screened\ subjects} * 100$$

Charts and graphs were plotted in Microsoft Excel version 2021.

Quality Control Measures

Precautions against contamination were taken. DNA extraction and PCR steps were carried out in a laboratory designated for PCR protocols. Filtered-carrier tips were always used, and plastics and reagents (when appropriate) were all UV-treated prior to use.

RESULTS

Collection of *Brassica oleracea* with Signs of Fungal Infestation

Fourteen (14) different *Brassica oleracea* were obtained from Obio Akpor and Port Harcourt L.G.As, Rivers State. The vegetables showed varying degrees of signs and symptoms of fungal diseases. The observed signs in all the vegetables were pale yellow angular spots, followed by circular and dark spots. The description of the colonial morphology and microscopic characteristics of the fungal isolates are shown in Table 2 and Figure 3.

Table 2: Macroscopic and microscopic characteristics of fungal isolates from *Bassica oleracea*

Isolate ID	Macroscopy	Microscopy
Sf2a	White dry with rough mycelia that are circular in shape, measuring about 1.2cm.	Presence of septat, macroconidia; scattered blue coloured with spindle edge.
Sf5	Circular grey to green flat and smooth mycelia	Presence of conidiophores with Septate hyphae
MF 1	White aerial mycelia with rough surface and circular margin. Reserse side is crack white.	Presence of thick walled macroconidia
MF 4	Green with yellow margin dry and rough mycelia with growth zonation	Thin hyphae with brush like conidiophores

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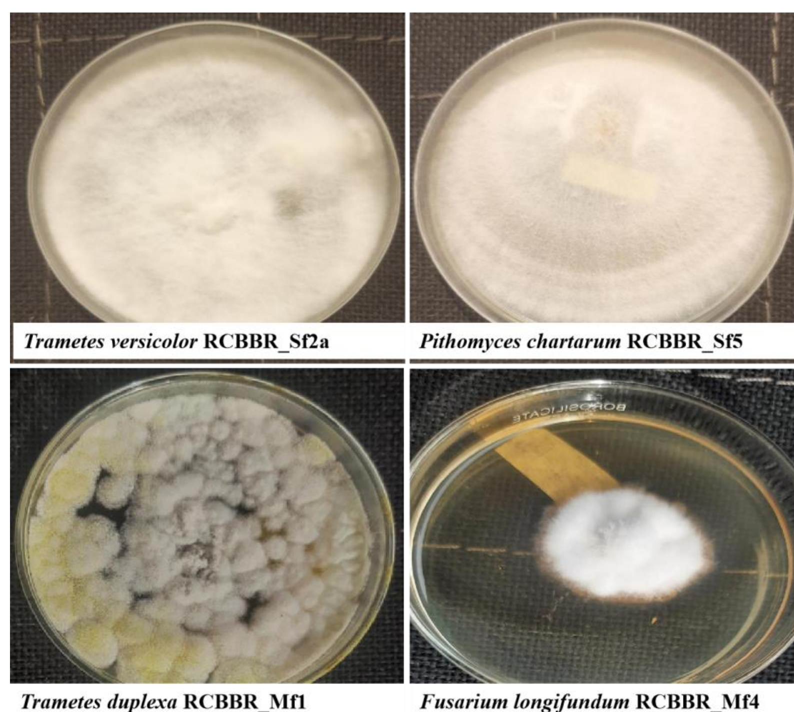
Trametes versicolor RCBBR_Sf2a
Pithomyces chartarum RCBBR_Sf5
Trametes duplexa RCBBR_Mf1
Fusarium longifundum RCBBR_Mf4

Figure 3: Colonial morphology of mycotoxin-producing fungi grown on SDA

Screening Characteristics of the Fungal Isolates from *Brassica oleracea*

Out of 14 fungi isolated from the vegetables, 4 were positive for mycotoxin. The positive isolates included Sf5, Sf2A, Mf1 and Mf4. The 3 mycotoxins observed were trichothecene, aflatoxin and ochratoxin. Isolates Sf5 and Mf4 were positive for trichothecene while isolates Mf1 and SF2A were positive for aflatoxin and ochratoxin, respectively. The screening result and prevalence are presented in Table 3 and Figure 4, respectively.

Table 3: Summary of mycotoxigenic gene screening characteristics of the fungal isolates

S/N	Isolate code	Result	Type of mycotoxin
1	SF8	-	-
2	SF19B	-	-
3	SF2B	-	-
4	SF5	+	Trichothecene
5	SF6A	-	-
6	SF2A	+	Aflatoxin
7	SF15B	-	-
8	Mf1	+	Ochratoxin
9	Mf4	+	Trichothecene
10	Sf16	-	-
11	Mf8	-	-
12	Mf2	-	-
13	Sf24	-	-
14	Sf25	-	-

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Legend: + = positive; - = negative

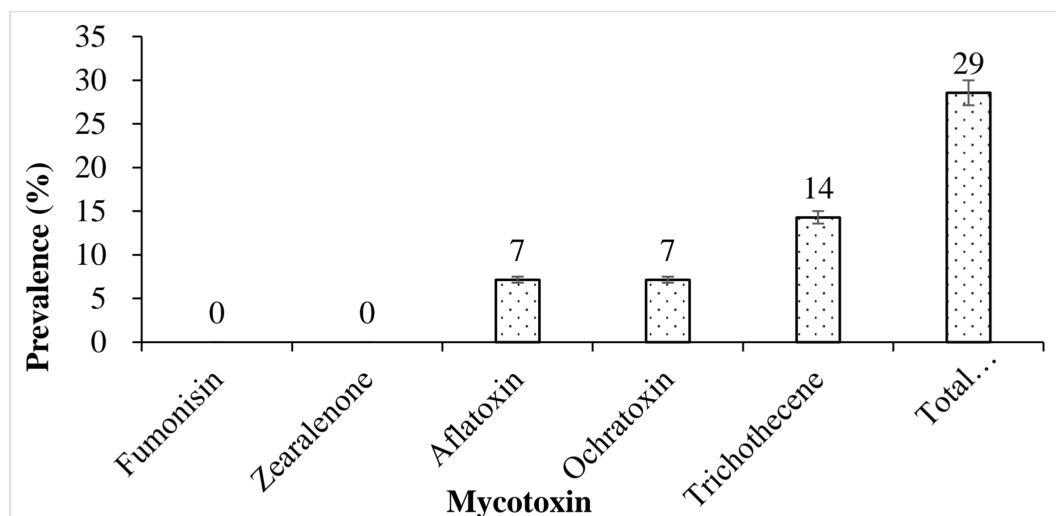

Figure 4: Prevalence of mycotoxin-producing fungi in *Brassica oleracea*

Molecular Identification of the Mycotoxin-producing Fungi Based on Its Gene Sequences

Polymerase chain reaction (PCR) amplification of the ITS gene in the 4 isolates were all successful, with the bands lying between 500 bp and 600 bp mark on the DNA ladder (Figure 5). The four mycotoxin-producing fungi were classified as *Pithomyces chartarum* strain RCBBR_Sf5, *Trametes versicolor* strain RCBBR_Sf2a, *Trametes duplexa* strain RCBBR_Mf1 and *Fusarium longifundum* strain RCBBR_Mf4 (Table 3). The sequences have been deposited under the accession numbers OR816040 and OR816043-OR816045. The phylogenetic tree of the four fungal isolates are presented in Figure 6.

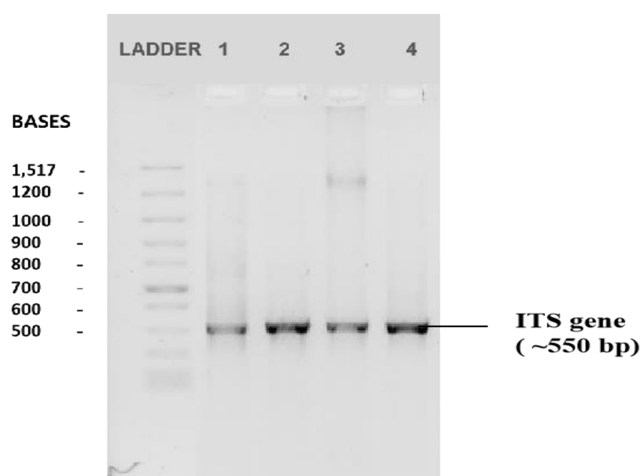

Figure 5: Agarose gel electrophoresis of the ITS rRNA gene amplicons from the mycotoxin-producing fungal isolate

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Table 4: Classification of mycotoxin-producing fungal isolates recovered from different leafy and fruit vegetables

Isolate code	% identity	Closest GenBank Match	Remark	Accession No
Sf5	**99.8**	***Pithomyces chartarum* NCBC 3.3.1**	***Pithomyces chartarum***	**OR816040**
Sf2a	**91.7**	***Trametes versicolor* CFMR FP-135156-Sp**	***Trametes versicolor***	**OR816043**
Mf1	**90**	***Trametes duplexa* BJFC Dai9343**	***Trametes duplexa***	**OR816044**
Mf4	**99.8**	***Fusarium longifundum* NRRL 36372**	***Fusarium longifundum***	**OR816045**

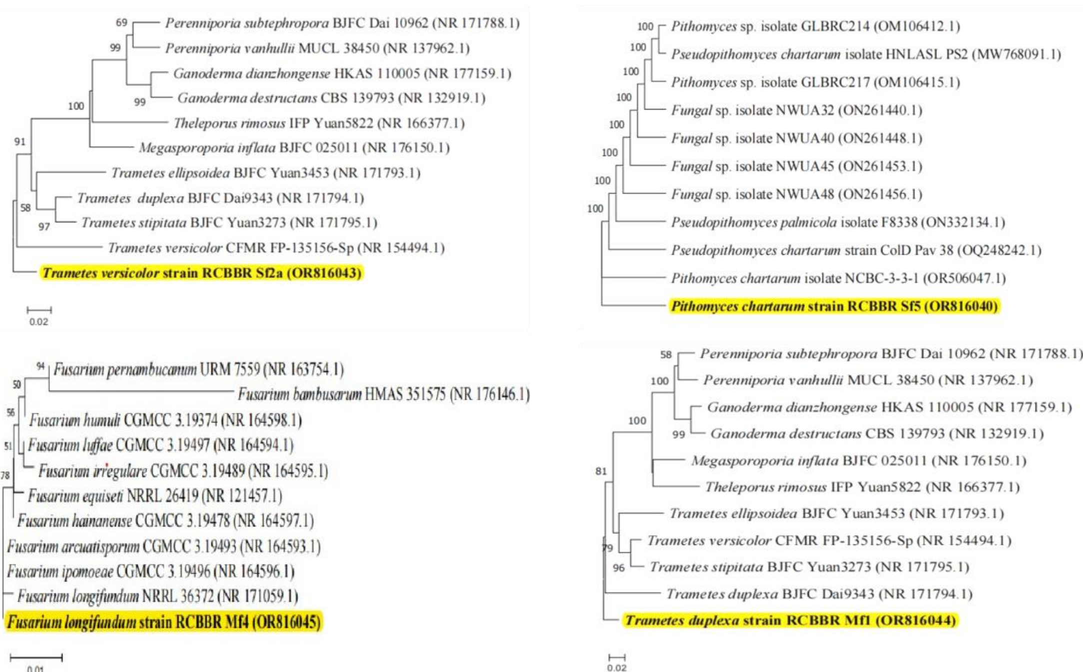

Figure 6: Phylogenetic tree of the mycotoxin-producing fungi

DISCUSSION

This study investigated the prevalence of mycotoxin-producing fungi in *Brassica oleracea* sold in Port Harcourt Metropolis, Nigeria. The importance of *Brassica oleracea* has been reported by studies across the country (Nadabo *et al*., 2022). The economic and nutritional importance of the vegetable justifies the reason for its study especially since the vegetable is mostly eaten raw as salad, suya vegetable, sauce etc. (Ike & Ogwuegbu, 2020; Nadabo *et al*., 2022). Therefore, there is a need to ensure that they do not contain toxic substances such as mycotoxins. Different mycotoxins have been detected in different fruit and vegetables. Fernández-Cruz *et al*. (2010) reported that the most frequently detected mycotoxins in fruits

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and vegetables include aflatoxins, ochratoxin A, patulin and the Alternaria toxins. Their report corroborates an earlier report by Barkai-Golan and Paster (2011), implicating mycotoxins as a major safety concern in the consumption of fruits and vegetables.

In this study, the mycotoxin-producing fungi were isolated from the leafy and fruit vegetables and screened for mycotoxigenic genes using PCR techniques. Many techniques have been successfully deployed in the detection of mycotoxins production by microorganisms. However, their wide range of structural diversity, high chemical stability, and low concentrations in tested samples make mycotoxins detection challenging, thereby requiring robust, effective, and comprehensible detection methods (Janik *et al*., 2021). Each of these techniques have their merits and demerits. The use of sensitive PCR-based assays allows researchers to identify the contamination of mycotoxigenic fungi in complex food and feed matrices and define the potential risk for human and animal health (Rahman *et al*., 2019).

While PCR can successfully detect the presence of mycotoxigenic genes in microorganisms, the technique is not without its limitations. A primer is usually needed for the assay and thus a minor change in the configuration of the gene sequence in an organism can result in lack of detection. Primers for multiplex PCRs generally must be highly selective for target sequences with consistent melting temperatures that produce amplicons of uniform length. This is important as primers failure can be erroneously termed absence of the desired gene. And primer design requires careful computation, as if designed improperly, primers form homo- or hetero-dimers, or amplify off-target sequences (Guan *et al*., 2022). This argument is important as the non-detection of some genes could mean inability of the primers to anneal to a corresponding DNA sequence arising from configuration issues rather than the presence or absence of the gene in the organism.

The mycotoxins found in the isolates included Ochratoxin A, Trichothecene and Aflatoxin. Trichothecene was produced by *Pithomyces chartarum* RCBBR_Sf5 and *Fusarium longifundum* RCBBR_Mf4 while aflatoxin and ochratoxin A were produced by *Trametes duplexa* RCBBR_Mf1 and *Trametes versicolor* RCBBR_Sf2a, respectively. The ITS gene sequences of the four fungi have been deposited in GenBank under the accession numbers OR816040; OR816043-OR816045. Among the fungal isolates identified in this study as mycotoxin producer, *Fusarium* species have been more frequently reported. *Fusarium* species such as *Fusarium graminearum* and *Fusarium culmorum* are the most frequently reported mycotoxin producers of the genus (Perincherry *et al*., 2019). Also, mycotoxin production by the fungus *Pithomyces chartarum* has been reported. The mycotoxin produced by *Pithomyces chartarum* has been linked to facial eczema (Le Bars *et al*., 1990). There seems to be dearth of information on recent implication of the fungus in mycotoxin production. So, this study stands out as it is one of the few recent studies reporting the production of mycotoxin by *Pithomyces chartarum*. As for *Trametes duplexa*, there is limited information on their ability to produce mycotoxin. However, a few studies have even reported the ability of *Trametes versicolor* to control mycotoxin-producing fungal genera, such as *Fusarium*, *Aspergillus* and *Penicillium* (Yazid *et al*., 2023).

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CONCLUSION

This study has demonstrated that mycotoxin-producing fungi are prevalent in *Brassica oleracea* sold in Port Harcourt Metropolis. And this observation raises both public health and food security concerns. Vegetables must be screened regularly to ensure that the outbreak of mycotoxigenesis does not occur. Moreover, vegetables with signs and symptoms of fungal infestations should be properly processed prior to consumption to avoid ingestion of mycotoxins.

Informed Consent

Not applicable.

Ethical Approval

The ethical guidelines for plants & plant materials are followed in the study.

Conflict of Interests

The authors declare that there are no conflicts of interests.

Funding

The study has not received any external funding.

Data and Materials Availability

All data associated with this study are present in the paper.

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