Bio-fabrication of ZnO nanoparticles in *Trachyspermum ammi L.* Sprague seeds Extract and its Application to control Rot Disease of Okra Pods

By

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DEPARTMENT OF PLANT SCIENCES QUAID-I-AZAM UNIVERSITY ISLAMABAD-PAKISTAN

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Bio-fabrication of ZnO nanoparticles in *Trachyspermum ammi L.* Sprague seeds Extract and its Application to control Rot Disease of Okra Pods

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In

Plant Sciences

By

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DEPARTMENT OF PLANT SCIENCES QUAID-I-AZAM UNIVERSITY

ISLAMABAD-PAKISTAN

2024

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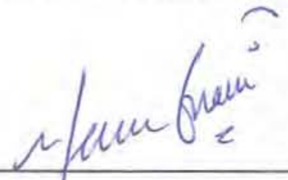

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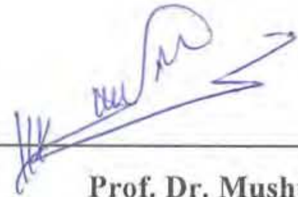

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DEDICATION

I dedicate my work

To my beloved Prophet Muhammad (S.A.W) for whom I recite from the core of heart.

To my beloved parents who put their efforts and always supported me in my studies. This humble work is a sign of love to you.

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All glory be to **ALLAH ALMIGHTY**, the Creator of the world and the Master of the Hereafter Who has shown His love for His creations by bestowing infinite kindness on them. Peace and unaccountable salutation upon the **Holy Prophet (Peace Be Upon Him)** Who has taken humanity from the days of cruelty and ignorance to the ages of peace and safety.

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LIST OF ABBREVIATIONS

Zn	Zinc
ZnNPs	Zinc Nanoparticles
$AgNO_3$	Silver Nitrate
ANOVA	Analysis of variance
AOA	Antioxidant Activity
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
Cm	Centimeter
CaM	Calmodulin
CNTs	Carbon Nanotubes
CTAB	Cetyl Trimethyl Ammonium Bromide
$CuSO_4$	Copper Sulphate
°C	degree Celsius
DHP	Disodium Hydrogen Phosphate
DSP	Dibasic Sodium Phosphate
DW	Dry Weight
DNA	Deoxyribonucleic Acid
Dntp	Deoxyribonucleotide Triphosphate
EDTA	Ethylenediamine Tetraacetic Acid
FW	Fresh Weight
Fig	Figure
FTIR	Fourier Transform Infrared Spectroscopy
FeNPs	Iron Nanoparticles

G	Gram
μg	Microgram
μl	Microliter
H	Hour
HCRSV	Hibiscus Chlorotic Ring Spot Virus
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric Acid
ITS	Internal Transcribed Spacer
IR	Infrared
JCPDS	Joint Committee on Powder Diffraction Standards
KBr	Potassium Bromide
μg	Microgram
μg/g	Microgram per Gram
μl	Microliter
MDP	Monosodium Dihydrogen Phosphate
MSP	Monobasic Sodium Phosphate
Mm	Milli molar
Mg	Milligram
mg/ml	Milligram/Milliliter
Ml	Milliliter
Mm	Millimetre
Mm	Millimolar
Min	Minutes
Nm	Nanometre

Na_2CO_3	Sodium Bicarbonate
NaOH	Sodium Hydroxide
NPs	Nanoparticles
NCBI	National Centre for Biotechnology Information
OD	Optical Density
%	Percentage
PCA	Principal Component Analysis
PC1	Principal Component 1
PC2	Principal Component 2
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
POD	Peroxidase
Psi	Pounds per square inch
REL	Relative Electrolyte Leakage
Rrna	Ribosomal Ribonucleic Acid
ROS	Reactive Oxygen Species
RWC	Relative Water Content
Rpm	Revolutions per minute
SEM	Scanning Electron Microscopy
SOD	Superoxide Dismutase
Trna	Transfer Ribonucleic Acid
ToMV	Tomato Mosaic Virus
TSS	Total Soluble Sugar
Sec	Seconds

UV	Ultraviolet
UV-vis	UV-visible
USA	United States of America
V/V	Volume/Volume
WHO	World Health Organization
W/V	Weight/Volume
XRD	X-ray Diffraction
ZnO	Zinc oxide

Abstract:

Quality of okra pods is affected by different biotic stresses during growth, harvesting and storage. Due to fungal attack, huge losses of its quality and quantity are observed. The present research was designed for the diagnoses and management of postharvest rot disease of okra pods. Infected okra pods were collected, and the disease-causing agent was identified as *Fusarium oxysporium*. To control this disease *Trachyspermum ammi* L. Sprague seeds extract mediated nanoparticles were used. Here in metabolites filtrate of *T. ammi* was used to reduce zinc acetate into zinc oxide nanoparticles (ZnO NPs). The physiochemical and morphological characters of NPs were determined. Before their application to control *Fusarium* rotting of okra pods, fabricated ZnO NPs were characterized. X-ray diffraction depicted Nano-size *T. ammi* seeds extract mediated ZnO NPs (26.7). UV vis spectroscopy displayed absorption peak of ZnO NPs on 390 nm indicating successful reduction of zinc acetate in metabolites of plants seeds extract. Fourier transform infrared (FTIR) spectroscopy showed the presence of reducing and stabilizing chemical compounds (alcohol, carboxylic acid, amines, and alkyl halide. NPs showed variable antifungal activities at four different concentrations (0.25, 0.50, 0.75 and 1.00 mg/ml). Diseases control and postharvest changes in okra pods were analyzed for 15 days. Among all treatments, 1.00 mg/ml concentration of ZnO NPs and 0.75 mg/ml concentration of ZnO NPs Showed the strongest antifungal activity. Application of ZnO NPs reduce okra pods decay, weight and preserved firmness of disease okra pods These nanofungicides were made in powder form and they can be easily transferred and used in the field to control *Fusarium* rotting of okra pods. Our results conclude that *T. ammi* seeds extract based synthesized ZnO NPs can efficiently control fruit rot, enhance shelf life, and preserve the quality of okra pods.

CHAPTER 1

INTRODUCTION

1. Introduction

1.1. NANOTECHNOLOGY

The use of nanotechnology in farming has changed the speed, and nanoparticles are becoming more significant in the control of plant diseases. More attention is being paid to creating novel antimicrobial agents as a result of the outbreak of resilience to drugs in bacteria and fungi. The introduction of new metallic nanoparticles with improved antibacterial properties requires the use of novel techniques. Owing to their distinct chemical and physical characteristics, large surface area with regard to volume proportion characteristics, nanoscale materials have been developed as creative antibacterial agents in the contemporary environment. Nanotechnology term was given by Norio Taniguchi in 1974.As anti-microbial agents, nanoparticles (NPs), which normally have a size between 1 and 100 nanometers (nm), are employed (Taniguchi, 1974). Processing, separating, consolidating, and deforming substances by one atom or one molecule constitute the main functions of nanotechnology.

Following the development of cluster science and the scanning tunneling microscope (STM), nanotechnology and nanoscience achieved significant strides in the 1980s (Iancu, 2010). Planning, controlling, and using materials at the nanoscale is the field of nanotechnology, a relatively new and emerging science. "Nanoscience" is the study of materials and their properties at the nanoscale. When referring to materials whose particle sizes are smaller than 100 nanometers, or one billionth of a meter, the term "Nano" (meaning dwarf in Greek) is used. Between one and one hundred nanometers (nm) is the usual range of nanoparticles (NPs) (Madkour & Madkour, 2019). Because they are so small—less than 100 nm—NPs have extraordinary physicochemical properties. Elements analytical clarity, crystalline nature, electrical properties, absorption, surface appearances, impact of deposited chemicals, form, and accumulation are the features of nanoparticles (NPs). These unique characteristics result from three factors: (i) quantum dots; (ii) electronic transitions caused by the size effect; and (iii) an increase in the surface area to mass ratio of NPs. These factors lead the behavior of atoms on the NPs surface to differ from that of atoms on the inside of the particles (Nel, Xia, Madler, & Li, 2006).

In every aspect of life, including health, food preservation, fiber coating, biological applications, environmental treatments, and various pharmaceutical and miscellaneous

uses, nanotechnology is a potent tool (Azizi-Lalabadi, Garavand, & Jafari, 2021). The pharmaceutical sector uses nanoparticles (NPs) because of their antimicrobial qualities. Nanomaterials differ from other technologies in that they can be used to: (i) explain nanomaterial composition and structures; (ii) design synthesis methods; (iii) provide guidelines for protected schemes; (iv) increase the advantages of using nanomaterials and their products (Hutchison, 2016). Nanotechnologies is classified into following three categories.

1.1.1. Wet Nanotechnology

Wet nanotechnology involves studying living things, most of which are found in water. Important elements of nanostructures in wet nanotechnology include biological systems, membranes, genetic material, and enzymes. The fact that there are biological entities whose structure, functions, and development are dictated by the reactions of nanocrystals attests the efficacy of today's nanotechnology.

1.1.2. Dry Nanotechnology

Dry nanotechnology, which has its roots in surface sciences and physical chemistry, focuses on the synthesis of inorganic materials, silicon, and carbon structures like carbon nanotubes (CNTs). Semiconductors and metals are used in dry technology. In a moist environment, the materials' electron transmission becomes excessively intense, but in a dry environment, these electrons provide the nanostructures their electrical, magnetic, and optical characteristics.

1.1.3. Computational Nanotechnology

Computational nanotechnology enables the intricate synthesis of nanoparticle. The predictive and quantitative power of computation is essential for the success of nanotechnology: while it took nature hundreds of millions of years to create an operational "wet" nanotechnology, knowledge gained from computation may enable the production of an operational "dry" nanotechnology in a few decades, with potential implications for the "wet" side as well. Each of the three nanotechnologies is interconnected. Techniques are put into practice, and each sees notable improvements (M. Singh, Singh, Prasad, & Gambhir, 2008).

1.2. Approaches for Nanoparticles Synthesis

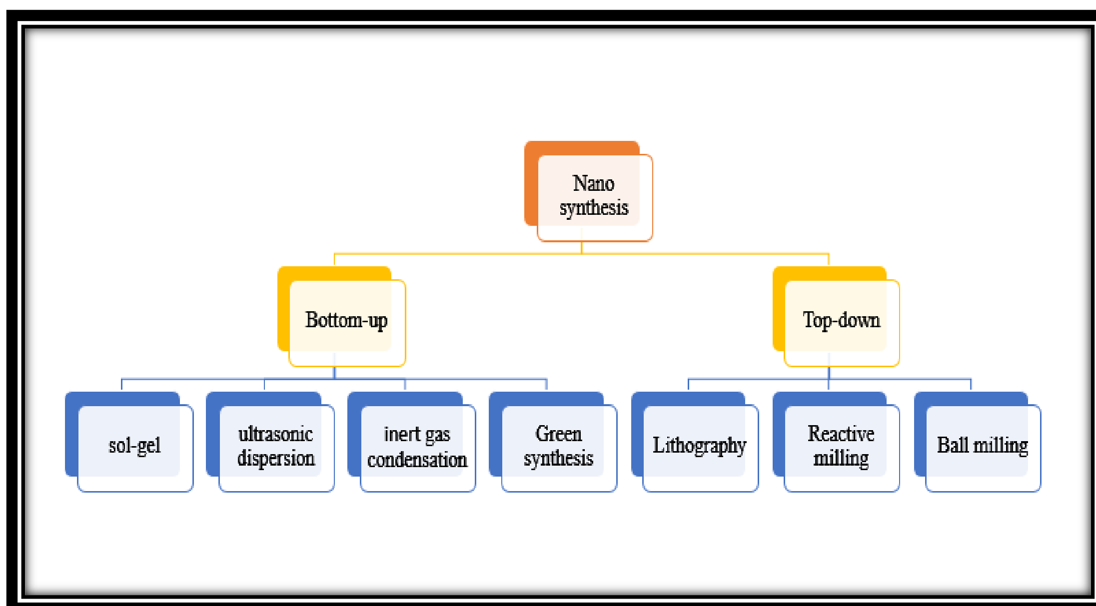


Fig:1.1. Process of NPs synthesis

There are primarily two kinds of methods for synthesizing nanoparticles: top-down and bottom-up techniques. Chemical procedures are often bottom-up, whereas physical ones are typically top-down (Behzad, Naghib, Tabatabaei, Zare, & Rhee, 2021). Green synthesis is categorized as a bottom-up methodology.

1.2.1. Constructive method

A constructive method known as "bottom-up" creates nanoparticles by joining small atomic-sized particles (Khan, Saeed, & Khan, 2019). It consists of inert gas condensation, sol-gel, green synthesis, solvo-thermal, sonochemical, and supersonic distribution (Tulinski & Jurczyk, 2017). The bottom-up approach involves creating nanoparticles (NPs) from small units like molecules and atoms, or else using a range of chemical and biological processes to cause atoms to self-assemble into nuclei and subsequently evolve into NPs (Jadoun, Arif, Jangid, & Meena, 2021)(Fig. 1.2).

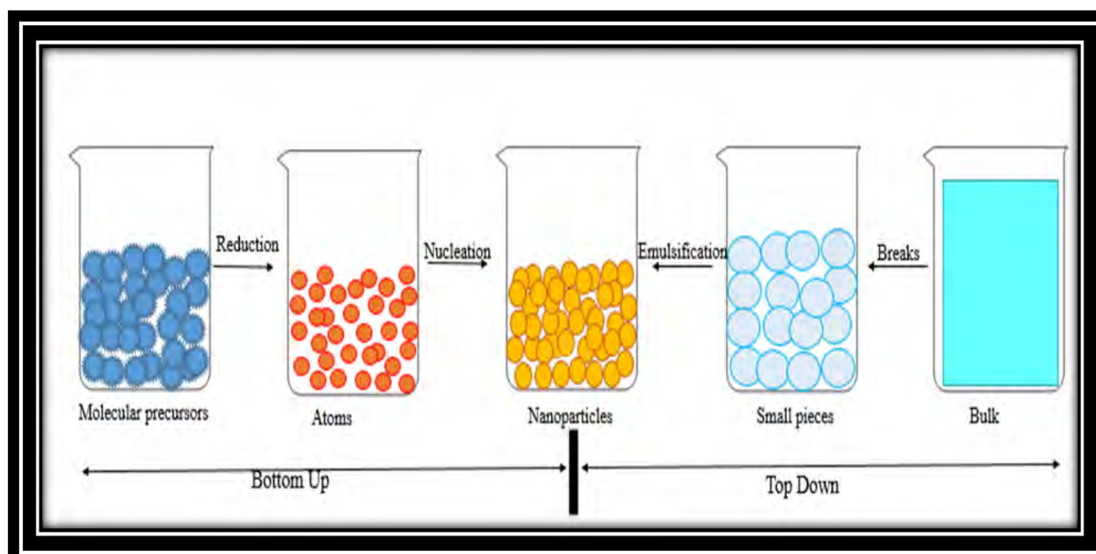


Fig: 1.2. The Top down and bottom-up processes.

1.2.2. Destructive method

A destructive method known as "top-down" breaks down large molecules into smaller ones, which ultimately results in the creation of useful nanomaterials (Khan et al., 2019). The top-down method frequently makes use of technologies like micromachining, reactive milling, physical alloying, deformation of plastic, lithography, etching, and high-energy ball milling (Tulinski & Jurczyk, 2017). In this approach, NPs are created by a size reduction process that uses lithography to convert bulk material into microscopic units through crushing, spitting, and grinding (Jadoun et al., 2021).

1.3. Methods of Nanoparticles Synthesis

NPs synthesized via physical, chemical and biological methods (Luechinger, Grass, Athanassiou, & Stark, 2010).

1.3.1. Chemical Method

Photo induced reduction, Nano emulsion, chemical reduction, irradiated process, UV triggered photo reduction, the microwave assisted synthesis, and electrochemical synthetic technique are some of the chemical procedures uses to create nanoparticles (Raza et al., 2016). It requires the employment of a variety of chemicals, including hydrazine and sodium borohydride, as reducing agents. While stabilizing compounds like thiophenol, mercapto-acetate, thiourea, and others are typically used to regulate the shape and avoid nanoparticle agglomeration. In addition, these chemicals are more

expensive than green alternatives, use more energy for manufacturing, utilize more chemicals, are not ecologically friendly, and can produce dangerous byproducts (Gomathi, Rajkumar, Prakasam, & Ravichandran, 2017). Sodium borohydride and other natural solvents have a water-resistant capping action. (NaBH4), dimethyl form amide (DMF), and hydrazine were initially employed as environmentally acceptable synthesis procedures. Using fluorinated ligands or supercritical CO2 as preservative or capping substance was also thought to be environmentally beneficial However, these environmentally friendly methods are made more difficult by the following: It is difficult to isolate nanoparticles using a CO2-philic surfactant. Micro-emulsions contain hazardous chemicals.). The inhibitors indicate a significant biological and environmental risk. The bulk of currently existing methods are evidently costly and ineffective as a result, necessitating the creation of a safe, clean, and environmentally acceptable synthesis process (Ahmad et al., 2003).

1.3.2. Physical Methods

The two most popular physical techniques are laser ablation and evaporation condensation. The uniform dispersion of nanoparticles and the avoidance of solvent contamination are advantages of using physical procedures over chemical ones. A number of variables affect the laser ablation procedure, such as the liquid media, wavelength, pulse duration, flux, and ablation time. Using the evaporation condensation process, very tiny NPs with sizes ranging from 6.2-21.5 nm are created. The evaporation condensation process makes it feasible to obtain a wavelength range of 1.23–1.88 nm. But the top-down (physical) method is time-consuming, expensive, and energy-intensive (energy is a limited resource needed to raise the working temperature) (Iravani, Korbekandi, Mirmohammadi, & Zolfaghari, 2014).

1.3.3. Biological Method

Natural alternatives to physical and chemical ways for the fabrication of tiny particles (NPs) include biological methods that use microbes (Konishi et al., 2007), enzymes (Willner, Baron, & Willner, 2006), fungi (Vigneshwaran, 2007), algae, plants or plant extracts, roots, and leaves. Highly regulated assembly processes are used to create plant-based biomolecules that are perfect for the fabrication of metal nanocrystals (R Nawab et al., 2022) Numerous biological materials, such as fruit peels and fungi, have been investigated in an effort to find appropriate reducing agents. Green ingredients include things like mango peel, clove (Syzygium aromaticum), aloe Vera, lemongrass

extract, flame lily (Gloriosa uperba), and neem. Various bacteria include Bacillus subtilis, Shewanella, Rhodococcus, which are utilized for gold, Pseudomonas Stutzeri, Lactobacillus, which are used for silver, and Desulfobacteriaceae, which are used for zinc sulphide (Ahmed & Ogulata, 2022). Alternanthera sessilis, which had a particle domain of 5–25 nm and an inhibitory zone of 21–29 mm for several other plants, was thought to have the finest extracts among the 16 plants tested by the researchers (Firdhouse & Lalitha, 2016). Additionally, the reduction process is employed to create the nanoparticles using biomolecules from plants and microorganisms, such as proteins and enzymes. Plants provide as a reliable supply of bioactive substances needed to produce metal nanoparticles. This method is economical and lessens the quantity of pollutants created. It is among the most straightforward, effective, affordable, sustainable, and environmentally acceptable methods of producing metal nanoparticles without the use of hazardous chemicals (R Nawab et al., 2022).

Table: 1.1. Synthesis of NPs by Fungi

Fungi	**Nature**	**Size**	**References**
Phoma sp.	Ag	71.06–74.46	(Hasan, 2015)
Fusarium oxysporum	Au	20–40	(Hasan, 2015)
Verticillium sp.	Ag	25 ± 12	(Hasan, 2015)
Aspergillus fumigates	Ag	5–25	(Hasan, 2015)
Trichoderma asperellum	Ag	13–18	(Hasan, 2015)
Phaenerochaete chrysosporium	Ag	50–200	(Hasan, 2015)

Table: 1.2. Synthesis of NPs by Bacteria

Bacteria	**Nature**	**Size**	**References**
Bacillus subtilis	Au	5–25	(Beveridge & Murray, 1980)
Rhodopseudomonas capsulate	Au	10–20	(He, Zhang, Guo, & Gu, 2008)

Stenotrophomonas malophilia	Au	40	(Sharma et al., 2012)
Lactobacillus sp	Ag	15–30	(FU, LIU, & GU, 2000)
Pseudomonas aeruginosa	Ag	13	(C. G. Kumar & Mamidyala, 2011)
Escherichia coli	CdS	2-5	(Sweeney et al., 2004)
Streptomyces sp	$MnSO_4$, $ZnSO_4$	10-20	(Waghmare, Deshmukh, Kulkarni, & Oswaldo, 2011)

Table: 1.3. Synthesis of NPs by Yeast

Yeast	Nature	Size	References
P. jadinii	Au	<100	(Agnihotri, Joshi, Kumar, Zinjarde, & Kulkarni, 2009)
Yarrowia lipolytica	Au	15	(Agnihotri et al., 2009)
Saccharomyces cerevisiae	TiO_2	12	(Jha, Prasad, & Kulkarni, 2009)

1.3.3.1. Green Synthesis

Plant extracts are utilized in the environmentally benign, biocompatible, and sustainable process of "green synthesis" to create nanoparticles for biological purposes (Razavi et al., 2015). Plant phyto-constituents serve as capping and dipping factors, and plant extracts are more suited for the synthesis of NPs (R Nawab et al., 2022). Plant extracts are combined with metal precursors at ambient temperature in a straightforward process called "green synthesis." Organic acids, or phyto-constituents, such flavonoids, amides, terpenoids, and ketones, are crucial for synthesis. While there are a number of hypotheses, the precise mechanism is still unknown. The biosynthetic process is a sustainable, ecologically acceptable method of producing nanoparticles for biological applications using microorganisms and plants (Razavi et al., 2015). Costs are increased when using microorganisms since careful handling and preservation are required (Vijayakumar et al., 2017). There are several disadvantages to employing bacteria, algae, and fungi in the production of nanoparticles, despite all of their advantages (Ijaz,

Shahid, Khan, Ahmad, & Zaman, 2017). The incubation procedure, bacterial toxicity, and microbial isolation present the most obstacles.

Plant extracts are therefore a great source of metal and oxide of metal nanoparticles (NPs). Plant materials such as leaves, bark, stem, seeds, fruit, and root extracts have been used in numerous environmentally friendly and quick production of nanoparticles (NPs) that have been documented in the past (Rabia Nawab et al., 2022). As lowering and capping agents, phyto-constituents produce efficient and enhanced performance. Plant extracts stabilize the production of nanoparticles. Particularly, plant extracts react considerably faster than other biological materials. For instance, it takes 24 to 120 hours for bacteria or fungus to become effective, yet it just takes a few hours for (Calotropis leaf) extract (R Nawab et al., 2022).

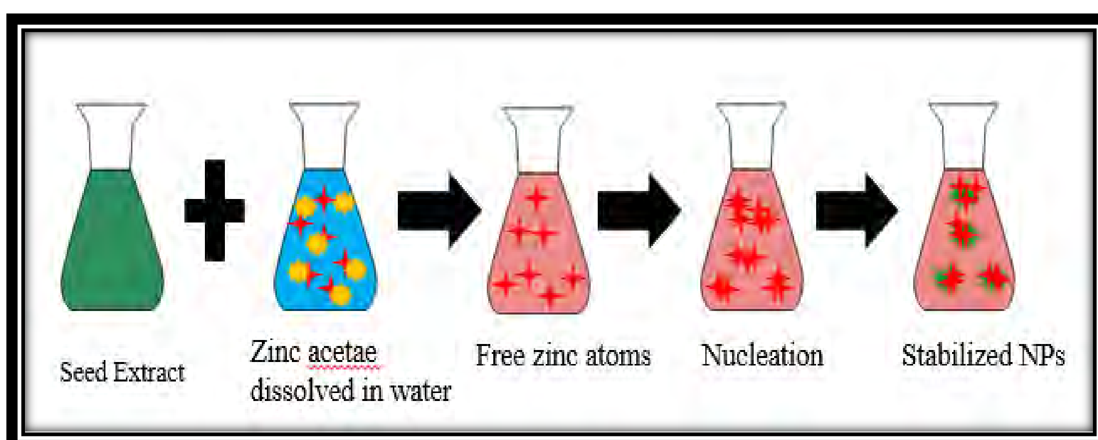


Fig:1.3. Synthesis Scheme

Table: 1.4. Synthesis of NPs by Plants

Plant	**Nature**	**Size**	**References**
Jatropha curcas	Ag	10-20	(Bar et al., 2009)
Cycas	Ag	2-6	(Jha & Prasad, 2010)
Prosopis farcta	Ag	10.8	(Miri, Sarani, Bazaz, & Darroudi, 2015)
Aloe vera	Ag	70	(Medda, Hajra, Dey, Bose, & Mondal, 2015)
Ferula persica	Au	37.05	(Hosseinzadeh et al., 2020)

Phragmites australis	Au	18	(El-Borady, Fawzy, & Hosny, 2021)
A. halimus	Au	2-10	(Hosny, Fawzy, Abdelfatah, Fawzy, & Eltaweil, 2021b)
C. amperosidies	Au	40	(Hosny et al., 2021b)
Punica granatum	Cu	40	(Ghidan, Al-Antary, & Awwad, 2016)
Aloe barbadensis Miller	Cu	15-30	(Gunalan, Sivaraj, & Venckatesh, 2012)

Nucleation/Growth

Fluctuations in free energy lead to nucleation. When additional surfaces appear, a solution's free energy shifts. The energy shift will therefore result in nucleation. At this point, the previously formed atoms are accumulated by this free energy (Burda, Chen, Narayanan, & El-Sayed, 2005).

Limiting Aggregation (Stabilization)

A small nucleation phase is necessary to produce NPs with a consistent size. In this grade, the smaller particles develop more quickly than the larger ones because of the higher free energy. To obtain the intended NPs, aggregation is avoided. If not, the bigger particles will enlarge significantly. Because of its flexible connections and plenty of phytochemicals, green synthesis inhibits agglomeration (Li et al., 2007). Plant chemicals' functional groups function as a polar head and the remaining portion as a nonpolar tail. Steric stability will be attained as a result. NPs with a high antioxidant activity (AOA) are tiny in size. Consequently, there is an increase in the rate of NP creation and stability.

Gold nanoparticles are an example of a man-made nanomaterial, whereas volcanic ashes are a naturally occurring material.

1.4. Types of Nanoparticales

Several metallic nanoparticles (NPs) that have been discovered, including copper (Cu), zinc (Zn), titanium (Ti), gold (Au), magnesium (Mg), alginate, and silver Ag. (Hasan (2015).

1.4.1. Zinc

Several metal nanoparticles, such as silver, gold, iron zinc, and others, have been described to have antibacterial and antifungal properties (Vahabi, Mansoori, & Karimi, 2011). Silver and gold nanoparticles are rarely used on broad scales due to their high cost, however zinc oxide nanoparticles (ZnO NPs) are considered to be less expensive and more effective (Vahabi et al., 2011). Zinc oxide and its various forms have also been classified as "safe compounds" by the US Food and Drug Administration (Raghupathi, Koodali, & Manna, 2011). ZnO NPs possess antibacterial and antifungal activities (Zheng & Shetty, 2000). Metal oxide's harmful effects must yet be investigated before they may use in the field (Patra & Biswas, 2017). The precise process of the antifungal capability of nanoparticles is not fully understood (de Carvalho Paulino et al., 2022). Plant diseases, such as those caused by fungi, are often treated with zinc; however, nanoparticles of zinc oxide (ZnO-NPs) are far more effective at preventing the spread of plant fungal infections. Previous research has shown that ZnO-NPs have the capacity to suppress fungal plant diseases such as rotting by directly inhibiting fungal growth by disrupting the developing mycelia and also by removing microbes. Nanoparticles may also have an impact on the formation of reactive oxygen species, which are the cause of the breakdown of lipids and, ultimately, the destruction of the microbial cell (Mohamed et al., 2019). ZnO NPs exhibit significant antifungal properties against a broad spectrum of mycotoxin-producing fungi, including Aspergillus flavus, Fusarium oxysporium (Raghupathi et al., 2011). ZnO NPs hinder mycelial development and disrupt cellular activities in a variety of fungi (Kalsa & Abebie, 2012).

1.4.2. Copper

Numerous biochemical and physiochemical reactions in plants depend on copper. It's one of the most crucial trace factors for plant growth (Ghaderian & Ravandi, 2012). As it is a cofactor for many different enzymes, it is necessary for the proper operation of several vital proteins and enzymes, such as plastocyanin, cytochrome c oxidase, and amino oxidase (Sifri, Burke, & Enfield, 2016). Copper oxide nanoparticles (CuO NPs) were produced using an extract from the peel of *Punica granatum* (Ghidan et al., 2016).

Aloe barbadensis Miller is a valuable medicinal plant from which Gunalan et al. successfully produced CuO NPs (Gunalan et al., 2012). It has also been discovered that *Cordia sebestena* flower extract is useful for creating CuO NPs. The spherical, 20–40 nm-sized nanoparticles were described by Prakash et al. (2018). Additionally, it has been shown that CuO NPs can be synthesized from the leaves of *Alternanthera sessilis*, *Olea europaea*, and *Citrofortunella microcarpa* (Waris et al., 2021).

1.4.3. Gold

Using gold nanoparticles, immunochemical studies can identify protein interlinkage. (AuNPs). They are used as a lab marker in DNA profiling to show that DNA is present in a sample. Using AuNPs, aminoglycoside medications like gentamycin, neomycin, and streptomycin are also identified. Au Nano rods are excellent for identifying and diagnosing cancer because they can detect tumors (Tomar & Garg, 2013). Numerous studies have proven that AuNPs have outstanding optoelectronic capabilities, a high ratio of surface to volume, and minimal toxicity. The production of AuNPs was investigated in extracts from *Potamogeton pectinatus* (AbdelHamid, Al-Ghobashy, Fawzy, Mohamed, & Abdel-Mottaleb, 2013), *Ferula persica*, and *Phragmites australis* (El-Borady, Fawzy, & Hosny, 2023). It is expected that these extracts from wild plant species would provide a range of sizes and forms for various uses. *Atriplex halimus* and *Chenopodium amperosidies* are two rapid, easy-to-use, and environmentally beneficial ways to moderate AuNPs. The diameters of *A. halimus* AuNPs range from 2 to 10 nm, whereas the sizes of *C. amperosidies* can reach up to 40 nm (Hosny, Fawzy, Abdelfatah, Fawzy, & Eltaweil, 2021a).

1.4.4. Alloy

Alloy nanoparticles differ structurally from their parent materials. Condensing vaporized Cu and Ag in an inert environment produced the Ag-Cu NPs. Copper's solubility in silver increased due to the particles' accelerated solidification. An ideal evaporation temperature when solubility reaches its maximum for a fixed carrier gas pressure. Since AgNPs have the highest electrical conductivity of all metal fillers and, in contrast to other metals, its oxides have higher conductivity, AgNPs are the most often used metallic filler (Hasan, 2015). Both metals influence the characteristics of bimetallic alloy nanoparticles, which have greater advantages than regular metallic NPs (Mohl et al., 2011).

1.4.5. Silver

The exceptional conductivity, chemical strength, speed booster, and antibacterial characteristics of silver nanoparticles (AgNPs) have garnered significant interest in recent years (Frattini, Pellegri, Nicastro, & De Sanctis, 2005). Silver nanocrystals (AgNPs) have been widely used in many biological and pharmacological applications because of their unique anti-microbial capabilities. AgNPs are nontoxic, inexpensive, easy to process, sustainable, and beneficial to the environment (R Nawab et al., 2022). The anti-microbial effectiveness of silver nanoparticles against bacteria, viruses, and other eukaryotic microorganisms makes them highly effective (Gong et al., 2007). Silver nanoparticles are among the most frequently used nanoparticles in the medical field, nutriment conservancy, textile coating, water management, health industry, sterilizing medical equipment, household items, medicine, pollution, and solar energy conversion. AgNPs have been extensively studied for their anti-microbial efficacy and are bio-genically synthesized from microorganisms and plant extracts. AgNPs are produced when a variety of biomolecules, including proteins, aldehydes, flavonoids, ketones, tannins, carboxylic acids, and phenolic acids, oxidize Ag+ to Ag0 in plant extracts (Allafchian et al., 2016). Plants that have been used to synthesize AgNPs include Cycas (Jha & Prasad, 2010), Calotropis procera (R Nawab et al., 2022) and Jatropha curcas (Bar et al., 2009). AgNPs with mean range of 10.8 nm at 25 oC were created by Miri et al. using an extract from Prosopis farcta (Miri et al., 2015).

1.4.6. Magnetic Nanoparticles

Biocompatibility has been established for attractive tiny entities such as Fe_3O_4 (magnetite) and Fe_2O_3 (maghemite). They are being intensively researched for directed medicals delivery, gene treatment DNA analysis, magnetic resonance imaging (MRI), stem cell sorting and manipulation, targeted cancer therapy (magnetic hyperthermia), and guided drug delivery (Fan, Chow, & Zhang, 2009).

1.5. NANOTECHNOLOGY IN AGRICULTURE

The only way to enhance agriculture in light of the declining availability of water and fertile land is to increase resource utilization while minimizing harm to the agro-ecological system through the right deployment of advanced technologies. Nanotechnology could have applications in water management, energy generation, biosciences, agricultural systems, and environmental science protection. By addressing

problems that have hitherto remained unsolved, nanotechnology—which operates with the tiniest particles—holds great potential for improving agricultural productivity. There are initiatives underway to increase productivity in terms of management. In terms of management, the release of fixed nutrients via Nano clays and zeolites is what maximizes the productivity of the fertilizer and restores crop yields. Studies on smart seeds with Nano polymer coatings that are intended to sprout in ideal circumstances is yielding encouraging results. The agricultural sector and efficient farming resource requirements of crops are identified with the use of Nano biosensors and satellite systems. According to needs, essential quantities are delivered at the proper time and location in a regulated environment. The creation of Nano-herbicides is currently being used to address the decline of the plant bank and recurrent weed control issues. Through techniques such as site-specific delivery, controlled release, and conditional release, nanostructured formulations were able to more precisely release their components in response to biotic demands and environmental stimuli. Studies show that using Nano-fertilizers lowers soil toxicity, lessens the likelihood of overdosing and its potential harmful effects, as well as increasing nutrient use efficiency and reducing treatment frequency. Therefore, there is great potential for achieving sustainable agriculture with nanotechnology, especially in underdeveloped nations (Manjunatha, Biradar, & Aladakatti, 2016).

The need to feed the projected 9 billion people by 2050 has sparked advances in nanotechnology, and in the near future, research on the interaction of nanotechnology, food, and agriculture will take precedence. To increase agricultural productivity, plant scientists have been employing a range of metal oxide nanoparticles as Nano fertilizers. Significant growth in plant dry biomass (12.5 percent), shoot length (15.1%), root length (4.2%), root area (24.2%), chlorophyll content (24.4%), total soluble leaf protein (38.7%), and enzymatic activity of acid phosphatase (76.9%), alkaline phosphatase (61.7%), phytase (322.2%), and dehydrogenase (21%) as a result of applying Nano-fertilizer, while 37.7% of crop yields increased when the crop reached full maturity. Rahore and Mahawar (2014). Plants can absorb more light, minerals, and water when nanoparticles are present. To increase production and raise the caliber of agricultural goods, Nano biotechnology is gradually being applied in agriculture (Raliya, Nair, Chavalmane, Wang, & Biswas, 2015).

1.6. GEOGRAPHIC ORIGIN AND DISTRIBUTION OF OKRA

The okra plant, often known as lady finger, was formerly a member of the family Malvaceae and genus Hibiscus, section Abelmoschus (Charmantier & Müller-Wille, 2014). It was therefore suggested that the section Abelmoschus be elevated to the status of separate genus (Medicus, 1787). The taxonomy and modern literature later acknowledged the broader usage of Abelmoschus. The calyx of the genus Hibiscus is spathulate, has five small teeth, connates to the corolla, and becomes caduceus after flowering (Terrell & Winters, 1974). Originating in the Ethiopian region, okra was first domesticated by the Egyptians by the 12th century B.C. Its cultivation extended over North Africa and the Middle East (Tindall, 1983). The most comprehensively recorded studies of the genus Abelmoschus are those resulting from the taxonomical revision that was started by Borssum and colleagues (van Borssum Waalkes, 1966)and continued by Bates (D. M. Bates, 1965). An updated classification was adopted at the 1990 International Okra Workshop held at the National Bureau of Plant Genetic Resources (NBPGR), using van Borssum Walkers' classification as a starting point. Eight are the most often accepted species out of the approximately 50 that have been described (Perret, 1996). Around the world, okra is cultivated, particularly in tropical and subtropical nations. This crop can be cultivated as a garden crop or on a huge commercial farm (Rubatzky & Yamaguchi, 2012). Many nations, including Bangladesh, Afghanistan, Pakistan, Myanmar, Malaysia, Thailand, India, SSBrazil, Ethiopia, Cyprus, Turkey, Iran, India, and Western Africa, as well as the Southern United States, cultivate okra economically (Benchasri, 2012; Benjawan, Chutichudet, & Kaewsit, 2007).

1.6.1. STRUCTURE AND PHYSIOLOGY

Worldwide, warm temperate and tropical countries produce Abelmoschus esculents for its spherical, white seeds found in fibrous fruits or pods. It is among the most thermal and adaptable to drought kinds of vegetables on the planet., and while it can withstand thick clay soils and sporadic wet spells, cold can harm the pods. Before planting the seeds to a depth of 1-2 cm in culture, they are soaked for the entire night. When seeds are wet, germination takes place in six to three weeks. Seedlings consumed plenty of water. After the fruit has been pollinated for one week, the seed pods must be collected in order for them to become edible as they quickly become woody and fibrous. When

the fruits are still young, they are harvested and consumed like vegetables (P. Singh et al., 2014).

1.6.2. CYTOGENETIC RELATIONSHIP

In the genus Abelmoschus, there are notable differences in the ploidy levels and chromosomal counts of the various species. A. angulosus has the lowest number reported (2n=56) and A. manihot var. caillei has the highest number reported (approximately 200) of chromosomes [29, 30, 31]. A. esculents has chromosome numbers of 2n = 72, 108, 120, 132, and 144, which are in a regular series of polyploidy with n = 12 (Owolarafe & Shotonde, 2004).

1.6.3. Scientific classification

Kingdom:	**Plantae**
Division:	**Magnoliophyta**
Class:	**Magnoliopsida**
(Unranked):	**Rosids**
Order:	**Malvales**
Genus:	**Abelmoschus**
Species:	**A.Esculentus**
Binomial name:	**Abelmoschus esculents**

1.6.4. Chemical composition:

A multilayered fiber called okra bast was examined, and the approximate typical chemical makeup of OBF (Abelmoschus esculents variety) is given below

67.5 %	a-cellulose
15.4 %	Hemicelluloses
7.1 %	Lignin
3.4 %	pectin matter
3.9 %	fatty and waxy matter

2.7 %	aqueous extract

It is evident that the primary components of OBF are lignin, a-cellulose, and hemicelluloses; the remaining ingredients are present in very small amounts and therefore have minimal effect on the structure of OBF. Thus, the composition of lignin, a-cellulose, and hemicelluloses, as well as the ways in which they combine, dominate the structure of OBF.

1.6.5. BIOCHEMICAL COMPOSITION OF OKRA

In every 100 grams of edible portion, okra pods contain the following:

Water	88.6 grams
Energy	144.00 kJ; 36 kcal
Protein	2.10 grams
Carbohydrate	8.20 grams
Fat	0.20 grams
Fiber	1.70 grams
Calcium	84 mg
Potassium	90.00 mg
Iron	1.20 mg
beta-carotene	185.00 µg
Riboflavin	0.08 mg
Thiamin	0.04 mg
Niacin	0.60 mg
ascorbic acid	47.0 mg

Okra is one of the significant ingredient of human diet and contains protein, sugars and vitamin C (Lamont, 1999). Eating young, immature okra pods is beneficial for your health since they are one of the many forms that you can eat (Kochlar & Joseph, 1986). The fruit of the okra plant is mostly eaten raw or cooked. It is rich in minerals, vitamins A, B, and C, iron, and iodine. It is also a significant vegetable source of dietary fiber, but it is low in cholesterol and saturated fat (Adebooye & Oputa, 1996; Kendall & Jenkins, 2004; P. Singh et al., 2014). It has also been reported that Fe, Zn, Mn, and Ni are present (Moyin-Jesu, 2007). Okra is a good source of minerals, including vitamins, calcium, potassium, and other elements that are frequently deficient in diets in

developing nations (Joshua, 2011). The highest amount of nutrients is found in fresh okra pods that are no more than seven days old or younger. (Agbo, Gnakri, Beugre, Fondio, & Kouamé, 2008). In every 100 grams of edible portion, okra leaves contain the following:

Water	81.50 grams
Energy	235.00 kJ; 56.00 kcal
protein	4.40%
Fat	0.60%
carbohydrates	11.30 grams
Fiber	2.10 grams
calcium	532.00 mg
potassium	70.00 mg
Iron	0.70 mg
ascorbic acid	59.00 mg
beta-carotene	385.00 µg
thiamin	0.25 mg
riboflavin	2.80 mg
Niacin	0.20 mg

Mucilage is the primary form of carbohydrates (Agnihotri et al., 2009; Liu, Liou, Lan, Hsu, & Cheng, 2005). You can eat the flowers and leaf buds as well. Okra seeds have a 20% protein content and a 20% oil content. There is a chance that okra seed oil lowers cholesterol (Srinivasa Rao, Udayasekhara Rao, & Sesikeran, 1991). There is a lot of potential for okra to be widely cultivated for cake and edible oil (Udayasekhara Rao, 1985). It is also possible to fortify cereal flour with okra seed flour. Okra meal, for instance, boosts the amount of protein, ash, oil, and fiber in maize (Akingbala, Akinwande, & Uzo-Peters, 2003). In nations like Egypt, okra seed flour has long been used in place of corn flour to produce dough of higher quality. Some nations grind and roast its ripe seeds and use them in place of coffee. The paper industry uses mature fruits and stems with crude fiber. Pressed from okra seeds, greenish-yellow edible okra oil has a pleasant taste and aroma and is rich in unsaturated fats like linoleic and oleic

acid. Certain seed varieties have a relatively high oil content—roughly 40%. Crops of okra also yield a lot of oil. Okra oil was found to be suitable for use as a biofuel in a 2009 study (Anwar, Rashid, Ashraf, & Nadeem, 2010). Okra stems and roots are used to clarify sugarcane juice, which is then used to make brown sugar or gur. ***Parts used***: fruit, leave seed, root20

1.6.6. Medicinal use

According to reports, it has medicinal value in treating hemorrhoids and ulcers (Ghanem, 2003). A number of sources related to herbal and traditional medicine mention the 1898 report (Adams, 1975) that certain plant parts had diuretic qualities. Medical applications of okra include blood volume expansion and plasma replacement (Savello, Martin, & Hill, 1980). Along with other beneficial compounds for medicine, it is also a good source of iodine, which helps treat simple goiter (P. Singh et al., 2014). The treatment of spermatorrhoea, chronic dysentery, and genitourinary disorders is highly beneficial (Nadkarni, 1927). Studies carried out in China indicate that an alcohol-based extract derived from okra leaves has the potential to eradicate oxygen free radicals, mitigate renal tubular interstitial diseases, lower protein urea levels, and enhance renal performance (Agnihotri et al., 2009; Liu et al., 2005). There has been a report from 1898 (P. Singh et al., 2014)that certain plant parts had diuretic properties; this information can be found in many sources related to herbal and traditional medicine. Research on okra extract as a diabetes remedy is presently being planned.

1.6.7. Propagation and cultivation

Because of its fibrous fruits or pods that contain spherical, white seeds, Abelmoschus esculents is grown all over the world in both warm temperate and tropical climates .It's among the vegetables that can withstand the most heat and drought worldwide. species, and it can withstand thick clay soils and sporadic wet spells, while cold can harm the pods. Before planting, the seeds are soaked for the entire night in 1-2 cm of water for cultivation. Seeds that have been soaked for six days to three weeks will germinate. Water is essential for seedlings. After the fruit is pollinated, the seed pods quickly become woody and fibrous, and they must be collected not more than seven days in order to be edible. When the fruits are still young, they are picked and consumed as vegetables

1.6.8. Trachyspermum ammi l Sprague

Trachyspermum ammi (L.) Sprague, commonly known as Ajwain, is an annual herbaceous plant that is a valued member of the highly significant Apiaceous family of medicinal plants (Shuja et al., 2022) It's claimed that the herb is commonly planted in semi-arid and arid areas. Where there is a significant salt content in the soil (Joshi et al., 2000) Ajwain possesses a striate, upright stem with glabrous or minute pubescent characteristics that can reach a height of 90 cm tall (Chatterjee ASC., 1995) Ajwain is grown and distributed widely indifferent areas, including Afghanistan, Pakistan, Iran, and Although it is native to Egypt, it is also found in Europe and India (Shojaaddini et al., 2008). Typically, the plant is grown in October through November. ought to be picked in May or June (Chauhan B et al., 2012). Typically, gray Ajwain fruits or brown seeds are thought to be used for dietary and medical reasons (Aggarwal et al., 2011).

1.7. AIMS AND OBJECTIVES

- Collection of diseased pods of Abelmoschus esculents
- Isolation, identification and characterization of disease causing agent
- Synthesis and characterization of green synthesized of ZNO NPs

In vivo and in vitro antifungal activity of synthesized NPs against the rotting caused by fungus

CHAPTER 2

MATERIALS & METHODS

2. Material and Methods

2.1.Sample Collection

In May and June 2023, okra fruit with disease spots was procured from the regional markets of Rawalpindi, Pakistan. In order to isolate the fungus that cause infections, these harvested sample were placed in plastic stacks and brought to the lab.

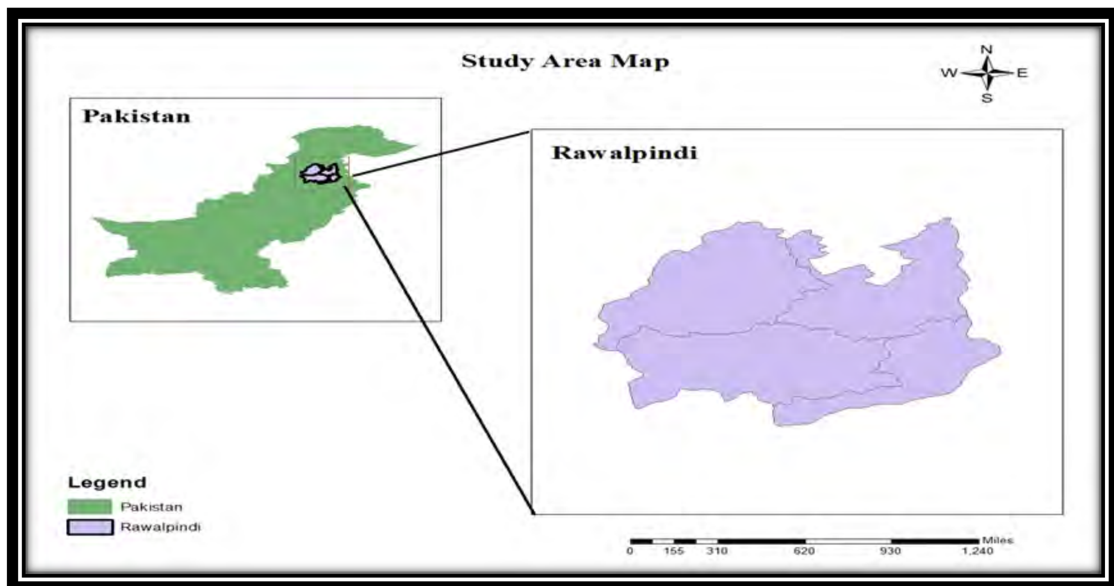


Fig: 2.1. Study Area Map

2.2. Isolation of disease-causing pathogen from okra

The tissue planting method was employed to isolate the pathogen from the infected okra pods. For two minutes, 70% ethanol was used to surface sterilize the infected sample. The specimen from the affected area was cleaned, sliced with a sterilized blade, and placed in a Petri plate with potato dextrose agar (PDA)—potato substance was prepared by simmering 250 grams of potatoes in 500 milliliters of water... Potato extract was mixed with 20 grams of dextrose and 15 grams of agar. Give it a good stir, then add distilled water to bring the volume up to one liter. For fifteen minutes at fifteen PSI, autoclave at 121° C. Fill cell culture dishes with the media, then leave them to gelatinize. After using ParafilmTM to seal the petri plates, they were nurtured at 27° for seven days. The mycelia from the fungal colony's margins were moved to PDA plates in order to obtain pure culture. Cultural characteristics were seen after the pure culture was incubated for seven days.

2.3. Chemical reagents

The analytical reagent-grade chemicals used in the experiment were purchased from Sigma Aldrich in Germany

2.4. Microscopic analysis of disease-causing Pathogen

Hyphae and reproductive organs of the fungus that was isolated were examined under a light microscope for identification. A small amount of lactophenol blue and lactic acid droplets were placed on the slide. On the slide, mycelium was selected from the margins of the fungal culture. After the cover slip was carefully placed, taking care to prevent air bubbles, the slide was examined under a light microscope with a magnification of 100 ×.

2.5. Molecular identification of Pathogen

A standard protocol was followed to isolate fungal DNA (Jin, Lee, & Wickes, 2004). Calmodulin (CaM) gene amplification was done using polymerase chain reaction (PCR) and CMD5/CMD6 primers. In a similar manner, tubulin primers (Bt2a/Bt2b) were also used to amp up the tubulin gene (Liaquat, Munis, Arif, Che, & Liu, 2019). Each primer (1 µl), genomic DNA (1 µl), Taq DNA polymerase (1.5 µl), dNTPs (6 µl), and 10× polymerase buffer (5 µl) were added to the reaction mixture. After four minutes of reaction time at 94°C, there were thirty-two cycles of 94°C for sixty seconds, 58°C for one minute, and 72°C for sixty seconds. After 35 cycles for terminal extension, the PCR was kept at 72 °C for 10 minutes. The PCR product was sequenced, utilized on the NCBI database (http://www.ncbi.nlm.nih.gov) for BLAST analysis. Nucleotide sequences were aligned using MEGA 7.0 (Tamura, Nei, & Kumar, 2004), and 1,000 bootstrap replications were used to build the phylogenetic tree.

2.6. Phylogenetic Analysis of Isolated Fungus

MEGA version 7 was used for the phylogenetic and evolutionary analyses (S. Kumar, Stecher, & Tamura, 2016). To deduce the evolutionary connection of all linked fungal strains, the neighbor-joining technique was applied (Lombard, Sandoval-Denis, Lamprecht, & Crous, 2019). To compare evolutionary distances, the maximum composite likelihood method was applied (Lombard et al., 2019).

2.7. Pathogenicity evaluation by following Koch's postulate.

Koch's postulates were adhered to, using a set procedure, in order to study the pathogenicity of isolated fungi (Kaur et al., 2019). Eight healthy pods were pierced with sterile needles in order to achieve this (6 mm deep). As a control, four uninfected pods were inoculated with conidial suspension (5 mL of water) and four fruits with 1 106 conidia per ml. Pods that had been inoculated underwent a five-day incubation period at 27 °C while being wrapped in sterilised muslin cloth to monitor disease symptoms. Finally, the pathogen that caused the sickness was re-isolated on SDA media and compared to the pathogen that had had been isolated initially.

2.8. Collection of Plant Material

Trachyspermum ammi L Sprague fresh seeds were bought from Punjab Cash and Carry Islamabad Pakistan. After washing the Islamabad seeds twice under running water and then twice with distilled water to remove any dirt or other impurities from their surface, the leaves were shade-dried at room temperature.

2.9.Preparation of Plant Extract

A solution of dry seeds (500) was prepared and then heated to 80°C in a water bath for half an hour. After allowing the solution to cool to room temperature, a muslin cloth and sterilized What- man filter paper no. 1 were used to filter the mixture and extract the water. Plant extract's pH was measured, and it was stored at 4C until needed again.

2.10. Preparation of Trachyspermum ammi L. Sprague seeds mediated Zinc Oxide Nanoparticles (ZnO NPs)

Purified filtrate and zinc acetate solution (5 mM) were combined in a beaker in a 1:1 ratio to produce ZnO NPs. In a shaking incubator, the mixture was shaken for 24 to 48 hours at 150 rpm and 40 C. The change in color of the solution was used to determine the shape of reduction of ZnO NPs. Samples were centrifuged for 15 minutes at 10,000 rpm. The pallet was retrieved, washed, and stored at 40 C overnight. The nanoparticles were heated in a furnace for two hours at 500 C.

2.11. Characterization of reduced ZnO NPs

2.11.1. UV-Visible Spectroscopic Analysis

The UV-vis spectra of the solution were analyzed using a UV-vis spectrophotometer (Shimadzu model UV-1601) in the 300 to 800 nm region to confirm the reduction process of ZnO NPs.

2.11.2. Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectroscopy was performed on *Trachyspermum ammi* L. Sprague seeds mediated ZnO NPs using the KBr pellet method to ascertain the kind and nature of distinct structural features and functional groupings. A scan range of 400 to 4000 cm-1 was employed for this (Mohamed et al., 2019).

2.11.3. XRD ANALYSIS

Where D is used to calculate the average crystalline size, β is FWHM (full width at the half maximum), K denotes the factor of shape, λ represents the X-ray wavelength, and θ denotes the angle of diffraction (Ali, Haroon, Khizar, Chaudhary, & Munis, 2020). To study resulting data, X'Pert High Score software was used.

2.11.4. SEM

By sonicating a nanoparticle suspension in double distilled water for 7 minutes, the elemental compositions and morphologies of nanoparticles were measured. A little amount of the sonicated suspension was dried under a lamp on conductive tape with a double carbon covering. A (SEM) thermionic emission system was employed for SEM evaluation.

2.11.5. In Vitro Study f or Mycelial Growth

The ZnO NPs' antifungal activity was assessed via the tainted food strategy. (R Nawab et al., 2022). PDA media having different doses (0.25 g/mL, 0.50 g/mL, 0.75 g/mL, and 1.0 g/mL) of ZnO NPs were used for this purpose. 20-25 mL of media were put into Petri plates and solidified. Fungal plugs (5 to 7 mm) from a 6-day old *F. oxysporum* culture were inserted in the middle of these Petri plates and cultured at 24-28 C for 7

days. The media without ZnO NPs suspension served as a control. Metalaxyl + mancozeb fungicide antifungal activity was also evaluated at the same concentrations [(0.25 g/mL (T1), 0.50 g/mL (T2), 0.75 g/mL (T3), and 1.0 g/mL (T4)]. The following formula was used to calculate the inhibition of mycelial growth:

$$\text{Growth inhibition percentage} = (C - T) / C \times 100 \quad (1)$$

C = Mean of mycelial growth in control petri dishes,

T = Mean of mycelial growth in treated petri dishes

2.12. *In vivo study for control of disease on fruits*

Okra decay was controlled using four different dosages of the *Trachyspermum ammi* L. Sprague seeds mediated -ZnO NPs (0.25, 0.50, 0.75, and 1.0 mg/ml) (Kaur et al., 2019). A sterile needle was used to pierce 30 healthy okra pods for the purpose of inoculating them with a specific fungal solution (1 106 conidia per ml). Two days after the inoculation, the inoculated pods were sprayed with the four NP concentrations stated above, whereas the control fruit received a double distilled water spraying. Each fruit's disease area was assessed after each fruit was incubated at 26 °C for a week

2.13. *BIOCHEMICAL PARAMETERS OF PLANTS*

2.13.1. *ESTIMATION PROLINE CONTENT OF PODS*

The Proline stuff of okra pods was ascertained using a standard procedure (L. S. Bates, Waldren, & Teare, 1973). Four milliliters of a 3% sulfo salicyclic acid aqueous solution were used to grind up fresh plant material (0.1 g). The samples were centrifuged for five minutes at 3000 rpm. Next, 2 ml of acidic ninhydrin solution was added to 2 ml of supernatant. This solution was made by dissolving 1.25 g of ninhydrin in 30 ml of glacial acetic acid and 20 ml of 6 M phosphoric acid. Samples were then allowed to cool after an hour of incubation at 100 °C in a water bath. The mixture's absorbance was measured at 520 nm, and the Proline content was computed in µg/g. The following formula was used to determine the Proline content:

Proline Content= k value x dilution factor x absorbance / fresh sample weight

Where:

k value = 17.52

dilution factor = 2

sample weight = 0.5g

2.14. PHYSIOLOGICAL PARAMETERS

Relative Water Content (RWC)

The fresh weight (FW) of the okra pods was measured in order to compute RWC. The turgid weight (TW) of the pods were measured after they were submerged in water for 24 hours. The pods were dried in a hot air oven until their dry weight (DW) was constant. The given formula was used to define RWC (Wheatherley, 1950).

RWC (%) = [(FW – DW) / (TW – DW)] x 100

Relative Electrolytic Leakage (REL)

A standard method was used to calculate the relative electrolytic leakage (REL) (Lutts, Kinet, & Bouharmont, 1996). In test tubes containing d.H2O, 0.1 grams of pods discs for each treatment were used for this purpose. REL was determined after pods were submerged in a water bath at 40°C for 30 minutes (C1). The EL was then recorded once more using the same pods sample in a water bath at 100 °C for ten minutes (C2).

Formula: Membrane stability index was calculated.

Membrane Stability Index = [1 – C1/ C2] x 100

2.15. ANTIOXIDANT ENZYMES ASSAYS

ESTIMATION OF POD

With a few minor adjustments, an optimized protocol (Vetter, Steinberg, & Nelson, 1958) was used to measure peroxidase activity (Gorin & Heidema, 1976). Using a pre-cooled mortar and pestle, 0.2 grams of plant material were ground in 3 milliliters of 100 milliphosphate buffer. The mixture was then centrifuged at 10,000 rpm for 15 minutes at 4°C. Following that, the absorbance was measured at 436 nm after mixing 100 µl of the supernatant with the reference solution. POD was computed using the subsequent formula:

POD (U/I) = Vr × Vt × 1000 / ε × L × Δt × 0.100

Where:

V_r = Total volume of reaction mixture (2.18 ml)

V_t = Crude enzyme solution in test cuvette (100 µl)

ε = Extinction coefficient at 436 nm (6.39 cm^2/ µmol)

L = Light path (1 cm)

Δt = Time duration mandatory for expansion in extinction to 0.100

0.100 = 1 unit of POD defined as amount of enzyme that increases 0.100 of absorbance at 436 nm/minute.

- Phosphate Buffer (100 mM, pH 7.0)

(a) Dissolved 13.61 g KH_2PO_4 in 1000 ml d.H_2O.

(b) Dissolved 22.82 g $K_2HPO_4.3H_2O$ in 1000 ml d.H_2O.

Made buffer (100 ml) by mixing solution a (39 ml) with solution b (61 ml).

- Guaiacol (20 mM)

Dissolved 249 mg guaiacol in 100 ml d.H_2O.

- H_2O_2 (12.3 mM)

Diluted 0.14 ml 30% H_2O_2 in 100 ml distilled water.

Superoxide Dismutase (SOD)

The protocol that had already been established was followed to assay superoxide dismutase (Beauchamp & Fridovich, 1971). 0.2 grams of plant material were ground in 4 milliliters of solution (a), and 10,000 rpm centrifugation was used. The supernatant's volume was increased to 8 ml (pH = 7.0). For this activity, three assays were conducted using supernatant as an enzyme source (Table 2.1).

Assay for Superoxide Dismutase Activity (Table 2.1)

Subsequently, the reaction mixture samples were placed in a light chamber for twenty minutes, while the reference samples were kept in complete darkness. A spectrophotometer was used to record the absorbance at 560 nm. The unit of SOD activity was units/100 g F.W.

Formula:

Following measurements were made:

R_1 = O.D of reference

R_2 = O.D of blank

R_3 = O.D of sample

$R_4 = R_3 - R_2$

$A = R_1 (50/100)$

Final = R_4 / A

☐ Phosphate Buffers

❖ Monosodium dihydrogen phosphate (MDP): 15.6 g was mixed in 500 ml d.H_2O

❖ Disodium hydrogen phosphate (DHP): 53.65 g was mixed in 600 ml d.H_2O

Phosphate Buffer of pH 7: 117 ml of MDP solution was mixed with 183 ml of DHP solution. Total volume was raised to to 600 ml with d.H_2O.

- ❖ Phosphate Buffer of pH 7.8: Mixed 25.5 ml of MDP solution with 275.5 ml of DPH solution. Total volume (300 ml) was raised to 600 ml with d.H_2O.
- ❖ Solution (a): 1 g PVP and 0.0278 g Na_2EDTA were dissolved in 100 ml of PB (pH = 7.0).
- ❖ Solution (b): 1.5 grams of Methionine, 0.04 grams NBT, and 0.0278 Na_2EDTA were dissolved in 100 ml of PB (pH = 7.8).
- ❖ Solution (c): 10 ml of solution (b) was raised to 50 ml with PB (pH = 7.8).
- ❖ Solution (d): 0.0013 grams of Riboflavin was mixed in 100 ml of PB (pH = 7.8).

Solution (e): 20 ml of solution (d) was elevated to 50 ml with

2.16. STATISTICAL ANALYSIS

All experiments, unless otherwise noted, were run in triplicate. With Excel 2016, the means and standard errors were calculated. One-way ANOVA was carried out using Statistix version 8.1, and Tukey's least significant difference analysis was then carried out. Principal component analysis was employed to compare various experimental treatments using XLSTAT 2016.

CHAPTER 3

RESULTS & DISCUSSION

3. RESULTS

3.1.Isolation and Identification of Pathogen

Okra pods with the infection were seen in the local marketplace of Rawalpindi (Figure 3.1A). The pathogen was isolated on PDA media (Figure 3.1B), and after a week, its colonies became slightly pink to whitish (Figure 3.1C). Mycelia had beautiful septa and walls. A septate, reniform, or spherical micro conidia were seen (Figure 3.1D). This pathogen was identified as Fusarium oxysporum f. sp. ciceris based on the information of conventional conidial and morphological characteristics [20].

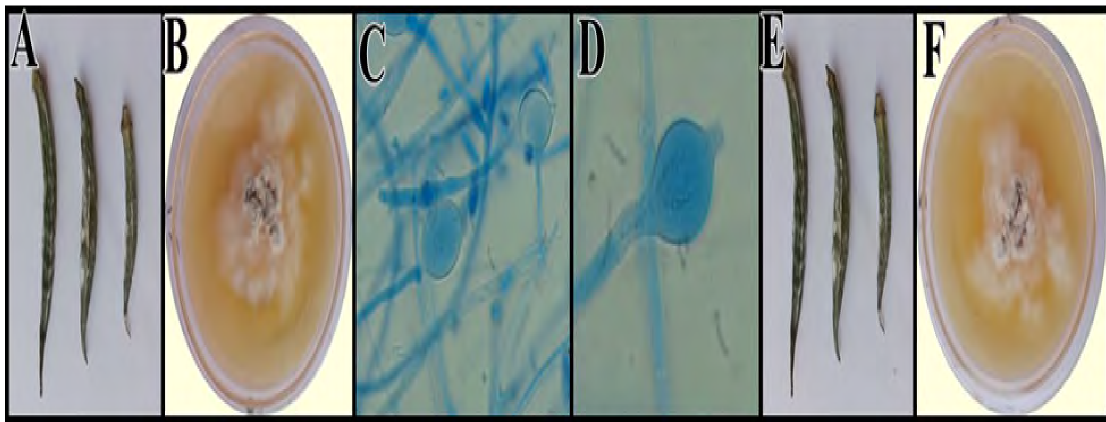


Fig:3.1. Infection was seen on okra pods(A). Disease causing pathogen were isolated on PDA media and observed on front side of petri plate (B). Under light microscope Mycelia observed pink to whitish (C). Mycelia had beautiful septa and wall and identified as Fusarium Oxysporum(D). Pathogen re inoculated into the healthy pods to confirm Koch postulate(E)Confirmation of Koch postulates(F).

3.2.Molecular Identification of Fungus3.1.

When compared to *Fusarium Oxysporium* (Accession No. MT649544), the PCR sequence that was produced was exactly the same. Our isolate and a particular strain of *F. Oxysporium* have an evolutionary link, according to phylogenetic analysis.

>FO_44
AGCGTATATTAAAGTTGTTGTGGTTAAAAAGCTCGTAGTTGAACCTTGGGC
CTGGCTGGCCGGTCCGCCTCACCGCGTGTACTGGTCCGGCCGGGCCTTTCC
CTCTGTGGAACCCCATGCCCTTCACGGGTGTGGCGGGGAAACAGGACTTT
TACTGTGAAAAAATTAGAGTGCTCCAGGCAGGCCTATGCTCGAATACATT
AGCATGGAATAATAGAATAGGACGTGTGGTTCTATTTTGTTGGTTTCTAGG
ACCGCCGTAATGATAATAGGGACAGTCGGGGGCATCAGTATTCAATTGTC
AGAGGTGAAATTCTTGGATTTATTGAAGACTAACTACTGCGAAAGCATTG
CCAAGGATGTTTTCATTAATCAGGAACGAAAGTTAGGGGATCGAAGACGA
TCAGATACCGTCGTATCTTAACCATAAACTATGCCGACTAGGGATCGGAC
GGTGTTATTTTTTGACCCGTTCGGCACCTTACGAGAAATCAAAGTGCTTGG
GCTCCAGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGAAATTGACGG
AAGGCACCACCAGGGGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGG
AAACTCACCAGGTCCAGACACAATGAGGATTGACAGATTGAGAGCTCTTT
CTTGATTTTGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATT
TGTCTGCTTAATTGCGATAACGAACGAGACCTTAACCTGCTAAATAGCCC
GTATTGCTTTGGCAGTACGCTGGCTTCTTAGAGGGACTATCGGCTCAAGCC
GATGGAAGTT

Fig: 3.2. FASTA format of amplified sequence

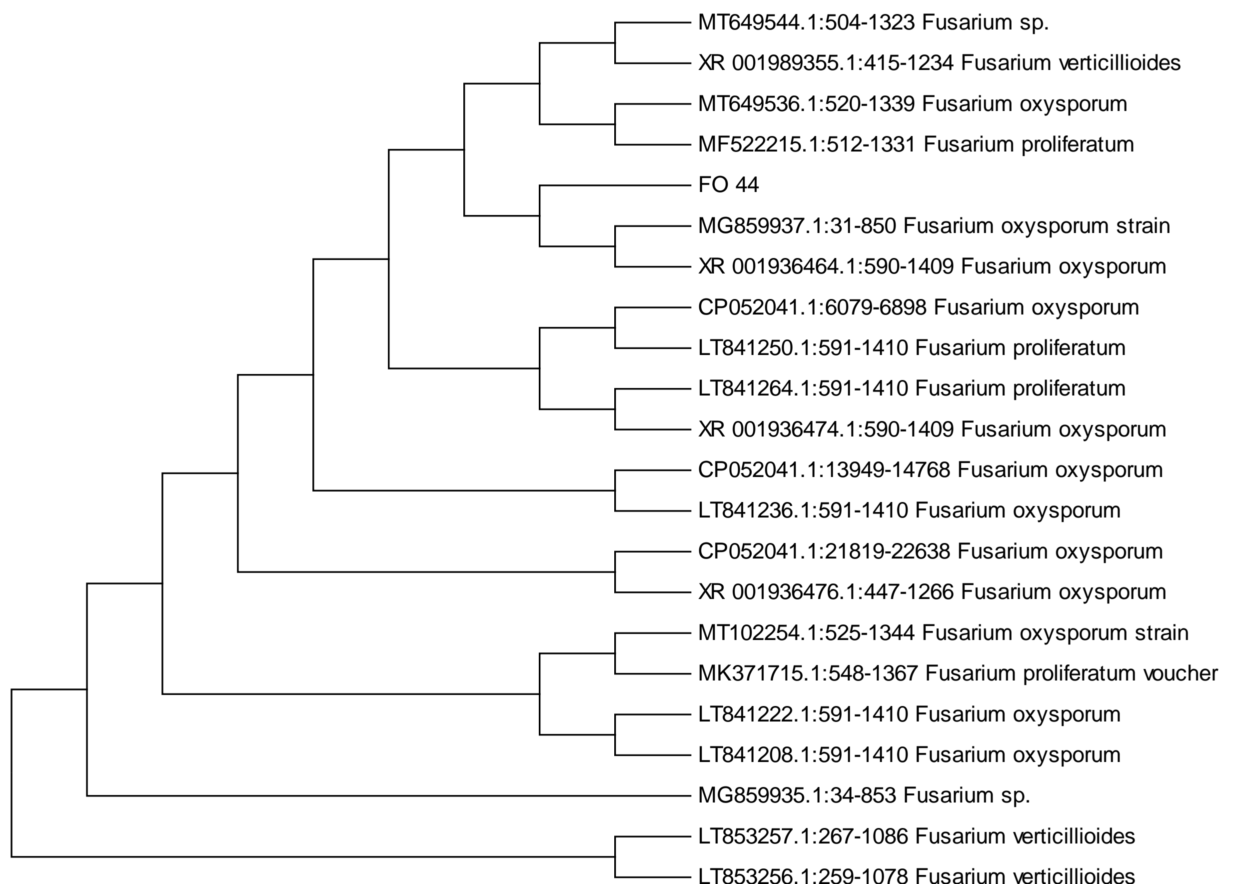


Figure: 3.3. Phylogenetic analysis of isolated pathogen.

3.3.Uv-Visible Spectroscopy

Resonant surface plasmon figures proved that ZnO NPs were generated efficiently at 340 nm (Figure 3.2). The ZnO NPs were produced as a result of the reduction of zinc acetate by secondary metabolites in the cell-free filtrate, which caused the color of the cell-free culture to change from yellow to whitish (Kalpana et al., 2018). Additionally, the difference between the shapes of the absorbance peaks for zinc oxide and filtrate indicated the transformation of zinc acetate salt into ZnO NPs. Zinc oxide had previously been found to have a comparable peak of absorption at 390 nm.

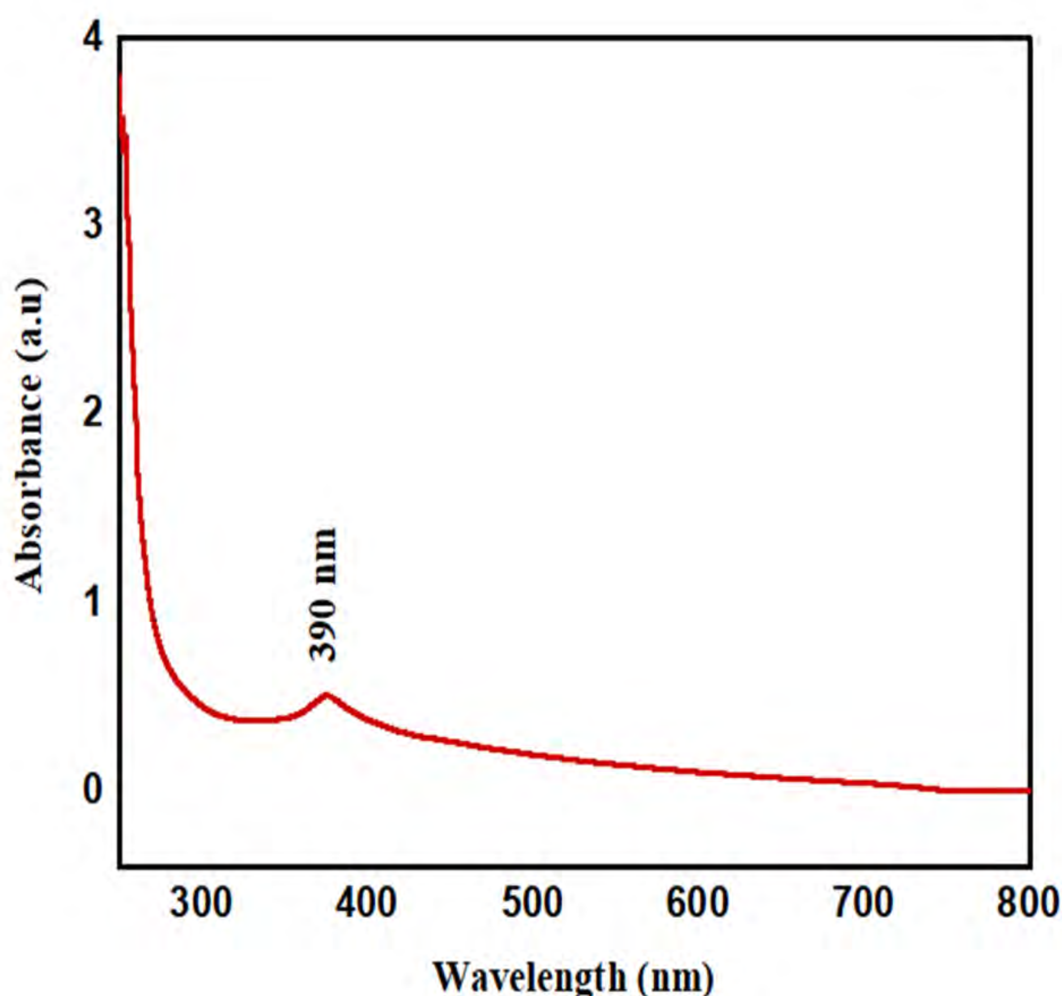


Fig:3.4. UV/Vis of ZNO NPs showing the synthesis of ZNO NPs

3.4.FTIR Analysis for Various Functional Groups

FTIR spectrum of ZnO NPs depicted the presence of various functional groups that aids in stabilizing Zinc oxide nanoparticles. FTIR spectra showed sharp peak at 1550.90cm-

1, which represents the presence of nitro compound and shows N-O stretching. At 1406.96cm-1, stretching of S=O depicted the presence of sulfonyl chloride. The peak 1339.47cm-1 represented strong S=O stretching of sulfonate. Peak at 1022.86cm-1 signified the involvement of amine salt C-N stretching. In the reduction process, peak at 670.26cm-1 represented C=C bending, attributed to the presence of alkene compound. Peaks at 614.86cm-1 and 526.67cm-1 showed C-Br stretching of halo compound. Peak at 534.86cm-1, 574.44cm-1, 584.00cm-1, 547.27cm-1, 561.82cm-1 and 591.60cm-1 represented C-I stretching of halo compound. Peak at 517.32cm-1 depicted the presence of C-Br stretching of halo compound. Organic compounds of seed extract showed the presence of these peaks, which shows significant roles in stabilization and reduction of ZnO nanoparticles.

TABLE 3:1 FTIR ANAYLSIS REVEALED CHARACTERISTIC PEAK NUMBER, FUNCTIONAL GROUP, APPEARANCE AND CLASS OF COMPOUND IN ZNO NPS PREPARED IN TRACHAYSPERMUM AMMI L SPRAGUE SEEDS EXTRACT

Standard Peak	**Sample Peak**	**Appearance**	**Group**	**Compound Class**
1550-1500	1550.90	Strong	N-O stretching	Nitro compound
1410-1380	1406.96	Strong	S=O stretching	Sulfonyl chloride
1350-1300	1339.47	Strong	S=O stretching	Sulfonate
1250-1022	1022.86	Medium	C-N stretching	Amine
730-665	670.26	Strong	C=C stretching	Alkene

690-515	614.86	Strong	C-Br stretching	Halo compound

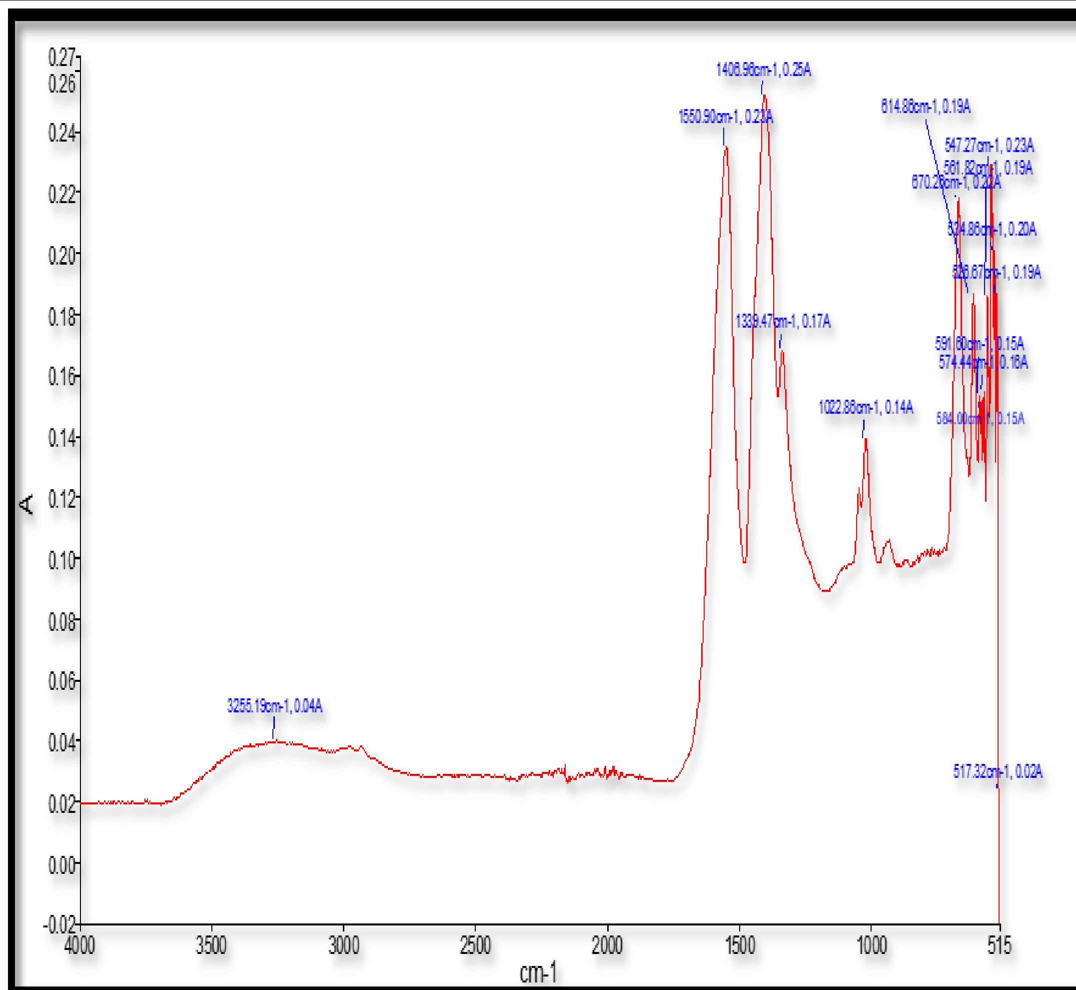


Fig: 3.5. FTIR spectra of *Trachyspermum ammi* L Sprague mediated ZNO NPs showing peaks at different numbers.

3.5. XRD

The X-ray diffraction (XRD) examination provided information about the size and crystal structure of ZnO NPs. Figure 3 shows the XRD spikes at the measurement angle (2θ), with the significant spikes indicating the diffraction planes. at 32°, 34°, 36.5°, 46.5°, 57.3°, 63.8°, 66.3°, 67°, 69.1°, and 75.1°. ZnO NPs have hexagonal crystal geometry, as shown by (100), (002), (101), (102), (110), (103), (200), (112), (201), and (202). Using the Scherrer equation, the mean crystal size was determined to be 26.7 nm.

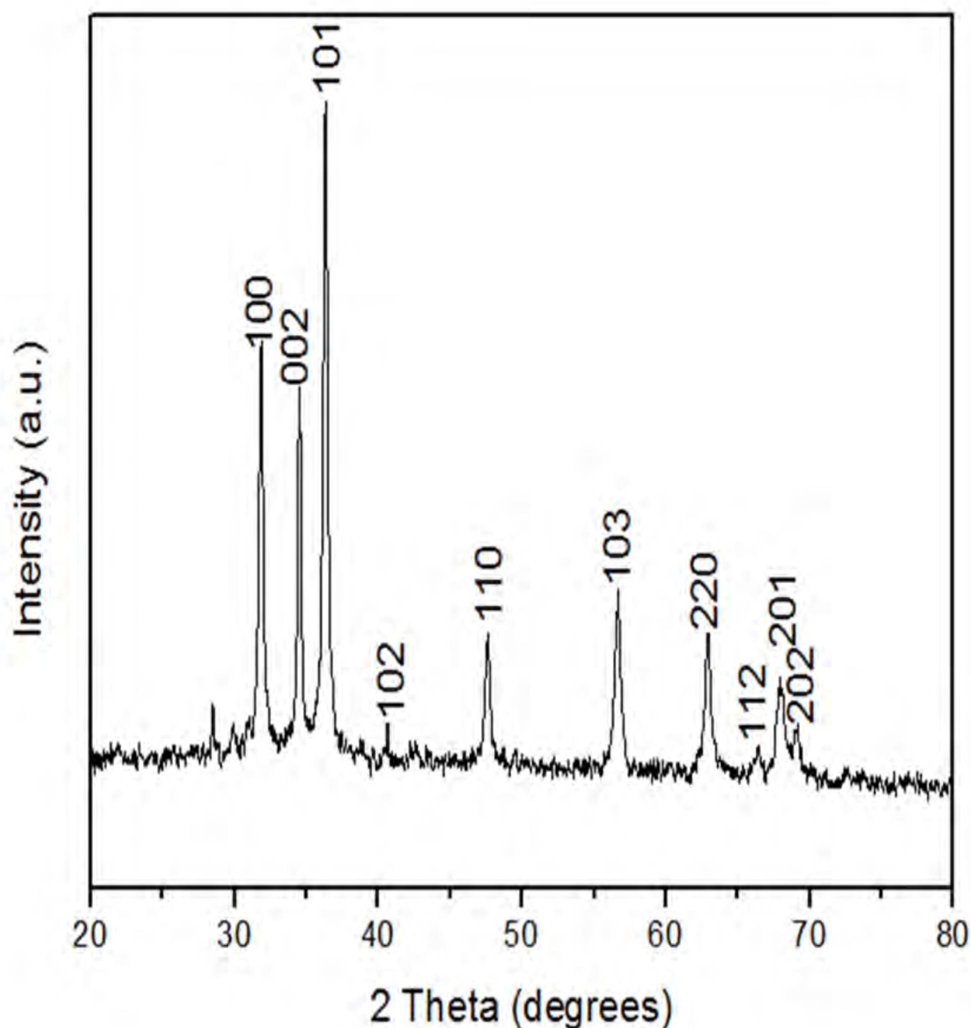
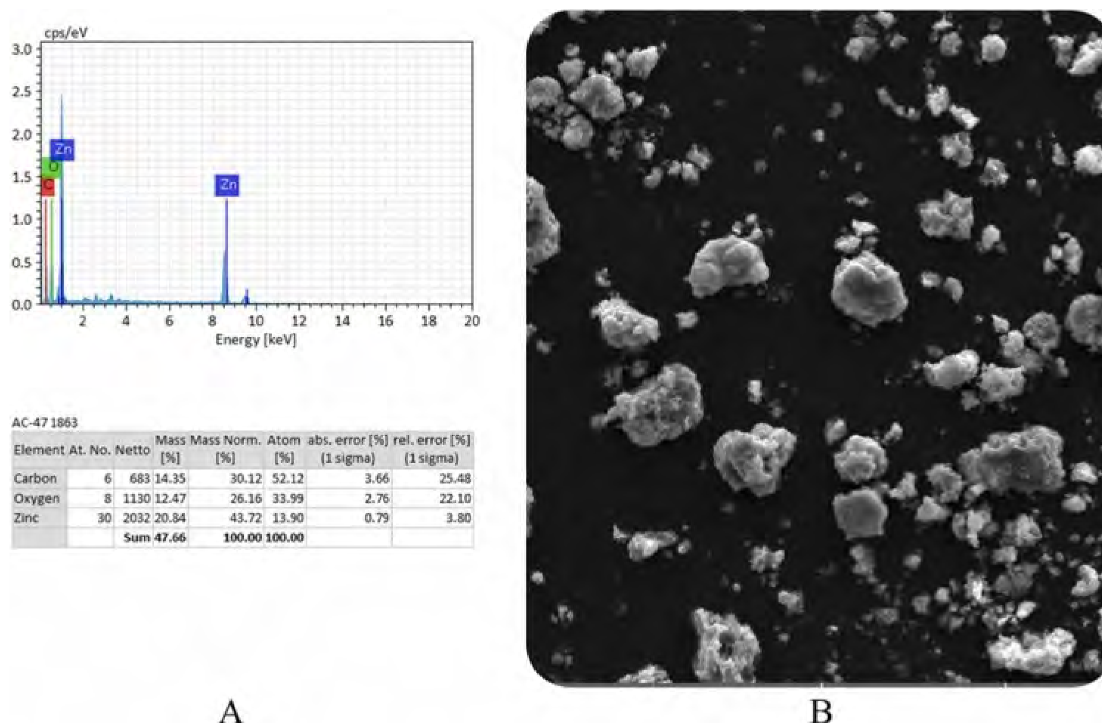


Fig:3.6. XRD anaylsis of *Trachyspermum ammi* L Sprague ZNO NPs showing characteristic at 2θ values 32°, 34°, 36.5°, 46.5°, 57.3°, 63.8°, 66.3°, 67°, 69.1°, and 75.1°

3.6. SEM EDX ANAYLSIS OF ZNO NPs

EDX spectra of NPs exhibited the dominant presence of zinc (43.72%), carbon (30.12%) and oxygen (26.12%) (Fig: 3.7). The EDX analysis also showed optical absorption peaks of NPs which indicate their surface Plasmon resonance effect. The origin of these elements lies in the components of phyto-extracts (Wei et al., 2009).



Element	At. No.	Netto	Mass [%]	Mass Norm. [%]	Atom [%]	abs. error [%] (1 sigma)	rel. error [%] (1 sigma)
Carbon	6	683	14.35	30.12	52.12	3.66	25.48
Oxygen	8	1130	12.47	26.16	33.99	2.76	22.10
Zinc	30	2032	20.84	43.72	13.90	0.79	3.80
		Sum	47.66	100.00	100.00		

Fig: 3. 7. SEM anaylsis of *Trachyspermum ammi* L

3.7. In Vitro Antagonism of F. oxysporum by Trachyspermum ammi L. Sprague) Mediated ZnO NPs

Variuos quantities of ZnO NPs and a conventional fungicide (metalaxyl + mancozeb) inhibited growth of *F. oxysporum* (Figure 10). The results of this study revealed that all doses of ZnO NPs can limit the growth of *F. oxysporum* in a varied manner (Table 1). Allong all investigated doses, 1.00 g/mL produced the best outcomes (76.2%), followed by 0.75 g/mL (74.1%). NPs inhibited mycelial development at a concentration of 1.00 g/mL before (Zaki et al., 2022) In order of chemical fungicide, 1.25 g/mL doses demonstrated the highest mycelial inhibition (76.0%). Conclusive investigations demonstrated that highest dose of ZnO NPs have a strong potential for inhibiting fungal growth, making them superior to chemical fungicides.

Table: 3.2. Growth inhibition at Different Concentrations of ZNO NPs

Inhibition in mycelial growth (%)	
Doses (mg/ml)	ZnO Nps
D1 0.25mg/ml	59 ± 0.4
D2 0.50mg/ml	73 ± 0.3
D3 0.75mg/ml	74 ± 0.3
D4 1.00mg/ml	76 ± 0.2

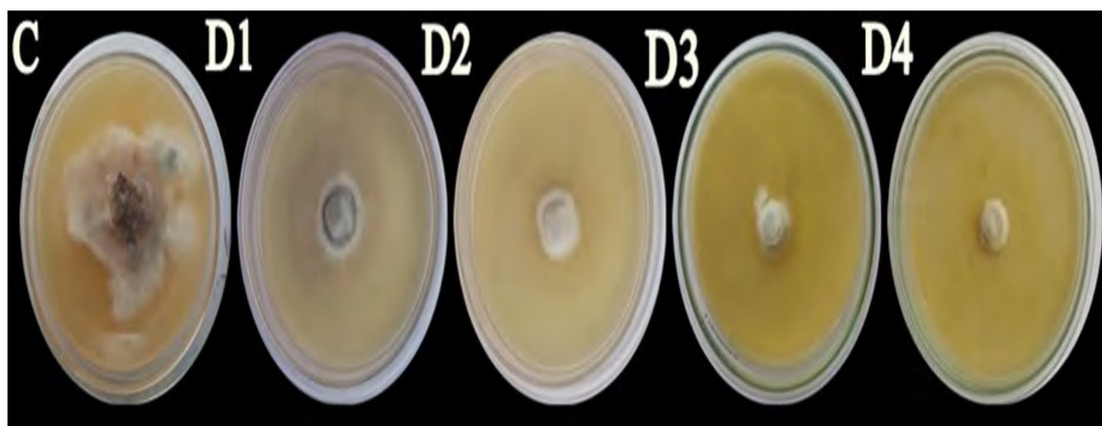


Fig: 3.8. Effect of different doses of Trachyspermum ammi L Sprague synthesized zinc oxide nanoparticles on fungal growth(C) CONTROL, (D1) 0.25mg/ml concentration, (D2) 0.50mg/ml concentration (D3) 0.75mg/ml concentration (D) 1.00mg/ml concentration.

3.8.DISEASED CONTROL ASSAY IN VIVO

Okra decay was controlled using four different dosages of the *Trachyspermum ammi L.* Sprague seeds mediated -ZnO NPs (0.25, 0.50, 0.75, and 1.0 mg/ml) (Kaur et al., 2019).

A sterile needle was used to pierce 30 healthy okra pods for the purpose of inoculating them with a specific fungal solution (1 106 conidia per ml). Two days after the inoculation, the inoculated pods were spurted with the various NP doses stated above, whereas the control fruit received a double distilled water spraying. Each fruit's disease area was assessed after each fruit was incubated at 26 °C for a week

Trachyspermum ammi L. Sprague seeds mediated -ZnO NPs gave excellent results to control okra pods decay. In ZNO NPs treated pods (D-4) decay symptoms were observed while control (C) one show significant damage to the okra pods.

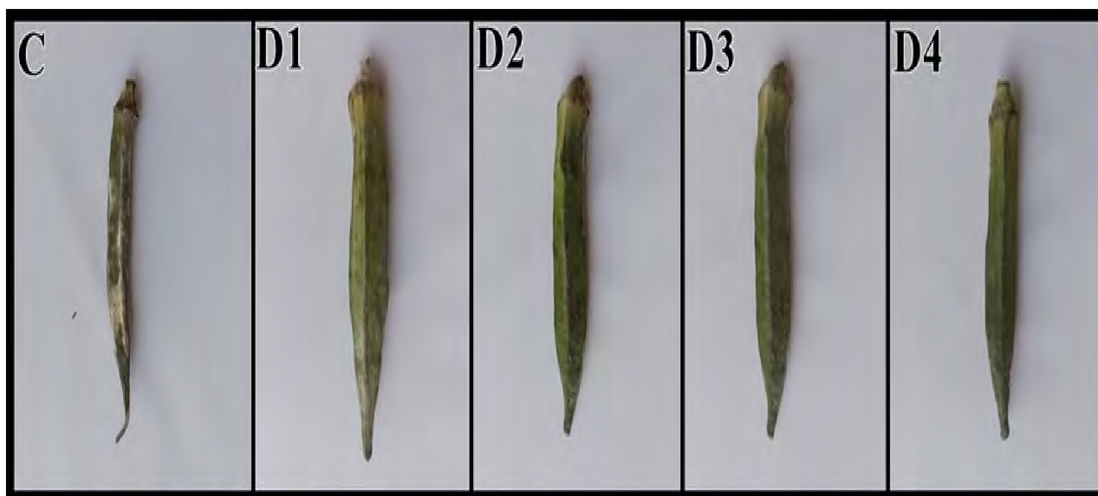


Fig: 3.9. Effect of different doses of Trachyspermum ammi L Sprague synthesized zinc oxide nanoparticles On fungal growth(C) CONTROL, (D1) 0.25mg/ml concentration, (D2) 0.50mg/ml concentration (D3) 0.75mg/ml concentration (D) 1.00mg/ml concentration

3.9.PHYSIOLOGICAL AND ANTIOXIDANT PARAMETERS OF OKRA PODS

Study of various physiological parameters (Table 3.3) and antioxidant enzyme assay (Table 3.4) assisted to comprehend the defense mechanism of treated okra. A remarkable increase in the Proline and sugar content was observed in 1g treated okra,

when compared with other concentrations treatments. Application of 1g ZnO NPs increased RWC and REL in 1g treated concentration. Assays for antioxidant enzymes showed clear differences in the number of enzymes accumulated. Plants subjected to 1g ZnO NPs showed increased accumulation of SOD, POD, and CAT.

Table: 3.3. Study of physiological parameters in different concentrations.

Treatments	**Proline (µg/g)**	**Sugar (µg/g)**	**RWC (%)**	**REL (%)**
Control	0.364±0.017	0.364±0.032	13.22±0.15	15± 2
0.25	0.534±0.014	0.49± 0.018	21.23±0.35	17±1
0.5	0.703±0.023	0.868±0.022	48.39±0.19	21.25±1.08
0.75	0.93±0.021	0.756±0.032	66.03±0.47	24±2.64
1	1.192±0.112	1.156±0.015	72.23±0.43	33±3.60

Table: 3.4. Estimation of antioxidant enzymes in different concentrations

Treatments	**SOD**	**POD**	**CAT**

Control	0.094±0.002	0.126±0.002	0.185±0.003
0.25	0.116±0.008	0.222±0.008	0.203±0.007
0.5	0.294±0.011	0.411±0.011	0.328±0.025
0.75	0.533±0.015	0.485±0.015	0.443±0.023
1	0.646±0.025	0.573±0.025	0.531±0.025

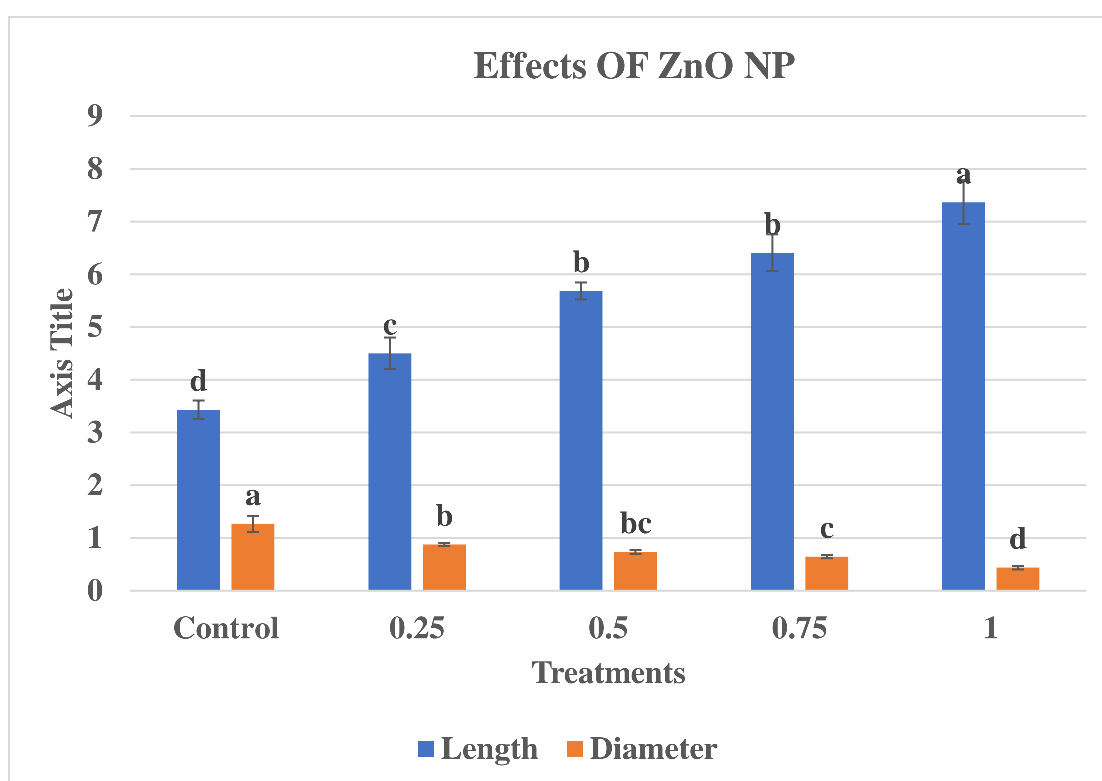


Fig: 3. 10. Effects OF ZnO NP on length and diameter of Okra PODS

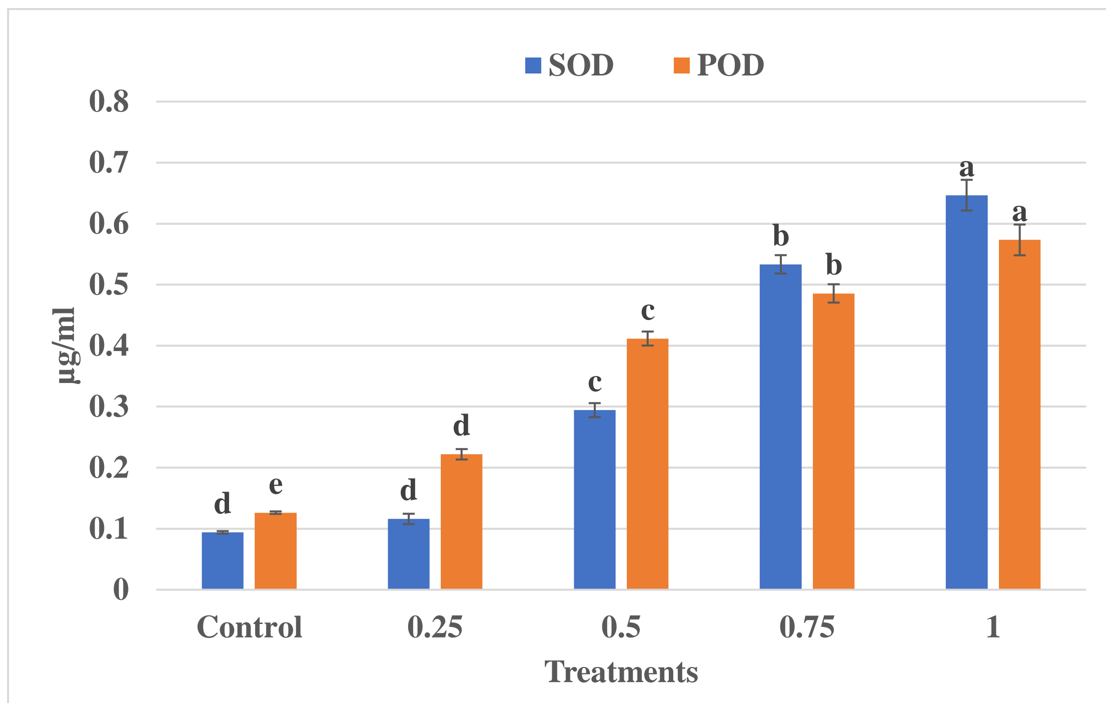


Fig: 3.11. Effects OF ZnO NPs on SOD and POD of OKRA PODS

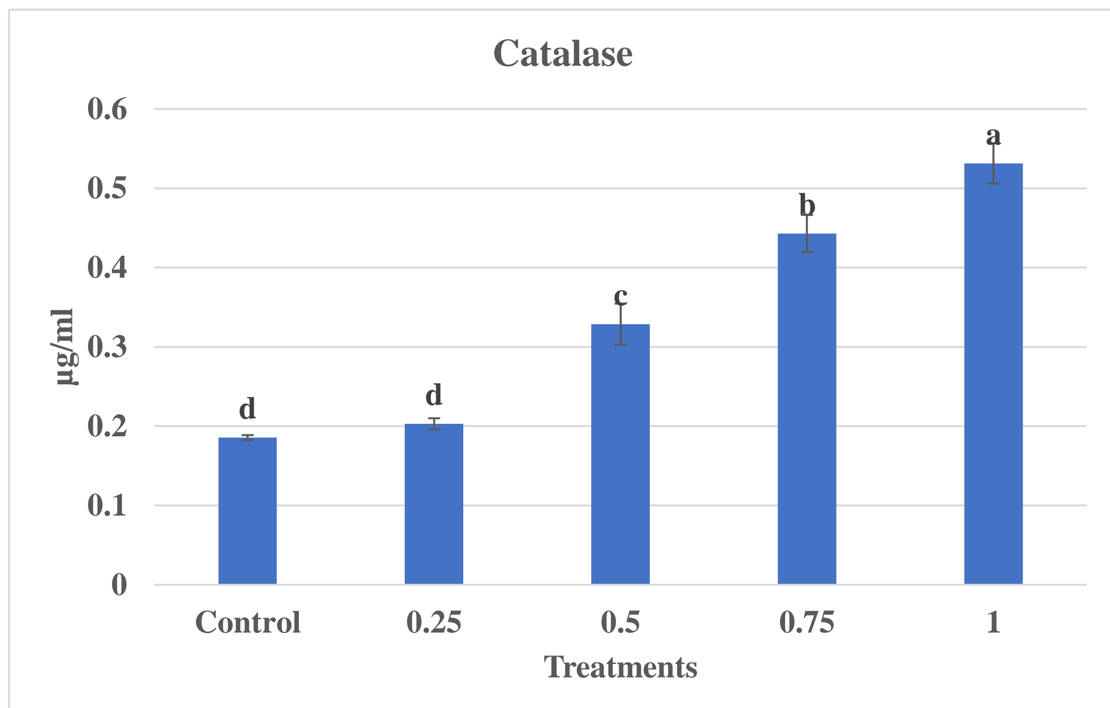


Fig:3.12. Effects OF ZnO NPs on Catalase of OKRA PODS

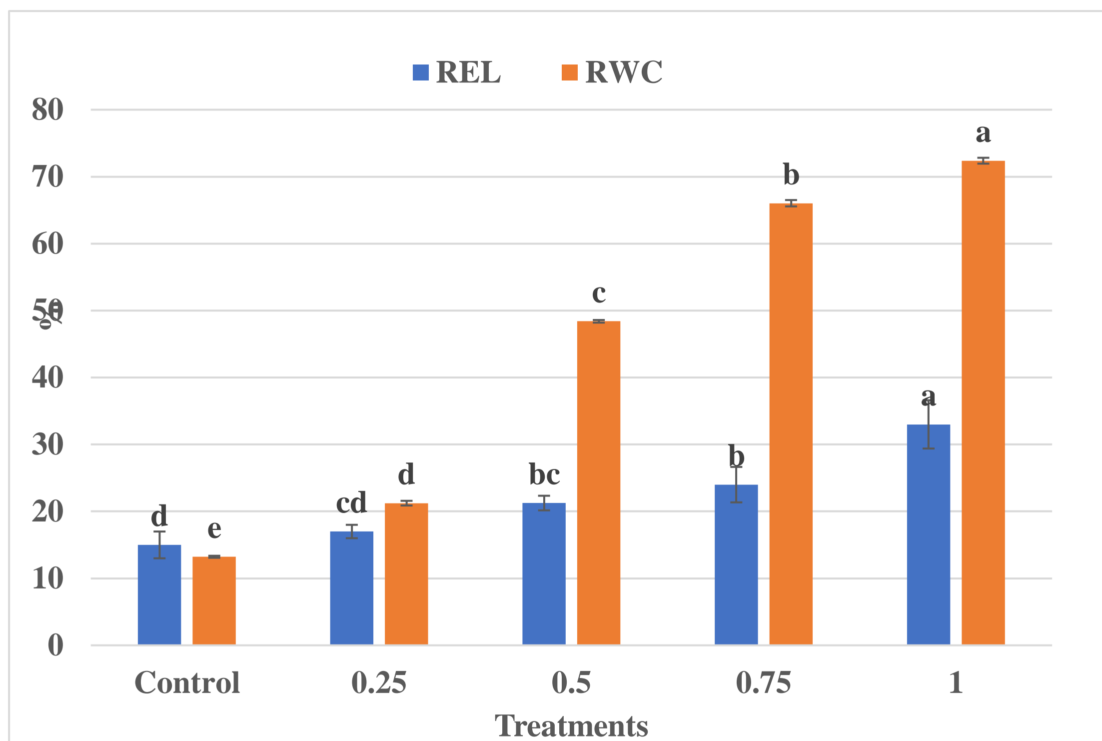


Fig: 3.13. Effects OF ZnO NPs on REL and RWC of OKRA PODS

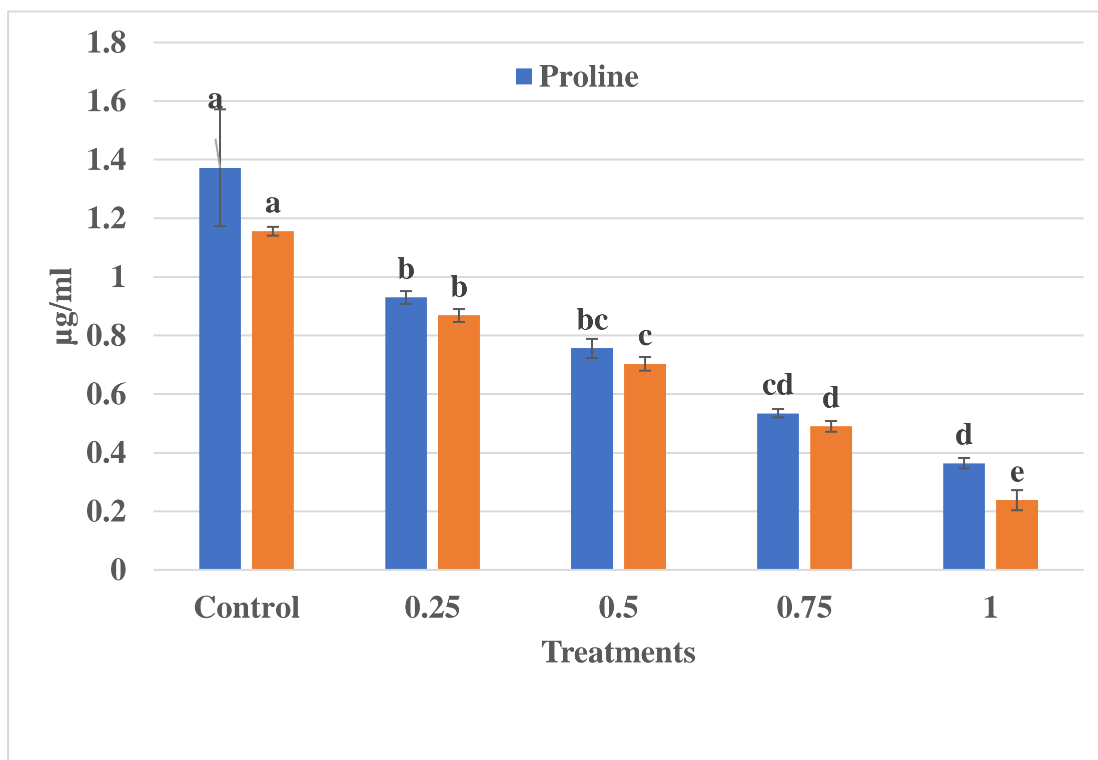


Fig:3.14. Effects OF ZnO NPs on Proline and Sugar content of OKRA PODS

4. Discussion

Fungi are now considered as the most destructive pathogen, accounting for around 50% of the losses of economically significant fruits (Shuping et al., 2017) In this research *Fusarium oxysporium* was discovered to be a following harvest disease causing agent of okra fruit. This research also indicates the control of this disease-causing agent using plant based-fabricated nanoparticles. Nanotechnology has emerged as the most successful and commonly used integrative strategy for controlling plant diseases and preserving crop quality. Nanomaterials are employed efficiently to improve Plant physiology, plant protection, dietary intake, and preservation (Guleria et al., 2023) Plant extracts may be the ideal approach for the biosynthesis of nanoparticles since they can create a huge amount of nanoparticles in a very less period of time with high efficiency and cheap production cost. Several metal nanoparticles, such as silver, gold, iron zinc, and others, have been stated to have antibacterial and antifungal properties (Rabia Nawab et al., 2022). Silver and gold nanoparticles are rarely used on broad scales due to their high cost, however zinc oxide nanoparticles (ZnO NPs) are thought to be less expensive and more effective (Cruz et al., 2020) (ZnO NPs possess antibacterial and antifungal activities (Pillai et al., 2020) Zinc is extensively used to prevent plant ailments like those caused by fungi, however zinc oxide nanoparticles (ZnO-NPs) are far better at inhibiting the growth of plant pathogenic fungi (Khan et al., 2019). Past study has shown that ZnO-NPs have the capacity to suppress fungal plant diseases such as rotting by directly inhibiting fungal growth by disrupting the developing mycelia and also by removing microbial (Yehia and Ahmed 2013).

Several methods used in this study suggested that the ZnO NPs that were produced using plants were stable. The FTIR approach aids in identifying chemical bonds and functional groups that operate as reducing and capping agents (Sidhu et al., 2022) The size and crystalline makeup of the synthesized NPs in the current work were made clear by XRD analysis. Numerous research has discussed the significance of NPs' crystal structure and size in giving them their antifungal effects (Sun & Le, 2018).

The results of this work showed that *Trachyspermum ammi L* Sprague mediated ZnO NPs have antifungal action, both in vitro and in vivo. A novel family of versatile antibacterial agents called ZnO NPs has evolved (Verma et al., 2021).ZnO NPs initially interact with microbial cells and cling to the plasma membrane's outer surface. ZnO

Nps build up and impede fungal cell division(Mani at al., 2022) They also promote the production of hydrogen peroxide (Hou & Zhang et al.,2020 Additionally, ZnO NPs assist in mediating singlet oxygen and hydroxyl radicals, which finally result to cell necrosis. ZnO NPs are thought to be a flexible and productive antibacterial agent for the production of superoxide radicals and hydrogen peroxide, as well as for extending the life span of economically significant fruits and vegetables (Zare et al.,2022)

By applying NPs, the quality of the okra fruit was preserved, and fungal growth was suppressed. Prior research has also highlighted the beneficial effects of ZnO NPs in sustaining fruit quality and preventing weight loss (Sharifan et al., 2021) Another crucial physical characteristic that influences consumer approval is the firmness of fruit, which ZnO NPs also preserved (Anugrah et al., 2020) When a fruit ripens, enzymes, particularly -galactosidase, polygalacturonase, and pectin methylesterase, weaken the cell wall's ability to resist mechanical forces, which causes the fruit to lose its firmness (Kunzek et al., 1999)In order to prolong the shelf life of fruit, firmness must be maintained. Moisture loss that occurs as it passes through the epidermis of the fruit and into the environment has an impact on the freshness and shelf life of the fruit. Fruits that had been NP-treated lost less moisture, stayed firmer, and displayed fewer disease symptoms.

Compared to control pods, treated pods displayed no signs of the disease. Minerals accumulate as a result of NPs therapies, increasing Proline content. The application of NPs raised the sugar content, which is crucial for the making of callose and lignin associated with defense. (Khizar et al., 2021). RWC concentration increases in ZnO NPs treated fruit. Highest level of REL in 1mg/ml shown the death of diseased cells as described earlier (Khizar et al., 2021).

Different quantities of ZnO NPs and a conventional fungicide (Metalaxyl + mancozeb) inhibited growth of *F oxysporum*. Pesticides of various types are regularly employed to combat fungal infections. Pathogens develop resistance to these synthetic chemical-based substances gradually (Lahlali et al., 2022) Moreover, these fungicides are reactive in nature and are hazardous to the biological biosphere (Lushchak et al., 2018) Plant extracts may be the ideal approach for the biosynthesis of nanoparticles since they can create a huge amount of nanoparticles in a short period of time with high efficiency and cheap production cost (Srikar et al. 2016). They include phytochemicals that can

replace very poisonous, costly, and environmentally hazardous chemical reduced factors such sodium citrate, sodium borohydride (NaBH4), and ascorbate (Ahmed et al. 2016). Plant extracts may be the ideal approach for the biosynthesis of nanoparticles since they can create a huge amount of nanoparticles in a short period of time with high efficiency and cheap production cost (R Nawab et al., 2022).

Conclusion

This study has convincingly demonstrated the beneficial function of *Trachyspermum ammi* L. Sprague seeds fabricated ZnO NPs in disease prevention and preserving okra fruit quality during storage. Extensive analyses enabled us to figure out the best concentration of optimal *Trachyspermum ammi* L. Sprague-ZnO NPs (1 mg/ml) in order to prevent okra postharvest rot. Using these ZnO NPs, be applied in the arena to effectively control the disease on a wide scale.

CHAPTER 4

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