Original article

Mycoflora within black plum (*Vitex doniana* sweet) fruits

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Abstract — **Introduction**. Black plum (*Vitex doniana* Sweet) is a plant widely used by various communities in Nigeria for many purposes, including production of wine and jam. Ripe mature black plum fruit for food use commonly collected from the ground instead of plucked which is more hygienic. An investigation was conducted to find out the mycoflora on the surface and within the fruits of black plum fruits. **Materials and methods**. Mycoflora associated with the surface, skin, pulp and juice of healthy intact fruits of black plum was isolated in malt extract agar and potato dextrose agar and identified. Pathogenicity test was carried out with three pathogens of the fruit. **Results and discussion**. Fungi with incidence of 20–100% of occurrence were classified as major components and those below 20% of occurrence were regarded as minor components. Yeasts making up the major components in the ripe fruits were *Saccharomyces cerevisiae* and *Candida albicans*, while the minor yeast components were *Rhodotrula* sp., *S. coreanus* and *Pichia* sp. Filamentous fungi constituting major components were *Aspergillus niger* and *Penicillium* sp. whereas minor components were *Botryodiplodia theobromae* and *Penicillium chrysogenum*. Spoilage of fruits was caused by *B. theobromae, Aspergillus* sp. and *A. niger*. **Conclusion**. Yeasts and, occasionally, filamentous fungi occur within apparently healthy fruits of *Vitex doniana* sweet. Stages of development when the yeasts enter the pulp of the fruit need to be thoroughly investigated.

Nigeria / *Vitex doniana* (fruits) / plant diseases / yeasts / pathogens / microbial flora

Mycoflore de la prune des savanes (*Vitex doniana* sweet).

Résumé — **Introduction**. La prune des savanes (*Vitex doniana* sweet) est une plante très répandue au Nigéria ; elle est utilisée à différentes fins, dont la production de vin et de confiture. Pour sa consommation, le fruit mûr de la prune des savanes est généralement ramassé à terre au lieu d'être cueilli, ce qui serait plus hygiénique. Des études ont été entreprises pour analyser la mycoflore présente sur la surface et à l'intérieur de ce fruit. **Matériel et méthodes**. Les mycoflores associées à la surface, à la peau, à la pulpe et au jus de fruits intacts et sains de prunes des savanes ont été isolées dans de l'agar d'extrait de malt et de l'agar de dextrose de pomme de terre, puis identifiées. La pathogénicité a été testées sur trois pathogènes du fruit. **Résultats et discussion**. Les champignons observés à la fréquence de 20 à 100 % ont été classés comme principaux composants et ceux intervenant dans moins de 20 % des cas ont été considérés comme composants mineurs. Les levures identifiées comme principaux composants des fruits mûrs ont été *Saccharomyces cerevisiae* et *Candida albicans*, alors que *Rhodotrula* sp., *S. coreanus* et *Pichia* sp. se sont révélées en être des composants mineurs. Parmi les champignons filamenteux identifiés, les principales espèces trouvées ont été *Aspergillus niger* et *Penicillium* sp. tandis que *Botryodiplodia theobromae* et *Penicillium chrysogenum* ont été classées comme composants mineurs. Une détérioration des fruits a été provoquée par *B. theobromae, Aspergillus* sp. et *A. niger*. **Conclusion**. Des levures et, occasionnellement, des champignons filamenteux se développent dans des fruits apparemment sains de prune des savanes. Les stades de développement propices à la pénétration des levures dans la pulpe du fruit devront être plus précisemment déterminés.

Nigeria / *Vitex doniana* (fruit) / maladie des plantes / levure / agent pathogène / flore microbienne

Received 7 December 1999
Accepted 11 April 2000

Fruits, 2001, vol. 56, p. 85–92

***Resumen Español**, p. 92*

1. Introduction

Vitex doniana sweet (black plum) of the family Verbanaceae is abundant and widespread in open places, especially in farm land area in savanna regions [1, 2]. With growing emphasis on upgrading traditional plant food resources in Nigeria, there is the need for better understanding of existing plants including the currently underutilized species. *V. doniana* represents one of our neglected untapped forest resources [3].

Fruits play an important role in the diet of many people in the tropics providing essential minerals and vitamins and adding flavor, color and variety to what would otherwise be a monotonous carbohydrate-rich diet. They also contribute protein and calories. Economic losses caused by postharvest diseases are heavier than is generally realised because fresh fruits and vegetables deteriorate in unit value while passing from the field at harvest to the consumer [4–5]. *Vitex doniana* is a plant widely used by various communities in Nigeria for many purposes [3, 6]. The wine produced locally using rural dwellers' technology is of immense social and nutritional significance [7]. Black plum fruit has been fermented to produce wine of high palatable quality and good flavor [7] and jam has also been produced from the fruit [6].

The tissue of normal healthy fruits is generally, without experimental evidence, considered to be sterile [8]. However, some workers have reported the occurrence of bacteria within fresh, healthy cucumbers and tomatoes [9, 11]. The potential for faecal and other forms of contamination of the surface of fruits is very high in developing countries due to the generally poor standard of sanitation. Ripe mature black plum fruit for food use commonly collected from the ground instead of plucked which is more hygienic. It is desirable in this study to know both the surface mycoflora and mycoflora within the fruits with a view controlling the rot causing organisms. The present study will contribute to knowledge of the important subject of fruit hygiene in Nigeria. This paper reported mycoflora both on the surface and within the fruits of black plum.

2. Materials and methods

2.1. Collection of samples and storage

Black plum (*Vitex doniana*) ripe fruits were collected from the rain forest zone of Ojoto in Eastern Nigeria. Harvesting was done by picking ripe fruits that fell on the ground, and by placing ripe and unripe branches from the plant into sterile polyethylene bags; they were used the next day. The ripe fruits were known by their black colors and softness. Collections were done from August to November 1990, with a daily temperature of 28 ± 2 °C and samples were stored in the refrigerator at 4 °C for 1 or 2 d before analysis. The fruit was not treated with any pesticide before storage.

2.2. Isolation, characterization and identification of microflora

Microorganisms were isolated from the extracted juice, pulp, skin and washings from the skin. Five serial dilutions of the juice and the skin wash were prepared using sterile peptone water, plated on yeast malt extract agar (Difco) and potato dextrose agar, and incubated at 28 °C for 48 h. The identification of yeasts and filamentous fungi was done using standard procedures [12–16].

2.3. Determination of microorganisms on the skin

Ten fruits of black plum harvested from the plant were transferred into a sterile 2-L flask containing 0.5 L sterile distilled water. The fruits were then washed by shaking the contents of the flask vigorously, several times. The wash was transferred into another empty sterile 1-L flask. Three serial dilutions (1×10^{-3} dilution) were prepared and spread-plated on yeast extract-malt extract agar (acidified) for yeast, and potato

dextrose agar with streptomycin incorporated for moulds. This procedure was mainly for the determination of the microflora on the surface of the skin.

For the determination of the microflora inside the skin, sterile gloves were worn and with the aid of a sterile forcep the skin of the washed fruit was peeled off into a sterile petri dish. Discs of the peeled skins were then made with the aid of a sterile cork borer (1 cm in diameter). The discs were dried on the sterile filter papers and plated on appropriate media as employed for the skin wash. Duplicate plates were used and three discs were plated on each. Incubation was at 28 ± 2 °C for 3 d for yeast and moulds. This method was also used for ripe fruits collected from the ground and for unripe fruits plucked from the top of the tree.

2.4. Pulp microorganisms

Ten fruits were washed thoroughly with a sterile water after which the surface of the skin was sterilized with 70% ethanol. With the aid of sterile forceps, strips weighing 1 g each of the pulp of two fruits were obtained aseptically and put separately into sterile test tubes containing 9 mL sterile peptone water, and shaken vigorously. One mL of each washing was then serially diluted (1×10^{-4} dilution) using sterile peptone water, and 0.1 mL plated on the above mentioned media. Smaller strips of the washed pulps were then made and plated (three strips to a plate). Pulps from each fruit were plated separately. Control plating was with sterile peptone water. This was done for ripe fruits collected from the ground and those from the top of the plant.

2.5. Juice microflora

About 3 mL of the fruit juice were expressed from one of the washed fruits. Before juice extraction, the outer skin was surface-sterilized with 70% ethanol. The juice was expressed through a tiny opening at the fruit's tip into a sterile test tube. Serial dilution (1×10^{-3}) was done on one mL of the juice, using sterile peptone water, and 0.1 mL was plated on the above media. Control plating was with sterile peptone water. This was done for ripe and unripe fruits.

2.6. Isolation of microogranisms

From the above plates, distinct colonies were picked and subcultured on the different media for purity.

2.7. Identity of air borne microorganisms

The identity of air borne microorganisms in the inoculating room was determined by exposing plates of potato dextrose agar and yeast extract-malt extract agar for 6 h. Triplicates were made of the agar plates.

2.8. Pathogenicity test

The cultures of fungi isolated from infected tissue of the fruit of *Vitex doniana* were all used for this study. The fungi used for this test were *Aspergillus niger*, *Aspergillus* sp. and *Botryodiplodia theobromae*. The spore suspension was prepared by centrifuging and resuspending the spores in three changes of sterile distilled water. The healthy black plum fruits that were used for the pathogenicity test were surface-sterilized with 70% ethanol and then cleaned with sterile distilled water. The surface of the skin was either sprayed with the spore suspension from fungus using rocking sprayer or a cork borer was used to bore a hole in the ripe and unripe fruits and spore suspension inoculated inside. The inoculum was 5 mL of conidial suspension of 30,000 spores × mL^{-1} prepared in potato dextrose broth (PDB) from 5-d old PDA culture of a text fungus.

Three replicates of the experiment were set up on different laboratory benches at room temperature (28 ± 2 °C). Healthy ripe and unripe fruits were sprayed with sterile distilled water and set aside as control. All the fruits used were covered with sterile cellophane bags for 2 d to allow the spores to establish and were thereafter exposed to natural conditions. The fruits were inspected daily to check for symptoms of

disease development or any other effect of the pathogen. Pieces of affected tissues removed from the edge of resulting symptoms were plated on potato dextrose agar (PDA) for reisolation and reidentification of the causal organisms with the aid of manuals [13–15].

3. Results

3.1. Effects of storage on fruits

The fruits of *Vitex doniana* that were collected and stored for 14 d in the refrigerator remained in good condition, but there was a slight change in the colour of the epicarp. The fruits stored in a frozen state exhibited cracking and, on thawing, there was an increase in the intensity of the color; the color changed from light brown to deep brown and the fruits became softer.

3.2. Mycoflora of *Vitex doniana* fruit

Fifteen isolates were obtained from the skin, pulp and juice. The skin wash of fruits collected from the ground had the highes microbial counts for moulds (3×10^2 colony forming units $\times$ mL^{-1}) and for yeasts 5×10 yeast cells $\times$ mL^{-1}). The fruits collected from the tree had mould counts of 1×10^2 cfu $\times$ mL^{-1} and yeasts, 2×10 cells $\times$ mL^{-1}.

The microflora of the fruits were divided into groups on the basis of percentage incidence of the yeast and filamentous fungi *(tables I–III)*. The major yeast or filamentous fungus components had a minimum incidence of 20% while those which had less than 20% incidence were designated a "minor yeast component". Incidence was expressed as percentage of replicate peels or parts of a treatment group from which a given fungus was isolated.

The dominant moulds found on the surface of fruits collected from the ground were *Aspergillus niger*, *Fusarium miniliforme* and *Botryodiploidia theobromae*. The minor components of filamentous fungi were *Aspergillus* sp., *Rhizopus stolonifer*, *Penicillium chrysogenum* and *Mucor* sp. The major yeast components were *Saccharomyes cerevisiae*, *Pichia* sp. and *Torulopsis* sp. The minor yeast components were S*chizosaccharomyces* sp., *Candida albicans* and *Rhodotorula* sp.

Table I.
Yeasts and filamentous fungi found on ripe black plum fruits (*Vitex doniana*) collected from the ground underneath the plant.

Part of fruits	Yeast constituting		Filamentous fungus constituting	
	Major components 20–100% incidence	Minor components < 20% incidence	Major components 20–100% incidence	Minor components > 20% incidence
Surface	*Saccharomyces cerevisiae* *Pichia* sp. *Torulopsis* sp.	*Schizosaccharomyces* sp. *Candida albicans* *Rhodotorula* sp.	*Aspergillus niger* *Fusarium moniliforme* *Botryodiplodia theobromae*	*Aspergillus* sp. *Rhizopus stolonifer* *Penicillium chrysogenum* *Mucor* sp.
Pulp	*Torulopsis* sp. *Saccharomyces cerevisiae*	*Schizosaccharomyces* sp.	*Botryodiplodia theobromae*	*Rhizopus stolonifer* *Fusarium moniliforme*
Juice	*Saccharomyces cerevisiae*	*Torulopsis* sp.	–	*Penicillium* sp. *Aspergillus niger*
Skin	*Saccharomyces cerevisiae*	*Candida albicans* *Schizosaccharomyces* sp.	*Fusarium moniliforme* *Botryodiplodia theobromae*	

Table II.
Yeasts and filamentous fungi found on ripe black plum fruits (*Vitex doniana*) collected on top of the tree.

Part of fruits	Yeast constituting		Filamentous fungus constituting	
	Major components 20–100% incidence	Minor components < 20% incidence	Major components 20–100% incidence	Minor components > 20% incidence
Skin	*Saccharomyces cerevisiae*	*Candida albicans*		*Fusarium moniliforme*
Surface	*Saccharomyces cerevisiae* *Candida albicans*	*Rhodotorula* sp. *Saccharomyces coreanus*	*Aspergillus niger*	*Botryodiplodia theobromae*
Pulp	*Saccharomyces cerevisiae*	*Saccharomyces coreanus* *Candida albicans*	–	*Penicillium chrysogenum* *Botryodiplodia theobromae*
Juice	–	*Saccharomyces cerevisiae* *Saccharomyces coreanus*	–	*Penicillium chrysogenum*
Skin	*Saccharomyces cerevisiae*	*Candida albicans* *Pichia* sp.	*Penicillium* sp.	–

The major component of moulds in the pulp was *B. theobromae.* The minor mould components were *Rhizopus stolonifer* and *Fusarium moniliforme.* The pulp of fruits collected from the ground had the major yeast components as *Torulopsis* sp. and *S. cerevisiae* while the minor yeast component was *Schizosaccharomyces* sp.

The juice had *S. cerevisiae* as a major yeast component and *Torulopsis* sp. as minor. *Penicillum* sp. and *Aspergillus niger* were found as minor mould components.

The moulds found in the skin had more than 20% incidence and these were *Fusarium moniliforme* and *Botryodiplodia theobromae.* The dominant yeast was *S. cerevisiae* and the minor yeast components were *Candida albicans* and *Schizosaccharomyces* sp. *(table I).*

The surface of the ripe fruits plucked from the top of the tree had lesser microorganisms than the ones collected from the ground (*table II*). The dominant yeasts on the surface were *Saccharomyces cerevisiae* and *Candida albicans.* The minor yeast components were *Rhodotorula* sp. and *Saccharomyces coreanus. Aspergillus niger* was the major components while *Botryodiplodia theobromae* was a minor component. The pulp had *S. cerevisiae* occuring in large numbers while the minor yeast components were found to be *Candida albicans* and *S. coreanus.* The incidence of occurrence of filamentous fungi was less than 20% and the fungi found were *Penicillium chrysogenum* and *B. theobromae.* The fruit juice had *S. cerevisiae* and *S. coreanus* occuring in less than 15% incidence, also the only filamentous fungus found was *P. chrysogenum* which occurred as a minor component. The skin had only *S. cerevisiae* as a major yeast component while *Candida albicans* and *Pichia* sp. occurred as a minor components. The mould found was *Penicillium* sp. which occurred in large numbers.

The surface of the unripe fruit had *Rhodotorula* sp. and *Pichia* sp. as minor yeast components (*table III*). The filamentous fungi that occurred at above 20% frequency were *Aspergillus niger* and *Rhizopus stolonifer.* The minor fungus components were *Penicillium chrysogenum* which was found in the pulp and skin. The juice also contained *Rhodotorula* sp. as a minor yeast component and *Penicillium* sp. occurred as a minor component.

Table III.
Yeasts and filamentous fungi found on different parts of unripe black plum fruits (*Vitex doniana*).

Part of fruits	Yeast constituting		Filamentous fungus constituting	
	Major components 20–100% incidence	Minor components < 20% incidence	Major components 20–100% incidence	Minor components > 20% incidence
Surface	–	*Rhodotorula* sp. *Pichia* sp.	*Aspergillus niger* *Rhizopus stolonifer*	*Penicillium chrysogenum*
Pulp	–	*Rhodotorula* sp.	–	*Penicillium chrysogenum*
Juice	–	*Rhodotorula* sp.	–	*Penicillium* sp.

3.3. Pathogenicity test

The result of the pathogenicity test showed that the necrotic spots found on the skin of the fruits were caused by *Botryodiplodia theobromae*. The skin showed a light brown lesion which later developed dark brown spots. The results with unripe mature fruit showed an anthracnose. The lesion on the unripe skin of the fruit were circular at first but, later, became irregular in shape and involved the entire surface of the affected fruit. The lesion on the fruit first appeared as a small yellow spot which later enlarged and turned light brown. The ripe fruits infected with *Aspergillus niger* and *Aspergillus* sp. showed fruit rot. There was decaying of the fruit due to the fungi.

4. Discussion

The fruits of black plum are seasonal in nature and the microflora associated with plum fruits were grouped according to their percentage incidence. Filamentous fungi constituted the major microflora on the epidermis of the black plum fruits that were collected from ground. This may be due to the rich soil fungal flora of the field where fruits were collected. The fruits collected on top of the tree showed that yeasts were the major components of the epidermis. Although no attempt was made to ascertain the reason for the high density of the yeast types, it is probable that the organisms constitute a major component of the air microflora in the field and the fruits served as a good substrate for the yeasts. Local variations of microorganisms on the fruits were expected because of the differences in location and dissimilarities in the conditions under which the fruits were found.

The microbial count results showed there were more than three times yeasts on the ripe fruits, epidermis than on unripe fruits. The richness of ripe fruits in species diversity and the population density of yeast could be due to changes in sugar content as the fruit ripens [17] or changes in animal visitors to the fruits. Apart from ants and other insects, rats and other mammals are usually attracted by the beautiful black color of a ripe fruit [3]. Comparing the percentage incidence of the yeasts found on the epidermis of the ripe and unripe fruits, it seems that *Rhodoturala* sp. is the major inhabitant of the surface. Reports of some workers [18] showed that many microorganisms found on the fruit surface occurred on the flowers of the plants. It is probable that insects that pollenate the flowers influence the composition of the microflora on fruits. *Botryodiplodia theobromae* was isolated mostly on the parts of the fruit skin that have necrotic spots. *B. theobromae* has been reported to cause necrotic spots on the fruits and leaves of some plants. The pathogenicity test carried out showed that *B. theobromae, Aspergillus niger* and *Aspergillus* sp. were found in greater numbers in the fruits which appeared to have suffered fungal attack than in those fruits that appeared quite healthy. *Aspergillus niger, Aspergillus* sp., *Rhizopus stolonifer,*

and *Mucor* sp., which are common laboratory contaminants, were isolated from the fruits. The source of these microorganisms could be the atmosphere from where the fruits were collected. The air microflora within the laboratory revealed the presence of *Aspergillus* sp. and *Rhizopus stolonifer* in abundance.

Previous works showed that the juice from fruits of black plum infected with *Aspergillus niger* decreased in protein content, pH, ascorbic acid and total sugars in infected fruit [5]. However, the titratable acidity of infected juice increased and this was attributed to microorganisms utilizing the nutrients for metabolism. The increase in titratable acidity was usually as a result of conversion of the sugars into organic acids by the microorganisms and this led to a decrease in the pH [5].

The results reported in our work show that in addition to bacteria, yeasts and, occasionally, filamentous fungi can be present within fruits. The occurrence of microorganisms in healthy fruits have been reported [11] and no information is yet available concerning their location, method of entry nor their function [11]. Internal infection of fruits could be due to contamination of the gynoecium by yeasts and filamentous fungi from air-borne infection or insect carrier during pollination [19]. During fruit growth and development, the microorganisms become sealed in.

5. Conclusion

Within the limits of the precautions taken in these experiments, it can be concluded from the results obtained that yeasts and, less frequently, fungi occur within apparently healthy fruits. There were more yeasts on ripe fruits' epidermis than on unripe fruits and black plum fruits also suffer fungal attacks. However, fruits need to be tested at various stages of development to determine when the fungi entered the pulp. Also, the mycoflora of the soil around the fruits need to be investigated and the pathogenic fungi should be grown at 37 °C to ascertain whether they are pathogenic to humans.

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Micoflora de la ciruela de la sabana (*Vitex doniana* sweet).

Resumen — **Introducción**. La ciruela de la sabana (*Vitex doniana* sweet*)* es una planta que está muy extendida en Nigeria; entre los diferentes usos destaca la producción de vino y mermelada. En vez de realizar la recogida en el árbol para garantizar una mejor higiene, el fruto maduro de *Vitex doniana* sweet utilizado para la alimentación se suele recoger en el suelo. Se han iniciado estudios para analizar la micoflora presente en la superficie y en el interior de este fruto. **Material y métodos**. Las micofloras asociadas a la superficie, la piel, pulpa y jugo de frutos intactos y sanos de *Vitex doniana* sweet fueron aisladas en agar malta y agar de dextrosa de patata y, seguidamente, identificadas. Se probó la patogenicidad en tres patógenos del fruto. **Resultados y discusión**. Los hongos observados con una frecuencia del 20 al 100% se clasificaron como componentes principales y los que presentaban una frecuencia inferior al 20 % como componentes menores. Las levaduras identificadas como componentes principales de los frutos maduros fueron *Saccharomyces cerevisiae* y *Candida albicans*, mientras que *Rhodotrula* sp., *S. Coreanus* y *Pichia* sp. se mostraron como componentes menores. Entre los hongos filamentosos identificados, las principales especies encontradas fueron *Aspergillus niger* y *Penicillium* sp., mientras que *Botryodiplodia theobromae* y *Penicillium chrysogenum* se clasificaron como componentes menores. *B. theobromae*, *Aspergillus* sp. y *A. niger* provocaron un deterioro de los frutos. **Conclusión**. Las levaduras y, ocasionalmente, los hongos filamentosos se desarrollan en frutos aparentemente sanos de *V. doniana* sweet. Las fases de desarrollo favorables a la penetración de las levaduras en la pulpa del fruto tendrán que determinarse de forma más precisa.

Nigeria / *Vitex doniana* (fruta) / enfermedades de las plantas / levadura / organismos patógenos / flora microbiana