

**Antifungal effect of Ag/Cu bimetallic nanoparticles against selected mycotoxin
producing fungi on maize**

By

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DECLARATION

I, Ongeziwe Sinazo Wutu, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

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ABSTRACT

Eastern Cape has the highest levels of maize contamination, which is accelerated by poor agricultural practices; therefore monitoring the spread of the contamination is crucial. The aim of this study was to investigate the antifungal effects of Ag/Cu bimetallic nanoparticles (BMNPs) against the selected mycotoxin causing fungi in maize. Stored maize samples with visible contamination were collected from Centane region in the Eastern Cape. Pathogens were isolated and their molecular identification was done using the Polymerase chain reaction (PCR). *Fusarium verticillioides* (*F. verticillioides*), *Fusarium oxysporum* (*F. oxysporum*) and *Aspergillus flavus* (*A. flavus*) were obtained. The nanoparticles were synthesized using chemical reduction method and characterized using ultra-violet visible spectroscopy (UV-Vis) and transmission electron microscopy (TEM) prior evaluation. The antifungal activity of Ag/Cu bimetallic nanoparticles against *F. verticillioides*, *F. oxysporum* and *A. flavus* was investigated through an agar diffusion and spiral gradient methods. Data collected was analysed statistically using Analysis of Variance (ANOVA) on GenStat 20th edition.

The mycelial growth was observed after 7 days and the investigations from this study resulted into nanoparticles (NPs) of an average size of 26, 10, ~5 and 6 nm for Ag, Cu, Ag/Cu and Cu/Ag, respectively. The antifungal activity of Ag NPs was found to be slightly stronger than that of the both controls (AgNO_3 and Cu(OA)_2) against *F. verticillioides*. Moreover, BMNPs showed improved percentage inhibition (61.0 %) against *A. flavus* as compared to their counterparts' monometallic NPs (42.0 % and 36.0 %). Cu/Ag BMNPs revealed to have almost equal efficacy in comparison with Ag/Cu BMNPs regardless of their size. The minimum inhibitory concentration of Cu NPs against *F. oxysporum* was observed to be 17.61 $\mu\text{g/mL}$, while that of Cu/Ag BMNPs was observed to be 12.89 $\mu\text{g/mL}$. The percentage difference between treatments was also evaluated in order to determine the strength of the NPs to each other. This study demonstrated that NPs exhibited reasonable antifungal activity against the tested crop fungal pathogens. Therefore, they may benefit the agricultural sector for the management of fungal pathogens, particularly those which are associated with post-harvest storage.

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DEDICATION

To the memory of my late grandmother, Nokhombile Nocawe Jako intombi yakwaTshezi.

ABBREVIATIONS AND ACRONYMS

AFB	Aflatoxin B1
AFM	Aflatoxin M1
AFG	Aflatoxin G
<i>A. flavus</i>	<i>Aspergillus flavus</i>
BMNPs	Bimetallic nanoparticles
CTAB	Cetyltrimethyl ammonium bromide
DON	Deoxynivalenol
EDTA	Ethylenediaminetetraacetic acid
FB1	Fumonisin B1
FB2	Fumonisin B1
<i>F. oxysporum</i>	<i>Fusarium oxysporum</i>
<i>F. verticillioides</i>	<i>Fusarium verticillioides</i>
IFI	Industrial Facilities Initiative
NPs	Nanoparticles
NS	Nanosphere
OTA	Ochratoxins
PVP	Polyvinyl pyrrolidone

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CHAPTER ONE INTRODUCTION AND BACKGROUND

This chapter contains the background, the aims, and objectives of the study.

1.1 Introduction

Maize (*Zea mays* L.) is one of the most important cereals and ranks the third largest produced cereal in the world after wheat and rice, serving different purposes of economic significance. In the rural areas of South Africa maize is used as a staple dietary meal, moreover, as a key component in animal feed formulations, largely for poultry, swine, and dairy cattle (Goodla & Panda, 2020; Kierończyk et al., 2021). However, maize crops are prone to fungal infections, especially those caused by the *Fusarium* spp. Maize contamination happens through agricultural practices, during storage and transportation. High temperatures and moisture conditions, unseasonal rains during harvest and flash floods lead to the spread of fungal contaminations (Ngum et al., 2022). Therefore, the success of maize production is crucial and requires proper management from contamination caused by insects and pathogens (Chomonyo et al., 2020; Diko & Jun, 2020).

Mycotoxins are a serious threat to human life, as a result there are specific regulations for different mycotoxins levels allowed in food stuff (Gulbis et al., 2016). Mycotoxins are not only carcinogenic but can affect enzymes that are involved in DNA replication causing birth defects and development malformations (Kyei et al., 2020; Gönenç et al., 2020; Tesfamariam et al., 2022). For example, in Kenya consumption of mycotoxin contaminated maize was associated with the incidence of liver cancer (Ngure et al., 2021). Moreover, cases of chronic exposure to mycotoxins and acute poisoning have been reported in Nigeria, Ghana, Tanzania, Somalia, China and South Africa (Arapcheska et al., 2015; Wielogorska et al., 2019; Massomo, 2020; Edgar et al., 2021; Mamo et al., 2021; Fru et al., 2021; Eskola et al., 2020).

In 2017, over 400 types of mycotoxins were recorded but the major mycotoxins that affect animals, plants and humans are Aflatoxins, Fumonisin, Ochratoxins (OTA), Zearalenone (ZEN), Deoxynivalenol (DON), Trichothecenes (TCs), Fusarin (Alshannaq & Yu, 2017; Awuchi et al., 2021; Achparaki et al., 2021). Fumonisin and aflatoxins are the most prevalent mycotoxins in maize crops. In a study that was conducted in South Africa in the Eastern Cape Province fumonisin was reported to cause oesophageal cancer in human, while aflatoxins were

associated with liver cancer in both human and animals (Sewram et al., 2016). This necessitates constant monitoring, mapping out of all communities that are contaminated, and manage organisms responsible for mycotoxin production, hence this study focuses on managing the organisms responsible. The most prevalent fungal species causing fumonisins and aflatoxins in maize are *F. verticillioides*, *F. oxysporum* and *A. flavus* hence these pathogens were selected for this study. It is a global challenge to control fungal growth and mycotoxin contamination in maize thus, requires exploration of new antifungal strategies. Moreover, commercial farmers can implement various strategies to reduce their losses, subsistence farmers on the other hand tend to rely on inadequate resources and skills when it comes to ensuring the quality of their crops. This can lead to poor harvests and fungal infection.

Nanoparticles (NPs) are gaining a lot of interest as antifungal agents due to their properties such as a broad-spectrum antimicrobial activity, small size and large surface area (Yah & Simate, 2015; Horky et al., 2018). In recent years, metallic nanoparticles have gained widespread interest among researchers due to their antimicrobial properties. However, bimetallic nanoparticles (BMNPs) are even more interesting due to the manipulation of the two different metals. Therefore, this study presents the antifungal activities of Ag/Cu BMNPs on the inhibition of *F. verticillioides*, *F. oxysporum* and *A. flavus* isolated from contaminated maize collected from Centane in the Eastern Cape Province. Cu and Ag nanoparticles have been studied against a wide range of pathogens, however, most of the studies employ the monometallic form of these nanoparticles.

1.2 Problem statement

Poor resource farmers are major contributors of maize production in rural areas in Centane region of the Eastern Cape Province. To sustain the production system, these farmers utilize maize seeds which had been stored from previous cultivation season. During storage, the seeds are contaminated particularly with *Fusarium* and *Aspergillus* pathogens. Such pathogens are among major causes of mycotoxins and if left untreated may cause serious yield losses. Moreover, these mycotoxins do not only affect yield levels but humans as well as livestock. According to (Poroşnicu et al., 2023; Yu & Pedroso, 2023; Visagie et al., 2024) fumonisin has been reported to cause liver and kidney injury in cattle, impairment of cardiac function and pulmonary oedema in pigs. While aflatoxin causes hepatotoxic, carcinogenic, mutagenic and immunotoxic effects (Shibabaw et al., 2023; Pożarska et al., 2024). (Sydenham et al., 1990) first reported the outbreak of oesophageal cancer in South Africa related to contaminated maize by fungi in 1990. Thereafter studies have

concentrated in quantification of levels of contamination while a few have focused in monitoring the levels of mycotoxins and finding remedies beyond the traditional remedies. Hence, innovative, and advanced systems are needed to enhance efficiency of plant disease control as well as the control of mycotoxin producing pathogens. As a result, there is a need to introduce the use of NPs as possible way to alleviate such a problem. One of the advantages of using NPs is the increased surface to volume ratio, which makes them highly effective for antifungal activities.

Recently, bimetallic nanoparticles, formed via integration of two different metals, have appeared particularly promising with antifungal efficiencies greater than that of monometallic counterparts due to synergistic effects. However, there is a current lack of studies focusing on the bimetallic nanoparticles of Ag and Cu as antifungal agents against *F. verticillioides*, *F. oxysporum* and *A. flavus*. Therefore, the aim of this study was to investigate a synergistic antifungal effect of bimetallic nanoparticles of silver and copper against selected mycotoxin producing fungi in maize.

1.3 Aims and Objectives

The aim of this project is to investigate the antifungal effects of Ag/Cu BMNPs against the selected mycotoxin causing fungi in maize. The objectives are:

- To synthesise and characterise Ag and Cu monometallic nanoparticles.
- To synthesise and characterise Ag/Cu and Cu/Ag bimetallic metallic nanoparticles.
- To isolate *F. verticillioides*, *A. flavus* and *F. oxysporum* from the contaminated maize.
- To investigate the in vitro antifungal activity of monometallic and BMNPs of Ag/Cu against *F. verticillioides*, *F. oxysporum* and *A. flavus*.

1.4 Hypothesis

Ag will have a higher inhibition efficacy than Cu. Ag/Cu bimetallic nanoparticles will have a higher inhibition efficacy than monometallic nanoparticles. Cu/Ag core-shell will have a lower inhibitory ability than Ag/Cu inverse core-shell.

1.5 Delimitations

- The study was limited fungal pathogens from stored maize samples only.
- Nanoparticles were synthesized by chemical method only.
- Antifungal activity was limited to *in vitro* studies only.

1.6 Outcomes of the study

- The study will generate new knowledge on potential methods of controlling the fungal infections in maize. Thus, it will contribute on the scientific community immensely as nanotechnology is further explored and applied.
- New knowledge on the application of Ag/Cu BMNPs in fungal plant pathogen management.
- Publication of research articles and a thesis for academic qualification is envisaged.

CHAPTER TWO LITERATURE REVIEW

This chapter contains the literature review based on the work that has been conducted on the topic.

2.1 Maize

Maize (*Zea mays* L.) is the most used staple in South Africa (SA) and the world. This crop has around 50 species and its grains have varying shapes, textures, and colors. Most eaten by humans are the yellow, white, and red varieties. The domesticated version of maize was first discovered in Mexico around 1,500 BC. In developed countries, maize is mainly used as a second cycle produce, such as dairy products and meat. In developing regions, it is a staple food, and it is regarded as a breakfast cereal. Although it is commonly regarded as a breakfast food, it can also be used as fuel and starch (Plessis, 2003).

It was introduced to Africa around 1500 AD and is regarded as the continent's most important crop (Ranum & Pe, 2014; Hossain et al., 2016). In sub-Saharan Africa, maize has become the main food crop. It is used by around 300 million people to produce various food products, and it accounts for about 50% of the region's low-income households' expenditures. About 30% of the calories consumed by people in the region come from maize (Pradyot Ranjan, 2021; Wang et al., 2021).

The maize industry is a vital part of Southern Africa's economy as it serves as the main source of carbohydrates for both animals and humans. South Africa is the largest producer of maize in the Southern African Customs Union (SACU), and it has an average production of around 10 to 12 million tons annually (Chomonyo et al., 2020). It is estimated that around 9 000 producers in SA grow the majority of the country's maize crop, while the remaining thousands are small-scale producers. The correct application of the right inputs, such as pesticides and fertilizers, can help sustain the environment and improve agricultural production. These include the establishment of suitable seed varieties, the use of soil tillage, and the control of pests and diseases (Deb et al., 2020; Prasad et al., 2022; Czarnecka et al., 2022).

The Free State, Northwest, Mpumalanga, and KwaZulu-Natal provinces are the leading producers of maize in SA. The major maize-growing area in these regions is in the western and central parts of the Northwest, Free State, Gauteng, and the southern region of Mpumalanga. It has been

estimated that the world will need to increase its agricultural production by 60% by 2050 to meet the increasing demand for food. According to some projections, the demand for maize will outstrip the supply of other cereals. To meet this demand, the world will need to increase its agricultural production by around 2.4% per year (Soare et al., 2018; Global market analysis, 2022).

2.2 Challenges affecting maize cultivation and production

Climate change is a serious deterrent that hinders the ability of farmers to produce adequate crop yields. Moreover, it limits the ability of small farmers provision to their families. It is therefore important that the public, researchers, institutions, and policymakers take action to prevent its effects (The UN Refugee Agency, 2017). According to the Intergovernmental Panel on Climate Change (IPCC), the increasing temperatures that are expected to occur in the future are expected to decrease the global agricultural production. This could also affect the timing of the harvest and planting of crops (Easterling et al., 2007). The increase in days of maturity and flowering, as well as the loss of root architecture is a window to several losses. These are some of the factors that can affect the yield in tolerant and susceptible lines.

In addition, the reduction in growth and biomass, as well as the reduction in water status, are also contributing factors to the reduction in yield. The reduction in the plant height, anthesis, and silking dates can also affect the yield. Furthermore, the reduction in growth parameters can also affect the water status and nutrient content in the plant (Moharramnejad et al., 2019; Bojtor & Nagy, 2022). The loss of chlorophyll content and the gas exchange parameters can also affect the plant's performance. The reduction in the plant's dry and fresh weight and transpiration can also affect the yield. The increase in the days that it takes to silking can also decrease the plant's yield (Ao et al., 2020).

The variations in the yield of maize in different countries are most apparent in Africa. For instance, some countries are experiencing decreases in their annual yields of only over 7%. In Sub-Saharan Africa, the average yield of maize is currently at around 1.5 tons per hectare. This is less than what the leading producers can achieve (Cudjoe et al., 2021). According to the Industrial Facilities Initiative (IFI), although cereal yields in Sub-Saharan Africa are expected to increase by 2020, they are still expected to be less than the high yields in East Asia (United Nations Environment Programme, n.d.). A report released by the UK government earlier this year indicated that the

potential yield of maize in Sub-Saharan Africa could be over 60% higher than what's currently being achieved.

In South Africa, (Kibirige & Obi, 2015) reported that some of the factors that have been identified as contributing to the decline in agricultural productivity within the small-scale farmers in the Eastern Cape province include the lack of access to land, inadequate financial capital, and uncertainty regarding the farm risks associated with climate change. Due to the effects of climate change, South Africa's maize production has been affected by various factors. This has prompted farmers to adapt their activities (Kori, 2018; Remilekun et al., 2021; Choruma et al., 2022).

This is therefore to say that there is a need to improve the yields in agriculture in order to make it more profitable and sustainable for small-scale farmers, such as those in sub-Saharan Africa (Mulungu & Ng'ombe, 2019). This region is heavily dependent on maize for food, which has low levels of productivity and vulnerability to natural disasters (Barton et al., 2019). The lack of training and resources for small-scale farmers (Hajdu et al., 2020; Kom, 2022) has been one of the main factors that has held back the growth of maize production. These constraints include the availability of high-quality seeds, poor distribution of agricultural practices, and the cost of pesticides and fertilizers. In addition, other factors such as the lack of storage space and poor post-harvest management are also contributing to the decline of maize production (Sabagh et al., 2020). Moreover, the weather patterns greatly affect the production of food and are likely to cause pests and diseases to proliferate (Ongoma, 2014).

2.3 Mycotoxin contamination in maize: Causes and Risks

Maize is exposed to mycotoxins, which are secondary metabolites produced by fungal species, such as *Aspergillus*, *Fusarium*, and *Penicillium* and many others. According to (Jard et al., 2011) about a quarter of the world's agricultural products are contaminated with mycotoxins that can exceed the European Union's safety standards and *Codex Alimentarius* limits. The World Health Organization (WHO) stated that over 500 million people, mainly children in Sub-Saharan Africa, are exposed to unsafe levels of mycotoxins. The exposure may lead to increased morbidity and mortality. Due to the prevalence of mycotoxin contamination, implementation of the Sustainable Development Goals in African nations is threatened. **Table 2.1** illustrates mycotoxins that were found in the different provinces of SA. The eastern part of the Eastern Cape (EC) is one of the poor areas in SA threatened by mycotoxin contamination, with more than 95% of households'

farm mainly for subsistence purposes (Perret, 2002). In this region Fumonisin B1, B2 and Deoxynivalenol (DON) were found (Alberts et al., 2019; Fru et al., 2021; Price et al., 2024). In addition, mycotoxins are classified according to their toxicity with highest being FB1 in fumonisins and AFB1 in aflatoxins (Santos et al., 2022).

Table 2.1: Mycotoxin contamination of maize in different provinces of South Africa.

Province	Type of fungi/ mycotoxin	Conc (µg/kg)	Reference
Northwest	AFs ^a	9.34	(Ekwomadu, et al., 2021)
	AFB ₁ ^b	4.96	
	AFB ₂ ^c	4.92	
	FB ₁ ^d	1566.7	
	OTA ^e	19.44	
Northern Cape	DON	174	(Chukwudi et al., 2021)
	FB	719	
	ZEA ^f	19.6	
Free State	FB ₁ + FB ₂ ^g	39440	(Fru, et al., 2021)
Eastern Cape	FB ₁ + FB ₂	1142	(Alberts, et al., 2019)
	FB ^h	1.4x 10 ⁸	(Fru, et al., 2021)
	DON ⁱ	7273	(Price, et al., 2024)
Western Cape	FB ₁	7125.3	

	FB1	125.1	(Mokubedi et al., 2019)
	FB ₁	115.1	
	AFB ₁	0.3	
	FB ₁ + FB ₂ + FB ₃ ^j	2206	
KwaZulu Natal	AFB ₁	133	(Rheeder & van der Westhuizen, 2024)
Limpopo	FB ₁	8514	(Mangqawa, et al., 2015)

Abbreviations: ^aAFs—total aflatoxin, ^bAFB₁—aflatoxin B1, ^cAFB₂—aflatoxin B2, ^dFB₁—Fumonisin B1, ^eOTA—ochratoxin A, ^fZEA—Zearalenone, ^gFB₂—Fumonisin B2, ^hFBs—total fumonisin, ⁱDON—Deoxynivalenol and ^jFB₃—Fumonisin B3.

Mycotoxin producing fungi that are common in maize in the area includes *F. verticillioides*, *F. oxysporum* and in rare instances *A. flavus*. The infection starts in the field through to transportation and subsequently storage. It is further promoted by grain moisture levels that are higher than 12% in the harvested maize storage. Therefore, poor harvest practices and inappropriate storage facilities contribute immensely in mycotoxin contaminations (Sserumaga et al., 2015). Moreover, post-harvest remedies such as sorting, milling and thermal processes have been reported to partly remove the fungi. These fungal metabolites are carcinogenic and have been associated with various cancer conditions such as oesophageal cancer (Sewram et al., 2016). The occurrence of mycotoxins in food and their severe toxicity, carcinogenic and estrogenic properties have been known to be the possible hazard to human health (Zöllner & Mayer-Helm, 2006). The most common mycotoxins are Fumonisin B (FB) and Aflatoxin (AF) which, are produced mainly by *Fusarium proliferatum*, *Aspergillus sp.* and *Fusarium verticillioides* (Misihairabgwi et al., 2019). These are the most harmful mycotoxins that are found in maize crops.

2.4 Mycotoxin-producing fungi

2.4.1 *Fusarium* species

Fusarium species are pathogens that cause diseases such as vascular wilt, root rot, stem rot, seed rot, ear rot and kernel rot on maize (Meissle et al., 2010; Oldenburg et al., 2017). This group includes a wide variety of species that vary in morphological and has phylogenetic characteristics. They are mainly endophytic and epiphytic organisms that are part of the maize's pathogenic microbiota. The damage they can do to a plant is caused by their presence in the xylem, which can lead to vascular obstruction (Bryła et al., 2022). *Fusarium* species are soil-borne pathogens that are most important phytopathogenics and toxin-producing fungi. Some species are parasites and some produce mycotoxins in plants. Several classes of the species *Fusarium* possess phytopathogenic and toxigenic properties that affect the quality and quantity of the produce, nutritious value and hygienic quality of agricultural products from arable crops worldwide (Rana et al., 2018) . Alongside with the progression of infection, the harvests may be contaminated by *Fusarium* mycotoxins, which can cause toxic effects on plants, animals (Abbas et al., 2013).

The development and production of *Fusarium* in maize can be caused by various factors. These include the genetic predispositions of the plant to resist fungal pathogens, the manufacturing practices utilized, and the weather conditions. In this study, the *Fusarium* species that were selected were *Fusarium verticillioides* and *Fusarium oxysporum*. *F. verticillioides* is a major pathogen in maize crop in most maize cultivating regions (Abbas et al., 2006). It can produce mycotoxins such as Fumonisin B1, B2 and B3 (Ramaswe, 2017). *F. oxysporum* (a soil-borne pathogen) is another species of *Fusarium* that is known to cause lesions, necrotic or chlorotic marks on the maize (Bahadur, 2016).

2.4.2. *Aspergillus flavus*

Aspergillus flavus is a fungus found in soils and may infect many plants causing different diseases such as maize ear rot. It grows very well in temperatures between 25 - 35 °C (Visagie & Houbraken, 2020). Maize losses in yield and economic returns are not primarily based on *A. flavus* ear rot but rather the production of the fungal metabolite aflatoxins (Fountain et al., 2014). *A. flavus* and *Aspergillus parasiticus* produce aflatoxins (AFB1) in the field not only prior to harvest but even at post-harvest if the crop is not dried and stored in a dry cool place (Hedayati et al., 2007). AFB1 targets the liver and lungs and is the most dominant naturally occurring aflatoxin listed Group 1 human carcinogen (IARC, 1993; Turner et al., 2009). Aflatoxins, ochratoxins and

fumonisin are three of the five mycotoxins that are significant in agriculture and they are produced by *Aspergillus* isolates (Amaiike & Keller, 2011). The highest levels of aflatoxins observed were 14.4 µg/kg in 2015 (Rheeder, 2017). Aflatoxins are heat resistant, they may remain stable and resist cooking processes (Binder et al., 2007; Marin et al., 2013). They have been reported to cause enlarged organs in broilers fed with 3000 ppb aflatoxin contaminated maize for 42 days (Delia et al., 2014). (Patel et al., 2015) suggested that the most effective way to reduce aflatoxin contamination in susceptible crops is particularly by stopping or reducing the growth of the causative toxigenic fungi.

2.5 Strategies for reducing mycotoxins

2.5.1 Conventional methods

The mycotoxin problem is a common food safety challenge that requires a comprehensive response. This involves addressing the various aspects of the food value chain, from farms and households to industries and regulators. There are many steps involved in addressing this issue, such as establishing effective storage systems and improving the quality of food. Various strategies can be used to address the issue, such as using post-harvest resistant crops, improving agricultural practices, biocontrol, and storage (Anyanwu et al., 2004; Winter & Pereg, 2019; Illueca et al., 2021). More efforts are needed to promote the harmonization of regional standards and the establishment of public-private partnerships. Pre-harvest measures that can be taken to combat the spread of mycotoxins include the use of appropriate seed material, crop rotation, fertilization, and sowing period. Good crop management can also help increase the resistance of plants to biotic and abiotic stress factors.

Post-harvest interventions, such as hand-sorting, separation, washing, heating, solvent extraction, irradiation and nixtamalization (Federica et al., 2017; Peng et al., 2018; Marisabel & Heinzl, 2019; Leslie et al., 2021) can also be implemented. These techniques reduce the contamination of mycotoxins in the food supply chain and can also be carried out at the household level. Unfortunately, mycotoxin control measures are still poorly implemented due to various factors, such as the lack of awareness about their importance and the weak implementation of regulations.

In order to reduce the toxic load in animal feed, certain clay binding agents such as Montmorillonite, Zeolite, and bentonite have been shown to bind effectively to mycotoxins. Contrary to binding, an adsorption method known as the yeast cell wall where β-D-glucans are

employed due to their adsorption capacity (Jouany et al., 2005; Solovyov et al., 2020). (Greco et al., 2022) obtained a mycotoxin-adsorber by combining of tri-octahedral smectite and a lignocellulose-based material against AFB1, ZEA, OTA, and FB1. The mixture revealed the potential to remove mycotoxins in feed.

2.6 The use of pesticides and antifungal agents in agriculture

Antifungal agents are able to control fungal diseases, these include inorganic, organometallic and organic composites (Jampilek, 2016). The wall of a fungal cell is (**Fig 2.1**) composed of mannoseproteins, chitin, α - and β -linked glucans, and it serves a variety of functions. Some of these functions include providing cell shape and rigidity, metabolism, and interaction with host defences. Some of the antifungal drugs that can interfere with this function include polyenes, echinocantins, allyl amine, and azoles (Thompson et al., 2009). These antifungal agents can penetrate the cell through a phospholipid bilayer. This layer is responsible for maintaining the cell's shape and preventing foreign materials from entering it.

The azole antifungal agents are inhibitors of P450- dependent enzymes, which breakdown the fungal cell structure and function. Allyl amine are broad-spectrum antifungal compounds that interfere with the 2,3-epoxidase which reduces the ergosterol (Garg et al., 2020). Antifungal agents are applied to crops before the development of a diseases, this is done to prevent any diseases that may appear during growth, harvest, or storage.

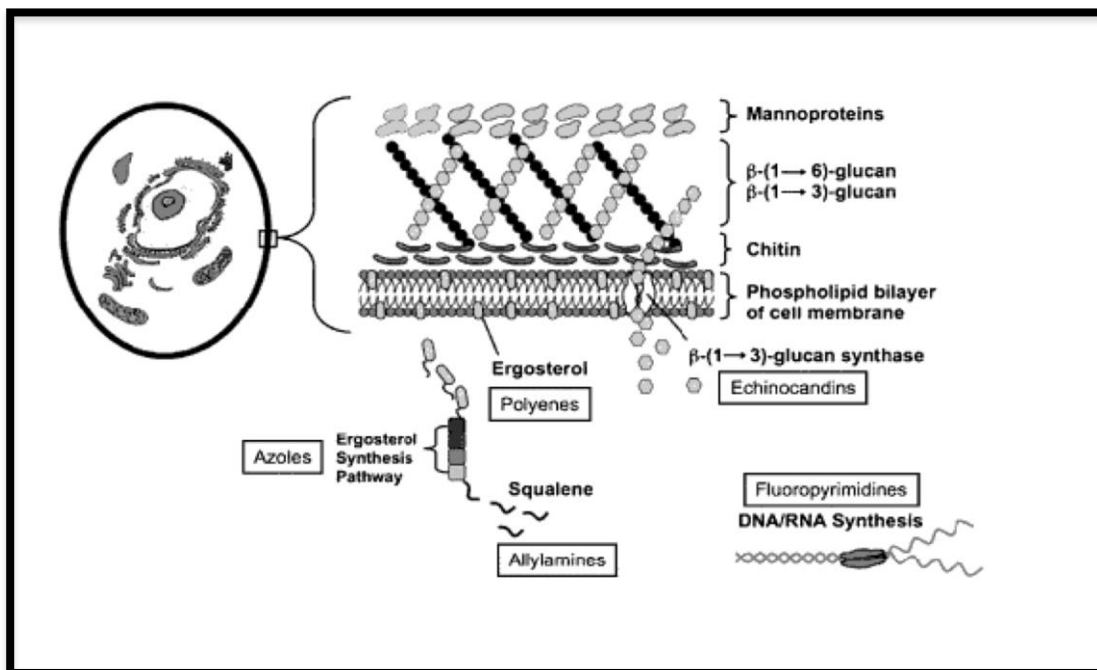


Fig 2.1: The functions of antifungal agents when in contact with the cell (Ashley et al., 2006).

Some of these reagents encounter resistance from pathogens and have to be incorporated into high levels in order to be effective and they are capable of reducing the nutrients and nutritional value of animal feeds (Hamad et al., 2022). Therefore, highly effective antimicrobial agents are needed to control the antibiotic resistant pathogens. Recently, nanoparticles have a huge potential to be applied as antifungal agents.

2.7 Nanoparticles: Properties and synthesis routes

Nanoparticles are materials that have their sizes ranging between (Patel et al., 1970; Sánchez-López et al., 2020). When working with materials and devices that are less than 100 nm in size, it is feasible to create lighter, cheaper, and more qualified products. Doing so helps minimize the energy consumption of the device and makes it easier to use. Owing to their high effects on quantum size, nanoparticles are widely used in various applications. Due to their exceptional properties, many pharmacological and technological products are produced using the synthesis of nanoparticles. This process involves the preparation of catalysts, optical materials, drug carriers, diagnostic tools, superconductors, and surfactants. Compared to bulk materials or

micromaterials, the surface characteristics of nanomaterials are different. These include their large surface area, which allows for higher particle count per mass unit; the number of atoms at the surface increases; and the atoms located at the surface have fewer neighbours (Joudeh & Linke, 2022; Sachin et al., 2023). These physical and chemical properties change as a result of the various differences between nanomaterials and their bigger counterparts. For example, the reduction in the number of direct neighbours for the atoms located at the surface leads to a decrease in the binding energy of the nanomaterials. This effect can also affect their melting temperature. The dispersibility of nanomaterials is an important factor that affects their surface effects. When the strong interactions between particles lead to aggregation and agglomerations, this can negatively affect their properties and their surface area. To prevent this, one can increase the zeta potential of nanomaterials, increase their repulsive force, or improve their hydrophobic or hydrophilicity (Du et al., 2022; Srivastava et al., 2022).

However, first let us discuss the different types of nanoparticles and how these nanostructures are synthesised. There are different types of nanoparticles such as, metallic, lipid-based, ceramic, semi-conductors, polymeric and carbon-based. Metallic NPs include those of Ag, Au, Ni, Pt to name a few. The optical properties of these NPs are attributed to the free electrons' resonance, which occurs when light interacts with them. One of the intriguing properties that metallic NPs possess is the number of atoms arranged in a complete cluster structure which is determined by the arrangement of these components in the atomic nucleus. The concept of this concept refers to the number of protons or neutrons that are arranged into the structure (Harish et al., 2022). Number of atoms can be derived from rare gas clusters. If the clusters are big, the surface atom gets reduced because the central atoms are more surrounded, while those in the inner shell are kept covered by other atoms (Khan & Sharma, 2022).

When exposed to a magnetic field, a magnetic nanoparticle can exhibit various features. These include a ferromagnetic or ferri-type behaviour. The most common configurations are those involving oxides, which are also known as ferrites. A classification of magnetic nanostructures is also carried out, such as those with shells or ferrites. The use of iron oxide nanomaterials in research is widely regarded as a promising area of activity in the field of nanotechnology. For instance, when a magnetic structure is superparamagnetic, it can be prevented from aggregation by the effects of temperature. The surface chemistry of nanostructures becomes particularly important at the nanoscale. For instance, the ferrite nanostructures' surface is relatively inert due to the presence of a coating, which is mainly silica, and the resulting ferrites, which are typically

made with shells. In order to passivate pure metallic nanoparticles, various processes, such as oxidation, can be utilized.

Through the use of tailored nanoparticles, one can create various useful and interesting applications such as biomedical, agricultural, and catalytic effects. The ability to combine these with other materials or molecules provides an avenue for developing new innovative and efficacious applications. There are two kinds of nanoparticles: organic and inorganic. The former consists of metals such as silver, platinum, gold, palladium, copper, while the latter is made up of semiconductors such as zinc, titanium oxide (Sibhatu et al., 2022; Yanar et al., 2023). They can be synthesized via two approaches (**Fig 2.2**) known as; the top-down and the bottom-up approach. In the top-down approach bulk materials are grounded into powder to form NPs by applying physical methods such as laser ablation and arch discharge (Jamkhande et al., 2019; Modan & Plaiasu, 2020). The bottom-up approach involves the transformation of atoms to clusters which results to NPs using the biological methods or the chemical methods (Christian et al., 2008; Tan & Cheong, 2013; Ijaz et al., 2020). The bottom-up approach is usually preferred by many researchers because of the ease of controlling the size and shape of the nanoparticles. On the other hand, the top-down approach which involves using either a fabrication technique or a chemical process to get the desired particles (Nguyen Hoang & Nguyen Hoang, 2020). The acid-based method is commonly used to get the required substances. There are three different types of procedures used in this process are: biological, physical, and chemical methods (Mir et al., 2021). The process of biologically synthesizing nanoparticles is known as the green synthesis. It is conducted through the use of various biological agents, such as fungi, bacteria, yeast, and viruses. The biological assisted method is categorized into three categories. These include biogenic synthesis through microorganisms, biogenic synthesis by using biomolecules, and biogenic synthesis by using plant extracts (Zhang et al., 2020; Prakash et al., 2022).

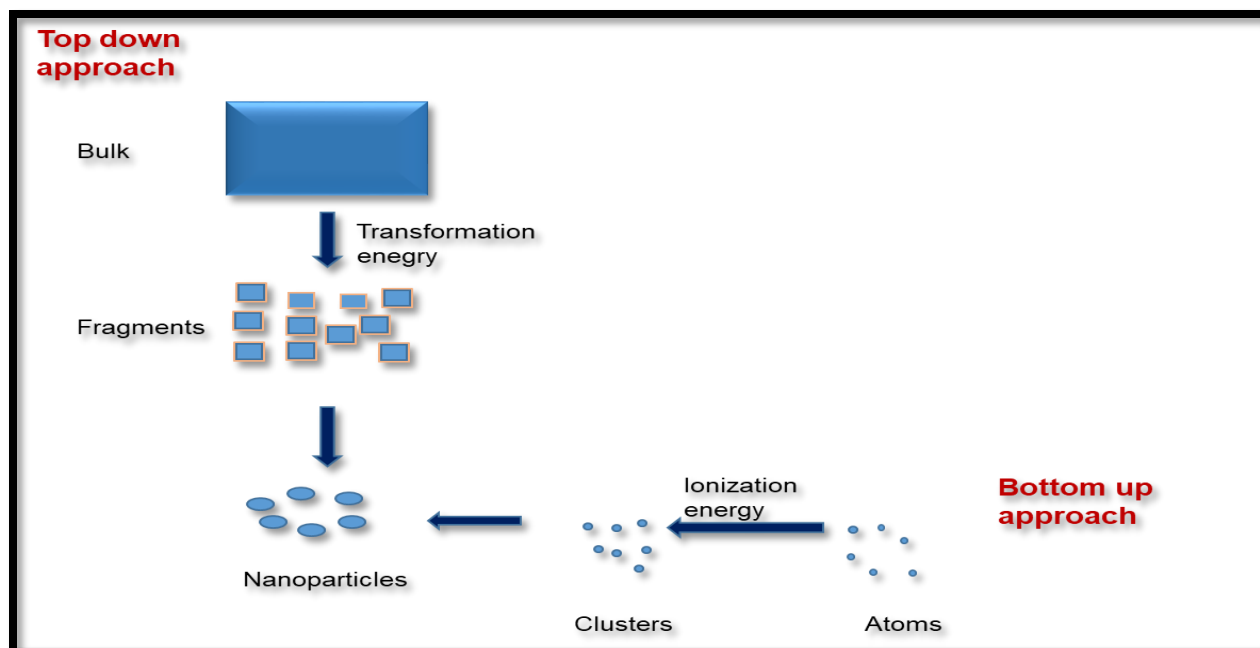


Fig 2.2: Top-down and bottom-up methods of synthesizing nanoparticles (Rico et al., 2014; Patil & Chandrasekaran, 2020).

Some of the prominent techniques used for the process of physical nanoparticle production include laser ablation, laser pyrolysis, electrospraying and ball milling. Lastly, the chemical reduction method which includes various preparation techniques such as Sol-gel method, hydrothermal synthesis, reverse micelle, chemical reduction method, solution-based synthesis, solvothermal, co-precipitation and polyol synthesis (Hachem et al., 2022). In the current study, we will be focusing on the chemical method of preparing nanoparticles. The chemical method involves the reduction of a metal ion in a solution using reducing agents in the presence of a capping agents or stabilizers to achieve the desired shape and size (Hossain et al., 2018). The three essential stages involved in chemical reduction approach is:- the reduction of the metallic salts, stabilization of the complex, and the controlling of the size. The capping agent, which is a stabilizing agent, helps in controlling the size of the finished product. High surface energy and the unstable properties of nanoparticle surfaces are the main causes of nanoparticle aggregation (Alkholief et al., 2022; Zhou et al., 2023). This process can be prevented by using a stabilizer which serves as a protective barrier that prevents nanoparticle surfaces from getting too heated and exposed to different chemicals. The formation of large surface energy particles by the interaction of two metallic nanoparticles leads to the thermodynamically advantageous bulk

particle. In order to maintain their spatial confinement, it is important that the two metallic particles are stabilized. This can be done through either electrostatic or steric exclusion. Steric stabilization (**Fig 2.3**) which is a process of adsorbing large molecules using polymers or surfactants on a nanoparticle's surface is another role for stabilizers. Steric stabilization works by attaching macromolecules to the nanoparticle's surface through a process known as chemisorption. These components are composed of long hydrocarbon chains and functional groups, and the former has a combination of catalytic and physical-grafting properties. The various functional groups that are involved in the process of steric stabilization help to anchor the macromolecules on the surface of a nanoparticle. In addition, the long hydrocarbon chain of the macromolecule can be extended from the surface to the solvent. These moieties act as a repellent barrier between the nanoparticles, which helps keep them away from each other (Petersen et al., 2022; Chan et al., 2022).

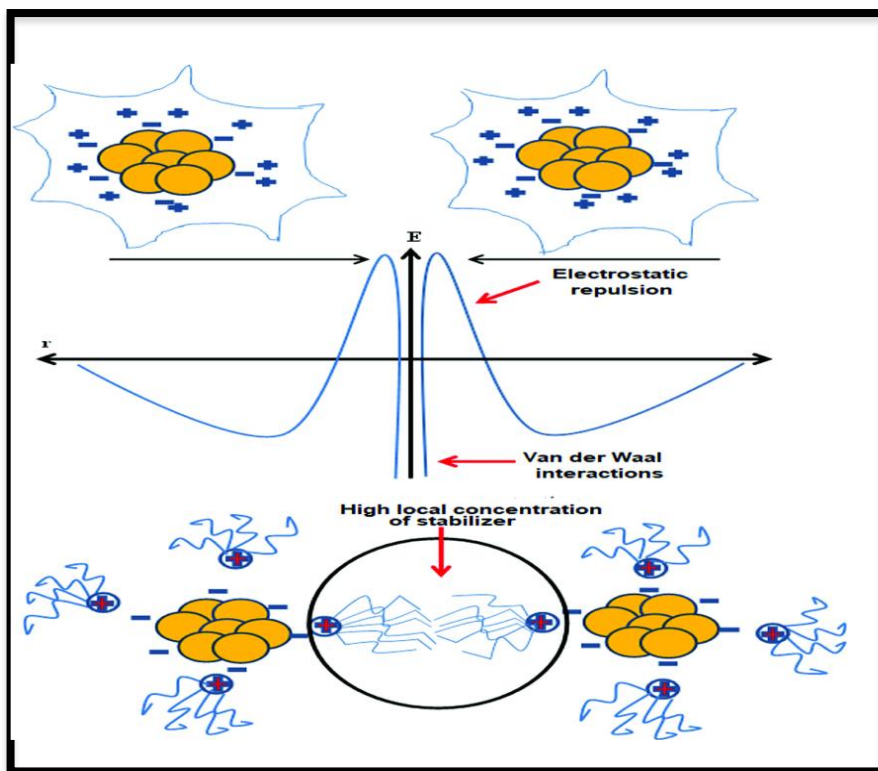


Fig 2.3: Schematic representation of the stabilization of steric and electrostatic mechanism of nanoparticles (Amina & Guo, 2020).

The capping and reducing agents are added at different times during the reaction. In some cases, a single compound can also act as both a capping and a reducing agent. Some of the commonly used reducing agents include sodium borate, toluene, and ascorbic acid (Christian et al., 2008; Tan & Cheong, 2013; Elgorban et al., 2016). Nanoparticles can be synthesized in a lot of different shapes as shown in **Fig 2.4**.

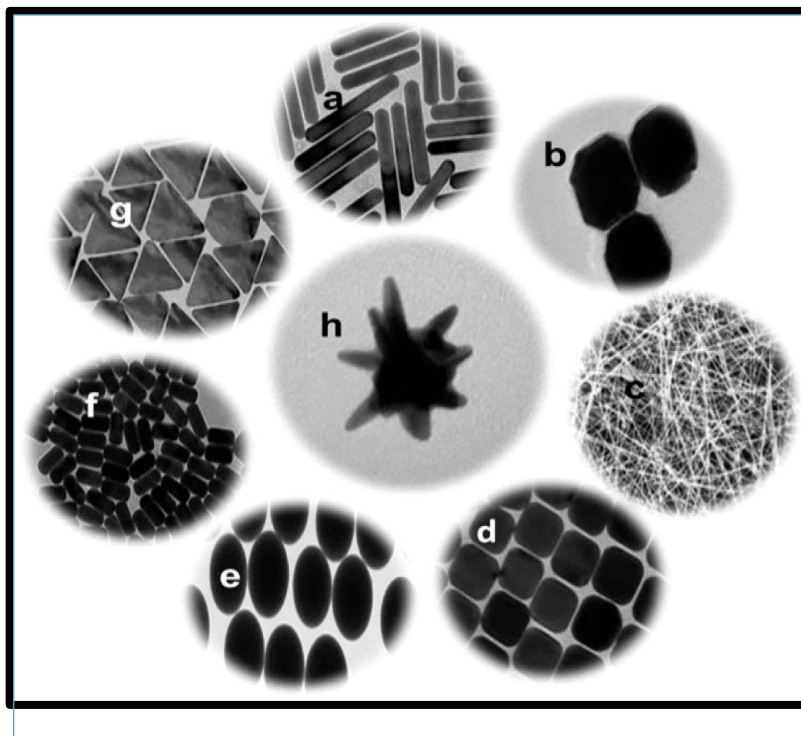


Fig 2.4: Shapes of NPs **(a):** Nanorods **(b):** Truncated cube NP **(c):** Nanowires **(d):** Nanotubes **(e):** Nanosphere **(f):** Nanorice **(g):** Nanoprisms **(h)** Nanoflower (Loiseau et al., 2019; Boselli et al., 2020).

The stability of nanoparticles can be improved through the addition of ligands such as Ethylenediaminetetraacetic acid (EDTA), polyethylene glycol (PEG), Cetyltrimethyl ammonium bromide (CTAB) and polyvinyl pyrrolidone (PVP) (Javed et al., 2020; Sidhu et al., 2022) which act as capping agents that create a steric hindrance on the shared bond. The capping agents has impact on the size, shape and functionality of the NPs. The process can be further classified depending on the energy source used and the device utilized for the reaction. The natural compounds or microorganisms that can trigger a reduction in an oxidation state can be utilized

as substrates. The chemical reduction process allows the creation of nanoparticles with varying morphologies. This process also allows for the strict control of their size (Szczyglewska et al., 2023).

2.7.1 Bimetallic NPs

Bimetallic nanoparticles are formed by integration of two different metals. The essence of BMNPs is to increase the chemical and physical abilities of NPs by introducing two metals with each having their own properties. In addition, (Abd El-Aziz et al., 2023) utilized carbohydrate polymeric polysaccharides to synthesise ZnO-Au bimetallic nanoparticles as biological antioxidant, anti-inflammatory, and wound-healing agent. In another study, (Amiripour et al., 2021) created functionalised porous Au-Ni bimetallic nanoparticles. Their electrochemical properties were evaluated by recording cyclic voltammograms in the absence and presence of glucose at 50 mV s⁻¹. The bimetallics presented an excellent sensing performance for glucose detection in the range of trace concentrations (1–1900 µM) with high sensitivity and low limit of detection (0.063 µM). In 2019, a study conducted by Mansouriieh and colleagues revealed that they used a Fe/Ni bimetallic nanoparticle as a catalyst to degrade the profenofos. The researchers were able to accelerate the reaction by halting the surface corrosion on nZVI particles using Ni. The various factors that affected the degradation process, such as pH, the number of NPs, and the profenofos concentration, were also considered. The highest removal rate was achieved when the pH was at 5.12 (Mansouriieh et al., 2019) . In 2017, Gao and his team created dual-function gold nanoparticles (Au NPs) with a platinum surface. The first phase of the plasma is retained by the nuclear energy source, which is a Pt shell. The catalytic activity of this component is very high (Gao et al., 2017).

The properties and structure of metal atoms are dependent on their arrangement. There are two approaches to synthesize bimetallic nanoparticles, which is simultaneous or sequential reduction of appropriate precursors. This kind of synthesis of two metals with different properties is an advantage as it does not only improve the properties (therefore expanding the use and application) of the bimetallic nanoparticles but limitations of monometallic nanoparticle are subjugated (Weir et al., 2010; Sharma et al., 2016) . For example, Cu oxidizes when exposed to air but Ag is stable, this makes the BMNPs of Ag and Cu stable due to the stability of Ag (Rodríguez-iznaga et al., 2022) . This was observed in the formation of Ag-Cu alloy nanoparticles by Tan and Cheong, where Ag was added to prevent oxidation of Cu nanoparticles and therefore increasing its stability in the atmosphere (Tan & Cheong, 2013) . The advantages include

distinctive size dependent optical, thermal, catalytic, electronic and magnetic effects (Shah et al., 2012), and enhanced plasmonic properties. The same theory applies in the case of Fe^0 which has the tendency to agglomerate and form an oxide layer (Lee et al., 2014; Ulucan et al., 2022; Wahab et al., 2022).

The advanced physiochemical properties of BMNPs are not only size and shape dependent but the arrangement of atoms is an enormous contributing factor (Min & Wang, 2020; Hu et al., 2020; Freire et al., 2021). BMNPs can be mixed as core-shell, multi-shell, alloys, and sub-clusters. Alloys are formed when both atoms have a consistent distribution in a particle (**Fig 2.5**). The core-shell structure consists of a shell of one atom surrounding a core of the other atom. A core-shell structure is an ordered structure that consists of a metal A and a shell B. The inner core is completely covered with a layer of chemical bonds. When two metal ions' precursors have different reducing capabilities, the former can be reduced to a metal atom and form a nucleation center. The latter can then be deposited on the center, which eventually forms the core shell. Seed-mediated growth is the most efficient way to create a complex core-shell structure. It involves the reduction of the metal A to a specific extent, which allows the emergence of a distinct nucleating site on the B atom for the subsequent thermal decomposition or chemical reduction processes (Pankaj et al., 2022). For example, Au-core and Ag-shell were synthesized when Ag^+ was added to Au nanoparticle, the inverse created hollow Au and Ag core (Krishnan et al., 2017). The various structures that are derived from the core-shell, including the multi-core structure, the yolk-shell, and the onion-shell, can affect their catalytic properties. When the shell metal only has one atomic layer, electronic and strain effects are severe, which can affect its selectivity and activity. The system achieves the lowest surface cost due to the high utilization of the shell atoms. When the multiple (2-6) atomic layers of the shell metal expand, the electronic and strain effects of the core and shell atoms will still be present (Chen et al., 2021; Pajor-Świerzy et al., 2022). Heterostructured bimetallic can occur when a metal atom is deposited on a substrate's surface in three different growth modes. The first is the layer-layer growth, the second is the film-island growth, and the third is the island growth. When the A-A bond energy of the metal A exceeds that of the metal B, the second is the island growth mode. The island growth allows the B to reduce the total surface-free energy of the metal A and the lattice stress between the two. A multi-level structure can be formed when a metal atom preferentially produces nanocrystals with a specific morphology. It can also be formed when another metal atom grows on the crystal in an island growth mode (Javed et al., 2020; Wu et al., 2023).

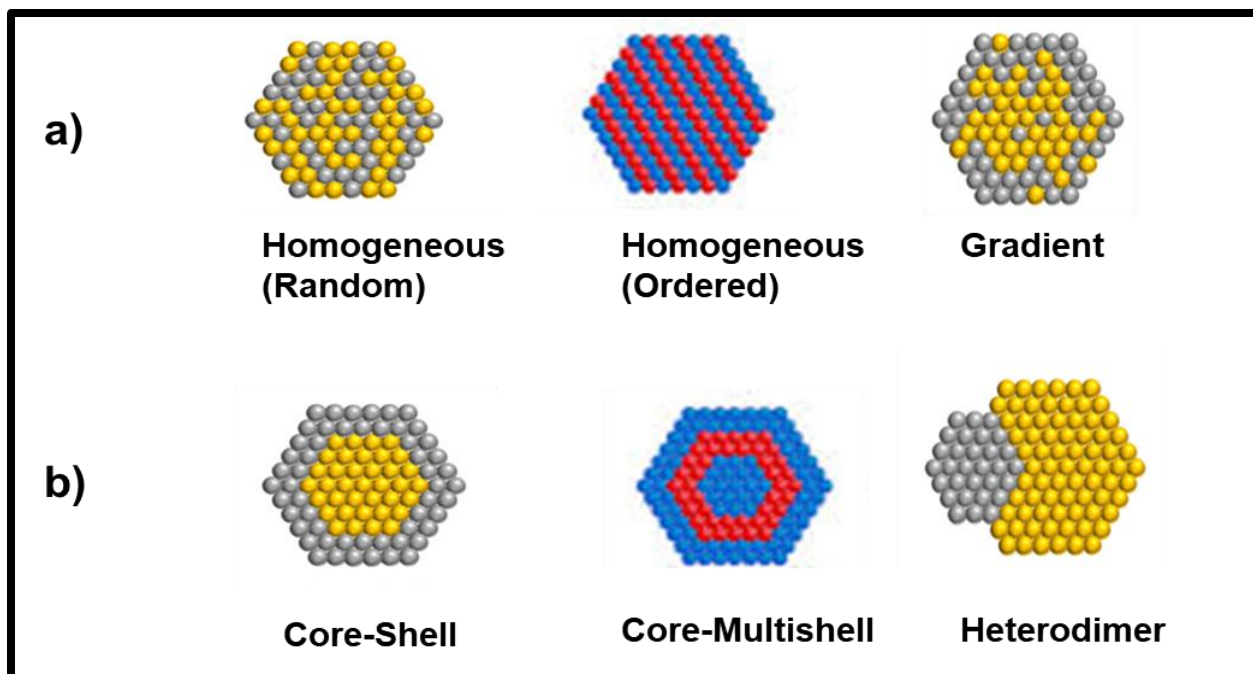


Fig 2.5: Arrangement of atoms in the bimetallic nanoparticle synthesis, **(a)** Alloyed bimetallic nanoparticles. **(b)** Heterostructure bimetallic nanoparticles is observed (Dang-Bao et al., 2017; Cruz-Luna et al., 2021).

Bimetallic nanoparticles are synthesised using the same methods as monometallic nanoparticles. For instance, in this study, (Liang, 2023) utilized a laser-induced vaporization process to create a solid palladium-platinum composite consisting of particles with varying total composition. This method was able to achieve a synthesis without using thermal processes or reducing agents. In a similar study (Ali et al., 2023) , a deposition procedure based on laser ablation was utilized to create bimetallic Ag-Ni nanoparticles. On the other hand, Pd-Ag core-shell nanoparticles were synthesized by pulsed laser ablation in distilled water and were found to exhibit higher antimicrobial effect against Gram-positive and Gram negative. While Pd NPs did not have a notable anti-bacterial activity (Hasan et al., 2023) .

Bimetallics of Au and Ag are the most widely investigated bimetallics. (Al-Zaban et al., 2022) recently developed core-shell NPs that can be used for the trans-esterification of fungal isolates. They also studied the reducing properties of these NPs when combined with TiO₂. In another study, (Aazam & Zaheer, 2021) utilized the photo-catalysed conversion of carbon dioxide using water. (Azmat & Zafar, 2023) employed the green synthesis to produce Ag/Cu bimetallic nanoparticles, this was achieved by using different fractions of the methanolic extract of *Trigonella*

foenum-graecum leaves. In this study, the BMNPs fabricated using ethyl acetate contained anisotropic microplates and various sizes of truncated shapes. On the other, those made using n-hexane extract exhibited spherical structures in clusters.

Due to the two metal precursors involved in the creation of bimetallic nanostructures, the first step of synthesis is to reduce one of them using a reducing agent. This process is followed by the addition of another metal precursor with a surfactant to prevent the overgrowth of the second one on the seeds' well-defined surface. The physical parameters that influence the development of bimetallic nanostructures play a significant role in its structural growth. These include the correlation of surface energy levels, the lattice match between the two metals and their reduction potentials (Duan et al., 2020; Ahmad et al., 2022).

2.8 Antimicrobial effects Nanoparticles

The increasing antimicrobial resistance of both fungi and bacteria has led to the interesting research on the development of new antimicrobial agents (Paiva et al., 2018; Chybowska et al., 2020). Antimicrobial nanoparticles have various characteristics, such as their biocompatibility, stability, and low toxicity. They can also be used for low doses and are ideal for controlled release (Unal, 2022). The various physical factors that affect the effectiveness of nanoparticles in terms of their antimicrobial activity are their size, morphology, and environmental conditions. Besides these, other factors such as the composition of the nanoparticle medium and the environment are also taken into account to determine their chemical activity (Sukhanova et al., 2018; Sharifi-Rad et al., 2020). An antimicrobial activity can be achieved by certain nanoporous enzymes, which can overcome various resistance mechanisms, such as cell permeability modification, excessive pumping of pumps, and activation of enzymes. These can be useful in treating various conditions, including those caused by antibiotic drugs.

Metal nanoparticles such as Ag, Au, Fe, Zn, Ti, Pd, Pt, Al and Cu, have emerged as the potential antimicrobial agents against bacteria, fungi and viruses. The synthesis of Ag₂O NPs was carried out on glass surfaces with a particle size of 1500 nm. The results indicated that the treated surfaces exhibited significantly lower titers of SARS-CoV-2 after 24 and 1 h. The Ag₂O NPs also caused the death of all the studied bacteria (Hosseini et al., 2022). A study carried out by (Chausov et al., 2022) revealed that Ag₂O NPs doped in borosiloxane matrix with a particle size of 65 nm exhibited bactericidal properties and bacteriostatic capabilities through the generation

of ROS, but did not induce cytotoxicity against living cells. Highly valuable in developing effective and versatile antimicrobial agents, Au NPs are characterized by their low toxicity, diverse effects, and easy detection. Compared to other types of NPs, they do not exhibit remarkable antimicrobial activity. Nevertheless, numerous studies have revealed the remarkable efficacy of these Au NPs in combination with small molecules, including vaccines, antibodies, and drugs (Wang et al., 2022; Al Hagbani et al., 2022). The stability and adaptability of Fe_3O_4 , Fe_2O_3 NPs were the main factors that prompted the FDA to approve them for clinical use. Compared to other nanomaterials, iron oxides offer better biocompatibility and toxicity. They also exhibit exceptional stability and paramagnetism in aqueous solutions. Although their antimicrobial effect can only be observed at elevated concentrations, it can be altered by varying their surface potential, functional groups and iron oxidation state (Malhotra et al., 2020; Ribeiro et al., 2023). Exceedingly versatile and advantageous in creating effective and versatile antimicrobial compounds, Cu NPs are widely studied due to their biocompatibility and antimicrobial properties. Unlike Ag, Cu is inexpensive and ready to be manufactured. However, its high oxidation susceptibility makes its synthesis difficult. Also, copper is prone to air oxidation (El-Naggar et al., 2022; Sharma et al., 2022). In addition, Cu NPs can be utilized in various applications, such as dental implants, adhesives, orthodontic arch wires, restorative materials, and resins (Ma et al., 2022; Xu et al., 2022). One of the most popular methods to synthesise Cu NPs has been the one-pot process which is a leading method for chemical transformations as it eliminates the need for different steps and allows for a high-atom economy while saving chemical resources. In this study, Cu NPs were synthesised by using diethylenetriamine as a protective agent in chemical reduction method. The minimum inhibition concentration and inhibit percentage using agar well diffusion method was employed against *S. aureus*, *E. coli*, *Klebsiella sp.*, *E. faecalis* and *P. aruginosa*. In addition, (Rajamohan et al., 2023) synthesised CuO NPs using the one-pot method and found that *E. coli* and *S. aureus* were susceptible to the NPs with MIC values of 50.0 $\mu\text{g}/\text{mL}$ and MBC 100 $\mu\text{g}/\text{mL}$, respectively. Moreover, agar diffusion tests revealed a clear zone of activity against two kinds of bacteria. ZnO NPs, which are inexpensive and can be used for various applications, have bactericidal properties and are biocompatible with human skin. They are regarded as promising MNPs. ZnO NPs are becoming more prevalent in the field of agronomy due to their ability to enhance the yield and growth of different plants, as well as their resistance to drought stress. They can be utilized as pesticides, fertilizers, and fungicides (Kalra et al., 2022; Chandrasekaran et al., 2022).

Numerous studies (Yorghlanlu et al., 2021; Younis, 2023) have been conducted on the antimicrobial properties of titanium dioxide nanoparticle (TiO_2) materials. These materials are

known for their favourable chemical stability and their ability to resist various forms of biomaterials. It has also been revealed that TiO₂'s photocatalytic effect can inactivate microorganisms by producing radical oxygen species. However, its activation mechanism can be limited by the energy needed to light its surface. The use of dopants has been suggested as a strategy to improve the TiO₂'s effectiveness in fighting against microorganisms. In addition, the effect of non-illuminated TiO₂ NPs on their antibacterial properties was studied. Only the PEG- and NH₄⁺-modified NPs exhibited a reduction effect. The use of semiconductors in the industry as effective antimicrobial agents has been linked to various factors. Some of these include the ROS attack on bacterial cells, the effects of metal ions on cellular proteins, and the mechanical damage to membranes caused by the interactions between the two (Yorghlanlu et al., 2021; Krakowiak et al., 2022; Raza et al., 2022; Alhashmi Alamer & Beyari, 2023).

Aluminium (Al) has also been explored in the nanoparticle form as Al₂O₃, these Al₂O₃ NPs are inexpensive and readily available to handle and effectively combat harmful microbes, such as multi-drug-resistant bacteria (MDR). However, their blood toxicity, as well as neurotoxicity, highlight their limitations. Therefore, novel engineering approaches need to be utilized to overcome these issues (Ghelani & Faisal, 2022). The inhibition of bacterial growth by catalysing the production of ATP is achieved through the use of Pt NPs. Although their potential is acknowledged, further studies are needed to confirm their effectiveness. The use of Pt NPs in combination with other materials can help develop new applications that require the control of bacterial growth. Studies (Lee et al., 2020; Yohannes et al., 2022) have shown that the properties of Pt NPs as antimicrobial agents are similar to those of standard drugs currently in use. New studies are required to analyse the different mechanisms of action and toxicity of these nanostructures. The use of nanoparticles alongside bacteriocins is a phenomenon that highlights the advantages of combining these two components. Bacteriocins, which are generally used in food preservation, are ribosomal-based peptides that are designed to kill harmful bacteria that cause food spoilage. Unfortunately, due to their various disadvantages, such as their high purification cost, low stability, and easy degradation, they do not perform well. The combination of Ag NPs and bacteriocins led to an enhanced anti-bacterial effect against certain types of bacteria, such as *Staphylococcus flexneri* and *S. aureus* (Sidhu & Nehra, 2020).

There have been limited studies on the BMNPs especially relating to their use in agriculture. (Kamli et al., 2021) explored the antifungal effects of Ag-Cu BMNPs against *Candida albicans* (*C. albicans*), the results indicated that BMNPs have a significant inhibitory effect on phospholipase secretion. They also revealed that the doses of NPs that are most effective at inhibiting the growth

and viability of cells can be up to 100%. These compounds are known to hinder the development of yeast to hyphae conversion, the formation of a bacterial community, and the secretion of hydrolytic enzymes. (Kamli et al., 2022) prepared semi-spherical Ag-Ni BMNPs which exhibited enhanced antifungal properties against the *Candida albicans* causing cell membrane disruption.

2.8.1 Antifungal effects of Ag Nanoparticles

The antifungal effects of Ag NPs against *C. albicans* was investigated by (Gibała et al., 2021). This study revealed that larger Ag NPs are biocidal, than those with smaller sizes. It was also observed that positive charged Ag NPs are less toxic compared to negative charged counterparts (Javed et al., 2020; Długosz et al., 2020). In another study Ag NPs with a concentration of 75 mg/L, caused significant changes in the structure of the hyphae of *F. verticillioides*. The results of the study revealed that the treatment resulted in the formation of mycelial growth, loss of evenness, and hyphae rupture. It was concluded that Ag NPs have antifungal and anti-fumonigenic properties against *F. verticillioides* (Pérez-de León et al., 2020).

Moreover, the size to concentration ratio was reported to have a major effect on the antimicrobial efficacy of the Ag NPs (Marinescu et al., 2022) against *Staphylococcus aureus* (*S. aureus*), *Enterococcus faecalis* (*E. faecalis*), *E. coli*, *Pseudomonas aeruginosa* (*P. aeruginosa*) and *C. albicans*.(Akpınar et al., 2021; Marinescu et al., 2022). As the size of the NPs decreased and the concentration increased the more the growth was inhibited against *F. oxysporum* f. sp. *radicis-lycopersici* (FORL) strains. (Żarowska et al., 2019) reported that at 107.2 mg/l Ag NPs with an average diameter of 20 nm inhibited the growth of *Paecilomyces variotii*, *Penicillium pinophilum*, *Chaetomium globosum*, *Trichoderma virens*, and *Aspergillus brasiliensis*.(Baker et al., 2021) synthesized Ag NPs from *Alternaria* sp. against the strains of *A. flavus* (MTCC 1475), *A. niger* (ITCC 1617), *Trichoderma viridens* (MTCC167), and *F. oxysporum* (NCIM No. 100). The highest inhibition was against *Trichoderma viridens* while the lowest inhibition was observed against *F. oxysporum*. Silver NPs are widely explored NPs for antifungal, antimicrobial, antibacterial, anticancer due to their antibacterial efficacy of Ag NPs (Halder et al., 2021). This study revealed that trisodium citrate coated Ag NPs had a high reactivity against *E. coli* and *S. aureus* than trisodium uncoated Ag NPs. It should be noted that NPs potential to inhibit fungal growth cannot be a blanket approach however rather the effect of the NPs differs from fungal species to another. In a study by (Bayat et al., 2021) the same concentration of Ag NPs hindered *B. cinerea* by 65.36% while it hindered the growth of *P. concavum* by 28%.

2.8.2 Antifungal effects of Cu Nanoparticles

For centuries, copper has been utilized as a biocide. In the past, Hippocrates, who lived during the 4th century BC, prescribed copper to treat pulmonary ailments. The "holy water" that Hindus offer to their devotees, which is referred to as Gangajal, is kept in copper pots to keep its sparkling clean. In the 1880s, a mixture containing copper sulphate, water, and lime was used to kill off various types of mildew on grapes in France and the US (Ingle et al., 2014). In recent year, the antimicrobial effects of Cu NPs have been investigated against a number of microorganisms including *E. coli*, *P. aeruginosa*, *Shigella sp.*, *Staphylococcus aureus* and *salmonella typhi* (Alao et al., 2022). (Martínez et al., 2021) reported high antifungal activity of Cu NPs and Cu nanowires against *C. albicans*. and Cu NPs. (Chalandar et al., 2017) was able to observe the antifungal effects of Cu and CuO against Penicillium on oranges. Colloidal Cu NPs were found to inhibit *F. oxysporum* and *Phytophthora capsica* after one day and 3 days, respectively (Pham et al., 2019). In another study, suspended Cu NPs capped with aminosilane showed antimicrobial affects against Gram-positive bacteria (*Bacillus subtilis*, *S. aureus* and *Listeria monocytogenes* (*L. monocytogenes*)), Gram-negative (*E. coli* and *P. aeruginosa*), yeast - *Candida tropicalis* (*C. tropicalis*) and the mould - *F. verticillioides* (Porta et al., 2019). Antifungal activity of Cu NPs was investigated by (Bayat et al., 2021) using agar dilution method against *B. cinerea*. It was found that no significant inhibitions were observed in the samples with either 1 ppm or 10 ppm concentrations. But, in those with 100 ppm Cu NPs, there was a 25% inhibition. This suggests that the increase in Cu NP concentrations to 100 ppm had a negative impact on the colony radius and *B. cinerea's* growth inhibition percentage. The effect of Cu NPs on the growth rate of *Aspergillus niger* (*A. niger*) as also investigated by (Maqsood et al., 2020). It was found that the inhibitory effect of 1.5% copper nanoparticle concentration on the growth of *A. niger* was significant. It also indicated that Cu NPs have an antifungal activity at dose or concentration.

2.9 Parameters affecting the properties of nanoparticles during synthesis

2.9.1 Temperature

Temperature also affects the nucleation and the crystal growth of the NPs is affected (Veerasamy et al., 2011; Lizunova et al., 2020; Gerosa et al., 2021). (Mountrichas et al., 2014) synthesized gold under different temperatures and the results show that the higher the temperature, the faster the process of synthesis, and the more uniform the resulting NPs. In addition, kinetic studies

conducted displayed that the process takes place in three steps at elevated temperatures while the steps taken at lower temperatures are more complex. The temperature increase during the induction reaction period and the growth phase of Pt NPs can decrease the duration of the process. It was observed that the higher the temperature, the faster the reaction and the smaller in size are the NPs.

(Paramo et al., 2020), reported that the size of the Ag NPs decreased at elevated temperatures than at lower temperatures. This was not the case with the findings by (Jiang et al., 2011) where temperatures of 0 to 55 °C slowed down the growth and formation of the NPs and it was also observed that the reaction rate and particle size increased with increased temperatures. Moreover, the size of the Ag Nanospheres increased from 25 to 48 nm, while the edge length of the NPs increased from 90 to 180 nm. In addition, the colour of the NPs changes with increasing temperature. (Melkamu & Bitew, 2021) recorded that the different temperatures used changed the colour of the Ag NPs from light yellow to dark-brown. It was also observed that maximum absorption to be at 40 °C with maximum wavelength of 402 nm and the absorption intensity was slightly increased in comparison with the temperatures. This concluded that at lower temperatures the plasmon peak showed a bathochromic shift at 60 °C and 80 °C. This bathochromic shift indicated that the mean diameter of the NPs decreased.

(Kumari et al., 2016) stated that it is important to stop the growth of nuclei in order to obtain monodispersed nanospheres. This was achieved by exposing the nanospheres to high temperature and pH at a rate of around 30 °C. Moreover, the repulsion between the charged and negatively charged moieties was higher at a pH level of 30 °C. While (Lade & Shanware, 2020) suggested that the increase in the reaction mixture's temperature can lead to a decrease in the production rate of NPs and a rise in their stability. In addition, small silver nanoparticles can be produced at higher temperatures.

For the production of CeO₂ NPs, the temperature ranges of 40 -90 °C was observed, this resulted in a decrease in the CeO₂ NPs' crystal size. At elevated temperatures of 65 °C to 90 °C, the opposite was observed. This is because when the temperature increases, the crystal growth rate exceeds the nucleation process. This therefore means that the size of the particles increases (Mehrooz et al., 2022). On another study by (Mountrichas et al., 2014), it was observed that high temperatures were required for well-defined and small uniform Au NPs to be produced. In addition, the results of the study revealed that the surface plasmon resonance and light scattering intensity were increased simultaneously at elevated temperatures.

2.9.2 Concentration

The concentration of the precursor salt, stabilizer and reducing agent plays a crucial role on the particle size of the NPs. (Yazdani et al., 2021) suggested that a ratio of 1.5:1 (reducing agent to precursor) was used to produce the largest Au NPs with a diameter of 38 nm. However, when the pH was increased by adding more trisodium citrate dihydrate (reducing agent), the resulting NPs had a smaller size. The concentration of the stabilizing agent is crucial as it can restrict the growth and coalescence of NPs (Mavani & Shah, 2013; Ahmad et al., 2018). Therefore, varying its amount can lead to both increasing and decreasing particle sizes. One of the most critical factors that can affect the size of these particles is the ratio of the precursor to the stabilizer (Das et al., 2021). An increase in the number of NPs and a gradual increase in the size of the particles was observed when the concentration was increased (Torras & Roig, 2020). This was likely to occur concurrently due to the increase in the number of Ag nuclei in a solution following the Ag(I) reduction when the concentration is increased.

The effect of concentration was also observed for bimetallic oxides of ZrO_2 -ZnO (Ayanwale & Reyes-López, 2019) where it was found on the XRD analysis that the formation of various types of crystal structures, such as monoclinic ZrO_2 , hexagonal cubic ZrO_2 , and amorphous ZrO_2 , was observed at lower concentrations of zinc oxide against ZrO_2 . In a study on the synthesis Pd@Au core-shell, it was suggested that increasing the concentration of Au precursor in the solution could lead to the formation of a thick gold shell (Jung et al., 2019). These findings reveal the importance of precursor concentration is to the synthesis of NPs and BMNPs.

2.9.3 Reaction time

A number of studies have revealed that there is an increase in the size of nanoparticles when the reaction time is increased (Sundarrajan et al., 2018). (Mehrooz et al., 2022) observed that the increasing stirring time during the reaction solution's stirring phase result in the crystal size of synthesized nanoparticles being continuously increased. This phenomenon is because the higher the stirring time, the more likely it is that the aggregation of the nanoparticles will occur. This will lead to a larger cluster of these particles. The size is not the only factor that is affected by time, the shape of the NPs is affected also (Aji et al., 2020). (Torras & Roig, 2020) evaluated that during a short reaction time (10s and 1 min), the particles can exhibit various shapes, such as hexagons and triangles with particle size of 300 nm and 200 nm respectively. When the reaction time was 2 min, the size of the particles was reduced by up to 200 nm. Most of the population consisted of spherical, monodisperse, and small NPs.

(Pris et al., 2008) synthesized Ag NPs and it was revealed that when the stirring time was extended from 30 - 60 min, the solution turned darker green, and Ag flakes were visible in it. The prolonged stirring process increased the possibility of aggregation. Moreover, an increase in polydispersion and the particle diameter led to the emergence of a wider SPR peak. As the process was extended, it started to shift toward longer wavelengths.(Manzoor et al., 2015) observed using the XRD that the sizes of the particles were varying from approximately 41 ± 11 , 84 ± 21 and 135 ± 95 nm for stirring time of 0, 2 and 8 min respectively. The influence of intermediate stirring improved the control over the size of ZnO NPs. The XRD results indicated that the crystallinity of the samples decreased with the decrease in particle size. This suggests that a compromise is needed between the smaller size of the NPs and the defects.

Certain control methods can be utilized to prevent the spread of plant diseases. These include implementing sustainable farming techniques, destroying infected plant tissue, and using chemicals that can prevent pathogens from infecting other plants or sites. Although multiple techniques can be used to minimize losses, two or more approaches are necessary. These approaches may increase the resistance of the fungi. However, there is an endless possibility to nanomaterial synthesis for example, they can be made of organic and inorganic materials such as polymers, liposomes, and ceramics. They can also be composed of nanostructures made of pure carbon such as graphene and carbon nanotubes. In addition, the literature review indicates that nanotechnology has greatly affected the field of science, leading to the development of new materials with outstanding potential for various applications. It also observed that when new materials are incorporated, their properties increase. It was also noted that factors that affect the synthesis of NPs need to be taken into consideration to maximise the efficacy of the NPs.

Conclusion

Certain control methods can be utilized to prevent the spread of plant diseases. These include implementing sustainable farming techniques, destroying infected plant tissue, and using chemicals that can prevent pathogens from infecting other plants or sites. Although multiple techniques can be used to minimize losses, two or more approaches are necessary. These approaches may increase the resistance of the fungi. However, there is an endless possibility to nanomaterial synthesis for example, they can be made of organic and inorganic materials such as polymers, liposomes, and ceramics. They can also be composed of nanostructures made of pure carbon such as graphene and carbon nanotubes. In addition, the literature review indicates

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CHAPTER THREE

MATERIALS AND METHODS

This chapter describes the materials and methods used in this study. The isolation of pathogens from maize samples is described in this chapter. The synthesis of the monometallic and bimetallic particles has also been described. In addition, the details of the characterisation techniques for the nanoparticles are found herein. Finally, the methods used to perform antifungal assays have also been included.

3.1 Materials and chemicals

The ZR Fungal DNA Kit™, Nimagen, Zymoclean™ Gel DNA Recovery Kit, BrilliantDye™ Terminator Cycle Sequencing Kit and the ZR-96 DNA Sequencing Clean-up Kit™ were all purchased at Zymo Research. OneTaq® Quick-Load® 2X Master Mix was purchased from New England Biolabs and the ABI 3500xl Genetic Analyser was purchased at ThermoFischer. A (CuSO₄·5H₂O), (C₆H₈O₆), (PVP, (C₆H₉NO) n.), (AgNO₃), (Na₃C₆H₅O₇) and (EG, C₂H₆O₂), were obtained from Merck. All the chemicals were used as-received without any further purification and de-ionized water was obtained using an ultra-filtration system (Milli-Q, Millipore) with a measured conductivity of above 35 mho cm⁻¹ at 25 °C.

3.2 Instrumentation

PerkinElmer LAMBDA 25 UV-visible spectrophotometer was used to obtain the absorption spectra of the nanoparticles. The TEM images were measured on a TECNAI F20 HRTEM.

3.3 Site for maize collection

White maize with visible infection were collected from subsistence farmers from two communities, namely, Mazeppa bay (32.4742° S, 28.6416° E) and Centane (32.5066° S, 28.3170° E), in the Eastern Cape. The maize was stored in silver tanks prior to collection.

3.4 Identification of the pathogens

3.4.1 Isolation of the pathogen

Prior to inoculation of maize kernels, Potato dextrose agar was prepared as a growing medium of choice by weighing 39 g of PDA media and dissolving it in 1000 ml autoclavable bottle. The bottle was autoclaved at 121 °C for 15 min and allowed to cool. The PDA was then poured in 90 mm petri dishes and allowed to solidify (Merck, 2006). The maize samples were slightly surface disinfected so as to eliminate possible contaminants (Davoudpour et al., 2020) however, not to but eliminate the visible fungi. The sterilization was carried out with ethanol for 5 min prior to plating onto the solidified PDA. One maize kernel was inoculated per petri dish and incubated for 7 days at 25 °C (**Fig 3.1**). Isolation of *Fusarium* was done according to (Choi et al., 1999; Summerell et al., 2011; Noman et al., 2018) and the hyphal tipping method was used for isolating *A. flavus* where a dissecting microscope was employed was isolated using a hyphal tipping method (Singh et al., 2004). Briefly, a dissecting microscope is used to view the species of interest at high magnification. Locate individual strands of hyphae of fungus. The scalpel was flamed, and the end of the hyphal strand was cut at about 1 mm from the end and placed on to a new agar plate. Several plates were prepared as this procedure is susceptible to contamination and interferences.

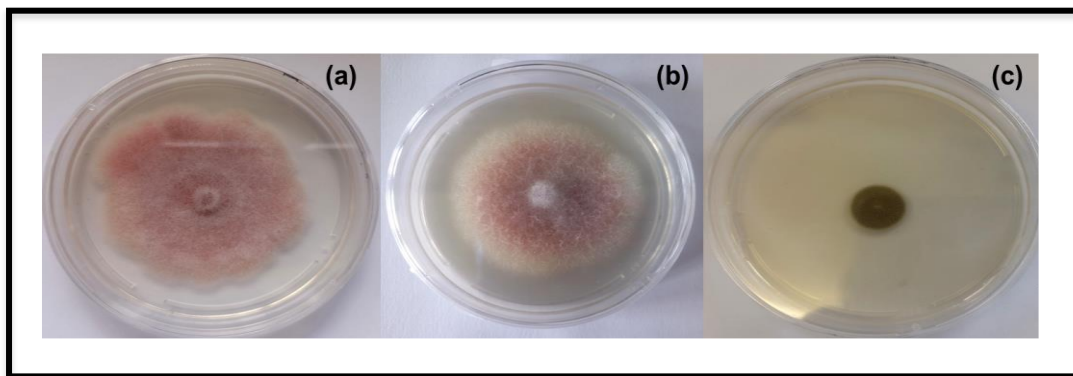


Fig 3.1: Fungi of (a) *Fusarium verticillioides*, (b) *Fusarium oxysporum* and (c) *Aspergillus flavus* isolated from maize and allowed to grow in potato dextrose agar plates.

3.4.2 Polymerase Chain Reaction (PCR) Analysis

PCR is one of the best tools used for identification of fungal pathogen at a molecular level due to their sensitivity. Results obtained from the samples above are summarised in **Table 3.1**. In brief, genomic DNA was extracted from the cultures using the ZR Fungal DNA Kit™ (Zymo Research, 2021b). The internal transcribed spacer (ITS) target region was amplified using OneTaq® Quick-Load® 2X Master Mix (Vazquez-Muñoz et al., 2019) with the ITS 1 and ITS 4 primers. The PCR products were run on a gel and gel extracted with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, 2021a). The extracted fragments were sequenced in the forward and reverse direction (Nimagen, 2017) and purified. The purified fragments were analysed on the ABI 3500xl Genetic Analyser (Applied Biosystems, ThermoFisher Scientific) for each reaction for every sample, as listed in section 1. CLC Bio Main Workbench v7.6 was used to analyse the.ab1 files generated by the ABI 3500XL Genetic Analyser and results were obtained by a BLAST search (NCBI) (White et al., 1990; Sena et al., 2011). A 50 µL working reaction mixture consisting of 275 µL sterile-distilled water, 100 µL 10 x amplication buffer, 100 µL dNTP stock mixture (2 mM each dNTP), 10 µL excess primer (50 µM stock), 10 µL limiting primer (1 µM stock) and 5 µL Taq polymerase (5 units/ µL) was added into test tubes. The DNA samples were diluted in water to 0.1 ng per 50 µL and 50 µL of the diluted sample was added into each test tube followed by two drops of mineral oil. The solution was then placed in a microcentrifuge. Cycling parameters were as follows; initial denaturation for 2 - 3 minutes at 95 °C, annealing for 30 seconds at 50 ° to 60 °C, extension for 0.5 to 2 minutes at 72 °C, denaturation for 30 seconds at 95 °C and the final extension for 10 minutes at 72 °C.

The amplification was performed as 25 cycles for double-stranded samples, using 50 pmol of each primer and 35 cycles for single-stranded samples, a primer ratio of 50: 2.5 pmoles was used. After completion, the tubes were centrifuged and 5 µL samples were taken for analysis by minigels. Prior to the single-stranded sequencing, the oil was removed by extraction in chloroform and the unincorporated nucleotides were removed while concentration of the DNA (by Centricon-30 centrifugal filtration) took place. The primers ITS1 were used with lower dNTP concentrations (35 µM each dNTP).

Table 3.1: The BLAST results correspond to the similarity between the sequence queried and the biological sequences within the National Center for Biotechnology Information database.

Sample name	Request ID	Predicted Organism	GenBank Accession
FVLP	0TG0KBBX01R	<i>Fusarium oxysporum</i>	MK849925.1
0TFY27UU013	ANNTR5	<i>Aspergillus flavus</i>	MK788223.1
PDA LP 1	5PD6BTKP016	<i>Fusarium verticillioides</i>	M T180471.1

3.5 Synthesis of Ag and Cu nanoparticles

Ag nanoparticles

The Ag NPs were prepared by a seed-mediated technique, the process contained a two-step reaction. The summary of the possible mechanism of synthesis is illustrated in **Fig 3.2**. The following solutions were prepared in separate volumetric flasks; 0.08495 g AgNO_3 in 50 mL, 0.0189 g NaBH_4 in 50 mL, 9.99 g NaOH 250 mL, 9.067 g CTAB in 250 mL and 1.76 g ascorbic acid in 100 mL. This preparation resulted in 0.01 M AgNO_3 , 0.01 M NaBH_4 , 0.1 M ascorbic acid and 1 M NaOH . A 250 μL AgNO_3 was added to 7.5 mL of CTAB, 600 μL of NaBH_4 was added into the mixture. This gave rise to the Ag seed solution (Jana et al., 2001; Kawamura et al., 2008; Monga et al., 2017).

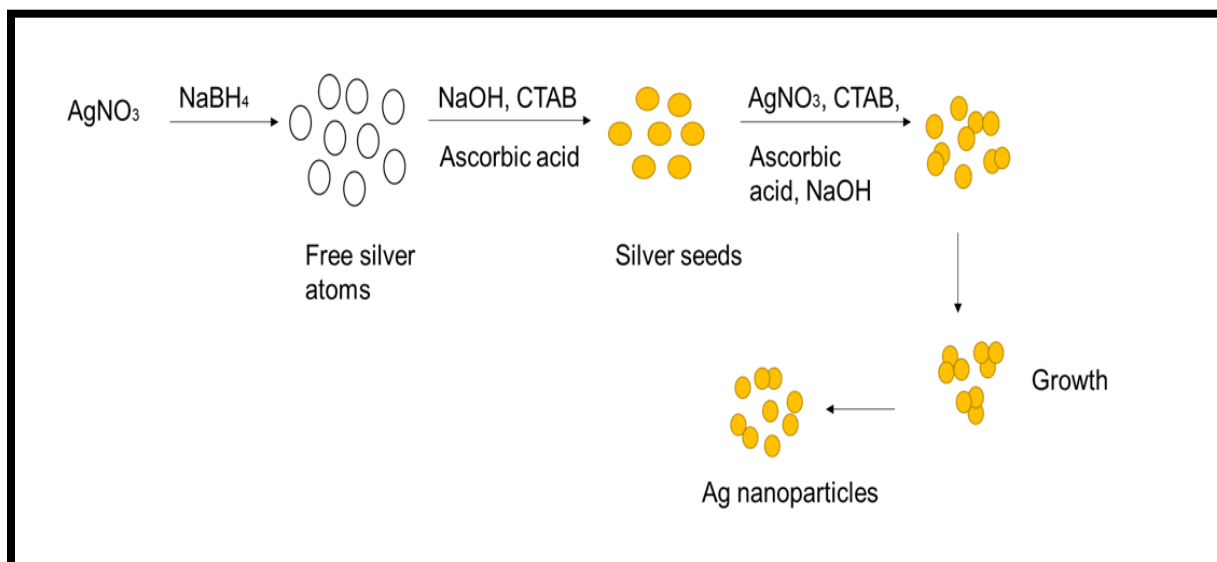


Fig 3.2: Possible mechanism of synthesis of Ag nanoparticles.

Synthesis of Copper nanoparticles

The synthesis of Cu nanoparticles was carried out by mixing the solution of PVP (0.3 M, 10 mL), Cu(AOC)₂ (1 wt %), 3 mL) and ascorbic acid (0.25 M, 3 mL) in EG at 140 °C. The appearance of dark reddish-brown colour indicated the formation of CuNS (Wang et al., 2006; Monga et al., 2017). In this method 3.33 g PVP, 1.997 g Cu(AOC)₂, 4.403 g ascorbic acid were all prepared in 100 ml volumetric flasks. The solution for the study was stirred using a magnetic bar, which was heated to 140 °C in a beaker. Ascorbic acid and Cu(AOC)₂ solutions were added dropwise into the hot PVP solution. After the first injection, the reaction mixture turned blue and on continuous addition turned orange. The reaction system started to turn turbid as more solutions were added, resulting in a dark red wine colour indicating the formation of Cu NPs as shown in **Fig 3.3**.

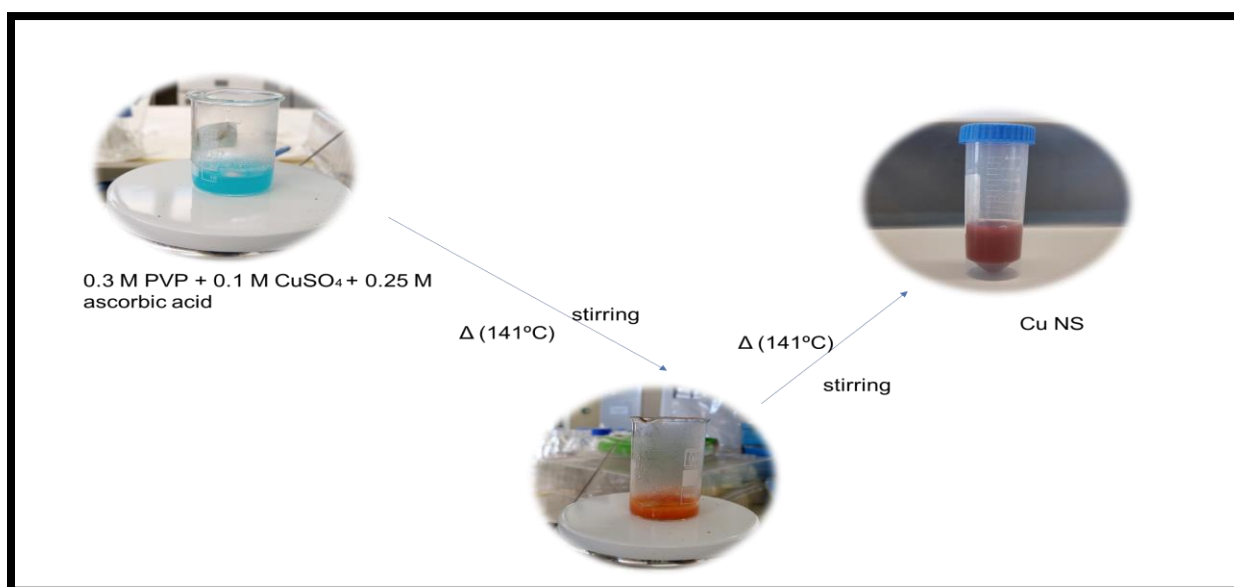


Fig 3.3: Synthesis of copper nanoparticles by chemical reduction process.

3.6 Preparation of Bimetallic nanoparticles

Ag/Cu bimetallic nanoparticles

The previously synthesized Ag Ns was used as a seed for coating of Cu to give rise to the core-shell nanostructure (**Fig 3.4**). 400 μL (0.1 M) of Cu(AOC)₂ was added to 4 ml of PVP which was

under a stirrer, thereafter, 11 μL (1 M) NaOH and 5 μL hydrazine were added to start coating of the Cu shell on Ag surface. The resulting ratio of Ag/Cu was approximately 0.8 and 0.1 respectively.

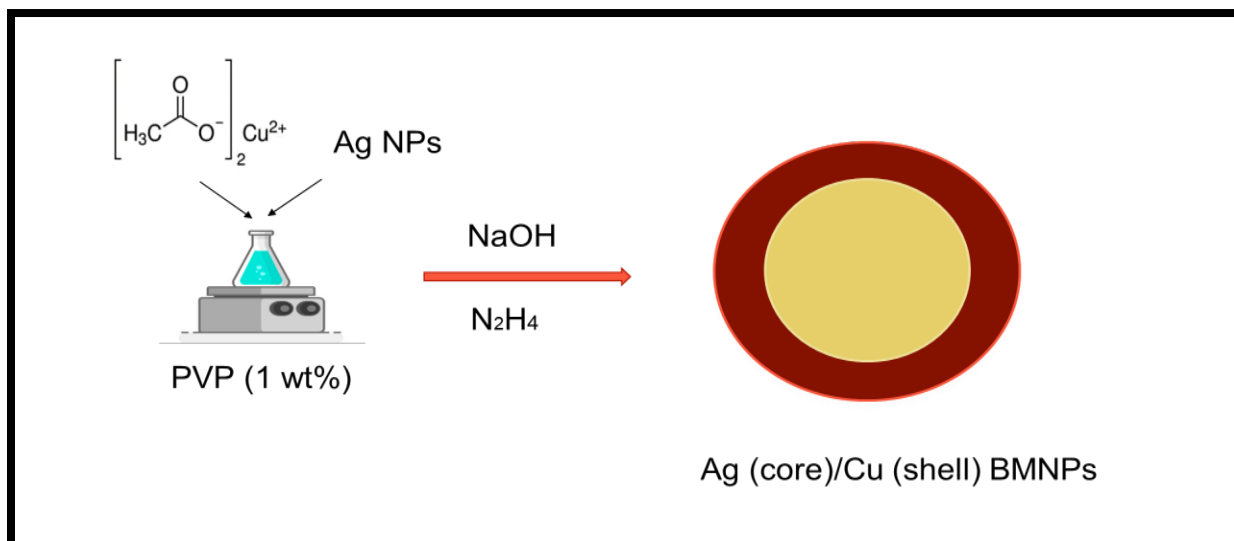


Fig 3.4: Formation of Ag/Cu bimetallic nanoparticles by depositing a $\text{Cu}(\text{Ac})_2$ onto the surface of Ag nanoparticles.

Cu/Ag bimetallic nanoparticles

The previously synthesized Cu Ns was used as a seed for coating of Ag to give rise to the core-shell nanostructure (**Fig 3.5**). Each set of the pre-synthesized Cu NS (400 μL) diluted with an aqueous solution of PVP (4 mL, 1 wt%) was treated an aqueous solution of AgNO_3 (100 μL , 0.01 M). After this, ascorbic acid (0.1 mL, 0.1 M) and NaOH (0.2 mL, 0.1 M) was added to initiate the reduction of Ag on the surface of Cu NS resulting in the formation of Cu/Ag NCs. The resulting ratio of Ag-Cu was approximately 0.8 and 0.1, respectively.

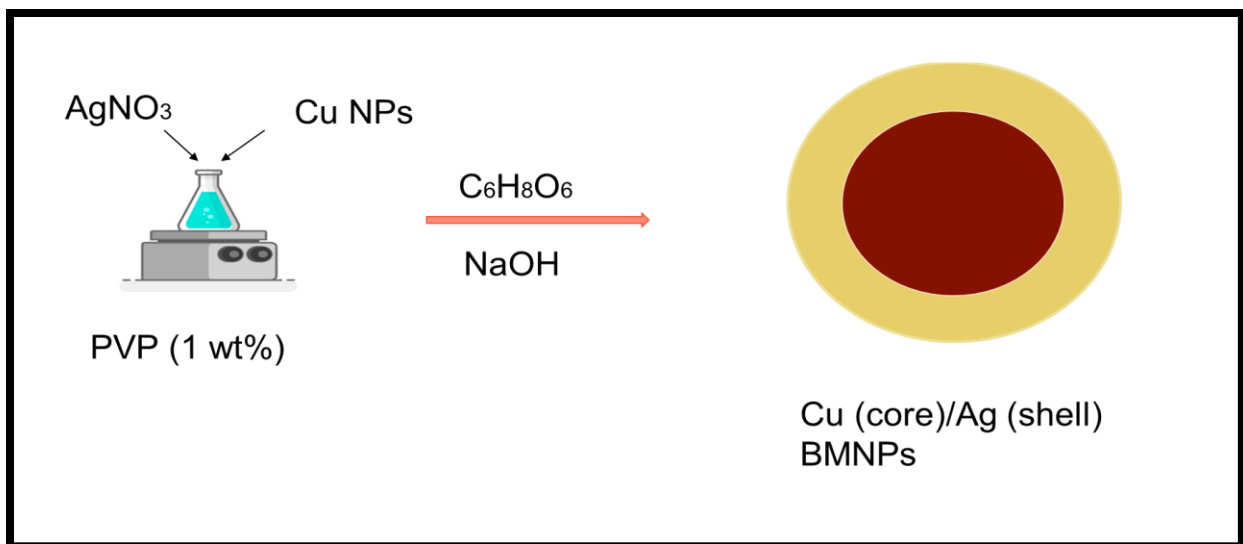


Fig 3.5: Formation of Cu/Ag bimetallic nanoparticles by depositing AgNO₃ precursor salt into Cu nanoparticles.

3.7 Characterisation of Nanoparticles

3.7.1 UV-VIS spectrophotometry

The absorbance of the colloidal nanoparticle samples was measured using the UV-Vis spectroscopy. In this process a sample containing water was used as a blank sample to set a baseline. A cuvette containing the nanoparticle sample was also placed in the UV-Vis spectroscopy and absorbance measured. Concentrated samples were diluted at a ratio of 1:3.

3.7.2 Transmission Electron Microscopy

The samples were deposited samples onto TEM grids coated with a thin carbon or support film. A small drop of solution was placed onto a grid and allowed to evaporate under vacuum. The sample was imaged, taking digital pictures of several locations on the grid to obtain a representative set of images. It is worth noting that, a lower magnification (2000 to 25000x) was used to obtain the size and shape of the particles. This provided a typical of 50 to 500 particles per image.

3.8 Determination of Antifungal activity of nanoparticles against selected mycotoxin producing pathogen

3.8.1 Agar diffusion method

Potato Dextrose Agar (PDA) was used throughout this study as the agar to grow fungal pathogens. A 19.5 g of PDA was weighed into 500 mL autoclave bottle and filled up to the mark with deionised water. The PDA solution was autoclaved at 121 °C for 15 min and allowed to cool at approximately 25 °C. About 100 mL of the antifungal agent (AgNO₃, Cu(OAc)₂, Ag, Cu, Ag/Cu and Cu/Ag NPs) was added into 250 mL PDA agar, and then mixed well. After the mixture was poured into Petri dishes, the medium was then subjected to pre-incubation where a mycelia disc with a diameter of 5 mm was placed at the centre of the petri-dish. After the incubation period of 7 days, the diameter of the fungal growth in the control and sample plates is measured, and the effect of the antimicrobial is estimated.

$$\text{Antifungal activity \%} = \frac{D_c - D_s}{D_c} \times 100$$

Where D_c is the diameter of fungal growth in control plate with no antifungal agent and D_s is the diameter of growth in the plate containing tested antifungal agent. Percentage inhibition was then calculated as per the formula above. This method can also be used to test the effect of the antimicrobial agent on fungal growth (Balouiri et al., 2016).

3.8.2 Spiral gradient dilution Assay

PDA plates were inoculated with a *F. verticillioides*, *F. oxysporum* and *A. flavus* and incubated for 7 days at ± 25 °C. The spore suspension of 1.164×10^4 CFU/mL for *A. flavus* was prepared in deionised water. There were three inoculated plates for each pathogen. Hydrophilic cellophane was cut into strips (4 × 35 mm), the strips were put to hang in an autoclavable glass jar and sterilized by autoclaving for 15 minutes at 121 °C. The cellophane strips were allowed to cool and using a sterile forceps, the strips were dipped into hot PDA media (this is done to avoid sticking into the agar after the growth of the pathogen). The hydrophilic cellophanes were placed across the PDA plate (**Fig 3.6**), the fungi were inoculated on the tape and allowed growth for 7 days at 25 °C.

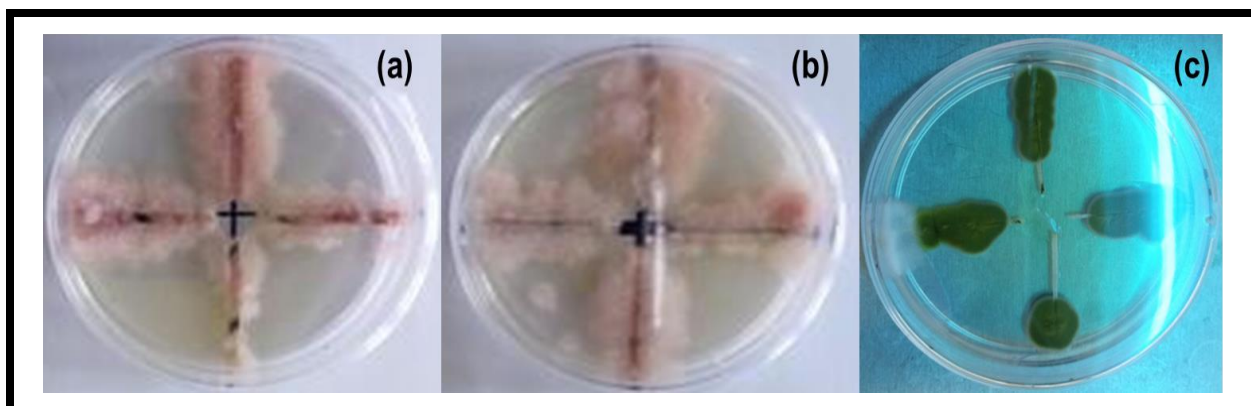


Fig 3.6: Cellophane tapes containing (a) *Fusarium verticillioides*, (b) *Fusarium oxysporum* and (c) *Aspergillus flavus* inoculated in potato dextrose agar plates.

Growth was observed after seven days and fresh PDA plates were prepared. The PDA plates were spirally inoculated with AgNO_3 , $\text{Cu}(\text{OAc})_2$, Ag, Cu, Ag/Cu and Cu/Ag NPs using an easySpiral@N° 30010-0 spiral plating machine and allowed to diffuse in the agar for a few minutes. The hydrophilic cellophanes containing the pathogen were placed on top of the antifungal agent. Growth of the pathogen was observed after seven days and a representation of how the materials and methods were conducted is depicted in **Fig 3.7**.

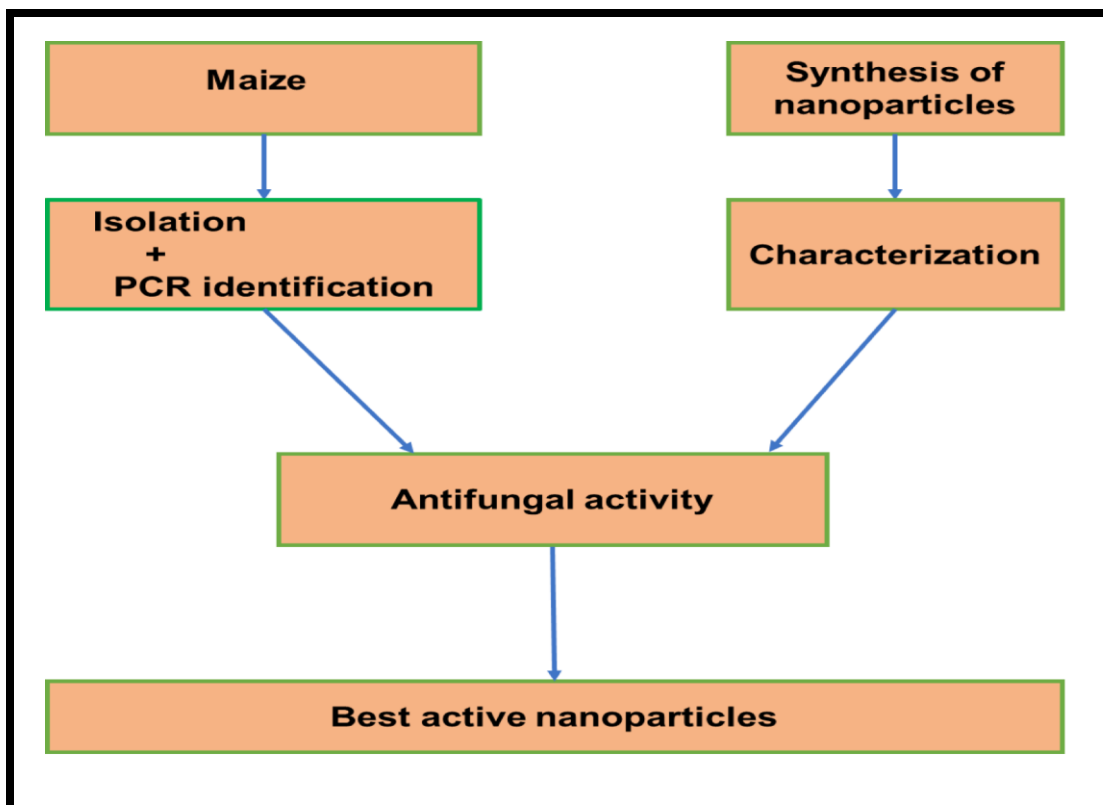


Fig 3.7: Schematic summary of methods.

3.9 Data collection and analysis

Mycelial growth length was measured using an electronic calliper and percentage inhibition calculated. The percentage inhibition and MIC of the NPs towards the three pathogens was analysed statistically using Analysis of Variance (ANOVA) on GenStat 20th edition. Means were separated at 5% significance level. All experiments were repeated twice.

CHAPTER FOUR

CHARACTERISATION AND APPLICATION OF THE SILVER-COPPER AND COPPER-SILVER BIMETALLIC NANOPARTICLES

*This chapter encompasses the discussion of the optical, size, morphological characterization of monometallic and bimetallic nanoparticles. Additionally, the results of antifungal activity of the monometallic Ag and Cu nanoparticles as well as the Ag/Cu and Cu/Ag bimetallic nanoparticles have been included. The metal salts used in the preparation of nanoparticles (copper acetate and silver nitrate) were also tested as controls and compared to the nanoparticles. The antifungal activity of the nanoparticles was evaluated by two methods thus, Agar diffusion method and spiral gradient method. The data was analyzed using Genstat 20th edition statistical package. All the nanoparticles were tested against the three types of pathogens (*Fusarium verticillioides*, *Fusarium oxysporum* and *Aspergillus flavus*).*

4.1 Characterization of monometallic and bimetallic nanoparticles

4.1.1 Optical properties

The UV-vis spectra of Ag, Cu, Ag/Cu and Cu/Ag nanoparticles is shown in **Fig 4.1**. Ag NPs had a surface plasmon resonance (SPR) peak at 453 nm, while Cu NPs the exhibit SPR at 607 nm. The values are comparable to the literature reported SPR values for Ag NPs and Cu NPs (Gomaa et al., 2019). The deposition of Ag⁺ ions on Cu NPs surface resulted on a blue shift of the SPR peak from 453 nm to 443 nm due to the formation of a thick shell around the copper NPs (**Fig 4.1 (c)**). These results are in agreement with those obtained by (Kaur et al., 2023) where the addition of Ag to Cu NPs caused a blue shift in the absorption band from 596 nm to 404 nm. The phase shifts in clusters are caused by the varying factors that affect their size, such as the high-order multipoles' influence and the electromagnetic waves' retardation. On the other hand, in smaller clusters, the spill out of electrons from the conduction causes a drop in their electron density (Manikandan et al., 2003). The SPR of the copper NPs was not visible in the bimetallic sample, which is further evidence of the successful synthesis core-shell type of the bimetallic with a thick shell. Meanwhile, UV-Visible spectrum of Ag/Cu bimetallic NPs gave a broad absorption peak between 500 – 700 nm, which is the SPR range for the Cu NPs (**Fig 4.1 (d)**). The broadening of the peak indicates that the particles are polydispersed. The absence of two plasmon peaks in the

spectrum indicates that these NPs were not a mixture of individual metal NPs. The resulted colour of the bimetallic NPs matched the descriptive colours of the shell nanoparticles as can be observed in the inserted images.

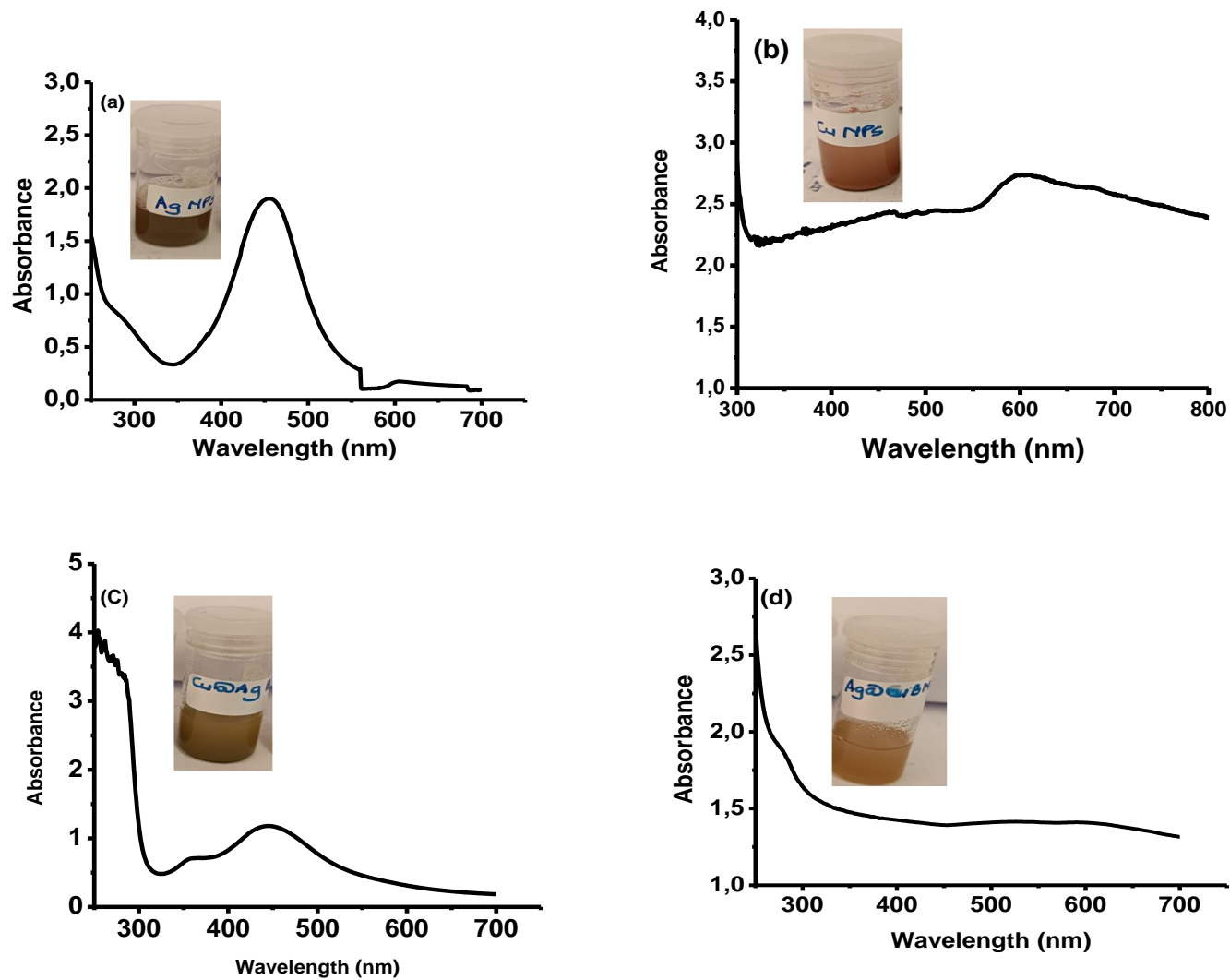


Fig 4.1: UV-Vis absorption spectra of **(a)** Ag NPs, **(b)** Cu nanoparticles, **(c)** Cu/Ag bimetallic and **(d)** Ag/Cu bimetallic nanoparticles.

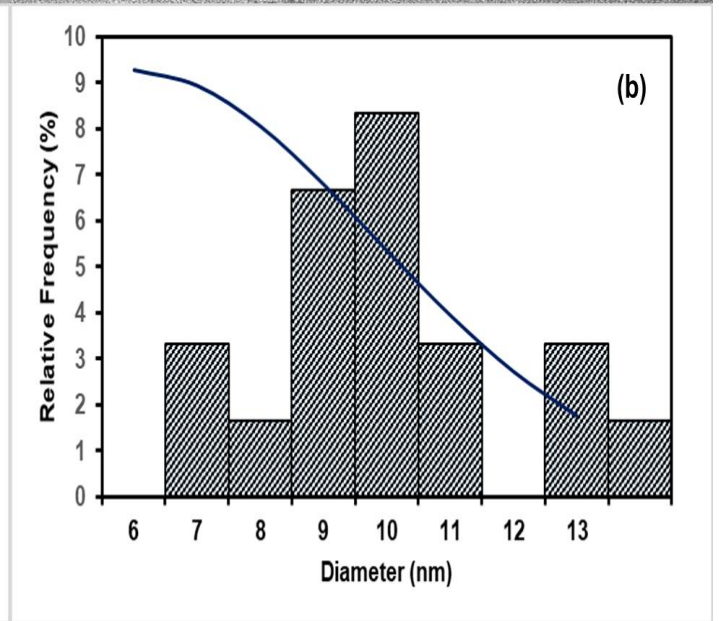
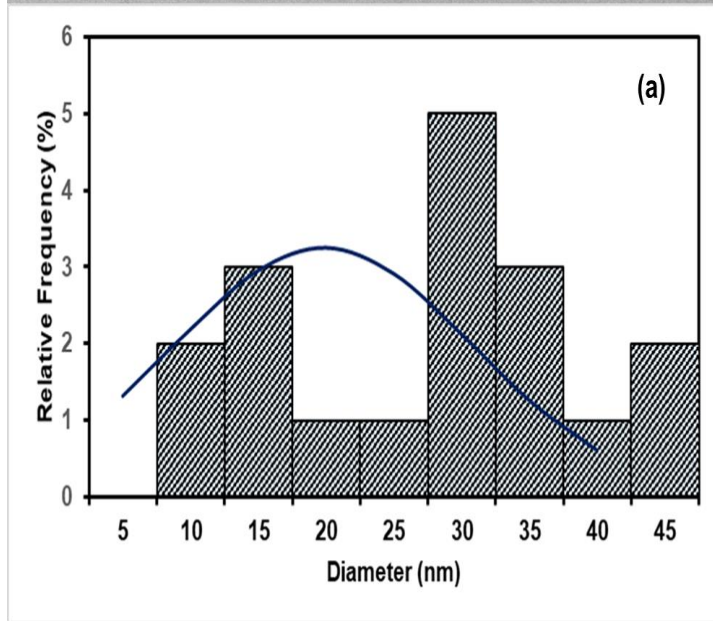
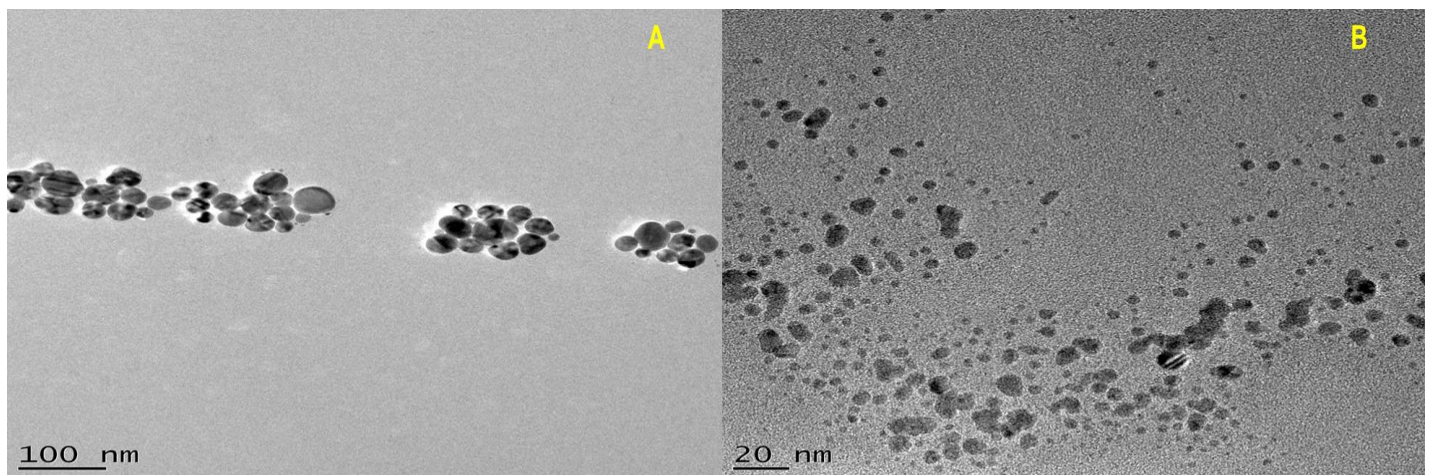
4.2 Morphological and size analysis

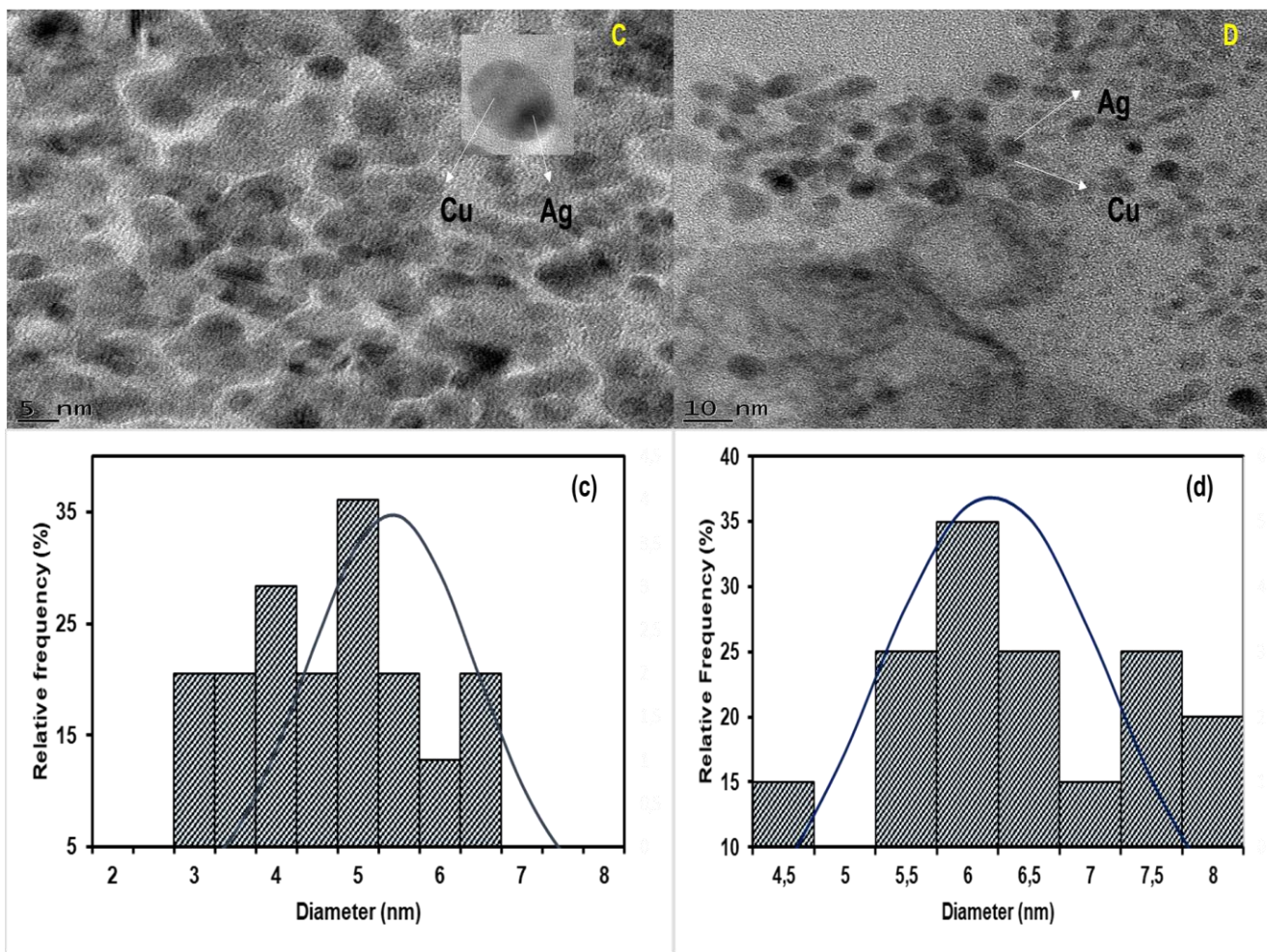
The shape and particle distribution of the as synthesized nanoparticles was investigated by the TEM and the particle size was measured using imageJ software. Ag NPs displays spherical NPs with an average particle size of 26 nm. The results in our study reveal that the most dominating shape amongst both monometallic and bimetallic NPs to be spherical as observed in **Fig 4.2**. The TEM image of Cu NPs is shown in **Fig 4.2 (b)**, which displays spherical NPs with particle size ranging from ~7 to 14 nm and an average particle size of 10 nm. The mono dispersed Ag/Cu obtained a particle size range of 3 to ~7 nm and an average size of 5 nm, while Cu/Ag were also mono dispersed with a particle size range of 4.5 to 8.5 nm and average particle size of 6 nm. Comparing the monometallic sizes, the addition of a precursor salt to the nanoparticle seems to decrease the overall size of the nanoparticles. (Camposeco et al., 2022) also found that the addition of Pt to Pd led to a reduction in the particle size and increased the homogeneity of the support. In the same experiment, the size of bimetallic Pt-Pd nanoparticles was 9 nm, while that of single Pd was 14 nm. Similar smaller sized Ag/Cu NPs were prepared by (Rahman et al., 2015) by mixing AgNO₃ and CuCl₂ in the presence of polyethylene imine (PEI) and later used a sensors to detect 2-butanone. In addition, (Bogatyrenko et al., 2023) formed Ag/Cu nanoparticles with a size range of 5 to 8nm on an amorphous carbon film by the physical vapour deposition technique. Due to the varying electron densities of Cu and Ag, the brighter particle is identified as Cu. On the other hand, the darker one is referred to as Ag.

Bimetallic synthesis revealed that the addition of a metal precursor to the nanoparticle resulted into core-shell arrangement as depicted in **Fig 4.2 C and D**. Following the initial synthesis of a core monometallic particle, the surface is modified by adding a secondary metal. Through two-step procedures, the control over particle morphology, size, and composition can be refined. The final size of a nanoparticle can be adjusted by choosing core particles that are of varying sizes. In addition, (Jung et al., 2019) stated that after synthesizing a monometallic core, carefully controlling the reaction conditions, and adding a second metal, the shape of this core can be preserved. It should be noted that, although the metal in excess can make a significant contribution to the solution's overall colour, the colloidal dispersion delivers a distinct colour based on the material's distribution and composition.

The Energy Dispersive X-ray spectroscopy (EDS) was used for the elemental characterisation. The analysis was performed to confirm the composition of the Cu/Ag composite. The peaks were studied and the different areas of the spectrum where they were focused were analysed. The

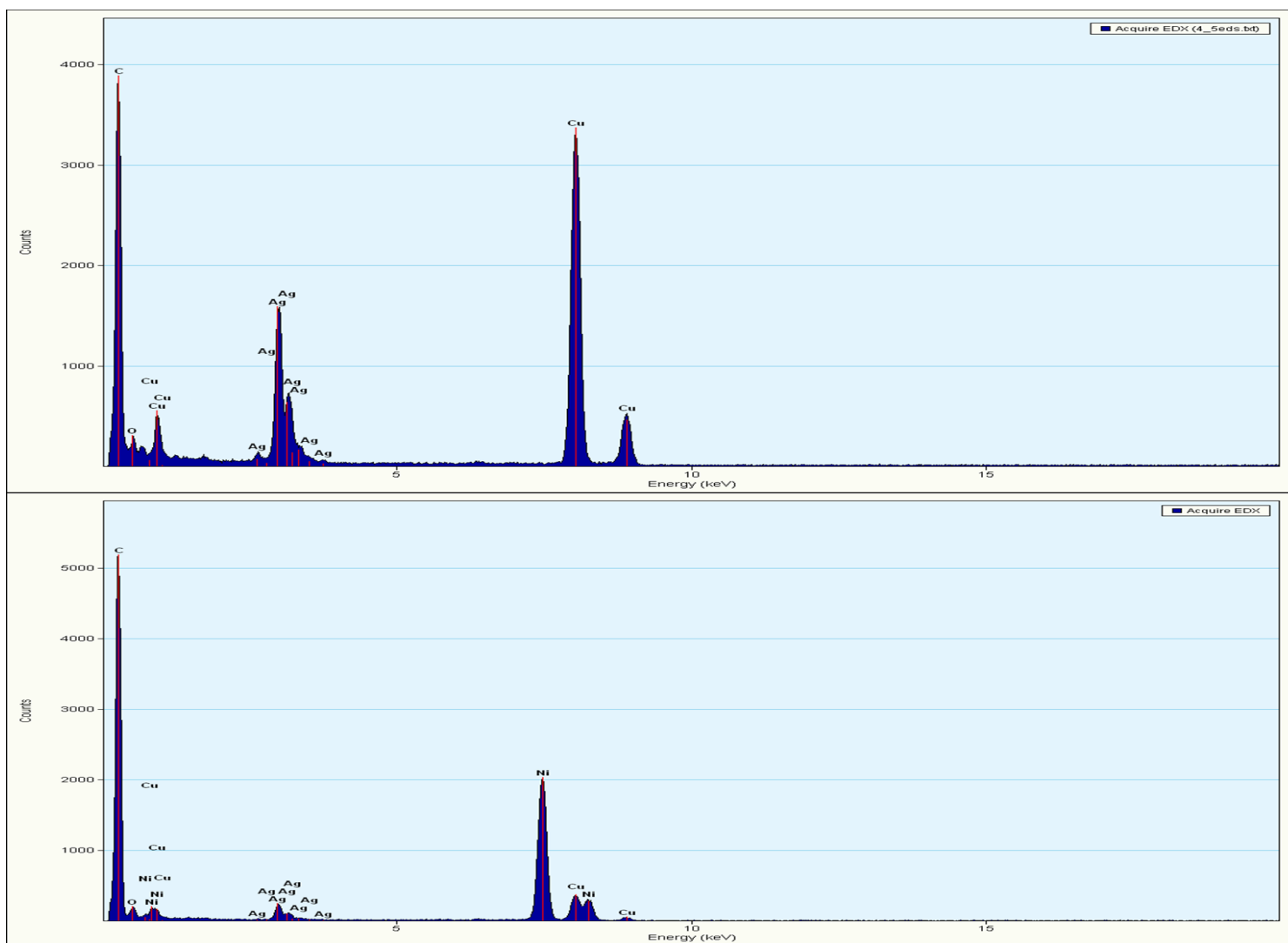
synthesized structures of the composite EDS spectrum clearly show the presence of Ag and Cu. The Energy Dispersive X-ray (EDX) results (**Fig 4.3**) confirmed the presence of both Ag and Cu.





2

Fig 4.2: Transmission Electron Microscopy images and histogram of the monometallics of (a) Cu NPs, (b) Ag NPs, (c) Ag/Cu BMNPs and (d) Cu/Ag BMNPs.



3

4 **Fig 4.3:** The Energy Dispersive X-ray spectrum of Ag/Cu and Cu/Ag bimetallic nanoparticles.

4.3 Efficacy of nanoparticles against *Fusarium verticillioides*, *Fusarium oxysporum* and *Aspergillus flavus*: Agar Diffusion method

All the agar diffusion experiments were performed in triplicates and the data was subsequently subjected to statistical analysis. The samples were incubated at ± 25 °C for 7 days and results are shown in **Fig 4.4**. The *A. flavus* showed a steady growth while both fusarium species were showing fast growth. According to these results, Cu NPs appear to inhibit the growth of *F. verticillioides* and *F. oxysporum* effectively compared to Ag NPs. However, Ag NPs were more effective than the Cu NPs against *A. flavus*. The bimetallic Ag/Cu and Cu/Ag are observed to hinder all pathogens almost to no significant growth. The higher antifungal activity of these nanoparticles is due to the synergistic effect of the two combined. The corresponding salts were also tested against the same pathogens and used as controls. The inhibition percentage by Cu (OAc)₂ and AgNO₃ below 50 % for all the tested organisms (**Table 4.1**).

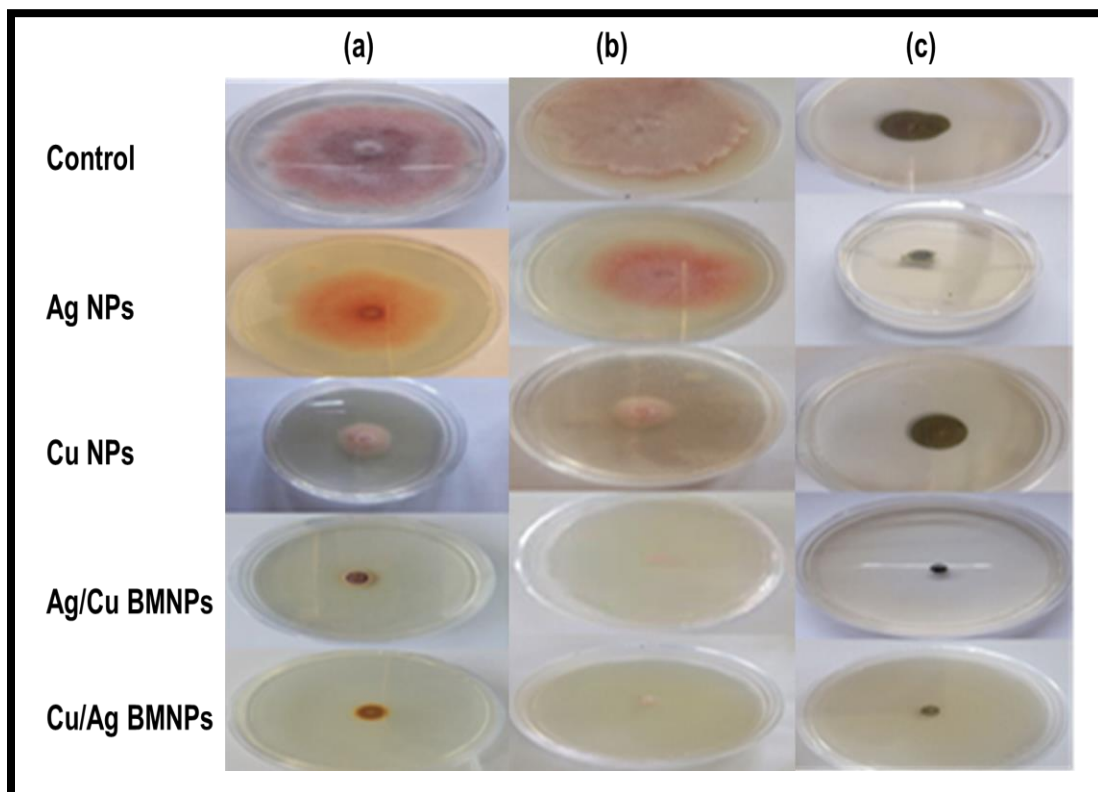


Figure 4.4: Representative plates of inhibition potential of the nanoparticles against (a) *F. verticillioides*, (b) *F. oxysporum* and (c) *A. flavus*.

Fig 4.5 shows the results of the growth inhibition percentage as a result of treatment with nanoparticles. The fungal growth of *F. verticillioides* and *F. oxysporum* was inhibited by 50% when treated with Ag NPs with a concentration of 25 mg/mL and a size of 30 nm (**Fig 4.5**). The growth inhibition of these pathogens shows the efficacy of Ag NPs because both *F. verticillioides* and *F. oxysporum* are known to be resistant to known antifungal agents such as azoles (Al-Hatmi et al., 2016). On the other hand, Cu NPs (25 mg/mL, 10 nm) achieved more than 80% inhibition against *F. verticillioides* and *F. oxysporum*. The bimetallic nanoparticles were also tested against these pathogens. Regarding the bimetallic nanoparticles, Ag/Cu (10 mg/mL, 5 nm) inhibited *Fusarium verticillioides*, *Fusarium oxysporum* and *Aspergillus flavus* pathogens by 89.88 %, 91.05 % and 61.07 %, respectively. Meanwhile, Cu/Ag bimetallic nanoparticles at a concentration of 10 mg/mL and size of 6 nm achieved a growth inhibition of 92.77%, 92.46% and 69.20% against *Fusarium verticillioides*, *Fusarium oxysporum* and *Aspergillus flavus*, respectively. The inhibition effect of the bimetallic NPs can be attributed to the possession of synergic effect between the metals (Ribeiro et al., 2022; Hao et al., 2023). In overall, the results show that the antifungal activity of the nanoparticles was in the order of Cu/Ag BMNPs > Ag/Cu BMNPs > Cu NPs > Ag NPs. The bimetallic nanoparticles showed higher antifungal activity in comparison to monometallic counterparts. The differences between antifungal activities of Ag/Cu and Cu/Ag may be due to the differences on the metals on the surface in core-shell structures. This proves that the reactions of a metal surface are affected by its composition and the arrangement of its atoms.

Nanoparticles have been reported to activate the formation of soluble compounds by disrupting the lipid-bound membranes and enzymes of fungal cells in the microorganisms. This process involves binding of NPs to external proteins, which could result in the creation of pores on the cells resulting in cell death. Other possible mechanism includes the development of reactive oxygen species (ROS) like superoxide anions, hydroxyl radicals, and hydrogen peroxide (Hwang et al., 2012; Radhakrishnan et al., 2018; Elamawi et al., 2018).

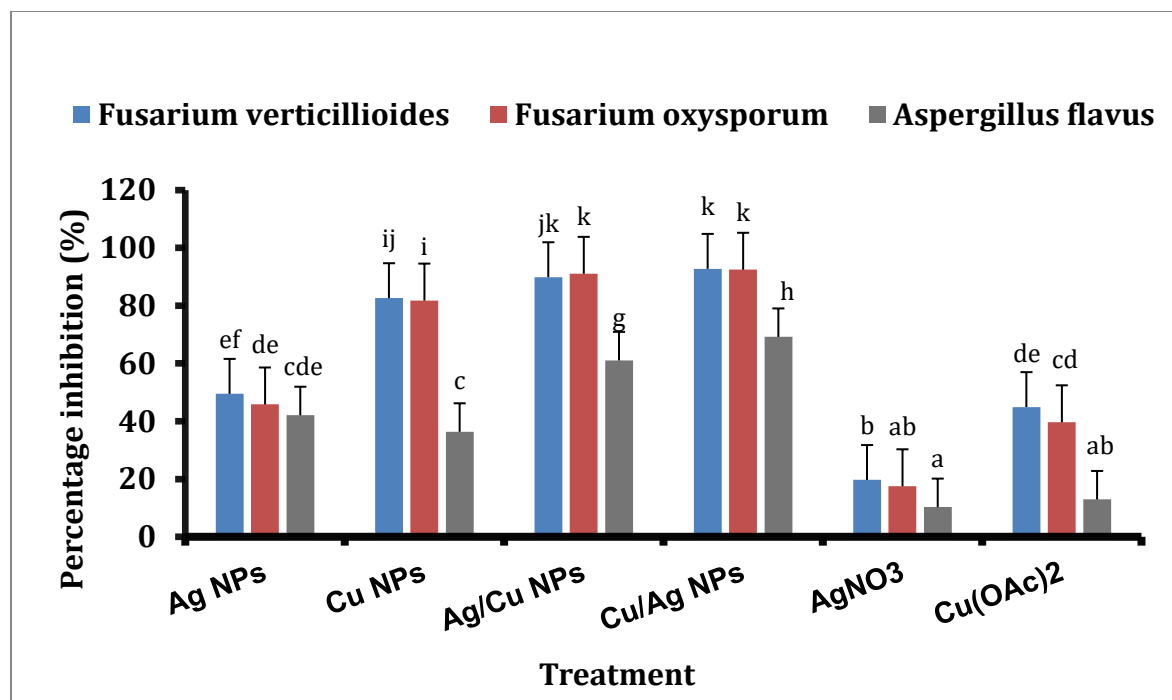


Fig 4.5: Percentage inhibition of the synthesized NPs (monometallic and bimetallic) and antifungal agents against *F. verticillioides*, *F. oxysporum* and *A. flavus*.

In the past few years, the field of nanotechnology has gained more interest due to the potential in controlling phytopathogens, making them an ideal candidate for agriculture as an alternative to chemical pesticides. In **Table 4.1** below, the results on the inhibition efficacy of Ag, Cu and Ag/Cu from this study were compared to the literature studies performed against *F. verticillioides*, *F. oxysporum* and *A. flavus*. Our results were comparable with literature studies, showing *A. flavus* to the least inhibited by the nanoparticles amongst all three pathogens (**Table 4.1**). Previous studies have shown that several factors such as the concentration, size distribution, shape, composition and surface chemistry have an influence over their antifungal activity. Among the metallic nanoparticles, Ag and Cu nanoparticles have been the most studied as antifungal agents. These nanoparticles have shown promising activity against different species of phytopathogenic fungi. However, the combination of these metals to form bimetallic nanoparticles shows a great potential due to the synergistic antifungal affect.

Table 4.1: Comparison of literature studies on the inhibition efficacy of Ag, Cu, Ag/Cu and Cu/Ag nanoparticles against *Fusarium verticillioides*, *Fusarium oxysporum* and *Aspergillus flavus*.

Nanoparticle	Size	Concentration	Inhibition (%)	Method of synthesis	Pathogen	Reference
Ag NPs	2–17 nm	60 mg/mL	42%	Green synthesis	<i>A. flavus</i>	(Mohamed El-Kadi et al., 2018)
Ag NPs	26 nm	25 mg/mL	49.50, 45.82 and 42.08%	Chemical Reduction	<i>F. verticillioides</i>, <i>F. oxysporum</i> and <i>A. flavus</i>.	Current Study
Ag NPs	30–50 nm	-	96.4%	Green synthesis	<i>F. verticillioides</i>	(Elamawi et al., 2018)
Ag NPs	9- 13 nm	-	70%	Green synthesis	<i>F. verticillioides</i>	(Akyüz et al., 2023)
Ag NPs	5 to 30 nm	150 ppm	43.35 to 54.8 %	Green synthesis	<i>A. flavus</i>	(Al-Othman et al., 2014)
Cu NPs	10 nm	25 mg/mL	82.62, 81.79 and 36.35 %	Chemical Reduction	<i>F. verticillioides</i>, <i>F. oxysporum</i> and <i>A. flavus</i>.	Current Study
Cu NPs	35 – 70 nm	300 ppm	100%	Chemical reduction	<i>F. verticillioides</i>	(Dorjee & Gogoi, 2022)
Cu NPs	26.5 nm	≥ 80ppm	100%	Chemical reduction	<i>F. oxysporum</i>	(Truong et al., 2023)
Cu NPs	53 nm	30 ppm	100%	Chemical reduction method	<i>F. oxysporum</i>	(Pham et al., 2019)
Cu NPs	15.6 – 34.3 nm	100 µg/mL	60%	Green synthesis	<i>A. flavus</i>	(Gomaa et al., 2019)

Cu NPs	2-10 nm	2400 ppm	76%	Green synthesis	<i>F. oxysporum</i>	(Mali et al., 2020)
Ag/Cu BMNPs	5 nm	10 mg/mL	89.88, 91.05 and 61.07 %	Chemical Reduction	<i>F. verticillioides, F. oxysporum and A. flavus</i>	Current Study
Ag/Cu BMNPs	16 nm	50 mg/mL	65% and 31%	Green synthesis	<i>F. oxysporum</i> and <i>A. flavus</i>	(Adewale & Similoluwa, 2019)
Cu/Ag BMNPs	6 nm	10 mg/mL	92.77, 92.46 and 69.20 %	Chemical Reduction	<i>F. verticillioides, F. oxysporum and A. flavus</i>	Current Study

4.4 Evaluation of antifungal activity using spiral dilution assay

The antifungal activity of the nanoparticles against *Fusarium verticillioides*, *A. flavus* and *Fusarium oxysporum* were also investigated using a spiral dilution assay method. In this method, the minimum concentrations at which the nanoparticles were able to hinder the growth of the pathogens was investigated (Fig 4.6). The antifungal activity of the synthesized Ag NPs at a final concentration of 21.16 µg/mL and 20.85 µg/mL was observed to reduce fungal growth of *Fusarium verticillioides* and *Fusarium oxysporum*, respectively. These findings are comparable for *F. oxysporum* but rather opposite for *F. verticillioides* in a study conducted by (Ribeiro et al., 2023). Ag NPs had an MIC value of 11.93 µg/mL against *A. flavus*. These results are also comparable with those discovered by (Jogee et al., 2017) where synthesized Ag NPs had an MIC of 20 µg/mL against *A. flavus*. Furthermore, MICs of Ag/Cu NPs against *Fusarium verticillioides*, *Fusarium oxysporum* and *A. flavus* were 13.99, 16.83, and 14.00 µg/mL, respectively. For the Cu/Ag NPs the MICs were 11.27, 12.89 and 7.60 mg/mL against *Fusarium verticillioides*, *Fusarium oxysporum* and *A. flavus*, respectively. These results were in agreement with the results of the Agar Diffusion method.

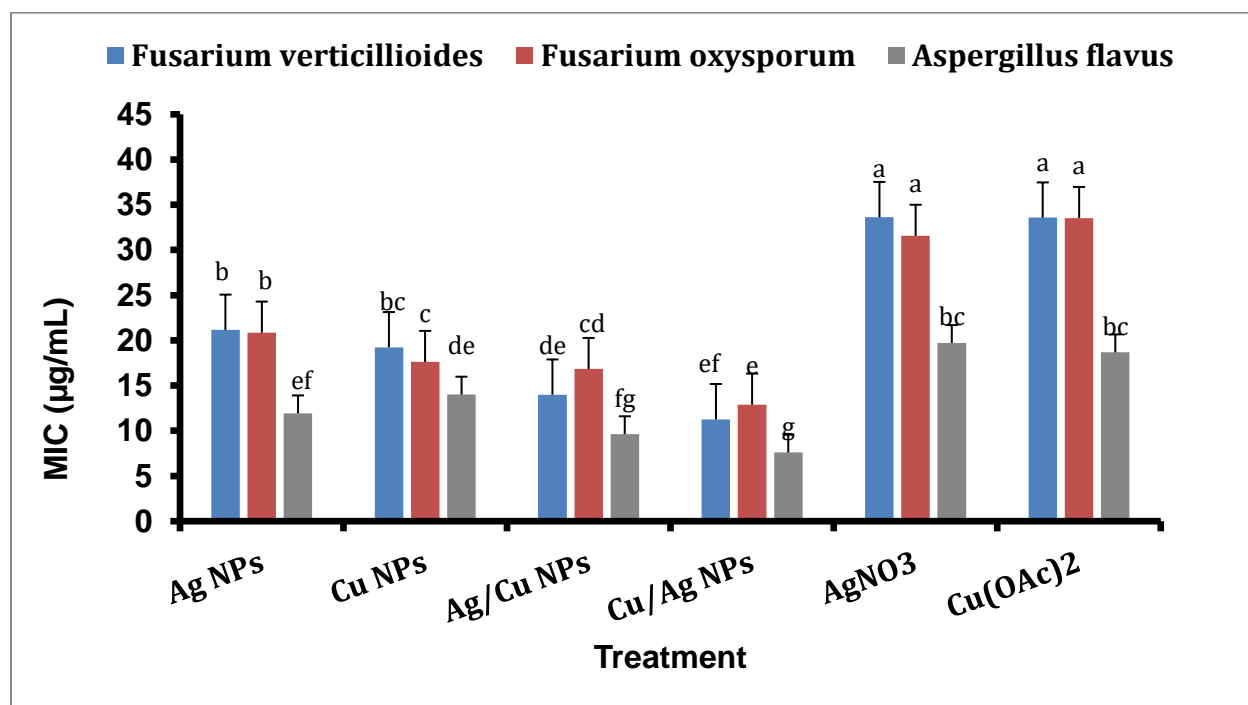


Fig 4.6: MIC of Ag, Cu, Ag/Cu and Cu/Ag nanoparticles against the isolated maize pathogens.

4.5 Comparing study between nanoparticles and their corresponding salts as Antifungal Agents

The antifungal strength of nanoparticles was compared against their salts (**Table 4.2**). Comparing Ag against its salt AgNO₃, it was found that AgNO₃ was less effective with a percentage of 37, 34 and 39% compared to Ag NPs against *F. verticillioides*, *F. oxysporum* and *A. flavus*, respectively. Ag NPs again were compared with the Ag/Cu and was found to have a percentage difference of 21, 13 and 12% against *F. verticillioides*, *F. oxysporum* and *A. flavus*, respectively. This was achieved using the formula:

$$\text{Percentage difference} = \frac{A - B}{A} \times 100$$

Where A is the least inhibiting antifungal agent and B is the highest inhibiting antifungal agent. 0

There was a slight significant difference with the activity of Cu NPs against *F. verticillioides* and *F. oxysporum*. *Fusarium oxysporum* revealed to be the most sensitive fungus to a concentration of 17.61 µg/mL. In a study by (Chandel, 2015), different strains of *F. oxysporum* had an MIC range of 30 to 60 µg/mL. This variation was explained by (Schuurmans et al., 2009) where according to their results, variations of up to 8 can easily be introduced in the measurement method by making slight changes. This means that different methodologies can produce variations of comparable results even against the same pathogen. A percentage difference of 43, 47 and 25% was observed when comparing the efficacy of Cu NPs and its salt, Cu (OA)₂ against *F. verticillioides*, *F. oxysporum* and *A. flavus*, respectively.

In comparison with Ag/Cu BMNPs, Cu NPs had a percentage difference of 15, 3 and 24% against *F. verticillioides*, *F. oxysporum* and *A. flavus*, respectively. In addition, the comparison with Cu/Ag BMNPs resulted to a difference of 23, 15 and 34% against the above-mentioned fungi. The Cu/Ag BMNPs revealed to inhibit the growth of the fungi even at the lowest of concentrations against *A. flavus*. Bimetallic nanoparticles showed tremendous opportunities for agricultural uses in controlling and managing crop pathogens tested in this study. Over the years literature (Ruparelia et al., 2008; Zain et al., 2014; Sharma et al., 2017; Hamouda et al., 2023) has focused mainly on employing nanoparticles especially bimetallic nanoparticles against bacteria and medical fungi hence there are few studies that investigate their effects against crop pathogens.

Table 4.2: The percentage difference between treatments of the same salts, including percentage difference between mono and bimetallic nanoparticles.

Pathogen	Antifungal agent	AgNO ₃	Cu(OAc) ₂	Ag	Cu
<i>F. verticillioides</i>	Ag	37% ^a	-	-	-
	Cu	-	43% ^b	-	-
	Ag/Cu	58% ^a	58% ^b	21% ^c	29 % ^c
	Cu/Ag	66% ^a	66% ^a	15% ^d	23% ^d
<i>F. oxysporum</i>	Ag	34% ^a	-	-	-
	Cu	-	47% ^b	-	-
	Ag/Cu	47% ^a	50% ^b	13% ^c	25% ^c
<i>A. flavus</i>	Cu/Ag	59% ^a	62% ^b	3% ^d	15% ^d
	Ag	39% ^a	-	-	-
	Cu	-	25% ^b	-	-
	Ag/Cu	51% ^a	49% ^b	12% ^c	22% ^c
	Cu/Ag	61% ^a	59% ^b	24% ^d	34% ^d

Note: *a* denotes the strength that Ag⁺ would need in order to have the same efficacy as the antifungal agent on the left. *b* denotes the strength of Cu⁺ would need in order to have the same efficacy as the antifungal agent. *c* denotes the strength that Ag NP would need in order to have the same efficacy as the antifungal agent. *d* denotes the strength that Cu NP would need in order to have the same efficacy as the antifungal agent.

CHAPTER FIVE

Conclusions and Recommendations

The study shows that the crucial attributes of nanoparticles, such as their size, shape and surface charge can be tuned to enhance their performance against plant fungal pathogens. These NPs were characterized by the following techniques: UV-vis spectroscopy, TEM and EDX. Based on our results, it was concluded that Ag/Cu and Cu/Ag bimetallic nanoparticles have shown promising antifungal activity results against *F. verticillioides*, *F. oxysporum* and *A. flavus* maize pathogens, which has limited information in literature. Bimetallic nanoparticles exhibited strong antifungal activity compared to monometallic nanoparticles. However, Ag/Cu BMNPs did not exhibit higher inhibition effect than Cu/Ag BMNPs as hypothesised. In addition, Ag NPs did not have a greater inhibition efficacy than Cu NPs. The growth inhibition of all the NPs against all tested pathogens revealed to increase with increasing incubation time regardless of the rate. Ag/Cu and its inverse Cu/Ag bimetallic nanoparticles were synthesised using chemical reduction method. Ag and Cu monometallic nanoparticles were successfully also synthesized and tested in comparison to the bimetallic nanoparticle.

It is recommended that an understanding of the interactions and combined antimicrobial mechanisms of Ag/Cu nanoparticles be investigated so that more potent formulations against a broader spectrum of plant pathogens will be possible. Furthermore, transitioning from laboratory studies to field trials will be an essential step in the development of bimetallic nanoparticle-based solutions. Future studies exploring the practicality, efficacy, and potential ecological impacts of these nanoparticles in real-world agricultural settings are necessary. Moreover, the effect of BMNPs containing other metal constituents on other fungal pathogens and bacteria should be investigated. This will provide clarity on the efficacy of NPs as it is observed that pathogens are not influenced by antifungal agents the same. A dual culture technique can be employed as one of the methodologies for bacterial species. Illustrations of the extent of the hyphae deformation may be added.

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