# INVESTIGATION OF THE MICROORGANISMS ASSOCIATED WITH SOURSOP SEEDS (*Annona muricata*).

**BY**

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**BENIN CITY, NIGERIA**

**JANUARY 2023**

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**A PROJECT REPORT SUBMITTED TO THE DEPARTMENT OF CROP SCIENCE, FACULTY OF AGRICULTURE, UNIVERSITY OF BENIN, BENIN CITY, NIGERIA, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF BACHELOR OF AGRICULTURE (B.AGRIC) IN CROP SCIENCE.**

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## CERTIFICATION

This is to certify that the work titled "Investigation of the Microorganism associated with soursop seeds(*Annona muricata)"* was carried out by Miss Loveth IMOISILI with Matriculation number, AGR1600334 in the Department of Crop Science, Faculty of Agriculture, University of Benin, Benin City, Edo State, Nigeria.

**………………………..**
**Prof. A.T Adekunle**

**Date…………………..**

**……………………**
**Prof K.E Law-Ogbomo**
**Head of Department**

**Date…………………**

# DEDICATION

This work is dedicated to God Almighty for his mercy throughout the period of my academic program.

## ACKNOWLEDGEMENTS

I want to thank Almighty God for his love, mercy, wisdom, knowledge, guidance and grace throughout this program and the completion of my academic program and project work.

I wish to express my utmost gratitude to my project supervisor, Prof A.T. Adekunle, I couldn’t have asked for a better supervisor, I am indeed grateful. To Mr. Joseph Osagie, thank you for the numerous times you had to answer our questions and correct us when we were going wrong, and to Prof. C.N.C Nwaoguola for your immense help on this project.

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My sincere appreciation to my mum Mrs Imoisili Agens for her unwavering support throughout the period of my academic program and my wonderful siblings (Collins, Edna, Mrs. Mable, Mr. innocent, Mr. destiny, Mr. Douglas, Mr and Mrs Ehis) for being pillars to rest on in difficult times.

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## ABSTRACT

This study was designed to evaluate the microorganisms associated with soursop seeds obtained from New Benin market, Benin City. The objectives of the study were to ascertain the percentage of infection of microorganism associated with soursop seeds; isolate and identify microorganisms associated with soursop seeds, and determine the microbial diversity associated with soursop seeds.

The experiment was carried out in the Department of Crop Science Laboratory, Faculty of Agriculture, University of Benin, Edo State. The Incubation Technique (Agar plate method) was employed during the course of the study. Soursop seeds (seedcoat and endosperm) were chopped into tiny bits, surface sterilized, and placed on filter paper to drain off moisture, and cultured with Potato Dextrose Agar and nutrient agar (PDA and NA). Microbial identifications were done using morphological and biochemical tests for bacteria, while microscopic features examination was carried out for fungi. During the course of the study which lasted for 9 weeks, three (3) bacteria species (*Proteus* sp., *Streptococcus sp.,* and *Bacillus sp*.) and three (3) fungi species (*Aspergillus sp., Phoma sp.,* and *Yeast cell*) were isolated from the seed of soursop.

Results of the study showed that *Aspergillus sp. Phoma sp. Yeast cell, Proteus sp. streptococcus sp.* and *Bacillus sp*., known to be pathogenic, were disseminated predominantly by seeds, and was observed that the level of bacteria infection was higher in seed coat when compared to endosperm while the level of fungi infection was higher in the endosperm when compared to the seed coat. The incidence of bacteria and fungal organisms on the seed parts of soursop has been demonstrated by this study. High incidence of some of these organisms can result in far reaching consequences such as total crop failure.

It was therefore recommended to carry out seed tests by agar plate method (Incubation Technique). This is because the agar plate method provides an efficient tool for the quick identification of specific seed infections; plant pathogen management should be carried out in form of plant products (plant extract) that show good bioactivity against bacterial and fungal isolates associated with soursop seed or other related crops.

# CHAPTER ONE

# INTRODUCTION

*Annona muricata* commonly called soursop and belonging to the family *Annonaceae* is a tropical fruit tree and one of the minor crops that is gaining popularity because of its economic and medicinal importance (Okoli *et al*., 2016). It is a native of the warmest tropical area in South and North America and is widely distributed throughout tropical and sub-tropical parts of the world including India, Malaysia, and Nigeria (Moghadamtousi *et al*., 2015)

*Annona muricata L.* fruits are generally rich in carbohydrates and sugars and are also sources of some vitamins and minerals. Various parts of A*nnona muricata L.* plants are known for their medicinal potential and have been used traditionally in many parts of the world to cure a variety of diseases and conditions. *Annona muricata L.* plants are sources of very interesting classes of natural compounds known as acetogenins localized in the leaves, stem, seeds, and fruits. Acetogenins possess a broad spectrum of biological activities that include anticancer, cytotoxic, antiparasitic, immunosuppressive, antiviral, antimalarial, and antidiabetic properties. Acetogenins are considered as potential candidates for the future generation of antitumor drugs (Padmanabhan and Paliyath, 2016).

Seeds represent one of the most crucial stages of a plant's life history. In agricultural systems, seeds serve to initiate a new crop cycle, are most commonly produced commercially, heavily handled, processed, and uniformly planted across large geographic areas. However, in natural ecosystems, seeds not only serve to initiate the life cycle and reproduce the species, but also to facilitate dispersal, adaptation to, and persistence in new environments (Fenner and Thompson, 2005).

Germinating seeds and seedlings are especially vulnerable to mortality from drought, granivores, and fungal seedborne and soil pathogens (Bever *et al*. 2015). Even following germination, seedlings continue to face threats to their establishment from pathogens and herbivores, but also to resource limitation and deficiencies in overall habitat suitability, making the seed-to-seedling transition in both natural and agricultural systems one of the most important bottlenecks in a plant's life cycle (Leck *et al*. 2008). Therefore, the nature and impact of microbial interactions that take place before and during these vulnerable stages in plant development are critical in setting the trajectories for plant population and community dynamics in natural systems and crop success or failure in agricultural systems. Microbes interact with seeds at all stages of plant development. These interactions may be casual or intimate, yet they all contribute to varying degrees to an evolving seed microbiome that may carry over to other developmental stages (Hardoim *et al*. 2015).

Microorganisms exist in nearly all environments, and since microbes occupy the base of the food chain, microbes are the first organisms to react to changes in the environment. Microbes contribute to soil nutrient levels, plant processes and functions, and overall crop health and productivity. Plant seeds carry embryonic plants and nutrients for early stages of seedling growth; some plant seeds also carry small communities of symbiotic microbes (primarily bacteria and fungi) that are needed for defense from pathogens, modulation of plant development, and nutrient acquisition in seedlings (Gond *et al*., 2015; Doty 2017).

## 1.1 Justification of the Study

The soursop fruit and other parts of the tree are considered to be underutilized. Information on the composition, nutritional value, medicinal uses, and toxicology of the soursop fruit and plant is limited and scattered (Badrie and Schauss, 2010).

*Annona muricata L.* (Soursop) is the largest-fruited of the family Annonaceae and belongs to the tropical region. Soursop is a multipurpose plant with acceptable nutritional value as food products, sources of medicinal and industrial products as well as contributing directly to food security and supplementary household income for small and medium-scale farmers (SCUC, 2006). Presently there is a noticed change in attitude in the past decade among policy makers and the public in respect of the quality of life as related to the quality and diverse sources of food. Vitamins and other micronutrients are for instance being searched in crops and plant species with greater emphasis than in the past in recognition of their role in combating diet imbalances. In developing countries such as Nigeria, the problem of 'hidden hunger' in children and older people as more vulnerable social groups is increasingly being recorded. With the current strong consumer demand for soursop fruits and other parts and its increased popularity in this part of the world due to some medicinal benefits and cure for cancer (FAO, 1997; SCUC, 2006).

Germinating seeds and seedlings are especially vulnerable to mortality from drought, granivores, and fungal seedborne and soil pathogens (Bever *et al*. 2015). Even following germination, seedlings continue to face threats to their establishment from pathogens and herbivores, but also to resource limitation and deficiencies in overall habitat suitability, making the seed-to-seedling transition in both natural and agricultural systems one of the most important bottlenecks in a plant's life cycle (Leck *et al*. 2008). Therefore, the nature and impact of microbial interactions that take place before and during these vulnerable stages in plant development are critical in setting the trajectories for plant population and community dynamics in natural systems and crop success or failure in agricultural systems. Microbes interact with seeds at all stages of plant development. These interactions may be casual or intimate, yet they all contribute to varying degrees to an evolving seed microbiome that may carry over to other developmental stages (Hardoim *et al*. 2015). Hence, that the study to examine the microorganism (microbes) associated with soursop seed was carried out.

## 1.2 Aim and Objectives of the Study

The aim of this research was to determine the microorganisms associated with soursop seed.

The specific objectives of the study were to:

i. ascertain the percentage infection of microorganisms on the soursop seed,

ii. isolate and identify microorganisms associated with soursop seed; and

iii. determine the microbial diversity of the seed.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1.1 Seed Pathogen

Seed pathology involves the study and management of diseases affecting seed production and utilization, as well as disease management practices applied to seeds. Seed pathology as a sub-discipline of plant pathology, though relatively new, encompasses all we need to improve productivity, longevity, and production of disease-free breeds of seeds. The discipline is responsible for the dependence on chemicals and other techniques for improvement of seeds (Besri, 1989). In the past, seed improvement depended on application of chemicals and natural conventional means of seed maintenance like crop rotation and shifting cultivation (Besri, 1989), but the discipline has since evolved to the use of modern molecular biological techniques such as genetic engineering and polymerase chain reaction technology (Nameth, 1998).

Paul Neergaard is considered the father of seed pathology. Paul Neergaard is considered as the father of seed pathology, and he defined seed pathology as the study of diseases and deterioration of seeds caused by bacteria, fungi, nematodes, viroids and viruses, and physiological and mechanical disorders (Neergaard, 1979). Many others, after him, have defined the term seed pathology in different other ways, but all the various shades of definition promulgated could be summed up to say that

Seed Pathology is the study and management of disease disorders arising from seeds, or affecting seed production and utilization (Agarwal and Sinclair, 1997; Munkvold, 2009).

### 2.1.2 Definition of Seed Pathology and It's Importance.

The entire discipline of Seed Pathology revolves around the 'biological entity' called seed. Its structure, development and function are necessary in understanding Seed Pathology in the general concept of plant pathology. Generally, the word 'seed' often refers to anything that is sown in the ground to produce a plant. However, this broad definition is rather ambiguous and grossly misleading because not everything that can be sown is actually a seed.

Like the term seed pathology, several authors have defined seed differently, essentially to mean the same thing. Some have defined a seed as the product of a ripened ovule of plants such as gymnosperms and angiosperms resulting from fertilization and grows within the mother plant (Cain and Shelton, 2001). A seed is therefore a matured ovule; consisting the young embryo, nutrients for the embryo and is covered with a protective coat. In other words, seeds are fertilised ovules containing embryos surrounded by an integument (Maude, 1996). All these different versions of definitions put forward by different workers could be summarized to mean that a seed is a mature ovule containing an embryo, durable seed coat and endosperm. In biological terms, a seed is a small embryonic plant enclosed in a covering called the seed coat, usually with some stored food in the cotyledon.

## 2.2 Seed

### 2.2.1 Part making up a seed

A seed is an important part of a flowering plant. They give rise to a new plant. They may be of different shapes, colours and sizes. They may be round, wrinkled, winged or hairy. They are in a dormant condition until they receive adequate sunlight, water, and soil. The growth of the plant from a seed is known as germination. A seed has three parts:

a) Seed Coat

b) Endosperm

c) Embryo

a) **Seed Coat**

A seed coat protects the internal parts of a seed. The seed coat has two layers. The outer layer is thick and known as the testa. The inner layer is thin and known as tegmen. A thick seed coat protects the seed from sunlight and water. It prevents the loss of water and entry of parasites within the seeds. The hard seed coats prevent germination during unfavourable environmental conditions. An opening in the integument of the ovule is known as the micropyle and is visible on some seed coats.

The hilum is also visible which is equivalent to the naval in humans where the umbilical cord is attached.

b) **Endosperm**

The endosperm contains the nutrients stored in it. It provides nutrients to the seed in the form of starch, carbohydrates and proteins to support the embryo during germination. It is located below the seed coat. The seeds remain viable with the intake of nutrients until germination.

The endosperm may be mealy, continuous or ruminated. An endosperm has a triploid chromosome complement.

In corns and other cereals, endosperm constitutes a major portion of the seed. In seeds like beans, the endosperm is utilized in the embryo development and is absent in the seed. Coconut is the liquid endosperm.

c) **Embryo**

The embryo is the most important part of a seed. It is diploid, developed from the fertilized egg. All the cells that need to develop into a mature embryo are present within the embryo. An embryo comprises the following parts:

i. Epicotyl

ii. Hypocotyl

iii. Radicle

iv. Cotyledons

i. **Epicotyl** is a small shoot which gives rise to the entire plant shoot system.

ii. **Hypocotyl** is the primary root that emerges first during germination. It anchors the plant firmly in the soil.

iii. **Radicle** is a small embryonic root.

iv. **Cotyledons** provide nourishment to different parts of the embryo. It emerges as a tiny or fleshy leaf from the soil with the seedling during growth. It stores food in the form of starch and protein.

The embryonic leaves are the first to appear above the ground. An embryo develops from a fertilized egg.

### 2.2.2 Types of Seeds

There are two types of seeds:

a) Monocotyledonous seeds

b) Dicotyledonous seeds

a) Monocotyledonous Seeds

These comprise a single cotyledon emerging from the seeds on germination. For example, rice.

b) Dicotyledonous Seeds

These comprise two cotyledons emerging from the seeds on germination. For example, tomato.

### 2.2.3 Uses of Seed

Man has continually depended on seeds, using them in variety of ways; as food, fibers and drugs. They are also used as ornaments of beauty, feed for livestock, raw materials for industry, and planting material for cultivation of crops (Maude, 1996; Judd *et al*, 2002). Apart from being used as planting materials, seeds are very good and viable sources of cooking oils, beverages, spices and other important food additives. Some are even used as beads in necklace and rosaries (Judd *et al*., 2002). Seeds are also used as toys by children, such as for the game conkers. As industrial raw materials, seeds of maize and other plants are used to produce industrial chemicals such as grain alcohol (ethanol) through fermentation (Judd *et al*., 2002).

As important as seeds are to human livelihood, their nature exposes them to microorganisms; some of which are pathogenic and adversely affect the physiology and metabolism of seeds, resulting to low yield and/or quality. Consequently, seed health testing, prior to sowing, is a vital prerequisite to maximize yield (International Seed Testing Association (ISTA),1985).

Furthermore, seeds in storage are prone to insect infestation, microbial attack, unfavourable temperature conditions, etc. The incidence and severity of seed deterioration during storage problems depends on the type of storage equipment and conditions, as well as biotic factors (Vanek, 1992). Storage conditions such as high temperature and humidity encourage rapid growth of seed pests and microbial pathogens (Maude, 1996), and the activities of these organisms play very significant roles in the spoilage and loss of seeds in storage. Some fungal pathogens such as Aspergillus, Penicillium, Fuasrium species etc. while attacking seeds sometimes produce toxic substances or mycotoxins which may be harmful to man and animals that ingest such contaminated seeds (Judd *et al*., 2002).

## 2.3 Some Seed-Borne Diseases, Pathogens and their Effects

A variety of crops are grown in Nigeria, including cereals such as maize, wheat, rice and millets; legumes such as beans, soybeans; oil seed crops such as groundnut and other food crops are adversely affected by various seed-borne diseases (Oluwatoye, 2003). Depending on the nature of pathogen, seeds and prevailing environmental conditions, seeds may be infested or infected by a microorganism. Seed and seed-borne pathogens include: fungi, bacteria, viruses and nematodes (Walkey, 1991; Agarwal and Sinclair, 1997).

Some microorganisms simply stick to superficial layers of seed tissues or are mixed with a seed lot. Examples of such organisms include fungi (Ascochyta pinodella in pea; Drechslera sorokiniana and D. oryzae in rice) and bacteria (Pseudomonas syringae pv. phaseolicola in bean, Pseudomonas. syringae pv. tomato in tomato and C. michiganense pv. michiganense in pepper) (Nome *et al*., 2002). These sorts of seeds with microorganisms occurring just superficially on its surface is often said to be infested or simply contaminated. In contrast to infestation or microbial contamination, some microorganisms infect seeds. In this case, the microorganism is considered to be a pathogen, and grows deep into the tissues of the seed. A seed is therefore said to be infected when a pathogen is able to penetrate deep into the tissues. Infection can be further categorized into systemic and non-systemic (Nome *et al*., 2002). An infection is said to be systemic if the seed-borne pathogen persists and develops with the plant as the latter progresses from the seedling stage to a fully grown plant. On the other hand, an infection is considered to be non-systemic if the pathogen remains within the seed where it causes a localized infection onthe seedling at the stage of pre or post emergency (Agarwal and Sinclair, 1987).

Many environmental factors directly or indirectly affect the establishment of seed-borne pathogens. Temperature, moisture, light and pH are known to be the most important environmental factors which influence the infection of plants (Tarr, 1989). Environmental factors modify the transmission of disease pathogen and their effect on host plant by modulating the action of the soil microbial community, and in the process renders a soil disease suppressive or conducive (Etebu and Osborn, 2012; Maude, 1996). The effects of seed-borne pathogens and diseases are varied, depending on the pathogen involved, the seed itself and the prevailing environmental or storage conditions. Some of these effects are seed discolouration, seed abortion, seed rot, seed necrosis, seedling damage, crop yield and adverse effect on seed viability, germination capacity and nutritive value.

## 2.4 Sources/Transmission of Seed-Borne Pathogens

The mode of transmission of pathogens depends on the type of infection or infestation. Microorganisms that occur only superficially on the outer embryo tissues of a seed do not affect the female gametes that form the new plant and as a result, the pathogen(s) do not persist via the new plant to affect the developing seeds thereafter (Shetty, 1992). With this sort of seed-borne infection, transmission is majorly influenced by environmental factors (Shetty, 1992). In contrast, some pathogen especially those that cause systemic infection, are transmitted directly through the tissues of the mother plant to the developing seeds. A diseased seed would usually produce a diseased seedling if the seed-borne pathogens are deep seated and the embryo area of the seed is affected (Maude, 1996). In such instances, the role of environmental factors is insignificant as the pathogen literally persists and develops with the plant as the latter progresses from the seedling stage to a fully grown plant. Sometimes infectious seed-borne pathogens are transmitted from diseased plants to infect healthy plants through indirect means. For example, a pathogen located in the inflorescence of the mother plants could be transmitted by insects, wind, rain and other means of transmission to affect the seeds and other healthy parts of the plants (Maude, 1996; Walkey, 1991). Other means of transmission are seeds contaminated with whole organisms or fungal resting bodies such as sclerotia, infected seed coats or pericarps, infected embryos, plant debris etc. (Maude, 1996).

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Experimental Location

The experiment was carried out in the Crop Science Laboratory of the Faculty of Agriculture University of Benin, Edo State, Nigeria.

## 3.2 Materials and Equipment

The seeds of soursops (Annona muricata l.) used for the study were obtained from New Benin Market, Benin city. The seed coat and the Endosperm of the seeds were use.

**Media:** Potato Dextrose Agar (PDA) and Nutrient Agar (NA)

**Other materials:** Sodium hypochlorite, Sterile Distilled water, Ethanol, Cotton wool, Aluminum foil, Filter paper, masking tape, Perti dishes and Spirit lamp.

**Equipment used were:** Inoculation Chamber (Laminar flow), Autoclave, Weighing Scale, Microscope, Incubator.

## 3.3 Sterilization of Equipment and Materials.

The Inoculation Chamber was swabbed with cotton wool and 98% ethonol. Sterilization of the Petri dishes, other glass wares and filter paper were carried out by wrapping them in aluminum foils and placed in a hot air oven at 160°C for 2hours.

Sterile distilled water was prepared by filling up glass Amber bottles with distilled water and autoclaved for 15min at 121°C.

**Surface sterilization of seeds**

The seeds were randomly selected from the sample and seed coat and endosperm were separated. Both seedcoat and endosperm were chopped into tiny bits and then surface sterilized in 1% sodium hypochloride for 60 seconds and rinsed in three changes with sterile distilled water. The Seed coat and endosperm were then placed on sterile filter paper for absorption of moisture.

## 3.4. Media Preparation and Plate Pouring

Media was prepared according to manufacturer's instruction by weighing 3.9g of potato Dextrose Agar (PDA) and 2.8g of Nutrient Agar (NA) into separate conical flasks and 100ml of distilled water was added.

These were thoroughly stirred using the stirring rod to allow it dissolve. The flasks were then corked with cotton wool and wrapped with aluminum foil. They were placed in the autoclave at 121°C for 15 minutes for sterilization. The media (PDA and NA) were transferred to inoculation chamber and allowed to cool to about 40°C before pouring. About twenty (20) milliliters of each medium is poured in individual petri dishes and allowed to gel. The plates were then sealed with masking tape to prevent contamination.

## 3.5. Plating of seeds, isolation and purification of culture

Plating was done by picking soursop seed (seed coat and endosperm) from sterile filter paper using forceps sterilized by flaming to red hot and then placed directly to petri dishes already containing media (PDA/NA) respectively. Observation for bacteria growth was done 24 hours after inoculation on plates. After 72 hours of plating, observation was made for fungal growth.

## 3.6. Sub-culturing of microorganisms

Freshly prepared NA and PDA was poured into already sterilized Petri dishes, it was allowed to solidify. From the old plates containing the microorganism, the streaking method was used to isolate the individual bacteria cultures into a fresh plate for further studies. The fungal cultures was sub-cultured by carefully taking a fresh growing portion with a sterilized needle into a fresh PDA plates. Sub culturing was done to obtain pure culture.

## 3.7. Identification of the fungal isolates

Fungi associated with the seed samples were then subcultured into a freshly prepared PDA on Petri dishes, after growth it was then examined and identified based on their cultural and microscopic characteristics according to the methods described by Barnett and Hunter (1998) and Nyongesa *et al.* (2015).

The fungal isolates were identified base on their cultural characteristics such as the colony colour, margins, elevation and colony reverse colours. Microscopic features were examined under a microscope fitted with a camera (Motic B1 Digital camera) using the cover-slip method in which a little quantity of each culture was transferred onto the base of cover slips buried in PDA. The fungal isolates were identified using described by Barnett and Hunter (1998) and Nyongesa *et al* (2015).

## 3.8. Characterization and identification of bacterial isolates

### 3.8.1 Cultural characteristics

For the bacterial isolates, cultural characteristics will be observed on Nutrient agar plates. The cultural characteristics include. Size, shape, surface, opacity, texture, elevation and pigmentation will be determined by visual observation.

### 3.8.2 Gram reaction

A wire loop was sterilized in Bunsen burner and allowed to cool then a loopful of growth was collected from the agar plate and applied on a clean grease-free slide then a drop of normal saline was added, emulsified and heat fixed by passing over a flame three times. The smear was flooded with crystal violet for 30-60 seconds and then covered with iodine for 30-60 seconds and then washed off; it was decolorized with acetone until no colour runs off the slide and rinsed immediately. The slide was covered with safranin for 1 minute and then washed off with clean water. The slide was kept in a rack to air dry after wiping the back with cotton wool. The stained smear was then examined microscopically under oil immersion at 100 magnification objective lens. Gram-positive bacteria appeared dark purple while gram- negative bacteria appeared pink.

### 3.8.3 Motility test

Motility test was done by stabbing a slant with the isolated bacteria. If bacteria is not motile, there will only be growth along the stab line.

### 3.8.4 Catalase test

This test was used to identify organism that would produce the enzyme catalase. The procedure include sterilization of working area with cotton wool and 98% ethanol.

Three milliliters (3ml) of hydrogen peroxide solution was poured into a sterile test tube. Then a sterile glass rod was used to collect several colonies of the test and inoculate into the hydrogen peroxide solution. It was observed for immediate active bubbling for positive test.

### 3.8.5 Coagulase test

This test is used to identify bacteria isolates capable of synthesizing the enzyme coagulase which clots blood plasma. The procedures include the sterilization of working area.

A drop of sterile distilled water was placed on each end of a sterile slide. Then a colony of the test organism was emulsified on each spot to make two thick suspensions. A loopful of plasma was added to one of the suspensions and mixed gently. The slide was examined for clumping or doting of the organisms within 10 seconds. Plasma was not added to the second suspension which serves as control.

### 3.8.6 Oxidase test

This test is used to identify bacteria isolates capable of synthesizing of the enzymes cytochrome oxides. The procedure include sterilization of working area. A piece of filter paper was placed in a clean Petri dish and 2-3 drops of fresh or nascent oxidase reagent was added. A colony of test organism was collected using a glass rod and smeared on the filter paper and observed. Blue-purple color within few a seconds showed a positive test.

### 3.8.7 Urease test

This test is used to identify isolates which has the ability to hydrolyze area with the enzyme urease. The procedure include sterilization of working area. The test organism was heavily inoculated onto Christensens urea broth in a bijou bottle using a sterile wire loop and incubated at 35°C- 37°C for 18- 24hours and examined, thereafter a pink color in the medium showed positive test.

### 3.8.8 Citrate test

This test is used to identify bacteria isolates which has the ability to utilize citrate as a source of energy. The procedures involve the sterilization of working area. Simon's citrate agar medium was prepared in a slant bijou bottle, then using a sterile wire loop was used to inoculate the test organism onto the slant medium and incubated at 35°C for 48 hours after which it was examined for color formation. A bright blue color in the medium gave a positive citrate test. *K. pneumonia* and *E. coil* were employed as positive and negative controls respectively.

### 3.8.9 Indole test

This test is used to identify bacteria isolates which has the ability to decompose the amino acid tryptophan to indole. The procedures include the sterilization of working area. A sterile wire loop was used to inoculate a colony of test organism into 2ml of peptone water containing tryptophan. The tube was stoppered and incubated at 37°C for 24 hours. Kovac's reagent was added to the medium. Observation of red coloration on the surface layer within 10 minutes showed a positive result.

## 3.9 Percentage Infection

After the preparation of the media, plating was done by picking soursop seed (seed coat and endosperm) from sterile filter paper using forceps sterilized by flaming to red hot and then placed directly to petri dishes already containing media (PDA/NA) respectively. Having 2 plate of NA for endosperm, 2 plate of NA for seedcoat, 2 plate of PDA for endosperm and 2 plates of PDA for seedcoat. Observation for growth was done after 72 hours

### 3.10 Experimental Design and Analysis

The experiment was laid out using Completely Randomized Design.

# CHAPTER FOUR

## RESULTS

The percentage seed infection of organism in the seed coat and endosperm shown on Table 1. The percentage infection for seed coat plated with PDA and NA was 100% while Endosperm was 50%.

A total of three fungi isolate comprising of three genera namely: *Aspergillus sp., Phoma sp***.** yeast cell And three bacteria was also isolated namely: *Proteus sp., Streptococcus sp., Bacillus sp.* Were isolated from seed part of soursop seed. (Table 2) *Streptococcus sp. a*nd *Aspergillus sp*. occurred in both seedcoat and endosperm.

Frequency distribution of microbial diversity of the isolates from the seed parts of soursops seed was *Bacillus sp., Streptococcus sp., Proteus sp., Aspergillus sp*., *phoma sp., and yeast cell* was isolated in both the seedcoat and endosperm (Table 3).

Table 4 shows the cultural and microscopic characteristics of fungi isolates from the seed part of soursops. Three fungi isolates were identified. The identified fungi isolate in soursops seed include *Aspergillus sp., Phoma sp*., and Yeast cells. *Aspergillus sp. was* a black dense mycelia growth colony with a rough texture of conidia, glucose conidia shape, and a length of 3.2µm with a diameter of 2.1µm. *Phoma sp*. was a dark brown mycelial growth colony with rough conidia texture, oval in conidia shape with 4.10µm conidia length. *Yeast cell* was white spotted growth colony with smooth conidia texture having a *pseudohyhae* with 0.2µm conidia length.

**Table 1. Percentage Infection**

| | PDA | | | | NA | | | |
|---|---|---|---|---|---|---|---|---|
| | **Seedcoat** | | **Endosperm** | | **Seedcoat** | | **Endosperm** | |
| **No. of Seed** | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| **Infection** | 4/4 | 4/4 | 2/4 | 2/4 | 4/4 | 4/4 | 2/4 | 2/4 |
| **% Infection** | 100% | 100% | 50% | 50% | 100% | 100% | 50% | 50% |

**Table 2. fungi and bacteria isolated from seed part of soursop seed from week 1, 3, 6 and 9**

**Fungi**

| | Week 1 | | Week 3 | | Week 6 | | Week 9 | |
|---|---|---|---|---|---|---|---|---|
| Isolates | Seed coat | Endosperm | Seed coat | Endosperm | Seed coat | Endosperm | Seed coat | Endosperm |
| Aspergillus sp | - | + | - | + | + | - | + | - |
| Phoma sp | + | - | - | - | - | - | - | + |
| Yeast cell | - | + | - | + | - | + | - | + |

**Key:** + = Present; - = Absent

**Bacteria**

| | Week 1 | | Week 3 | | Week 6 | | Week 9 | |
|---|---|---|---|---|---|---|---|---|
| Isolates | Seed coat | Endosperm | Seed coat | Endosperm | Seed coat | Endosperm | Seed coat | Endosperm |
| Streptococcus sp | + | - | + | - | + | - | - | + |
| Proteus sp | - | - | + | - | + | - | - | - |
| Bacillus sp | - | - | + | - | - | + | - | - |

**Key:** + = Present; - = Absent

**Table 3: Frequency distribution of Microbial diversity of isolates from Weeks 1, 3, 6 to 9**

| Isolates | Weeks | Frequency |
|---|---|---|
| Aspergillus sp | 1, 6, and 9 | 3% |
| Phoma sp | 1 | 1% |
| Yeast cell | 1, 3, 6, and 9 | 4% |
| Streptococcus sp | 1, 3, 6 and 9 | 4% |
| Bacillus sp | 3 and 6 | 2% |
| Proteus sp | 3 and 6 | 2% |

**Table 4. Cultural and microscopic characteristics of fungi isolates from soursop seed**

| **Cultural characteristics** | **1** | **2** | **3** |
|---|---|---|---|
| Colony description | Black dense mycelia growth | Dark brown mycelia growth | White-spotted growth |
| Texture of conidia | Rough | Rough | Smooth |
| Conidia shape | Glubose | Oval | Pseudohyhae |
| Conidia length | 3.2µm | 4.10µm | 0.25µm |
| Conidia diameter | 2.1µm | 0.16µm | |
| **Fungi isolated** | *Aspergillus sp.* | *Phoma sp* | *Yeast cells* |

Table 5 shows the morphological and biochemical test of bacteria isolates from seed part of soursop. Cultural characteristics evaluated include the shape. *Proteus sp*. *Streptococcus sp*. *Bacillus sp*. was the bacteria found in the seed part of soursop studied. Streptococcus sp. was cocci shape with positive gram stain reaction, *proteus sp*. was rod shape with negative gram stain reaction while *Bacillus sp*. was rod shape with positive gram stain reaction.

**Table 5. Morphological and Biochemical Characteristics of Isolation Bacteria from Soursop Seed**

| **Cultural Characteristics** | **1** | **2** | **3** |
|---|---|---|---|
| Gram stain | +V | -V | +V |
| Cell type | Cocci | Rod | Rod |
| Catalase | _ | + | + |
| Oxidase | _ | _ | + |
| Citrate | + | + | _ |
| Coag | + | + | _ |
| Lac | + | + | + |
| Swarm | + | + | + |
| Gas | + | + | _ |
| H2s prod | + | + | + |
| Motility | _ | + | _ |
| Urea | _ | _ | _ |
| *Identified isolate* | *Streptococcus sp.* | *Proteus sp.* | *Bacillus sp.* |

**Key:** + = Present;  - = Absent

# CHAPTER FIVE

# DISCUSSION

Obsolete agricultural practices, poor storage facilities and unfavourable environmental conditions during pre and post harvest handling of the crops are responsible for the severe contamination, infection, and colonization by microbes (Janardhana *et al*., 1999).

The experiment showed that fungal and bacterial species were found to be associated with soursop seed obtained from New Benin Market, Benin City. In this experiment, species of *Aspergillus sp. Phoma sp. Yeast cell, Proteus sp. streptococcus sp.* and *Bacillus sp*. Known to be pathogenic, we disseminated predominantly by seeds and was observed that the level of bacteria infection was higher in seedcoat when compared to endosperm while the level of fungi infection was higher in the endosperm when compared to the seed coat. This confirm the fundings of Osaigbovo *et al*. (2016) and Adekunle (2014) who observed that fungal and bacterial species was also isolated from seed of Dialium guineense and citrus species in Benin and other parts of Nigeria respectively.

## Conclusion

It could be concluded that the high incidence of some of these organisms can result in far reaching consequences such as total crop failure. Emechebe and Shoyinka

(1985) reported great losses in cowpea production due to seed decay and seedling damping off.

It is therefore recommended to carry out seed test by agar plate method (incubation technique). The agar plate method provides an efficient tool for quick identification of specific seed infection. The method is relatively quick and can be used for testing for bacteria or fungi, or mixed spores.

It can also be recommended that plant pathogen management should be carried out. This could be in form of the use of plant products (plant extract) which shows a good bioactivity against bacteria and fungal isolates associated with soursop seed or other related crop.

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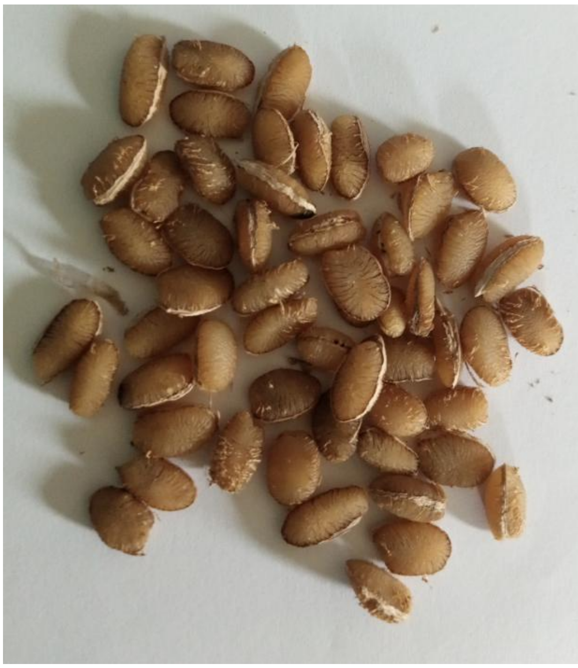
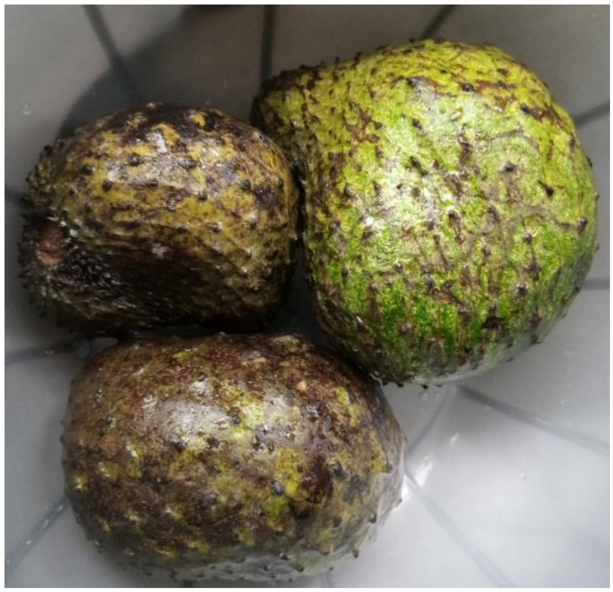
**APPENDIX**

Soursop seeds

Soursop seed coat



Soursop Endosperm



Soursop