DEVELOPMENT AND CHARACTERIZATION OF COPPER SULFIDE NANOPARTICLE CONJUGATED MANCOZEB FORMULATION

Dissertation submitted in fulfillments of the requirements for the Degree of

Master of Science in Biotechnology

By

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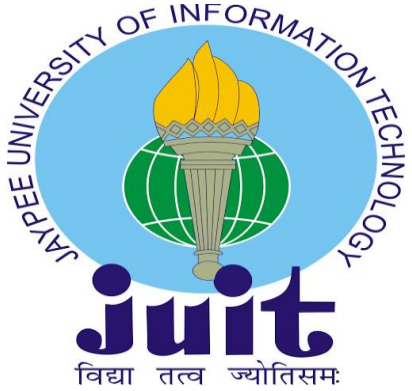

DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY
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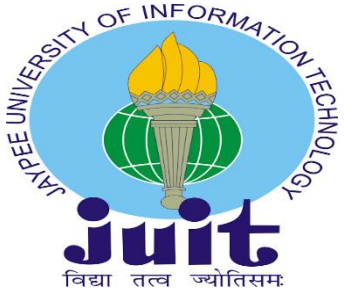

CERTIFICATE

This certificate that the project work, which has been designated "**DEVELOPMENT AND CHARACTERIZATION OF COPPER SULFIDE NANOPARTICLE CONJUGATED MANCOZEB FORMULATION**" by **Akanchha Kumari** during semester. Under my direction, the conditions required for the **Jaypee University of Information Technology**, Solan Master of Science degree in Biotechnology has been successfully completed. No other university or organization has received a partial submittal of this work as a substitute for a degree or other accreditation.

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DECLARATION

I hereby declare that the project presented in this report entitled " **DEVELOPMENT AND CHARACTERIZATION OF COPPER SULFIDE NANOPARTICLE CONJUGATED MANCOZEB FORMULATION** " in slightly fulfillment of the requirements for the award of the degree of **Master of Science in Biotechnology** submitted in the Department of Biotechnology & Bioinformatics, Jaypee University of Information Technology, Waknaghat (Solan) is an authentic record of my own work carried out over a period from August 2023 to May 2024 under the supervision of **Dr. ANIL KANT** Associate Professor, Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Solan, Himachal Pradesh.
The dissertation contents have not been offered for approval for any other degree or accreditation.

AKANCHHA KUMARI, 225111024

This is to certify that the above statement made by the candidate is true to the best of my knowledge.

Supervisor Name: **Dr. ANIL KANT**

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Date: 17.06.2024

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LIST OF ABBREVIATIONS AND ACRONYMS

CuS-NPs	Copper sulfide nanoparticles
XRD	X-ray diffraction
TEM	Transmission electron microscopy
FTIR	Fourier-transform infrared spectroscopy
CuNPs	Copper nanoparticle
Np	Nanoparticle
AgNPs	Silver nanoparticles
FeNPs	Iron nanoparticles
PVA	Polyvinyl alcohol
Fe3O3	Iron [III] oxide
ZnO	Zinc oxide
PBS	Phosphate buffered saline
PBST	Phosphate-buffered saline with Tween 20
AU	Arbitrary unit

Abstract

This research investigates the fabrication and evaluation of an innovative composition incorporating Mancozeb with copper sulfide nanoparticles (CuS-NPs). The principal aim was to optimize the antifungal effectiveness of mancozeb through the utilization of CuS-NPs' distinct characteristics. Utilizing methods including ultraviolet–visible spectrophotometry (UV–VIS) and Fourier-transform infrared spectroscopy (FTIR) will be employed to reveal insights into the nanostructure and chemical composition of the CuS NP-conjugated mancozeb mixture, the nanocomposite was produced and thoroughly described.

The antifungal activity of the CuS-NPs coupled with the mancozeb formulation was assessed using a collection of bioassays. In these bioassays, the well diffusion method was employed to measure the inhibition zones against specific fungal pathogens. The well diffusion method involves depositing the formulation in wells cut into agar plates inoculated with the test fungi. The formulation was expected to diffuse through the agar, inhibit fungal growth, and create clear zones of inhibition around the wells.

However, it was noted that the CuS-NPs conjugated mancozeb formulation did not adequately diffuse through the agar medium. This restricted diffusion led to the absence of clear inhibition zones, indicating no significant antifungal activity under these experimental conditions. This finding implies that the diffusion limitations of the nanoparticle conjugate formulation in the well diffusion assay must be addressed to effectively exhibit its potential antifungal properties. The nanocomposite bioassay appears as a trustworthy tool for determining its antifungal activity

Through integrating Mancozeb with CuS-NPs, agronomic practices might be significantly improved and environmental effects could be minimized. Mancozeb's stability, effectiveness, and precise delivery are all intended to be improved. This work advances the field of agronomy by putting forth a novel formulation that takes advantage of the complementary qualities of CuS and Mancozeb NPs, opening the door to increased crop yields with less negative effects on the environment.

CHAPTER 1

INTRODUCTION

Introduction

1.1 Introduction

In contemporary agronomy, the effective management of phytopathogens remains a significant obstacle, necessitating continual advancements in biocide concoctions. The advent of nanoscale technology has presented a promising avenue for addressing these issues through the creation of innovative nanosubstance-based solutions [1]. Among these, copper sulfide nanoparticles (CuS-NPs) have attracted substantial interest due to their distinctive properties and potential applications in crop cultivation. Mancozeb, a broad-spectrum fungicide, has been extensively used to counteract fungal infections in various crops. However, traditional mancozeb mixtures often encounter drawbacks such as diminished efficacy, ecological concerns, and the development of resistance in target organisms. Consequently, the conjugation of mancozeb with copper sulfide nanoparticles offers an intriguing strategy to surmount these limitations and enhance the performance of this essential agronomic tool. This research project concentrates on the creation and assessment of a copper sulfide nanoparticle-conjugated mancozeb mixture, aiming to leverage the synergistic effects of nanoscale science and agrochemicals for superior phytopathogen control in crops. The production of CuS-NPs and their conjugation with mancozeb will be rigorously examined, utilizing various methods to optimize the mixture's physicochemical attributes and stability. Synthesis of (CuS-NPs). Several crucial processes are involved in the chemical synthesis of (CuS-NPs). Chemical precipitation is the initial process used to create (CuS-NPs). [2,3]. These methodologies result in the production of nanoparticles suitable for integration into agricultural formulations. Moreover, thorough assessment studies will be undertaken to elucidate the morphology, structure, and surface characteristics of the developed mixture [4]. Sophisticated analytical techniques such as, ultraviolet–visible spectrophotometry (UV–VIS) and Fourier-transform infrared spectroscopy (FTIR) will be employed to reveal insights into the nanostructure and chemical composition of the CuS NP-conjugated mancozeb mixture [5,6]. Through a combination of empirical investigations and analytical evaluations, this project aspires to contribute to the expanding body of knowledge in nanoscale-enabled agronomic innovations. By elucidating the mechanisms underlying the augmented performance of CuS NP-conjugated mancozeb, this research endeavor aims to furnish valuable insights for the development of next-generation biocide mixtures with enhanced efficacy, sustainability, and ecological compatibility.

The sector of agronomy has been confronting a multitude of obstacles, including erratic climate variations, soil contamination by various detrimental environmental pollutants such as fertilizers and pesticides, and significantly escalating food demands driven by a burgeoning global populace. Recent years have witnessed considerable advancements in the formulation of nano-pesticides and nano-fungicides for agronomic applications. A principal benefit of these nano-pesticides and nano-fungicides is their capacity to infiltrate plant tissues, leading to enhanced absorption and efficacy. The utilization of nanomaterials in agronomy aims to curtail nutrient losses to augment yields, diminish the quantity of products required for plant protection, and reduce production costs to optimize output [7].

Crop afflictions present a substantial obstacle in contemporary agronomy, underscoring the necessity for more efficacious strategies in pathogen management. Conventional pesticide formulations, such as mancozeb, are linked with limitations including diminished potency, environmental hazards, and the evolution of resistance in target organisms. There is an urgent need for continuous advancements in pesticide development to rectify the deficiencies of traditional methods and enhance pathogen control in crops. The advent of nanotechnology provides promising opportunities for the creation of innovative pesticide formulations with superior potency, stability, and environmental sustainability. CuS-NPs have garnered attention due to their unique characteristics and potential uses in agronomy, indicating they may address current obstacles in pathogen control. The study will involve meticulous examination of the production and integration of CuS-NPs with mancozeb, utilizing advanced analytical methodologies to refine the formulation's physicochemical attributes and durability. This research aspires to make a substantial contribution to the realm of nanotechnology-driven agricultural advancements by elucidating the mechanisms behind the improved performance of CuS NP-conjugated mancozeb. This will pave the way for the development of next-generation pesticide formulations with enhanced efficacy, sustainability, and environmental responsibility [8,9].

1.2 Objectives

- Synthesis of copper sulfide nanoparticle and their characterization
- Development of methods to conjugate mancozeb fungicide with CuS particles and characterization of conjugates
- Testing fungicidal effect of synthesized fungicide-nanoparticle conjugates on selected fungi

CHAPTER 2

REVIEW OF LITERATURE

Review of literature

2.1 Review of literature

Advanced material science has emerged as a transformative field with extensive applications in agronomy. Central to this field is the manipulation of materials at the nanoscale, typically ranging from 1 to 100 nanometers [1,2]. At this level, materials exhibit unique physical, chemical, and biological properties distinct from their macroscopic counterparts. In agronomy, advanced material science offers innovative solutions to longstanding challenges in crop cultivation, pest control, soil enhancement, and food preservation. Nanoscale materials hold substantial potential for augmenting crop protection against pests, diseases, and environmental stressors. Nanoscale pesticide formulations, or nano-pesticides, provide several benefits over traditional formulations, including heightened efficacy, reduced environmental impact, and controlled release kinetics. Various types of nanoscale materials, including metal nanoparticles (e.g., silver, copper), metal oxide nanoparticles (e.g., zinc oxide, sulfate, titanium dioxide), and carbon-based nanoparticles (e.g., graphene, carbon nanotubes), have been investigated for their pest control properties [10]. Advanced material science enables precise control over nutrient delivery and soil management practices, thus optimizing plant nutrition and improving soil health. Nanoscale fertilizers and soil amendments exhibit enhanced nutrient uptake efficiency, extended nutrient release, and reduced nutrient leaching, leading to increased crop productivity and resource utilization. Additionally, nanoscale materials such as nanoclays and nanosensors provide novel approaches for soil enhancement, contaminant detection, and precision agronomy applications. Nanoscale delivery systems offer a platform for targeted delivery and controlled release of plant growth regulators (PGRs), hormones, and bio stimulants. Nanoencapsulation, nanosuspensions, and nanogels facilitate efficient encapsulation of active ingredients, protecting them from degradation and enhancing their uptake by plants [11]. Nanoscale-based PGR formulations provide precise control over plant growth processes, including germination, root development, flowering, and fruit ripening, leading to improved crop yield and quality.

2.2 Nanotechnology

The focus of this investigation lies in the meticulous exploration of nanotechnology applications within agricultural practices shown in (Fig 2.1), particularly concerning the synthesis

and characterization of a composite formulation involving Mancozeb and copper sulfide nanoparticles (CuS-NPs). Synthesis of CuS Nanoparticles: Various innovative methodologies, aqueous based methods have been systematically studied for the fabrication of CuS-NPs. These nanoscale materials serve as potential carriers or adjuncts for Mancozeb, displaying promising characteristics for integration into agricultural formulations.

- **Characterization Techniques:** Rigorous examinations employing diverse analytical tools, such as ultraviolet–visible spectrophotometry (UV–VIS) and Fourier-transform infrared spectroscopy (FTIR) investigations into the intermolecular interactions between CuS-NPs and fungicide, have been meticulously conducted. These analyses provide crucial insights into the physicochemical properties, structure, and behaviors of the synthesized nanoparticles, fundamental for formulating stable and efficacious composites.
- **Agricultural Implications:** The amalgamation of CuS-NPs with Mancozeb proposes innovative pathways for enhancing the stability, targeted delivery, and potency of agricultural formulations. This research holds the potential to revolutionize agricultural practices by offering improved delivery systems and potentially reducing the ecological footprint associated with traditional pesticide applications.

This scholarly pursuit aims to contribute substantively to the agricultural industry by harnessing the potential of nanotechnology in creating efficient and sustainable solutions for crop protection and yield enhancement. [8,12]

The quest for novel formulations through the fusion of Mancozeb with CuS-NPs denotes an innovative stride toward revolutionizing agricultural technology.

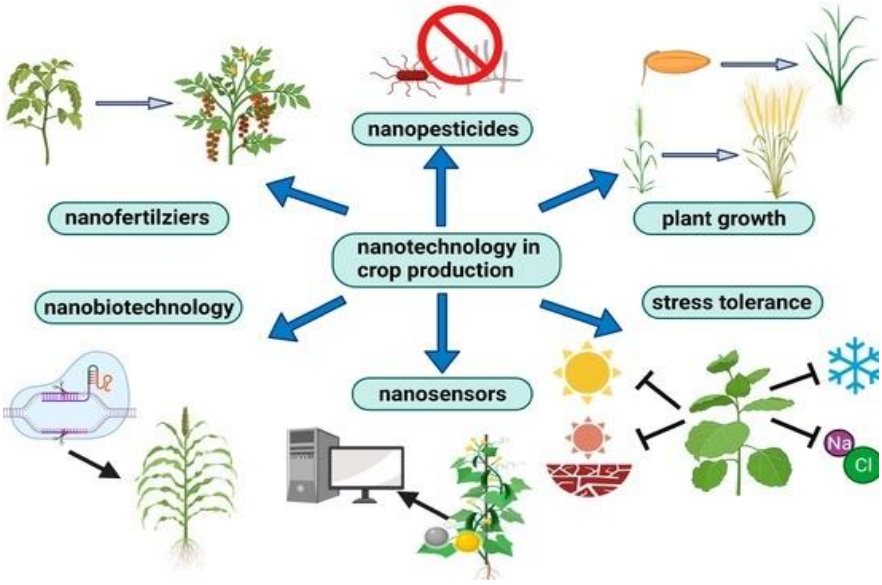


Fig 2.1: Advancement of nanotechnology in crop production [72]

2.3 Nano-pesticide

This investigation focuses on the synthesis and characterization of a nanoparticle-based pesticide formulation involving the amalgamation of Mancozeb, a widely used fungicide, with (CuS-NPs). The research delves into diverse methodologies including innovative approaches like chemical precipitation (Polyol Process), Sol-Gel method, Microemulsion method, green Synthesis using in-situ Conjugation employed for synthesizing CuS-NPs conjugate mancozeb. Characterization techniques, such as ultraviolet–visible spectrophotometry (UV–VIS) and Fourier-transform infrared spectroscopy (FTIR), optical and structural analyses, as well as in-depth studies concerning the interactions between CuS-NPs. The literature presents various applications of copper nanoparticles, demonstrating their potential in sensor development and antimicrobial activities across diverse domains [13]. Combining Mancozeb with CuS-NPs seeks to enhance the stability, efficacy, and targeted delivery of Mancozeb, envisaging significant advancements in agricultural practices while potentially mitigating environmental impacts. This research endeavors to introduce a novel nanoparticle-based pesticide formulation that harnesses the collective advantages of Mancozeb and CuS-NPs. This innovative approach holds promise for improving agricultural productivity while minimizing adverse environmental repercussions [14].

2.4 NPs interaction with flora

The interaction dynamics between (CuS NP)-conjugated Mancozeb formulations and plants constitute a pivotal area for agricultural research.

- **Uptake Mechanisms:** Investigations on CuS NP uptake by plants involve exploring root absorption pathways, translocation processes, and cellular internalization mechanisms.
- **Physiological Responses:** The study evaluates plant responses upon exposure to CuS NP-conjugated Mancozeb, observing alterations in metabolic pathways, stress responses, growth parameters, and potential effects on plant health and productivity.
- **Distribution and Wash-off:** Research focuses on the distribution patterns and susceptibility to wash-off of CuS NP-treated plants, elucidating the impact of formulations on plant surfaces [15].
- **Antifungal Activity:** Assessments of the antifungal properties of copper oxide nanoparticles synthesized through green methods, demonstrating their effectiveness against plant pathogens, offer insights into their application potential.

Understanding the nuanced interactions between CuS NP-conjugated Mancozeb and plants is crucial for optimizing agricultural practices, ensuring minimal environmental impact, and maximizing crop yield.

2.4.1 Size-Dependent NP Uptake

Size Variability and Uptake Mechanisms: Investigating the size-related impact on CuS NP uptake by plants delves into the complex interplay between nanoparticle size variations and the diverse mechanisms governing their absorption, translocation, and cellular internalization within plant systems.

- **Transport and Translocation Pathways:** Scrutinizing how the diverse sizes of CuS-NPs influence their transport through root uptake pathways and translocation mechanisms across plant tissues aids in comprehending the intricate dynamics of nanoparticle movement shown in (Fig 2.2) and accumulation within various plant compartments [16].
- **Physiological Implications:** Examining the physiological implications arising from size-dependent CuS NP interactions encompasses probing alterations in plant metabolism, stress responses, and potential ramifications on growth parameters, shedding light on the nuanced impact of nanoparticle size on plant health and development shown in (Table 2.1).

- **Environmental Relevance:** Grasping the significance of size-related interactions between CuS Ns and plants extends to understanding their fate, bioavailability, and potential ecological implications, contributing to the broader discourse on nanoparticle-related environmental safety [17].

Table 2.1: Size reliant on translocation

	0.1 nm	1nm	10 nm	100 nm	1000 nm
Cell wall		✔	✔		
Extracellular space		✔	✔		
Cell membrane			✔	✔	
Plasmodesmata		✔	✔		
Cuticles	✔				
Pit membranes			✔	✔	✔
Vasculature				✔	✔

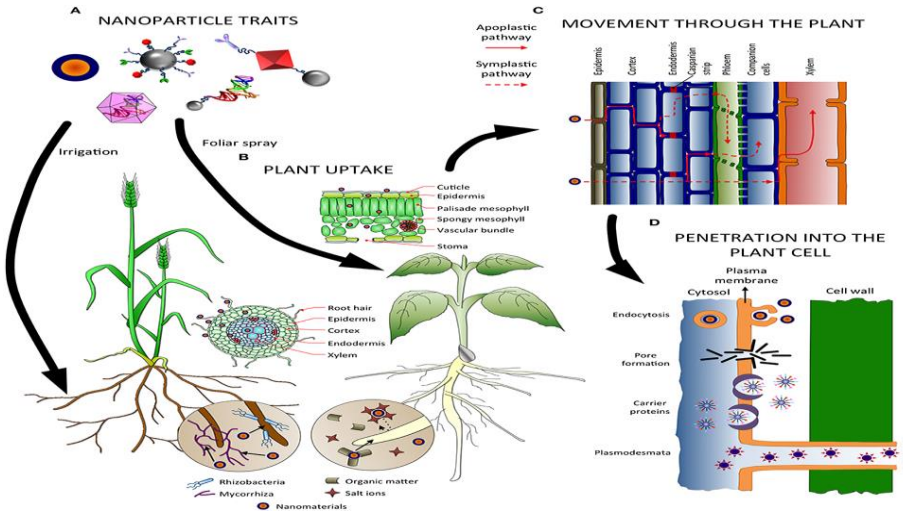


Fig 2.2: Variables affecting a plant's ability to absorb, absorb, transport, and penetrate nanoparticles [73]

2.4.2 Charge-Dependent Surface Absorption of NPs

Surface Charge Influence: Studies indicate that nanoparticles possessing diverse surface charges exhibit distinct plant uptake patterns. Negatively charged nanoparticles, owing to electrostatic interactions, might demonstrate higher affinity towards plant root systems. Conversely, positively charged nanoparticles might evoke altered uptake mechanisms or binding affinities within plant tissues, necessitating comprehensive investigations.

- **Uptake Dynamics:** Understanding the intricacies of surface charge-dependent uptake dynamics involves probing the routes and mechanisms governing nanoparticle entry into plant systems. Studies exploring root uptake pathways, translocation mechanisms, and cellular internalization processes shed light on these phenomena, essential for unraveling the nuances of plant-nanoparticle interactions (Fig 2.3).
- **Physiological Implications:** Assessing the physiological repercussions of negatively and positively charged CuS NP-conjugated Mancozeb formulations on plant growth, metabolism, and stress responses provides critical insights into their potential effects on plant health and productivity [18].

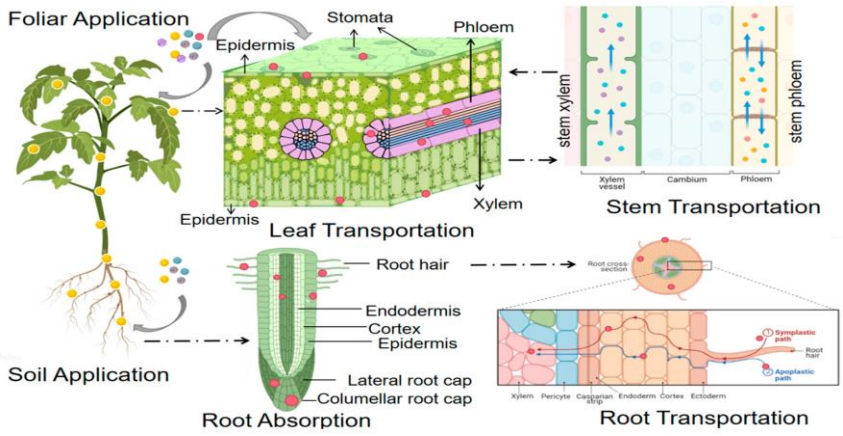


Fig 2.3: External Charge-Dependent Uptake of NPs: Because of their electrochemical interactions, NPs with varied surface charges have varying rates of uptake and translocation within the plant system [74]

2.5 Response of plants' physiochemistry to conjugated mancozeb formulation including copper sulfide nanoparticles

Understanding the physicochemical interplay between plants and the composite formulation of (CuS-NPs) conjugated with Mancozeb requires an in-depth exploration with an academic perspective.

- **Uptake Dynamics:** Investigating the mechanisms underpinning the uptake and transport of CuS NP-infused Mancozeb within plant systems is pivotal. This involves deciphering root uptake pathways, translocation mechanisms, and intracellular transportation processes.
- **Metabolic and Molecular Effects:** Delving into the alterations in plant metabolic pathways, gene expression, and cellular responses upon exposure to CuS NP-conjugated Mancozeb elucidates the physiological impact on plants.
- **Toxicological Implications:** Rigorous assessments of the potential toxicological effects encompassing cellular toxicity, alterations in enzymatic activity, and impact on growth parameters shed light on the safety and potential risks associated with the formulation.[19]
- **Environmental Impacts:** Evaluating the fate and persistence of CuS NP residues in soil, potential leaching, and broader ecological implications are essential to comprehend the environmental footprint and sustainability of this formulation in agricultural settings.

2.5.1 NPs-mediated abiotic stress response

The integration of (CuS-NPs) into Mancozeb formulations has garnered attention for potential roles in abiotic stress management within agricultural ecosystems. While the precise mechanisms are diverse and complex, research has indicated promising indications of NPs' capability in abiotic stress alleviation shown in (Table 2.2)

- **Synthesis and Characterization:** Studies focusing on the synthesis and characterization of copper sulfide nanoparticles elucidate their structural and physicochemical attributes, laying the groundwork for their application in stress mitigation.

- **Biological Impacts:** Investigation into the electrochemical properties and bandgap energies of copper sulfide nanoparticles suggests their potential in conferring superior stress-alleviating attributes, although further studies on their specific biological interactions are necessary.
- **Green Synthesis:** The realm of green synthesis for copper nanoparticles highlights sustainable approaches, a crucial aspect in ensuring environmentally friendly formulations for stress management.[20]

Table 2.2: Abiotic stress response in plants is mitigated by the types of NPs, their application, concentration, and relevance.

S.No	Nanoparticle	Size(nm)	Plants	Application	Response	Stress types	References
1	Copper oxide nanoparticle and Chitosan-PVA	95 nm	Tomato	Hydroponics	Increased levels of lycopene, carotenoids, SOD, vitamin C, and chlorophyll a and b	Saline	[21]
2	Copper oxide nanoparticle	30–40 nm	Maize	Plants priming	Higher amounts of anthocyanin, chlorophyll, and carotenoid contents, total seed count, grain yield, and plant biomass and leaf water content	Drought	[22]
3	Silver nanoparticles	15–30 nm	Bread wheat	Pre-sowing seed preparation	Wheat germination, growth, and reduced ABA as well as the induction of IBA, NAA, and BAP contents	Salinity	[23]
4	Iron (III) oxide	20–40 nm	Moldavian balm	Foliar spray	Elevated leaf area, phenolic, flavonoid, and anthocyanin content, along with improved enzyme activitie of catalase, guaiacol peroxidase, ascorbate peroxidase, and glutathione reductase	Salinity	[24]
5	Iron nanoparticles	Size(nm)	Bread wheat	Potting mix	Higher photosynthesis, increased growth and physiology, higher concentrations of Fe, and lower levels of cadmium	Cadmium and drought	[25]

2.6 Analysis of the impact of mancozeb formulations infused with nanoparticle in agriculture

A significant improvement is the inclusion of (CuS-NPs) to Mancozeb connects suited for agronomic purposes. These nanoscale formulations display considerable promise in transforming contemporary agronomic practices [26]. Embedding CuS-NPs into Mancozeb mixtures enhances the overall effectiveness of pest control agents. The nanoscale particles offer increased surface area, leading to improved adhesion and uptake on crop surfaces, which can result in superior pest and disease management. Precise delivery of nanoscale pesticide mixtures reduces overall pesticide usage. Enhanced targeting capabilities necessitate less pesticide per application, thereby minimizing environmental pollution and reducing harmful effects on non-target organisms and ecosystems. Nanoscale pesticide formulations often exhibit heightened stability and longevity on crops due to their advanced chemical properties [27]. This can extend the efficacy of pesticide treatments, reducing the frequency of applications and associated costs. Despite these advantages, the introduction of nano-pesticides necessitates thorough risk assessments. Key variables to take into consideration are assessments of the materials' lasting impact on ecosystems, environmental destiny, and human toxicity. Using cutting-edge materials in pesticide formulations, such CuS NP-enhanced Mancozeb compositions has tremendous potential to address contemporary problems while promoting sustainable farming methods.[28]

2.7 Pesticides based on nanoparticle interaction with plants:

The interaction dynamics between nanoparticle-formulated pesticides and plants encompass multifaceted aspects deserving scholarly exploration. Nano-pesticides, exemplified by formulations like (CuS-NPs) conjugated with Mancozeb, prompt comprehensive studies to elucidate their implications within plant systems.

Uptake Mechanisms: Investigations into the mechanisms underpinning nanoparticle absorption by plants, including root uptake pathways, translocation mechanisms, and cellular internalization processes, are paramount for understanding their uptake dynamics.

Physiological Effects: Studying the physiological responses of plants exposed to nano-pesticides entails observing alterations in metabolic pathways, stress responses, growth parameters, and potential implications for plant health and yield [29].

Biological Fate and Toxicity: Rigorous assessments of the fate and toxicity profiles of nanoparticle residues within plant tissues, encompassing persistence, biotransformation, and potential ecological impacts, are crucial for ensuring environmental safety.

Efficacy and Targeting: Evaluating the efficacy of nano-pesticides in mitigating crop diseases while minimizing non-target effects on beneficial organisms demands systematic investigations. Understanding these intricate interactions between nano-pesticides and plants fosters a more comprehensive grasp of their agricultural applicability, environmental implications, and potential optimization in sustainable farming practices [30]

2.8 Bioassays and bio-efficacy of nano fungicide

2.8.1: In vitro assays

Before applying nano-fungicides to a variety of crops, their efficacy can be tested against a specific pest. Copper sulfide (CuS) and other various nanomaterials, together with bioactive pesticidal chemicals, can be used to develop these nano-fungicides. Using the agar diffusion well technique, the lowest inhibitory concentration can be used to test the toxicity of nano-fungicides. This technique makes it possible to precisely measure the number of pests that are dead or surviving. In order to assess the biological tests and bio-efficacy for nano-fungicides, laboratory tests are an essential starting point. They offer a controlled setting in which to ascertain the activity of the compounds against fungus. These assays reveal significant data on the inhibitory effects and modes of action of nano-fungicides by utilizing a range of approaches to assess their antifungal characteristics. Among the main methods is a disk diffusion assay. This technique involves cultivating fungal pathogens on plates of agar to produce a homogenous growth layer. The agar surface is covered with paper disks that have been treated with nano-fungicides in varying amounts [31]. Zones of resistance around the disks are looked for on the plates after incubation. Greater reduction in fungal growth is shown by larger zones, and the measurement of these zones represents the efficiency of the nano-fungicide. In addition to the disk diffusion assays, dilution of broth assays is widely used to assess the curative effects of nano-fungicides quantitatively. In this case, the nanomaterial is supplied in different concentrations while fungal spores are being grown in a liquid media. Spectrophotometric or visual methods are used to track the growth of fungi throughout time. A study's effectiveness and dose-response relationship can be evaluated by computing the level at which the nano-fungicide suppresses fungal growth by a given percentage

(IC50, for example). With the use of this technique, one can assess fungicidal activity precisely and compare various nano-formulations and concentrations. A high-throughput method for assessing the impact of nano-fungicides against several fungal strains at once is through microplate clinical trials. Growth medium-containing multi-well plates are infected with fungal spores. The nanomaterial is added in varying amounts to each well. Following incubation, the development of fungi is evaluated visually or by utilizing a microplate reader to measure optical density. With the help of this technique, numerous nano compositions and concentrations may be quickly screened, helping to identify potential candidates for additional testing.

Time-kill kinetics investigations shed light on the dynamic interactions that occur over time between fungal pathogens and nano-fungicides. Candida spores are exposed to a set concentration of the nanomaterial in this experiment, and samples are obtained for colony counting at regular intervals. Researchers can better understand the mechanism of action of the nanomaterial by characterizing the kinetics of fungicide action and tracking the pace and degree of fungal growth inhibition. Time-kill kinetics tests provide valuable information regarding he length of exposure required to achieve efficient fungus control and can aid by enhancing the nano-fungicide leadership procedures. All things thought of, laboratory assays are an essential stage in the assessment of nano-fungicides, offering insightful information about their antifungal characteristics and directing further study in greenhouse and field habitats [32,33].

2.8.2: Field experiments or greenhouse trials

Field testing and greenhouse testing are essential stages of testing the biological experiments and bio-efficacy of nano-fungicides, offering vital information about how well they function in real-world conditions. In order to assess how well nano fungicides work against fungal infections, these studies have to be precisely established and carried out, taking consideration of crop safety, application techniques, and their impact on the environment. As an intermediate phase between laboratory experiments and field trials, greenhouse trials provide controlled circumstances that mimic natural surroundings and enable more accurate variable monitoring and modification. Typically, in these studies, plants are grown in greenhouse facilities in pots or trays, with the humidity, temperature, and light intensity specifically monitored to replicate field conditions. To determine if nano fungicides are effective in avoiding or suppressing fungal infections, they are given to the plants using an assortment of methods, including foliar sprays and

seed treatments. Through visual inspections, assessments of the severity of the disease, and/or molecular investigations to quantify fungal biomass, the course of the disease is tracked over time [34,35]. Researchers can find possibilities by using greenhouse research to acquire important data on the effectiveness of nano fungicides to under tightly monitored circumstances formulations along with methods of application to be explored further in field tests. Before being traded in, field trials are the last step in assessing the bio-efficacy for nano fungicides and deliver useful knowledge for their effectiveness as they behave in actual agricultural environments. These tests are carried out in actual environments, where the efficacy of the nanomaterial may be altered by environmental elements like the type of soil, climate, and insect pressure. In field experiments, nano fungicides are usually applied to larger land areas, either as a treatment on their own or in contrast to standard fungicides or controls that have not been treated [36]. Complete block designs that are randomized are frequently used to reduce inevitability and provide robust statistical investigation of the data. Data on variables including incidence, disease severity, and agricultural yield are gathered during the growing season to evaluate. The severity and course of fungal diseases, and also the quantity and health of crops. Field trials not only assess the effectiveness of nano fungicides but also shed light on the potential impacts on ecosystem dynamics, non-target organisms, and soil health.

When evaluating the bio-efficacy for nano fungicides, field trials and greenhouse trials complement each other by enabling controlled environments for initial assessment and real-world confirmation of the products' effectiveness. Researchers can obtain a thorough grasp of the efficacy, safety, and viability of nano fungicides that as permanent options for the control of fungal diseases in agriculture by combining data from both kinds of experiments. These experiments are important phases in the creation and exploitation of nano fungicides, which are eventually environmentally friendly technologies [37,38].

2.9 Current Studies on Fungicidal Properties of Nanoparticles

The literature pertaining to the development and characterization of a formulation combining (CuS-NPs) with Mancozeb encapsulates a range of scholarly investigations elucidating various facets of nanoparticle-based agricultural technologies.

Synthesis Approaches: Scholarly works have delineated diverse methodologies for CuS-NPs synthesis, such as microwave-induced heating, biomolecule-assisted routes, and polyol methods [39,40]. These techniques offer insights into controlled synthesis and optimal properties.

CHAPTER 3
MATERIALS AND METHODS

MATERIALS

3.1.1 Media and other Chemicals

Media and chemicals used in experiments are Copper Sulfate (Merk), Ammonium Sulfate (Iso chem), Ammonia Solution (SRL), Thiourea (Fisher Scientific), 2-mercaptoethanol (SRL), Mancozeb (Sigma-Aldrich), Potato Dextrose Agar (Himedia), Sodium Chloride (SRL), Potassium Chloride (Pure Chems), Sodium Phosphate Dibasic (SRL), Potassium Phosphate Monobasic (Loba Chemie).

3.1.2 Instruments used

Instruments that are used in experiments are Centrifuge Machine (Eppendorf), 4°C storage (Blue-Star), Incubator Shaker (Labnet), UV-VIS Spectrophotometer (Thermo Scientific), Weighing Balance (Citizon), Magnetic Stirrer Hot Plate (Texcare), Laminar Air Flow Cabinet, pH meter (Eutech), Fourier Transform Infrared Spectrophotometer (FTIR) (Agilent cary630).

3.1.3 Fungal strains

The fungal strain *Rhizopus oryzae* and *Aspergillus flavus* (BT01) was procured from Genomics lab of JUIT Solan.

3.2 Methodology

3.2.1 Preparation of Copper sulphate nanoparticles (CuS-NPs)

Copper Sulfate (metal precursor) and Ammonium Sulfate (Complexing agent) were added in distilled water and mixed well using hot-plate magnetic stirrer at 50°C (Fig. 3.1). The pH of solution was adjusted at 8.2 using ammonia solution and temperature of the solution was maintain at 50°C. Thiourea (sulfur source) and 2-mercaptoethanol (at a dosage of 5%) were used as capping agents to enhance the stability of the nanoparticles. The solution was kept on magnetic stirrer for 3 h for constant stirring. After stirring, the solution was centrifuged at 12000 rpm for 30 min to collect the nanoparticles. Pellets of nanoparticles was dried for 12 h at 50°C in an oven. The confirmation of synthesis of CuS-NPs were done by performing the UV-VIS and FTIR of the samples.

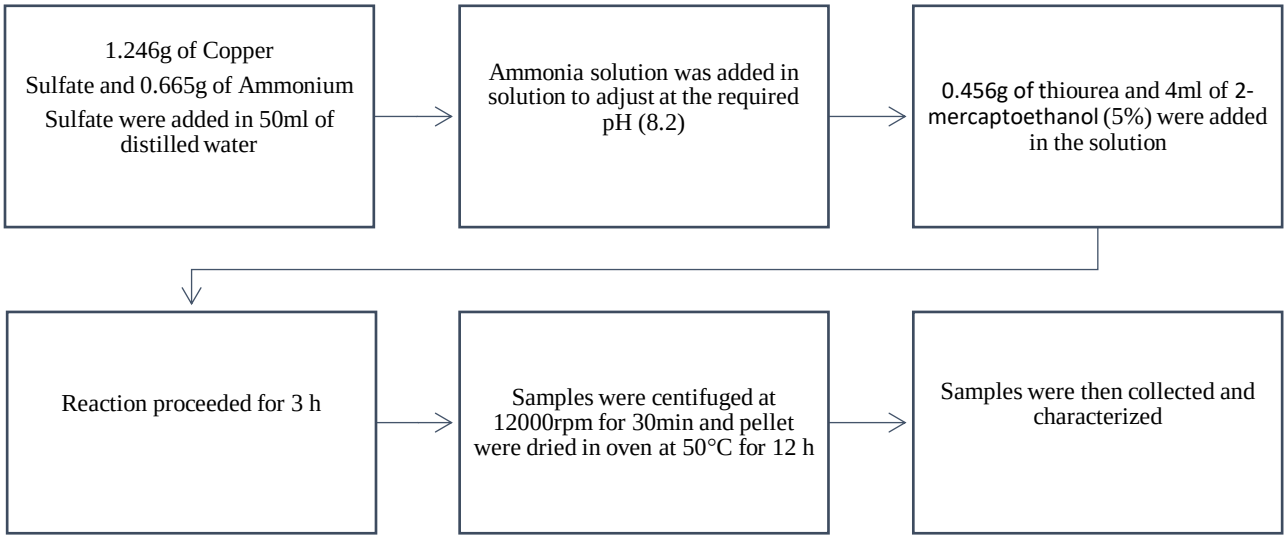


Fig 3.1: Processes of synthesis of CuS-NPs

3.2.2 Preparation of conjugate mancozeb fungicide with CuS-NPs

The mancozeb conjugated CuS-NPs was developed by using two methodologies i.e. pre-transformation and post-transformation approach.

3.2.2.1 Pre-transformation Approach

3.2.2.1.1 Treatment A

Copper Sulfate and Ammonium Sulfate with 10 mg of mancozeb were added in distilled water and mixed well using hot-plate magnetic stirrer at 50°C (Table 3.1). The pH of solution was adjusted at 8.28 using ammonia solution and temperature of the solution was maintain at 50°C. Thiourea and 2-mercaptoethanol (at a dosage of 5%) were used as capping agents to enhance the stability of the nanoparticles. The solution was kept on magnetic stirrer for 2h 30min for constant stirring.

3.2.2.1.2 Treatment B

Copper Sulfate and Ammonium Sulfate were added in distilled water and mixed well using hot-plate magnetic stirrer at 50°C (Table 3.1). The pH of solution was adjusted at 8.23 using ammonia solution and temperature of the solution was maintain at 50°C. After 5-10sec 10 mg of mancozeb will be incorporated. Thiourea and 2-mercaptoethanol (at a dosage of 5%) were used as

capping agents to enhance the stability of the nanoparticles. The solution was kept on magnetic stirrer for 2h 30min for constant stirring.

3.2.2.1.3 Treatment C

Copper Sulfate and Ammonium Sulfate were added in distilled water and mixed well using hot-plate magnetic stirrer at 50°C (Table 3.1). The pH of solution was adjusted at 8.23 using ammonia solution and temperature of the solution was maintain at 50°C. Thiourea with 10 mg of mancozeb will be incorporated and 2-mercaptoethanol (at a dosage of 5%) were used as capping agents to enhance the stability of the nanoparticles. The solution was kept on magnetic stirrer for 2h 30min for constant stirring.

3.2.2.1.4 Treatment D

Copper Sulfate and Ammonium Sulfate were added in distilled water and mixed well using hot-plate magnetic stirrer at 50°C (Table 3.1). The pH of solution was adjusted at 8.25 using ammonia solution and temperature of the solution was maintain at 50°C. Thiourea and 2-mercaptoethanol (at a dosage of 5%) were used as capping agents to enhance the stability of the nanoparticles. 10 mg of mancozeb will be incorporated after thiourea or 2-mercaptoethanol (5%). The solution was kept on magnetic stirrer for 3 h for constant stirring.

After stirring, the solution was centrifuged at 12000 rpm for 30 min to collect the nanoparticles. Pellets of nanoparticles was dried for 12 h at 50°C in an oven. The confirmation of synthesis of CuS-NPs were done by performing the UV-VIS and FTIR of the samples.

3.2.2.2 Post-transformation Approach or Treatment E

Copper Sulfate and Ammonium Sulfate were added in distilled water and mixed well using hot-plate magnetic stirrer at 50°C (Table 3.1). The pH of solution was adjusted at 8.25 using ammonia solution and temperature of the solution was maintain at 50°C. Thiourea (sulfur source) and 2-mercaptoethanol (at a dosage of 5%) were used as capping agents to enhance the stability of the nanoparticles. The solution was kept on magnetic stirrer for 3 h for constant stirring. After stirring, the solution was centrifuged at 12000 rpm for 30 min to collect the nanoparticle. 10 mg of Mancozeb will be included in collected nanoparticle with distilled water. The reaction will continue for 24 hours in shaker incubator at a temperature of 25°C and 120 rpm for the synthesis of CuS-NPs conjugate mancozeb. The solution was again centrifuged at 12000 rpm for 30 min to collect the nanoparticle. Pellet of nanoparticles was dried for 12 h at 50°C in an oven. The

confirmation of synthesis of CuS-NPs were done by performing the UV-VIS and FTIR of the samples.

Table 3.1: Sample description

Treatment	Precursor	Complexing agent	Temperature	pH Adjustment	Ph	Sulfur source+ Capping agent	Proceeds Reaction	Mancozeb incorporation
Treatment A (pre)	Copper Sulfate (1.246g)	Ammonium Sulfate (0.665g)	50°C	Ammonia Solution	8.28	Thiourea (0.456g) + 4ml of 2-ME (5%) conc	2hour30min	10mg of mancozeb with Ammonium Sulfate
Treatment B (pre)	Copper Sulfate (1.246g)	Ammonium Sulfate (0.665g)	50°C	Ammonia Solution	8.23	Thiourea (0.456g) + 4ml of 2-ME (5%) conc	2hour30min	10mg of mancozeb after Ammonia Solution
Treatment C (pre)	Copper Sulfate (1.246g)	Ammonium Sulfate (0.665g)	50°C	Ammonia Solution	8.23	Thiourea (0.456g) + 4ml of 2-ME (5%) conc	2hour30min	10mg of mancozeb with Thiourea or 2-ME (5%) conc
Treatment D (pre)	Copper Sulfate (1.246g)	Ammonium Sulfate (0.665g)	50°C	Ammonia Solution	8.25	Thiourea (0.456g) + 4ml of 2-ME (5%) conc	2hour30min	10mg of mancozeb after Thiourea or 2-ME (5%) conc
Treatment E (post)	Copper Sulfate (1.246g)	Ammonium Sulfate (0.665g)	50°C	Ammonia solution	8.25	Thiourea (0.456g) + 4ml of 2-ME	2hour30min	After the synthesis of CuS-NPs Mancozeb incorporation

						(5%) conc		and reaction will proceed for 24 hours in orbital shaker

3.2.3 Characterization of nano-particles

Fourier Transform Infrared (FTIR) spectroscopy and UV-VIS spectroscopy are two methods that are used for the characterization of nanoparticles.

3.2.3.1 UV-VIS Spectroscopy

UV-VIS spectroscopy quantifies a sample's transmittance or absorbance of UV and visible light. Nanoparticles have special optical properties, because of their small size. The absorbance is detectable between 200 and 1000 nm of copper sulfate nanoparticles and it's shifting toward infrared wavelength.

3.2.3.2 Fourier Transform Infrared (FTIR)

FTIR spectroscopy counts how much infrared light a sample can absorb, which results in molecular vibrations. FTIR spectra show distinctive peaks, such as C=O, C-H, O-H, and N-H stretches, that correspond to various functional groups. This may assist in determining the surface composition of the nanoparticles chemically.

3.2.4 Testing antifungal bioassay of synthesized conjugate mancozeb fungicide with CuS-NPs

3.2.4.1 UV sterilization of Nanoparticles

To testing fungicidal effect nanoparticle were sterilized by UV sterilization method. Placed the Mancozeb formulation samples that were conjugated with CuS-NPs for sterilizing. The samples were put in sterile Eppendorf tubes, and placed them under UV light for 2 hours. The antifungal bioassay was done by using two methods i.e. disk diffusion method and nanocomposite assay method.

3.2.4.2 Disk diffusion method for Antifungal bioassay

3.2.4.2.1 Preparing the culture media

Cultures of *Aspergillus flavus* (BT01) and *Rhizopus oryzae* were grown on potato dextrose agar plates (PDA). The agar medium was prepared by dissolving PDA powder in distilled water according to mention amount (Table 3.2). The culture media was autoclaved at 121°C for 15 - 20

min and poured on petri plates under sterile conditions. A small disc of fungal cultures was placed on solidified PDA media and incubated at 25°C for 48 h for the growth of fungi.

Table 3.2: Composition of the culture media

Component	g/l
PDA	39.0g
Distilled water	1000ml

3.2.4.2.2 Spore suspension preparation

10ml of PBST (Phosphate Buffered Saline with Tween 20) solution was added to the culture plate to wash the fungal colony and kept on stationary position for 5 min. The spores were gently scraped from the media using pipette tips. Fungal spores were carefully transferred into sterile falcon tube. 2ml of PBST was added in the spores and centrifuged for 5 minutes at 6000 rpm. Then the supernatant was discarded without disturbing the pellet. The pellet was washed with 5ml of PBST and again centrifuge for 5 minutes at 6000 rpm. The supernatant was removed and spores were stored in 1ml of PBS at 4°C till further use. To count the spores, a manual hemocytometer was employed. With this technique, the concentration of spores in the suspension could be accurately counted. The preparation of PBST & PBS mention in (Table 3.3).

Table 3.3: Preparation of PBS & PBST

PBS	**PBST**
137mM NaCl (Sodium chloride)	137mM NaCl (Sodium chloride)
2.7mM KCl (Potassium chloride)	2.7mM KCl (Potassium chloride)
10mM Na2HPO4 (Sodium phosphate dibasic)	10mM Na2HPO4 (Sodium phosphate dibasic)
1.8mM KH2PO4 (Potassium dihydrogen phosphate)	1.8mM KH2PO4 (Potassium dihydrogen phosphate)
Maintain the Ph 7.4 and makeup the volume	Maintain the Ph 7.4 and makeup the volume
	To the 1L of PBS add 50 µL of Tween 20

Autoclaved in 121°C for 15 to 20 min	Autoclaved in 121°C for 15 to 20 min

3.2.4.3 Cells count hemocytometer

A hemocytometer was used to count the number of viable cells in the sample (Fig 3.2). Spore suspension of *Aspergillus flavus* (BT-01) and *Rhizopus oryzae* was prepared. Using a micropipette, 10-20 µL of the spore suspension adding and gently placed the coverslip over the counting surface. The spores were counted in four squares of the hemocytometer and estimated using the following formula.

$$\text{concentration of cells in original mixture} = \frac{\text{number of cells counted}}{\text{proportion of chamber counted} \times \text{volume of square counted}} \times \frac{\text{volume of diluted sample}}{\text{volume of original mix of sample}}$$

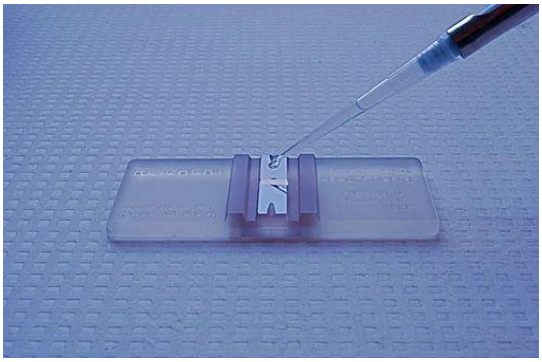


Fig 3.2: Hemocytometer (75)

3.2.4.4 *Aspergillus flavus* (BT01)

Cultures of *Aspergillus flavus* (BT01) were grown on potato dextrose agar plates (PDA). The agar medium was prepared by dissolving PDA powder in distilled water according to mention amount (Table 3.2). The culture media was autoclaved at 121°C for 15 - 20 min and poured on petri plates under sterile conditions. An autoclaved distilled water was used to prepare a suspended form of the fungal spores. To standardize the quantity of spores, the concentration of the fungal suspension was used 10µL in which 4 lakh spores were present in each plate. The fungal inoculum was evenly distributed across the agar plate's surface using a sterile L-shaped spreader. Wells were made in the agar plates and 100mg of the CuS-NPs conjugate mancozeb samples were added. The inoculated plates were incubated at 25°C for 48 h for the growth of fungi and the antifungal diffusion.

3.2.4.5 *Rhizopus oryzae*

Cultures of *Rhizopus oryzae* were grown on potato dextrose agar plates (PDA). The agar medium was prepared by dissolving PDA powder in distilled water according to mention amount (Table 3.2). The culture media was autoclaved at 121℃ for 15 - 20 min and poured on petri plates under sterile conditions. An autoclaved distilled water was used to prepare a suspended form of the fungal spores. To standardize the quantity of spores, the concentration of the fungal suspension was used 15µL in which 3 lakh spores were present in each plate. The fungal inoculum was evenly distributed across the agar plate's surface using a sterile L-shaped spreader. Wells were made in the agar plates and 100mg of the CuS-NPs conjugate mancozeb samples were added. The inoculated plates were incubated at 25℃ for 48 h for the growth of fungi and the antifungal diffusion.

3.2.4.6 Nanocomposite antifungal bioassay

3.2.4.6.1 *Aspergillus flavus* (BT01)

75 mg of sterile CuS-NPs Mancozeb conjugates (Treatment A, Treatment B, Treatment C, Treatment D, Treatment E) were added in 150ml of sterile PDA media and mixed evenly before pouring in the petri plates. Culture plates containing PDA devoid of the nanocomposite were used as control of the experiment. Small fungal discs of *Aspergillus flavus* (BT01) of same size were cut and placed on each treatment plate. Inoculated plates were kept at 25°C for 72h. The analysis of antifungal activity of each treatment was done by comparing the area of the fungus colony on treated and control plates. Diameter was measured at two different sites in each colony using a measuring scale. The formula used to calculate the colony area of the fungus is given below:

$$Colony\ area = \frac{\pi}{4} \times (A)(B)$$

Here A and B are 2 diameters of irregular colony.

3.2.4.6.2 *Rhizopus oryzae*

75 mg of sterile CuS-NPs-Mancozeb conjugates (Treatment A, Treatment B, Treatment C, Treatment D, Treatment E) were added in 150ml of sterile PDA media and mixed evenly before pouring in the petri plates. Culture plates containing PDA devoid of the nanocomposite were used as control of the experiment. Small fungal discs *Rhizopus oryzae* of same size were cut and placed on each treatment plate. Inoculated plates were incubated at 25°C for 24 h. The analysis of antifungal activity of each treatment was done by comparing the area of the fungus colony on treated and control plates. Diameter was measured at two different sites in each colony using a

measuring scale. The formula used to calculate the area of the fungus colony is given in section 3.2.4.6.1. The overall procedure was mention in (Fig 3.3)

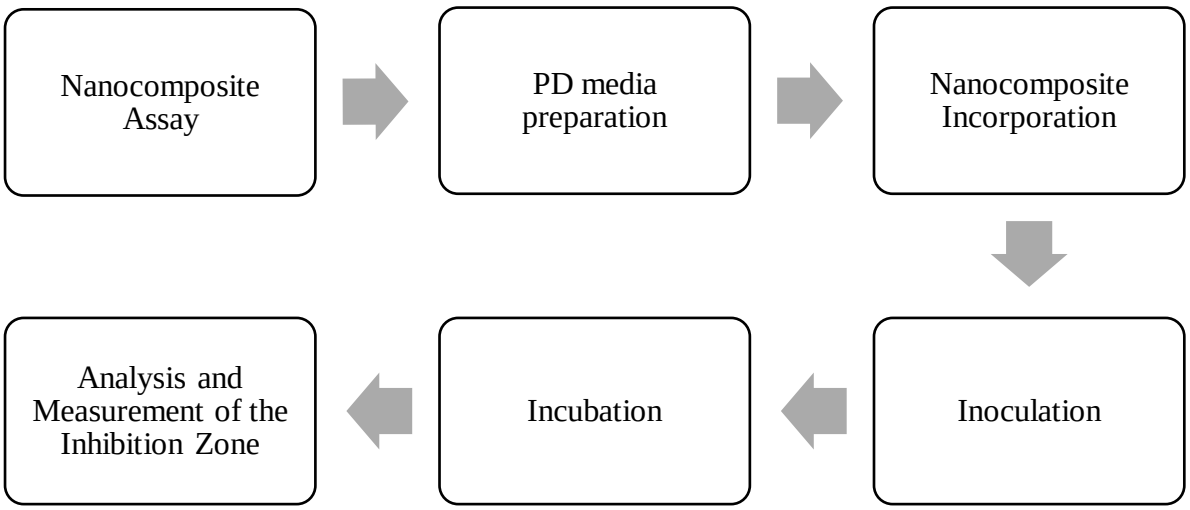


Fig 3.3: Procedure for the Antifungal bioassay

CHAPTER 4

RESULTS

Results

4.1 Formation of copper nano-particles

Copper sulfate was taken as a precursor for copper nanoparticle preparation. Ammonium sulfate was used as complexing agent, Thiourea for sulfur source and for capping 2-ME (5%) was used. Change in color of solution from Carolina blue- dark green color confirmed the presence of copper nano-particles (Fig 4.1).

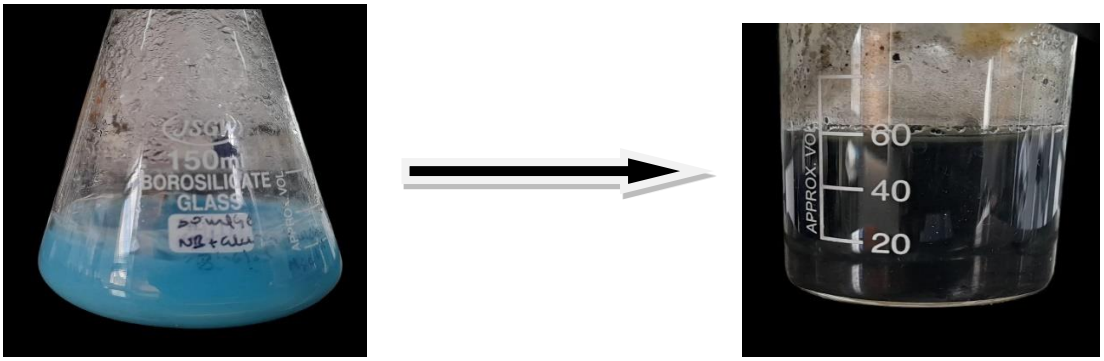


Fig 4.1: Color of reaction mixture before and after 3 hours that reaction will proceed for CuS-NPs synthesis.

4.2 Characterization of copper nano-particles

4.2.1 UV-VIS Spectroscopic analysis

UV-VIS spectroscopy was used to confirm the synthesis of copper nanoparticles. It was observed that CuS-NPs prepared from its precursor has shown a strong absorption band peak at 400-800 nm. as shown in (Fig 4.2). The y-axis shows the sample's absorbance, and the x-axis most likely indicates the light's wavelength in nanometers (nm). The electronic transition of the CuS-NPs may be the cause of the peak at approximately 300 nm. The CuS nanoparticle's surface plasmon resonance may be the cause of the large peak at about 550 nm. The optical evaluations by UV-VIS spectrophotometer depicts spectral shift of copper nano-particles and its precursor that confirms the formation of copper nano-particles.

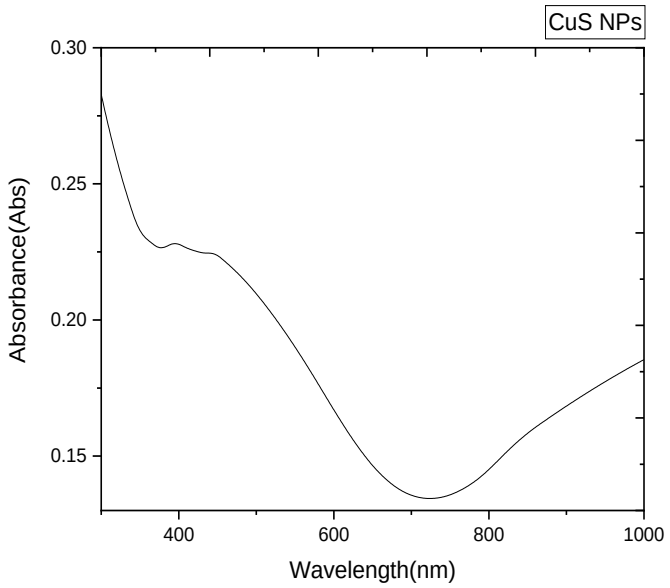


Fig 4.2: CuS-NPs 'UV-VIS' diffuse reflectance spectrum

4.2.2 FTIR results

The technique of Fourier Transform Infrared Spectroscopy (FTIR), that examines the sample's absorption of infrared radiation, is used to assess copper nanoparticles. Wavenumber (cm^{-1}) is displayed on the x-axis, while transmittance (%) is displayed on the y-axis. In (fig 4.3) shows that the large peak at approximately 3400 cm^{-1} indicates the existence of stretching of O-H vibrations, which could be caused by hydroxyl groups or water molecules. It is possible that C=O stretching vibrations, which show the existence of carbonyl groups, are responsible for the peak at about 1640 cm^{-1}. The peak at approximately 1100 cm^{-1} may be related to vibrations caused by C-O stretching. The peak at about 600 cm-1 might be the result of vibrations in Cu-S.

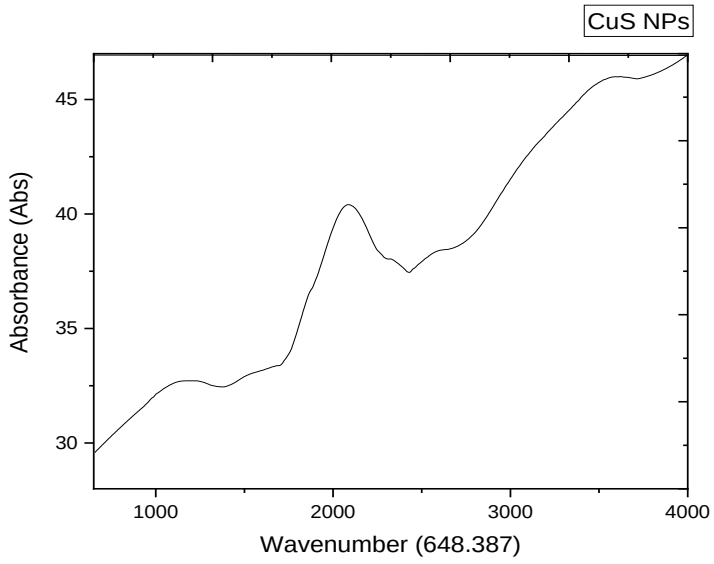


Fig 4.3: FTIR analysis of powdered synthesized copper nanoparticles

4.3 Assessment of mancozeb conjugated copper nano-particles.

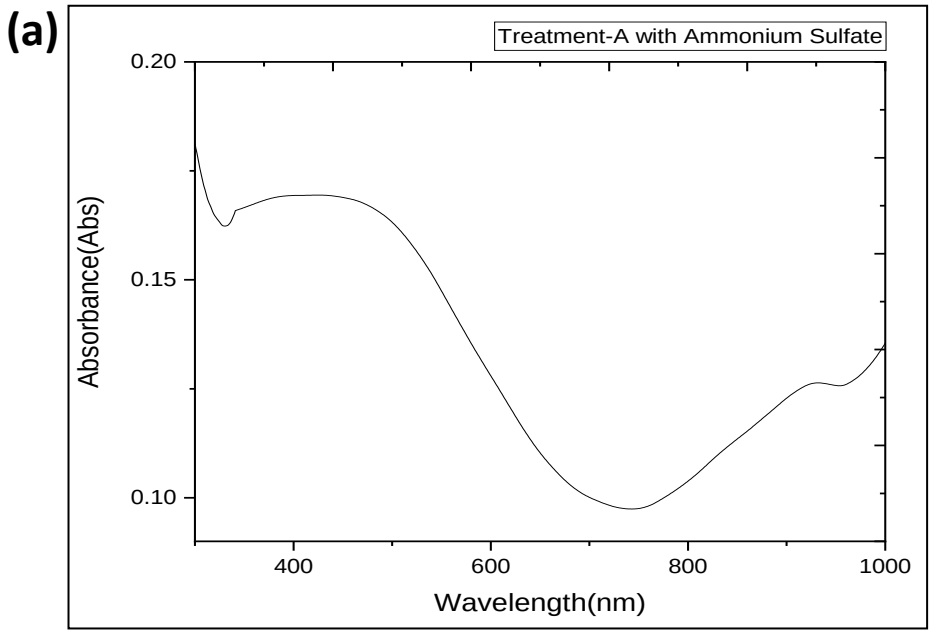
4.3.1 Conjugated system formulation

In order to increase the compound's antifungal efficacy, the optimized copper nanoparticles were employed to conjugate copper nanoparticles with mancozeb (fungicide). During the chemical synthesis of the CuS-NPs, the fungicide Mancozeb (0.02%) was added either concurrently or after a certain amount of time, a process known as pre- or post-transformation. The mixture was stirred for two to three hours.

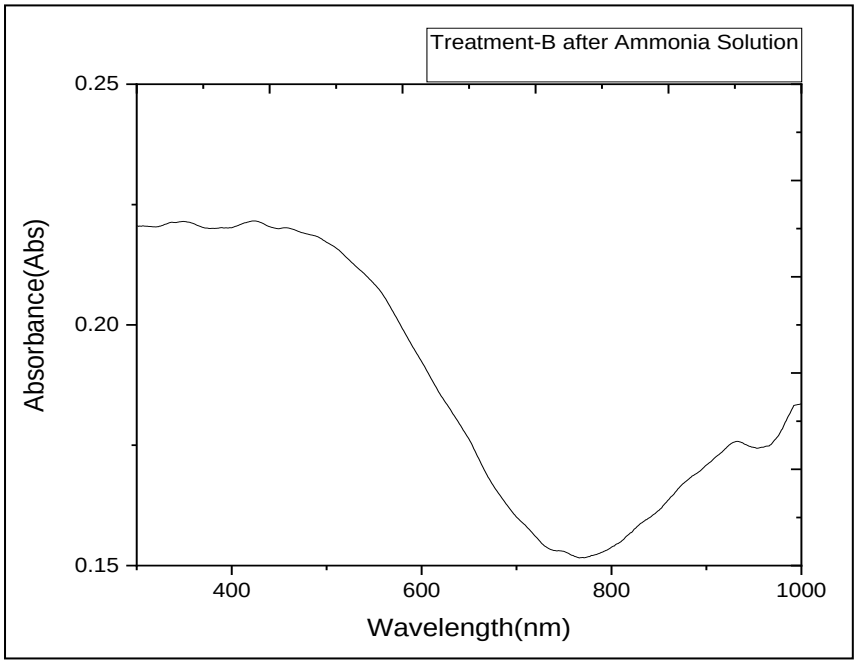
4.3.2 Characterization of conjugated system

The optical properties of synthesized copper nanoparticles conjugate with mancozeb (Fungicide) were determined by UV-VIS spectrometry (Thermo-scientific). The UV-VIS the maximum absorbance spectra of Cus-NPs conjugate mancozeb were observed in the range 700 nm to 800 nm. The y-axis shows the sample's absorbance, and the x-axis most likely indicates the light's wavelength in nanometers (nm). In Treatment A shows the wavelength at which the absorbance peak is highest, roughly 550 nm, it may indicate the presence of nanoparticles that are between 40 and 50 nm in size. In Treatment B shows the graph demonstrates a peak absorbance value at approximately 550 nm 0.70 arbitrary units (AU), suggesting that the nanoparticles and light at this wavelength are significantly interacting. The wavelength peak at 550 nm is associated

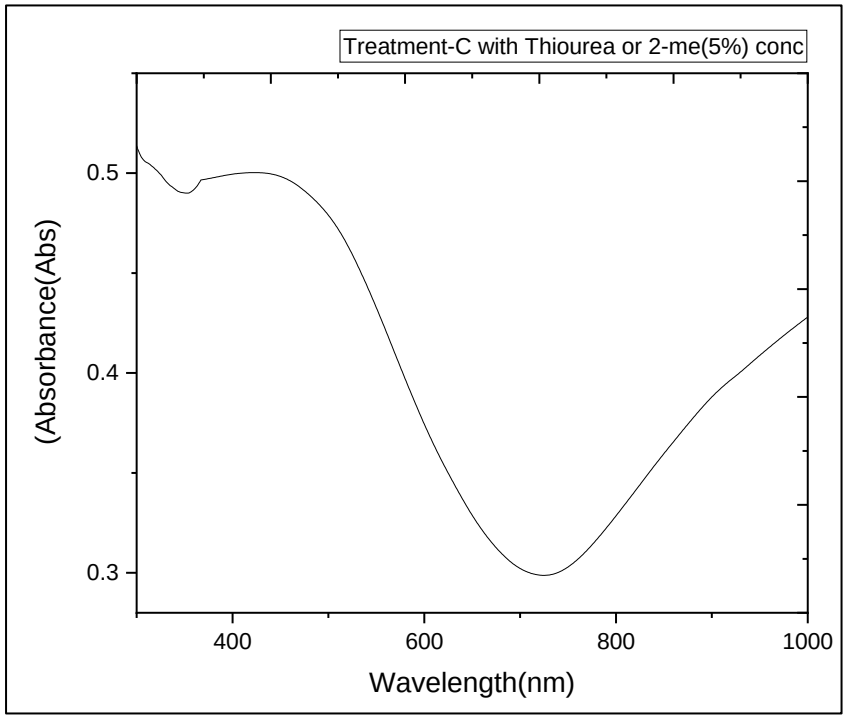
with particles that are between 40 and 50 nm in size. The absorbance pattern for nanoparticle is characterized by an increase in absorbance from 250 nm to 550 nm. In Treatment C shows that the conjugate Mancozeb sample of CuS-NPs absorbs light strongly, with a peak absorbance of about 1.2–1.5 at a wavelength of about 420–440 nm. The wavelength at which the absorption peak is centered, 430 nm, is typical of CuS-NPs. The particle size of the CuS-NPs can be approximated to be between 20 and 30 nm based on the absorption spectrum. In Treatment D shows the graph shows that there are CuS-NPs present because of the peak absorbance that occurs between 550 and 570 nanometers (nm). There may be a considerable concentration of CuS-NPs in the sample due to the comparatively high absorbance value at this peak wavelength, which is likely between 1.5 and 2.5 (AU). It is possible that the peak's full width at half its maximum (FWHM) is between 50 and 70 nm, indicating a range of particle sizes. In Treatment E shows that the Mancozeb sample treated with CuS-NPs has a peak at 420 nm in its UV-VIS absorption spectra, with a highest absorbance of 1.2. At 450 nm, the curve's shoulder has an absorption value of 0.8. According to the peak wavelength and width of the spectrum, the particle size is estimated to be between 20 and 30 nm. At decreasing wavelengths, the absorbance values progressively drop, reaching a minimum at 300 nm. All the result shown in (Fig 4.4).



(b)


Treatment-B after Ammonia Solution
0.25
0.20
0.15
Absorbance(Abs)
400
600
800
1000
Wavelength(nm)

(c)


Treatment-C with Thiourea or 2-me(5%) conc
0.5
0.4
0.3
(Absorbance(Abs)
400
600
800
1000
Wavelength(nm)

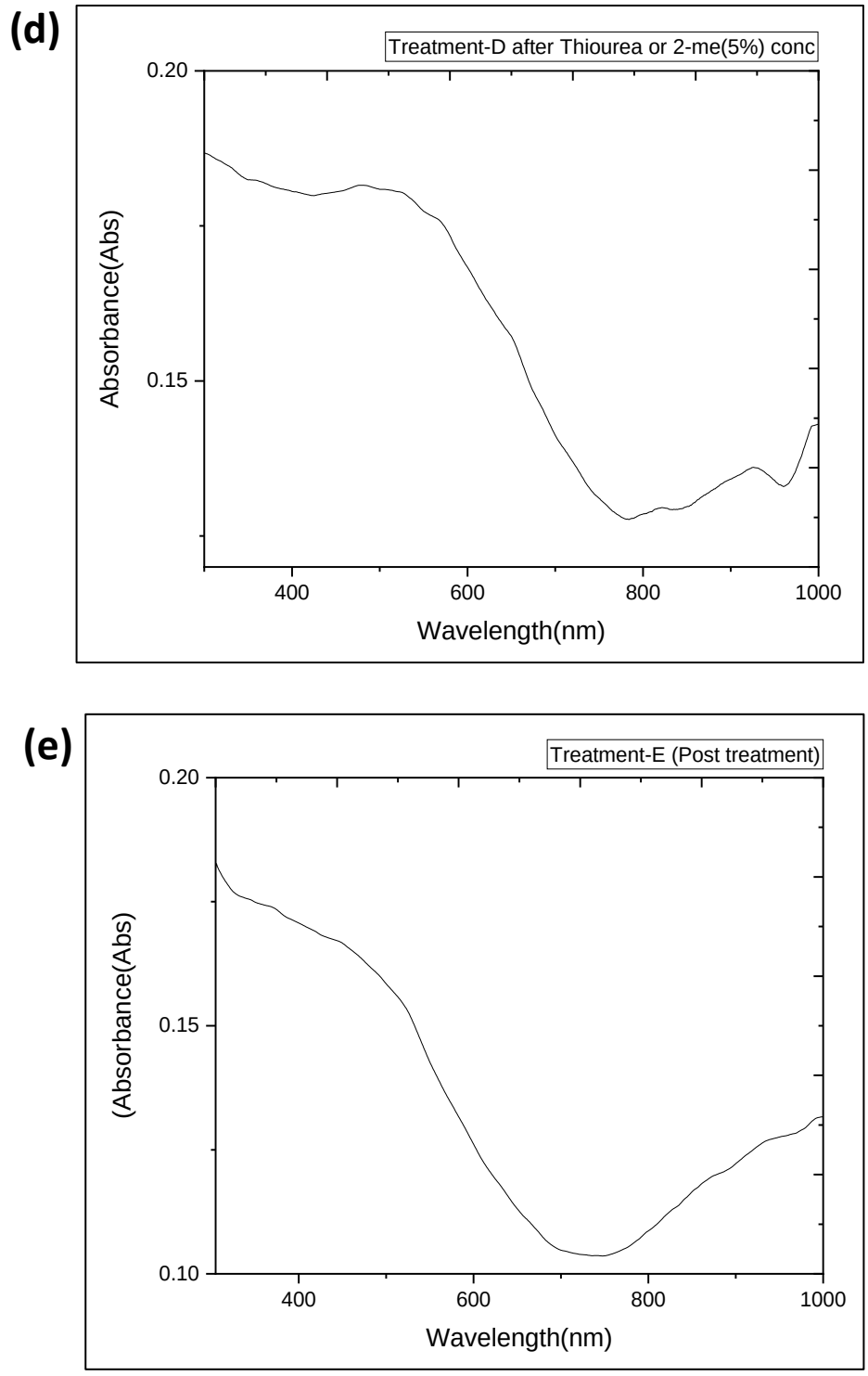


Fig 4.4: UV-VIS graph of conjugate mancozeb fungicide with CuS-NPs, Treatment A (a) Treatment B (b), Treatment C (c), Treatment D (d), Treatment E (e)

4.4 Growth of *Aspergillus Flavus* (BT01) and *Rhizopus oryzae* on PDA culture media

The *A. flavus* (BT01) forms an irregular shaped colony on PDA media. The yellowish green color of the colony was due to the color of spores. The development of spores on *A. flavus* (BT01) were initiated after 24 h of incubation (Fig. 4.5 A).

The *R. oryzae* forms an irregular shaped white colony on PDA media. The spore of the *R. oryzae* give dark greyish-black color to the colony. *R. oryzae* is a fast-growing fungus and forms fungal load within 24 hrs of incubation. (Fig. 4.5 B).

(A) (B)

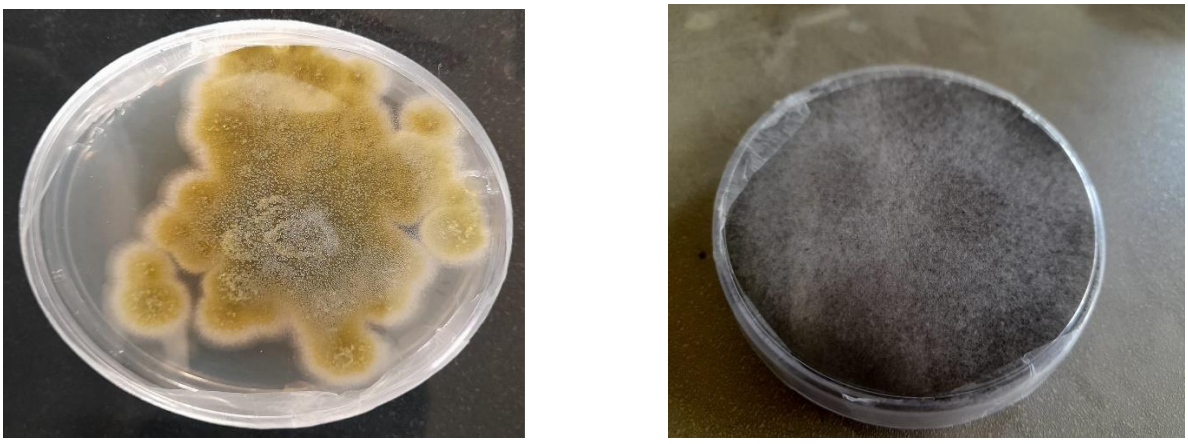


Fig 4.5: Growth of fungus on PDA culture media (A) *A. flavus* (BT-01) and (B) *R. oryzae* in PDA plate after 2 days

4.4.1 Spore harvesting from fungal culture

The mature fungal cultures of *A. flavus* (BT01) and *R. oryzae* was used for harvesting of spores. The harvested spores were stored at 4°C in 1ml of PBS solution (Fig 4.6). The spore counting was done by using hemocytometer it counts the number of viable spores in each fungal colony. For spore quantification 40X objective lens was used (Fig 4.7A) and (Fig 4.7B). In sample A has 2515×10^4 spore in per ml and in sample B has 4120×10^4 spore in per ml. The dilution ratio was used for both the sample (*A. flavus* (BT01) and *R. oryzae)* was 1:4 where in each dilution 1 part was spore and 4 parts was autoclaved PBS.

Result: (A) has 2515×10^4 spore in per ml

(B) has 4120×10^4 spore in per ml

(A) (B)

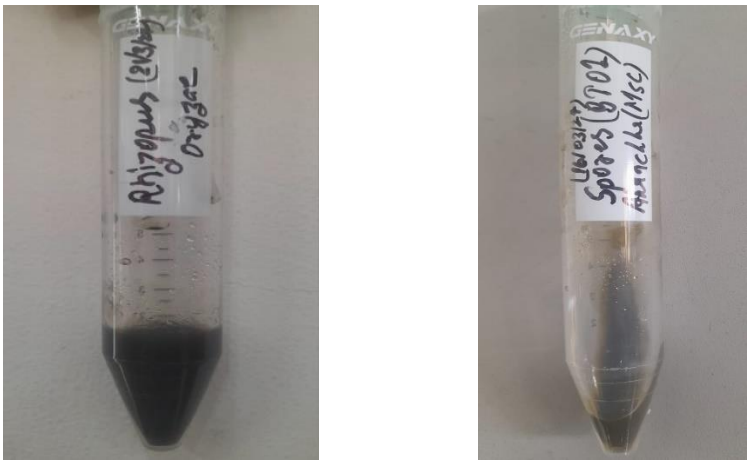


Fig 4.6: Spore harvesting of (A) *R. oryzae* (B) *A. flavus* (BT-01)

(A) (B)

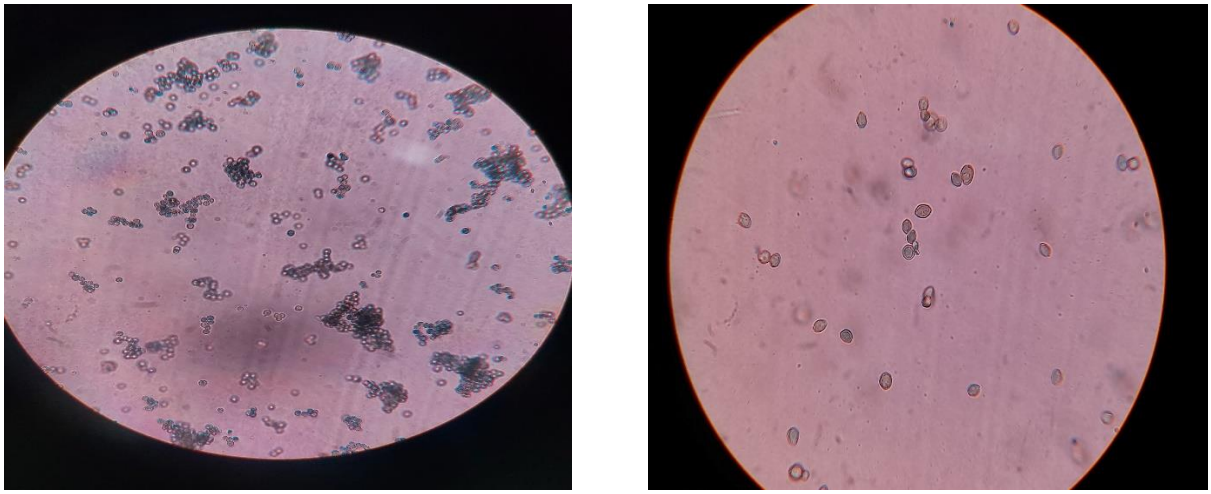


Fig 4.7: (A) *R. oryzae* and (B) *A. flavus* (BT01) spore quantification under 40X objective

4.4.2 Disc diffusion antifungal bioassay

Copper sulfate nanoparticles (CuS-NPs) coupled with mancozeb were evaluated for their antifungal efficacy against *Rhizopus oryzae* and *Aspergillus flavus* (BT01) using the disk diffusion method. Agar plates were inoculated with 3 lakh spores of *Rhizopus oryzae* and 4 lakh spores of *Aspergillus flavus* (BT01). Three wells were created in each inoculated plate and100 mg of different treatments were added in each well an incubated at 25 ℃. Measured the zones of

inhibition were assessed after incubation. No zone of inhibition was observed in treated as well as control plate (Fig 4.8).

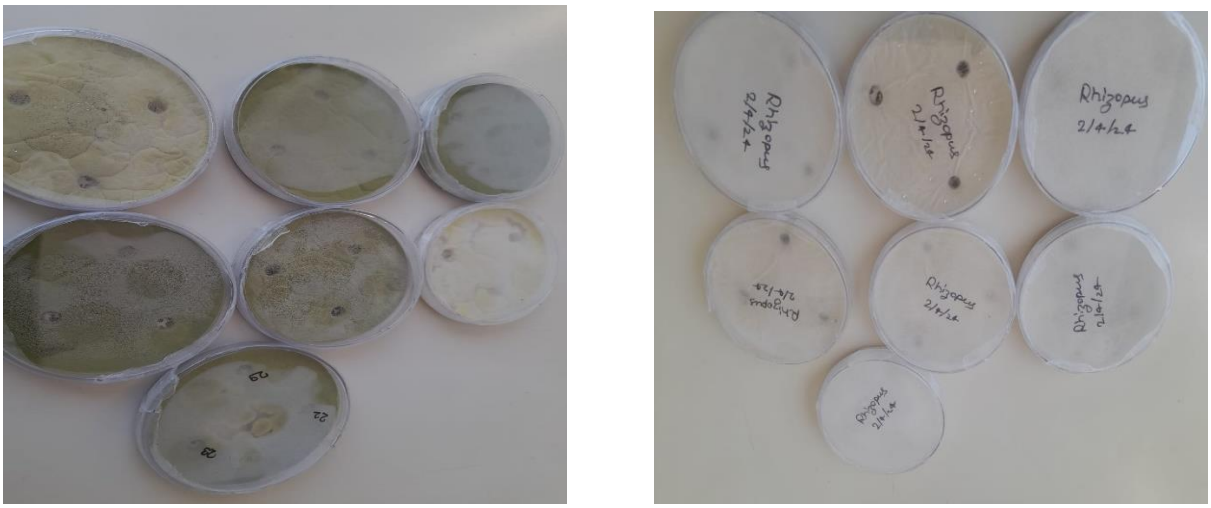


Fig 4.8: (A) R. oryzae and (B) A. flavus (BT 01) zone of inhibition shown by synthesized fungicide-nanoparticle conjugates

4.5 Nanocomposite antifungal bioassay

The antifungal activity of conjugated system was evaluated by nanocomposite assay method. Antifungal Bioassay of nanoparticles were examined against *Aspergillus flavus* (BT-01) and *Rhizopus oryzae*. Antifungal assay employed eight treatment groups, including one positive control and one negative control. (Fig 4.9) & (Fig 4.10). The maximum growth inhibitory effect of various treatments in case of *Rhizopus oryzae* and *Aspergillus flavus* (BT-01) was shown in Treatment B (Table 4.1) & (Table 4.2) respectively.

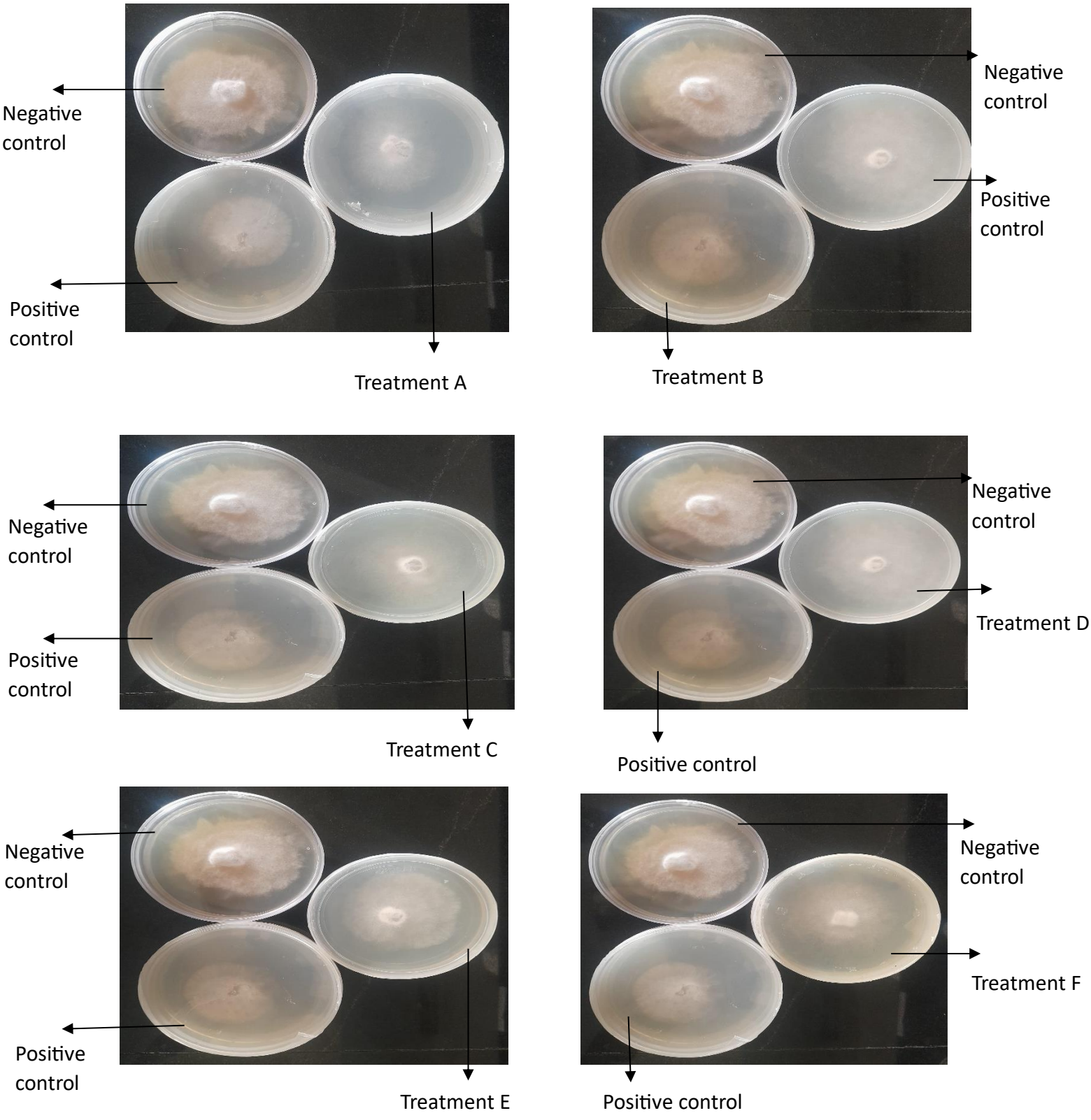


Fig 4.9: Growth inhibitory effect of colony area shown by synthesized fungicide-nanoparticle conjugates against *R. oryzae*

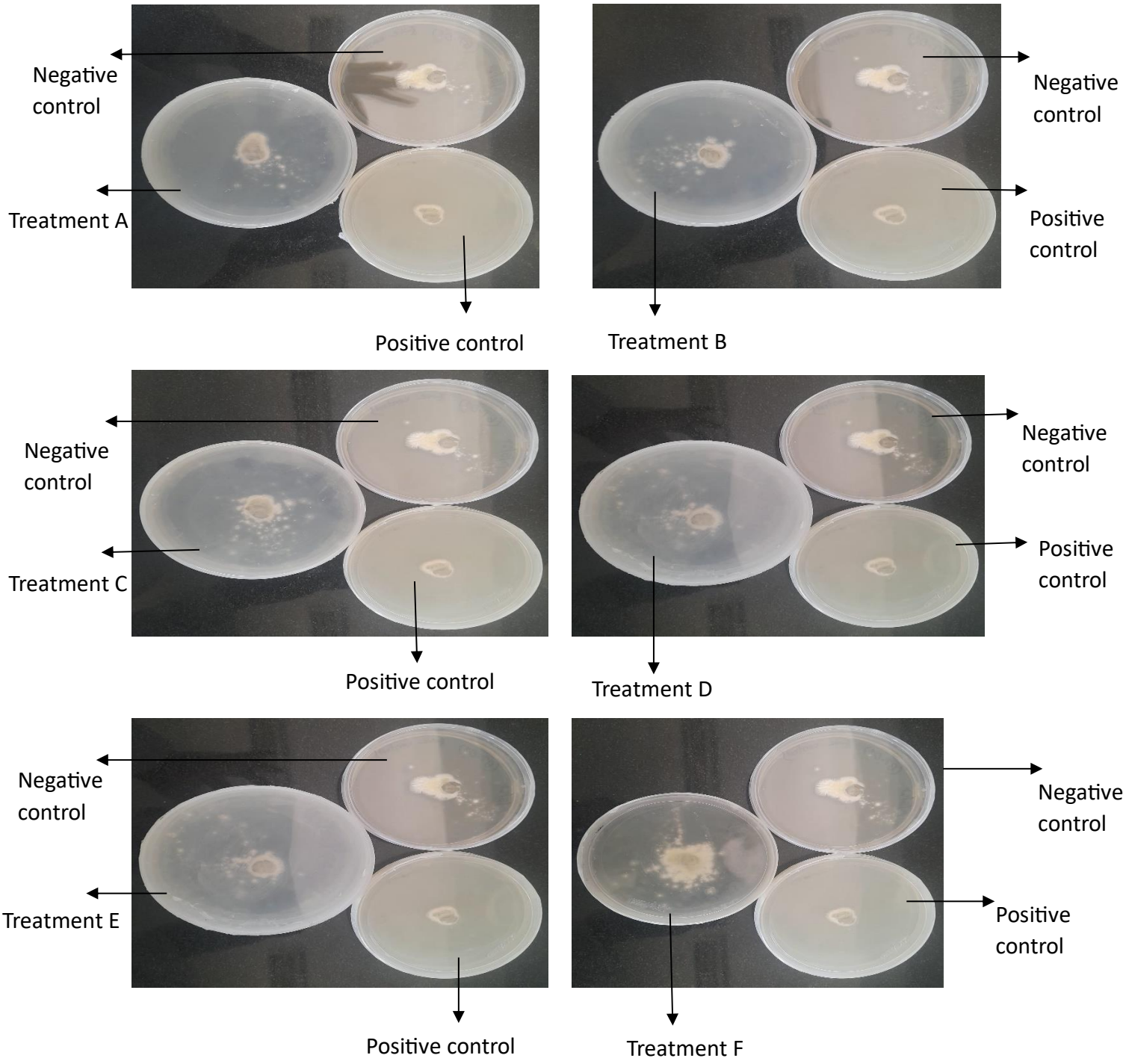


Fig 4.10: Growth inhibitory effect of colony area shown by synthesized fungicide-nanoparticle conjugates against *A. flavus* (BT-01)

Table 4.1: Growth inhibitory effect of various treatments in case of *Rhizopus oryzae*

Treatment	Average colony area (cm^2)	Growth inhibition (%)
Positive control	26.01	-
Negative control	72.13	-
Treatment A	52.21	28.61
Treatment B	43.91	39.96
Treatment C	59.08	19.22
Treatment D	66.25	9.41
Treatment E	60.45	17.34
Treatment F	66.84	8.61

Table 4.2: Growth inhibitory effect of various treatments in case of *A. flavus* (BT-01)

Treatment	Average Colony area (cm^2)	Growth inhibition (%)
Positive control	4.004	-
Negative control	27.36	-
Treatment A	18.84	31.15
Treatment B	16.67	39.08
Treatment C	18.45	32.57

Treatment D	19.23	29.72
Treatment E	17.66	35.46
Treatment F	26.29	3.92

CHAPTER 4
DISCUSSION AND CONCLUSION

Discussion

In the area of agricultural nanotechnology, the effective synthesis and analysis of the copper sulfate nanoparticle-conjugated mancozeb formulation mark a noteworthy development. We have shown the viability and effectiveness of this innovative formulation for use in agriculture through an array of methodical trials and analysis. First, characterization methods including Fourier-transform infrared spectroscopy (FTIR), and ultraviolet–visible (UV–VIS) spectrophotometry verified that the synthesis process produced homogenous and stable copper sulfide nanoparticles. These analyses shed light on the nanoparticles' structural characteristics by revealing their crystalline form, shape, and chemical makeup [64,65]. Following that, it was successful to conjugate these tiny particles with mancozeb and a fungicide that is frequently used in agriculture. In order to efficiently load mancozeb on the surface of the nanoparticle while preserving the stability & integrity of the formulation, the conjugation procedure was carefully adjusted. Through both in vitro and in vivo tests, the effectiveness with CuS-NPs-conjugated the mancozeb formulation was assessed. Strong antifungal activity was shown in vitro against a variety of phytopathogenic fungal organisms, including those that are immune to common fungicides. This shows that the formulation may be able to help with the expanding issue of resistance to fungicides in agriculture. In addition, compared to treatments and commercially available formulations, in vivo testing carried out on agricultural plants under controlled conditions demonstrated significant enhancements in preventing disease and yield protection [66,67]. The formulation that was coupled with nanoparticles demonstrated improved adhesion and penetration qualities, which resulted in improved dispersion and preservation of the active component on plant surfaces.

The study's findings demonstrate the intriguing potential of the mancozeb formulation conjugated with copper sulfide nanoparticles as a long-term and successful approach to disease control in agriculture. Prospective study avenues could encompass field experiments to evaluate the formulation's efficacy in real-world scenarios, in addition to studies examining the formulation is enduring environmental consequences and suitability for current farming methods.

This study's antifungal bioassay demonstrated the formulation of mancozeb conjugated with copper sulfide nanoparticles had a promising activity against a variety of phytopathogenic fungi. The mixture demonstrated strong inhibitory efficacy, outperforming traditional mancozeb formulations and indicating its potential as an effective fungicidal drug for use in agriculture.

Crucially, the formulation's increased antifungal activity indicates that it can be used to fight fungal illnesses that seriously jeopardize crop quality and output. Furthermore, a notable development in agricultural nanotechnology is the use of copper sulfide nanoparticle as mancozeb carriers. The nanoparticles offered further advantages such prolonged release kinetics and enhanced adhesion to plant surfaces in addition to facilitating the effective delivery of the mancozeb to the intended pathogens [68,69]. These characteristics are essential for optimizing fungicidal treatments' effectiveness and durability while reducing the environmental damage brought on by repeated pesticide applications. The need for environmentally friendly alternatives to traditional fungicides in agricultural disease prevention is critical given the environmental concerns surrounding these products. Conventional fungicides frequently show non-specific toxicity, which has a negative impact on ecosystem health and non-target organisms. Furthermore, the establishment of pesticide-resistant strains and the continuous application of new chemicals are exacerbated by the appearance of tolerance in fungal populations, which calls for their continual introduction [70].

The efficacy of the nanocomposite was assessed using various bioassay methods. One of the techniques utilized was the nanocomposite bioassay, where CuS-NPs conjugated with mancozeb were aseptically combined with Potato Dextrose Agar (PDA) media. Previously synthesized fungal colonies were excised and introduced into the PDA media containing the nanocomposite. This approach exhibited significant fungal growth suppression, indicating the efficacy of the CuS-NPs conjugated mancozeb composite in controlling fungal proliferation.

Conversely, the well diffusion method, another bioassay technique employed in the study, revealed constraints in the diffusion of the CuS-NPs conjugated mancozeb composite. In this procedure, the nanocomposite was introduced into wells formed in an agar medium seeded with fungal spores. However, the composite did not adequately disperse from the wells, resulting in an absence of a clear inhibition zone around the wells. This poor diffusion implies that although the composite may possess antifungal properties, its physical attributes impeded effective dispersion within the agar medium. Furthermore, the synergistic effects of mancozeb and copper sulfide nanoparticles demonstrate the possibility of lowering the total pesticide load necessary for efficient disease management, therefore lowering the environmental impact of agricultural operations [71].

Conclusion and Future prospects

A notable development in the realm of agricultural nanotechnology is the creation and characterization of the sulfide of copper nanoparticle-conjugated mancozeb formulation. This work has addressed a crucial need for long-term disease control methods in agriculture by demonstrating the formulation's viability and effectiveness in treating phytopathogenic fungi. Through the utilization of nanoparticles' distinct characteristics and the combined benefits of copper sulfide & mancozeb in this mixture presents a viable substitute for traditional fungicides, exhibiting increased effectiveness and a diminished ecological footprint. This discovery opens up a number of new directions for future study and development. First, in order to optimize the formulation's effectiveness in field settings, more optimization is necessary. Field tests carried out in various crop systems and agroecological zones will offer important insights into how well the formulation performs in practical situations and whether or not farmers would use it widely. Furthermore, evaluating the formulation's general environmental sustainability including ecological compatibility will need research on the formulation's long-term impact on microbial communities, non-target organisms, and soil health. Additionally, efforts ought to be focused on clarifying the mechanisms that underlie the CuS-NPs-conjugated the mancozeb formulation's antifungal efficacy. Comprehending the molecular interactions of nanoparticles that fungicidal ingredients that act, and target pathogens will aid in the logical development of next-generation formulations that exhibit enhanced efficacy and specificity. Additionally, investigating synergistic pairings with additional bioactive substances or nanomaterials may result in the creation of multifunctional formulations that can solve several. The CuS-NPs-conjugated the mancozeb formulation has antifungal qualities, but it may also be used in agriculture to improve plant development, nutrient uptake, and stress tolerance. Examining these possible advantages will increase its usefulness and aid in the creation of sustainable farming methods. An encouraging development in the realm of nanopesticides is the creation and characterization of the conjugated mancozeb formulation of CuS-NPs. While the difficulties encountered when using the wells diffusion approach emphasize the significance of taking into account the practical application of bioassay methods when evaluating nanopesticides, the nanocomposite bioassay appears as a trustworthy tool for determining its antifungal activity. To ensure the formulation's effectiveness

in actual agricultural contexts, more study is required to modify it for better diffusion and antifungal activity. Furthermore, in order to translate scientific discoveries into workable solutions and ensure their responsible application in the agricultural sector, interdisciplinary interactions between investigators, business interests, and governments will be crucial.

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