Assessment of biological application of green synthesized Ag-ZnO nanocomposite

A Dissertation for

MIC-651 Discipline specific dissertation

Credit:16

Submitted in partial fulfilment of Master Degree

Msc. In Microbiology

by

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "Assessment of biological application of green synthesized Ag-ZnO nanocomposite" is based on the results of investigations carried out by me in the (MSc. Microbiology) at the School Of Biological Sciences and Biotechnology /Dept of Microbiology, Goa University under the Supervision of Mr/Ms/Dr/Prof. Dr Trupti Asolkar and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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Date: April 2024

Place: Goa University

COMPLETION CERTIFICATE

This is to certify that the dissertation report "Assessment of biological application of green synthesized Ag-ZnO nanocomposite" is a bonafide work carried out by Ms. Bhavana Falgun Gawas under my supervision in partial fulfilment of the requirements for the award of the degree of (MSc. Microbiology) in the Discipline (Microbiology) at the School Of Biological Sciences and Biotechnology /Dept of Microbiology, Goa University.

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CONTENT

PREFACE

Pathogenic Microorganism are increasingly exhibiting resistance to antibiotic and pesticides, thereby placing strain on the conventional microbial control methods Utilizing nanoparticle to combat this problem will exert less pressure on the environment .Green synthesized nanoparticle can be of great benefit since the traditional methods are expensive and exert hazardous effect on the resources. Nanoparticle synthesized from green technology are eco-friendly and non-polluting in nature . Use of nanoparticle in plant growth promotion and antagonism against pathogen can help the farmer to improve their yield and economic status.

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LIST OF TABLES

LIST OF FIGURE

ABBREVATION USED

Entity	Abbreviation
µg	microgram
µl	microlitre
Ag	Silver
gm	grams
L	Litre
MBC	Minimum Bactericidal Concentration
mg	Milligram
MIC	Minimum Inhibitory Concentration
ml	mililitre
NA	Nutrient Agar
NP	Nanoparticle
PDB	Potato Dextrose Agar
ZnO	ZincOxide

ABSTRACT

This study investigated the antimicrobial, anti-inflammatory, and seed germination activities of 10% and 15% Ag-ZnO nanocomposites, focusing on their potential for plant pathogen control. The results showed that 10% nanocomposite exhibited strong antibacterial activity against Gram-positive and Gram-negative bacteria. The nanocomposite displayed antifungal and anti-inflammatory properties.

It also promoted seed germination, with optimal concentrations observed at 50 and 75 mg/l, while higher concentrations displayed toxicity. Furthermore, the nanocomposite showed promise in reducing wilt in tomato seedlings caused by *Ralstonia solanacearum*, suggesting its potential for disease management in agriculture.

SEM analysis revealed cell lysis and reduced bacterial populations upon treatment with 10% Ag-ZnO, confirming its antibacterial effects. Overall, the findings emphasize the versatile potential of green-synthesized Ag-ZnO nanocomposites for agricultural and biomedical applications.

Keywords; Nanocomposite , seed germination, antagonistic effect, Anti-inflammatory activity, Antimicrobial, Ag-ZnO

CHAPTER :1

INTRODUCTION

1.1 BACKGRAOUND

In the modern era, the rise of antibiotic-resistant bacteria and escalating resistance of phytopathogens to traditional pesticides has emerged as a significant global problem. These resilient strains demonstrated the capacity to persist and flourish even in the presence of antimicrobial agents and pesticides that were formerly efficacious against them. This presents a notable peril for human health and agricultural efficiency. (Agarwal et al.,2017).

Conventional strategies for combating these resistant strains entail the utilization of synthetic compounds, including antibiotics and pesticides, which have disadvantages and can engender detrimental repercussions on the environment and non-target organisms, potentially fostering the further development of resistance. (Chen et al., 2014)

To overcome this problem, many scientists have investigated easy and cost-effective methods to develop new strategies to combat the resistance of these microorganisms. Such problems and needs have led to the utilization of nanoscale materials such as nanoparticles because of their high zone-to-volume proportion and remarkable concoction and physical properties (Savi et al., 2013).

Nanomaterials show great potential as supplementary agents to antibiotics in combating bacteria, attracting significant attention owing to their ability to address the limitations often encountered by antibiotics. (Beyth et al., 2015)

Nanoparticles are particles at the atomic scale, ranging from to 1-100 nm, with at least one dimension smaller than 1 μm. Nanoparticles can be broadly categorized as metallic nanoparticles (for example, Au, Ag, Cu, Fe, and Zn), metal/non-metal oxides (for example, FeO, AlO, ZnO), semiconductor nanoparticles (ZnS, CdSe, ZnSe, CdS), and carbon

nanoparticles. Nanoparticles exist in various forms, such as quantum dots, nanotubes, nanowires, nanorods, and nanobelts. (Prabhu et al., 2014).

Compared with bulk materials, nanoparticles exhibit distinct properties, including size, distribution, and morphology alterations. Several physical and chemical possess drawbacks like high energy intensity in physical methods and generation of hazardous waste in chemical routes, making both conventional physical and chemical synthesis unfavorable due to the use of harmful substances.(Kinnear et al.,2017)

The utilization of green synthesis of nanoparticles has come to the forefront in this context. Green synthesis of nanoparticles pertains to the fabrication of nanoparticles through eco-friendly and sustainable means. These methodologies employ natural sources, such as plant extracts, microorganisms, or even waste materials, to orchestrate nanoparticles with distinct characteristics. This methodology offers numerous benefits over the traditional approaches. Primarily, green synthesis of nanoparticles is acknowledged for their environmentally conscious nature. The integration of natural resources diminishes dependency on harmful substances and curtails the ecological impact. (Shukla et al.,2010)

Green synthesized nanoparticles demonstrate heightened effectiveness against resistant bacteria and phytopathogens. They possess the ability to disrupt the cellular configuration of these microorganisms or impede their metabolic functions, resulting in their suppression or annihilation. They are used in coatings, films, and sprays for targeted and regulated dispensation. This allows accurate and effective distribution of nanoparticles to the designated area, augmenting their efficacy while minimizing their environmental footprint. (Ahmad et al., 2006)

Green synthesis of nanoparticles has exhibited promising results in reducing resistance development. Unlike traditional chemicals, which frequently exert substantial pressure on microorganisms, nanoparticles can operate through multiple mechanisms, making it challenging for bacteria and phytopathogens to evolve resistance.

The application of nanoparticles in agriculture is a strategic technique for enhancing existing crop management practices. Nano capsulated pesticides have been used effectively to release chemicals in a controlled and targeted way, making pest management safer and easier (Barman et al.,2013)

1.2 AIM AND OBJECTIVE

This study was carried out to evaluate the In –Vitro application of Green synthesized Ag-ZnO nanocomposite .

1) To evaluate the antibacterial and antifungal activity of Ag-ZnO nanocomposite.
2) To evaluate the anti-inflammatory activity of Ag-ZnO nanocomposite.
3) To assess the potential of Ag-ZnO nanocomposite in seed germination ,plant growth and antagonism against *Ralstonia solanaceum.*

1.3 HYPOTHESIS

Chemically synthesized antibiotic and pesticides pose a pressure on the environment as it gets bioacummulated .Utilizing nanoparticle can be a great alternative to minimize ecological footprint .green synthesized minimize the reliance on chemical and physical methods

Green synthesized nanoparticle have antimicrobial ,anti-inflammatory ,seed germination and antagonist activity .providing a alternative approach to agriculture sustainability and in biomedical sectors.

1.4 SCOPE

Green synthesized Ag-ZnO Nnanocomposite has a wide range of potential in agriculture and biomedical application. The antimicrobial, antifungal, and seed germination activity of these nanoparticles could have significant impacts on agriculture, healthcare and food security. The antagonistic activity of Ag-ZnO nanocomposite could have implications for disease treatment and prevention. Studying the green synthesized nanocomposite has the potential to make significant contributions to the fields of nanotechnology, biotechnology, and agriculture

CHAPTER: 2

LITERATURE REVIEW

Nanotechnology is a growing discipline of research that has application in health as well as agriculture for plant disease management (Rodríguez et al., 2013) because of its increased antimicrobial activity and decreased ecological toxicity (Neal, 2008) Synthesizing nanoparticles using plant extracts is more effective and environmental friendly approach compared to traditional physical and chemical methods This is because plant phytochemicals show greater reduction and stabilization.(Medda et al.,2015)

Nanoparticles (NPs) have demonstrated a broad spectrum of antibacterial activities against Gram-positive and Gram-negative bacteria as well as antifungal activity against broad range of phyto-pathogenic fungi (Shinde et al.,2012),

Zinc Oxide and silver are the most prominently used metallic and metal oxide nanoparticles (NPs) in a variety of sectors like medicine, renewable energy, cosmetics, textile production, environmental clean-up, electronics, surface disinfection, and agriculture. (Pati et al., 2016)

Owing to the strong binding energy of ZnO-NPs, remarkable piezoelectric qualities, biocompatibility, and lack of toxicity, their increased industrial potential has attracted considerable interest (Amanda et al., 2010). Moreover, the exceptional antibacterial activity of Ag-NPs makes them one of the preferred inorganic nanomaterials (Singh.,et al 2008). ZnO NPs have been shown to inhibit Staphylococcus aureus growth, and Ag NPs have concentration-dependent antibacterial action against Pseudomonas aeruginosa and Escherichia coli.

The antibacterial efficacy of Ag-NPs correlates with their particle size, with greater antimicrobial effectiveness observed at smaller sizes. (Morones et al., 2005),However, as the size of the Ag-NPs drops, NP aggregation becomes an issue, and the antibacterial activity of the NPs is reduced. As a result, in order to stop the cytotoxicity and aggregation Ag-NPs have

recently been hybridised and incorporated into several other nanomaterials (Agnihotri et al., 2013)

2.1 SILVER NANOPARTICLE

Silver has strong conductivity, stability, and antibacterial action when it is in its colloidal form. Silver is lethal to microorganisms, and the use of silver nanoparticles has the potential to increase the toxic effect .Silver ions have several applications, including seed priming, antimicrobial agent in air sanitizer spray, bactericidal coating in filters, and wet wipes and various other products (Prabhu ,2012).

The Susceptibility of silver to bacterial activity was shown to be directly related to silver content and inversely proportional to crystalline size of the silver (Aher et al., 2013).

The first green production of silver nanoparticles was reported by Wallen and coworkers, using beta-D-glucose and soluble starch as non-toxic stabilising and reducing agents. When an Ag+1 ion is reduced to Ag^0, or zero, silver nanoparticles are created. Plant phytochemicals, such as aldehydes, flavonoids, carboxylic acids, amides, terpenoids, and ketones, participate in the process of silver reduction.(Sharma et al.2020)

2.1.1 Mechanism of action

Interaction of silver nanoparticle with the thiol group required by the enzyme ,result in cell death.Silver is also known to cause damage to DNA by reacting with sugar and phosphorus-based DNA bases,which has an an impact on DNA replication (Rai and Bai 2011).

Ag ions is dependent on their positive charge,so the antibacterial activity is achieved by the electrostatic interaction between the positive charged of nanoparticle and the negative charged cell membrane of the microbe. (Muthukrishnan et al.,2015)

The antibacterial efficacy of silver nanoparticles against Gram-negative bacteria is contingent upon their concentration and is intimately linked to the development of soils within the bacterial cell wall. When silver nanoparticles build up in the bacterial membrane, they produce permeability, which kills the cells. (Sondi & Salopek-Sondi, 2004).

The attachment of silver nanoparticles to the cell wall resulted in the buildup of envelope protein precursor, which led to the dispersion of the protein motive force. Silver nanoparticles cause the plasma membrane to burst and the outer membrane to become unstable, which depletes intracellular ATP. (Song et al., 2006)

2.2 ZINC OXIDE NANOPARTICLE

The zinc oxide nanoparticle (ZnO NP) is a metal oxide that is commonly used in biosensore sunscreens ,solar cells and in cosmetics industry (Kumar et al.2008).

ZnO nanoparticles derived from plant extract has shown a strong antibacterial activity against both Gram-positive S. aureus and Gram-negative E. coli, with a high minimum inhibitory concentration (Bala, 2020).

The result obtained in the study by Zhang et al 2007 show that the presence of ZnO nanoparticle leads to damage of the membrane wall of E.coli. This damage may be due to direct interaction between ZnO nanoparticle and bacterial membrane (sabi).

According to a different research published the antibacterial activity of green synthesised ZnO rises proportionately with NP content. ZnO synthesized from leaf extract of Costus pictus demonstrated antifungal activity against Aspergillus niger and Candida albicans .this study reveal the potential antifungal activity of ZnO nanoparticle (Liu et al., 2020).

In a related study, ZnO synthesized from green tea leaves showed potent antifungal activity against Aspergillus niger ,According to the study the antifungal activity is due the electrostatic attraction between the nanoparticle and the cell membrane leading to the membrane disruption and cell death(Malachová, Praus, Rybková, & Kozák, 2011)

2.2.1 Mechanism of action

A study revealed which that the mechanism of antimicrobial activity of ZnO-NPs involves multiple metabolic pathways mechanism which includes (i) the destruction of cell integrity, (ii) ROS formation and (iii) the release of Zn(II) (Gong et al., 2012)

ZnO nanoparticles (NPs) are known to disrupt cellular processes, induce morphological changes in fungal hyphae, and impede fungal growth (He et al., 2011).

The bactericidal efficacy of ZnO primarily arises from the production of exceedingly reactive oxygen species (Anita et al., 2010). The interaction between ZnO NPs and bacteria triggers the continuous release of membrane lipids and proteins, altering the membrane permeability of bacterial cells and ultimately leading to cell lysis (Zhang and Chen, 2007).

Zinc is an important trace element that is required for the proper functioning of numerous enzymes that are involved in maintaining the balance between oxidative and anti-oxidative activities.disruption in this process result in inflammation, leading to serious consequences for human body (Pati et al., 2016).

Zinc plays a vital role in the immune system, in the normal development and functioning of immune cells. some studies have revealed that zinc can enter target cells and block

inflammatory signaling pathways . Zinc has been proven to significantly reduce pro-inflammatory cytokines and inhibit macrophages.(Kumar et al.,2010).

2.3 SEED GERMINATION ACTIVITY OF ZINC AND SILVER

Germination is important for assesing the density of the plant. AgNPs have been applied to agriculture to increase crop productivity.

Silver nanoparticles are known to have potential to promote photosynthesis and seed germination (Wahid et al., 2020).Application of silver nanoparticles on seeds are dose-dependent ,low concentration increase seed germination and yield whereas high concentration reduce seed germination (Alabdallah and Hasan 2021). The use of AgNPs on plants may vary depending on their size and duration of exposure (Aher et al., 2013)

Due to the ability of zinc oxide to generate reactive oxygen and its optical, physical and antimicrobial activity it has application in enhancing the agriculture production. (Jain et al.,2020). The positive influence of zinc oxide's antioxidant defence on the growth of rice and barley crop plants have been studied (Rajput et al.,2020).

ZnO NP(average size ~25 nm) when used in the peanut seed germination showed enhance growth parameters(Prasad et al., 2012). ZnO nanoparticle are reported to have higher bioavailability due to its nano size and lower water solubility which is responsible for higher yields of plants. (Noginov et al., 2007)

At high concentration Zinc is known for its widespread contamination in soil and toxicity at although ,Zinc (Zn) is an also essential micronutrient for plants to perform several enzyme activities and metabolic processes , also the deficiency of zinc in plants, results in low yield.

CHAPTER : 3

MATERIALS AND METHODS

AgZnO nanocomposite were provided by Miss. Mamata Prabhugaonkar,Assistant Professor,St Xaviers College Mapusa. Two concentration namely, 10% and 15 % AgZnO nanocomposite were provided in this study.

3.1 ANTI-BACTERIAL SCREENING OF Ag-ZnO NANOCOMPOSITE

The unidentified Gram positive bacteria and Gram negative phytopathogen *Ralstonia solanaceum* & *Xanthomonas oryzae* were obtained .The strains were kept on Nutrient agar and BG agar plates at 4ºC.

3.1.1 Preparation of inoculum

Active Gram positive & Gram negative culture for test were set up by inoculating a single 24hrs old colony from stock culture plate into Nurtient broth & BG broth flask which were then incubated for 24hr at 37ºC on incubator shaker at 110 rpm.

3.1.2 Broth dilution method

The Agar well diffusion method's accuracy was hampered by observed nanocomposite settling , hence broth dilution approach was adopted to assure uniform dispersion of nanocomposite and its direct interaction with bacterial cells.

3.1.3 Preliminary test

25µl Ag-ZnO nanocomposite (10mg/ml) from stock was added to 75µl of 24hr old Gm +ve and Gram –ve culture and incubated for 3hrs on incubator shaker . After 3hrs , the mixture was added to 5 ml Nutrient and BG broth tubes and incubated for 24hrs on incubator shaker at 110 rpm to observe the growth.

DMSO was used as a negative control and bacterial culture was used as positive control. After 24hr absorbance was measured by recording the O.D at 600nm where uninoculated broth was kept as a blank.

3.1.4 Minimum inhibitory concentration

Antimicrobial Efficacy of Ag-ZnO nanocomposite was studied by standard broth method with minor modification . *Ralstonia solanaceum, Xanthomonas oryzae* and an unidentified Gm +ve isolate was used for the study. *Ralstonia solanaceum* was grown in BG media and Nutrient Broth was used for *Xanthomonas oryzae* & Gm+ve isolate . Ag-ZnO nanocomposite concentration ranging from 20 µg to 100 µg shown in table 3.1 was mixed with 375 µl of overnight grown bacterial cells and incubated for 3hr for the bacterial cell to interact uniformly .After 3hrs the content was transferred to Test tube containing 5 ml of Nutrient and BG broth and incubated for 24hrs at 37ºC in incubator shaker at 110 rpm. The culture control and DMSO control were maintained. The inhibition in growth was recorded at 600nm by comparing with the control containing DMSO. The lowest concentration of Ag-ZnO nanocomposite which showed spectrophotometric inhibition/reduction in optical densiy (turbidity of bacterial cells)were considered as the minimum inhibitory concentration (Bala, 2020).

Table 3.1 MIC Concentrations of Ag-Zno nanocomposite

Concentration Of Ag-ZnO nanocomposite (µg)	Volume of Nanocomposite	Volume of DMSO	Volume of Bacterial culture	Volume of broth	Total Volume
20	20 µl	100 µl	375 µl	5 ml	5.495ml
40	40 µl	80 µl	375 µl	5 ml	5.495ml
50	50 µl	70 µl	375 µl	5 ml	5.495ml
60	60 µl	60 µl	375 µl	5 ml	5.495ml
70	70 µl	50 µl	375 µl	5 ml	5.495ml
80	80 µl	40 µl	375 µl	5 ml	5.495ml
90	90 µl	30 µl	375 µl	5 ml	5.495ml
100	100 µl	20 µl	375 µl	5 ml	5.495ml
+ve control	-	-	375 µl	5 ml	5.375 ml
DMSO control	-	120 µl	375 µl	5 ml	5.495ml

3.1.5 Minimum bactericidal concentration determination

Ag-ZnO nanocomposite in concentration ranging from 100 µg to 180 µg shown in table 3.2 was mixed with 320 µl *Ralstonia solanaceum* cells and incubate for 3hr.After 3hrs the content was transferred to Test tube containing 5 ml of BG broth .The tubes were incubated for 24hrs at 37ºC in incubator shaker at 110 rpm. After incubation the optical density of the tubes were recorded and 100 µl from tubes showing lowest absorbance were spread plated on BG agar plates and incubated for 24hr at 37ºc .the plates were checked for the presence or absence of bacterial colonies .

Table 3.2 MBC Concentration Of Ag-zno nanocomposite

Concentration Of Ag-ZnO nanocomposite (µg)	Volume of Nanocomposite	Volume of DMSO	Volume of Bacterial culture	Volume of broth	Total Volume
100	100 µl	80 µl	320 µl	5 ml	5.5ml
110	110 µl	70 µl	320 µl	5 ml	5.5ml
120	120 µl	60 µl	320 µl	5 ml	5.5ml
130	130 µl	50 µl	320 µl	5 ml	5.5ml
140	140 µl	40 µl	320 µl	5 ml	5.5ml
150	150 µl	30 µl	320 µl	5 ml	5.5ml
160	160 µl	20 µl	320 µl	5 ml	5.5ml
170	170 µl	10 µl	320 µl	5 ml	5.5ml
180	180 µl	-	320 µl	5 ml	5.5ml

3.1.6 SEM analysis for the *Ralstonia solanaceum* :

The effect of Ag-ZnO nanocomposite on bacteria was observed by scanning electron microscope .

SEM analysis slide preparation**:**

A clean glass slide (1 cm × 1 cm) was loaded with 5 µl of treated and untreated *Ralstonia solanaceum* cells respectively. The slides were air dried,fixed with 2.5 % glutraldehyde & maintained overnight for fixation. After 24hrs ,glass slide was washed with distilled water followed by successive dehydration with 20%, 40%, 60%, 80%, 90% ethanol for 10 min each and 100% ethanol 30 min. the slides were complete air dried. Then the cut slides were

attached to a tub and sputter coated with gold .the cells were observed under scanning electron microscope (Evo 18,Carl ZIESS, Germany) with magnification from 5KX to 25KX.

3.2 ANTI-INFLAMMATORY ACTIVITY OF Ag-ZnO NANOCOMPOSITE

Test group- 100µg, 200 µg, 300 µg, 400 µg concentration (25 µl, 50 µl, 75 µl, 100 µl,) of 10 % & 15% Ag-ZnO was taken in 5 test tube. 2 ml of of 1% bovine serum albumin (BSA) was added to each test tube. 475 µl, 450 µl, 425 µl, 400 µl distilled water was added to test tube containing 10% & 15% Ag-ZnO

Control group-2 ml of DMSO was added to 2 ml of BSA solution.

Standard group-100µg, 200 µg, 300 µg, 400 µg concentration (25 µl, 50 µl, 75 µl, 100 µl,) diclofenac was taken in 5 test tube . 2 ml of 1%BSA was added to each test tube .The test tube was incubated for 10 minutes at room temperature .then the tubes were incubated in water bath at 55 Cfor 10 mins .Absorbance was recorded at 660 nm .The % inhibition was calculated by the following formula.

$$\text{Percent inhibition} = \frac{\text{Control O.D- Treated O.D}}{\text{Control O.D}} \times 100$$

3.3 ANTIFUNGAL ACTIVITY OF Ag-ZnO NANOCOMPOSITE

Fungal strain: Fungal Phytopathogens *Fusarium oxysporum.sp.solani, Fusarium oxysporum.sp.niveum, and Sclerotium_ sp* were obtained .

3.3.1 Disc diffusion assay

The agar plate disc approach was used to assess the impact of green synthesised Ag-ZnO nanocomposite on plant fungal pathogens. In order to do this, 200µl from10 mg/ml Ag-ZnO stock of 10% and 15% Ag-ZnO nanocomposite was spread-plated onto potato dextrose agar plates. Plant fungal pathogen plugs *of Fusarium oxysporum.sp.solani, Fusarium oxysporum.sp.niveum* and *Sclerotium spp*. were subsequently placed onto the plates.DMSO control and culture control was maintained for each of the pathogen and incubated at Room Temperature for 4 days until the mycelial development .Later the plates were checked for the growth by comparing with negative and positive control and measuring their radial mycelium(He et al., 2011).

3.3.2 Broth based assay

A broth-based fungal assay was performed for *Sclerotium sp, Fusarium oxysporum.sp.solani,* and *Fusarium oxysporum.sp.niveum* .The 20 ml of PDB broth was used in each of the 12 conical flasks, (4 for each fungal species). Flasks were labelled Culture Control, DMSO Control, 10%, and 15% Ag-ZnO respectively. Each conical flask was inoculated with a fungal disc containing cultured fungi. 200 µl from10 mg/ml of 10% & 15% nanocomposite was added to 10% and 15% labelled conical flask, respectively, while 200 µl of DMSO was added to the DMSO control. The flask was incubated for 24 hours at 37 °C on an incubator shaker set to 110 rpm. Following incubation, the flask was removed out and each component was individually filtered through Whattman No. 1 filter paper in order to determine the wet weight.

3.4 SEED GERMINATION AND PLANT GROWTH ACTIVITY OF Ag-ZnO NANOCOMPOSITE

Brinjal seeds were surface sterilize by rinsing it with 70% ethanol for 2 minutes and immediately washing it again thrice with distilled water . Seeds were washed with 1.5% sodium hypochlorite solution for 5 minute. And then rinsed with distilled water 4 times.

3.4.1 Seed germination activity of Ag-ZnO

40 seeds per nanocomposite concentration (25mg/L, 50mg/L, 75mg/L, 100 mg/L) were subjected to 5hrs incubation period in an incubator shaker .Following the incubation the seeds were utilized to perform both Roll towel method and the soil- based seed germination method .

3.4.2 Roll towel method

For the seed germination roll paper towel method was followed where the paper were initially moistened with water to achieve the desired moisture level .Subsequently 20 treated seeds were placed equidistant from each other on moistened paper. Another moist paper was placed over the seeds, the assembly was then rolled carefully and secured with rubber band at both ends and then wrapped in polythene .This was then incubated at room temperature for 4-5 days . Nanocomposite was used in the following concentration: 25mg/L, 50mg/L, 75mg/L, 100 mg/L & 200 mg/L. Control were kept with untreated seeds. Following the incubation, seed germination percentage, height of seedling & seedling vigor, was recorded.

3.4.3 Soil-based seed germination

Soil based method was employed to observe whether the varying concentration of nanocomposite yield divergent outcomes compared to those obtained from roll paper towel method

The experiment was carried out by sowing the 20 treated seeds in seed germination pot filled with soil mixture composed of 3 parts soil, 1 part sand and 1 part vermicompost and designated as 25mg/L, 50mg/L, 75mg/L, 100 mg/L .The seeds were then sown into the soil, watered appropriately and the seeds were allowed to grow for a period of 7-8 days. Control was kept without seed treatment. After seed germination parameters such as seed germination percentage ,height of seedling and seedling vigor was measured .

3.5 ASSESSMENT OF Ag-ZnO NANOCOMPOSITE PLANT GROWTH AND ANTAGONISTIC ACTIVITY.

In this experimental protocol ,10 pots, each containing 5 tomato seedling designated as control, 200mg/L, 150mg/L, 100 mg/L ,75 mg/L were subjected to treatement with different concentration of nanocomposite .Following a 30 mins incubation period ,each plant was inoculated with 10 ml of 0.1 O.D *Ralstonia solanaceum* culture. Control was maintained by inoculating *Ralstonia solanaceum* only. The pots were left for incubation and wilting result was recorded after 4 days of incubation .

CHAPTER: 4

ANALYSIS AND CONCLUSION

4.1 ANTI-BACTERIAL SCREENING OF Ag-ZnO NANCOMPOSITE

4.1.1 Preliminary test

The preliminary test was performed to assess the antibacterial activity of 10% and 15% Ag-ZnO nanocomposite against Gram-positive and Gram-negative bacteria.. The result are tabulated in table 4.1.

Table 4.1 Preliminary test absorbance reading at 600nm

Treatment	Absorbance at 600nm		
	Gram positive bacteria	Gram negative bacteria (Ralstonia solanaceurum)	Gram negative bacteria (Xanthomonas oryzae)
10 %	0.221	0.087	0.16
15%	0.258	0.100	0.5
DMSO control	0.934	0.487	0.449
+VE control	1.047	0.962	0.56

Gram positive and gram negative bacteria were evidently susceptible to the Ag-ZnO 10% and 15% nanocomposite. 10% Ag-ZnO nanocomposite demonstrated a significant reduction in absorbance at 600 nm for both gram positive and gram negative bacteria, indicating that it is more efficient than 15% Ag-ZnO. Additionally, compared to gram positive bacteria, gram negative bacteria had a greater susceptibility to 10% and 15% Ag-ZnO.

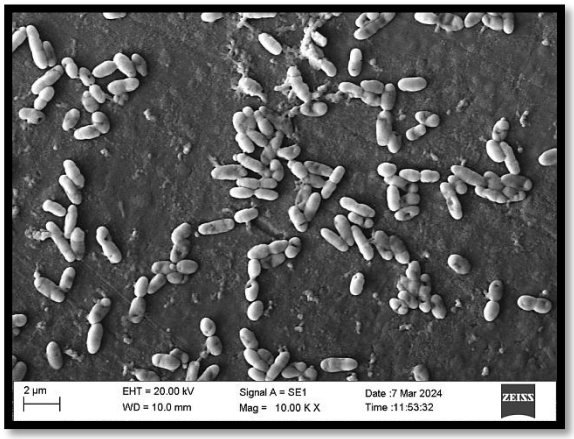
4.1.2 Minimum inhibitory concentration

Standard broth dilution method was carried out using 10 % Ag-ZnO nanocomposite against gram positive and gram negative bacteria by selecting a concentration range of 20 µg to 100 µg. The result are tabulated in the table 4.2 .

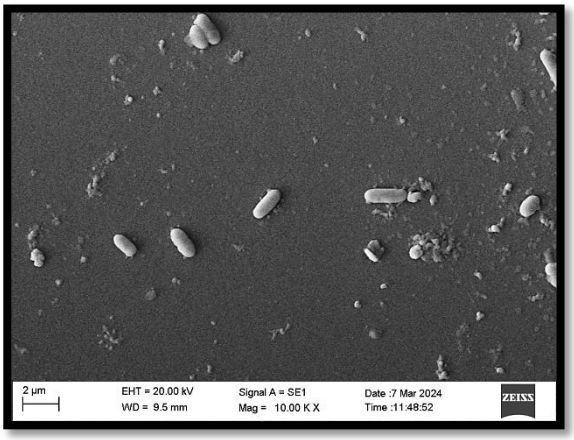
Table 4.2 MIC absorbance reading at 600nm

Concentration of Nanocomposite (µg)	Absorbance at 600 nm			
	Gram positive bacteria	Gram negative bacteria (*Ralstonia solanaceurum*)	Gram negative bacteria (*Xanthomonas oryzae*)	
20	1.084	1.038	-	0.534
40	1.064	0.789	-	0.519
50	0.880	0.677	-	0.408
60	0.782	0.536	0.701	-
70	0.702	0.492	0.654	0.151
80	0.682	0.470	0.647	0.021
90	0.592	0.375	0.593	-
100	0.492	0.322	0.470	0.293
DMSO Control	1.175	1.037	0.732	0.474
+VE control	1.200	1.3205	0.904	0.584

As the concentration of nanocomposites increased, the absorbance values obtained at O.D 600 nm for both gram positive and gram negative bacteria showed a decrease in absorbance, indicating a dose-dependent suppression of bacteria. Gram positive bacteria were found to have a minimum inhibitory concentration (MIC) at 50 µg with a absorbance of 0.880, where as *Ralstonia solanaceurum* had a MIC at 40 µg with a absorbance of 0.789 & *Xanthomonas oryzae* had MIC at 60 µg with a absorbance of 0.701 The findings indicate that 10% Ag-ZnO nanocomposites are more efficient against gram negative bacteria.



A



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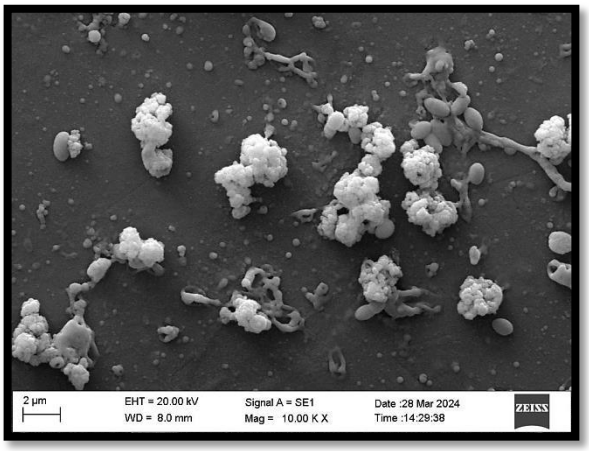

C

Figure 4.1 : Scanning Elevtron Microscopy Image of *Ralstonia solanaceurum* treated with Ag-ZnO (A) Control (B) & (C) Ag-ZnO treated .

Ag-ZnO nanocomposite treated *Ralstonia solanaceurum* cells show a decrease in population compared to the control as well as it shows change in the structure of the cells due to the antibacterial effect of silver and zinc ions that interact with cell membrane of bacteria .Furthermore, damage to the cell membrane is evident as a result of bactericidal effect of Ag-ZnO with heterogeneous response among cells, and some undamaged cells .The damage caused to these cells is irreparable and it is the reason for reduction in OD after overnight incubation.

4.1.3 Minimum bactericidal concentration (MBC)

The Minimum Bactericidal Concentration was used to assess the bactericidal action of Ag-ZnO by selecting a concentration range of 100 µg to 180 µg .The lowest concentration of nanoparticle that effectively prevents bacterial growth is known as MBC . The result are tabulated in the table 4.3

Table 4.3 MBC absorbance at 600nm for *Ralstonia solanaceurum*

Concentration of nanocomposite (µg)	Absorbance at 600nm
100	0.341
110	0.331
120	0.311
130	0.281
140	0.246
150	0.196
160	0.096
170	0.040
180	0.033
+VE control	1.498

As per the result, MBC was determined to be between the concentration 160 µg and 180 µg which can be visually observed in Figure 4.2. Spread plating was used to determine the viable count for the final three concentrations that showed a decrease in O.D. As the concentration increased there was a drastic reduction in the growth of *Ralstonia solanaceum* .At concentration of 160 µg colony count was found to be 780 cfu/ml and at concentration of 170 µg colony count was found to be 270 cfu/ml , indicating that 160 µg and 170 µg nanocomposite concentration are bacteriostatic rather than bactericidal. At 180 µg

concentration, no colonies were seen, indicating complete inhibition or bactericidal action Figure 4.3.

Figure 4.2 MBC tubes showing visible reduction in bacterial growth

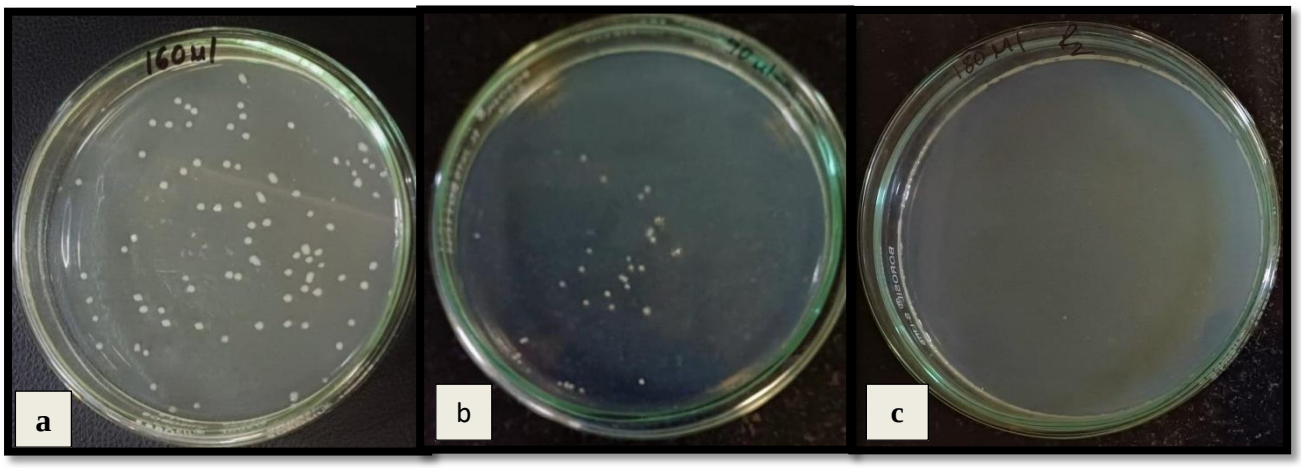

Figure 4.3:MBC spread plating (a)160 µg, (b)170 µg (c)180 µg

4.2 Anti-inflammatory activity of Ag-ZnO

Bovine serum albumin(BSA) was used to examine the anti-inflammatory effects in vitro. The standard of reference was diclofenac sodium. Percent inhibition of BSA at various Ag-ZnO concentrations of 10% and 15% is shown in table 4.4

Table 4.4 Percent inhibition of Ag-ZnO & Sodium diclofenac Standard

Concentration of Nanocomposite (µg)	Percent inhibition		
	Sodium Diclofenac	10% Ag-ZnO	15 % Ag-ZnO
100	78.34 %	77.36 %	41.33 %
200	82.2 %	80.31 %	46.06 %
300	84.25 %	86.01 %	65.74 %
400	90.1 %	89.36 %	79.33 %

Higher inhibition rates were consistently demonstrated by the 10% Ag-ZnO nanocomposite at all concentration (100µg, 200 µg, 300 µg, 400 µg) ,outperforming the 15 % Ag-ZnO nanocomposite. The 10 % Ag-ZnO nanocomposite displayed a greater anti-inflammatory effect than the standard sodium diclofenac at a concentration of 300 µg (86.01 %) which is more effective than the standard sodium diclofenac (65.74 %) seen in the Figure 4.4

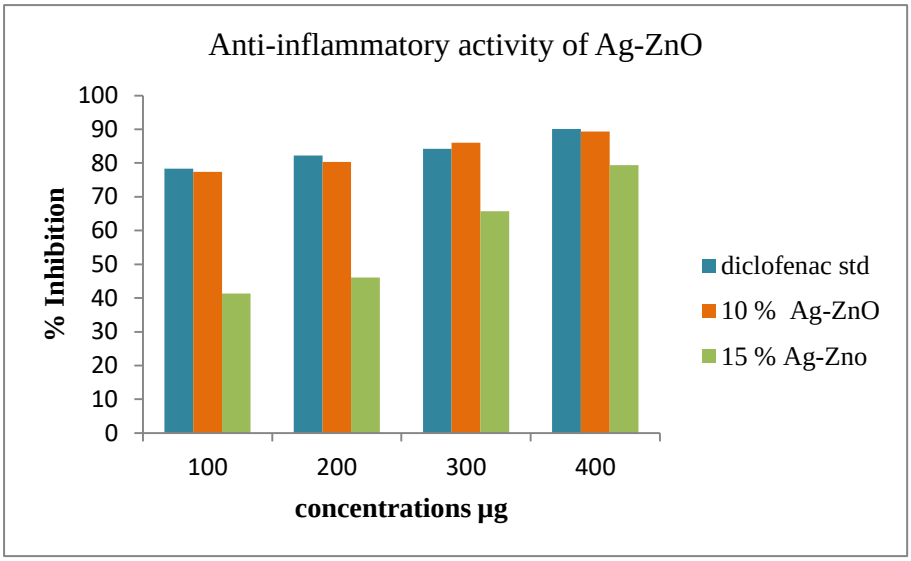

Figure: 4.4. Anti-inflammatory activity of 10 % & 15 % Ag-ZnO

From the obtained result ,it can be concluded that the 10 % Ag-ZnO is more effective than the standard sodium diclofenac at 300 µg concentration.

4.3 ANTIFUNGAL ACTIVITY OF Ag-ZnO NANOCOMPOSITE

4.3.1 Disc diffusion Assay

In this agar disc assay of green synthesized 10% & 15 % Ag-ZnO nanocomposite were evaluated against phyto-pathogen fungi, *Fusarium oxysporum.sp.solani*, *Fusarium oxysporum.sp.niveum, and Sclerotium_ sp*in comparison with their DMSO and culture control. The zone of inhibition is tabulated in table 4.5

Table 4.5 Zone of inhibition of fungal phytopathogen

Treatment	Zone of inhibition		
	Sclerotium_ sp	*Fusarium oxysporum.sp.niveum,*	*Fusarium oxysporum.sp.solani*
10% Ag-ZnO	36 mm	50 mm	39 mm
15% Ag-ZnO	33 mm	52 mm	45 mm
DMSO	54 mm	54 mm	50 mm
+VE Control	84 mm	67 mm	57 mm

The mycelial growth of, *Sclerotium_ sp, Fusarium oxysporum.sp.solani, Fusarium oxysporum.sp.niveum* with 200 µl of 10mg/ml of 10 % & 15% Ag-ZnO nanocomposite treatment is shown in Figure 4.5

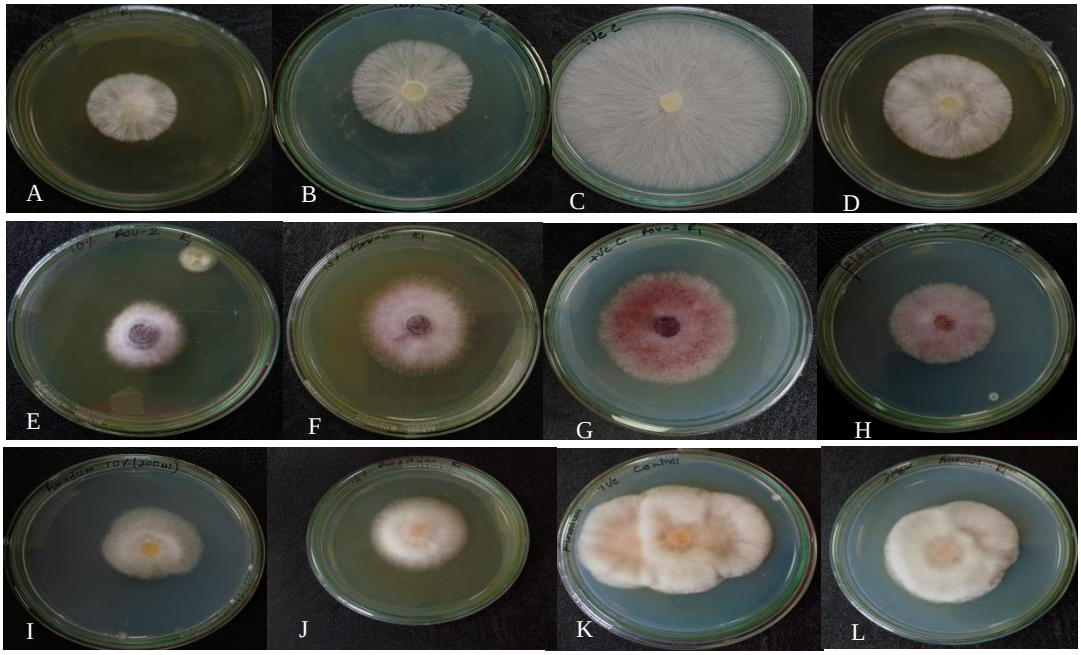

Figure 4.5: Mycelial growth of *Sclerotium_ spp* (A)treated with 10 % Ag-ZnO ,(B)treated with 15% Ag-ZnO , (C)Culture control, (D)DMSO control. Mycelial growth of *Fusarium oxysporum.sp.niveum*, (E)treated with 10 % Ag-ZnO ,(F) treated with 15% Ag-ZnO , (G) Culture control, (H) DMSO control & mycelial growth of *Fusarium oxysporum.sp.solani* (I)treated with 10 % Ag-ZnO ,(J)treated with 15% Ag-ZnO , (K) Culture control, (L)DMSO control.

The above Figure clearly show significant antifungal activity of 10 % & 15% Ag-ZnO nanocomposite against tested fungi strains .This is evident by the observed zone of inhibition around treated agar disc which are substantially larger than the control and DMSO control.

Sclerotium_ sp shows better result in terms of inhibition with 10% & 15% concentration of Ag-ZnO nanocomposite compared to *Fusarium oxysporum.sp.solani and Fusarium oxysporum.sp.niveum.* 10% Ag-ZnO nanocomposite shows great inhibition against *Fusarium*

oxysporum.sp.solani, Fusarium oxysporum.sp.niveum, and Sclerotium_ sp compared to 15% Ag-ZnO nanocomposite.

4.3.2 Broth based assay .

Antifungal activity of phytopathogen fungi *Fusarium oxysporum.sp.solani, Fusarium oxysporum.sp.niveum, and Sclerotium_ spp*. evaluated by inoculating it in PBD along with 200 µl of 10mg/ml 10% & 15% Ag-ZnO nanocomposite is depicted in the table .The wet weight obtained on incubation is tabulated in table 4.6. Growth reduction was observed in the fungal strains treated with Ag-ZnO nanocomposite .In case of *Sclerotium_ sp* treated with 10% Ag-ZnO showed 76% reduction and with 15% Ag-ZnO nanocomposite showed 38% reduction in wet weight. *Fusarium oxysporum.sp.niveum* showed 61% reduction with 10% Ag-ZnO nanocomposite and 37% reduction with 15% Ag-ZnO nanocomposite. subsequently *Fusarium oxysporum.sp.solani* showed 62% reduction with 10% Ag-ZnO and 46% reduction with 15%.Ag-ZnO nanocomposite.

Table 4.6 wet weight of fungal strains

	Wet weight (in gram)			
Fungal strains	Control	DMSO control	Treated with 10%	Treated with 15%
Sclerotium_ spp	4.682	3.892	1.110	2.875
Fusarium oxysporum.sp.niveum	5.875	5.211	2.254	3.654
Fusarium oxysporum.sp.solani	3.989	3.102	1.500	2.120

4.4 SEED GERMINATION AND PLANT GROWTH ACTIVITY OF Ag-ZnO NANOCOMPOSITES

4.4.1 Roll towel method

Efficacy of different concentration of Ag-ZnO nanocomposite treatment on seed germination of Brinjal were evaluated Figure 4.6 and The data is recorded in table 5.3.1

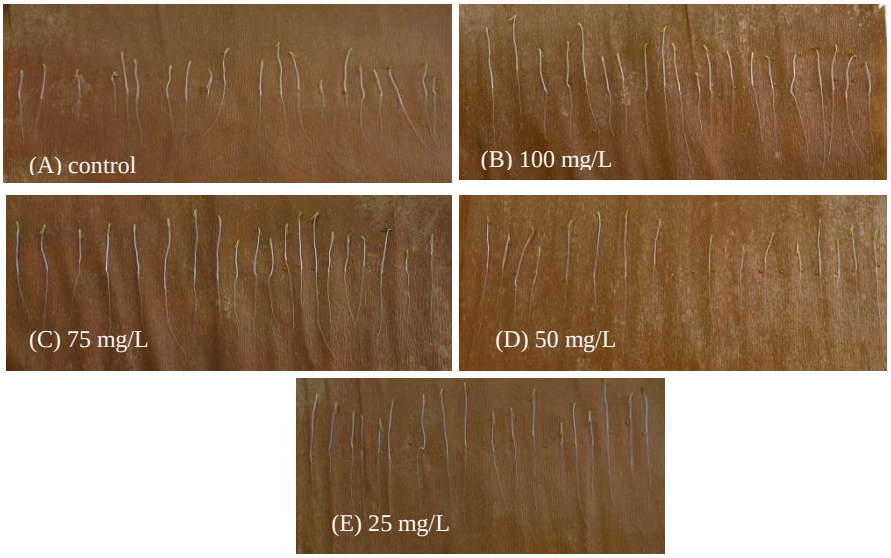

Figure 4.6 Brinjal seed germination activity of Ag –ZnO Of Set-up 2 (A) Control, (B) 100 mg/L treatment, (C) 75 mg/L treatment,(D) 50 mg/L,(E)25 mg/L treatment.

Table 4.7.Seed germination activity Set-up 1

Treatment	Seedling Height	Germination %	Seedling Vigour	Standard Deviation	Standard error
Control	8.17	95 %	7.76	2.022	0.4639
200 mg/L	7.18	85%	6.10	2.024	0.4920
150 mg/L	6.38	90 %	5.74	2.544	0.6101
100 mg/L	7.70	100 %	7.7	2.562	0.5729
50 mg/L	8.91	95 %	8.46	1.905	0.4370

Table 4.8 Seed germination activity Set-up 2

Treatment	Seedling Height	Germination %	Seedling Vigour	Standard Deviation	Standard error
Control	8.97	100 %	8.97	2.27	0.509
100 mg/L	9.95	100%	9.95	1.75	0.393
75 mg/L	10.36	100 %	10.36	1.94	0.434
50 mg/L	10.06	80 %	8.04	1.92	0.481
25 mg/L	9.272	90 %	8.34	1.74	0.410

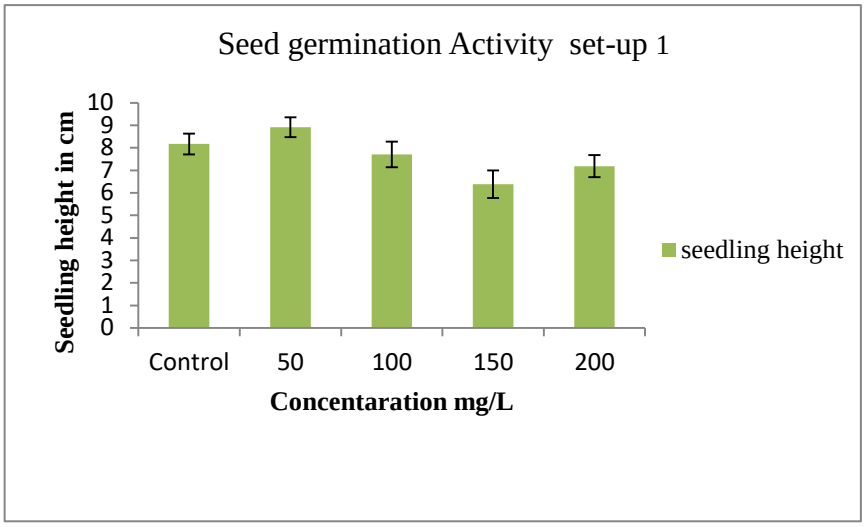

Figure 4.7 Seed germination activity Set up-1

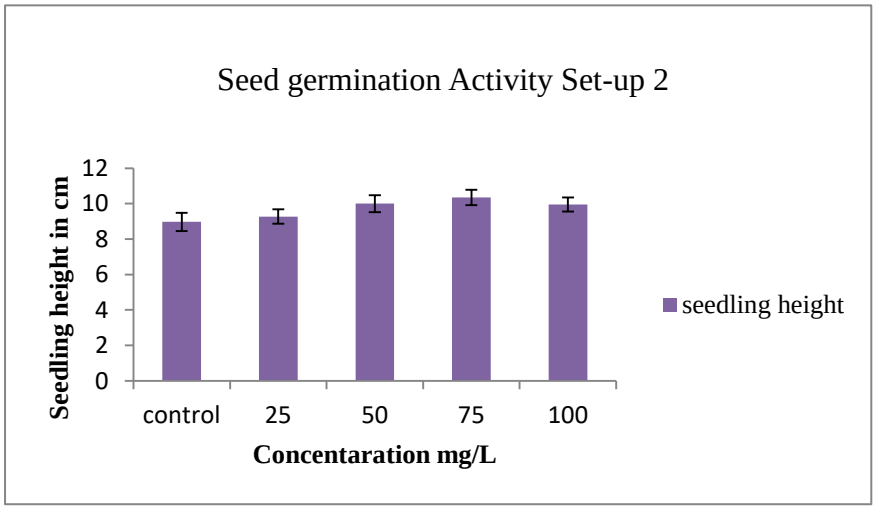

Figure 4.8 Seed germination activity Set up-2

As seen from the Figure 4.7 set 1 show enhanced seedling growth at a concentration of 50mg/L compared to the Control indicating a potential stimulating effect on the development of seedlings, . The seedling height decreased at 100mg/L, 150 mg/L, 200 mg/L suggesting a inhibitory effect on seed germination.

On the other hand Figure 4.8 set 2 show apparent trend of increase in seedling height with increase in Ag-ZnO concentration compared to control, indicating a potential stimulating effect on the development of seedlings. For both 75 mg/L and 100 mg/L, the germination percentage and seed vigour remained high.

4.5 Soil based seed germination

A Soil-based approach was used Figure 4.9 to see if different nanocomposite concentrations would produce different results from the roll paper towel method. The data is recorded in the table 4.8

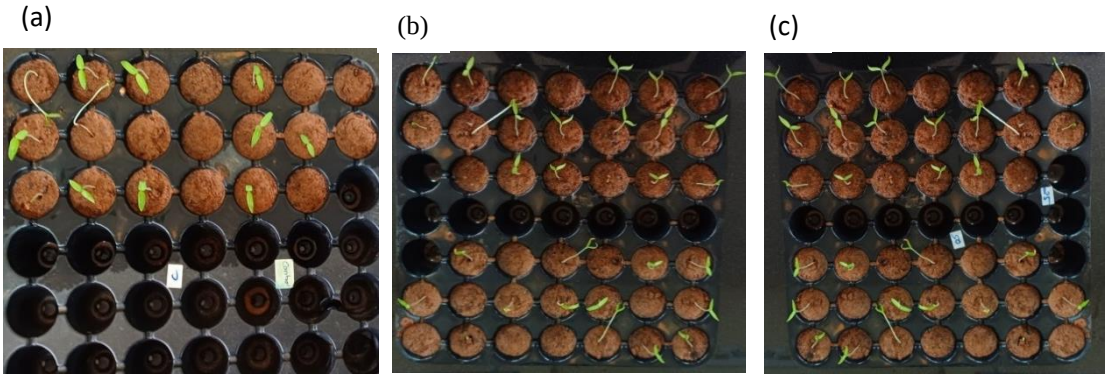

Figure 4.9 Soil based seed germination (a)Control (b)100 mg/L & 75 mg/L (c)50 mg/L & 25 mg/L

Table 4.9 Soil based seed germination activity of Ag-ZnO

Treatment	Seedling Height	Germination %	Seedling Vigor	Standard Deviation	Standard error
Control	5.675	95 %	5.391	1.849	0.533
100 mg/L	7.675	100 %	7.675	0.994	0.287
75 mg/L	6.966	100 %	6.966	1.225	0.353
50 mg/L	6.025	100 %	6.025	1.145	0.330
25 mg/L	5.636	100 %	5.636	2.147	0.647

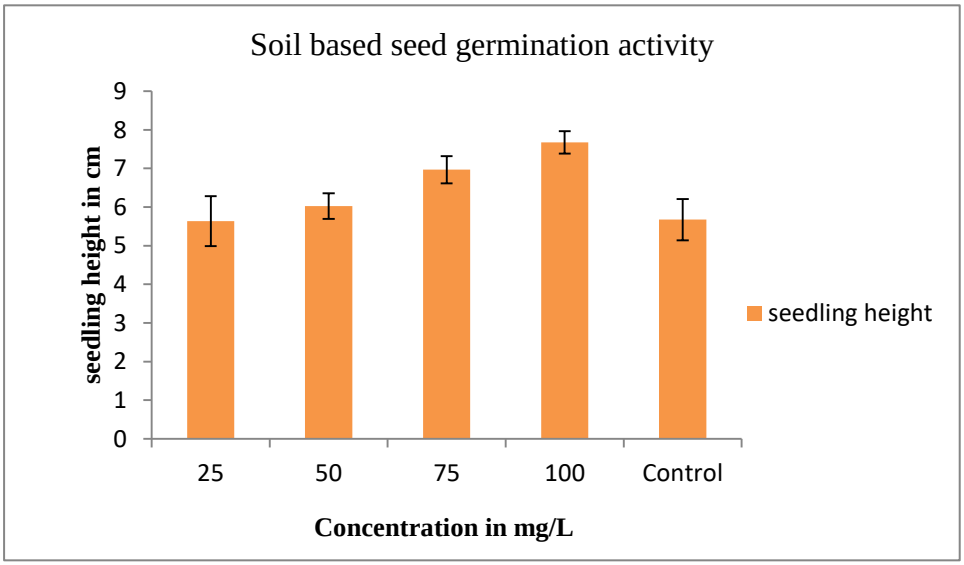

Figure. 4.10: Soil based Seed germination activity of Ag-ZnO

The germination percentage was high for all Ag-ZnO concentration compared to control . As the Ag-ZnO concentration increased there was increase in the seedling height .

4.6 ASSESSMENT OF Ag-ZnO NANOCOMPOSITE PLANT GROWTH AND ANTAGONISTIC ACTIVITY.

After 5 day of inoculation the control showed complete wilting Figure 4.10, it is evident that as the concentration of Ag-ZnO increased, there was delayed wilting in tomato plants. table 4.10

Figure.4.11: Wilting in Tomato plants (A) control, (B)75 mg/L Ag-ZnO ,(C)150 mg/L Ag-ZnO, (D)100 mg/L Ag-ZnO, (E)200 mg/L Ag-ZnO

Table.4.10 Biocontrol activity of Ag-ZnO nanocomposite against *Ralstonia solanaceum* on Tomato

Treatment		No of plants	Days of Observation						
			Day 5	Day 6	Day7	Day 8	Day 9	Day 10	Day 11
Control	R1	5	5	5	5	5	5	5	5
	R2	5	5	5	5	5	5	5	5
200 mg/L	R1	5	1	2	2	3	4	5	5
	R2	5	-	1	1	3	3	4	5
150 mg/L	R1	5	4	5	5	5	5	5	5
	R2	5	5	5	5	5	5	5	5
100 mg/L	R1	5	1	2	2	3	4	5	5
	R2	5	2	2	3	3	4	4	4
75 mg/L	R1	5	4	4	5	5	5	5	5
	R2	5	3	3	5	5	5	5	5

This study aimed to evaluate the antimicrobial, anti-inflammatory, seed germination and plant growth activities of 10% and 15% Ag-ZnO nanocomposites.

This line of work was more oriented towards evaluating the potential of Ag-ZnO nanocomposites against plant pathogens; hence, gram-positive bacterial cultures were used to check only the preliminary antibacterial activity of the nanocomposite.

The preliminary test suggested that 15% Ag-ZnO displayed less effective result against gram positive and gram negative bacteria, hence it was not used for further testing of antibacterial efficacy.

The antibacterial efficacy of the 10% Ag-ZnO nanocomposite was evaluated using MIC and MBC. This antibacterial activity can be attributed to the synergistic effect of Ag and ZnO NPs (Gunalan et al., 2012). Ag-ZnO nanocomposite increased the production of oxidative stress, thereby improving antibacterial activity (Liu et al., 2020).Analysis using liquid-based media allows direct interaction between the pathogen and the test molecules, and therefore has an advantage over the agar diffusion approach, wherein it is not affected by the diffusion property of the tested item or media. It also allows direct interactions between particles to penetrate the pathogen cell membrane.

The antibacterial efficacy of AgNPs against gram-negative bacteria is dose dependent, leading to the development of pits within the bacterial cell wall. The buildup of silver ions in the bacterial membrane makes it permeable, leading to cell death (Sondi & Salopek-Sondi, ,2004).In addition, reactive oxygen species such as superoxide and hydroxyl radicals destroy bacterial membranes by binding to proteins containing sulfur due to their electrostatic attraction, causing Ag and ZnO NPs to penetrate and disrupt bacterial cells. ZnO and Ag

hinder bacterial growth by damaging the cell membrane, leading to cytoplasmic disruption and eventual death of the cell (Divyapriya et al., 2014).

Even at low concentrations, Ag-ZnO-Nanocomposite has been shown to be a highly effective antibacterial agent against resistant microbial strains (Kim et al., 2007) because of the intracellular destruction of biological pathways in microbial cells, leading to long-lasting cell growth inhibition within a short period of time (Gunalan et al., 2012) Furthermore, it has been demonstrated that Ag and ZnO hybrid NPs exhibit much greater antibacterial activity in comparison to the individual properties of Ag & ZnO (Gupta et al., 2017).

The synergistic effect of Ag and ZnO on phytopathogenic fungal strains has not yet been studied extensivly .Our study clearly shows that the synergistic effect of the green-synthesized Ag-ZnO nanocomposite was effective against phytopathogenic fungal strains. Antifungal activity may be due to the electrostatic attraction between the nanoparticles and the cell membrane, leading to membrane disruption and cell death (Ouda.,2014).Zinc oxide nanoparticle exhibit size dependent antifungal activity by production of reactive oxygen species (Suyana et al., 2014) Zinc oxide is known to disrupt fungal hyphae and limit fungal spore. It shows dose-dependent fungicidal activity (He et al.,2011)

Ag-ZnO showed excellent anti-inflammatory activity comparable to the standard sodium diclofenac.10% AgZnO showed strong anti-inflammatory activity at a concentration of 300 µg with 86.01% inhibition percentage where standard sodium diclofenac showed 84.24%.Green synthesized zinc oxide are known to show evident suppression of albumin denaturation depicting the strong and effective anti-inflammatory activity of the zinc oxide. (Pati et al., 2016)

Ag-ZnO nanocomposite showed increased seed germination activity compared to the control in both the roll towel and soil based seed germination methods. As the concentration increased, the seed germination activity also increased. At 100 mg/L of Ag-ZnO, seedling height increased, but beyond 100 mg/L concentration, seedling height did not increase, indicating toxic effects of silver and zinc at high concentrations. In set-up 1 at concentration of 50 mg/L there is increase in seedling height where as in set-up 2 there is decrease in seedling height this can be due to manual error during the experiment. Silver and Zinc can potentially increase seed germination at low concentrations (Vinay et al. 2021).As a result, 50 mg/L and 75 mg/L can be administered to increase seed germination.

In this study, a highly susceptible variety of tomato was used, which displayed complete wilting in the seedling within five days after inoculation. Treatment with the Ag-ZnO nanocomposite resulted in mild resistance in tomato seedlings. The efficacy of the resistance improved with increasing concentration of the Ag-ZnO nanocomposite. To obtain significant improvement in the results, we need to use a moderately resistant variety of tomatoes.

Ag-ZnO nanocomposite with a ability to cause mild reduction in wilt has proven to be efficient against *Ralstonia solanaceum* as a result it can be administered to moderately resistant tomato variety to *Ralstonia solanaceum* .A glass-house based experiment must be conducted to obtain the same results.

Based on all the result obtained it can be concluded that Ag-ZnO nanocomposite have potential antimicrobial, anti-inflammatory, seed germination, plant growth and antagonistic activity. Overall, the findings emphasize the versatile potential of green-synthesized Ag-ZnO nanocomposites for agricultural and biomedical applications.

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APPENDIX I

MEDIA COMPOSITION

Nutrient agar media (100ml)	
Peptone	1g
NaCl	0.5g
Beef extract	0.3g
Agar	3g
Distilled water	100ml
pH	7.2

Nutrient broth (100 ml)	
Peptone	1g
NaCl	0.5g
Beef extract	0.3g
Distilled water	100ml
pH	7.2

Potato dextrose agar (100ml)	
Potatoes, infusion	20 g
Dextrose	2
Agar	1.5
Distilled water	100ml
pH	5.6

Potato dextrose broth (100 ml)	
Potatoes Dextrose broth granulated	2.4g
Distilled water	100ml
pH	5.1

BG Agar (100ml)	
Peptone	1g
Yeast extract	0.1g
Tryptone	0.1g
Glucose	0.5g
Agar	1.5g
Distilled water	100
pH	7.2

BG broth (100ml)	
Peptone	1g
Yeast extract	0.1g
Tryptone	0.1g
Glucose	0.5g
Distilled water	100ml
pH	7.2

APPENDIX II

Reagent preparation

A. Stock preparation 10 µg/µl

0.01 gm of Ag-ZnO nanocomposite in 1 ml DMSO solvent ,mixed uniformly

B. TBS (Tris Buffer Saline) Solution
0.87gm Nacl & 0.121 g Tris base ,in 100 ml distilled water,pH adjusted to 6.2 .

C. BSA 1 % solution
1gm of BSA in 100 ml TBS in volumetric flask.

D. Diclofenac Standard
0.05 gm sodium diclofenac in 5 ml DMSO solvent