Original Research Article

Pathogenicity of fungi isolated from spoilt sweet oranges (*Citrus sinensis*) and from the air in the environment of the orange section of the Gamboru market in Maiduguri, Nigeria.

ABSTRACT

Aim: Though Oranges are very important crops for man both economically and in terms of nutrition, they have a short life span due to poor handling during harvesting, transporting, and storage. The ubiquitous nature of fungi makes them ready contaminants for these oranges especially when their skin is broken. The aim of this study is to isolate, identify and determine the pathogenicity of fungi responsible for spoilage in sweet oranges and investigate the presence of fungi in the air of the environment where these oranges are sold.

Place and duration of study: Department of Microbiology Faculty of Science University of Maiduguri between September and October 2020

Methodology: A total of 100 samples of ripe oranges with signs of spoilage (n=100) were collected randomly from 10 selling points in the Gamboru fruit market in Maiduguri, Borno state of Nigeria, and analyzed for fungal isolates using morphometric techniques. Two mycological media Potato Dextrose Agar and Malt Extract Agar (PDA and MEA) were used for isolation, by deploying direct plating method. Petri dishes containing prepared PDA and MEA were also exposed to the air in the shops for some minutes. Morphometric analysis was carried out to identify the fungal isolates using macroscopic and microscopic observed features. Pathogenicity tests were carried out to ascertain the ability of these isolates to cause spoilage.

Results: The results of the morphometric characterization revealed the presence of seven (*Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger,* and *Penicillium oxalicum, Rhizopus oryzae*, *Cladiosporium sphaerospermum* and *Scopulariopsis brevicaulis)* different fungi from the air and from the deteriorating oranges. Four of the isolates which were also positive for pathogenicity and observed to be responsible for causing spoilage in the oranges were also found to be the same as those isolated from the air, while three other organisms isolated from the air in the sales environment were not isolated from the spoilt oranges. *Penicillium oxalicum* was isolated from 41% of the oranges making it the most predominant organism causing spoilage in the oranges while Aspergillus fumigatus was isolated from only 10% of the samples.

Conclusion: The findings of this study showed that fungal contaminants were responsible for the spoilage of oranges in this market and many of them were present in the air in the shops where these oranges were sold and stored leading to serious economic losses for both farmers and consumers.

Keywords: Environment, spoilage, fruits, economic loss. nutritional value, fungi

1. Introduction

Fruits are energy-dense foods that play an important role in human nutrition and health, particularly as sources of vitamin C, thiamine, niacin, pyridoxine, folic acid, minerals and dietary fiber and, other bioactive compounds [1]. Fruits are an important component of a balanced daily diet in humans that can help to sustain a healthy life. They contain sufficient quantity of potassium, which is important for the reduction of bone loss and the occurrence of kidney stones [2].

Some of the benefits that can be obtained from consumption of fruits are longer life span, [3] improved mental health better cardiovascular health [4] reduced risks of some cancers, and weight management [5]. The economic value of fruits is limited by the relatively short shelf-life which is attributed to decay by pathogens during post-harvest handling [6]

Citrus sinensis, which is also known as sweet orange belongs to the group of fruits call citruses, it is the most popular of the citrus fruits, and other members of the family include lemons, grapes, and tangerines. It is widely cultivated in most regions of the world [7]. They contain flavonoids, which are antioxidants that could neutralize free radicals, protect from heart diseases and improve blood flow through the coronary arteries [8]. Sweet Oranges contain a high quantity of vitamin C, folate, and thiamin. Vitamin C protects the body from free radicals that could destroy the body and helps in wound healing and holding blood vessels, tendons, ligaments, and bones together [9]. Sweet oranges are subject to a high rate of economic loss, these losses are due to a number of factors, including post-harvest fungal diseases which have been implicated as a major cause, and they become vulnerable to post-harvest fungal infection especially when their skin barrier has become compromised. A study by [10] showed that fungal infection of sweet oranges varied from 29.9 to 43.8%.

A large portion of the sweet oranges sold within the Maiduguri metropolis are transported into the state from other parts of the country and the poor road network coupled with insecurity has extended the length of time the fruits spend in transit and the level of hazards they are exposed to [11].

A very important factor that influences both the economic and health values of sweet oranges is the relatively short lifespan. They begin to deteriorate shortly after harvest and sometimes they do not reach consumers at optimal quality after the rigors of post-harvest handling and marketing. Factors such as temperature, relative Humidity, and storage atmospheric conditions affect the rate of deterioration. The high level of sugars and nutrients present in sweet oranges in addition to their low pH value also makes them very susceptible to fungal spoilage, especially during storage [12].

The spoilage of fruits by fungal contaminants is a source of serious potential health hazard to man due to the mycotoxins they produce which can result in mycotoxicoses in man when ingested. Severe economic

loss to farmers, traders and the society in general also occurs as a result of fungal spoilage of sweet oranges.

This present study was carried out to isolate, identify and determine the pathogenicity of fungi isolated from spoilt sweet oranges and investigate the presence of these fungal isolates in the air of the environment where these oranges are sold.

2. MATERIALS AND METHODS

2.1 Sample collection

One hundred oranges with visible signs of spoilage (soft, mushy texture, brown or black spots) were collected from 10 different shops in Gamboru fruit market in Maiduguri, Borno state. They were stored in sterile polythene bags separately, and they were transported to the Microbiology laboratory, for fungal isolation. All the glass wares used were properly washed, dried, and sterilized. The workbench was also disinfected with 95% ethanol to curtail contamination. Petri dishes containing prepared PDA were also taken to the orange shops and exposed to the air.

2.2 Isolation of fungi from Orange

The fungal isolation technique was carried out using the method of [13] modified. Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA) were prepared following the manufacturer's instruction and 1mg/ml of chloramphenicol was added to prevent bacterial growth. Both media were aseptically poured into separate Petri-dishes and allowed to solidify. samples were first surface sterilized by washing under running tap water in order to remove dirt and sand. The infected portions of the various samples were excised and cut into 2 mm pieces with a flamed surgical blade, surface sterilized with 1% NaOCl and rinsed four times successively in sterile distilled water. The excised infected portions were then plated onto the prepared Petri dishes placing them on three extreme ends of the Petri- dish. These plates and those that were exposed to the air in the Orange sales environment were incubated at 28^{o}C for five days. The fungal colonies that developed were sub-cultured repeatedly on PDA to obtain pure cultures.

2.3 Macroscopic and microscopic identification

Seven days old pure cultures of the fungal isolates were identified on the basis of macromorphological characteristics such as colony colour, shape, size, and colour of the reverse side of the culture. micromorphological observations were made by mounting small portions of the fungal growth on a glass slide, teasing it out, and staining it with a drop of lactophenol in cotton blue solution which was then observed under X10 and x40 objective lens of a light microscope. The presence of septa, the shape of spores, and other microscopic features were observed [14].

2.4 Pathogenicity of Isolated Fungi

Using a standard method, eight healthy oranges were surface sterilized with 90% ethanol, and incisions were made on them using a sterile 4mm cork borer; a similar sterile cork-borer was used to cut pellets of agar containing the cultures of fungal mycelia of each of the four isolates. These fungi were then inoculated into the hole created on the healthy fruits in a laminar flour chamber. The inoculated wound

was sealed with petroleum jelly. Two controls with incision but no inoculation were established. The inoculated fruits and the controls were placed in clean zip lock polyethene bags (one fruit per bag) each moistened with-wet balls of absorbent cotton wool to create a humid environment as it exists in the Gamboru market and incubated at 28^{o}C for 5 days. After 72 hours, the inoculated fruits were observed for symptom (soft, mushy texture, brown or black spots) development. The fungal agents were re-isolated from the infected fruits and compared with the original isolates.

3. RESULTS

A total of seven fungal isolates were recorded in this study as shown in table 1. Four of these fungi were isolated from the spoilt sweet oranges, they include *Aspergillus fumigatus* infecting 10% of the oranges, *Aspergillus flavus,* infecting 23% while *Aspergillus niger,* and *Penicillium oxalicum* were the two prominent ones occurring in 26% and 41% of the oranges respectively as shown in figure 1. Table 2 presents the number of oranges that were contaminated by the various fungal isolates with respect to the number of orange samples that were collected. Eighty-two of the 100 orange samples were contaminated with *Penicillium oxalicum* making it the most prominent contaminant while only 20 oranges were contaminated by *Aspergillus fumigatus.* All the fungi isolated from the spoilt fruits were also isolated from the air in the shops while three fungal species were isolated from the air in the sales environment was not isolated from the spoilt oranges. These fungal species that are peculiar to the air in the shops include *Aspergillus terrus,* Cladiosporium *sphaerospermum* and *Scopulariopsis brevicaulis* the pathogenicity test showed that *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger* and *Penicillium oxalicum* were all responsible for spoilage in the oranges. The healthy oranges developed the observed symptoms and these organisms were re-isolated from the newly infected oranges. The macroscopic and microscopic features of the fungal isolates are shown in table 1 while the pictures are presented in fig.1(A-

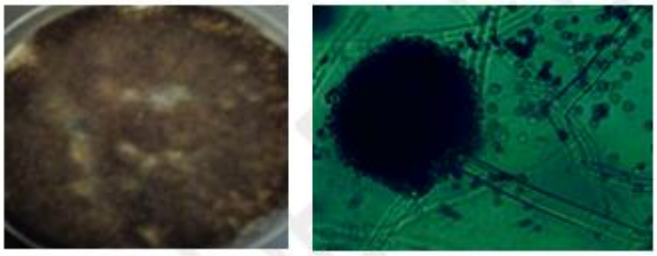
Table 1: Macroscopic and Microscopic description of fungal isolates

S/N	Macroscopic Observations	Microscopic Observations	Name of Isolate
1.	Colonies on Potato dextrose agar attained a diameter of 4-5 cm within 7 days. It consists of a compact white or yellow basal felt with a dense layer of dark brown to black conidiophores.	Conidiophore stipes are smooth-walled, hyaline, and brown in colour. Phialides are borne on metulae. Conidia globose and rough-walled.	*Aspergillus niger*
2.	Colonies on Potato dextrose agar attained a diameter of 4 cm within 7 days. It appears blueish green in colour with white edges becoming powdery with age.	Conidiophore stipes are smooth-walled, hyaline, and tetraverticillate. Metulae is smooth-walled and cylindrical bearing 2-4 phialides each. Phialides and conidia are	*Penicillium oxalicum*

	The reverse is uncoloured.	cylindrical	
3.	Colonies on Potato dextrose agar attained a diameter of 3-5 cm within 7 days consisting of a felt of dark green conidiophores mixed with aerial hyphae.	Conidiophores are short, smooth-walled, and green. Vesicles are clavate. Phialides are directly borne on the vesicle. Conidia are globose.	*Aspergillus fumigatus*
4.	Colonies on Potato dextrose agar attained a diameter of 3-5 cm within 7 days. It consists of a dense felt of yellow-green conidiophores.	Conidiophores are hyaline and coarse, vesicles are globose. Phialides are borne directly on the vesicle. Conidia are globose to sub globose pale green and echinulate.	*Aspergillus flavus*

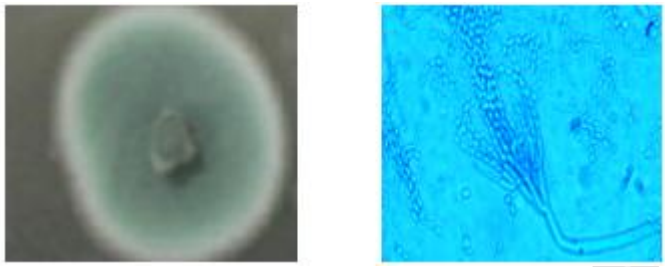
Table: 1 continued: Macroscopic and Microscopic description of fungal isolates

S/N	Macroscopic Observations	Microscopic Observations	Name of Isolate
5.	Colonies on potato Dextrose Agar attained a diameter of 3.5-5.0cm at 7 days consisting of a dense felt of yellow-brown conidiophore,	Conidiophores are hyaline and smooth-walled. Vesicles are subglobose and phialides are borne on metulae. Conidia are globose, hyaline and smooth.	*Aspergillus terrus*
6.	Colony on PDA attains a diameter of 2cm in 10 days, velvety and olivaceous brown. The reverse is greenish-black.	Conidiophores arise laterally and terminally from the hyphae bearing several branched conidial chains. Dark olivaceous- brown and smooth-walled.	*Cladosporium sphaerospermum*
7.	Colonies on potato Dextrose Agar attained a diameter of 3cm in 7days light rose brown at first becoming dark brown,	Conidiogenious cells are borne singly on ariel hyphae or in groups of 2-10 on short conidiophores. Conidiospores are globose with a truncate smooth-walled, olive to brown in colour	*Scopulariopsis brevicaulis*



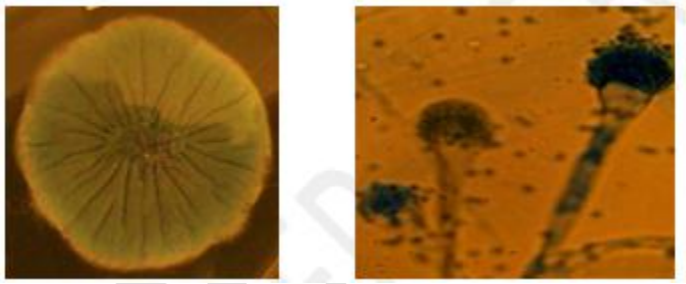
Obverse view Micrograph (X40)

Fig.1 A: *Aspergillus niger* isolated from oranges and the air in the orange sales environment



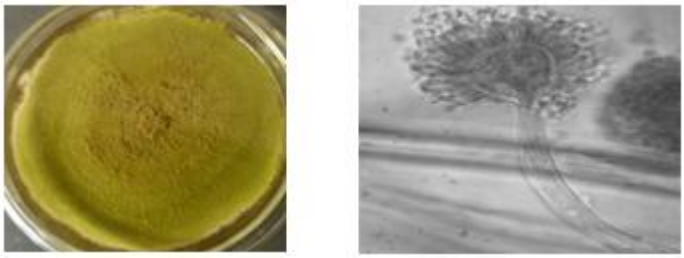
Obverse view Micrograph (X40)

Fig. 1 B: *Penicillium oxalicum* isolated from Oranges and the air in the orange sales environment.



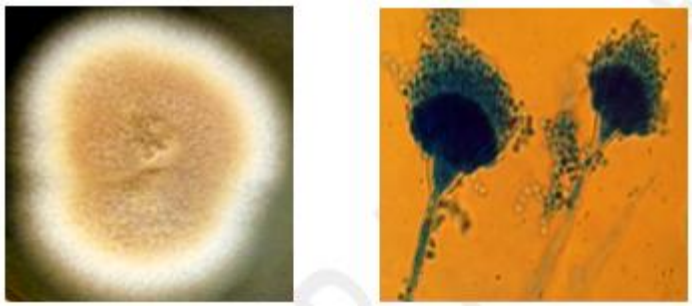
Obverse view Micrograph (X40)

Fig.1 C: *Aspergillus fumigatus* isolated from oranges and the air in the orange shops



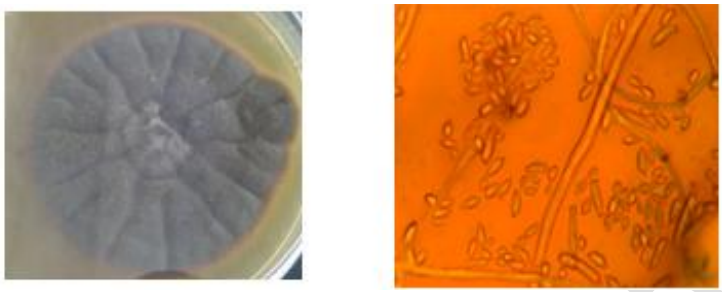
Obverse view Micrograph (X40)

Fig.1 D: *Aspergillus flavus* isolated from Oranges and the air in the orange sales environment



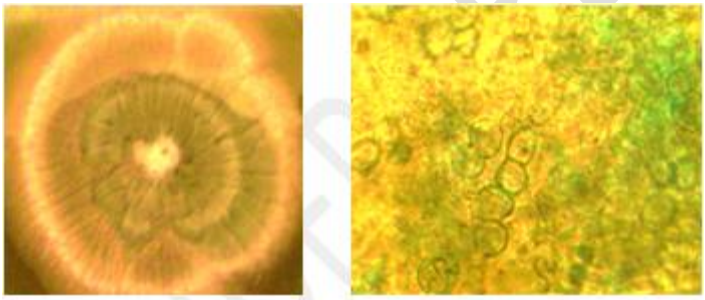
Obverse view Micrograph (X40)

Fig.1 E: *Aspergillus terrus* isolated from the air in the orange shops



Obverse view Micrograph (X40)

Fig.1 F: *Cladosporium sphaerospermum* isolated from the air in orange shops



Obverse view Micrograph (X40)

Fig.1 G: *Scopulariopsis brevicaulis* isolated from the air in orange shops

Table:2 Rate of fungal occurrence in orange samples

S/N	Fungi	Total number of samples	Number of samples it occurred in
1.	*Penicillium oxalicum*	100	82
2.	*Aspergillus niger*	100	53
3.	*Aspergillus flavus*	100	47
4.	*Aspergillus fumigatus*	100	20

The number of oranges that were infected by each fungal isolate is presented in Table 2, indicating that *Penicillium Oxalicum* is the highest contaminant and *Aspergillus fumigatus* as the lowest.

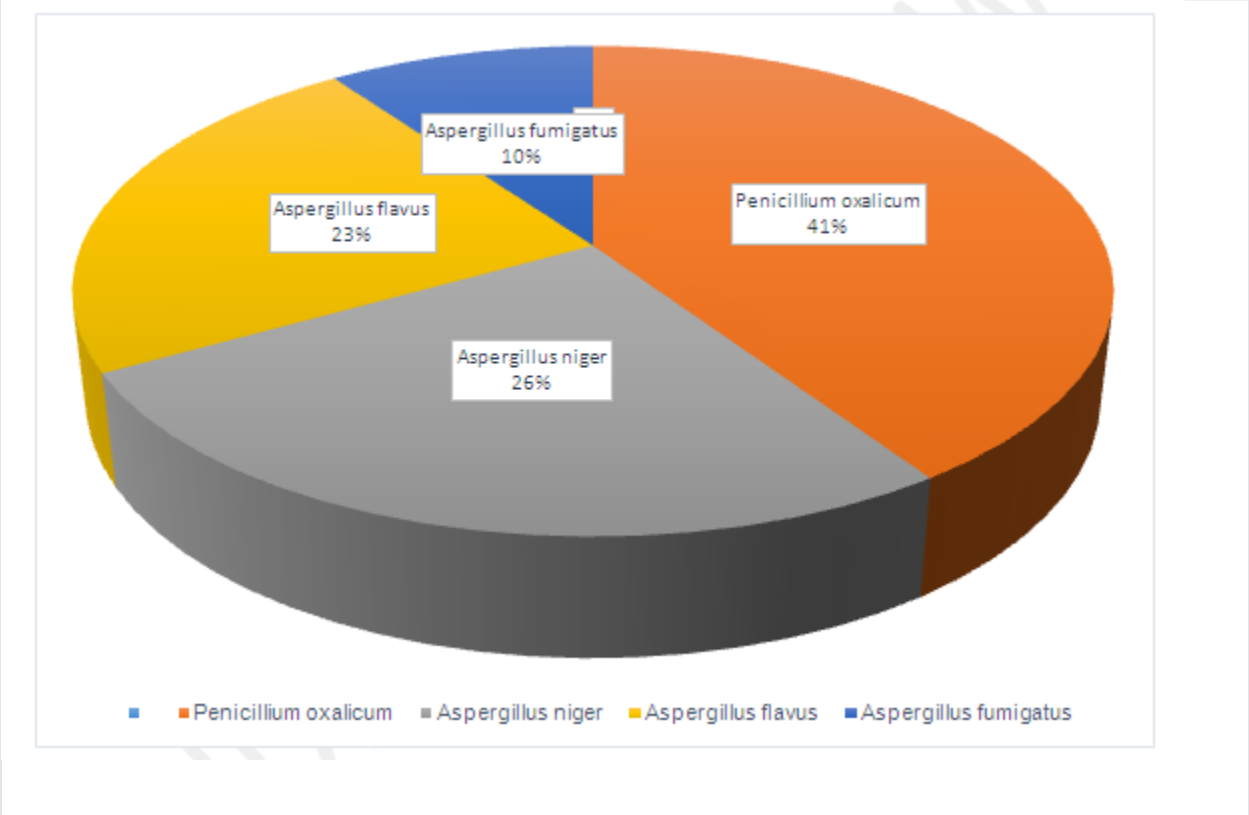


Figure 2: Percentage occurrence of each fungal species in one hundred samples (%)

Figure 1: Shows in terms of percentage that 41 percent of the oranges sampled were infected by *Penicillium oxalicum*, 26 percent were infected by *Aspergillus niger*, 23 percent by *Aspergillus flavus,* and 10 percent by *Aspergillus fumigatus*.

DISCUSSION

When fruits are transported or stored at suboptimal conditions, fungi grow, cause spoilage and a number of them produce mycotoxins (Aflatoxins, Ochratoxin) which are responsible for mycotoxicoses in man. According to Bukar, *et al.,* [15] The Isolation of these fungi in sweet oranges could be as a result of the

ability of fungi to produce resistant spores. They noted that spores of Aspergillus are quite resistant to high temperatures, such that exists in the northeastern part of Nigeria where the Gamboru market is located, creating an atmosphere that is conducive for the growth of these organisms.

The results obtained in this study revealed that most of the spoilage occurring in sweet oranges in the Gamboru market was caused by *Penicillium oxalicum* as it was the most prevalent of the four isolates on the sweet oranges, it was isolated from eighty-two (Table 2) of the orange samples. This organism was also isolated from the air in the shops where these oranges are sold.

This is in line with the study of [16] who identified Penicillium species as one of the fungi responsible for post-harvest rot in sweet oranges in Yemen. They also reported the isolation of *Aspergillus niger, Aspergillus flavus,* and *Aspergillus fumigatus.*

This result also agrees with the findings of [17] also reported the isolation of *A. niger. A flavus* and *A fumigatus* as being responsible for the spoilage of sweet oranges (*Citrus sinensis* L) in Sokoto state. In their study, they isolated *Rhizopus stolonifer* which was not isolated in this study. *Penicillium oxalicum* is a widespread species associated with oil seeds, cereals, fruit, and vegetables. It has been identified as a known producer of mycotoxins such as Oxaline, Secalonic acid D, and roquefortine C [14]. *Aspergillus niger* which was isolated in this study is a common contaminant occurring on a wide range of substances such as plants, fruits, vegetables, and nuts, some strains of this species have been implicated in the production of ochratoxins. *A flavus* is the principal producer of aflatoxins, it is widely distributed in nature; it grows on several agricultural crops before harvest or during storage and its growth is affected by the temperature and relative humidity of the environment [18]. *Aspergillus fumgatus* which is also widely distributed in nature produces versicolorins, sterigmatocystin, and tremorgenic mycotoxins among others [19]. Also, the results obtained from the air sampling carried out in the shops where the sweet oranges are sold, revealed the presence of all the fungal isolates reported in this study. Even though these fungi and their spores can contaminate the oranges from the farmland, during the harvesting process, especially when there is unprofessional handling of the fruits by the workers, while transporting or in storage, these results also suggest that the air could also be the source of the contaminants that cause spoilage in these oranges, it could also suggest that the contaminants which have spores that are readily airborne release their spores into the air and these can serve as inoculum to the next batch of oranges that are brought in for sales. A study by [20] also reported Aspergillus, Penicillium, and Cladosporium as some of the major fungal isolates found to be present in the air in some fruit markets in India which is also in agreement with the results obtained from this study. According to a study carried out in China by [21]. Cladosporium was one of the fungi frequently isolated in the air which agrees with the findings of this study.

4. CONCLUSION: This study has shown that the spoilage of oranges resulting in economic loss to both farmers and consumers is majorly caused by fungi species present in the air around the facilities where the oranges are sold and stored, and these fungi have been found to be pathogenic, thereby predisposing the sweet oranges to fungal spoilage. This calls for further research to investigate the possibility of

fumigating the fruit markets using fungicides periodically so as to minimize the loss occurring from these fungal contaminants.

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