Journal Homepage: - www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)

Article DOI: 10.21474/IJAR01/13324
DOI URL: http://dx.doi.org/10.21474/IJAR01/13324

RESEARCH ARTICLE

FUNGAL DETERIORATION OF LEMON (CITRUS LIMON BURN F.) AND VITAMIN C CONTENT OF INFECTED FRUITS FROM KEFFI, NASARAWA STATE

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Manuscript Info

Manuscript History
Received: 29 June 2021
Final Accepted: 30 July 2021
Published: August 2021

Key words:-
Fungi, Deterioration, Lemon, Vitamin C, Keffi

Abstract

This study was carried out in Keffi Metropolis to evaluate fungi associated with the deterioration of lemon (Citrus limonBurn F.) and the vitamin C content of the infected fruits. The lemon fruit samples were obtained from four selected marketing centres in Keffi metropolis. These includes Keffii main market, AngwanLambu, AngwanKaje and Angwan Fulani respectively. Out of 48 samples of lemon fruits examined, 34 had fungal species while 14 had no fungal species. The fungal analysis showed that Aspergillus niger, Rhizopus stoloniferand Penicillium digitatumwere associated with the spoilt lemon fruits (C. limon) with frequencies of occurrence of 61.76%, 17.65% and 20.59% respectively. Some fresh lemon fruits stored at a temperature of 25^{o}C and 30^{o}C showed no sign of decay. The incidence of fungal species in lemon fruits from the different sampling markets include Keffi main market (26.47%), AngwanLambu (20.59%), AngwanKaje (29.41%) and Angwan Fulani (23.53%)respectively. There is no significant difference (P>0.05) between species of fungi isolated from different sources (markets) in Keffi. Pathogenicity test showed that Penicillium digitatumwas not a mere contaminant of lemon fruits but a primarily causative organism (80%) followed by Rhizopus stolonifer(60%) and Aspergillus niger(60%). Vitamins C content of both the infected and uninfected lemon fruits showed that infected fruits gave 31.37mg/100ml of vitamin C while the uninfected fruits gave 32.47mg/100ml vitamin C. Consumption of deteriorated lemon fruits should be discouraged.

Introduction:-

Lemon (*Citrus limon*Burn F.) also known as “osanwewe” (in Yoruba language), “oromankiri” (in Igbo language) and “babbanleemu” (in hausa language), is a species of small evergreen tree that belongs to the genus *Citrus*, Rutaceae family to which belong also citrus fruits such as oranges, tangerine, grapefruit, bergamots, citrons (Taiwo, 2005).It is an ancient hybrid, native to Asia, typical of warm regions, halfway between the pomelo and citron, but since centuries an independent species which spreads through scions and grafts (Wilson *et al*., 1991). Lemons are grown globally and commercially, in Nigeria, greater percentages of them are produced and sold for income and a fraction of it used for human consumption.

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The fruits of lemon are oval or elongated, with pointed apexes. The skin is usually yellow, but there are also varieties of different colours, such as green or white. The skin is rich in essential oils and can be more or less thin. The flesh of lemon is generally bitter and juicy. The crisp and tangy aroma of lemon is unmistakable and it is often used to scent many different household and beauty products. Lemon are known for their high vitamin C content. This vitamin C, along with other vitamins and minerals in lemons has been shown to help fight infections, boost immune system, and even promote weight loss efforts (Ralp and Bender, 2000).The lemon contains the 71% of daily need for vitamin C for an adult person, 7% of potassium, 1% of calcium and the 9% magnesium requirement. The juices of lemons are often used to make tonics, a refreshing drink and for health purposes. Lemon juice is an acid (pH 2-3) as it is made up of about 5% citric acid (Rauf *et al*., 2014).

The common lemon "rough lemon" is widely grown from seed. The 'Meyer' lemon is easily reproduced by rooting large cuttings in the nursery and planting them directly in the groove. They fruit 2 to 3 years sooner than budded trees and have a long life, remaining in full production for over 30 years, perhaps much longer (Morton, 1987).A fungus(pluralfungi) is any member of the group of eukaryotic organisms that includes unicellular microorganisms such as yeasts and molds, as well as multicellular fungi that produce familiar fruiting forms known as mushrooms. These organisms are classified as a kingdom, Fungi, which is separate from the other eukaryotic life kingdoms of plants and animals. Many fungi produce biologically active compounds, several of which are toxic to animals or plants and are therefore called mycotoxins. Of particular relevance to humans are mycotoxins produced by molds causing food spoilage (Blackwell, 2011). Therefore, this research is aimed to evaluate fungi associated with the deterioration of lemon (*C. limon*) and the vitamin C content of the infected fruits from Keffi.

Materials and Methods:-

Study Area

The study was conducted in Plant Science and Biotechnology Unit laboratory Department of Biological Sciences, Nasarawa State University, Keffi. The survey was carried out in some selected markets in Keffi local Government Nasarawa State, Nigeria. These include; Keffi Main Market, AngwanLambu, AngwanKaje, and Angwan Fulani.

Sample Collection

A total of forty-eight(48) lemon fruits (*Citrus limon*) were obtained from four different markets in Keffi metropolis, randomly selecting twelve samples from each source (market); those that look sunken and shrivelled, those with different coloured lesions with water-soaked appearance around the wound and healthy ones were collected. Diseased lemon fruits were identified by physical examination following the method of Kutama*et al.* (2008). These lemon fruit samples were then transported immediately to Plant Science and Biotechnology Unit laboratory, Department of Biological Sciences, Nasarawa State University, Keffi for fungal analysis.

Preparation of Culture Medium

Potato dextrose agar was used for culturing of fungi from the deteriorated lemon fruits. Thirty-nine (39) grams of potato Dextrose Agar powder was weighed out and dissolved in 1L of distilled water in a sterile conical flask covered with cotton wool and aluminium foil paper. It was mixed thoroughly and autoclaved at 121^{o}C for 15 minutes under a pressure of 15 pounds per square inch (15lb/inch^{2}) (Downes and Itok, 2001). The medium was cooled after autoclaving to 50^{o}C and then dispensed aseptically into sterile Petri dishes. Streptomycin (30mg/l) was added to the medium to prevent the growth of Bacteria.

Isolation of Fungi

The infected lemon fruits were surface sterilized with cotton wool soaked in 70% alcohol. The fruits were then cut into two using sterilized scalpel. The segments of the infected fruits were then plated on solidified Potato Dextrose Agar plates (containing streptomycin 30 mg/l to prevent the growth of bacteria) aseptically using Onyeka*et al.* (2003) method. Inoculated plates were incubated at room temperature (28^{o}C) for 7days. From the incubated plates the differentcolorations observed include: (i) Brown (ii) Black and (iii) White, which signified the occurrence of different fungal colonies.

Identification of the Fungal Isolates

The pure cultures of the fungal isolates were identified using cultural and morphological features such as colony growth pattern, conidial morphology and pigmentation with reference to Domsch*et al.* (1980), Damson *et al.* (1984) and Rippon (1988). In all cases, a drop of 0.5% lactophenol cotton 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 mould culture. The mycelium was then spread evenly on the slide. Teasing was done to separate the mycelium in order to get a homogenous mixture.The mixture was then covered with a cover slip gently, after which it was viewed under the light microscope first with (x10) and then with (x40) objective lens to detect spores, hyphae and other special structures.

Data Analysis
Data obtained from the survey were subjected to Chi-square test.

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 was tested on healthy fruits for its ability to induce spoilage. Briefly, twenty (20) clean mature healthy lemon fruits were surface sterilized with 70% alcohol. A sterile 4 mm cork borer was used to make holes in each of the lemon fruits. A colony of fungal isolate (from each pure culture) was used to inoculate fifteen (15) of the fruits. The point of inoculation was sealed with petroleum jelly to prevent contamination. Controls of lemon fruits were wounded with sterilized cork borer but not inoculated. The inoculated lemon fruits and the controls were placed in clean polyethylene bag (one lemon fruit per bag) each moistened with wet balls of absorbent cotton wool to create a humid environment and incubated at $30 \pm 1^{o}C$ for 2-7 days for fungal growth.

Titrimetric Determination of Vitamin C (Ascorbic Acid) in Infected and Uninfected Lemons Fruits
Vitamin C was determined by acid-base reaction (oxidation-reduction). 2, 6-dichlorophenolindophenol(DCPIP) solution was used as an indicator for vitamin C.

Methods:-

0.2 g of ascorbic acid was weighed out and made up to 1 L of distilled water.The concentration of the ascorbic acid solution was calculated using the formula below:
Concentration of ascorbic acid = Mole/Molar mass/Volume (AOAC, 2016)
Approximately 0.24g DCPIP was weighed out and made up to 1 L of distilled water.
The concentration of DCPIP solution was calculated using the formula below:
Concentration of DCPIP solution = Mole/Molar mass /Volume (AOAC, 2016)
25 ml of 0.5% oxalic acid was measured and transferred into 250ml conical flask.
10 ml of standard ascorbic acid solution was added into the conical flask which contained oxalic acid.
A trial run of titration was carried out with a titration set. The ascorbic acid solution was titrated rapidly with DCPIP solution. The DCPIP solution was added through the burette and solution was vortex well. Colour change of DCPIP solution to pink was observed when the solution came in contact with the ascorbic acid solution and then became colourless after it was well shaken. After trial run, another three actual titrations to the ascorbic acid standard solution was conducted and the results were averaged. Then, DCPIP solution was added drop by drop carefully when the volme of DCPIP solution used was close to the end point volume.
The volume of DCPIP solution used was recorded.
The concentration of the DCPIP solution was calculated using the formula below;

CV (Ascorbic acid) = CV (DCPIP)
*C refers to Concentration
*V refers to Volume (AOAC, 2016)

Sixteen lemon fruits (eight infected and eight uninfected) were cut in half with knife and their juices squeezed out. The fruit juice was collected with the aid of a funnel and filter paper, the flesh (pulp) and seed was separated from the juice.

10 ml of the fruit juice was pipetted into a 250 ml conicl flask, which contained 25ml of 0.5% oxalic acid, and 10 ml of distilled water was added.

The fruit juice solution was titrated with the DCPIP solution in the burette to a pink end point.

The test was triplicated and results were averaged.The vitamin C concentration in the lemon fruit juice was calculated using the following formula:
Mole (Vitamin C) = CV (DCPIP solution)

Mass/Molar mass = CV
Mass= Mr (vitamin C) x C (DCPIP) x V (DCPIP)
*Mr refers to Molar mass
*C refers to Concentration
*V refers to Volume (AOAC, 2016)

Results and Discussion:-

From the results obtained 48 lemon fruits were sampled, out of which 34 lemon fruits had fungal species while 14 samples were without fungal species (Table 1). The species of fungi isolated and identified from the deteriorated lemon fruits were *Aspergillus niger*, *Rhizopus stolonifer*and *Penicillium digitatum*. Their frequencies of occurrence were 61.76%, 17.65% and 20.59% respectively (Table 2).

The effect of temperature on lemon fruits is presented in Table 3. Lemon fruits kept in cold storage (4^oC) showed no sign of decay while lemon fruits stored at temperatures 25^oC and 30^oC showed symptoms of decay.

The incidence of fungi species in the different markets in Keffi presented in Table 4. AngwanKaje had the highest incidence of fungi (29.41%) while AngwanLambu had the lowest incidence (20.59%).

There is no significant difference ($P>0.05$) between species of fungi isolated from different markets (locations) in Keffi (Table 5).

Pathogenicity test showed Penicillium digiatumhad higher percentage of infection (80%) after artificial inoculation while Rhizopus stoloniferand Aspergillus nigerhad lower percentages (60%) (Table 6).The vitamin C content of infected lemon fruits was less than that of uninfected lemon fruits (Table 7)

Table 1:- Numeric Representation of Lemon Fruits with and without Fungal Species from Different Sources (Markets) in Keffi.

Source (Market)	**Total Number of Samples**	**Number with Fungal Species**	**Number without Fungal Species**
Keffi main Market	12	9	3
AngwanLambu	12	7	5
AngwanKaje	12	10	2
Angwan Fulani	12	8	4
Total	**48**	**34**	**14**

Table 2:- Types and Frequency of Occurrence of Identified Fungi Isolated from Diseased Lemon Fruits Obtained from the Markets.

Source (Market)	***Aspergillus niger***	***Rhizopus stolonifer***	***Penicillium digitatum***	**Frequency of Attack**
Keffi Main Market	7	0	2	9
AngwanLambu	4	3	0	7
AngwanKaje	10	0	0	10
Angwan Fulani	0	3	5	8
Total	21	6	7	34
Percentage Total	**61.76%**	**17.65%**	**20.59%**	

Table 3:- Effect of Temperature on Fresh Lemon Fruits.

Duration	**Above Room Temperature –Oven (30^oC) (Observable Signs)**	**Room Temperature (25^oC) (Observable Signs)**	**Cold Storage – Refrigerator (4^oC) (Observable Signs)**
Day 2	Yellowish brown discoloration	Slightly discoloured spot	No decay
Day 4	Appearance of greyish fluffy mass	Loss Firmness	No decay
Day 6	Visible black spores	Slightly softening	No decay
Day 8	Crispy rind	Watery, sunken appearance	No decay
Day 10	Appearance of black mould rot	Olive green spores, following the	No decay

		appearance of white mycelium around the rind	

Table 4:- Incidence (%) of Fungal Attack on Lemon Fruits Obtained from Different Sources (Markets) in Keffi.

Source (market)	*Aspergillus niger*	*Rhizopus stolonifer*	*Penicillium digitatum*	Frequency of Attack	Mean	Percentage Incidence
Keffi Main Market	7	0	2	9	3.00	26.47
AngwanLambu	4	3	0	7	2.33	20.59
AngwanKaje	10	0	0	10	3.33	29.41
Angwan Fulani	0	3	5	8	2.67	23.53
Total	21	6	7	34	11.33	
Mean	5.25	1.5	1.75			
Percentage total	**61.76%**	**17.65%**	**20.59%**			

Table 5:- Chi-Square on the Relationship between Fungal Species in Lemon Fruits Obtained from Different Sources (Markets) in Keffi.

Source (Market)	Total Number of Samples	Number with Fungal Species	Number without Fungal Species
Keffi main Market	12	9 (8.5)*	3 (3.5)*
AngwanLambu	12	7 (8.5)*	5 (3.5)*
AngwanKaje	12	10 (8.5)*	2 (3.5)*
AngwanFualni	12	8 (8.5)*	4 (3.5)*
Total	**48**	**34**	**14**

H_0: There is no significant difference (P>0.05) between species of fungi isolated from different markets (sources) in Keffi.Since the Tabulated value 7.815 is greater than the calculated value 2.0 H_0 is accepted

Table 6:- Percentage of Infection of Healthy Lemon Fruits Artificially Inoculated with Fungi Isolated from Diseased Lemon Fruits.

Fungal Isolate	Number of Lemon Fruits Inoculated	Infection after 5 days of Inoculation (%)
Aspergillus niger	5	60
Rhizopus stolonifer	5	60
Penicillium digitatum	5	80

Table 7:- Vitamin C concentration of Uninfected and Infected *Citrus limon.*

Lemon condition	Vitamin C content (mg/100ml)
Uninfected	32.47
Infected	31.37

Discussion:-

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 pot-harvest storage and marketing conditions, or after purchasing by the consumers.Lemon fruits contain high levels of nutrients element and their low pH values make them particularly desirable to fungal decay (Singh and Sharma, 2007).

This study revealed the species of fungi which are responsible for the diseases of lemon fruits in Keffi Local Government Area of Nasarawa State, Nigeria. It is estimated that about 20-25% of the harvested lemon fruits can be deteriorated by pathogens during postharvest handling even in developed countries (Droby, 2006; Zhu, 2006). The presence and isolation of these fungal species depict that they are the causal agents responsible for the deterioration

of such an economical and medicinal plant. The fungal species isolated and identified include *Aspergillus niger*, *Rhizopus stolonifer*and *Pnicilliumdigitatum*.

Out of the three fungal species isolated and identified. *Aspergillus niger*had the highest (61.76%) frequency of occurrence followed by *Penecilliumdigitatum*(20.59%) and *Rhizopus stolonifer*which had the lowest (17.65%). These occurrences may be attributed to their ability to produce resistant spores, as reported by Hocking (2006), He stated that "Aspergilligenerally grow at higher temperatures or lower water activities than Penicillia and they usually grow more rapidly than Penicillia, although they take longer to sportulate, and produce spores which often are more resistant to light and chemicals."

Some fresh lemon fruits stored at a temperature of 4^oC showed no sign of decay while lemon fruits stored at temperatures 25^oC and 30^oC showed signs of decay, which are characteristics of fungal species. These signs include brown discolourations with sunken spots which is attributed to *Aspergillus niger*, water-soaked wrinkle appearance with a fluffy texture which is attributed to *Rhizopus stolonifer*and appearance of olive green mould which is a distinct feature of *Penicillium digitatum*.

AngwanKaje had more fungal attack than any other market while AngwanLambu had the least fungal attack. In conformity with these findings, Effiuvwevwere (2000) reported that contamination of fruits 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 fungi, poor storage condition, distribution, marketing practices and transportation. The percentage incidence of fungi attack in lemon fruits obtained from the four sampling markets showed that AngwanKaje had the highest (29.41%) while AngwanLambu had the lowest (20.59%), this could be due to little variation in soil type.

There is no significant difference ($P>0.05$) between species of fungi isolated from the different sources (markets) in Keffi, because they are all within the same geographical location.

In respect to the test on the concentration of vitamin C in lemon fruits carried out on both the infected and uninfected samples, the former had 31.37mg/100ml and the latter had 32.47mg/100ml respectively. This clearly shows that the effect of fungal attack actually reduced the vitamin C content in lemon fruits. This finding is in line with the findings of Olsen (2004).

*Penicillium digitatum*causes pulmonary infections in humans, this fungal species is associated with much higher mortality rates in patients with nosocomical infections or infections complicating organ failure (Chen *et al*., 2001). Extra measures have to be taken in the storage of lemon fruits so as to eradicate or minimize the incidence of fungi, because the present and subsequent spoilage due to these fungi if not checked, could lead to serious economic loss and possible health hazards when these fruits are consumed.

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