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Assessment of fungal pathogens associated with orange spoilage

Oviasogie, F. E.[1], Ogofure, A. G.[1*], Beshiru, A.[1], Ode, J. N.[1] and Omeje F. I.[2]

[1]Department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City, Edo State, Nigeria.
[2]Department of Biological Sciences, Faculty of Sciences, University of Otuoke, Bayelsa State, Nigeria.

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Citrus sinensis **also known as sweet orange is the most popular of the citrus fruits. It is widely cultivated in most regions of the world possessing a rich source of vitamin C, flavonoids, phenolic compounds and pectin. This research was conducted to investigate the assessments of fungal pathogens associated with orange fruit spoilage sold in five markets in Benin metropolis and the possible public health implications. Some pathogenic fungal species were isolated from all five markets used in this study.** ***Aspergillus*** **species had the highest frequency and distribution from all sampling points (80%).** ***Alternaria*** **and** ***Saccharomyces cerevisiae*** **had the least occurrence from all sampling points (40% apiece).** ***Candida, Mucor, Penicillium*** **and** ***Rhizopus*** **had 60% occurrences, respectively.** ***Candida tropicalis*** **and species of** ***Rhizopus***, ***Penicillium***, ***Aspergillus***, ***Alternaria***, **and** ***Mucor*** **produced same symptoms and signs as observed in the original spoilt orange fruits before isolation. All fungal isolates were able to re-infect the healthy orange fruits with the exception of** ***Alternaria*** **species and** ***Saccharomyces cerevisiae*** **which were not able to grow and produce spoilage condition on the inoculated healthy orange fruits after five days.** ***Aspergillus*** **spp. are known to produce several toxic metabolites, like aflatoxins and ochratoxins, which are very important toxins worldwide because of the hazard it poses to human and animal health.**

Key word: Pathogenicity test, *Aspergillus* sp., *Alternaria* sp., pathogens.

INTRODUCTION

Fruits and vegetables are very important and have high dietary and nutritional qualities. Consumption of fruit and vegetable products has dramatically increased by more than 30% during the past few decades (Barth et al., 2009). They are good sources of nutrients for growth, repair and control of body processes as most of them contain sugar, vitamins, mineral elements and small quantities of protein and oil (Zubbair, 2009). *Citrus sinensis,* also known as sweet orange, is the most popular of the citrus fruits. It is widely cultivated in most

*Corresponding author. E-mail: ogofureabraham@live.com.

regions of the world (Muhammad et al., 2013). Oranges form a rich source of vitamin C, flavonoids, phenolic compounds and pectins. The main flavonoids found in citrus species are hesperidine, narirutin, naringin and eriocitrin (Ghasemia et al., 2009). Just one orange provides 116% of the daily requirement for vitamin C. Vitamin C is the primary water-soluble antioxidant, which prevents free radical generation in the body and damage to the tissues in the aqueous environment both inside and outside cells (Milind and Diev, 2012). Drinking of orange juice without salt and sugar is associated with reduced severity of inflammatory conditions, like asthma, osteo-arthritis, and rheumatoid arthritis. Vitamin C is also necessary for the proper functioning of immune system. Vitamin C is good for preventing cold, cough and recurrent ear infections (Guanieri et al., 2007). These losses are due to many factors, among which post-harvest fungal diseases are considered as principal cause. Sweet orange are vulnerable to post-harvest diseases. It was observed in previous studies, that the extent of damage varied from 29.9 to 43.8% in sweet orange and 25.5 to 36.8% in acid lime (Reddy et al., 2008). Studies have shown that oranges have been found to protect the moderate consumer against cardiovascular diseases (Milind and Diev, 2012), possess anti-carcinogenic properties (Tanaka et al., 1997), reduce the risk of kidney stones (Honow et al., 2003), possess anti-ulcer properties (Simon et al., 2003), antianxiety effect (Fsaturi et al., 2010), anti-typhoid activity (Vivek et al., 2010), antibacterial activity (Milind and Diev, 2012) and antifungal activities (Neeta and Abhishek, 2008) amongst the many medicinal uses.

The improper handling, packaging, storage and transportation may result in decay and growth of microorganisms, which become activated because of the changing physiological state of the fruits and vegetables (Wilson et al., 1991). Fruit, due to their low pH, higher moisture content and nutrient composition are very susceptible to attack by pathogenic fungi, which in addition to causing rots, may also make them unfit for consumption by producing mycotoxins (Moss, 2002). The principle of spread of fungal infection in fruits supports that a single infected orange fruit can be the source of infection to other orange fruits during storage and on transit (Jay, 2003). Soil-infesting fungi and bacteria that cause loss of fleshy tissue typically infect plants at the time of or just before harvesting. Infection may occur, however, during post-harvest handling or storage. Common air molds such as *Penicillium* species may gain entry into the susceptible tissue and cause loss during packaging. *Penicillium digitatum* and *Penicillium italicum* causes green and blue mold diseases respectively which are universal post-harvest diseases of citrus. The extensive spore production by these pathogens ensures its presence wherever fruit was handled, including field, packing house, equipment, de-greening, storage rooms, transit containers and market place (Ismail and Zhang, 2004).

The aim of this study was to isolate and identify fungal pathogens associated with orange spoilage in Benin City metropolis, Edo state, Nigeria, using five markets as case study.

MATERIALS AND METHODS

Collection of samples

Healthy, viable orange fruits were purchased from different markets in Benin City, Edo State. The orange fruits were transported in sterile polyethylene bags to the laboratory for analysis.

Physical examination of sample

The physical examinations of spoiled or diseased oranges were identified using the method of (Balali et al. (1995) where various types of spoilt oranges were selected including those that were mechanically wounded or bruised, with purplish to dark brown rot, blue rot, green rot as well as those with black lesions on them.

Preparation of culture medium

Potato dextrose agar was used for isolation of fungi from the Citrus fruits and for the preparation of pure cultures. The medium was prepared from commercially produced dehydrated medium following the manufacturer's instruction. Thirty-nine (39) grams of Potato Dextrose agar powder was dissolved in 1 L of distilled water in a sterile conical flask covered with cotton wool and aluminium foil paper. It was mixed thoroughly and autoclaved at 121°C for 15 min under a pressure of 15 pounds per square inch (15lb/inch^2). The medium was cooled after autoclaving to 50°C and then dispensed aseptically into sterile Petri dishes. Streptomycin (0.3%w/v) was added to the medium to prevent the growth of bacteria.

Isolation of fungi

Two hundred (200) fruits samples were washed and sterilized with 70% ethanol. The borderline between healthy and infected tissue of surface fruits was cut with sterile razor blade. The cut portion of the lesion was disinfected with ethanol of 70% concentration for 2 min. These were then rinsed in three different changes of distilled water. Each excised portion of the infected part showing lesions were plated in Potato Dextrose Agar plates containing streptomycin (30 mg/l) to prevent the growth of bacteria. The plates were incubated at room temperature (28°C) for 72 h.

Identification of fungal Isolates

The fungi isolates were identified on the basis of macro-morphological and micro-morphological characteristics. The morphological characteristics which include colony growth and colour, presence or absence of aerial mycelium, presence or absence of wrinkles and furrows, presence or absence of pigmentation amongst others were observed under the microscope (Thiyam and Sharma, 2013; Barnett and Hunter, 1972) and recorded. In all cases, a drop of lactophenol blue stain was placed

on a clean grease-free sterilized glass slide after which a sterile inoculating wire loop was used to pick the mycelium unto the glass slide from the mold culture. The mycelium was then spread evenly on the slide. Teasing was done to separate the mycelium in order to get a homogenous mixture and the mixture was then covered with cover slips gently and then allowed to stay for some seconds before observing with the microscope under ×40 magnification lens. The microscope examination of actively growing mold was on the basis of structures bearing spores, presence or absence of septa.

Pathogenicity test

Pathogenicity test was carried out as described by Baiyewu et al. (2007) and Chukwuka et al. (2010) where each of the fungal isolates were tested on healthy fruits for its ability to induce spoilage. The methods by these authors are outlined below:

a) Clean mature healthy fruits were washed with tap water and rinsed with distilled water after which they were surface sterilized with 75% ethanol.

b) A sterile 4 mm cork borer was used to make holes in each of the fruits.

c) A colony of fungi isolate (from each pure culture) was used to inoculate the fruits and the core of the fruits were replaced.

d) The point of inoculation was sealed with petroleum jelly to prevent contamination.

e) Controls of orange fruits were wounded with the sterilized cork borer but not inoculated.

f) The inoculated fruits and the controls were placed in clean polyethylene bag (one fruit per bag) each moistened with wet balls of absorbent cotton wool to create a humid environment and incubated at 30 ± 1°C for 5 days.

e) After 72 h, the inoculated fruits were observed for symptom development.

f) The causal agents were re-isolated from the infected orange fruit and compared with the original isolates. This experiment was replicated three times.

RESULTS

The Table 1 shows the occurrence and distribution of each fungal isolates from the five sampling points. *Aspergillus niger*, *Mucor* species and *Rhizopus* species were fungal species isolated from New Benin market. *A. niger*, *P. chrysogenum*, *R. stolonifer*, *Alternaria*, *C. tropicalis* and *Mucor* species were fungal isolates from sweet orange from Oba market. Fungal isolates such as *A. niger*, *Alternaria* species, *R. stolonifer*, *C. tropicalis* and *Saccharomyces cerevisiae* were identified from sweet oranges obtained from Uselu market while Satanna market sweet oranges had fungal isolates such as *Penicillium digitatum*, *C. tropicalis* and *Mucor* species.

Table 2 shows the percentage occurrence and distribution of the fungal isolates from all sampling market points. While *Aspergillus* species had the highest percentage occurrence (80%), *Alternaria* species had the lowest percentage occurrence (40%).

Table 3 reveals the pathogenicity test on fresh healthy citrus fruit samples. From day 0 to day 5, *Alternaria* species and *Saccharomyces cerevisae* were not able to grow on the sample. However, from day 1 to day 5, *Rhizopus*, *Penicillium* and *Aspergillus* species were able to grow with similar growth characteristic features to the original diseased sample. More so, *Mucor* and *Candida* species were also able to grow with similar growth characteristic features to the original diseased samples from day 2 to day 5.

Table 4 describes the spoilage pattern on sweet orange (*Citrus sinensis*) produced by isolated fungal species. Overall, the fungal isolates include *A. niger*. *C. tropicalis*, *R. stolonifer*, *P*. chrysogenum, *P*. *digitatum* and *M*. species.

DISCUSSION

It is estimated that about 20-25% of the harvested orange fruits can be deteriorated by pathogens during post-harvest handling even in developed countries (Droby, 2006; Zhu, 2006). In developing countries, postharvest losses are often more severe due to inadequate storage and transportation facilities. Fungal fruits infection may occur during the growing season, harvesting, handling, transport and post-harvest storage and marketing conditions, or after purchasing by the consumer.

Orange fruits contain high levels of sugars and nutrients element and their low pH values make them particularly desirable to fungal decay (Singh and Sharma, 2007).

The seven fruit spoilage fungi were isolated from the two hundred (200) orange fruit samples and identified as *Aspergillus* species, *Mucor* species, *Penicillium*, *Rhizopus*, *C. tropicalis, S. cerevisiae* and *Alternaria* species. *Aspergillus* spp. are widespread among all examined spoilage fruits with the highest percentage occurrence of 80% from all sampling points. Fourty orange fruit were sampled in each market point. *Aspergillus* species, *Penicillium* species, *Mucor* species and *Rhizopus* species were able to cause spoilage on re-infection with healthy fruits, while *Alternaria* species showed no growth. Bukar et al. (2009) reported that *Aspergillus* species, *Mucor*, *Penicillium* species and *Rhizopus* sp, which are the same genus with those isolated from orange fruits in this study, as responsible for the soft rots of orange fruits in Nigeria. The spoilt sweet oranges sampled from the different markets in Benin City were found to be massively infected by different species of fungi. This is similar to the findings of Bukar et al. (2009) who reported that diseased oranges sampled from Na'ibawa yan Lemu Market in Kano were found to be massively infected with six genera of fungi namely *Fusarium, Aspergillus*, *Candida, Rhizopus, Penicillium* and *Mucor*. The occurrence of these organisms may be attributed to their ability to produce resistant spores, as reported by Hocking (2006) that “Aspergilli generally grow at higher temperatures or lower

Table 1. Occurrence and distribution of the fungal isolates from the sampling market points.

Fungal isolates	Ekiosa	New Benin	Oba market	Uselu market	Satanna market
Rhizopus species	-	+	+	+	-
Penicillium species	+	-	+	-	+
Aspergillus species	+	+	+	+	-
Alternaria species	-	-	+	+	-
Mucor species	-	+	+	-	+
Candida tropicalis	+	-	-	+	+
Saccaromyces cerevisiae	-	-	+	+	-

+, represent presence; -, represent absence.

water activities than Penicillia and they usually grow more rapidly than Penicillia, although they take longer to sporulate, and produce spores which often are more resistant to light and chemicals".

Akintobi et al. (2011) reported that *Aspergillus flavus, A. niger, Fusarium solani, Penicillium digitatum, R. stolonifer* and yeasts were found in fruits sold in major markets in Ibadan, Oyo State, South Western Nigeria. *P. digitatum*, *R. stolonifer*, and *A. niger* were found to be associated with spoilage or deterioration of orange fruits in Ibadan (Akintobi et al., 2011). This could be due to the presence of their spores, which in turn releases toxins into the oranges or even releases enzymes which could contribute to the deterioration of the oranges. Samples from Oba market had the highest occurrence of fungal isolates. This might be due to the high population density as the market is located at the heart of the city where virtually anyone from all classes can be found to carry out one economic activities. Sweet oranges from Satanna market had the least occurrence of fungal isolates and this could be as a result of improved personal hygiene of handlers or good storage methods. Taking the population of and size of the market into consideration, it is far smaller than Oba market, Uselu market and more likely, Ekiosa market. In conformity with this research, Effiuvwevwere (2000) reported that contamination of fruits and vegetables by fungi could be as a result of poor handling practices in food supply chain, damage inflicted on fruits at time of harvest creating a route for spores of pathogenic fungus, poor storage condition, distribution, marketing practices and transportation. Different spoilage types were observed on re-inflection of healthy oranges with pure isolate of fungi species. This could be as a result of the ability of the fungi species to survive in the oranges especially when the environmental conditions are favourable, producing spores, toxins and enzymes. This is similar to the findings of Bukar et al. (2009) who reported that different spoilage types were observed when the healthy oranges were re-inoculated with the pure isolates of the pathogens. Some however, did not cause spoilage on re-inoculation. *Aspergillus* species, *Penicillium*, *C. tropicalis*, *Mucor* species and *Rhizopus* species were the fungi that caused spoilage of the sweet oranges in this study. This is in conformity with Bukar et al. (2009) who revealed that *Aspergillus* species, *Penicillium* species, *Mucor* species and *Fusarium* species were the fungal that were able to cause re-infection in the healthy oranges after the pathogenicity tests.

Several fruit spoilage fungi from different region has been isolated and identified. *A. niger* and *C. tropicalis* were found associated with deterioration of orange; this is in line with the work of Nijis et al. (1997) who reported that *Aspergillus* species is the predominant organism associated with the spoilage of orange. The isolation of *R. stolonifer* and *Mucor* species from orange confirmed the studies of Bukar et al. (2009) who reported that *Mucor* species and *Rhizopus stolonifer* are responsible for the spoilage of orange.

Similarly, Voysey (2011) reported that *Alternaria* sp. causes black rot in citrus fruits, *Aspergillus* species causes brown rot of citrus fruits and pineapple, *Penicillium* species causes blue and green mould rots of citrus fruits, apples, grapes, pears and also brown rot of pineapple, *Aspergillus* species and *R. stolonifer* causes watery, soft rot of apples, pears, stone fruits and grapes, *Geotrichum* species causes sour rot of citrus fruits, *Trichoderma* species causes cocoa-brown to green rot of citrus fruits. The principle of spread of fungal infection in fruits supports that a single infected orange can be the source of infection to other oranges during storage and on transit (Jay, 2003).

The presence of the fungi or their resistant spores is most likely to have originated from the farms where the fruits were harvested and some from the stores due to horizontal contamination by the already spoilt fruits as Jay (2003) observed that most spoilage organisms may be present on fruits and vegetables from the farm, during harvest operations, and this may result in post-harvest contamination and spoilage of these fruits and vegetables. The present and subsequent spoilage due to these fungi, if not checked could lead to serious economic loss and possible health hazards when these

Table 2. Percentage of occurrence and distribution of the fungal isolates from all sampling market points.

Fungal isolates	Percentage occurrence (%)
Aspergillus species	80
Penicillium species	60
Mucor species	60
Rhizopus species	60
Candida tropicalis	60
Saccharomyces cerevisiae	40
Alternaria species	40

Table 3. Pathogenicity test on fresh healthy citrus fruit samples.

Fungal isolates	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Rhizopus species	-	+	+	+	+	+
Penicillium species	-	+	+	+	+	+
Aspergillus species	-	+	+	+	+	+
Alternaria species	-	-	-	-	-	-
Mucor species	-	-	+	+	+	+
Candida tropicalis	-	-	+	+	+	+
Saccaromyces cerevisiae	-	-	-	-	-	-

+ = Isolates grow with a similar growth characteristic features to the original diseased samples; - = Isolates not able to grow on the sample.

Table 4. Spoilage pattern on sweet orange (*Citrus sinensis*) produced by isolated fungal species.

Fungal isolates	Spoilage pattern produced
Aspergillus niger	Dark brown discoloration, sunken spots, fruits become spongy with gas production
Candida tropicalis	Fruit becoming spongy with gas production, sunken spots
Rhizopus stolonifer	Watery, soft rot wrinkled appearance with depression and yellowish in color
Penicillium chrysogenum	Wrinkled appearances, pale green-blue, exuding bright yellow pigment into the medium
Penicillium digitatum	Wrinkled appearances with sunken spots and ;live green in color
Mucor species	Whitish mycelia growth with a cream white color

fruits are consumed. Generally, spoilage fungi are considered toxigenic or pathogenic. Toxigenic fungi have been isolated from spoilt fruits (Stinson et al., 1981). During storage at room temperature, some moulds may produce mycotoxins (Tournas and Katsoudas, 2005). Pathogenic fungi, on the other hand, could cause infections or allergies (Monso, 2004). *Aspergillus* spp. are known to produce several toxic metabolites, such as malformins, naphthopyrones (Frisvad and Samson, 1991; Pitt and Hocking, 1997) and they can produce Ochratoxins (OTA), a mycotoxin which is a very important toxin worldwide because of the hazard it poses to human and animal health (Peraica et al., 1999; Petzinger and Weidenbach, 2002) thus extra care should be taken during personnel handling of these fruits; such as harvesting, cleaning, sorting, packaging, transport and storage.

The high prevalence of some fungi demand that appropriate control measures against infection, should be employed if farmers expect good performance of their produce. The fruits used in this study are not cultivated in the city but are transported to from distant villages in locally woven baskets and sacks under weather conditions that encourage the incubation of these contaminating microorganisms. It is therefore important that both the farmer who harvests the fruits into bags for transportation, the marketers and consumers take

necessary precaution in preventing contamination and also try to create an environment that will not encourage the growth or multiplication of microorganisms.

Conflict of interests

The authors did not declare any conflict of interest.

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