EFFECT OF INCUBATION PERIODS ON THE ANTIFUNGAL ACTIVITIES OF PHYTOSYNTHSIZED MAGNESUIM CHLORIDE AND ZINC OXIDES NANOPARTICLES AGAINST PATHOGENS ISOLATED FROM DISEASED CASSAVA PLANT (*Manihot esculenta* Crantz)

BY

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LSC1705791

DEPARTMENT OF PLANT BIOLOGY AND BIOTECHNOLOGY
FACULTY OF LIFE SCIENCES UNIVERSITY OF BENIN
BENIN CITY

NOVEMBER .2022

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A THESIS SUBMITTED TO THE DEPARTMENT OF PLANT BIOLOGY AND BIOTECHNOLOGY, FACULTY OF LIFE SCIENCES, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF BACHELOR OF SCIENCE (B.Sc .HONOURS) IN PLANT BIOLOGY AND BIOTECHNOLOGY, FACULTY OF LIFE SCIENCE, UNIVERSITY OF BENIN

NOVEMBER .2022

CERTIFICATION

This is to certify that this work was carried out by Favour Olohimarin AGBEDE (Miss) of the Department of Plant Biology and Biotechnology, Faculty of Life Science, University of Benin, Benin City, Edo State, Nigeria**.**

Mr. O. H. Obiazikwor --

(**PROJECT Supervisor**) Signature and Date

Prof. E. O. Akpaja --

Head of Department Signature and Date

External Examiner -- --

Signature and Date

DEDICATION

I dedicate this work to God for the physical, mental, and financial strength to make this work a success and for seeing me throughout the period of this research.

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Table of Contents

LIST OF TABLES

LIST OF PLATES

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LIST OF FIGURES

ABSTRACT

Cassava (*Manihot esculenta*) is one of the major staple food crops in Africa, overtime the constraints caused by Plant ravaging pathogen such as bacteria, viruses, fungi and other abiotic factors has led to decline in the yield of cassava produced per annum. In this study the effect of incubation period on the antifungal activities of zinc oxide and magnesium chloride nanoparticles against pathogens isolated from diseases cassava plants *(Manihot esculenta*) were determined. Zinc oxide (ZnO) and magnesium chloride (Mgcl) nanoparticles were synthesized using *Moringa oleifera* leaf extract and characterization of the nanoparticles was done using UV-Vis spectrometer; the peak of the absorbance was recorded at 350nm. Zinc Oxide nanoparticles showed a high anti-fungal potency on the test organism at shorter incubation period (24-72 hours). From this study it was observed that as incubation period increases the anti-fungal properties decreases.

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CHAPTER ONE

INTRODUCTION

1.1 Background of study

Nanoparticles are particles with at least one dimension less than 100 nanometers. Because of their small size, they have remarkable physical, chemical, and biological activity (Mariselvan *et al*., 2014). Nanoparticles are classified in several ways; carbon-based, lipid-based, metal-based, ceramic-based, polymeric-based, and semiconductor nanoparticles are all types of nanoparticles. Nanoparticles are synthesised using two methods: top-down (breakdown) and bottom-up (synthesis) from the ground up or build up (Patil *et al.,* 2021) Synthesis of nanoparticles using plant extract is thought to be a cost-effective way as a result, it is a cost-effective approach for large-scale production. Plant extracts have several advantages over microorganisms, including simplicity of maintenance and lower costs of microorganism isolation (Ahmed *et al.,* 2016). In this research Zinc oxide (ZnO) and magnesium chloride (MgCl) nanoparticles have been synthesised using *Moringa oleifera* leaf extract.

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Cassava plant (*Manihot esculenta*) is a woody shrub of the spurge family Euphorbiaceae. Although cassava is a perennial herb, it is often cultivated as an annual crop in tropical and subtropical regions due to the edible starchy tubers that are an important source of carbohydrates (Adera 2018). Cassava is mainly consumed in cooked form but is used in large quantities in the production of cassava starch called tapioca which is used for food, animal feed, and industrial purposes. Cassava is the third largest source of carbohydrates in the world after rice and corn (Vinduranga, 2018). Nigeria is the world's largest producer of cassava and Thailand is the largest exporter of cassava starch (Vinduranga, 2018) . Common names of cassava include yuca, tapioca, manioc, mandioca.

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1.2 NANOTECHNOLOGY AND NANOPARTICLES

Nanotechnology is the understanding and the control of materials on a nanoscale with dimensions of approximately 1 to 1000 nanometres and unique phenomena to enable new applications (Laurent *et al.,* 2010). It is one of the new areas of modern research dealing with nanoparticles composite, strategy, manipulation and their potential applications.

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Many technologies and industry sectors, including information technology, homeland security, medicine, engineering, transportation, drug-gene delivery, cosmetic, food and feeds, agriculture are benefitting from nanotechnology.

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The idea of nanotechnology was first materialized in 1959 with the help of Nobel Prize winner Richard Phillips Feynman's book "There's Plenty of Room at the Bottom". Nanotechnology is a promising diagnostic package, biomarker, labelling, antibacterial agent, drug transport structure and nano-agent for the treatment of many diseases (Marcato and Duran, 2008; Singh and Nalwa, 2011).

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The term nanoparticle is derived from the Greek words nanosì (dwarf) and particulium (particles). In scientific context, the term nano primarily refers to a specific order of magnitude, namely 10-9 in the metric system. This can refer to a volume, a weight, or a unit of time. Nanoparticles are particles possessing chemical, physical, and biological properties that are smaller than a nanometre. They are a broad category of material that contains particulate substances with size ranging from 1 to 100 nanometres.

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1.2.1 CHARACTERISTICS OF NANOPARTICLES

The features of different nanoparticles in comparison to their bulk equivalent are summarised below:

a. **Thermal properties:** The melting point of nanoparticles (less than 10 nm) is lower than that of bulk materials. Nanoparticles with a low boiling point such as polymer can be used to create electronic wire. (Source?)

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b. **Magnetic function:** As the particle diameter is reduced, the attraction force of a magnetic metal increases allowing soft-magnetic materials to be created as nanoparticle alloys. A permanent magnet can built if the nanoparticles are smaller than the magnet domain.

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c. **Catalytic activity:** Nanoparticles are used as heterogeneous catalysts (different phase catalysts) to operate as a binding/adsorption site or as a catalytic support, with their large active surface area being used for a variety of processes.

1.2.2 TYPES OF NANOPARTICLES

The size, shape, physical and chemical features of nanoparticles can be used to classify them into several categories. A few examples are carbon nanoparticles, ceramic nanoparticles, metal nanoparticles, semi-conductors nanoparticles, polymeric nanoparticles and lipid-based nanoparticles.

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a. Carbon nanoparticles

Carbon-based nanoparticles include fullerenes and carbon nanotubes (CNTs). Fullerenes comprise allotropic carbon nanomaterials in the shape of globular hollow cages. Carbon precursors, particularly atomic carbons, evaporated from graphite are commonly used in their production (Elliot *et al*., 2013).

b. Ceramic nanoparticles

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Ceramic nanoparticles are mostly composed of metal and metalloid oxides, carbides, phosphates, and carbonates such as calcium, titanium and silicon. Due to a variety of beneficial features including as strong heat resistance and chemical inertness, they have a wide range of uses. They can be found in amorphous, polycrystalline, dense, porous or hollow forms (Sigmund *et al*., 2006).

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c. Metal nanoparticles

Metal nanoparticles are submicron-scale entities made of pure metals (for example, gold, platinum, silver, titanium, zinc, cerium, iron and thallium) or their compound (e.g., oxides, hydroxides, sulfides, phosphates, fluorides and chlorides). Metal nanoparticles are used in a variety of scientific fields due to their superior optical characteristics.

D. Semi-conductor nanoparticles

Semiconductor Nanoparticles have characteristics that fall between metals and metalloids/non-metals. They are commonly employed in photocatalysis, electrical equipment and gadgets, and other applications. GaN, GaP, InP, ZnO, ZnS, CdS, CdSe, and CdTe are a few examples of these materials (Klaine *et al*., 2008).

1.2.3 SYNTHESIS OF NANOPARTICLES

Nanoparticles synthesis refers to methods for creating nanoparticles. Synthesis of nanoparticles can be achieved using two major methods which includes top-down approach and bottom-up approach (Khan *et al.* ,2017).

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Top-down approach

This can be said to be the breaking down of large particles into small nano materials. This can be done by Milling, Attrition and Electro exploding wire technique (Iravani, 2011). Although it is a quick manufacturing process, it requires more energy making it unsuitable for large productions

Bottom-up approach

The bottom-up approach refers to organising matter by molecule, atom by atom, and cluster by cluster (Iravani, 2011). During assembly, the physical forces acting on the nanostructures are used to bind the particles together into larger ones. For the synthesis of complex nanostructures, nanotechnologists generally prefer the bottom-up approach because the advantage of this approach lies in the precise control of particle size which leads to superior optical, electronic and other properties (Source)

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Common method used for a bottom-up approach are :

Sol-Gel Method: The sol-gel method is a well-established method for synthesising colloidal nanoparticles in liquid phase (Nilesh *et al.,* 2021). The main advantage of sol-gel technology is its versatility, low temperature process and flexible rheology which make it easy to mould. The sol-gel process is very suitable for the synthesis of oxide nanoparticles and composite nanopowders. The most commonly used precursors allow *in situ* pickup during processing with alkyl oxides due to their commercial availability and high ability to account for MOR bonding.

Co precipitation

This is a typical wet chemical process, also known as a solvent substitution process. Due to its simplicity, economic and reproducibility this method has been widely used. The main component required to produce nanoparticles are synthetic or natural polymer phases, polymer solvents usually acetone, ethanol, hexanes and non-solvent (Source?). Nanoparticles are created by the final mixing of polymer solutions which results in the rapid diffusion of the polymer solvent into the non-solvent polymer phase. The interfacial tension between the two phases creates a maximum surface area which can lead to spontaneous precipitation on nanoparticles. (Ahamed *et al.,* 2010)

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Green synthesis

Green synthesis of nanomaterials is the synthesis of metal nanomaterials using bioactive substances such as various biological wastes such as plant materials, microorganisms, plant wastes, eggshell wastes and agricultural wastes.

Factors affecting the synthesis of nanoparticles

1. Temperature
2. Pressure
3. Time
4. Particle size and shape
5. Cost of preparation

1.2.4 Effect of synthesis method on the activity of nanoparticles

Nanoparticles generally possess a unique biological, physical and chemical properties which can be easily configured to suite the desired applications (Feynman, 1991); Moreover in reconfiguring the properties of the nanoparticle a proper selection of the mode of synthesis have to be considered, Several studies have been carried out to investigate the effect of different process of synthesizing nanoparticle on the activity of nanoparticle (Parak *et al.,* 2003)described biological synthesis of nanoparticle as a bottom up approach that requires the use of simple unicellular to complex multicellular organisms such as bacteria, fungi and plant materials, generally in the use of plant extract the methodology adopted for the synthesis is important to standardize and optimize the nanoparticles to get the desired size, shape, and surface charges(Antonyraj *et al.,*2013). Plants material comprises of numerous active secondary metabolite such as phenols, alkaloids, flavonoids, terpenoids, saponins, tannins, polysaccharides, polyphenols vitamins, etc which reduce and stabilized the nanoparticles(P.Kuppusamy *et al .,* 2014) Proper and optimized use of biological entities for the synthesis of nanoparticle will produce well-characterized and highly stable nanoparticles, the stability of the nanoparticles is determined by pH, surface capping agents, and functionalization techniques.

Chemical synthesis can be performed using range of precursors, temperature, time, and concentration of reactants. Metal and metal oxide nanoparticles when embedded and coated on to surfaces can find immense applications in water treatment, synthetic textiles, biomedical and surgical devices, food processing and packaging (Gutierrez *et al.*, 2010). Chemical-mediated synthesis of nanoparticles is stable for a long duration; the colloidal stability of nanoparticles influences the absorption quotient into the targeted cells.

The antimicrobial activity of nanoparticles depends on the type of metals used, nanoparticles [size, shape, pH, charge, and coating agent] (Wang *et al.,* 2017). Nanoparticles generally, have a high surface to volume ratio compared to other different metallic element, this high surface to volume ratio possessed by nanoparticle enables them to easily interact with the cell membrane (Slavin *et al.,* 2017).

The nanoparticle synthesis method also influences the physicochemical properties of the nanoparticles, the physicochemical properties of nanoparticles play a vital role in the antimicrobial activity of metal oxide, different metallic nanoparticles exert multiple mechanisms including particle aggregation, crystal phase and surface modification to counteract the microbial activity. Numerous reports have postulated the effect of synthesis on the antimicrobial mechanism of nanoparticles but until now a precise mechanism has not yet been justified for the mechanistic action of nanoparticles (Surte and Garje, 2013).

1.2.5 APPLICATIONS OF NANOPARTICLES AND NANOTECHNOLOGY

Over the years, nanoparticles and nanotechnology have been applied in so many sectors which include: applications in medicine, engineering and agriculture

APPLICATION OF NANOPARTICLES IN MEDICINE

The application of nanoparticles or nanotechnology in medicine is called nanomedicine. It can be described as a branch of nanotechnology that applies to the field of health and medicine. It makes use of nano materials and nanoscale biosensors (Biosseau 2011). In the world today various diseases like cancer, cardiovascular diseases and so many other serious infectious diseases constitute of a large number of sickness affecting mankind. Nanomedicine has helped in early identification of diseases, better diagnosis, appropriate therapy and so on. Since the development of nanotechnology, such as the use of gold nanoparticles short DNA sequences can be utilized to tag particles and use them for genetic sequence in samples. Damaged tissues can be repaired or restored with the aid of nanotechnology. When employed in tissues engineering, so-called artificially stimulated cells have the potential to completely transform artificial implants or organ transplant (Nikaije 2015).

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APPLICATIONS OF NANOPARTICLES IN ENGINEERING

The application of nanotechnology/nanoparticles in engineering can be used to improve the functionality of certain products and impact the NOT COMPLETE MA

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APPLICATIONS OF NANOPARTICLES IN AGRICULTURE

Agriculture is one of the main sectors that provide food for humans, either directly or indirectly. Food demand is increasing due to the ever-growing human population. Nanotechnology is an emerging sustainable solutions to many agricultural problems . This technology has been applied in the following ways:

- Production of nanofertilizers to repair the chemical ecology of the soil due to excessive use of regular fertilizers thereby increasing available areas for crop production.
- Delivery systems: In particular, nanomaterials enables a site-specific, controlled supply of nutrients thereby making sure there is a minimal use of agricultural inputs

Nanoparticles has also been predicted by scientist to be able to reduce plant diseases in both greenhouse and in the field by reducing a large chuck of active metals entering the environment when compared to conventional metallic fungicide (Alaa *et al.,* 2019).

1.3 CASSAVA PLANT

Cassava plant (*Manihot esculenta*) is a woody shrub of the spurge family Euphorbiaceae. Although cassava is a perennial herb, it is often cultivated as an annual crop in tropical and subtropical regions due to the edible starchy tubers that are an important source of carbohydrates. Cassava is mainly consumed in cooked form, but is used in large quantities in the production of cassava starch called tapioca which is used for food, animal feed and industrial purposes. Cassava is the third largest source of carbohydrates in foods after rice and corn in the tropics. Nigeria is the world's largest producer of cassava and Thailand is the largest exporter of cassava starch. Cassava has been classified in the following way (Afedraru *et al.,* 2019).

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Kingdom:	Plantae
Sub-kingdom:	Tracheophytes
Super-division:	Angiosperms
Division:	Eudicots
Class:	Rosids
Order:	Malpighiales
Family:	Euphorbiaceae
Genus:	*Manihot*
Species:	*esculenta*
Bionomial name:	*Manihot esculenta* Crantz

Source: IITA, (1990).

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1.3.1 BOTANY OF CASSAVA

Cassava (*Manihot esculenta*) is a slightly woody, often shrubby plant that grows to a height of one to three meters (3-10 feet) (Katz and Weaver, 2003). The dark green leaves are nearly palmate (fan- or hand-shaped) and nearly palmate (fan- or hand-shaped). Cassava comes in approximately 5,000 different types, each with its unique set of characteristics ranging from low plants to bushes with many branches to unbranched trees.

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The cassava root is long and tapering, with a firm homogeneous flesh wrapped in a 1–millimetre- thick removable rind that is rough and brown on the surface similar to a potato. Commercial cultivars can have a top diameter of 5 to 10 centimetres and a length of 50 to 80 centimetres. Along the root's axis, a woody cordon runs. The flesh may be white or yellowish in colour.

1.3.2 CULTIVATION OF CASSAVA

Cassava tolerates more drought than most other crops and can reproduce in very poor acidic soils because of its symbiotic interaction with soil fungi mycorrhiza (Katz and Weaver 2003). Cassava is a high-yielding crop that can produce up to 13 million kcal per acre (Bender and Bender 2005). Small-scale farmers often grow cassava using traditional methods on soil that isn't suited for other crops (Katz and Weaver 2003). Cassava is propagated by cutting mature stems into 15-centimeter portions and sowing them before the rainy season. These plants require appropriate watering for the first two to three months but are drought tolerant after that (Katz and Weaver 2003).

After six to twelve months, the roots are ready to be harvested and they can be harvested at any time during the next two years giving growers a lot of flexibility (Katz and Weaver 2003). Cassava is harvested by elevating the bottom section of the stem and plucking the roots out of the ground before detaching them from the plant's base. Before harvest, the upper parts of the stems containing the leaves are removed. Roots decay in three to four days after harvesting; therefore, they are either eaten right away or processed into a form that can be stored longer (Katz and Weaver 2003).

Cassava root production was predicted to be 184 million metric tons in 2002. Most of the crop was cultivated in Africa, with 99.1 million metric tons produced, and followed by Asia with 51.5 million metric tons and Latin America and the Caribbean with 33.2 million metric tons. However, according to UN figures, Thailand is the world's largest exporter of dried cassava accounting for 77 percent of global exports in 2005. Vietnam is the second largest exporter, with 13.6 percent, followed by Indonesia (5.8%) and Costa Rica (2.1 percent).

1.3.3 USES OF CASSAVA

Cassava possess high starch content and is rich in calcium (50 mg/100g), phosphorus (40 mg/100g) and vitamin C (25 mg/100g). It is however deficient in protein and other nutrients. Fresh, peeled tubers may contain 30–35 percent carbohydrate, but only 1–2% protein and less than 1% fat. Despite having cyanide, cassava leaves are a good source of protein (23 percent) when supplemented with the amino acid methionine. Cassava protein is of reasonably high quality (Katz and Weaver 2003). Cassava can be prepared in a variety of ways. The soft-boiled root has a delicate flavour and can be used in place of boiled potatoes in a variety of dishes: purées, dumplings, soups, stews, gravies and more.

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Cassava flour also known as tapioca flour or tapioca starch can be used to replace wheat flour in recipes. Some persons with wheat allergies such as celiac disease utilize it in this way. Cassava root is used to make boba tapioca pearls. It's also used in grains and certain South

American tribes have made substantial use of it. Cassava cake, a popular confection, is also made with it. Cassareep is made from the bitter cassava juice that has been cooked into thick syrup and seasoned with spices. It is mostly used in tropical regions as a base for various sauces and as a culinary seasoning.

1.3.4. Diseases of cassava (causative agent, symptoms, and mode of transmission)

DISEASES	CAUSATIVE AGENT	SYMPTOMS	MODE OF TRANSMISSION	REFERENCE
Cassava bacterial blight (CBB)	*Xanthomonas axonopodis* pv. .manihosis	Small angular aqueous- looking leaf spots on the lower surface of the leaf blade	Rain splash, contaminated cultivation tools put the entire crop at danger ,because this method causes wounds in healthy cassava plants, *X. axonopodis* uses these wounds as an entry point	Alvarez *et al.,* 2012
Cassava mosaic disease	Cassava mosaic geminiviruses	Green to yellow mosaic distortion of leaflets	Cassava mosaic disease is propagated mostly by the propagation of stem cutting obtained from diseased cassava plants.	Graziosi *et al.,* 2016
Brown leaf spots(BLS)	*Cerocosporidium henningsii*	Uniform brown spots on the lea f	Airborne spores can spread the	Graziosi *et al.,* 2016

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		surface	fungus from one plant to another in the field.	
Root rot (RR)	*Pythium* spp. *Fusarium* spp.	Maceration of the root parenchyma ,colour changes of roots	Root rot can be spread from one plant to the other. The fungal spores are airborne and can be spread by insects and unclean gardening tools.	
Ring leaf spot	*Phoma* spp.	Large dark-brown leaf with undefined margin	Ring leaf spot is spread by wind, splashing rain or irrigation	

1.4. Plant Disease Management

Plant diseases are caused by pathogenic living organisms or environmental conditions that cause aberrant physiological functions. Initially, plants react to the diseases causing the production of chemicals at infection sites. Later on, the reaction becomes more extensive and histological alterations occur. Different forms of symptoms might be seen macroscopically because of these alterations to control or manage plant diseases, two major control methods have been employed

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- Control method that excludes the pathogens
- Control methods that eradicate or reduce pathogens

CONTROL METHODS THAT EXCLUDES THE PATHOGENS

No infection will develop as long as plants and pathogens are kept separate from one another. Many plants are produced in places of the world where some pathogens are still absent, hence

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making them disease free. Some regulatory controls have been applied to prevent import of pathogens into areas that they are absent. Some of these regulatory controls include:

- Quarantine and inspections
- Evasion or avoidance of pathogens
- Use of pathogen free propagating materials

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CONTROL METHODS THAT ERADICATE OR REDUCE PATHOGENS

This are methods that have been designed to eradicate the whole pathogens population or reduce them drastically. Such methods include physical and chemical methods. The physical method involves the use of physical agents such as temperature, unfavourable wavelengths etc. Cultivation in greenhouses also provides physical barriers to pathogens thereby protecting the plants from diseases. The chemical method on the other hand is use of chemical pesticides on the surface of already contaminated plants to eradicate the pathogens, sanitation of handling equipment's amongst others (Aqleem 2015).

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1.5 AIM AND OBJECTIVES OF THE STUDY

The aim of the study was to evaluate the effect of incubation period on antifungal properties of zinc oxide and magnesium chloride nanoparticles against phytopathogens isolated from disease cassava plant.

The objectives of the study were

- Isolation of pathogens from disease plants
- Identification of the pathogens
- Synthesis of nanoparticles

- Characterization of the nanoparticles
- Antifungal testing of nanoparticles against test pathogens

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials used:

The diseased cassava plants for this study were obtained from a cassava farm in Ovia North-East Local Government Area, Benin, Edo State (6ºN 5ºE). Fresh *Moringa oleifera* leaves were collected from a tree in an open location near JB LODGE EKOSODIN in Ovia North East, Edo State, Benin City, Nigeria (6ºN 5ºE). Conical flask, autoclave, forceps, weighing balance, inoculation loop, spectrophotometer, magnetic stirrer, inoculation chamber, and spectrophotometer were among the materials used in this study. Pyrex suppliers provided all consumables including the potato dextrose agar.

2.2. Experimental methodology:

The experimental methodologies were as follows:

2.2.1 Preparation Potato Dextrose Agar (PDA)

This study's medium, PDA was prepared according to the manufacturer's instruction; 39 g of powdered PDA was dissolved in 1 liter sterile distilled water to make the PDA. The medium was then autoclaved at 121°C for 20 minutes under pressure to sterilize it. It was sterilized, cooled to 45-50 degrees Celsius before being aseptically dispensed into sterile petri dishes.

To prevent bacteria growth, an antibiotic (250 mL chloramphenicol) was added to 250 mL PDA before dispensing.

2.2.2 Isolation of fungal pathogens from diseased cassava

The cassava samples were prepared by teasing the plant part used (leaves) into smaller pieces, followed by surface sterilization with alcohol to remove surface contaminants. Direct plating method of fungal isolation was used. The prepared samples were aseptically inoculated on the already dispensed medium using sterile forceps. This was then inoculated at room temperature (28±2°C) for 72 hours and observed for fungal growth. Pure cultures of the isolates were obtained by picking single isolated mycelia of the fungi with the help of sterilized wire loop and streaking on fresh PDA medium. The culture was then incubated at room temperature (28±2 °C) for 72 hours.

2.2.3 Identification of fungal isolates

Macroscopy and microscopy were used to identify the fungal isolates. The morphological features were obtained, observed, and characterized. After staining with lactophenol blue, the isolates were examined under the microscope. After putting a drop of lactophenol blue stain on a clean grease-free sterilized glass slide, a sterile inoculating wire loop was used to select the mycelium from the culture onto the glass slide. On the slide, the mycelium was evenly distributed. Teasing was used to separate the mycelium to obtain a homogeneous mixture and prevent clustering. The mixture was then gently covered with cover slips and left for a few seconds before it observed at x40 magnification under the microscope. This was then compared with a fungus identification manual in the lab.

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2.2.4 Preparation of *Moringa oleifera* leaf extract

The stalk was removed, and the leaves were washed in sterile distilled water. To remove impurities, the surface was sterilized with 70 percent ethanol. A weighing balance was used to weigh twenty (20) grams of washed leaves. Mortar and pestle were then used to macerate the leaves. The blended leaves were suspended in 100 mL of sterile distilled water, well mixed, and then boiled for 2 minutes. Whatman filter paper was used to filter the extract.

2.2.5 Synthesis of zinc oxide nanoparticles using *Moringa oleifera* leaf extract

This was carried out according to the modified method of Alavi *et al*. (2019). The precursor used for the synthesis of zinc oxide nanoparticles was zinc nitrate hexahydrate $(ZnO(NO_3)_2;6H_2O)$. A concentration of 0.1 M of the precursor solution was prepared. An aliquot of 10 ml of the prepared plant extract was then added to 50 ml of the prepared precursor solution. The resulted solution was stirred vigorously for an hour. Reduction of $(ZnO(NO_3)_2;6H_2O)$ with *Moringa oleifera* leaf extract resulted in yellow–dark black colour.

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2.2.6 Synthesis of magnesium chloride nanoparticles using *Moringa oleifera* leaf extract

The synthesis was done using modified method of Alavi *et al*. (2019). Magnesium chloride hexahydrate (Mgcl.6H20) was used as a precursor to synthesize magnesium chloride nanoparticles. The precursor solution was produced at a concentration of 0.1 M. 60ml of the prepared plant extract was taken and added to 60ml of the prepared precursor solution in a conical flask ,the resulting solution was vigorsly agitated for an hour.

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2.2.7 Characterization of nanoparticles using spectrophotometer

The intensity of absorption peaks and peak absorbance of phytosynthesized nanoparticles was examined by UV–vis spectrophotometer. This was carried out by measuring the absorbance at regular intervals (2, 24 and 48 hours after synthesis) within the wavelengths of 300-800 nm.

2.2.8 Antifungal testing of nanoparticles against test pathogens

The antifungal activities of *Moringa oleifera* leaf extract, precursor solution, zinc oxide and magnesium chloride nanoparticles against the test pathogens were carried out using the food poisoning method. Two different concentrations corresponding to 100 and 0% (control) of zinc oxide and magnesium chloride nanoparticles was prepared. The 100% concentration of each was taken to be the stock solution while, 0% was the control. An aliquot of 2 ml of the nanoparticles for the different concentrations of both magnesium chloride and zinc oxide was added to 20 ml of PDA after pouring under sterile conditions. This was then shaken carefully and allowed to solidify. After solidifying, the test pathogens were inoculated by picking a culture plug of the fungal culture and placing it at the centre of the solidified medium. This was then incubated at room temperature and fungal mycelia growth was measured for a period of 8 days. Only 100% concentration of the leaf extract and precursor solution was tested for their antifungal activities using the same food poisoning method.

Percentage inhibition of growth was calculated following the formula suggested by Sunder *et al* (1995).

$$\text{Inhibition Rate\%} = \frac{dc - dt}{dc} \times 100$$

dc= average increase in mycelia growth (control)

dt= average increase in mycelia growth in treatment

2.2.9 Statistical analysis

Each treatment was repeated in three replicate and the results were presented as mean ± standard error. The data from this study were subjected to descriptive statistics using Microsoft excel and parametric statistics using Statistical Package for Social Sciences (SPSS).

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CHAPTER THREE

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RESULTS

Table 1 shows the morphological characteristics of the fungal isolated from with diseased cassava plant. The isolated fungal pathogens both have flat elevations and filamentous margins. Plate 1 and 2 showed the pure cultures and photomicrograph of *Aspergillus* sp.and *Penicillium sp*. recpectively

Table 1: Morphological characteristics of fungal isolates associated with disease cassava plants

MORPHOLOGY	**ISOLATE 1**	**ISOLATE 2**
Margin	Filamentous	Filamentous
Elevation	Flat	Flat
Size	Medium	Large
Texture	Powdery	Velvety
Pigmentation	Blue green	White at margin, green at centre
Form	Irregular	Circular
Optical property	Transluscent	Opaque
Identified organism	***Penicillum sp.***	***Aspergillus sp.***

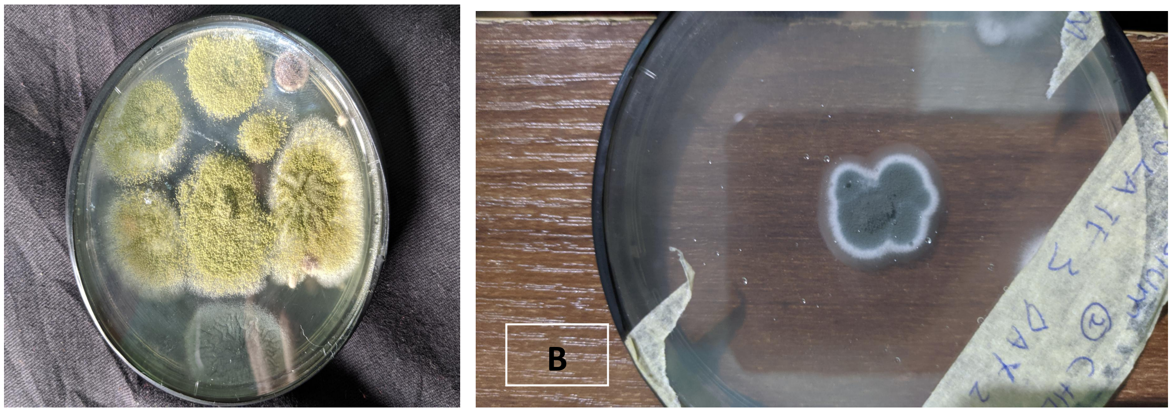

Plate 1: pure cultures of *Aspergillus sp*. and *Penicillium sp.* isolated from diseased cassava plant.
A; *Aspergillus sp*.   B: *Penicillium sp.*

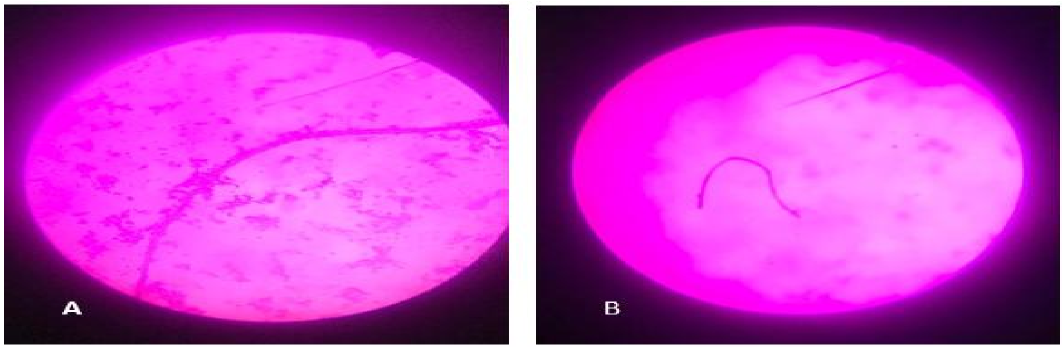

Plate 2: Photomicrographs of *Aspergillus sp*. and *Penicillium sp.* isolated from diseased cassava plant.

Legend;
- A: *Aspergillus sp*.
- B: *Penicillium sp.*

The absorbance values of the synthesized zinc oxide nanoparticles taken after 2, 24, 48hours of synthesis is shown in figure 1. The wavelength of the absorbance ranges from 300- 500nm and the peak of the absorbance was recorded at 400nm

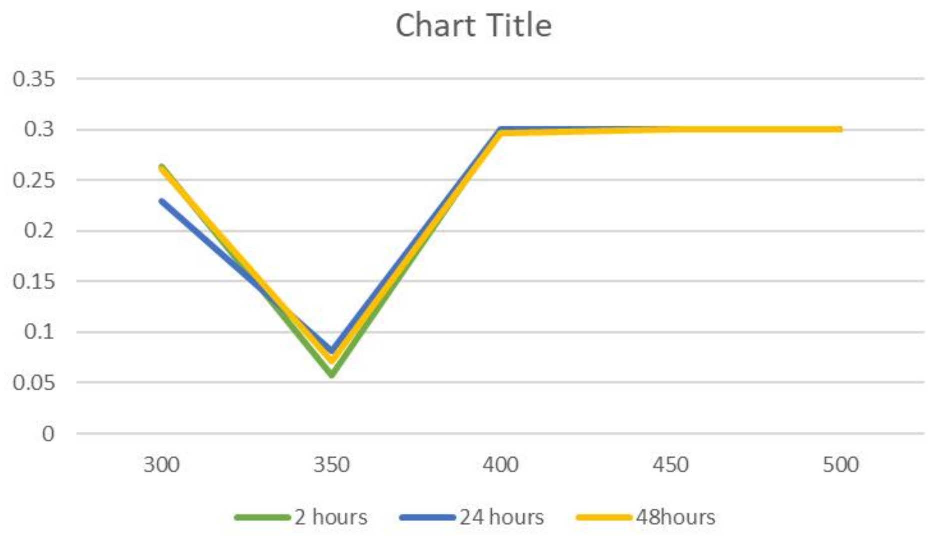

Figure 1: The absorbance values of biologically synthesized zinc oxide nanoparticles taken after 2,24 and 48 hours of synthesis.

The absorbance values of the synthesized magnesium chloride nanoparticles taken after 2, 24, 48hours of synthesis is shown in figure 2. The wavelength of the absorbance ranges from 300- 500nm. The peak absorbance was recorded at 350nm.

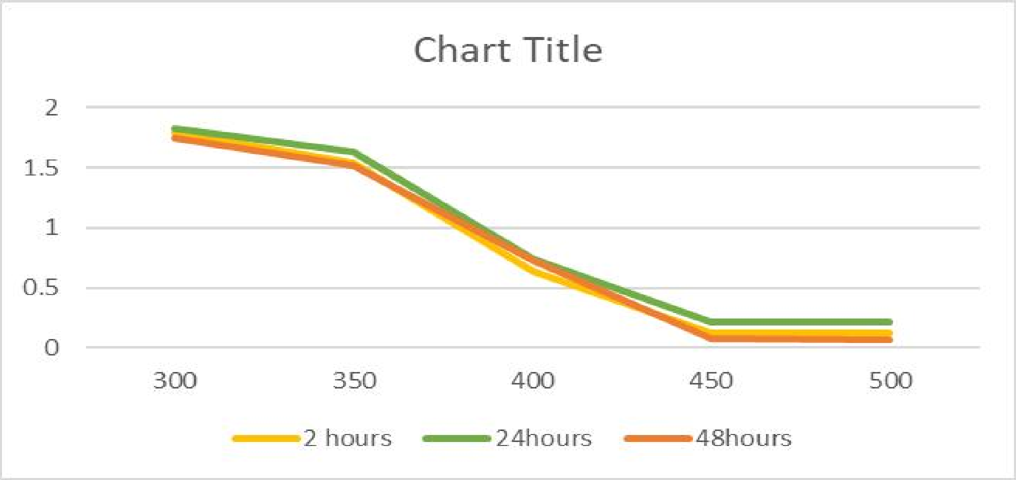

Figure 2: The absorbance values of biologically synthesized magnesium chloride nanoparticles taken after 2,24 and 48 hours of synthesis.

Table 2 shows the effect of the incubation periods of magnesium chloride nanoparticles on the mycelia growth of *Penicillium* of The 100% $MgCl_2$ nanoparticles treatment at 24 hours (Day 2) exhibited the lowest mycelia growth of 2.67±0.44. The highest mycelia growth, 36.83±2.05 was observed on day 8.

Table 2: Effect of incubation periods of magnesium chloride nanoparticles on the mycelia growth of *Penicillium* sp.

Treatments	**Mycelia Growth Measurement (mm)**			
	Day 2	**Day 4**	**Day 6**	**Day 8**
Control	$13.83^{c}\pm3.94$	$21.83^{d}\pm1.20$	$24.37^{c}+\pm3.87$	$33.73^{cd}\pm3.56$
24 hours	$2.67^{a}\pm0.44$	$18.67^{cd}\pm1.48$	$20.80^{abc}\pm1.33$	$22.50^{cd}\pm1.61$
Control	$6.33^{ab}\pm0.60$	$14.00^{ab}\pm0.50$	$21.50^{abc}\pm0.76$	$36.83^{d}\pm2.05$
48 hours	$4.67^{ab}\pm0.73$	$11.50^{ab}\pm1.61$	$15.17^{a}\pm1.67$	$25.27^{ab}\pm0.67$
Control	$6.67^{ab}\pm1.01$	$13.17^{ab}\pm2.52$	$17.83^{abc}\pm1.87$	$21.50^{ab}\pm1.61$
72 hours	$4.33^{ab}\pm0.44$	$13.80^{ab}\pm1.62$	$20.17^{abc}\pm0.88$	$27.00^{abc}\pm2.08$
Control	$7.83^{b}\pm0.17$	$12.17^{ab}\pm1.17$	$16.67^{ab}\pm1.45$	$22.00^{ab}\pm1.16$
96 hours	$4.33^{b2}\pm0.44$	$16.00^{bc}\pm1.16$	$23.33^{c}\pm2.03$	$19.67^{cd}\pm2.91$
Control	$5.67^{ab}\pm0.67$	$9.33^{a}\pm0.88$	$15.33^{a}\pm1.86$	$26.67^{a}\pm3.11$
120 hours	$7.70^{b}\pm1.23$	$11.67^{ab}\pm1.67$	$22.00^{bc}\pm2.08$	$26.35^{ab}\pm1.13$

Values are presented in mean ± standard error; figures bearing similar superscripts within columns are not significantly different using Duncan's Multiple Range (DMR) test at 0.05 level of significance.

Legend: Control Plate (0%) NPs
Treated Plate (100%) NPs

The effect of the incubation period of zinc oxide nanoparticles on the mycelia growth of *Penicillium* sp. (Table 3) indicated that there was no mycelia growth of the test organism subjected to the 120 hours of incubation treatment on day 2 and day 4. The mycelia growth of the test organism was observed to increase with increasing days of incubation.

Table 3: Effect of incubation period of zinc oxide nanoparticles on the mycelia growth of *Penicillium* sp.

Treatment	**Mycelia Growth Measurement (mm)**			
	Day 2	**Day 4**	**Day 6**	**Day 8**
Control	$2.67^{ab}\pm0.76$	$18.67^{e}\pm1.48$	$20.80^{cde}\pm1.33$	$22.50^{ab}\pm1.61$
24 hours	$4.83^{b}\pm0.44$	$4.83^{a}\pm0.44$	$4.83^{a}\pm0.44$	$4.83^{c}\pm4.41$
Control	$4.67^{b}\pm0.73$	$8.80^{bc}\pm4.08$	$15.17^{bc}\pm1.67$	$25.27^{ab}\pm0.67$
48 hours	$3.67^{b}\pm0.83$	$3.67^{a}\pm0.83$	$12.67^{b}\pm0.83$	$18.50^{a}\pm1.32$
Control	$4.33^{b}\pm0.44$	$16.5^{d}\pm1.44$	$21.67^{de}\pm1.88$	$27.00^{b}\pm2.08$
72 hours	$10.17^{c}\pm2.33$	$13.83^{cde}\pm0.67$	$18.83^{cde}\pm2.95$	$22.33^{ab}\pm2.19$
Control	$4.33^{b}\pm0.44$	$16.00^{d}\pm1.16$	$23.33^{e}\pm2.03$	$28.33^{b}\pm1.45$
96 hours	$10.00^{c}\pm0.58$	$12.83^{cd}\pm0.88$	$16.00^{bcd}\pm1.80$	$21.33^{ab}\pm1.20$
Control	$8.00^{c}\pm1.53$	$11.67^{cd}\pm1.67$	$22.00^{de}\pm2.08$	$26.33^{b}\pm1.57$
120 hours	$0.00^{a}\pm00$	$0.00^{a}\pm00$	$15.30^{bc}\pm2.31$	$23.00^{ab}\pm1.55$

Values are presented in mean ± standard error; figures bearing similar superscripts within columns are not significantly different using Duncan's Multiple Range (DMR) test at 0.05 level of significance.

Legend: Control Plate (0%) NPs
Treated Plate (100%) NPs

The effect of the incubation period of magnesium chloride nanoparticles on the mycelia growth of *Aspergillus* sp. is shown in table 4. There were significant differences in the growth of the test pathogen subjected to the different treatments in all the days observed. The lowest mycelia growth was observed on day 2 (5.40±1.31), while the highest mycelia growth was observed on day 8 (81.67±0.88) from the 72 hours incubation period treatment.

Table 4: Effect of the incubation period of magnesium chloride nanoparticles on the mycelia growth of *Aspergillus* sp.

Treatments	**Mycelia Growth Measurement (mm)**			
	Day 2	**Day 4**	**Day 6**	**Day 8**
Control	$16.89^{c}\pm1.66$	$34.00^{bc}\pm3.04$	$50.33^{bc}\pm5.49$	$80.33^{ba}\pm2.60$
24 hours	$9.83^{ab}\pm1.59$	$28.67^{b}\pm2.33$	$39.63^{a}\pm3.96$	$57.33^{a}\pm073$
Control	$17.00^{c}\pm1.89$	$34.33^{b}\pm2.46$	$57.67^{c}\pm1.20$	$81.67^{b}\pm0.60$
48 hours	$10.67^{b}\pm1.76$	$27.33^{b}\pm2.60$	$38.50^{a}\pm3.75$	$55.83^{a}\pm0.44$
Control	$14.83^{bc}\pm2.19$	$36.17^{bc}\pm1.26$	$51.00^{bc}\pm1.53$	$81.67^{b}\pm0.88$
72 hours	$10.33^{ab}\pm0.88$	$30.67^{b}\pm1.20$	$42.67^{ab}\pm1.45$	$53.67^{a}\pm2.02$
Control	$17.33^{c}\pm0.73$	$39.33^{c}\pm2.96$	$55.67^{c}\pm1.20$	$81.00^{b}\pm1.00$
96 hours	$10.00^{ab}\pm1.15$	$28.83^{b}\pm3.49$	$40.00^{a}\pm1.32$	$55.00^{a}\pm1.32$
Control	$15.67^{c}\pm1.88$	$34.50^{bc}\pm0.76$	$43.53^{c}\pm1.30$	$80.33^{b}\pm0.17$
120 hours	$5.40^{a}\pm1.31$	$21.83^{a}\pm1.04$	$35.00^{a}\pm1.53$	$57.07^{a}\pm0.99$

Values are presented in mean ± standard error; figures bearing similar superscripts within columns are not significantly different using Duncan's Multiple Range (DMR) test at 0.05 level of significance.

Legend: Control Plate (0%) NPs
Treated Plate (100%) NPs

Table 5 shows the effect of the incubation period of zinc oxide nanoparticles on the mycelia growth of *Aspergillus* sp. There were no significant differences within some groups, in the growth of the test pathogen subjected to the different treatments in all the days observed. The lowest mycelia growth, (5.40±1.31) was observed on day 2, while the highest mycelia growth, (81.67±0.67) was observed on day 8.

Table 5 Effect of the incubation period of zinc oxide nanoparticles on the mycelia growth of *Aspergillus* sp.

Treatment	**Mycelia Growth Measurement (mm)**			
	Day 2	**Day 4**	**Day 6**	**Day 8**
Control	9.83^{abc}±1.59	28.67^{bc}±2.33	39.63^{b}±3.96	57.33^{d}±0.72
24 hours	7.67^{a}±3.18	8.00^{a}±3.21	24.50^{a}±1.76	31.00^{a}±3.79
Control	10.67^{abc}±1.76	27.33^{bc}±2.60	38.83^{b}±3.47	55.83^{d}±0.44
48 hours	15.37^{cd}±1.84	24.67^{bc}±2.83	32.33^{ab}±2.40	43.00^{d}±3.18
control	13.33^{bcd}±1.76	22.70^{b}±3.10	35.00^{ab}±4.36	38.67^{bc}±1.45
72 hours	10.33^{abc}±0.88	30.67^{c}±1.20	38.33^{bc}±4.41	53.67^{d}±2.03
Control	17.33^{d}±1.20	21.17^{b}±1.09	31.67^{ab}±3.28	36.17^{ab}±2.13
96 hours	10.00^{abc}±1.15	28.83^{bc}±3.49	36.33^{b}±4.75	55.00^{d}±1.32
Control	13.20^{bcd}±1.67	21.33^{b}±1.20	31.67^{ab}±2.19	36.67^{ab}±1.67
120 hours	5.40^{a}±1.31	21.83^{b}±0.60	33.67^{ab}±1.67	57.07^{d}±0.99

Values are presented in mean ± standard error; figures bearing similar superscripts within columns are not significantly different using Duncan's Multiple Range (DMR) test at 0.05 level of significance.

Legend: Control Plate (0%) NPs
Treated Plate (100%) NPs

The result for the rate of inhibition (%) of Magnesium chloride nanoparticles on *Penicillium* is shown in Figure 3. Under different incubation treatments with respect to days, the inhibition rate varied for each group. At 24 hours incubation period, magnesium chloride nanoparticles exhibited the highest inhibition rate.

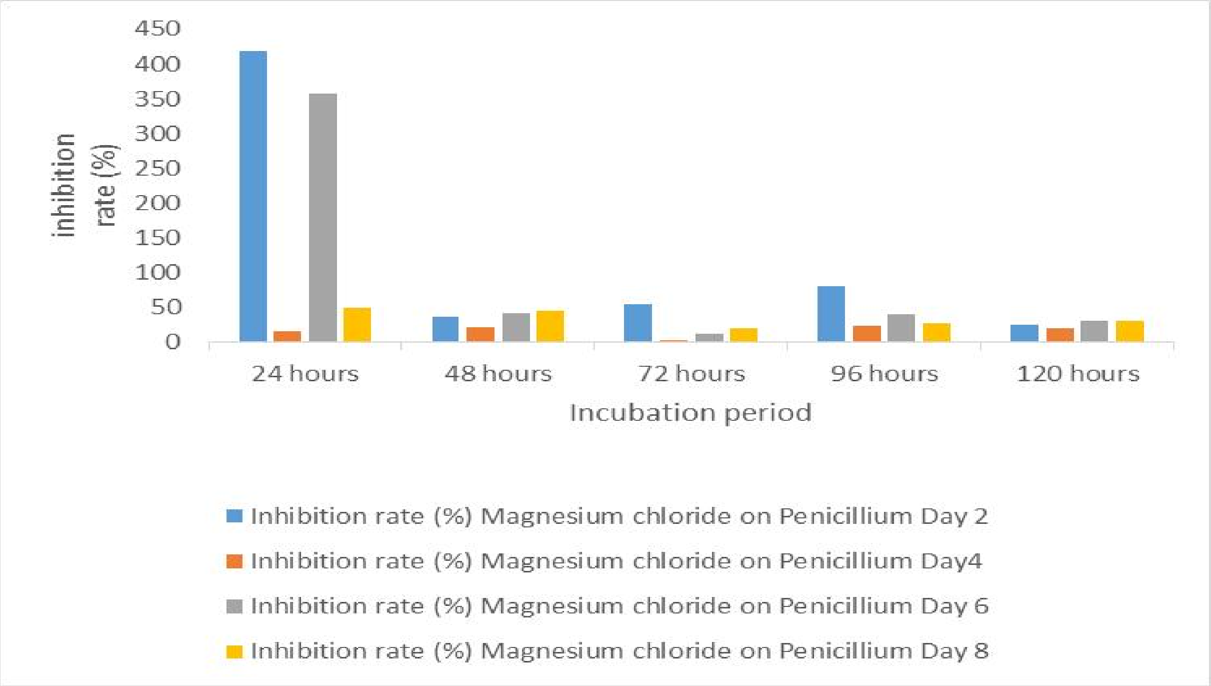

Figure 3: Rate of inhibition of magnesium chloride nanoparticles on *Penicillium* sp.

The rate of inhibition (%) of Zinc oxide nanoparticles on *Penicillium* sp. is shown in Figure 4. The treatments varied in each group; although there was no variation within the 24 hours incubation period, magnesium chloride exhibited the highest inhibition rate, at 96 hours and 72 hours of incubation treatments respectively.

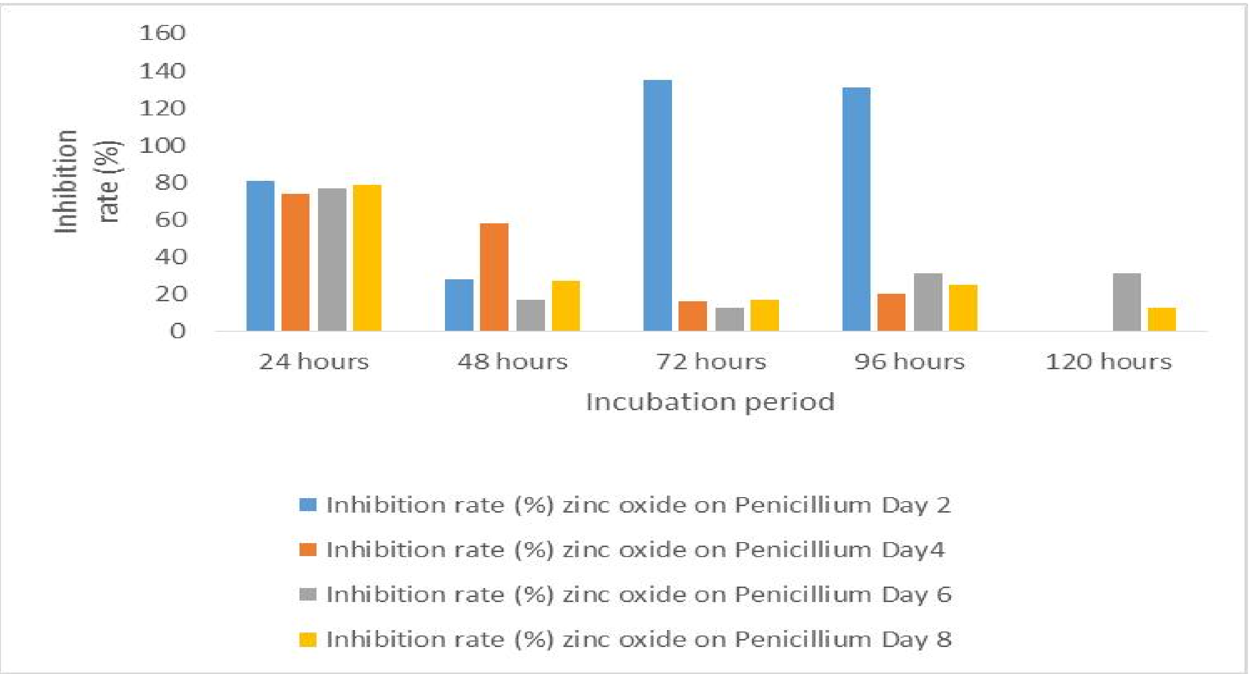

Figure 4: Rate of inhibition of zinc oxide nanoparticles on *Penicillium* sp.

The result for the rate of inhibition (%) of magnesium chloride nanoparticles on *Aspergillus* sp. is shown in Figure 5. The inhibition rate varied in each group; 185 % inhibition was obtained by 120 hours of incubation treatment against the other treatment which showed a lesser inhibition rate.

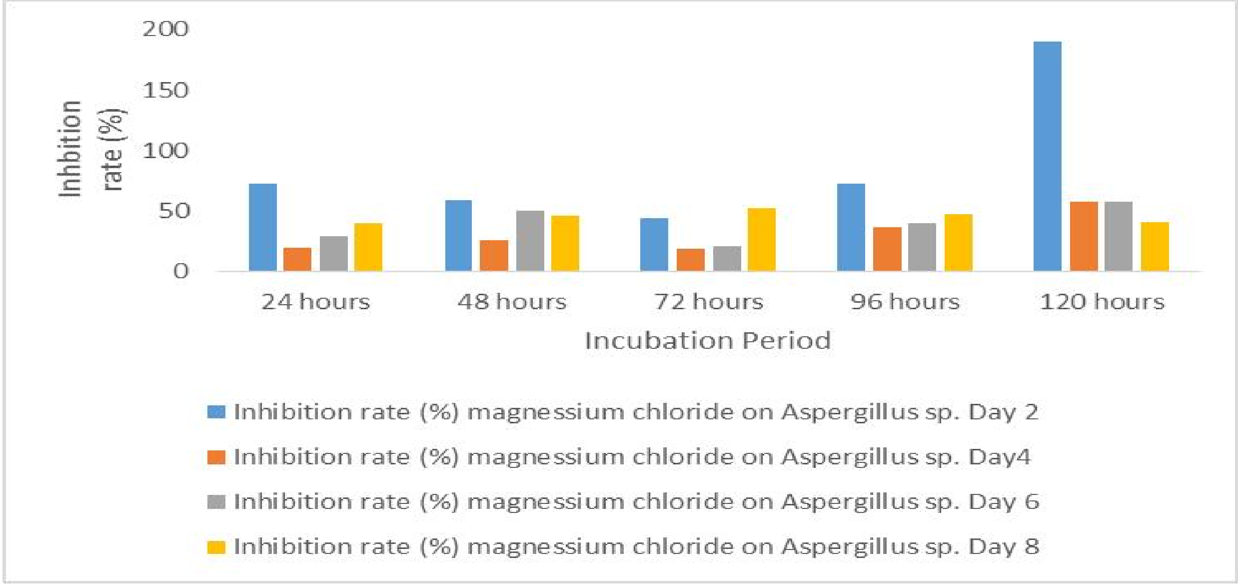

Figure 5: Rate of inhibition of magnesium chloride nanoparticles on *Aspergillus* sp.

Figure 6 shows the rate of inhibition of zinc oxide nanoparticles on the mycelia growth of *Aspergillus* sp. The highest rate of inhibition observed was 150% inhibition at day 2.

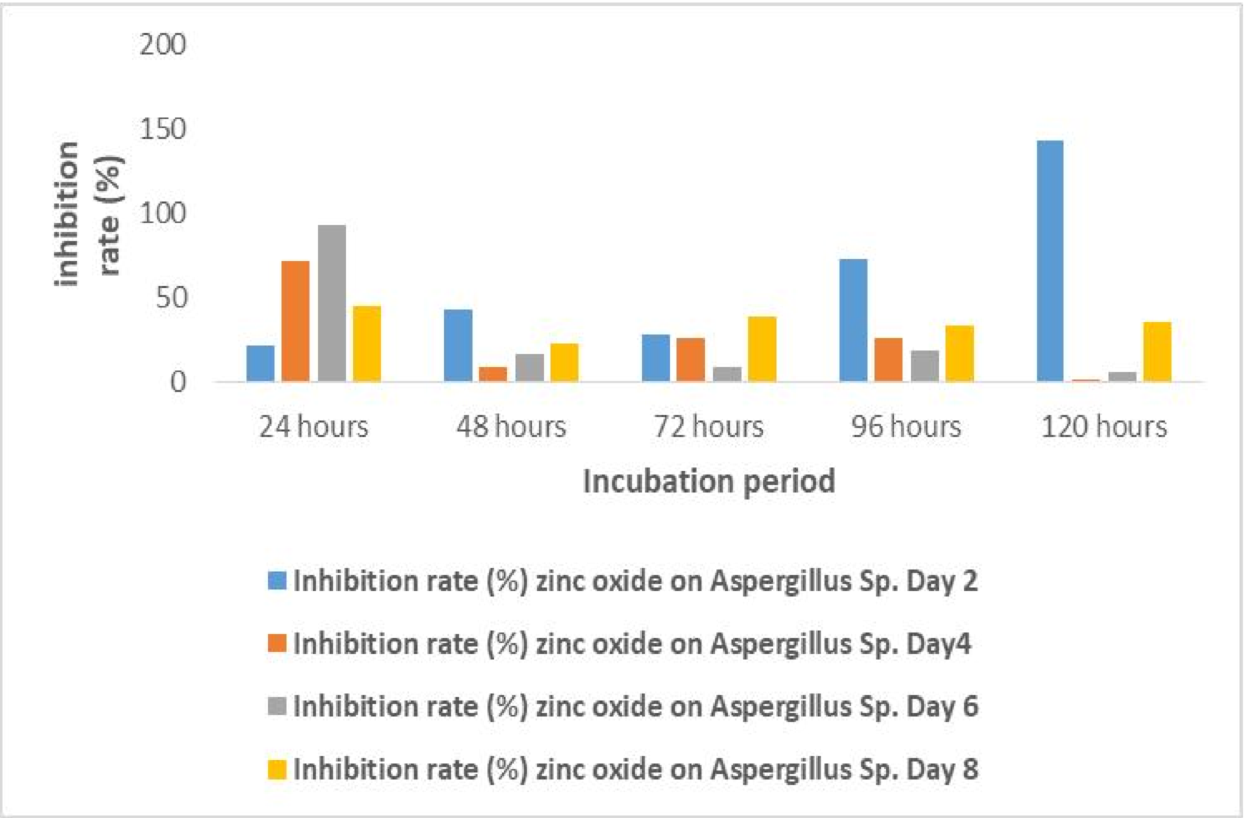

Figure 6: Rate of inhibition of zinc oxide nanoparticles on *Aspergillus* sp.

CHAPTER 4

DISCUSSION

The purpose of this study was to evaluate the effect of incubation period on the antifungal activity of magnesium chloride and zinc oxide nanoparticles against pathogens isolated from diseased cassava plant. Incubation is usually defined as the time required for multiplication of the parasite within the host to the threshold at which its population is large enough to produce symptoms (Armenian and Lilienfeld, 1983), incubation period has become one of the most neglected parameters in evaluating the antifungal activity of nanoparticles (Sartwell, 1950).

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The fungal isolates from the diseased cassava plant were identified to be *Penicillium* sp. and *Aspergillus* (Table 1). *Aspergillus* spp. is mainly saprophytes in soil, decaying vegetation, hay and grains undergoing microbiological deterioration and they can also be pathogenic to plants and animals including humans. Different factors influence the incidence of fungal infection to plants, these include the presence of invertebrate vectors, injury to the bark of the stem and root, tuber damage, inocula load, substrate composition, fungal infection levels, prevalence of toxigenic strains and microbiological interactions (Horn, 2003). *Aspergillus* sp. produces secondary metabolites such as aflatoxins, among all molds and mycotoxins found in cassava, only *Aspergillus flavus* and aflatoxin contamination remain complex, previous studies by (Brudzynski *et al.*, 1977; Bottalico *et al.,* 1980; Adegoke *et al.,* 1993) focused on the contamination of cassava by aflatoxin, and its presence was marked with different symptoms such as leaf rot, sunken stem, and tuber discoloration.

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Penicillium sp. causes numerous diseases such as cassava rot, concentric leaf spot in cassava (Ambe, 1994; Okigbo *et al.,* 2009). The occurrence of these diseases is usually during cold seasons temperature below $20^{o}C$ and during rainy season (CIAT, 1972), according to Edison (2002), this pathogen is an emerging problem in certain areas where cassava cultivation is intensive. The disease may cause severe defoliation in susceptible varieties and almost always produces stem dieback, a phenomena in which there is a progressive death of tuber roots, shoots and stem usually starting from the tips of the plant. Favourable conditions for the germination of their fungal spores occur at temperatures between ($20-25^0C$). While artificial inoculation, infection is only achieved when inoculated plants are kept for 48 hours at less than 24^0C and with 100% relative humidity (Lozano, 1979). *Aspergillus and Penicillium* are considered as the most important fungal genera because of their ability to produce mycotoxins such as fumonisin (fB), aflatoxin (Af), ochratoxin (OTA), ZEA and trichothecenes (Adaku *et al.,* 2012)

The absorbance value of zinc oxide nanoparticles synthesized using zinc nitrate hexahydrate ($ZnO(NO_3)_3.6H_2O$) after 2, 24, 48 hours of synthesis is shown in figure 1. The wavelength of the absorbance ranges from 300- 500nm, the highest absorbance peak was recorded at 400nm these values agrees with those obtained by (Zak *et al*., 2016). The plant extract and its concentration in biosynthesis reaction usually influence the morphology of ZnO nanoparticles, for instance extract containing functional groups such as alcohol, ketone, carboxylic acid and amine creates spherical-shaped ZnO nanoparticles, whereas extract with -OH (hydroxyl) group gives quasi-spherical agglomerates (Elumali and Velmurugan, 2015; Matinise *et al.,* 2017))

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The absorbance of magnesium oxide nanoparticles had its highest peak at 350 nm after 2, 24, and 48 hours of synthesis. Figure 2 showed the absorbance value of magnesium chloride nanoparticles using magnesium chloride hexahydrate ($MgCl_6H_2O$). According to the study carried out by Lopez-Tellez *et al*. (2018), it was observed that secondary metabolites such as terpernoids, flavones, ketone aldehyde, amines and carboxylic acids which are present in the plant extract used for synthesis acted as reducing and capping agents during the formation of magnesium nanoparticle and it also influenced the morphology of the particles.

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The antifungal properties of ZnO and $MgCl_2$ nanoparticle on the mycelia growth of *Penicillium* was investigated. The results obtained in (table 2)~~,~~ indicated that $MgCl_2$ nanoparticle showed antifungal activity against the test pathogen. There was significant difference between the control containing 0% nanoparticle and the culture media that was treated with 100% $MgCl_2$ nanoparticles. It was observed that the $MgCl_2$ nanoparticle showed the highest potency against the test pathogen during the first 24 hours of incubation period. The mycelia growth was highly inhibited and subsequently the sizes of the mycelium began to increase slightly over the following incubation period 48, 72, 96, 120, hours. Moreover, the increase in the mycelia growth rate was significantly dependent on the incubation period. The higher the incubation period the lesser the inhibition rate (figure 3); because with time the fungus pathogen tends to build up resistant against the antifungal agent; this findings is synonymous with the findings of (Whitelaw et al., 1997; Khan et al., 2008; Leitao and Enguita, 2016) in their study which made them conclude that genus *Penicillium* is one of the most versatile “mycofactories,” comprising species able to solubilized magnesium, when this solubilization reaction occur, magnesium is then dissociated.

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The effect of incubation period of ZnO nanoparticles on the mycelia growth of *Penicillium* is shown in table 3, A significant difference was observed in the growth of the pathogen subjected to different treatment . The mycelia growth observed in the control was higher than those treated with nanoparticles, which implies that the presence of ZnO nanoparticles affects the mycelia the growth of the pathogen. This study shows that for all the treatments carried out, the nanoparticles inhibited fungal growth to a high degree. This finding is consistent with the results of a study done by ~~(Gehan A.N., *et al.,* 2014)~~ Gehan *et al.* (2014), which confirmed the weak antifungal effect of ZnO on the mycelia growth of fungal pathogens at optimal incubation period.

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Table 4 and Table 5 shows the effect of incubation period of magnesium chloride nanoparticles and zinc oxide respectively on the mycelia growth of *Aspergillus* sp. a level of antifungal activity was observed although the mycelia growth of the organism increased during the extended incubation period, the level of antifungal activity of the two nanoparticles it was observed that ZnO has more antifungal activity compared to MgCl, this findings aligns with a study carried out by (Moraru *et al.,* 2003; Sewai and Yoshikawa, 2004). They reported that the antimicrobial effect of ZnO nanoparticle occur by two ways. The first is the formation of H_2O_2 on the surface of ZnO nanoparticles due to the possible formation of hydrogen bond between hydroxyl group of cellulose molecules of fungi with oxygen atom of ZnO nanoparticles leading to inhibition of the microbial growth, while the second is the release of Zn^{2+} which causes damages of cell membrane and interacts with intraocular contents. Nanoparticles generally both natural and synthesized have demonstrated strong antimicrobial properties through diverse mechanisms including photocatalytic production of reactive oxygen species that damage cell components and viruses (as ZnO), compromising the cell envelope, e.g. peptides, chitosan carboxyfullerene, carbon nanotubes, ZnO and interruption of energy transduction (Matei, *et al.,* 2010; Violeta *et al.,* 2011). This explains the reason for the different level of antifungal activity of ZnO and MgCl on the test pathogen.

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Th0e result for the inhibition rate (%) of magnesium chloride nanoparticles on *Aspergillus* sp. is shown in Figure 5. The inhibition rate varied in each group; 185 % inhibition was obtained by 120 hours of incubation treatment against the other treatment which showed a lesser inhibition rate. Mycelia growth was significantly inhibited in all four-incubation period, however in day 8 and day 10 when there was a reduction in the concentration of the PDA treatment, the antifungal activities was reduced, Verma and Dubey (1999), ascertained the effectiveness of plant synthesized nanoparticles as effective antifungal agents.

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The present investigation is an important step in ascertaining the effect of nanoparticles on diseased cassava plant *in-vitro,* before it is applied *in*-vivo; although slight differences may occur in the field application which will be different from the plate trial of the test nanoparticles because of unstable temperature in the field. Further investigation will be done for developing commercial formulation based on field trail and toxicological experiment

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CONCLUSION

The results obtained from this study suggest that the test nanoparticles were not effective against *Aspergillus* and *Penicillium* sp. in longer incubation period, Similar experiment can be carried out with same methodology a fresh treatment will be added to the incubation as the incubation period increases, because it was observed that as the incubation period increases the potency of the nanoparticle decreases, which could either be due to some R-genes fungus possess in their cell wall.

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Appendix

Inhibition rate (%) Magnesium chloride on isolate 3

Treatment	Day 2	Day4	Day 6	Day 8
24 hours	418	17	357	50
48 hours	36	22	42	46
72 hours	54	4	13	20
96 hours	80	24	40	28
120 hours	26	20	30	30

Inhibition rate (%) zinc oxide on isolate 3

Treatment	Day 2	Day4	Day 6	Day 8
24 hours	81	74	77	79
48 hours	28	58	17	27
72 hours	135	16	13	17
96 hours	131	20	31	25
120 hours	0	0	31	13

Inhibition rate (%) magnessium chloride on isolate 4

Treatment	Day 2	Day4	Day 6	Day 8
24 hours	72	19	29	40
48 hours	59	26	50	46
72 hours	44	18	20	52
96 hours	73	36	40	47
120 hours	190	58	58	41

Inhibition rate (%) zinc oxide on isolate 4

Treatment	Day 2	Day4	Day 6	Day 8
24 hours	22	72	94	46
48 hours	44	10	17	23
72 hours	29	26	9	39
96 hours	73	27	19	34
120 hours	144	2	6	36