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IDENTIFICATION PROFILE OF PREDOMINANT FUNGAL CONTAMINANTS OF DRY SEASON PRE-HARVEST GARDEN EGG CROP FROM UZO-UWANI, NSUKKA, NIGERIA FOR POSSIBLE CONTROL.

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ABSTRACT

In Nigeria, dry season garden eggs are often associated with higher fungal contamination than rain-fed garden eggs and most reports on the identified pathogens were on post-harvest crops. There is possibility that some of the implicated pathogens can be traced to the farm. Thus, the present study aimed to identify the predominant fungi contaminants of dry season pre-harvest garden egg for possible bio-control measures. Sampled pre-harvest garden eggs grouped into symptomatic and asymptomatic types were collected from three farms within January to March for two consecutive years. Standard phenotypic identification methods were adopted. The most predominant isolates were further subjected to ITS rRNA sequencing. The applied methods identified 7 common isolates across the farms in the ascending frequency of Fusarium thapsinum (6.67±10.34%) <Colletotrichum spp (11.10±17.20%) <Fusarium oxysporum (14.43±22.36%) <Fusarium solani (23.70±20.32%) <Penicillium citrinum (35.57±9.12%) <Pichia spp(52.20±7.52%) <Aspergillius niger (66.67±5.99%) which were all significant (P<0.05). Among the isolates, a higher variance of 44.5was observed within the three farms than within their months of recovery (14.8 variance). The fresh asymptomatic garden egg had 71.4% contaminants as against 100% from symptomatic ones. Molecular assay confirmed the most predominant isolates as Aspergillus niger 41 and Pichia kudriavzevii C101 with assigned NCBI accession numbers of ON417062 and ON417063 after their sequence deposition in Genbank. High percentage recovery of these isolates especially from asymptomatic garden eggs gave an insight of possible health problems associated with it on consumption and this calls for appropriate bio-control measures from the farm.

Keywords: Dry-season, Pre-harvest, Garden-egg, Fungi, Contamination, Identification

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INTRODUCTION

Garden egg (*Solanum melongena L*) locally called "bitter tomato" in some countries is a vegetable crop widely cultivated in the tropical regions including Nigeria. It consists of over hundred species in Africa, out of which twenty-five species are found in Nigeria (Ayodele, 2018). In Nigeria, it is known as 'Añara' (in Igbo), 'Igbagba' (in Yoruba) and 'Dauta' (in Hausa).

The edible species are widely used in the preparation of local dishes in Nigeria especially in the South East. Their utilization in indigenous medicine for various ailments has also been documented (Igwe *et al.*,2003;Orororo*et al.*,2018)and this could be due to their pharmacological properties ranging from ascorbic acid, phenols, anthocyanin, glycol-alkaloids to α- chaconine present in their cells (Eletta *et al.*,2017; Nwanna *et al.*,2014).It is an economic vegetable associated with health benefits due to its nutritive components(Nwanna *et al.*,2019) and the sales from it have been a source of income for many, especially the Nigerian market women.

The cultivation of garden egg in Nigeria is an annual activity but it is practiced more in raining than dry season in South East where modern irrigation facilities are rarely available. To engage in dry season irrigation in some parts of South East, high water volume of about1500L/ha is needed (Ikeh and Akpan, 2018). The few areas that engaged in dry season cultivation on a small and medium scale reside along water-ways which predispose them to using local irrigation technology. The irrigation increases the moisture content of the plant and its fruit development leading to high yield of garden egg (Şenyigit *et al.,* 2011; Ayas, 2017) and subsequent economic growth. However, the local irrigation practice may not provide much desired moisture content in the plants due to excessive dry weather condition (usually between January and March). The outcome is lesser water activity which booster the proliferation of fungi in the garden egg while still in the farm. This could account for the increase in fungal contamination of pre-harvest garden egg during the dry season than raining season. The scenario may not be different from dry season garden egg from Uzo-Uwani, Nsukka, where local irrigation is practiced. The Uzo-Uwani, is a local Government Area in Nsukka within Enugu State but has borders with Anambra and Kogi states. The region is known for agricultural activities including garden egg cultivation, even during the dry season, although in a small scale level.

In pre-harvest, the fungi attack garden egg and other vegetable crops in the field from various sources including soil and its organic manures and among the implicated common contaminants were *Aspergillus, Penicillum and Rhizopus* species (Tsado *et al.,* 2013; Massoud, 2013). However, there are only few available reports on garden egg spoilage traced to pre-harvest contamination as most reports were on postharvest contamination in Nigeria (Akwaji *et al.*,2016; Gambari and Chiejina, 2013; Yaji, *et al.,* 2016).

The contamination of the pre-harvest garden egg with common pathogenic fungi during the dry season can be more worrisome than that of the raining season as their surfaces often appear good but rotten inside. This calls for caution as the garden egg is often consumed from the field to the market, thereby exposing the consumers to heavy fungal contamination unconsciously and this is of public health importance. Besides, it affects both its yield and quality (Ikeh and Akpan, 2018; Nasiru and Dalhatu, 2020). Hence, the need to identify the fungal contaminants especially the predominant ones in order to necessitate their possible pre-harvest control or treatment measures.

Therefore, the present study was to assess the fungal profile of dry season pre-harvest garden egg from Uzo-Uwani, Nsukka for subsequent evaluation of their pre-harvest bio-control measures.

MATERIALS AND METHODS

Source of Pre-Harvest Garden Egg Samples

Contacts were made and agreement reached with the garden gg farm owners through an indigene from Adani in Uzo-Uwani prior to collection of the garden egg from three different farms. The farms were spaced by about 500m^{2}within the locality surrounded by thick forest, known as 'Agu-Ofia' in Igbo dialect. About 10 observed surfaced-spoiltgarden egg fruits described as 'Symptomatic' and 10surfaced-good ones described as 'Asymptomatic'in this study were randomly plugged from each farm per year for two consecutive years. Each collection was done in 3 batches per month within January to March periods. The 1st, 2nd and 3rd batches of the sample collection were done in every 2nd3rd and 4th weeks of each month. Each batch collection was respectively packaged with high quality China Stallion aluminum foil paper (sterilized at 80^{o}C for 2h) down to the laboratory for isolation.

Isolation of Resident Fungi

The external surfaces of each test garden egg were first washed with sterile water, air-dried, cleaned with 95% ethanol for 60sec and rinsed off with sterile water. The wet surfaces were dried with sterile white cotton material. Thereafter, each prepared garden egg sample was cut into about≥8 small units of different dimensions, making sure that all the cut parts were mixed with 20ml of sterile water and stirred to obtain stock garden egg solution.

The solution (0.3ml) was swabbed into Sabouraud Dextrose agar (SDA) (Oxoid) and Potato Dextrose agar (PDA) (Titan Biotech Ltd., India) supplemented with 0.05% ($^w/_v$) chloramphenicol and incubated at 28+2^{o}C for 5 days. The incubation at 28+2^{o}C was based on the report that the atmospheric temperature of tropical *Solanium melongena* is between 27 to 30^{o}C (Ikeh and Akpan, 2018). The pure cultures obtained were identified.

Phenotypic Identification

This identification was based on the growth rate, forward and reverse colour arrangement of mycelial appearances on the plate and microscopic examination using Lactophenol cotton blue staining as established by Brayford (1992), Barnett and Hunter (1972) and Watanabe (2010).

Selection of Predominant Isolates

The number of times each isolate was recovered from the sampled garden egg within the sampled periods were recorded and used to determine its percentage occurrences as stated below:

$$\% \text{ Occurrence/Recovery} = \frac{\text{The no. of times the isolate was recovered} \times 100}{\text{Total no. of times sampled}}$$

The two isolates with higher % occurrence were selected as predominant isolates and subjected to molecular characterization for further intended studies.

Molecular Identification

The chromosomal DNA of the 2 fungal isolates were fist extracted from their 24h PDA broth cultures with Zymos Research (ZR) DNA MINIPREP kits using modified method of Pitcher *et al.* (1989) as described by George-Okafor *et al*. (2018). The extraction included lysis with ZR lysing solution, several centrifugations (at 10,000 x g/min), washing with buffers and filtrations. The eluted DNA after centrifugation at 10,000 x g/ 30 sec was preserved at -20^{o}C for subsequent analysis which included the evaluation of the DNA concentration via agarose electrophoresis, DNA amplification and sequencing.

DNA Amplification

The amplification of the ITS gene of the extracted fungal DNAs through polymerase chain reaction (PCR) was conducted with universal primers; ITS1 (5'TCC GTA GGT GAA CCT GCGG-3') as forward and ITS4 (5'TCC TCC GCT TAT TGA CAT GS-3') as reverse (White *et al.,* 1990). The assay was carried out in a 25 µL PCR mix containing 12.5 µLTaq 2X Master mix (New England Biolabs, M0270), 1µL each of 10µM forward and reverse primer, 2µL of DNA template and 8.5µL Nuclease water. The cycling conditions involved initial denaturation at 94^{o}C/5min, followed by 35 cycles of denaturation at 94 ^{o}C /30sec, annealing at 54 ^{o}C /30sec and elongation at 72 ^{o}C /45sec. The elongation was further extended for 7min at 72 ^{o}C. Thereafter, their sizes of the PCR products were estimated by comparison with the mobility of a 50bp of DNA ladder (New England Biolabs) that was run alongside with amplified products in 2% ethidium bromide-stained agarose gel (Fisher Scientific).

Sequencing and Phylogenetic Studies

The amplified products were purified with 95% ethanol to remove PCR reagents before sequencing which was performed using Genetic Analyzer 3130x1 sequencer (Applied Bio-systems) with the Big-Dye terminator v3.1 cycle sequencing kit. The obtained sequence of each isolate was subjected to a BLAST search http://www.nci.nlm.nih.gov.for similar or related sequences of known species domiciled in NCBI (National Center for Biotechnology Information) database. Bio-Edit software and MEGA11 were utilized for all genetic analysis including editing, alignment and phylogenetic studies (Tamura *et al.,* 2004; Tamura *et al.,* 2021).

Statistical Analysis

Data obtained were analyzed using IBM Statistical Product and Service Solutions (SPSS), version 18. The results were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) with Duncan test for multiple comparison which was used to compare means across the groups. Mean values with $P < 0.05$ were considered statistically significant compared across and within the group.

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RESULTS AND DISCUSSION

Isolation and Phenotypic Characterization

In a total of 360 samples of garden eggs assayed per farm, only 7 predominant isolates were selected among others (result not shown). Their characterization through morphological features revealed colonies that ranged from dirty white, white, black, pale grey to greyish green mycelia at different growth rates and textures (Fig.1.0). Their microscopic observations on arrangement of conidiophore, phialides and spores as described on Table 1.0 further identified the 7 isolates as A; *Pichia* spp, B**;** *Fusarium solani*, C; *Colletotrichum* spp, D; *Aspergillius niger*, E, *Fusarium oxysporum*, F; *Fusarium thapsinum* and G*; Penicillium citrinum*. Although this phenotypic approach has been criticized as a reliable identification tool, yet it is still a common practice in routine laboratory work as it gives an insight of responsible pathogens for immediate treatment measures. Apart from routine analysis, phenotypic based identification had been used for many research studies including fungal biochemistry, biotechnology, bioremediation, physiology and plant pathology (Shanthi and Vittal, 2010; Sangeetha *et al.*, 2020; Zainab and Shinkafi, 2016).

Table 1.0: Microscopic Features of the Isolates

Isolates Type	Microscopic Features	Organism
A	Yeast-like structures (spherical spores singly arranged*).*	*Pichia* spp
B	Presence of conidiophore, mostly with long phialides unbranched bearing single conidia at the tip. Most conidia are smooth and cylindrical	*Fusariumsolani*
C	Presence of many thick cylindrical hyaline conidia with setae	*Colletotrichum* spp
D	Long conidiophore with vesicle and many phialides with many spores.	*Aspergillius niger*
E	The phialides are unbranched but the conidia were slender, ellipsoidal and pointed at their tips	*Fusarium oxysporum*
F	The phialides bore more than one conidia that appeared in chains. The conidia were slightly stout and ellipsoidal The phialides are conspicuously vertical.	*Fusarium thapsinum*
G	Presence of branched phialides that appeared like a funnel with long metulae. The phialides have round conidia in chains	*Penicillium citrinum*

The microscopic features were compared with those previously established.

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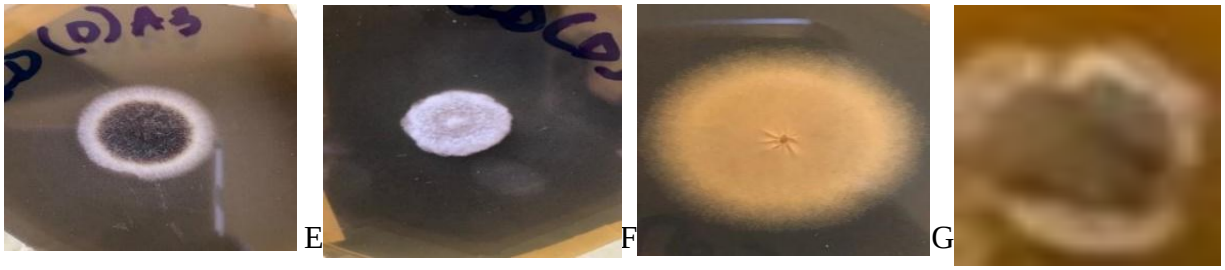
A Isolate on SDA B Isolate on SDA C Isolate on PDA



D Isolate on SDA E Isolate on SDA F Isolate on PDA G Isolate on PDA

Fig 1.0: Surface Colonial Images of Fungal Isolates from SDA and PDA Plates of Cultured Test Garden Eggs

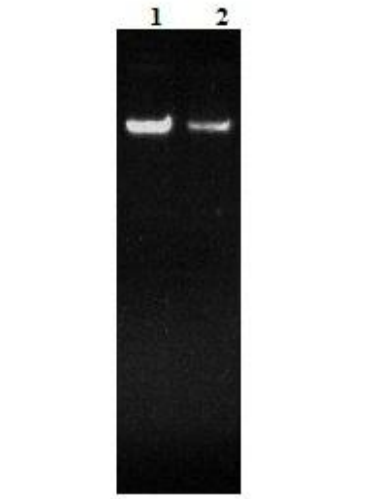
A. Pichia spp, B; Fusariumsolani C; Colletotrichum spp D; Aspergillius niger

E. Fusarium oxysporum F; Fusarium thapsinum G; Penicillium citrinum

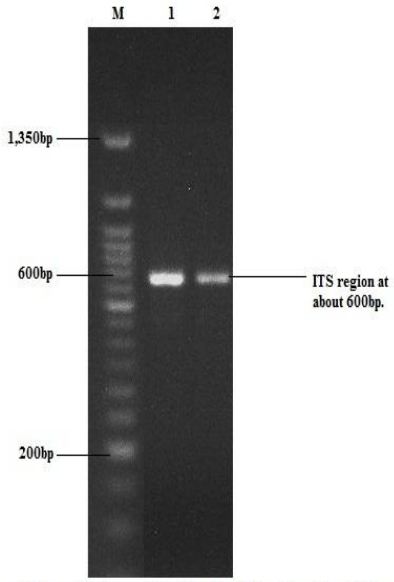
*The phenotypic identity of these organisms based on their colonial appearances were compared with those previously established as described in the text

Molecular Characterization

DNA Amplification: The two DNA molecules from the most predominant isolates (D and A) displayed two distinct bands (Fig.2a) with a molecular size of their PCR products at about 600bp of their ITS (Internal Transcribed Spacer) regions (Fig.2b). The PCR products of 600bp in size suggests the isolates as fungi since it is closer to DNA barcode of ITS region of fungi kingdom which has about 700 nucleotides in length (Nurrahmi, 2016)



Gel image showing genomic DNA extracted from fungal isolates.



Gel image showing amplification of the ITS region at about 600bp. M is 50bp Ladder NEB.

(a) **(b)**

Fig. 2a: Electrophoregram of Test Genomic DNA
Fig. 2b: PCR Products of the test genomic DNA
M: markers with their molecular weights; **1** and **2** are DNAs of the most predominant isolates coded D and A respectively.

DNA Sequencing and Phylogenetic Tree

The blast nucleotide sequence result showed that the two isolates had pairwise identity of 96.36% for *Aspergillus niger* 41 and 92.11% for *Pichia kudriavzevii* C101 (Table 2.0) and their nucleotide sequences have been deposited in Genbank with assigned accession numbers of ON417062 and ON417063 for *Aspergillius niger* and *Pichia kudriavzevii* respectively. The phylogenetic tree (Fig.3.0) also displayed their closely related species which clustered together as an indication of evolutionary related organisms. The result from molecular characterization in this study confirms our phenotypic characterization as a reliable tool for the isolates identified to genus as well as species levels for some of them. However, the inclusion of molecular approach resulted in specific identification of the two isolates up to sub-species levels (i.e.41 and C101 respectively). The molecular identification of the two isolates with the knowledge of their physiological characteristics would aid on the ongoing research on the possible bio-control measures in the farm. Thus, complementing phenotypic approach to molecular identification process stands to achieve a more reliable result (Muhammad, 2007).

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Table 2.0: Molecular Characteristics of Selected Predominant Isolates

Phenotypic Identity	NCBI Accession No	E- Score Value	Pairwise Identity (%)	Molecular Identity	*New Accession No
Aspergillus niger	MK414042	3e-13	96.36	*Aspergillus niger strain 41*	ON417062
Pichia spp	MG015976	4e-161	92.11	*Pichia kudriavzevii* strain C101	ON417063

Primers: ITS1: 5'TCC GTA GGT GAA CCT GCGG-3' as forward
ITS4: 5'TCC TCC GCT TAT TGA CAT GS-3' as reverse

The pairwise identity was considered based on the closest percentage similarity resulting from the closest type strain sequence.

***New NCBI Accession Nos. were assigned to our isolates after the deposition of their nucleotide sequences in the Genbank.**

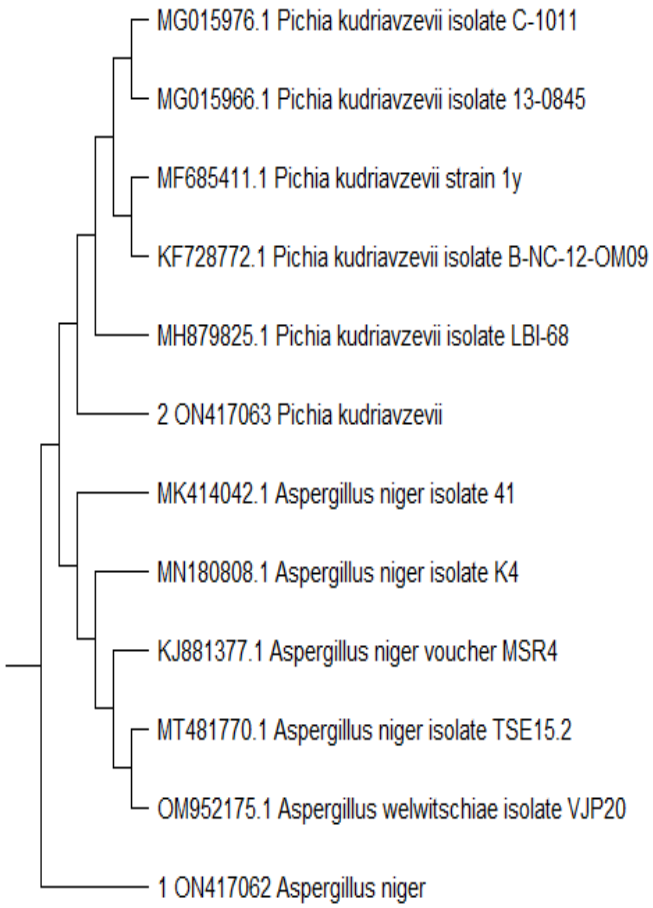

Fig. 3.0: Phylogenetic of *Pichia kudriavzevii* and *Aspergillus niger* isolated from Garden egg.

The evolutionary distances were computed using the maximum composite likelihood method and the analyses were conducted in MEGA There were a total of 605 positions in the final dataset and the accession numbers were listed along with the genus and species of related isolates.

Recovery Pattern of Fungal Isolates from the Garden Egg Farms

The mean percentage occurrence of the isolates across the three farms ranged from 6.67±10.34 to 66.67±5.99% with isolates variance of 44.51 at a significant difference of P<0.001 (Table 2.0). The three *Fusarium* spp and *Colletotrichum* spp were not recovered in every farm within the period of assay. The highest and least percentage of isolates were obtained in farm 2 and 3 respectively. *Aspergillus niger* (66.67±5.99%) and *Pichia kudriavzevii* (52.20±7.53%) were the most frequently isolated contaminants from the three garden egg farms.

Most of the isolated species especially *Aspergillus niger* and *Penicillum citrinum* have frequently been associated with post-harvest and not pre-harvest garden egg spoilage in Nigeria (Nasiru and Dalhatu, 2020; Jidda and Musa, 2016). However, few available literatures have implicated some fungal species including *Collectotrichum* spp as pathogenic contaminants of pre-harvest garden egg (Akwaji *et al.,* 2016) with more reports on other pre-harvest vegetable crops (Tsado *et al.,* 2013).

High prevalence of *Aspergillus niger* as contaminant in the three farms could be majorly from polluted soil with plants and animal manure leading to their accumulation in the soil. When absorbed into the plant system through transpiration, they are bound to move across the largest parts of the plant, causing infection. Contamination from irrigation water could be additional mainstream of pollution as its natural sources are often associated with high microbial load in dry season. Tsado *et al.* (2013) have implicated irrigation water as a key factor to the recovery of high fungal load from irrigated farms and open garden in Minna.

Pichia kudriavzevii, formerly known as *Candida krusei* also had high percentage occurrence and it is the first time it is associated with pre-harvest garden egg contamination in Nigeria and some literature had indicated soil, fruits and polluted carbon sources as its natural sources (Koricha *et al.,* 2019). Thus, there is possibility that polluted soil could also account for its presence in the three farms.

Periodic Influence on Fungal Contamination Profile of Pre-Harvest Garden egg

The frequency of isolates in relation to the month of recovery slightly varied with highest recovery in March (33.33±0.28 %**),** followed by February (30.48±0.14) and January (25.71±0.01%) respectively (Table 4.0). However, their variability was lower (variance of 14.82) than that (variance of 44.51) observed in relation to the farms of their recovery. Least recovery of *A.niger* and *F. oxysporum* in January, could be attributed to very low humidity and dryness associated with January harmattan period despite the applied irrigation practices. Thus, *A niger* and *F. oxysporum* can be inferred to be influenced by dry season climatic changes. Such climatic changes could have brought about specific physiological changes in them which could not have taken place significantly in other isolates such as *Colletotrichum* spp that seemed to be insensitive to the January observed climatic changes. Climate fluctuation was reported to have effect on *Aspergillus* contamination in crops with high frequency in humid warm climate and irrigated hot weather (Cotty and Jaime, 2007).

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Table 3.0: Recovery Pattern of Fungal Isolates from Different Garden Egg Farms

Isolates	% Recovery from Each Farm			Mean Total Recovery (%)
	*1	*2	*3	
Pichia kudriavzevi	60.00±0.71 [c]	53.30±0.28 [c]	43.30±0.42 [c]	52.20±7.53[C]
Fusarium solani	26.60±0.85 [b]	44.50±0.64 [b]	0 [b]	23.7±20.32 [b]
Colletotrichum gloeosporiodes	33.30±0.28 [c]	0	0	11.1±17.20 [b]
Aspergillius niger	66.70±0.71 [b]	73.30±0.57 [b]	60.00±1.27 [b]	66.67±5.99 [c]
Fusarium oxysporum	0	43.30±0.14[c]	0	14.4±22.36 [b]
Fusarium thapsinum	0	0	20.00±1.13[c]	6.67±10.34 [b]
Penicillium citrinum	46.70±0.57 [c]	26.70±0.28 [c]	33.30±0.14 [c]	35.57±9.12 [c]
% Recovery	33.33±0.28	34.44±0.57	22.37±0.03 [c]	30.05±0.03
Variance within the farms:	**44.51**			

*1: 1st Garden egg Farm *2: 2nd Garden egg Farm *3: 3rd Garden egg Farm
Data are mean ± standard deviation (SD) (n = 2). [a] =p < 0.05, [b] =p<0.01, [c] =p<0.001 as compared within and between the group (One-way ANOVA followed by Duncan t-test).

Table 4.0: Periodic Influence on Fungal Contamination Profile of Garden egg

Isolates	% Recovery for each Month within Two Years		
	January	February	March
Pichia kudriavzevii	56.67±0.28 [c]	66.67±0.41 [c]	33.30±0.41 [c]
Fusarium solani	23.33±0.42 [b]	16.72±0.78 [b]	26.66±0.04 [b]
Colletotrichum spp	16.67±0.03 [c]	10.00±0.07 [c]	6.67±0.01 [c]
Aspergillius niger	36.67±0.14 [c]	73.30±0.42 [c]	90.00±0.71 [c]
Fusarium oxysporum	6.67±0.28 [c]	10.00±0.03 [c]	26.67±0.28 [c]
Fusarium thapsinum	6.67±0.14 [c]	10.00±0.06 [c]	3.33±0.28 [c]
Penicillium citrinum	33.33±0.04 [c]	26.67±0.14 [c]	46.67±0.01 [c]
Percentage recovery	25.71±0.01 [c]	30.48±0.14 [c]	33.33±0.28 [c]
Variance within the months	**14.82**		

Data are mean ± standard deviation (SD) (n = 2). [a] =p < 0.05, [b] =p<0.01, [c] =p<0.001 as compared within and between the groups (One-way ANOVA followed by Duncan t-test).

Occurrence Pattern of Fungal Isolates from two groups of Pre-Harvest Garden egg

The extent of crops contamination and spoilage may not always be detected externally. Some contamination may only result in the production of toxins or devastation of internal tissues without any external changes in appearance, odour and texture especially when still in the farm.

This is a scenario of some of the test garden eggs termed 'Asymptomatic' in this study. They were observed to be fresh externally but slightly spoilt inside. The group was contaminated with 71.4% of the total isolates recovered from the farm, although its degree of contamination was moderate (Table 5.0). This pattern of spoilage could be species dependent as some organisms such as *F. oxysporum* and *F. thapsinum* were not recovered from them. The asymptomatic contaminated garden eggs seem to appear more in dry than raining season and climatic changes could also be a factor (Cotty and Jaime, 2007). The implication of this pattern of contamination is the exposure of consumers to food borne infections which is of public health concern. Although pathogenicity tests on the recovered isolates were not conducted but all the isolates have been recognized as human pathogens including *P. kudriavzevii* which was earlier reported as GRAS (Generally Recognized as Safe) organism (Nasiru and Dalhatu, 2020; Khodadadi, *et al.*, 2020; Nagarathnamma *et al.*, 2017). This calls for safe and adequate biological control measures from the farm.

Table 5.0: Occurrence Pattern of Fungal Isolates from two groups of Pre-Harvest Garden Egg

Isolates	*Surface Appearance of Garden Egg Groups	
	Symptomatic	Asymptomatic
Pichia kudriavzevii	++	+ +
Fusarium solani	+	+
Colletotrichum spp	+	+
Aspergillius niger	+ +	+ +
Fusarium oxysporum	+	--
Fusarium thapsinum	+	--
Penicillium citrinum	++	+
% of Recovered Isolates	100%	71.4%

--- Not present + moderately present ++ Highly present

* Surface Appearance of Garden Egg Groups were termed symptomatic and asymptomatic based on the surface appearances of the garden egg during collection as described in the text.

% of Recovered Isolates was calculated based on the total no. of isolates recovered which was 7.

CONCLUSION

The study revealed the presence of fungal contaminants includinga wild yeast at varied frequencies within the pre-harvest garden eggs with *A. niger*41 and *P.kudriavzevii*C101 as the most predominant species.

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