Mycosynthesis and Application of Fe_2O_3 Nanoparticles to Diminish Fruit Rot Diseases by Maintaining their Composition and Pertinent Organoleptic Properties

BY

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In the name of Allah, the Entirely Merciful, the Especially Merciful.
All the praise is due to Allah, Lord of worlds. The Entirely Merciful, the Especially Merciful, Sovereign of the Day of Recompense.
It is You we worship, and You we ask for help. Guide us to the straight path. The path of those upon whom You have bestowed favors, not of those who have evoked Your anger or of those who are astray.

Surah Al-Fatiha

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APPROVAL CERTIFICATE

This is to certify that the research work in this thesis, entitled **"Mycosynthesis and Application of Fe_2O_3 Nanoparticles to Diminish Fruit Rot Diseases, Maintaining Composition and Organoleptic Properties"** was conducted by **Ms. Mahnoor Akbar** under the supervision of **Dr. Muhammad Farooq Hussain Munis**. No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the **Department of Plant Sciences, Faculty of Biological Science, Quaid-i-Azam University, Islamabad** in partial fulfillment of requirements for the degree of **Doctor of Philosophy** in the field of **Plant Science**.

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DEDICATED TO

My Parents

Mr. Ghulam Akbar (Late) & Miss. Riffat Shaheen

This dissertation is dedicated to my father, whose memory lives on as a constant source of strength and inspiration. It was your dream to see me reach this milestone, and though you are no longer here, your motivation and belief in me have carried me forward every step of the way. I hope this work honors the vision you had for me. I carry you with me always.

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Mahnoor Akbar

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LIST OF ABBREVIATIONS

Abbreviation	Full Form
NPs	Nanoparticles
Fe_2O_3	Iron (III) Oxide
ROS	Reactive Oxygen Species
CuO	Copper Oxide
ZnO	Zinc Oxide
TiO_2	Titanium Dioxide
SiO_2	Silicon Dioxide
Ag NPs	Silver Nanoparticles
Au NPs	Gold Nanoparticles
PVP	Polyvinylpyrrolidone
PEG	Polyethylene Glycol
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
XRD	X-ray Diffraction
FTIR	Fourier Transform Infrared Spectroscopy
DLS	Dynamic Light Scattering
BET	Brunauer–Emmett–Teller (Surface Area Analysis)
EDS	Energy Dispersive X-ray Spectroscopy
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
AFM	Atomic Force Microscopy
XPS	X-ray Photoelectron Spectroscopy
SERS	Surface-Enhanced Raman Spectroscopy
PDA	Polydopamine
PANI	Polyaniline
PLGA	Poly(lactic-co-glycolic acid)
PLA	Polylactic Acid
PAA	Polyacrylic Acid
PCR	Polymerase Chain Reaction
GMO	Genetically Modified Organism
NCBI	National Center for Biotechnology Information
FAO	Food and Agriculture Organization
WHO	World Health Organization
EPA	Environmental Protection Agency
USDA	United States Department of Agriculture
R&D	Research and Development

Abbreviation	Full Form
UV	Ultraviolet
IR	Infrared
NIR	Near-Infrared
EPR	Electron Paramagnetic Resonance
MRSA	Methicillin-Resistant Staphylococcus aureus
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
NMR	Nuclear Magnetic Resonance
FRET	Förster Resonance Energy Transfer
RT-PCR	Real-Time Polymerase Chain Reaction
UV-Vis	Ultraviolet-Visible Spectroscopy
LC-MS	Liquid Chromatography-Mass Spectrometry
GC-MS	Gas Chromatography-Mass Spectrometry
AAS	Atomic Absorption Spectroscopy
MS	Mass Spectrometry
HPLC	High-Performance Liquid Chromatography
GC	Gas Chromatography
NAA	Neutron Activation Analysis
SPME	Solid Phase Microextraction
LOD	Limit of Detection
LOQ	Limit of Quantification
TOF	Time of Flight
MALDI	Matrix-Assisted Laser Desorption/Ionization
ELISA	Enzyme-Linked Immunosorbent Assay
ICP-AES	Inductively Coupled Plasma Atomic Emission Spectroscopy
BSA	Bovine Serum Albumin
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
ATP	Adenosine Triphosphate
GMO	Genetically Modified Organism
ATPase	Adenosine Triphosphatase
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
H_2O_2	Hydrogen Peroxide
N_2	Nitrogen Gas
O_2	Oxygen Gas

Abbreviation	Full Form
CO_2	Carbon Dioxide
NH_3	Ammonia
HCl	Hydrochloric Acid
HNO_3	Nitric Acid
H_2SO_4	Sulfuric Acid
NaOH	Sodium Hydroxide
KOH	Potassium Hydroxide
AgCl	Silver Chloride
$ZnCl_2$	Zinc Chloride
$FeCl_3$	Ferric Chloride
BOD	Biochemical Oxygen Demand
COD	Chemical Oxygen Demand
TSS	Total Suspended Solids
TOC	Total Organic Carbon
ORP	Oxidation-Reduction Potential
EMF	Electromotive Force
NPK	Nitrogen, Phosphorus, Potassium
IAA	Indole-3-Acetic Acid
GA_3	Gibberellic Acid
FPP	Fruit Preservation Process
MWCNT	Multi-Walled Carbon Nanotubes
SWCNT	Single-Walled Carbon Nanotubes
FRET	Fluorescence Resonance Energy Transfer
PAMAM	Poly(amidoamine) Dendrimers
ChNP	Chitosan Nanoparticles
PCL	Polycaprolactone
CNC	Cellulose Nanocrystals
GNRs	Gold Nanorods
NIR	Near Infrared
CNFs	Cellulose Nanofibers

ABSTRACT

Fruit rot diseases pose significant threats to the agricultural sector by impacting the production of fruits. The economy of Pakistan heavily relies on agriculture and the fruit rot diseases are particularly concerning, especially for high-value fruits like peaches, apples, and strawberries. These fruits hold substantial commercial value and contribute in export and local livelihoods. Addressing these challenges requires the adaptation of innovative technologies for the sustainable production of these plants. In the last decade, nanotechnology has become one of the most utilized technologies in the food and agricultural industry.

This study was planned to optimize the mycosynthesis and application of iron oxide nanoparticles (Fe_2O_3 NPs) to control fruit rot diseases of peaches, apples and strawberries. For better understanding, this study has been described in three parts.

In the first section, surveys were conducted to collect apple fruit with typical brown rot symptoms. Diseased fruit parts were cultured in Petri plates containing potato dextrose agar media. After 3-5 days, mycelial mass could be observed in Petri plates and based on its morphological, microscopic and molecular analyses, it was identified as *Fusarium oxysporum*. For the control of this disease, a known beneficial fungus (*Trichoderma harzianum*) was used to synthesize Fe_2O_3 NPs. For this purpose, $FeCl_3$ was mixed in the potato dextrose broth extract of *T. harzianum* and calcinated to synthesize Fe_2O_3 NPs. Before the application of these mycosynthesized Fe_2O_3 NPs to control brown rot disease of apple, sophisticated techniques were used for their characterization. FTIR spectroscopy showed the presence of different reducing and stabilizing compounds on the surface of Fe_2O_3 NPs. The average size (17.78 nm) of these mycosynthesized Fe_2O_3 NPs was revealed by X-ray diffraction (XRD) analysis. The purity of Fe_2O_3 NPs was determined by Energy-dispersive X-ray (EDX) which showed strong presence of iron, while scanning electron microscopy (SEM) exhibited a high degree of polydispersity. These characterized NPs were used for foliar application, and it resulted in significant reduction of brown rot symptoms of apple. Moreover, NPs-treated fruit maintained better biochemical composition and greater organoleptic properties than untreated fruit. The superior quality of NPs-treated apple fruit was evident by the higher presence of soluble solids, sugars and ascorbic acid. These results depicted the great potential of these NPs in controlling brown rot of apple.

In the second part of this study, aflatoxin contamination was assessed in infected peach (*Prunus persica* L.) fruit and this infection and aflatoxin production was controlled by the

application of mycosynthesized Fe_2O_3 NPs. For this purpose, diseased peach fruit were collected, and the disease-causing pathogen was isolated on SDA. By observing the morphology of isolated pathogen on Pteri plates and under microscope, and by analyzing its 16S sequence, the disease-causing pathogen was identified as *Aspergillus flavus*. To see the aflatoxin-producing ability of this isolated strain of *A. flavus*, *in vitro*, it was cultured in Petri plates, on SDA. After one week, fully grown fungus was observed under UV light at 365 nm wavelength. These Petri plates were then exposed to the vapors of ammonium hydroxide (31%), which changed the colony color to light grey, and confirmed the presence of aflatoxin. To control peach-fruit disease, Fe_2O_3 NPs were synthesized in the filtrate of a *T. harzianum* (as described in the previous section) and characterized. FTIR spectrum revealed the attachment of secondary amines, alcohol and alkyne with Fe_2O_3 NPs. XRD analysis confirmed their nano-size, and the SEM analysis revealed their spherical shape. The EDX spectrum displayed strong signals of iron (74.38%), indicating the successful formation of Fe_2O_3 NPs. These NPs inhibited mycelial growth in Petri plates, *in vitro,* and the highest growth inhibition (65.4%) was exhibited by 1 mg/ml concentration of NPs. The concentration performed best in controlling fruit rot of peach, *in vivo*. In infected NPs-treated peach fruit, higher amounts of soluble solids, sucrose, total sugar and ascorbic acid were observed than infected and untreated control fruit. Similarly, treated fruit displayed higher titratable acidity and firmness than untreated peach fruit. In these infected fruits, the production of aflatoxin was observed by using three reliable methods including thin layer chromatography (TLC), enzyme-linked immunosorbent assay (ELISA) and high-performance liquid chromatography (HPLC). All these techniques verified the variable production of aflatoxins in NPs-treated and untreated fruits. Treatment of peach fruit with Fe_2O_3 NPs not only decreased the disease infestation of peach fruit but also resulted in less production of aflatoxins. In infected and untreated control fruit, the maximum production of aflatoxins was detected.

In the third and last section of this study, Fe_2O_3 NPs were applied to increase the shelf-life of strawberries at room temperature. As described in the first section, Fe_2O_3 NPs were mycosynthesized in the cell-free extract of *T. harzianum.* UV–vis spectroscopy at 420 nm indicated the synthesis of NPs and FTIR spectroscopy showed the presence of amines, aromatics, and alkenes on the surface of Fe_2O_3 NPs. XRD analysis determined their nano-size, while EDX analysis confirmed the significant presence (68%) of iron. SEM microscopy verified the spherical flowery-crystalline morphology of Fe_2O_3 NPs. Healthy strawberries were treated with

variable doses (0.1, 0.25, 0.50, 0.75, 1.0, and 1.5 mg/ml) of Fe_2O_3 NPs and their effects on the postharvest quality attributes of strawberries were monitored for six days. Though all concentrations of superparamagnetic Fe_2O_3 NPs contributed to enhance their shelf life, 1 mg/ml concentration of Fe_2O_3 NPs performed best to inhibit the weight loss and preserve their titratable acidity. Application of Fe_2O_3 NPs also helped strawberries to sustain good quality by maintaining higher ascorbic acid contents and superior firmness.

Conclusive findings of this study signify the environment-friendly use of Fe_2O_3 NPs for the management of fruit rot diseases of peaches, apples and strawberries. *T. harzianum* is well known for its biocontrol activities. Using the metabolites of this fungus to synthesize NPs is a next-level utilization of this human-gut friendly fungus. Through the mycosynthesis process, these nanoparticles offer an eco-friendly alternative to conventional practices of disease control and management. The findings of this thesis encourage the use of nanotechnology in controlling fruit diseases and provide valuable insights for future research and practical applications. Fe_2O_3 NPs are produced in the powder form, which make their application very convenient in the field. As the world is continuously seeking innovative solutions to enhance its agricultural productivity and sustainability, Fe_2O_3 NPs could play a pivotal role in protecting valuable fruit crops and ensuring food security.

1. INTRODUCTION

1.1 Introduction to Fruit Rot Diseases and the Role of Nanotechnology

1.1.1 Fruit Rot Diseases Affecting Horticultural Crops Globally

Fruit rot diseases present a significant challenge to global agriculture, particularly in the horticultural sector. These diseases are caused by a variety of fungal, bacterial, and viral pathogens, and result in substantial losses in their yield and quality (Dowling et al., 2020). According to the Food and Agriculture Organization (FAO, 2021), post-harvest losses due to fruit rot can account for up to 50% of total fruit production in some regions, depending on climatic conditions and the type of fruit being cultivated (FAO, 2021). According to one study, *B. cinerea* (causes gray mold), *Penicillium spp.* (cause blue mold) and *Colletotrichum spp.* (cause anthracnose) are among the most prevalent fruit rot pathogens (Zakaria, 2023). These diseases can lead to even higher losses in developing countries, where agricultural practices are not properly regulated. Moreover, in tropical and subtropical climates, where humidity and temperature favor the growth of pathogenic fungi, fruit rot is a persistent problem. In tropical regions, *Fusarium oxysporum* is a major pathogen (Lastochkina et al., 2019) due to its robust life cycle (Figure 1.1). A study by Sharma et al. (2017) highlighted that in India, post-harvest losses of fruits due to rot can reach up to 40%, significantly impacting the economy and food security.

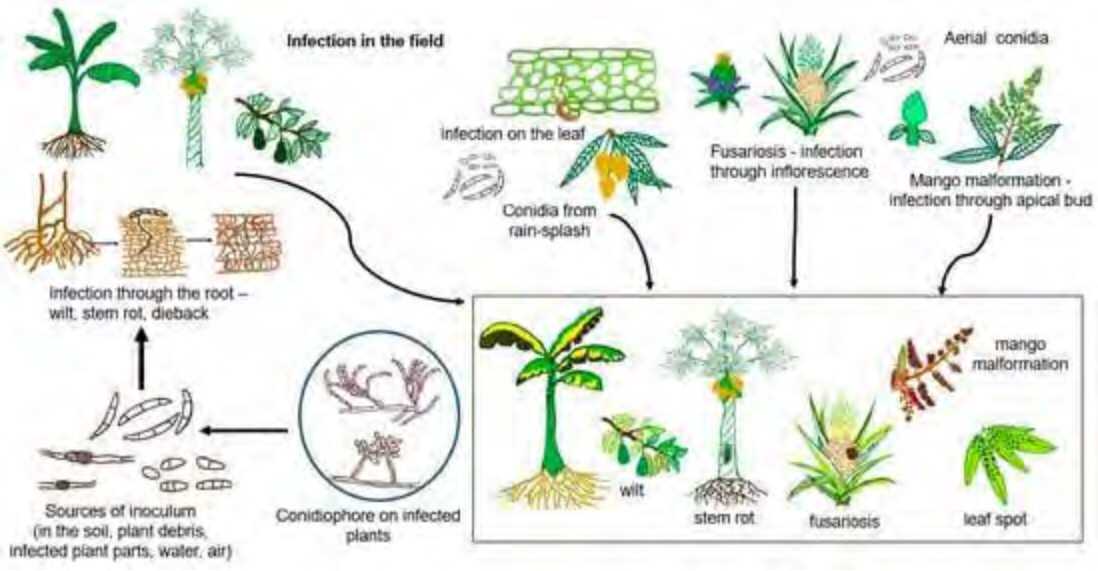

Figure 1.1. Disease cycle of Fusarium diseases (Lastochkina et al., 2019)

Fruit rot affects the marketable yield and quality of infected produce. Infected fruits often exhibit discoloration, softening, and off-flavors, making them unappealing to consumers. Moreover, some fruit rot pathogens produce hazardous mycotoxins (Roberto et al., 2019). The global economic impact of fruit rot is staggering, with billions of dollars lost annually due to reduced yields, lower fruit quality, and the costs associated with disease management and post-harvest treatments (Luo et al., 2022).

1.1.2 Importance of Addressing Fruit Rot Diseases in Pakistan's Agricultural Sector

In Pakistan, agriculture is a cornerstone of the economy, contributing approximately 19% to the national GDP and employing about 38.5% of the labor force (Pakistan Economic Survey, 2023). Horticultural crops, including fruits like peaches, apples, and strawberries play a vital role in this sector (Fahim, 2022). However, the productivity and profitability of these crops are severely threatened by fruit rot diseases (Palwasha & Fahim, 2022).

The climatic conditions in Pakistan, characterized by high humidity and temperature during the growing season, are conducive to the proliferation of fruit rot pathogens. According to the Punjab Agricultural Research Board (PARB), fruit rot diseases cause an estimated 20-30% loss in fruit production annually in Pakistan (Figure 1.2), with some regions experiencing even higher losses (Ali et al., 2018). For example, in the Swat Valley, a key region for apple and peach production, losses due to fungal pathogens such as *Botrytis* and *Colletotrichum* have been particularly severe (Ahmad et al., 2021). The economic implications of these losses are profound. Smallholder farmers, who constitute most of the farming population, are especially vulnerable (Ur-Rahman, 2018). They often lack access to modern disease management practices and rely on traditional methods, which are not always effective against persistent and adaptive fruit rot pathogens. As a result, fruit rot diseases not only diminish the income of these farmers but also threaten food security and export potential. In 2021, Pakistan exported fruits worth $431 million, but this figure could be significantly higher if post-harvest losses were minimized (Hussain & Usman, 2019).

Addressing fruit rot diseases is, therefore, critical for the sustainability and growth of Pakistan's horticultural sector. This involves adopting innovative disease management strategies that are both effective and environmentally sustainable.

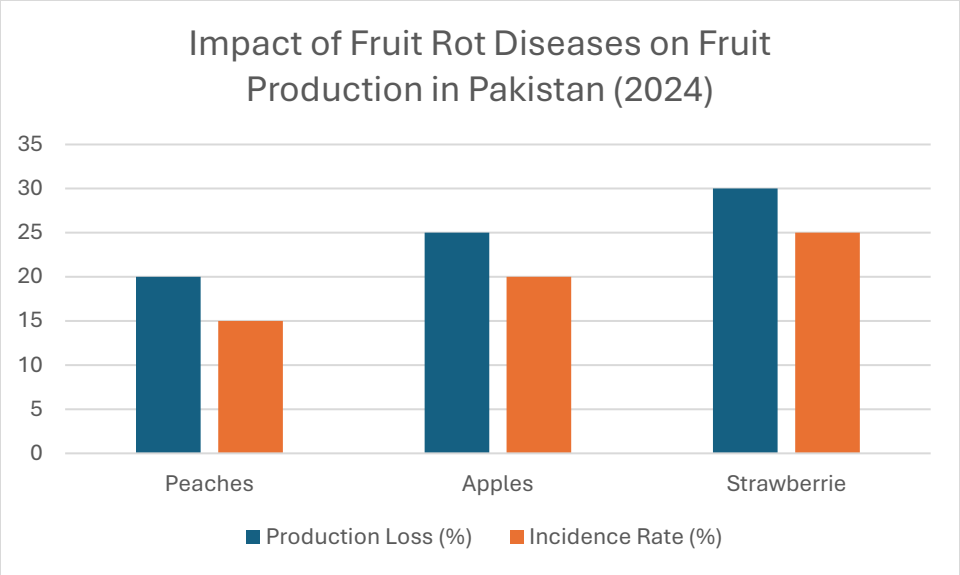

Figure 1.2 Impact of Fruit Rot Diseases on Fruit Production in Pakistan (2024).

1.1.2 Nanotechnology: A new avenue Plant Disease Management

For the last few decades, nanotechnology has been gaining the attention of agricultural scientists to improve crop yield and control diseases. Nanoparticles (NPs) keep small size and they can easily penetrate biological membranes, which make them highly effective in interacting with pathogens at cellular level (Worrall et al., 2018). In the context of fruit rot diseases, NPs can be employed in disease management practices (Fu et al., 2020). These days, nanotechnology is being applied to produce nano-fungicides. Unlike conventional chemical fungicides, which often require high doses and can have adverse environmental effects, nanoparticle-based formulations can deliver active ingredients more precisely and effectively (Thakur et al., 2018). NPs have been reported to possess strong antifungal properties against different fruit rot pathogens (Jo et al. 2015).

Nanotechnology has also been used in smart delivery systems, which can release antifungal agents for targeted protection against fruit rot pathogens (Farooq et al., 2021). Additionally, nanoparticles can be used for the encapsulation of beneficial microbes and their further delivery (Elmer et al., 2018; Farooq et al., 2021). Nanotechnology also offers potential solutions for post-harvest disease management. Nanoparticles can be kept in packaging materials to inhibit pathogen growth during storage and transport. This could be particularly beneficial to improve the shelf life of fruits (Bally et al., 2019). Application of nanotechnology holds

significant promise for improving the management of fruit rot diseases. However, potential risks such as environmental persistence and toxicity are often associated with the application of NPs. There is a need to keep these risks in consideration as well, while working on NPs.

Nanotechnology offers a new frontier in the fight against fruit rot diseases. By providing more effective, targeted and sustainable solutions, nanotechnology can help mitigate the losses caused by these diseases, thereby enhancing the productivity and profitability of horticultural crops in Pakistan and globally (Bally et al., 2019).

1.2 Peaches: Economic Importance, Health Benefits, and Nanoparticle Application

1.2.1 Economic Significance of Peaches in Pakistan’s Agricultural Economy

Peaches hold a prominent place in Pakistan's agricultural sector, both in terms of production volume and economic contribution (Fahim, 2022). Peaches are primarily grown in the provinces of Punjab, Khyber Pakhtunkhwa, and Baluchistan, regions known for their favorable climatic conditions that support peach cultivation. The Swat Valley in Khyber Pakhtunkhwa, in particular, is renowned for its high-quality peach production, contributing significantly to the local economy and livelihoods of farmers (Ullah et al., 2018). The crop's relatively short cultivation cycle allows farmers to generate revenue, which is particularly beneficial in regions with limited agricultural resources (Fahim, 2022). The peach industry also supports various ancillary sectors, including transportation, packaging, and processing industries, thereby creating employment opportunities and contributing to rural development.

The domestic market for peaches in Pakistan is robust, with a strong demand driven by the fruit's popularity among consumers. Peaches are commonly consumed fresh, but there is also a growing market for processed peach products such as juices, jams, and canned peaches. This demand has led to the establishment of small- and medium-sized enterprises (SMEs) focused on peach processing, which further adds value to the fruit and extends its market reach (Palwasha & Fahim, 2022). Exporting peaches is another area where Pakistan has significant potential. Although the country’s peach export volume is currently modest, there has been a growing interest in expanding into international markets, particularly in the Middle East and Central Asia, where Pakistani peaches are highly valued for their flavor and quality (N. Khan et al., 2019). In 2021, Pakistan exported approximately 3,500 metric tons of peaches, generating revenue of

around $4.5 million (Pakistan Bureau of Statistics, 2022). This figure, while still small compared to other fruit exports, highlights the untapped potential of peach exports (Khan et al., 2019).

The economic importance of peaches in Pakistan is also evident in the government's agricultural policies. Various initiatives have been launched to support peach cultivation, including the provision of subsidies for quality seeds, fertilizers, and pest control measures. Additionally, agricultural research institutions in Pakistan are actively involved in developing new peach varieties that are more resistant to diseases and have better yield potential, which is expected to further enhance the productivity and profitability of peach farming in the country (Rabbi et al., 2020). However, despite the economic significance of peaches, the sector faces several challenges that hinder its full potential. These include issues related to post-harvest handling, inadequate cold storage facilities, and limited access to international markets due to stringent phytosanitary requirements. Additionally, peach production in Pakistan is highly susceptible to climatic variations and disease outbreaks (Ullah et al., 2018). Addressing these challenges is crucial for ensuring the sustainability and growth of the peach industry in Pakistan.

In conclusion, peaches are a valuable agricultural commodity in Pakistan, contributing to the economy through domestic sales, exports, and the support of related industries (Shah et al., 2022). Continued investment in research, infrastructure, and market access will be essential for the future growth of Pakistan’s peach industry.

1.2.3 Nutritional and Health Benefits of Peaches

Peaches are not only economically significant but also highly valued for their rich nutritional profile and associated health benefits. This fruit contains essential vitamins, minerals, and antioxidants. The nutritional content and health-promoting properties of peaches are crucial for human health (Bento et al., 2022).

Peaches are excellent source of vitamin C (ascorbic acid), which has an important role in collagen synthesis and boost immune system (Hans et al., 2020). Vitamin C is an antioxidant, and it can neutralize free radicals in the body to resist cancer and heart disease. Peaches also contain vitamin A (beta-carotene), which is important for the health of vision, skin, and mucous membranes. Vitamin A supports immune function of the body and decreases the risk of aging (Hussain et al., 2021).

Peaches also contain significant amounts of dietary fiber, with a medium peach providing about 2 grams, which is roughly 8% of the daily recommended intake for adults. Dietary fiber is essential for proper digestion (Alasalvar et al., 2020). Moreover, high fiber intake also reduces the risks of heart disease (Yang et al., 2022). Peaches also contain a great amount of potassium, which is important for maintaining blood pressure and lowering the risk of hypertension (Abdelghafar et al., 2018). One of the key health benefits of peaches lies in their antioxidant capacity. Different phenolic compounds, such as chlorogenic acid, catechins, and flavonoids, are present in peaches, in ample amount and they possess anti-inflammatory properties (Abobatta, 2020). These compounds help reduce inflammation in the body and lower cholesterol levels. The consumption of peaches has also been associated with skin health. Moreover, the antioxidants of peaches protect skin from ultraviolet (UV) radiations (Mihaylova, et al., 2021).

Research has also indicated that the regular consumption of peaches may contribute to weight management. Peaches are low in calories, with a medium peach containing only about 60 calories, making them a healthy snack option for those looking to control their weight (Mihaylova et al., 2021). The health-promoting properties of peaches, including their potential to support immune function, improve skin health, and aid digestion, highlight their importance as a functional food in both traditional and modern diets.

1.2.3 Application of Nanoparticles in Managing Peach Diseases

Nanotechnology offers a promising approach to manage peach diseases, particularly those caused by pathogenic microorganisms that lead to significant crop losses. NPs act as efficient carriers of agrochemicals or as antimicrobial agents, thereby enhancing disease management strategies' efficacy in peach cultivation (Tahir, 2021). One of the major challenges in peach production is the control of brown rot, which is caused by *Monilinia spp.*, and it can devastate this crop. Traditional methods of controlling such diseases often involve the use of chemical fungicides, which, while effective, can damage human health and environment, due to their residue accumulation. Nanoparticles offer an alternative or complementary approach to conventional fungicides (Roberto et al., 2019).

NPs have shown significant efficacy in controlling fungal pathogens and they can effectively reduce the brown rot of peaches (Akbar et al., 2023). CuO NPs are another class of metal oxide nanoparticles with proven antifungal properties. CuO NPs are known to interfere

with the metabolic processes of fungal cells by disrupting enzyme activity and generating ROS. CuO NPs can display mycelial growth inhibition of *M. fructicola*, the pathogen responsible for brown rot in peaches. This nanoparticle-based approach not only minimizes the need for chemical fungicides but also enhances the overall sustainability of peach production (Saqib et al., 2020). Some studies have also demonstrated the use of chitosan and chitosan nanoparticles in controlling and managing peach diseases. Chitosan nanoparticles, derived from chitosan, a natural polysaccharide, exhibit antimicrobial activity. When applied to peaches, chitosan NPs can trigger defense-related enzymes and phenolic compounds, enhancing fruit's resistance to fungal infections. This biocompatible and biodegradable nanomaterial offers an eco-friendly alternative to synthetic chemicals in peach disease management (Kralova & Jampilek, 2023).

The use of nanoscale delivery systems for agrochemicals has emerged as a novel strategy to improve the targeting and efficacy of disease control agents. Encapsulating fungicides or biocontrol agents within nanoparticles allows for controlled release and increased bioavailability at the site of infection, reducing the required dosage and minimizing environmental impact. For instance, encapsulating fungicides in polymeric nanoparticles can protect the active ingredients from degradation, enhance their stability, and ensure sustained release, thereby providing prolonged protection against pathogens such as *Monilinia spp.* in peaches (Scortichini, 2022). Despite the promising results, the application of nanoparticles in agriculture, including peach disease management, has its own challenges. Issues such as the potential toxicity of nanoparticles to non-target organisms, which might be beneficial microorganisms, and risk assessments must be addressed to ensure useful application of nanotechnology in agriculture (Moreno, 2022). Applying nanoparticles in important to manage diseases and reduce reliance on chemical fungicides.

1.3 Apple: Economical and Health Benefits

1.3.1 Economic Importance of Apple in Pakistan

Apple production holds substantial economic importance in Pakistan, supporting the livelihoods of thousands of farmers and significantly contributing to the country's agricultural sector. The apple industry is particularly prominent in the provinces of Baluchistan, Khyber Pakhtunkhwa, and Azad Kashmir, where the climatic conditions are ideal for apple cultivation. Baluchistan, in particular, is renowned for its apple orchards, which benefit from the region's

temperate climate and fertile soil, making it a major apple-producing area in Pakistan (Alves et al., 2019). According to the Pakistan Bureau of Statistics (2022), the country produced approximately 640,000 metric tons of apples, making it a leading fruit crop in terms of both production volume and cultivation area. This production scale not only meets domestic consumption needs but also has significant implications for export potential. The apple sector provides income to thousands of farmers, where agriculture is a primary source of livelihood (Asma et al., 2023).

The economic impact of apple production extends beyond the direct revenues from sales. It encompasses the entire value chain, including farming, harvesting, processing, and distribution. In the apple-growing regions of Pakistan, the industry supports ancillary businesses such as cold storage facilities, packaging units, and transportation services. This broader economic ecosystem contributes to local employment and development, reinforcing the importance of the apple sector to the overall economic health of these regions. Export markets are a crucial aspect of the apple industry's economic significance. Pakistan exports apples to neighboring countries like Afghanistan and India, as well as to various Middle East countries (Naseer et al., 2018). The increasing demand for Pakistani apples abroad reflects the high quality and distinctive taste of the fruit, which is attributed to the unique growing conditions in Pakistan. In 2021, the country’s apple exports were valued at over USD 50 million, highlighting the fruit's role in generating foreign exchange and enhancing Pakistan's trade balance (Trade Development Authority of Pakistan, 2022).

Despite its economic benefits, the apple industry in Pakistan faces several challenges that threaten its sustainability and growth. One major issue is the reliance on outdated farming practices, which limits productivity and quality. Many apple orchards still use traditional methods, resulting in lower yields compared to modern agricultural techniques. Additionally, there is a significant gap in infrastructure, particularly in cold storage and transportation facilities. This inadequacy leads to post-harvest losses and reduce competitiveness in the market. Climate change also poses a threat to apple production in Pakistan. The sector is heavily dependent on water resources, which are becoming increasingly scarce due to erratic rainfall patterns and poor water management. Water scarcity not only impacts apple yields but also affects the overall health of the orchards (He et al., 2019).

To address these challenges, there is a need for modernization and innovation within the apple industry. Implementing advanced agricultural practices, improving infrastructure, and enhancing access to international markets are essential steps toward sustaining and expanding the economic benefits of apple production. Efforts on improving apple varieties, pest management, and post-harvest technologies could bolster the industry's resilience and profitability. Moreover, the economic importance of apple production in Pakistan is multifaceted, encompassing direct revenue from sales, contributions to rural employment, and export potential. However, the industry faces challenges that must be addressed through modernization and improved infrastructure to ensure its continued growth and sustainability. By overcoming these obstacles, Pakistan can further enhance the economic contributions of its apple sector and strengthen its position in the global market (Madbouly, 2021).

1.3.2 Health Benefits of Apple

Apples have numerous health benefits, making them a vital component of a healthy and balanced diet. Apple contains essential nutrients and fiber and contributes to overall well-being (Bhat et al., 2023). One of the most significant health benefits of apples is their role in promoting cardiovascular health. Apples contain a handsome amount of fiber, particularly soluble fiber in the form of pectin. Pectin is considered important to lower contents of low-density lipoprotein (LDL) cholesterol, which is also known as "bad" cholesterol (He & Hwang, 2016). Additionally, apples contain polyphenols, such as quercetin and catechins, which have been shown to improve vascular health by enhancing endothelial function and reducing inflammation (Kumar et al., 2020).

Apple also contains high antioxidant contents, which plays a crucial role in reducing oxidative stress and inflammation. Apples also contain flavonoids, including quercetin, epicatechin, and kaempferol, which possess strong antioxidant properties. (Abdel-Rahman et al., 2021). The consumption of apple fiber can improve gastrointestinal health by increasing stool frequency and improving stool consistency (Kumar et al., 2021). Furthermore, the prebiotic effects of apple fiber support the growth of gut microbiota, which can enhance overall digestion process (Bennet et al., 2014). Consumption of Apple also contributes positively to weight management. The fiber and water content of apples help promote satiety, reducing overall calorie intake. Apples consumed before meals experienced greater satiety and consumed fewer calories

compared to those who did not (Teshome et al., 2023). Apples contain a combination of fiber, water, and natural sugars in apples, which contribute to a feeling of fullness and help regulate appetite. The soluble fiber in apples slows down the absorption of glucose, leading to more stable blood sugar levels (Teshome et al., 2023)

Apples contain various nutrients essential for bone health, including vitamin C, potassium, and flavonoids. Vitamin C synthesizes collagen, which is important for healthy bones and tissues. Potassium helps to regulate calcium levels in the body, which is important for bone density. Studies have shown that higher apple consumption provides mineral to the bones and avoids osteoporosis (Jeyavishnu et al., 2021). Apples provide cardiovascular health and improve digestion and weight management. Their rich nutrient profile and antioxidant content make them important for human health.

1.3.3 Use of Nanoparticles in Preventing Apple Diseases

The use of NPs is a groundbreaking strategy to manage plant diseases, including those affecting apples. Nanoparticles offer innovative solutions to combat various phytopathogens, increase crop yield and enhance fruit quality (Singh et al., 2021). Apples are susceptible to various fungi, bacteria and viruses. Famous diseases of apples include apple scab and powdery mildew, which are caused by *Podosphaera leucotricha* and *Venturia inaequalis*, respectively. Traditional disease management practices often involve chemical fungicides, which are toxic to human gut and environment. The use of nanoparticles presents a promising alternative, offering targeted and efficient disease control with reduced environmental impact (Suhag et al., 2023).

Fe_2O_3 NPs have been reported to manage apple diseases by generating reactive oxygen species (ROS) and disrupting microbial cell membranes (Khan et al., 2020). Fe_2O_3 NPs have been described to inhibit the mycelial growth of *V. inaequalis*, the pathogen responsible for apple scab. NPs disrupt fungal mycelial growth and reduce spore germination, thereby controlling the spread of the disease (Khan et al., 2020). Another research confirmed that Fe_2O_3 nanoparticles generate ROS upon contact with fungal cells, which results in enhanced antifungal activity. This oxidative stress mechanism is highly effective against various fungal pathogens, making Fe_2O_3 nanoparticles a viable option for disease management in apple orchards (Tomar et al., 2020).

Fe_2O_3 nanoparticles also enhance plant resistance to different diseases. In lants, NPs induce systemic acquired resistance (SAR) and protect plants from pathogenic damages. Fe_2O_3 nanoparticles trigger SAR in apple plants, leading to increased expression of PR proteins and enhanced disease resistance (Zhang et al., 2021). This effect contributes to the overall health and resilience of apple trees, reducing the need for chemical interventions (Singh & Rattanpal, 2014). Furthermore, nanoparticles can improve the efficiency of fungicide application by enhancing the penetration and distribution of active ingredients. High surface area and reactivity of nanoparticles enable them to penetrate plant tissues more effectively than traditional fungicides. Fe_2O_3 nanoparticles, when used in combination with conventional fungicides, improve the efficacy of disease control by increasing the fungicide's absorption and retention on plant surfaces (Ndlovu et al., 2020). This collaborative effect enhances the overall performance of disease management strategies.

Nanoparticles might persist in the environment, which is their negative feature, and it must be addressed. However, ongoing research aims to optimize nanoparticle formulations, ensure environmental safety, and reduce production costs. Synthesis of non-toxic nanoparticle technologies will further enhance their applicability and acceptance in agricultural practices (Sekhon, 2014). The application of Fe_2O_3 nanoparticles offers a promising approach to managing apple diseases. Their antimicrobial properties, ability to induce plant resistance, and enhancement of fungicide efficiency make them valuable tools for disease control in apple orchards. Continued research is important to optimize nanoparticle applications, ensuring sustainable and effective disease management in apple production. Integrating nanoparticles into disease management strategies holds great potential for improving apple health and crop yield while reducing the reliance on chemical fungicides (Khan et al., 2019).

1.4 Strawberries: Economic Importance, Health Benefits, and Nanoparticle Application

1.4.1 Economic Role of Strawberry Cultivation in Pakistan

Strawberry cultivation holds significant economic value in Pakistan’s agricultural sector, contributing to both domestic consumption and export markets. The fruit, known for its high demand and profitability, is cultivated in various regions of Pakistan, including the Punjab and Khyber Pakhtunkhwa provinces. The economic role of strawberries in Pakistan can be examined through aspects such as production volume, market demand, and export potential. In Pakistan,

strawberry farming is a lucrative venture due to the fruit's high market value and relatively short cultivation period. According to the Pakistan Bureau of Statistics (PBS), the area under strawberry cultivation has been expanding, reflecting its increasing popularity among farmers. The fruit is typically grown in the winter season, which aligns with high demand periods in both local and international markets. The Punjab province, with its favorable climatic conditions, is the primary region for strawberry production, contributing significantly to the national output (Luksiene et al., 2020).

The economic benefits of strawberry cultivation are evident in the income generated by farmers. Strawberries are sold at premium prices due to their high quality and perishable nature. The fruit's market value is further enhanced by its high demand during the winter months. According to FAO, strawberries are lucrative fruit crops, with a return on investment that can exceed other agricultural products (Hemmati et al., 2020). In addition to domestic sales, strawberries have considerable export potential. Pakistan exports strawberries to various countries, including the Middle East and Central Asia. The country’s export of fresh strawberries has been growing steadily, driven by international demand for high-quality fruit. The Pakistan Horticulture Development and Export Company (PHDEC) reports that strawberry exports have increased by approximately 15% annually over the past five years, highlighting the fruit's importance in the export sector (Rahman et al., 2021).

The economic impact of strawberry cultivation extends beyond the farm level. Industry supports various ancillary activities, including packaging, transportation, and marketing. The development of a robust supply chain and marketing infrastructure has been crucial in enhancing the economic viability of strawberry farming. Investments in cold storage and transportation facilities are essential for fruit preservation, thereby increasing their export values (Khan et al., 2022). However, the strawberry industry faces challenges that impact its economic sustainability. Issues such as pest and disease management, high input costs, and climate variability can affect yield and quality. Addressing these challenges through innovative solutions, such as the use of nanoparticles for disease control, is crucial for ensuring the long-term profitability of strawberry farming in Pakistan. Strawberry cultivation plays a significant economic role in Pakistan, contributing to both domestic and international markets. The fruit’s high market value, coupled with its growing export potential, makes it a vital component of the agricultural economy.

Continued investment in production techniques, infrastructure, and disease management will be essential for sustaining the economic benefits of strawberry farming in Pakistan (Mozafari et al., 2018).

1.4.2 Nutritional and Health Benefits of Strawberries

Strawberries are renowned for their rich nutritional profile and various health benefits, making them a popular choice in fresh and processed forms. These benefits stem from their high content of essential nutrients, vitamins, and bioactive compounds. Strawberries particularly contain high vitamin C content (Mogazy et al., 2022). Strawberries also contain dietary fiber, particularly soluble fiber such as pectin. Fiber is essential for digestive health and maintain blood sugars (Mogazy et al., 2022). Strawberries are also packed with various phytonutrients, including flavonoids and phenolic acids. One of the most studied flavonoids in strawberries is quercetin, which has been associated with reducing inflammation (Mogazy et al., 2022). Another important compound, ellagic acid, has been linked to reduce oxidative damage (Mogazy et al., 2022). The high anthocyanin content in strawberries, which gives them their vibrant red color, has also been associated with several health benefits. Anthocyanins are known to enhance overall heart health and help lower the risk of heart attack and stroke by improving endothelial function and reducing arterial stiffness (McCullough et al., 2017).

Furthermore, strawberries are low in calories and contain negligible amounts of fat. The low caloric density combined with high fiber content contributes to their role in a balanced diet that supports weight management (Kendall et al., 2014). The fruit's natural sugars and unhealthy fats are often found in processed snacks and desserts. The antioxidants and vitamin C in strawberries contribute to the production of collagen, which controls wrinkle, acne and eczema (Garza-Alonso et al., 2022). High content of vitamins, minerals, fiber, and bioactive compounds supports various aspects of health.

1.4.3 Application of Nanoparticles in Combating Strawberry Diseases

Strawberries are susceptible to various diseases that can significantly impact their quality and yield. Nanoparticles are being extensively used for the control of fruit diseases, due to their small size, reduced surface properties, and ability to interact with biological systems at a molecular level. Nanoparticles can deliver active compounds directly to target sites with high

precision. This targeted delivery improves the efficiency of disease control measures and minimizes the impact on non-target organisms. For strawberries, nanoparticles have shown significant potential in managing diseases like gray mold, powdery mildew and anthracnose, which are caused by *B. cinerea, S. macularis* and *Colletotrichum* spp., respectively (Giampieri et al., 2014).

AgNPs have been shown to effectively control *B. cinerea,* a common pathogen responsible for gray mold. Research indicates that Ag NPs can inhibit the growth of this fungus by disrupting its cellular functions and reducing spore germination (Rai et al., 2017). Furthermore, Ag NPs have low toxicity to plants and beneficial microorganisms, making them a viable option for integrated disease management strategies. Copper nanoparticles are another promising nanomaterial for disease control. Copper is a well-known fungicide, and its efficacy is enhanced when delivered in nanoparticle form. Cu NPs have been reported to combat powdery mildew in strawberries (Khan et al., 2020). In strawberries, Fe_2O_3 NPs have been shown to reduce the incidence of anthracnose in strawberries (Tariq et al., 2019). The effectiveness of nanoparticles in disease management is largely attributed to their small size, which allows for better penetration and distribution within plant tissues, ensuring a more comprehensive treatment of infected areas. The surface modification of nanoparticles can also enhance their interaction with pathogens and improve their stability in the environment (El-Sayed et al., 2021).

Disease control in strawberry cultivation involves several practical considerations. The foliar, soil and see application of nanoparticles can influence their effectiveness and uptake. Additionally, the concentration and formulation of nanoparticles must be optimized to balance efficacy and safety. While nanoparticles offer significant advantages, their use in agriculture also presents challenges, including potential environmental impacts, regulatory issues, and the need for standardized application protocols (Khan et al., 2020). Nanoparticles have been reported useful in controlling key pathogens, enhancing plant resistance, and improving overall fruit quality.

1.5 Introduction to Nanoparticles: Properties, Types, and Characteristics

Nanoparticles, with 1-100 nm size, are tiny entities that possess unique features. The distinct behaviors of nanoparticles arise from quantum effects and size-dependent properties. This introduction will explore the fundamental characteristics of nanoparticles, including their

types, properties, and applications, providing a comprehensive understanding of their significance in various fields (Khan et al., 2019).

1.5.1 Fundamental Properties of Nanoparticles

1. **Size and Surface Area:** NPs are known for their small size, which imparts a large surface to volume area. This property enhances their reactivity and interaction with surrounding environments. This property is crucial in applications such as catalysis and drug delivery, where enhanced surface interaction can lead to improved performance (Joudeh & Linke, 2022).
2. **Quantum Effects:** Quantum effects arise due to the confinement of electrons within the nanoparticle, leading to unique optical, electronic, and magnetic properties. For example, quantum dots, which are semiconductor nanoparticles, exhibit size-dependent fluorescence. As the size of the quantum dots decreases, the emitted light shifts to shorter wavelengths (Chen et al., 2017). Similarly, nanoparticles scatter light at specific wavelengths (Jung et al., 2018).
3. **Optical Properties:** Nanoparticles interact with light and the phenomenon of surface plasmon resonance (SPR) is particularly notable. It occurs when conduction electrons in the nanoparticle resonate with incident light, leading to intense absorption and scattering. This property is used in biosensing and imaging (Modena et al., 2019).
4. **Magnetic Properties:** Magnetic NPs exhibit size-dependent magnetic properties. Superparamagnetism is a prominent feature of magnetic nanoparticles. This property is advantageous in magnetic resonance imaging (MRI) and targeted drug delivery (Kim et al., 2019).
5. **Chemical Reactivity:** The chemical reactivity of nanoparticles is significantly enhanced by their surface chemistry. Surface functionalization facilitates the attachment of various chemical groups on the surface of NPs. This property is useful for the catalysis processes, where nanoparticles serve as efficient catalysts due to their increased surface reactivity (Zhao & Stenzel, 2018).

1.5.2 Types of Nanoparticles

1. **Metallic Nanoparticles:** Metallic nanoparticles, including gold, silver, platinum, and copper, are commonly used nanomaterials. Au NPs are known for their distinct optical properties and are used in imaging, sensing, and therapeutic delivery. Silver nanoparticles (Ag NPs) possess antimicrobial properties and are employed in wound dressings and disinfectants. Platinum and copper nanoparticles are used in catalysis and environmental remediation (Sudha et al., 2018).
2. **Semiconductor Nanoparticles:** These NPs are synthesized from materials with semiconductor properties that exhibit size-dependent optical and electronic characteristics. Quantum dots emit specific wavelengths of light. Materials such as cadmium selenide (CdSe) and lead sulfide (PbS) are used to synthesize quantum dots (Jamkhande et al., 2019).
3. **Metal Oxide Nanoparticles:** Titanium dioxide (TiO_2), zinc oxide (ZnO), and iron oxide (Fe_2O_3) are metal oxide NPs and they have diverse applications due to their photocatalytic and antibacterial properties. TiO_2 NPs are used for photocatalysis and self-cleaning surfaces, while ZnO nanoparticles are employed in sunscreens and as antibacterial agents. Iron oxide nanoparticles are utilized in MRI imaging and environmental cleanup (Singh et al., 2020).
4. **Carbon-Based Nanoparticles:** This type of NPs include carbon nanotubes (CNTs), graphene, and fullerenes, These NPs possess exceptional mechanical and thermal properties (Geim & Novoselov, 2007).
5. **Polymeric Nanoparticles:** These NPs are composed of organic polymers which are biocompatible and degradable (Gref et al., 2014).

1.5.3 Characteristics of Nanoparticles

1. **Surface Modifications:** Surface modification of nanoparticles is crucial for enhancing their stability, functionality, and biocompatibility. Various methods, such as coating with surfactants, ligands, or polymers, can be employed to modify the surface of nanoparticles. These modifications increase the solubility of NPs and enable specific interactions with target molecules.

2. **Size Distribution and Shape:** These are very important traits of nanoparticles and define their further application. For example, spherical NPs are often used in imaging and sensing, while rod-shaped nanoparticles exhibit anisotropic optical properties that can be utilized in therapeutic applications (Jeevanandam et al., 2018)
3. **Toxicity and Environmental Impact:** These are very important considerations in the development and application of NPs. Studies on nanoparticle toxicity are essential for ensuring their safe use and minimizing potential adverse effects (Nel et al., 2006).
4. **Synthesis and Fabrication:** For the synthesis of NPs, various biological (using plants and microbes) physical, and chemical techniques are being used. Physical methods involve the direct manipulation of materials to produce nanoparticles. Chemical methods involve chemical reactions, and biological techniques offer environmentally friendly alternatives for nanoparticle synthesis (Wang et al., 2017).

1.6 Types of Nanoparticles Used in Agriculture

1.6.1 Commonly Used NPs in Agriculture

Nanoparticles are being used in agriculture to enhance germination, improve yield, positively influence physiological traits and control diseases. Following types of nanoparticles have been reported to be synthesized and applied in the field of agriculture.

1. Metallic Nanoparticles

Following metallic nanoparticles are being extensively used in agricultural practices due to their antimicrobial properties and potential to improve crop yield.

- Silver Nanoparticles (Ag NPs): These nanoparticles have been reported to control plant diseases and inhibit microbial growth in soil. They can effectively target bacteria, fungi, and viruses. Ag NPs are used in seed treatments to promote germination and protect seedlings from soil-borne diseases (Rai et al., 2017). Ag NPs can reduce disease incidence by preventing pathogen proliferation (Singh et al., 2021). These the one of the most used nanoparticles in the field of agriculture.
- Gold Nanoparticles (Au NPs): These are employed in agriculture primarily for their role in plant disease detection and monitoring (Ghosh et al., 2017). Additionally, Au NPs have

been explored for targeted delivery of agricultural chemicals, improving the efficiency of pesticide application and nutrient delivery (Chhipa, 2019).

- Copper Nanoparticles (Cu NPs): Copper nanoparticles are used for their fungicidal properties. Cu NPs are effective in controlling fungal diseases in crops and have been applied as an alternative to traditional copper-based fungicides. They exhibit enhanced efficacy due to their high surface reactivity, which improves their interaction with fungal cells (Sharma et al., 2019). These NPs deposit less chemical residues and lower environmental impact compared to conventional fungicides.

2. Metal Oxide Nanoparticles

These NPs have diverse applications in agriculture due to their photocatalytic, antibacterial, and magnetic properties. The following are the major metal NPs.

- Titanium Dioxide Nanoparticles (TiO_2 NPs): These nanoparticles are widely known for their photocatalytic properties to break organic pollutants in soil and water. TiO2 NPs can enhance soil health by degrading harmful chemicals and improving nutrient availability. Additionally, they are used in developing smart fertilizers and pesticide delivery systems, where their photocatalytic activity can control slow their release (Chen et al., 2020).
- Zinc Oxide Nanoparticles (ZnO NPs): These NPs are famous for their antimicrobial and UV-blocking properties. ZnO NPs are used to improve plant health by acting as antifungal agents and protecting plants from harmful UV radiation. They also act as a source of micronutrient to plants (Chavali & Nikolova, 2019). ZnO NPs are applied on seeds, in soil, and on foliage to deliver zinc in a bioavailable form, addressing zinc deficiencies in crops.
- Iron Oxide Nanoparticles (Fe_2O_3 NPs): These are magnetic NPs and these can be employed for the remediation of soil and water through magnetic separation. They also serve as carriers for delivering fertilizers and pesticides, providing controlled release and targeted application (Nikolova & Chavali, 2020).

3. Carbon-Based Nanoparticles

These types of NPs include carbon nanotubes (CNTs) and graphene, are explored for their mechanical strength, electrical conductivity, and versatility in agricultural applications.

- Carbon Nanotubes (CNTs): These are utilized in agriculture for their mechanical strength and ability to improve soil structure. They are incorporated into soil to enhance its physical properties, leading to better aeration and water retention (Liu et al., 2019).
- Graphene: Graphene nanoparticles have high electrical conductivity, and their surface area is also large. They are used in developing sensors for detecting plant diseases and monitoring soil nutrients. Additionally, graphene-based materials are incorporated into fertilizers and pesticides to enhance their effectiveness and reduce environmental impact (Zhu et al., 2020).

4. Polymeric Nanoparticles

These NPs are composed of organic polymers and are used in agriculture. They are very helpful in controlled release of fertilizers and pesticides.

- Poly (lactic-co-glycolic acid) (PLGA) NPs: These NPs are employed for their biodegradability and ability to encapsulate various agricultural chemicals. They are used to create smart fertilizers and pesticide formulations that release their active ingredients in a controlled manner, enhancing their efficiency and reducing the frequency of application (Gref et al., 2014).
- Polyvinyl Alcohol (PVA) Nanoparticles: PVA nanoparticles are utilized for their water solubility and biocompatibility. These NPs develop biodegradable films, which is further used for the coatings of seeds and soil (Singh et al., 2021).

In summary, nanoparticles offer a range of benefits in agriculture, including improved disease management, enhanced nutrient delivery, and environmental remediation. Their unique properties enable innovative solutions for addressing challenges in crop production and soil health. The continued exploration and development of nanoparticles hold promise for advancing sustainable agricultural practices.

1.6.2 Fe_2O_3 Nanoparticles: Synthesis, Properties, and Benefits

In agriculture, Fe_2O_3 NPs are increasingly recognized for their potential in enhancing crop production, managing soil health, and combating plant diseases.

1. Synthesis of Fe_2O_3 NPs

For the synthesis of Fe_2O_3 nanoparticles, the following methods are being used:

- Sol-Gel Method: It is one of the simplest methods for the synthesis of Fe_2O_3 NPs. Using this method, highly pure NPs are produced. In this process, iron salts (such as iron chloride or iron nitrate) are dissolved in a solvent to form a sol. This sol is then gelled by adding a gelling agent. The sol-gel method allows the formation of NPs with precise size and morphology (Lassoued et al., 2018).
- Hydrothermal Synthesis: Hydrothermal synthesis involves high-pressure and high-temperature conditions to produce Fe_2O_3 NPs. Using this method, nanoparticles of uniform size and high crystallinity are produced. These NPs exhibit enhanced stability and reactivity (Ramalingam et al., 2020).
- Chemical Vapor Deposition (CVD): This method produces NPs of precise size and shape. In this process, iron-containing gases are decomposed on a substrate to form Fe_2O_3 nanoparticles (Srinivasan et al., 2019).
- Green Synthesis: This method uses a variety of biological agents including plant extracts or microorganisms, to produce Fe_2O_3 NPs. This approach is environmentally friendly and reduces the use of hazardous chemicals. For example, plant extracts rich in polyphenols can act as reducing agents to convert iron salts into Fe_2O_3 NPs (Rao et al., 2020).

2. Properties of Fe_2O_3 Nanoparticles

Fe_2O_3 nanoparticles have the following unique properties:

- Magnetic Properties: Fe_2O_3 nanoparticles possess unique magnetic properties. These magnetic properties enable their use in soil and water remediation through magnetic separation. Magnetic Fe_2O_3 NPs can be easily recovered from soil or water using external magnetic fields, making them valuable for removing contaminants and pollutants (Karade et al., 2019).
- Photocatalytic Activity: Fe_2O_3 NPs exhibit photocatalytic activity to degrade organic pollutants in soil and water (Sivakumar et al., 2021). This property is particularly useful in developing sustainable agricultural practices.

- High Surface Area: This property of Fe_2O_3 NPs enhances their reactivity and interaction with other substances. It is useful in nutrient delivery and pesticide formulation (Fahmy et al., 2018).

3. Benefits of Fe_2O_3 Nanoparticles in Agriculture

Fe_2O_3 nanoparticles offer numerous benefits in agricultural applications, including disease management, soil improvement, and nutrient delivery. Their unique properties enable innovative solutions for enhancing crop productivity and sustainability.

- Disease Management: Fe_2O_3 nanoparticles have demonstrated efficacy in managing plant diseases. Fe_2O_3 nanoparticles can inhibit mycelial growth of pathogenic microorganisms that cause fruit rot and other plant diseases. Fe_2O_3 NPs can be applied as a coating on seeds or plants to protect them from soil-borne pathogens and reduce disease incidence (Choi et al., 2020).
- Soil Improvement: The ability of Fe_2O_3 nanoparticles to remove contaminants from soil through magnetic separation helps improve soil quality and fertility. Additionally, Fe_2O_3 NPs can enhance soil structure and aeration, leading to better root growth and nutrient uptake (Zhang et al., 2020).
- Nutrient Delivery: Fe_2O_3 NPs are important agents for slow delivery of fertilizers and pesticides. High surface area and reactivity of these NPs allow the encapsulation of agricultural chemicals, providing a slow and sustained release (Hosseini et al., 2021). Fe_2O_3 NPs can be enhance the effectiveness of fertilizers or pesticide (Terna et al., 2021).

In conclusion, Fe_2O_3 nanoparticles offer significant advantages in disease management, soil improvement, and nutrient delivery. These NPs contribute to sustainable agricultural practices and enhance crop productivity. As research continues to explore and refine the applications of Fe_2O_3 NPs, their potential for revolutionizing agricultural practices becomes evident.

1.7 Mycosynthesis of Fe_2O_3 Nanoparticles

1.7.1 Introduction to Mycosynthesis: Process and Advantages

Mycosynthesis refers to the formation of NPs using fungi, a process that harnesses the natural metabolic pathways of fungal organisms to produce nanomaterials. This method has

replaced traditional chemical synthesis techniques. It is an eco-friendly and cost-effective approach. In this process, metal ions are reduced through the enzymatic and metabolic activities of fungi.

Process of Mycosynthesis

The mycosynthesis process begins with the selection of suitable fungal strains capable of reducing metal ions. These fungi, such as *Fusarium oxysporum*, *Aspergillus niger*, and *Trichoderma viride*, are exposed to metal salt solutions under controlled conditions. This process can be executed by the following steps:

1. Fungal Cultivation: Selected fungal strains are cultivated in a nutrient-rich medium to achieve optimal growth.
2. Metal Ion Exposure: The grown fungal cultures are then exposed to solutions containing metal salts, which serve as precursors for Fe_2O_3 nanoparticles. Usually ferric chloride ($FeCl_3$) and ferric nitrate ($Fe(NO_3)_3$) are used for this purpose.
3. Reduction and Formation: Fungal enzymes such as reductases and metabolic products like secondary metabolites reduce metal ions to their NPs.
4. Recovery and Characterization: The nanoparticles are collected from the fungal biomass, and their properties are characterized using various techniques, like FTIR, TEM, SEM and X-ray diffraction (XRD) (Bhattacharya & Gupta, 2005).

Advantages of Mycosynthesis

Mycosynthesis offers several advantages over conventional chemical methods:

- Eco-Friendliness: Mycosynthesis utilizes biological processes, reducing the need for toxic chemicals and minimizing environmental impact. It also operates under mild conditions, lowering energy consumption (Mukherjee et al., 2008).
- Cost-Effectiveness: Fungi are relatively inexpensive and easy to cultivate, making the mycosynthesis process cost-effective compared to traditional methods that require expensive chemicals and equipment.

- Scalability: The mycosynthesis process can be scaled up for industrial applications. Fungal cultures can be grown in large bioreactors for their commercial scale production (Gade et al., 2008).
- Versatility: Fungi can synthesize nanoparticles with various sizes and shapes by adjusting growth conditions, which is beneficial for tailoring nanoparticles for specific applications (Nair et al., 2010).

1.7.2 Specific Techniques and Fungi Used in Fe_2O_3 NP Synthesis

The following techniques are used for the synthesis of NPs.

Techniques in Mycosynthesis

- Solid-State Fermentation (SSF): SSF is a technique where fungi are grown on solid substrates containing metal salts. This method allows large-scale production of NPs. SSF provides a high surface area for fungal growth and metal ion interaction, leading to efficient nanoparticle synthesis (Rai et al., 2009).
- Liquid Fermentation: In liquid fermentation, fungi are grown in a liquid medium containing metal salts. This technique facilitates better control overgrowth conditions and is commonly used for laboratory-scale synthesis. Liquid fermentation allows for the easy collection and purification of nanoparticles from the culture broth (El-Ghamry et al., 2014).
- Bioreactor Cultivation: Bioreactor cultivation involves growing fungi in controlled environments such as bioreactors, where parameters like temperature, pH, and aeration are regulated. This technique enhances the scalability of nanoparticle production and ensures consistent quality and yield (Khan et al., 2013).

Fungi Used in Fe_2O_3 NP Synthesis

- *Fusarium oxysporum*: This fungus is known for its ability to reduce metal ions into nanoparticles. *F. oxysporum* is widely used due to its rapid growth and high efficiency in nanoparticle synthesis. It produces Fe_2O_3 nanoparticles with well-defined sizes and shapes (Singh et al., 2011).

- *Aspergillus niger*: It is another commonly used fungus for nanoparticle synthesis. It is known for its high metal ion uptake capacity and ability to produce nanoparticles with enhanced stability and uniformity. This fungus has been used to synthesize various metal oxides, including Fe_2O_3 (Suresh et al., 2009).
- *Trichoderma viride*: This fungus synthesizes Fe_2O_3 nanoparticles due to its strong enzymatic activity. This fungus can reduce metal ions efficiently, resulting in nanoparticles with desirable properties for agricultural applications (Rai et al., 2010).

1.7.3 Applications of Mycosynthesized Fe_2O_3 Nanoparticles in Agriculture

- Mycosynthesized Fe_2O_3 NPs is an eco-friendly approach of their synthesis. These applications span across disease management, soil improvement, and nutrient delivery, contributing to sustainable agricultural practices.
- **Disease Management**

  Fe_2O_3 NPs synthesized using fungi exhibit antimicrobial properties that can be leveraged for managing plant diseases. The nanoparticles inhibit the growth of pathogens responsible for fruit rot and other plant diseases. For instance, studies have demonstrated that Fe_2O_3 NPs effectively control fungal pathogens (Rajendran et al., 2013). These NPs can protect plants from infection and enhance overall crop health.
- **Soil Improvement**

  In addition to disease management, mycosynthesized Fe_2O_3 NPs also improve soil properties. These NPs remove contaminants from soil through adsorption and magnetic separation contributes to soil decontamination and enhancement. These NPs bind with heavy metals and organic pollutants and reduce their availability and toxicity in the soil (Mohan et al., 2007). This property supports sustainable soil management practices by improving soil quality and fertility.
- **Nutrient Delivery**

  Fe_2O_3 NPs are also utilized for controlled nutrient delivery in agriculture. The nanoparticles can be used to encapsulate essential nutrients, ensuring their slow and sustained release to plants. Fe_2O_3 NPs can be incorporated into fertilizers or soil conditioners to improve their efficacy and minimize environmental impact (Li et al., 2012).

1.7.4 Antimicrobial Mechanisms of Fe_2O_3 Nanoparticles

Fe_2O_3 NPs are recognized for their antimicrobial properties, which have proven effective in combating a range of pathogens responsible for fruit rot diseases. Understanding these mechanisms is crucial to leveraging their full potential in agricultural applications.

Oxidative Stress Induction

A key mechanism through which Fe_2O_3 nanoparticles exert their antimicrobial effects is their ability to induce oxidative stress. Fe_2O_3 nanoparticles produce reactive oxygen species (ROS) to damage microbial cells (Khan et al., 2018). This oxidative stress is induced by Fenton reaction, which contributes to the antimicrobial efficacy of Fe_2O_3 nanoparticles (Cui et al., 2020). This continuous production of ROS enhances their effectiveness in controlling pathogens, making them a valuable tool in managing fruit rot diseases.

Disruption of Cellular Membranes

Fe_2O_3 nanoparticles also exert antimicrobial effects by disrupting microbial cellular membranes, which can lead to structural damage. This physical interaction and reactivity can destabilize the microbial membranes (Gao et al., 2021). This membrane disintegration by Fe_2O_3 nanoparticles compromises essential cellular functions, making it difficult for microorganisms to maintain their structural and functional stability (Ali et al., 2019).

Interaction with Microbial DNA

Another significant mechanism of Fe_2O_3 nanoparticles is their interaction with microbial DNA. This interaction can cause strand breaks and hinder the process of DNA replication and transcription, and cause cell death (Khan et al., 2020). This mechanism is crucial for the effective control of fruit rot pathogens, as it interferes with the growth and reproduction of the microorganisms, contributing to the overall antimicrobial efficacy (Liu et al., 2021).

Enhanced Uptake of Nutri ents and Compounds

Fe_2O_3 nanoparticles facilitate nutrients uptake by plants. By improving nutrient availability and uptake, these nanoparticles help strengthen plant defenses against pathogen infections. Better nutrient uptake positively influences the production of secondary metabolites and antimicrobial compounds by plants, providing additional protection against fruit rot diseases

(Zhang et al., 2020). The improved bioavailability of nutrients facilitated by Fe_2O_3 nanoparticles supports plant health and resistance, making it more challenging for pathogens to establish infections. This mechanism adds an additional layer of protection beyond the direct antimicrobial effects of Fe_2O_3 nanoparticles, contributing to their overall effectiveness in managing fruit rot diseases.

1.7.5 Specific Case Studies and Research Findings on Fe_2O_3 Nanoparticles

Research into Fe_2O_3 NPs has revealed their significant potential in managing fruit rot diseases, with numerous studies documenting their efficacy and mechanisms of action. This section delves into specific case studies and research findings, highlighting the practical applications and effectiveness of Fe_2O_3 NPs in agricultural disease management.

Case Study 1: Fe_2O_3 Nanoparticles Against *B. cinerea* in Strawberries

B. cinerea, commonly known as gray mold, is a prevalent pathogen responsible for significant losses in strawberry production. This pathogen can be controlled by the application of Fe_2O_3 NPs (Wang et al. 2019). The researchers applied Fe_2O_3 NPs at various concentrations to infected strawberry plants and monitored disease progression and fruit quality. The findings demonstrated a notable reduction in lesion size and spore germination. This disease control is related with the production of reactive oxygen species (ROS) (Wang et al., 2019).

Case Study 2: Fe_2O_3 Nanoparticles for Managing Apple Scab Disease

This disease, caused by *V. inaequalis*. has been reported to be controlled using Fe_2O_3 NPs (Sharma et al. 2020). The researchers applied Fe_2O_3 NPs to apple trees and assessed their impact on disease incidence and fruit yield. The study found that Fe_2O_3 NPs significantly reduced the incidence of apple scab, with a 50% reduction in disease severity. The authors noted that the Fe_2O_3 NPs effectively disrupted fungal cell membranes and induced oxidative stress. Additionally, the treated trees exhibited improved fruit quality and yield, highlighting the potential of Fe_2O_3 NPs as a fungicide (Sharma et al., 2020).

Case Study 3: Fe_2O_3 Nanoparticles in Peaches for Controlling *Monilinia fructicola*

Application of Fe_2O_3 NPs can manage brown rot of peaches, caused by *M. fructicola* (Lee et al. 2021). The researchers treated infected peaches with Fe_2O_3 NPs and evaluated their effectiveness in controlling the disease. Fe_2O_3 NPs markedly decreased (70%) brown rot

symptoms. Fe_2O_3 NPs were effective in inducing oxidative stress and disrupting fungal cells. Additionally, the study highlighted the potential of Fe_2O_3 NPs to improve post-harvest fruit shelf life by reducing disease-related rot (Lee et al., 2021).

Case Study 4: Comparative Study of Fe_2O_3 NPs and Traditional Fungicides

Patel et al. (2022) evaluated the efficacy of Fe_2O_3 NPs in comparison to traditional fungicides in managing fruit rot diseases of apples, peaches, and strawberries, and assessed the performance of Fe_2O_3 NPs against conventional chemical treatments. The study found that Fe_2O_3 NPs were more effective than traditional fungicides in controlling fruit rot diseases. The authors reported that Fe_2O_3 NPs offered several advantages, including reduced environmental impact, lower risk of pathogen resistance, and enhanced safety for consumers. The research highlighted the potential of Fe_2O_3 NPs as an environmentally friendly alternative to chemical fungicides (Patel et al., 2022).

Case Study 5: Fe_2O_3 Nanoparticles in Integrated Disease Management

An integrated disease management approach combining Fe_2O_3 NPs with other control measures was explored by Zhang et al. (2023). The findings revealed that combining Fe_2O_3 NPs with biological control agents, such as beneficial bacteria and fungi, resulted in enhanced disease suppression compared to individual treatments. This research underscored the potential of integrating Fe_2O_3 NPs with other management strategies to achieve comprehensive disease control (Zhang et al., 2023).

1.8 Literature Gap

The exploration of Fe_2O_3 NPs in managing fruit rot diseases has yielded significant insights into their potential as effective tools in agricultural disease management. The studies conducted on peaches, apples, and strawberries have consistently demonstrated the efficacy of Fe_2O_3 NPs in reducing disease severity and improving fruit quality.

Efficacy in Disease Management

Fe_2O_3 NPs have shown promise in controlling fruit rot diseases across multiple fruit crops. For instance, the application of Fe_2O_3 NPs to peaches infected with *M. fructicola* resulted in a substantial decrease in brown rot incidence, highlighting their effectiveness in managing this prevalent disease (Lee et al., 2021). Fe_2O_3 NPs can significantly reduce apple scab caused by *V.*

inaequalis, offering a viable alternative to traditional chemical fungicides (Sharma et al., 2020). Furthermore, research on strawberries reveal that Fe_2O_3 NPs can inhibit the mycelial growth of *B. cinerea*, the pathogen responsible for gray mold (Wang et al., 2019).

Mechanisms of Action

The antimicrobial mechanisms of Fe_2O_3 NPs rely on the production of reactive oxygen species (ROS). These compounds cause oxidative stress in pathogens and inhibit their cell membrane (Patel et al., 2022). Additionally, Fe_2O_3 NPs disrupt pathogen cell walls, enhancing their antimicrobial properties. This mode of action not only suppresses pathogen development but also reduces the potential for resistance, a common issue with conventional fungicides.

Environmental and Economic Implications

Application of Fe_2O_3 NPs offers several environmental and economic benefits. Firstly, Fe_2O_3 NPs are less harmful to the environment compared to traditional chemical fungicides. They decompose more rapidly and reduce the risk of long-term environmental contamination (Zhang et al., 2023). Economically, its application can reduce the reliance on expensive chemical treatments and enhance crop yield and quality, leading to increased profitability for farmers.

Integration with Other Control Measures

The integration of Fe_2O_3 NPs with other disease control strategies has shown promise in enhancing overall disease control. Studies have highlighted the synergistic effects of combining Fe_2O_3 NPs with beneficial microbes, which can lead to more comprehensive disease suppression and improved plant health (Zhang et al., 2023). This integrated approach not only maximizes disease control but also promotes sustainable agricultural practices by reducing the dependence on chemical inputs.

In summary, the key findings from the research on Fe_2O_3 NPs underscore their potential as effective and environmentally friendly tools for managing fruit rot diseases. Their ability to reduce disease severity, improve fruit quality, and offer economic benefits makes them a promising alternative to traditional fungicides. The mechanisms of action, environmental advantages, and potential for integration with other control measures further enhance their value in sustainable agriculture. As research on Fe_2O_3 NPs advances, several future directions and potential applications emerge, highlighting their role in enhancing sustainable agriculture. These

developments promise to further leverage the benefits of Fe_2O_3 NPs while addressing current limitations and expanding their applications.

Exploration of Mechanisms and Efficacy

Future research could investigate how different sizes, shapes, and surface modifications of Fe_2O_3 NPs affect their antimicrobial activity and selectivity. This deeper understanding could lead to the optimization of nanoparticle properties for enhanced efficacy (Joudeh & Linke, 2019).

Nanoparticle Formulation and Delivery Systems

The formulation and delivery systems of Fe_2O_3 NPs represent a critical area for future research. Current studies have primarily used suspension formulations, but exploring alternative delivery methods, such as encapsulation in biodegradable polymers or incorporation into seed coatings, could be more effective (Gao et al., 2022). Developing innovative delivery systems could improve the efficiency of Fe_2O_3 NPs in field applications, ensuring sustained protection against fruit rot diseases and minimizing the need for frequent applications.

Field Trials and Long-Term Impact Studies

Conducting extensive field trials is essential for evaluating the real-world efficacy and safety of Fe_2O_3 NPs. These trials should be conducted under varying environmental conditions to ensure that the nanoparticles perform effectively across different climates and soil types (Li et al., 2021). Additionally, long-term studies can help determine any potential accumulation or unintended environmental effects, ensuring that Fe_2O_3 NPs contribute positively to sustainable agriculture.

Integration with Integrated Pest Management (IPM)

Future research could explore the integration of Fe_2O_3 NPs into integrated pest management (IPM) systems. Combining Fe_2O_3 NPs with other pest and disease control strategies, crop rotation, and the use of resistant plant varieties, could enhance overall disease management and reduce reliance on chemical inputs. Research should investigate how Fe_2O_3 NPs can complement existing IPM practices and evaluate their effectiveness in combination with other control measures (Noman et al., 2022).

Economic and Environmental Assessments

Assessing the economic and environmental impacts of Fe_2O_3 NPs is crucial for determining their viability in commercial agriculture. Future studies should include cost-benefit analyses to compare the economic feasibility of using Fe_2O_3 NPs versus traditional chemical treatments. Additionally, research should evaluate the environmental impact of Fe_2O_3 NPs and their potential role in promoting environmentally friendly agricultural practices (Raza et al., 2022).

Exploration of New Applications

Finally, expanding the applications of Fe_2O_3 NPs beyond fruit rot diseases could open new avenues for their use in agriculture. Research could explore their potential in managing other crop diseases, enhancing soil fertility, or promoting plant growth. For example, Fe_2O_3 NPs might be tested for their ability to improve nutrient uptake or stimulate plant defense mechanisms. Investigating these new applications could further establish Fe_2O_3 NPs as versatile tools in sustainable agriculture (Nair et al., 2020).

The future research directions for Fe_2O_3 NPs in agriculture hold significant promise for advancing sustainable practices. By exploring the mechanisms of action, optimizing formulations, conducting field trials, integrating with IPM, assessing economic and environmental impacts, and exploring new applications, researchers can further harness the potential of Fe_2O_3 NPs to improve crop health and productivity while minimizing environmental impact.

1.9 Research aims and Objectives

This study was primary designed to manage fruit rot diseases of peaches, apples, and strawberries using Fe_2O_3 NPs. By focusing on this aim, the study seeks a sustainable solution for reducing fruit rot incidence, maintaining fruit quality, and contributing to the overall sustainability of agricultural practices. To achieve this, the study outlines several specific objectives that guide the research process and contribute to a comprehensive understanding of the topic.

Aims:

This study aims to assess the effectiveness of Fe_2O_3 NPs in managing fruit rot diseases in key horticultural crops i.e., peaches, apples, and strawberries. The study aims to provide insights into how Fe_2O_3 NPs can be utilized to mitigate the adverse effects of fruit rot, enhance fruit quality, and promote sustainable agricultural practices.

Objectives:

1. Evaluate the Efficacy of Fe_2O_3 Nanoparticles in Reducing Fruit Rot Incidence

The first objective of this study was to assess the efficacy of Fe_2O_3 nanoparticles for reducing fruit rot diseases of peaches, apples, and strawberries.

This objective involves:

- Designing and implementing experimental setups to test the application of Fe_2O_3 NPs on fruit crops.
- Monitoring and documenting disease incidence in treated and untreated samples.
- Analyzing the data to quantify the reduction in fruit rot incidence by using Fe_2O_3 NPs.

2. Assess the Impact of Fe_2O_3 Nanoparticles on Fruit Quality and Composition

This objective involves:

- Conducting sensory evaluations and laboratory analyses to assess the quality of treated fruits.
- Measuring changes in nutritional content, including vitamins, minerals, and antioxidants.
- Comparing the results with untreated control samples to determine any significant differences.

3. Investigate the Mechanisms of Action of Fe_2O_3 Nanoparticles

This objective involves:

- Conducting laboratory experiments to study the interaction between Fe_2O_3 NPs and fruit rot pathogens.
- Analyzing the production of ROS and other antimicrobial activities induced by Fe_2O_3 NPs.
- Reviewing existing literature and research findings to corroborate the experimental results.

4. Evaluate the Sustainability and Environmental Impact of Using Fe_2O_3 Nanoparticles

An important objective is to evaluate the sustainability of synthesized Fe_2O_3 NPs in agriculture. The study aims to ensure that the application of Fe_2O_3 NPs aligns with sustainable agricultural practices and does not pose any significant environmental risks.

This objective involves:

- Conducting soil and environmental impact assessments to measure any changes resulting from Fe_2O_3 NP application.
- Evaluating potential effects on beneficial microorganisms and soil fertility.
- Analyzing the overall environmental footprint of using Fe_2O_3 NPs in comparison to traditional disease management methods.

5. Explore the Potential for Integrating Fe_2O_3 Nanoparticles into other Systems

This aims involves investigating how Fe_2O_3 NPs can complement other pest and disease control strategies and identifying synergies between Fe_2O_3 NPs and existing IPM practices to enhance overall disease management.

This objective involves:

- Reviewing existing IPM practices and identifying potential integration points for Fe_2O_3 NPs.
- Conducting experiments to evaluate the combined effectiveness of Fe_2O_3 NPs and other control measures.
- Assessing the overall impact on disease management efficiency and sustainability.

By evaluating their efficacy, impact on fruit quality, mechanisms of action, sustainability, and integration with IPM systems, the study aims to provide valuable insights and practical recommendations for leveraging Fe_2O_3 NPs in sustainable agricultural practices. This research has the potential to contribute significantly to the field of agricultural nanotechnology and support efforts to enhance fruit production and quality while minimizing environmental impact.

2. MATERIALS AND METHODS

2.1 EXPERIMENT 1: Mycosynthesized Fe_2O_3 nanoparticles to diminish brown rot of apple

2.1.1 Collection of Diseased Samples

During the apple fruiting season (February 2021 to May 2021), two surveys were conducted in the apple orchards of District Mansehra (34°20′23.0″N 73°11′53.7″E). Throughout these surveys, a total of 30 diseased fruit samples were collected, exhibiting brown rot symptoms. These diseased samples were carefully gathered and shifted to the laboratory.

2.1.2 Growth and Examination of Pathogen

To isolate the pathogen responsible for the brown rot, the diseased fruits were first surface sterilized using 70% ethanol to eliminate any surface contaminants. Small sections (2–3 mm) from the diseased areas of the fruits were then excised and incubated in Petri plates with potato dextrose agar (PDA) media, which supports the growth of fungi. These plates were subsequently incubated at 25°C for 5–6 days to allow for the growth of any fungi present in the samples. At different intervals, the Petri plates were meticulously examined to observe the growth and morphology of the isolated fungus. This examination involved assessing the characteristics of the fungal colonies, including their color, texture, and pattern of growth. The observations made during this examination were crucial for identifying the specific pathogen responsible for the brown rot in the apple fruits.

The methodology employed ensured a sterile environment for the growth and isolation of the pathogen, allowing for accurate identification and further studies on the isolated fungus. This procedure was needed for understanding the nature of the pathogen and suggesting effective strategies for managing brown rot in apple orchards.

2.1.3 Pathogenicity Test

The protocol of Ali et al. (2021) was adopted to follow Koch’s postulates. In brief, the following steps were performed:

1. **Inoculation of Healthy Fruits:** To check the pathogenicity of isolated pathogen, 10 healthy apple fruit were wounded, using a sterilized needle to create an entry point for the pathogen.

2. **Inoculation:** A fungal conidial suspension (1×10^6 conidia per ml) was prepared. Five of the wounded healthy apple fruits were injected with 5 µl of this fungal conidial suspension. As a control, the remaining five healthy fruits were inoculated with 5 µl of distilled water.
3. **Incubation and Symptom Observation:** The inoculated fruits were then kept under suitable conditions for infection development. After 4–5 days, the fruits were examined to see disease symptoms. The presence of symptoms in the inoculated fruits and their absence in the control fruits would indicate the pathogenicity of the isolated fungus.
4. **Re-isolation of Pathogen:** To further confirm the pathogenicity, the pathogen was re-isolated on PDA. Small sections from the symptomatic areas of the inoculated fruits were excised and placed on Petri plates. These plates were kept at 25°C for 5–6 days to allow the growth of fungus.
5. **Comparison of Morphology:** After the incubation period, the morphology of the re-isolated fungus was compared with the original inoculated fungus. This comparison involved assessing characteristics such as colony color, texture, and growth pattern to ensure they were identical.
6. **Repetition of Experiment:** To ensure the accuracy and reliability of the results, the experiment was performed in triplicate. This repetition helped to confirm that the observed pathogenicity was consistent and not due to any experimental anomalies.

By following these detailed steps, the pathogenicity of the isolated pathogen was confirmed, establishing a causal relationship between the pathogen and the brown rot disease observed in the apple fruits.

2.1.4 Characterization of the Isolated Pathogen

Microscopic Identification: For the microscopic identification of the isolated pathogen, a standard slide culture protocol as described by Liaquat et al. (2021) was followed. Fungal mycelia were observed under a light microscope to study their morphological characteristics.

Molecular Characterization: To achieve molecular characterization, total DNA of isolated pathogen was extracted, using the CTAB method (Umesha et al. 2016), and its rDNA

sequence was amplified and analyzed. The isolated DNA served as the template for the polymerase chain reaction (PCR).

PCR Amplification: Specific forward primer ITS1 (TCCGTAGGTGAACCTGCGG) and the reverse primer ITS4 (TCCTCCGCTTATTGATATGC), were used to amplify desire DNA (White et al. 1990).

The reaction mixture for PCR was prepared in a total volume of 25 µl, comprising the following components:

- 0.5 µl of 2.5 mM dNTP,
- 0.25 µl of 5 U/µl *Taq* DNA polymerase enzyme,
- 1 µl of 50 ng/ml genomic DNA,
- 2.5 µl of 10× buffer, and
- 1 µl of each primer at a concentration of 10 pmoles/µl.

The PCR conditions were set as follows:

- Initial denaturation at 94°C for 4 minutes,
- 35 cycles of
    - ✓ denaturation at 94°C for 1 minute
    - ✓ Annealing at 57°C for 1 minute
    - ✓ Extension at 72°C for 1 minute

Sequencing and Analysis: The PCR product was then sequenced. The obtained sequence was aligned with the NCBI database using the BLASTn search program (http://www.ncbi.nlm.nih.gov) to identify the pathogen by comparing its sequence with known sequences in the database.

Phylogenetic Analysis: To explore evolutionary relationships, the resultant sequence was aligned with 15 related sequences using the MUSCLE program (Edgar, 2004). Following this alignment, a phylogenetic tree was constructed using MEGA 7 software (Kumar et al., 2016). This study provided insights into the evolutionary lineage and relatedness of the isolated pathogen to other known species.

This comprehensive characterization, combining microscopic and molecular techniques, ensured a precise identification and understanding of the isolated pathogen, contributing valuable information for further studies and management strategies.

2.1.5 Mycosynthesis of Fe_2O_3 NPs

Fe_2O_3 NPs were synthesized by the following way:

2.1.5.1 Isolation and Cultivation of T. harzianum

T. harzianum was provided by the 'First Fungal Culture Bank of Pakistan' (FCBP) at Punjab University, Lahore. Obtained strain was cultured in potato dextrose broth media and incubated at 28°C. After 7 days of incubation, the fungal biomass was filtered out and washed three times with distilled water to remove any residual media components.

2.1.5.2 Induction of Mycotoxin Production

To induce the production of mycotoxins, the fungal biomass was subjected to mild stress conditions. For this purpose, 35 grams of mycelial biomass was added to 150 ml of sterilized water and placed at 150 rpm and 40°C for 10 days. Following this stress period, the biomass was sonicated for 30 minutes to break down the cell walls and release intracellular contents, then filtered to separate the mycotoxin-containing filtrate from the biomass. The pH of the filtrate was adjusted to 7.2 to prepare it for further use.

2.1.5.3 Synthesis of Fe_2O_3 NPs

In a beaker, a mixture of the cell-free filtrate and a 5 mM solution of iron chloride ($FeCl_3$) was prepared in a 1:1 ratio. This mixture was placed at 150 rpm and 40°C for 24 to 48 hours. The change in the color of the solution indicated the reduction of iron ions to iron oxide nanoparticles.

2.1.5.4 Collection and Processing of Nanoparticles

For the collection of mycosynthesized NPs, solution was centrifugaed at 5000g for 20 minutes, after which the pellet was washed thoroughly to remove unreacted substances. The washed pellet was then dried overnight in an oven at 40°C. Finally, the dried nanoparticles were calcined in a furnace at 500°C for 2 hours to obtain the desired iron oxide nanoparticles (Fe_2O_3 NPs).

2.1.6 Characterization of Fe_2O_3 NPs

Synthesis of Fe_2O_3 NPs was determined by studying their following characteristics:

2.1.6.1 Fourier Transform Infrared (FTIR) Spectroscopy

Following the protocol of Griffiths (1983), the FTIR spectroscopy was performed. A PerkinElmer spectrometer was used to reveal the types and nature of functional groups present on the surface of NPs (Malaikozhundan et al., 2017a).

2.1.6.2 X-ray Diffraction (XRD)

Size and shape of Fe_2O_3 NPs was determined, using XRD and X'Pert HighScore software (Malaikozhundan et al., 2017b).

2.1.6.1 Scanning electron microscopy (SEM) and energy-dispersive X-ray (EDX) analyses

The shape and elemental composition were determined using SEM and EDX analyses (Malaikozhundan et al., 2020). Calcined Fe_2O_3 NPs were suspended in water and positioned on the double carbon coated conductive tape. The samples were dried and analyzed using a standard SEM system (VEGA3 TESCAN).

2.1.7. Mycelial growth inhibition assay, *in vitro*

Using "poisoned food method" (Liaquat et al., 2021), the potential of Fe_2O_3 NPs in inhibiting mycelial growth was determined. Various concentrations of Fe_2O_3 NPs (2 mg/ml, 1.5 mg/ml, 1 mg/ml, 0.75 mg/ml, 0.5 mg/ml, 0.25 mg/ml, and 0.1 mg/ml) were added in PDA media and no NPs were added in control Petri plates. Fungal discs (5 mm) of five days old fungal culture were placed on nanoparticle amended PDA media. All treated and control plates were placed at 25 °C for one week. The growth of fungus on control and NP amended PDA media was measured in millimeters (Ali et al., 2021). The mycelial growth inhibition percentage was observed by the following equation:

$$\text{Growth Inhibition \%} = (C - T) / C \times 100$$

Where: C = Average mycelial growth on control PDA media, T = Mycelial growth on treated (NP amended) media.

2.1.8. Disease control assay on fruit, *in vivo*

Based on *in vitro* assay, NPs with 1 mg/ml concentration and below were carried forward for *in vivo* antifungal activity analysis.

In vivo disease-control ability of mycosynthesized Fe_2O_3 NPs was determined using "wound inoculation method" (Ali et al., 2021). To follow this method, eighteen healthy apple fruit were wounded with sterilized needle and inoculated with 5 µl conidial suspension (1×10^6 conidia mL^{-1}). Inoculated fruits were placed at 25 °C for two days to let fungus penetrate and cause infection on apple fruit. Two days post inoculation; different concentrations of Fe_2O_3 NPs (0.1 mg/ml, 0.75 mg/ml, 0.5 mg/ml, 0.25 mg/ml, and 0.1 mg/ml) were sprayed (till run off) on randomly selected fruits. Three fruits were sprayed with each concentration of NPs and three fruits were sprayed with distilled water (control). All fruits were again placed at 25 °C. Seven days post inoculation, the diseased area of each fruit was measured.

2.1.9. Study of biochemical and organoleptic properties

After 10 days of inoculation with fungus, the quality of NP treated, and control apple fruit was determined by the assessment of their biochemical and organoleptic properties. A digital refractometer (Pam Abbe model PA203X, MISCO Refractometer, Solon, OH) was used to quantify soluble solids of diseased apple fruits for all treatments. Other quality related biochemical compounds including sucrose, and total sugars were measured using a standard protocol of Helrich (1990a). To measure Ascorbic acid in all fruit samples, the titration was carefully performed using 2,6-dichlorophenolindophenol sodium salt solution (Helrich, 1990).

The effects of fungus inoculation on the firmness of apple in all treatments were determined using TA.XTplus Texture Analyzer (Stable Micro Systems Ltd., Godalming, Surrey, UK). Firmness was tested by touching the surface and entering the probe into the sample to a depth of 10 mm by applying a force of 2 g (de Jesús et al., 2018).

2.1.10. Statistical Analysis

All the experiments were performed in three replicates and the data was analyzed using Microsoft Office Excel 121 (Microsoft, Redmond, WA, USA). Univariate analysis of variance (ANOVA) was performed to see the significant differences and presented as standard deviations at $p < 0.05$.

2.2 Experiment 2 Application of Fe_2O_3 NPs to inhibit the production of aflatoxins (B1 and B2) in peach fruit.

2.2.1 Sample collection and isolation of pathogen

Diseased samples of peach fruit showing fruit-rot symptoms were collected from Swat District (35.2227° N, 72.4258° E). Two different surveys were conducted, and 24 diseased fruit samples were collected in sterilized bags and brought to the laboratory. For the growth of fungus, Sabouraud dextrose agar (SDA) media was prepared. SDA (39 g) was mixed in 1000 ml water and autoclaved. After sterilizing peach fruit with 70% ethanol, the infected part was aseptically transferred to Petri plates containing SDA and incubated at 28 ºC. Pure isolates were obtained after 3-4 days and mycelial morphology was observed, microscopically and macroscopically. Morphological and microscopic traits of isolated pathogen were compared with the compendium of phyto-pathogenic fungi (Barnett and Hunter, 1972). For DNA sequence analysis of isolated pathogen, internal transcribed spacer (ITS) regions and intervening 5.8S rDNA was amplified and sequenced (White et al., 1990). Further, the evolutionary and phylogenetic analyses were carried out by using MEGA (version 7). By applying the Neighbor-joining method, the evolutionary history was inferred of all closely related sequences of fungal strains. To check the pathogenicity of isolated strain, Koch's postulates were followed (Fredericks and Relman, 1996).

2.2.2 Determination of aflatoxins production by cultural methods

The isolated pathogen was identified as *Aspergillus flavus*, which is one of the most important aflatoxin producing pathogens. As all the strains of *A. flavus* do not produce aflatoxins, two cultural methods (UV test and exposure to NH_4OH) were carried out for preliminary detection of aflatoxins production (Saito and Machida, 1999).

In UV test, back side of the SDA Petri plate with fully grown colonies of *A. flavus* strain, was observed under UV light (365 nm). Around colony margins, the presence of grayish or black boundary provided rough idea about the aflatoxigenic nature of the isolated strain. Whitish colonies depict the non-aflatoxigenic nature of mold.

In ammonium hydroxide (NH_4OH) vapor test, 2-3 drops of 31% NH_4OH were placed in the lid of Petri plate and the lower portion of the Petri plate (containing fungal colonies) was

placed on its lid. Results were drawn, because the changes in color from yellow to pink or plum red indicates aflatoxin synthesizing ability of the strain (Abbas et al., 2004).

2.2.3 Preparation of mycological Fe_2O_3 NPs

Fe_2O_3 NPs were synthesized by methodology described in section 2.1.5.

2.2.4 Characterization of Fe_2O_3 NPs

Successful synthesis of Fe_2O_3 NPs was determined by studying their following characteristics:

2.2.4.1 Fourier Transform Infrared (FTIR) Spectroscopy

Following the protocol of Griffiths (1983), the FTIR spectroscopy was performed using a PerkinElmer spectrometer (Malaikozhundan et al., 2017a).

2.2.4.2 X-ray Diffraction (XRD)

XRD described the size and shape of Fe_2O_3 NPs and X'Pert HighScore software described the size of NPs (Malaikozhundan et al., 2017b).

2.2.4.1 Scanning electron microscopy (SEM) and energy-dispersive X-ray (EDX) analyses

The shape and elemental composition were determined using SEM and EDX analyses (Malaikozhundan et al., 2020). Calcined Fe_2O_3 NPs were suspended in water and positioned on the double carbon coated conductive tape. The samples were dried and analyzed using a standard SEM system (VEGA3 TESCAN).

2.2.4.1 Fungal growth assay, in vitro

Performance of different concentrations of Fe_2O_3 NPs in reducing mycelial growth was determined with 'poisoned food method' (Ferhout et al.,1999). In an *in vitro* experiment, 15 mL PDA media was poured in Petri plates with variable amounts of Fe_2O_3 NPs (0.1 mg/ ml, 0.25 mg/ ml, 0.5 mg/ ml, 1 mg/ ml, and 1.5 mg/ ml). In control treatment, no NPs were added in the PDA media. Once solidified, all Petri plates were inoculated with fungal discs of 5 mm diameter. These fungal discs were placed in the center of each Petri plate and allowed to grow at 25°C. After a week, mycelial growth in NPs treated (T) and control (C) Petri plates was measured, and mycelial growth inhibition was determined in percentage.

2.2.5 Disease assay on peach fruit

Different amounts of Fe_2O_3 NPs (0.1 mg/ ml, 0.25 mg/ ml, 0.5 mg/ ml, 1 mg/ ml, and 1.5 mg/ ml) were sprayed to control fruit rot of peach. Initially, 18 healthy peach fruits of equal size were obtained and 5 µL of conidial suspension (1×10^6 conidia ml^{-1}) was injected. To assure infection, these fruit samples were placed at room temperature. After 48 h, three infected fruits were sprayed, till run-off, with each above-mentioned amount of Fe_2O_3 NPs. Three fruits (control) were not treated with NPs and sprayed with distilled water, only. All treated and controlled fruit were incubated, and the diseased area was measured after one week of incubation.

2.2.6 Detection of aflatoxin in diseased fruit

2.2.6.1 Thin layer chromatography (TLC)

To detect aflatoxins, TLC technique was used (Hoeltz et al., 2010). From each treatment, 50 g of fruit sample was blended in distilled water (50 ml) and acetone (200 ml). The mixture was filtered, and 150 ml of filtrate was taken as a representative sample. The filtrate was then spotted (two spots of 1 uL and 5 uL) on TLC plate. The lower 2 cm part of silica plate was scratched, and plate was developed in CH_3Cl - acetone (85:15) solution. After development, TLC plate was examined under long wave UV light (150-350 nm).

2.2.6.2 Enzyme-linked immunosorbent assay (ELISA)

Veratox 8030® aflatoxin test kit was used for ELISA. The peach fruit sample (50 g) was shaken in 70% methanol (250 mL) for 1 h at 200 rpm. The filtrate was obtained, and its pH was maintained (6-8). Following kit instructions, the conjugate was taken in the red-colored well and mixed with the calibration standard solution and the extract. The mixture was incubated in the antibody coated well and rinsed with deionized water. The substrate solution was incubated in the antibody coated well for 3 minutes, and after adding stop solution, the reading of the solution was obtained.

2.2.6.3 High Performance Liquid Chromatography (HPLC)

For HPLC, grinded fruit samples (50 g) were mixed in 80% methanol (100 mL). For 2 min, the sample mixture was blended, filtered through Whatman No. 4 filter paper and placed in

air-tight amber vials. Immunoaffinity columns (IACs) were used for sample cleaning. For this purpose, filtered extracts (2 ml) were mixed with PBS (14 mL) and dispensed in IACs. For standard cleaning, a flow rate of 1 drop sec^{-1} was maintained. For the washing of columns, 20 ml of PBS (pH 7.4) was passed through at 5 ml min^{-1} flow rate and air dried, rapidly. Aflatoxins were eluted from vials with methanol (Daradimos et al., 2000).

2.2.7 Fruit quality attributes

The quality of infected peach fruits was compared in different treatments. Fruit pulp (300 g) from each treatment was homogenized and filtered through Whattman Filter paper No. 1. The blended pulp was centrifuged at 3000 rpm for 15 min and the obtained clear juice was used to measure the following quality parameters:

2.2.7.1 Total soluble solids (TSS)

For the measurement of total soluble solids, a standard protocol was followed (Ying et al., 2005). From each treatment, 100 mL of juice filtrate(s) were used to determine TSS (%), using a hand refractometer.

2.2.7.2 Total Titratable acidity (TTA)

Following the protocol of Lobit et al. (2002), a clear filtrate of inoculated and un-inoculated peach fruit was used to determine TTA. After titration with NaOH (0.1 N), phenolphthaline was used as an indicator. The acidity percentage was measured as mg citric acid per 100 g fresh weight of peach fruit, by using the following equation:

Acidity % = ml of NaOH used × N of NaOH (0.1) × 0.064 / Sample volume of peach (ml)

2.2.7.3 Ascorbic Acid

Ascorbic acid contents were determined by following the protocol of Matias et al. (2016). Filtered juice was diluted with 3% metaphosphoric acid and titrated against 2, 6-dichlorophenol indophenol (until a steady light pink color developed), and ascorbic acid contents were determined in mol kg^{-1}.

2.2.7.4 Firmness

Following a standard protocol (Wang et al., 2013), firmness of each fruit was determined using TA.XTplus texture analyzer (Stable Micro Systems Ltd., Godalming, Surrey, UK).

2.2.7.3 Statistical Analysis

Each experiment was executed in triplicate and the experimental data was statistically analyzed by following one-way ANOVA. The significance of difference between treatments was tested by least significance difference (LSD) post hoc test ($\alpha = 0.05$). All data was expressed as mean ± standard error.

2.3 Experiment 3 Fe_2O_3 nanoparticles modulate titratable acidity, ascorbic acid contents, and firmness of Strawberries to enhance their shelf-life at room temperature.

2.3.1 Mycosynthesis of Fe_2O_3 NPs

Fe_2O_3 NPs were synthesized by methodology described in section 2.1.5.

2.3.2 Characterization of Fe_2O_3 NPs

For the characterization of Fe_2O_3, protocol mentioned in section 2.1.6 were followed.

2.3.3 Collection of Strawberry

Based on the outer peel color having a pinkish hue, 30 strawberries were harvested from Kallar Syedan during May-June 2023 (Figure 3.2.1). Ripe fruits were brought to the lab for further experimentation.

Figure.2.1 Fresh Strawberry fruits were collected and uniformly graded.

2.3.4 Application of Fe_2O_3 NPs on Strawberry

Foliar application of mycosynthesized Fe_2O_3 NPs was carried out, after sonication, on healthy fruits in 6 different concentrations (0.1, 0.25. 0.5, 0.75, 1 and 1.5 mg/ml). The strawberry fruits were sprayed, till runoff and stored at room temperature.

2.3.5 Post-harvest Physio-chemical Qualities of Strawberry fruit

Following physico-chemical and sensory characteristics of these fruits were determined on 7^{th} day of storage.

2.3.5.1 Measurement of fruit firmness

With the use of a penetrometer (GY-4, Zhejiang Top Instrument Co., Ltd, Chine), the firmness of strawberry fruit was measured. The measurement of firmness of the strawberries was taken by choosing the point straight from the upper side of the fruit. Using 4 mm diameter probe, the values were obtained and expressed in Newton (Nyamende, 2021).

2.3.5.2 Weight Loss percentage of Strawberry fruit

A group of fruit was weighed every day to obtain mass losses over a six-day period of sample storage. Samples were weighed in triplicates and the percentage of dry weight was calculated (Atukuri, 2017).

2.3.5.3 Titratable acidity (TA)

From the strawberry fruit tissues, juice was extracted by 10 ml water and placed in distilled water (Michailidis et al., 2017). Titration was performed with 0.1M solution of sodium hydroxide and phenolphthalein indicator. The TA value was then acquired by the estimation of malic acid in strawberries. Titrable acidity was measured by using the following formula:

$$TA = \frac{[NaOH\ (mL)] \times [0.1\ M\ NaOH] \times 0.067 \times 100}{Juice\ (mL)}$$

Where: 0.067= equivalent factor of malic acid

2.3.5.4 Total soluble solids

Strawberry fruit pulp (15 g) was mechanized, filtered and used for Brix assay using refractometer (DI VITTORI, 2018) .

2.3.5.5 Ascorbic acid Content

A standard procedure was used to assess ascorbic acid contents (Wojdyło et al., 2016). Briefly, 20 g of pulp was mechanically agitated in 85 ml of 3% HPO_4 and centrifuged for 25 minutes at 6000 rpm. Following the filtering of the supernatant, the dichlorophenol indophenol dye was titrated for approximately 20 seconds, or until a reddish colour was visible. Following Levy et al. (2019), ascorbic acid content were determined in milli grammes for every 100 grammes of fruit pulp.

2.3.5.6 Carotenoid Content

Carotenoid content was determined by following a standard (Basto et al., 2016). Around 10 g of strawberry puree was acquired and diluted with 10 ml phosphate buffer (pH 7.5) and 3 ml ethyl acetate. This mixture was centrifuged using homogenizer at 10,000 × g for 15 min, and the upper (lipophilic) fraction of the resulting mix was taken for carotenoids content analyses. Lipophilic fraction absorbance was recorded at 450 nm which was then detected by the UV-Visible Spectrophotometer (Rymbai et al., 2023). The results were displayed as mg β-carotene per 100 gm fresh weight.

2.3.5.7 Sensory evaluation

The sensory parameters were analyzed by means of the 9-points scale of hedonic (1-disliked extremely, 5-neither liked nor disliked and 9-liked extremely). To assess the overall acceptability (OAA) of strawberry, 5 panelists, who were partially trained in the laboratory setting, were used (Lucas et al., 2018).

2.3.5.8 Fruit iron oxide level analysis

In order to observe FeO release in strawberry fruit, each treated and controlled sample was dried in the oven at 80°C and crushed to powder form, in the mortar and pestle. About 2 g of crushed powder was burned at 100°C in a muffle furnace to provide white ash. The Fe contents of this pure white ash were determined using an atomic absorption spectrophotometer (Varian Spectra AA220, Australia) using extraordinary Fe concentrations, for the standard curve. The results were represented in mg kg^{-1} of dry weight. The ash was dissolved in 10 mL of 6 N HCl (Abbasi et al., 2020).

2.3.5.8 Shelf life of Strawberry

Shelf-life of strawberries was accessed by observing their colour, skin evenness, pulp level and aroma, for six days.

2.3.5.9 Statistical analysis

As described in section, 2.1.10, statistical analysis was performed.

3. Results

3.1 EXPERIMENT 1: Mycosynthesized Fe_2O_3 nanoparticles diminish the brown rot of apple whilst maintaining composition and pertinent organoleptic properties.

3.1.1 Isolation and identification of fungus

In the field, typical brown rot symptoms (tan-brown and circular spots) were observed on apple fruit (Figure 3.1a). The disease-causing fungus was successfully isolated, and its rapid growth was observed on PDA media. In 5–7 days, whitish colonies covered the entire Petri plate (Figure 3.1b). Mycelia were observed to be entangled and woolly to cottony in texture.

The pathogenicity test successfully described the virulence of the isolated pathogen. On wounded control fruit, no symptoms were observed after 2 days (Figure 3.1c) and 5 days (Figure 3.1d) of wounding. On inoculated fruit, typical symptoms could be observed after 2 days of inoculation (Figure 3.1e), and these symptoms progressed further after 5 days (Figure 3.1f). These symptoms were similar to the field symptoms. Fungus was isolated from these self-inoculated apple fruit. It displayed similar morphology to the inoculated fungus (Figure 1g). These findings fulfilled Koch's postulates and showed the involvement of isolated fungus in brown rot. Microscopic studies of this fungus revealed macro conidial septation (Figure 1h).

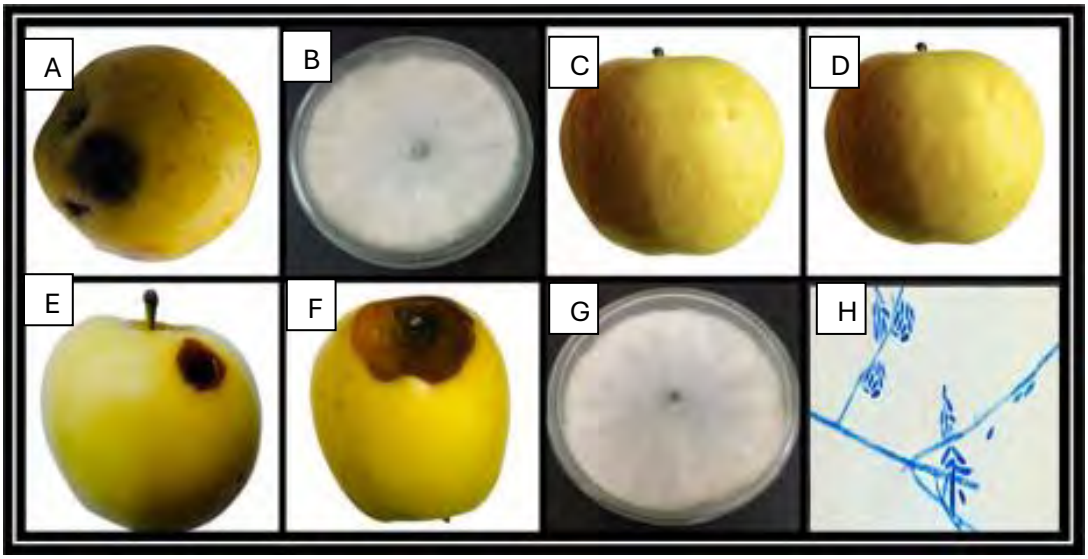

Figure 3.1 Apples with brown rot symptoms were collected from the field (a). The pathogen was isolated and grown on PDA media (b). Control apple fruit showed no disease symptoms after 2 (c) and 5 days (d), whilst inoculated fruit exhibited disease symptoms after 2 (e) and 5 days (f) of inoculation. The pathogen was re-isolated on PDA (g). Microscopic study efficiently helped to observe mycelial morphology (h).

3.1.2 rDNA sequence analysis and phylogenetic tree

BLAST alignment of the resultant PCR sequence demonstrated 100% similarity with *Fusarium oxysporum* strain FO_11 (Accession no. MT447552.1). This result verified our morphological and microscopic observations, which also described this disease-causing pathogen to be *F. oxysporum*. The phylogenetic tree also confirmed these results, and the isolated pathogen was present in the same clade with *F. oxysporum* (Figure 3.2).

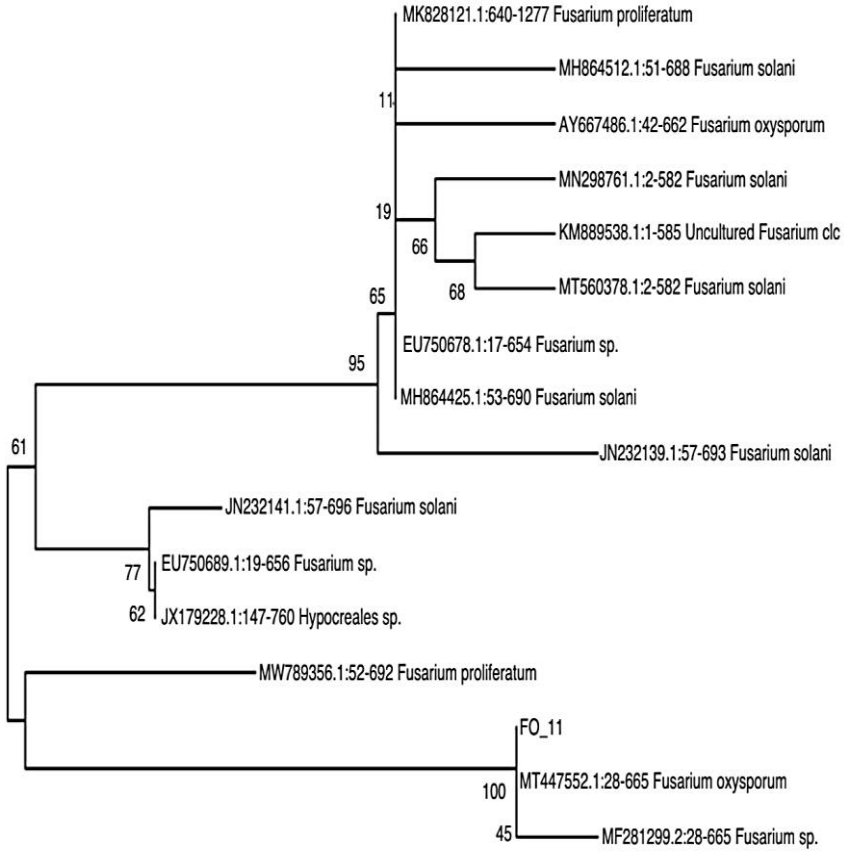

Figure 3.2. Evolutionary relationship of isolated F. oxysporum strain FO_11, with 15 related GenBank sequences

3.1.3 Characterization of mycosynthesized iron nanoparticles

3.1.3.1 FTIR analysis

FTIR spectrum showed specific peaks to represent different functional groups. Amongst these, peak at 3251.25 represented N-H stretching (secondary amine group), 3080.71 depicted O-H stretching (alcohol), 2181.60 demonstrated C=C bending (alkyne), 2166.69 showed N=N=N

stretching (azide), 1609.64 represented C-H bending (aromatic compounds) and 854.52 cm^{-1} presented C-Cl stretching (alkene). All these compounds act as capping and reducing agents for the synthesis of NPs.

3.1.3.2 XRD analysis

XRD pattern of synthesized NPs showed distinct diffraction peaks at 2θ 22.970, 30.480, 58.660, 65.060, and 67.700, corresponding to (013), (216), (2011), (034), and (221) planes of hexagonal with P3 space group, indicating iron oxide. The planes of XRD pattern are in good agreement with JCPDS (Joint Committee on Powder Diffraction Standards) number 01-076-1821. JCPDS provides a database to characterize powder. The average size of my synthesized iron oxide NPs was calculated to be 17.78 nm, and they were crystalline in nature.

3.1.3.2 SEM and EDX analyses

Iron salts were biologically reduced to form Fe_2O_3 NPs by *T. harzianum*. SEM of Fe_2O_3 NPs revealed a high degree of polydispersity, and they were crystalline and spherical in shape (Figure 3.3). The EDX spectrum of NPs significantly showed strong signals of iron (74.38%) and low signals of C (3.20%), O (15.39%), and Cl (7.03%).

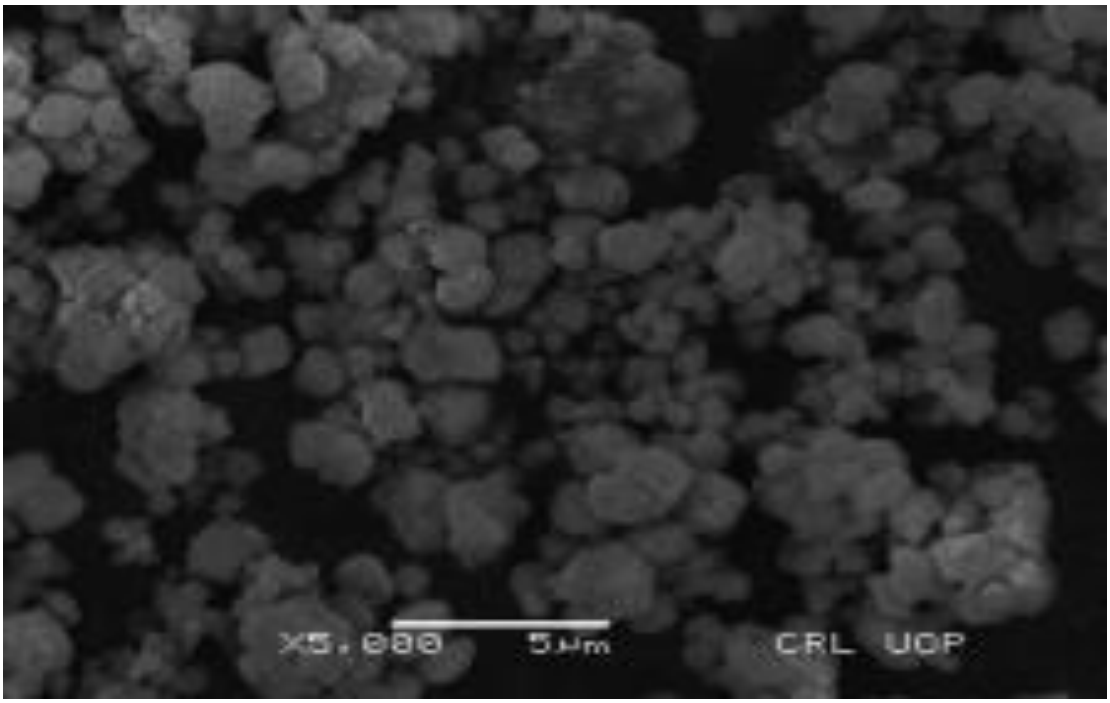

Figure 3.3 Scanning electron micrograph of mycosynthesized *Fe_2O_3* nanoparticles. Scale bar = 5 micron

3.1.4 Antifungal activity of iron oxide nanoparticles, *in vitro*

Different concentrations of mycosynthesized Fe_2O_3 NPs showed variable growth inhibition of fungal mycelia (Figure 3.4). The highest growth reduction was exhibited by 1 mg/ml concentration (65.4%) of mycosynthesized Fe_2O_3 NPs. Notable reduction in mycelial growth inhibition was also observed at lower concentrations like 0.75 mg/ml concentration (56.6%), 0.5 mg/ml con centration (12.4%), 0.25 mg/ml concentration (7.84%) and 0.1 mg/ml concentration (6.62%) (Table 3.1).

Interestingly, the very high concentration (1.5 mg/ml and 2 mg/ml) of NPs was also not effective for disease control.

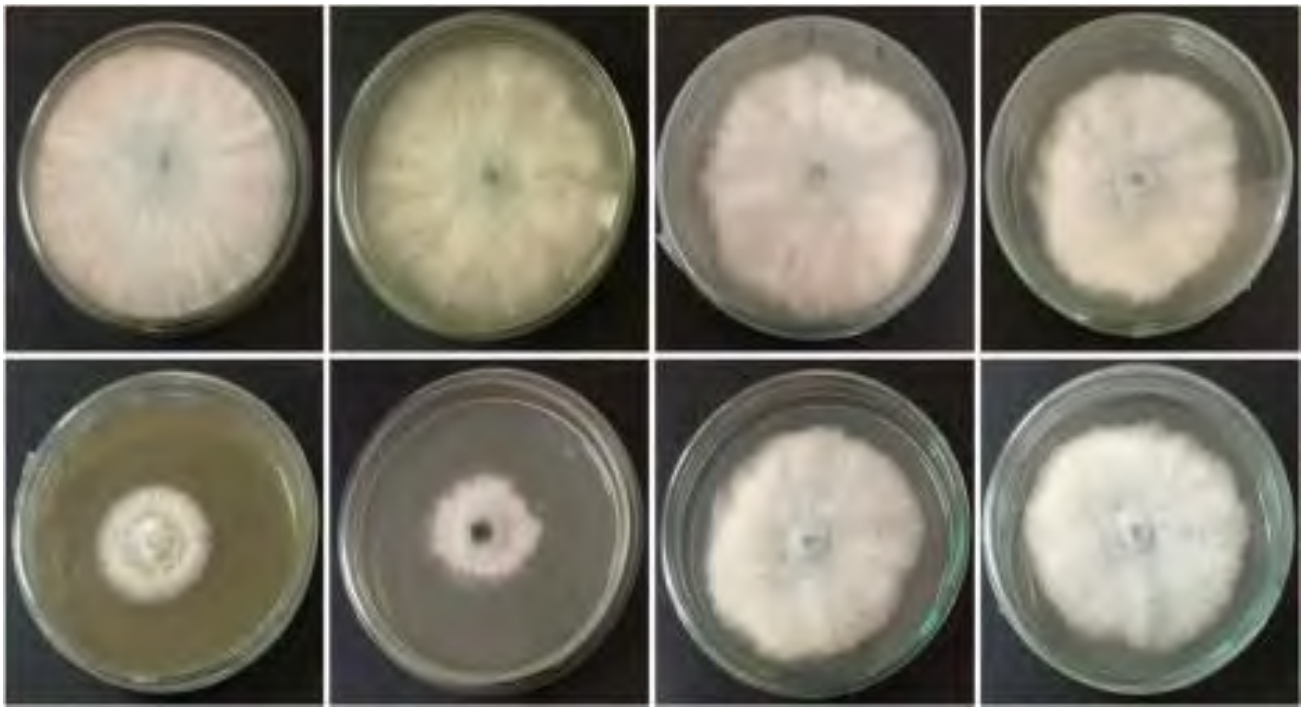

Figure 3.4 Effect of nanoparticles on the growth of *Fusarium oxysporum* strain FO_11. Seven different concentrations of NPs were compared with control (a). Fungus was grown on Petri plates amended with NP concentrations of 0.1 mg/ml (b), 0.25 mg/ml (c), 0.5 mg/ml (d), 0.75 mg/ml (e), 1 mg/ml (f), 1.5 mg/ml (g) and 2 mg/ml (h)

Table 3.1 Mycelial growth inhibition, *in vitro*, at different concentrations of NPs

Concentration (mg/ml)	Growth Inhibition * (%)
0.1	6.6 ± 1.3e
0.2	57.8 ± 1.1de
0.5	11.2 ± 1.3c
0.75	56.6 ± 2.9b
1.0	65.7 ± 3.6a
1.5	9.2 ± 1.2cd
2.0	8.5 ± 1.1d

* Values are described as mean and ±denotes standard error. Dissimilar alphabets represent significantly different values from each other.

3.1.5 Antifungal activity of iron oxide nanoparticles, *in vivo*

Inoculation of *F. oxysporum* resulted in typical brown rot symptoms. The application of Fe_2O_3 NPs successfully controlled apple brown rot disease and decreased disease area (Figure 3.5). Increasing concentration of NPs displayed reduced diseased area (Table 3.2). The maximum disease control was exhibited at 1.0 mg/ml concentrations of Fe_2O_3, and 91.5% less diseased area was observed than control.

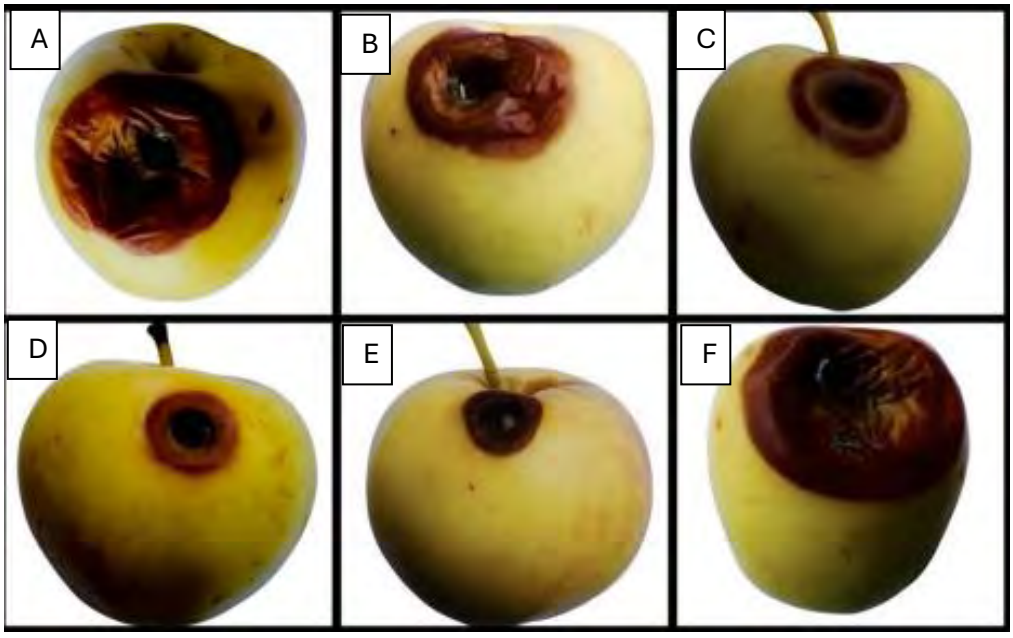

Figure 3.5 Apple fruits were infected and treated with 0.1 mg/ml (a), 0.25 mg/ml (b), 0.5 mg/ml (c), 0.75 mg/ml (d) and 1 mg/ml (e) concentration of NPs. No NPs were applied in control (f)

3.1.6 Biochemical and organoleptic changes

The inoculation of fungus severely affected the quality of fruit. Assessment of their biochemical and organoleptic characteristics helped us to understand the useful role of Fe_2O_3 NPs in mycelial growth inhibition and ultimately influencing the quality of inoculated fruit (Table 3.2). Application of Fe_2O_3 NPs helped to maintain higher soluble solids of diseased apple fruit. These higher amounts of soluble solids resulted in increased firmness of fruit. Higher soluble solids are related with the firmness and good quality of fruit (Peck et al., 2006). Analysis of diseased fruit also revealed decreased sucrose and total sugar contents in control fruit. Application of NPs helped plants to accumulate higher sucrose and total sugar contents. Sucrose is an important sugar which describes the sweet taste of apple and depicts its good quality (Cichowska et al., 2020). Many studies have described that the higher levels of sugars and lower percentage of acids indicate good quality of apple fruit (Sudheeran et al., 2018). In this study, application of NPs also influenced diseased fruits to maintain higher contents of ascorbic acid. In apples, ascorbic acid exhibits strong antioxidant properties to protect DNA and proteins from the damage of free radicals (Gorkom et al., 2019).

Table 3.2. Biochemical and organoleptic changes in diseased fruit at different NP concentrations

NP Concentration (mg/ml)	Diseased Area (mm^2)	Soluble Solids (%)	Sucrose (%)	Total Sugars (%)	Ascorbic Acid (mg/100 g)	Firmness (N/cm^2)
0.1	1406 ± 0.7b	13.1 ± 1.5c	2.2 ± 0.9c	10.7 ± 0.8b	7.2 ± 1.3c	179.1 ± 15.5c
0.25	780 ± 0.9c	13.2 ± 1.1c	2.2 ± 1.2c	11.8 ± 1.1ab	7.2 ± 1.1c	189.2 ± 17.7bc
0.5	399 ± 1.5d	14.4 ± 0.8bc	2.4 ± 0.7ab	11.7 ± 1.3ab	8.2 ± 2.3bc	209.5 ± 14.2b
0.75	225 ± 1.6e	15.4 ± 2.1ab	3.4 ± 0.8b	12.2 ± 1.7a	9.4 ± 1.3bc	219.3 ± 18.7b
1.0	132 ± 2.1f	15.1 ± 1.3ab	3.5 ± 1.1b	12.5 ± 0.9a	10.2 ± 1.4ab	288.4 ± 22.1a
Control	1554 ± 1.9a	13.2 ± 0.9c	2.0 ± 1.2c	10.8 ± 1.2b	7.1 ± 1.4c	145.6 ± 14.3d
Untreated Fruit	0	15.2 ± 0.9ab	3.4 ± 0.8b	12.4 ± 1.1a	10.1 ± 0.8ab	292.1 ± 18.1a

Values are described as mean and ±denotes standard error. Dissimilar alphabets represent significantly different values from each other.

3.2 Experiment 2 inhibition of the production of aflatoxins (B1 and B2) and regulate total soluble solids and titratable acidity of peach fruit by Fe_2O_3 nanoparticles

3.2.1 Isolation of disease-causing pathogen

Diseased peach fruit was brought to the laboratory (Fig. 3.6 A) and disease-causing pathogen was isolated in Petri plates. After one-week, greenish mycelia with white edges were observed on PDA media (Fig. 3.6 B). Light microscope revealed clear conidial heads on hyaline hyphae. Moreover, flask-shaped or elliptical phialides were also observed (Fig. 3.6 H). All these features indicated this pathogen to be *Aspergillus flavus* (Zubair et al., 2022). Koch's postulates successfully confirmed the pathogenicity of the isolated pathogen (Fig. 3.6 C–F). Sequence alignment displayed 100% similarity with the isolate of *A. flavus* (Accession No. OP218632.1). Phylogenetic analysis also confirmed the close relationship of the sequence of isolated pathogen with *A. flavus* (Fig. 3.7).

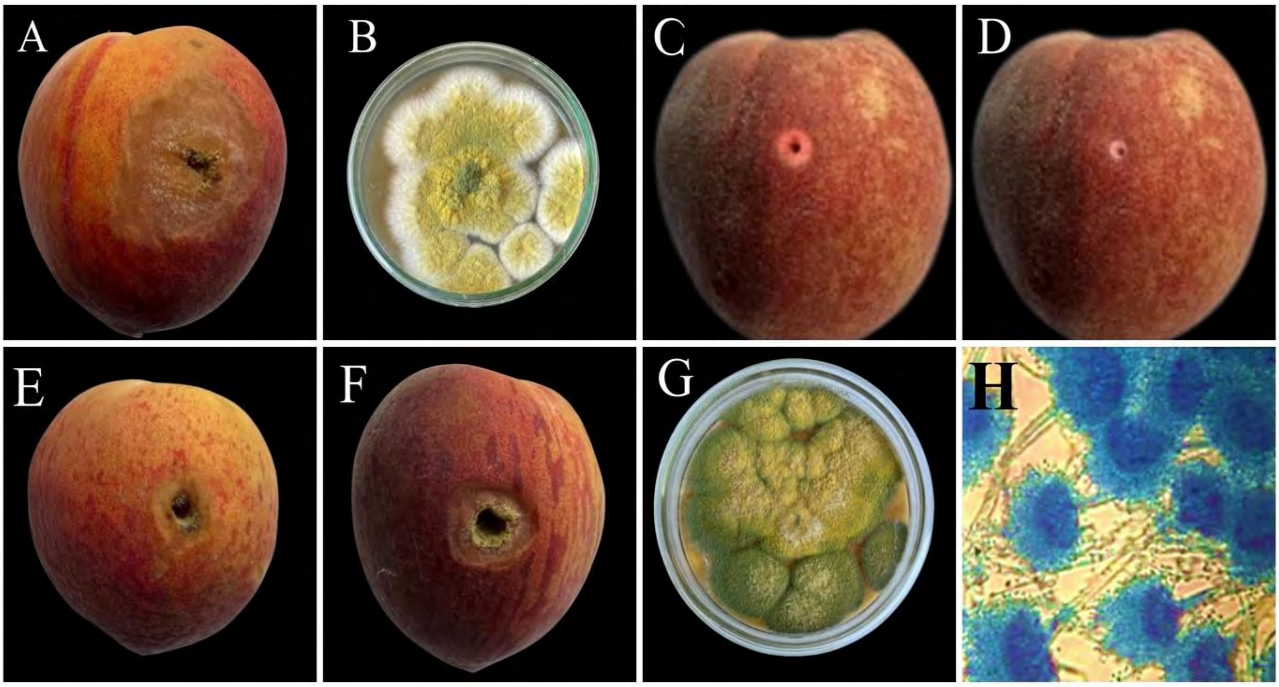

Figure 3.6 A. Peach fruit displayed fruit rot symptoms in the field (A). The morphology of pathogen was observed in Petri plates (B). Control fruit displayed no symptoms after 2 days (C) and 5 days (D) of inoculation. After fungal inoculation, distinct symptoms could be observed after 2 days (E) and 5 days (F). Similar morphology of re-isolated pathogen was observed on PDA (G) and observed under microscope (H).

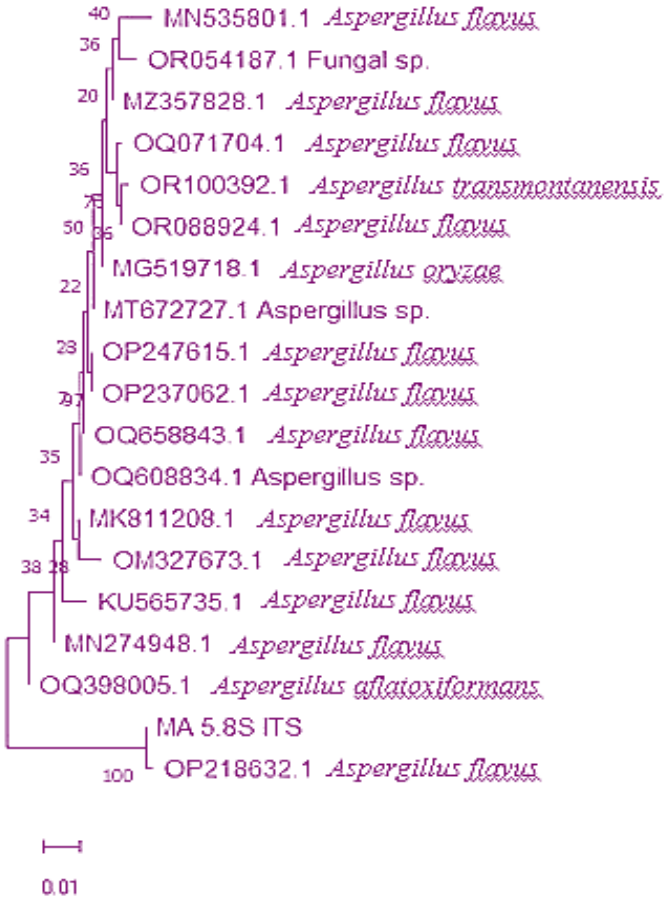

Figure 3.7. Phylogenetic tree depicting the taxonomic relationship of isolated fungus with 18 closely related fungal strains.

3.2.2 Determination of aflatoxins production by cultural methods

Isolated pathogen (*A. flavus*) was successfully grown on PDA media and observed from front and backside of Petri plates (Fig. 3.8 A-B). These plates were observed under UV light (365nm), which displayed greyish black boundaries around colony margin (Fig. 3.7 C-D) and provided preliminary evidence of aflatoxins production. By the application of 2-3 drops of ammonium hydroxide (NH_4OH) vapor, the underside color of the colony changed from yellow (Fig. 3.7E) to pink / plum-red (Fig. 3F), indicating the production of aflatoxins. Later, by the addition of 2-3 drops of glacial acetic, the colonies retained their original color, partially (Fig. 3.8 G-H).

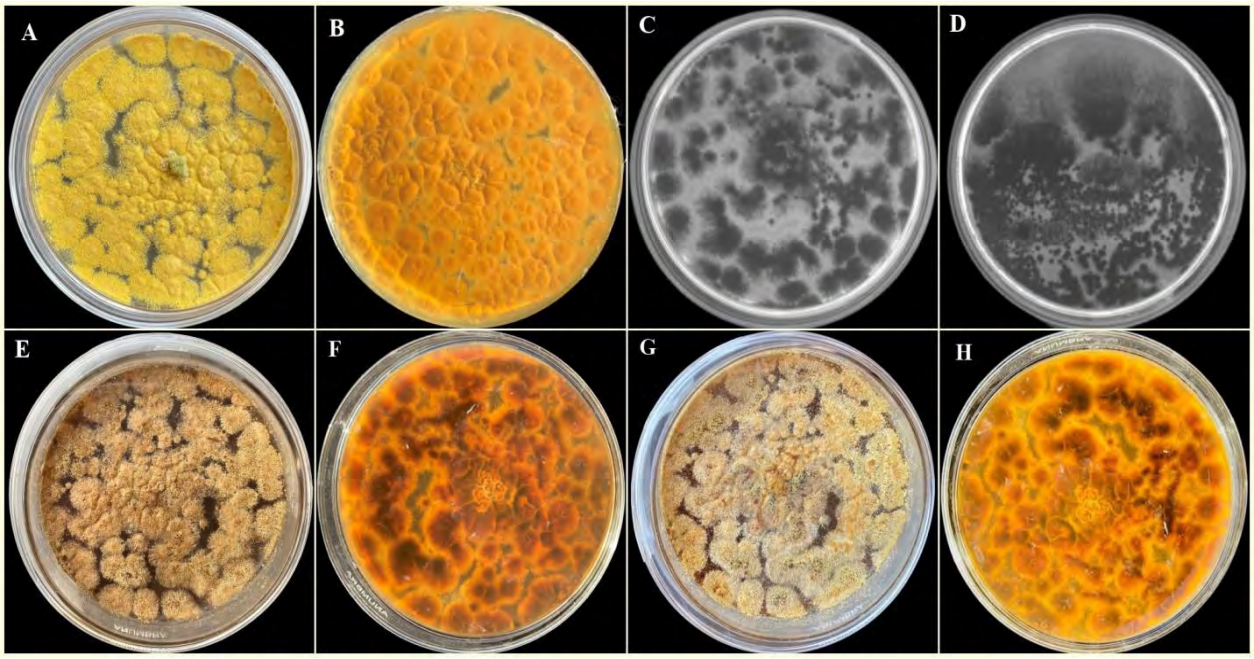

Figure 3.8 Appearance of pure fungal colonies of A. flavus on PDA media from front side (A) and back side (B) of Petri plates. Plates were observed under UV (C, D). Addition of NH_4OH changed colony color (E, F), which was restored, partially by the addition of glacial acetic acid (G, H).

3.2.3 Characterization of Fe_2O_3 NPs

The following results were obtained by the characterization of mycosynthesized Fe_2O_3 NPs.

3.2.3.1 Fourier transform infrared (FTIR) spectroscopy

Obtained FTIR spectra displayed peaks of specific functional groups (Fig. 3.9). A sharp peak at 3391.83 cm^{-1} showed N-H stretching of secondary amine group. Peaks at 3179.90 cm^{-1} determined C-H stretching of alkene, 2168.75 cm^{-1} described N=N=N stretching of azide, 1594.02 cm^{-1} displayed N-O stretching of nitro compound, and 1404.53 cm^{-1} indicated C-F stretching of floro compound. Other peaks (in the range of 500-580 cm^{-1}) presented C-Br stretching of halo compound. These functional groups indicated the capping and reduction of synthesized NPs.

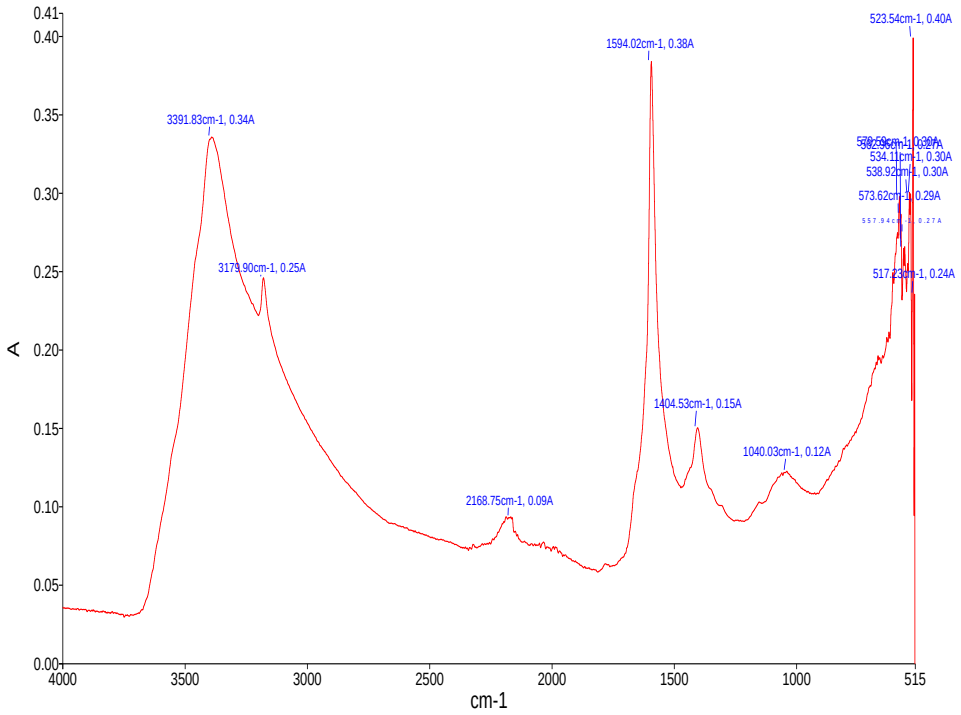

Figure 3.9 FTIR spectra of mycosynthesized Fe_2O_3 NPs showing the peaks of specific functional groups.

3.2.3.2 X-ray diffraction (XRD)

The average nanoparticle size was determined by XRD analysis as 7.78 nm (Table 3.3). XRD analysis also described noticeable pattern of Fe_2O_3 NPs peaks at 22.82, 56.18, 59.63, 59.99, and 67.55 degrees of 2θ, corresponding to hexagonal planes (Fig. 3.10). These results indicated the formation of magnetite iron oxide. The planes of XRD patterns corresponded to JSCPD number 01076-1821.

Table 3.3 XRD analysis to determine the size of *Fe_2O_3* NPs.

No. Peaks	Peak position 2 theta	FWHM	Crystallite size D (nm)	D nm (Average)
1	22.82	3.52	7.71	7.78
2	56.18	3.68	6.68	
3	59.63	3.60	6.72	
4	59.99	3.70	6.53	
5	67.55	2.06	11.25	

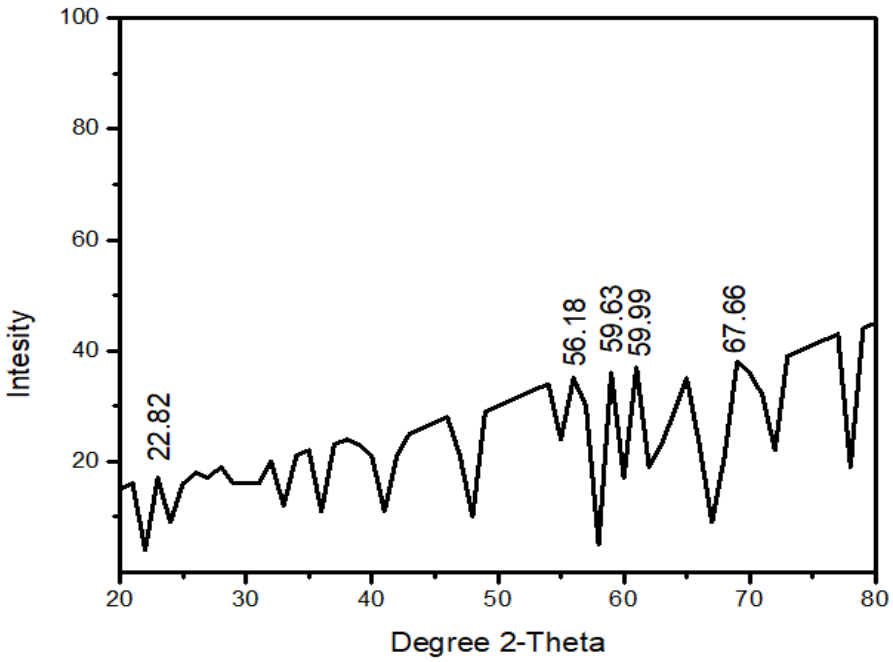

Figure 3.10 XRD spectra depicting noticeable pattern of Fe_2O_3 NPs peaks.

3.2.3.3 SEM and EDX analysis

SEM analysis displayed spherical shape and high polydispersity of biologically reduced Fe_2O_3 NPs (Fig. 3.11). In EDX spectrum, strong signals of iron were observed (Fig. 3.12), indicating the successful formation of Fe_2O_3 NPs.

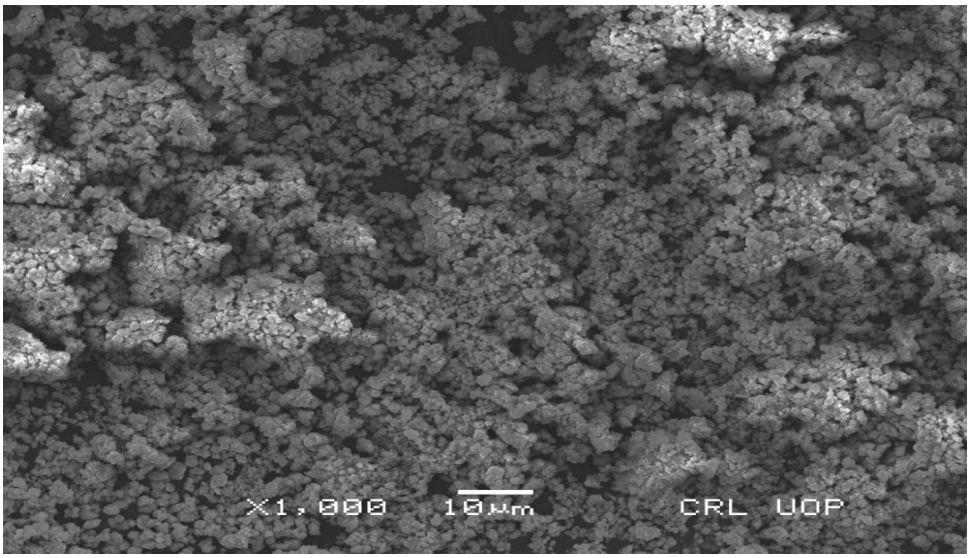

Figure 3.11 Scanning electron microscopy displayed spherical shape and high polydispersity of biologically reduced Fe_2O_3 NPs. Scale bar = 10 microns

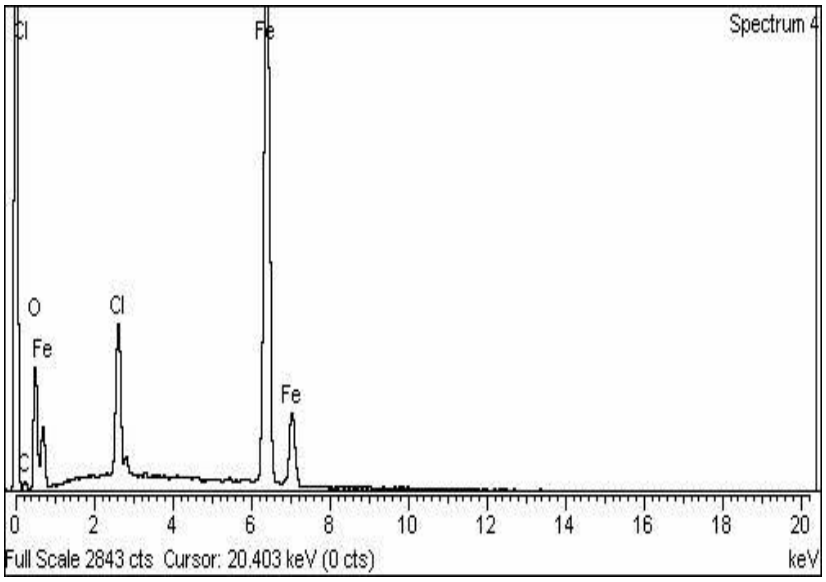

Figure 3.12 The EDX spectrum of mycosynthesized Fe_2O_3 NPs, indicating strong signals of Fe.

3.2.4. Fungal growth assay, *in vitro*

T. harzianum mediated Fe_2O_3 NPs showed variable mycelial growth inhibition, *in vitro* (Fig. 3.13). Though all concentrations of NPs inhibited mycelial growth, the maximum growth

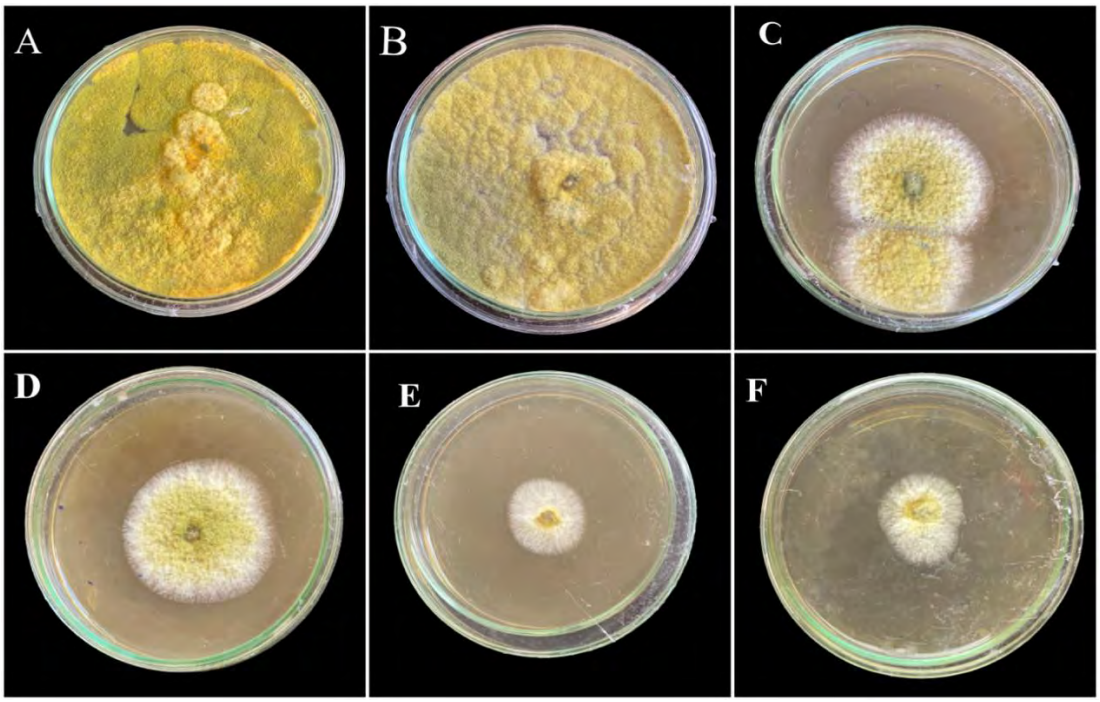

Figure 3.13 Fungus was grown on simple PDA (control) (A) and at different concentrations of NPs including 0.1 mg/ ml (B), 0.25 mg/ ml (C), 0.5 mg/ ml (D), 1 mg/ ml (E), and 1.5mg/ ml (F).

inhibition was observed at 1 mg ml^{-1} concentration of NPs (72.5 %). A reduced inhibition was noticed at 0.1 mg/ ml (12.2 %), 0.25 mg/ ml (26.5 %), 0.5 mg/ ml (49.1 %), and 1.5 mg/ml (64.2 %) concentrations. It has been described earlier that the efficiency of NPs decreases at very high concentrations due to the activation of diverse stress-compensation-pathways (Molina-Hernández et al., 2022).

3.2.5. Disease control of peach fruit, *in vivo*

Fe_2O_3 NPs efficiently inhibited fruit rot of peach fruit (Fig. 3.14). Increasing concentration of Fe_2O_3 NPs controlled disease and the least disease incidence was observed at 1.0 mg/ml concentrations.

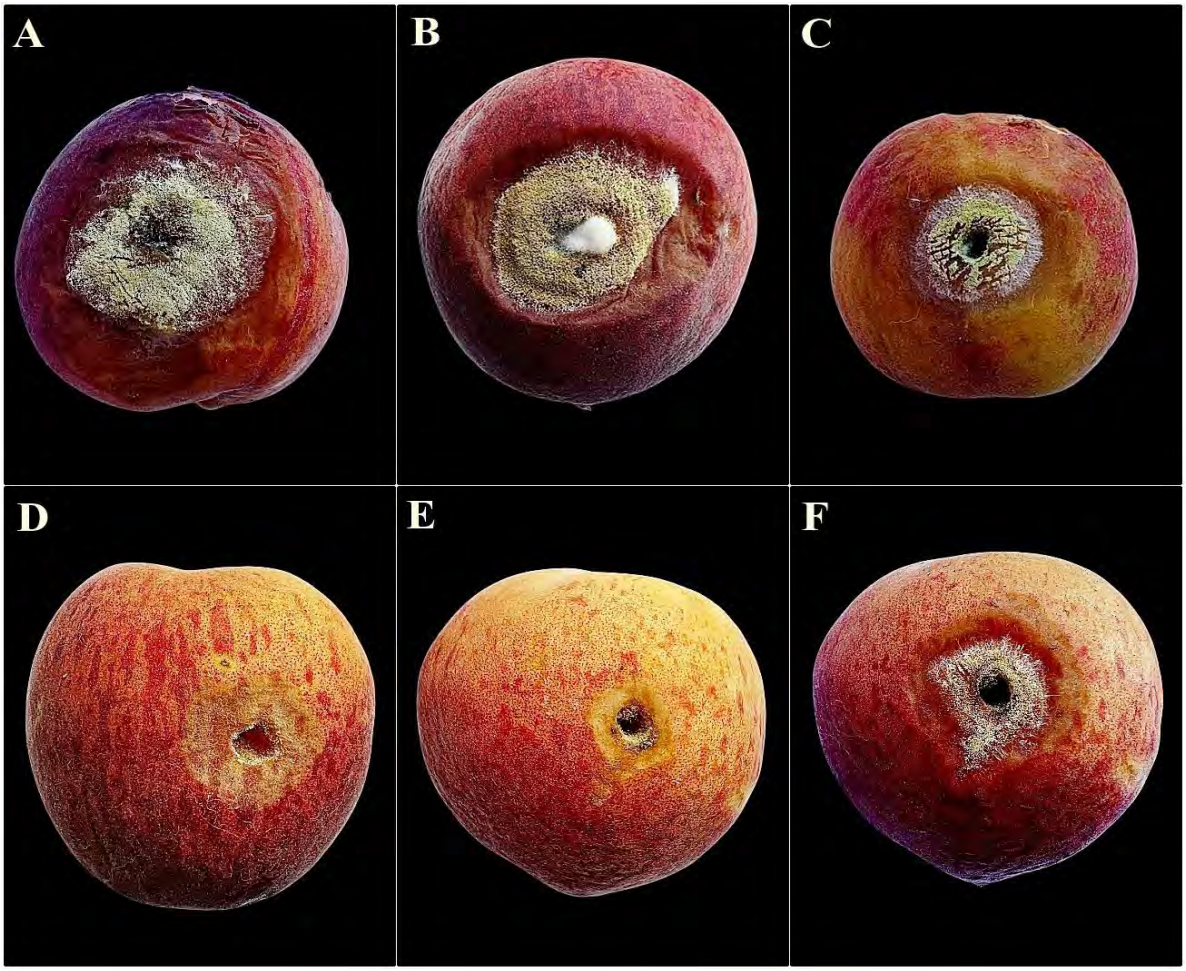

Figure 3.14 Inoculation of fungus displayed maximum disease in untreated peach fruit (A). Disease infestation was controlled with different concentrations of Fe_2O_3 NPs including 0.1 mg/ ml (B), 0.25 mg/ ml (C), 0.5 mg/ ml (D), 1 mg/ ml (E), and 1.5mg/ ml (F).

3.2.6. Fruit quality attributes

Fruit quality was severely affected by fungus inoculation. Different biochemical and organoleptic characteristics of fruit were preserved by the application of Fe_2O_3 NPs (Table 3.4). Along with soluble solids, treatment of Fe_2O_3 NPs enabled peach fruit to maintain higher titratable acidity, ascorbic acid contents and fruit firmness. Previous studies have described them as key fruit quality parameters (dos Santos et al., 2022). Ascorbic acid has been reported to play key role in protecting cellular DNA and proteins (van Gorkom et al., 2019).

Table 3.2 Fruit quality attributes after treatment with *Fe_2O_3* NPs.

Conc of NPs (mg/ml)	**Diseased area (mm2)**	**Soluble solids (%)**	**TTA (%)**	**Ascorbic acid (mg/ 100 g)**	**Firmness (N cm-2)**
0.1	1101±10.2 ab	12.1±1.3 d	2.1±0.5 c	7.1±0.8 c	232.7±18.2 c
0.25	552±6.8 c	14.2±2.1 c	2.7±1.0 b	7.7±1.1 c	218.6±13.3 d
0.5	232±3.4 de	15.3±0.7 bc	3.0±0.5 a	9.2±1.4 ab	246.3±18.0 c
1	124±2.1 f	18.1±1.6 a	3.3±0.7 a	10.2±1.2 a	302.2±16.8 a
1.5	258±4.2 d	16.3±1.1 b	2.9±0.5 ab	9.1±1.0 ab	266.7±14.2 b
Control	1204±9.5 a	11.3±0.8 de	1.8±0.7 d	6.6±1.1 d	188.3±12.9 e
Untreated fruit	0	18.6±1.2 a	3.4±0.5 a	10.8±0.8 a	311.5±12.2 a

Note: Values with same digit (a, b, c, d, e or f) are not significantly different from each other.

3.2.7. Detection of aflatoxin in diseased fruit

Aflatoxin contents in diseased fruit were successfully detected by the following three different techniques.

3.2.7.1. Thin layer chromatography (TLC)

The preliminary qualitative detection of aflatoxins (B_1, B_2, G_1 and G_2) was performed using TLC (Fig. 3.15). The results depicted that aflatoxins B_1 and B_2 were the major compounds in peach fruit. Light bands were observed in diseased fruits treated with 1.0 mg/ml concentration of Fe_2O_3 NPs (T4).

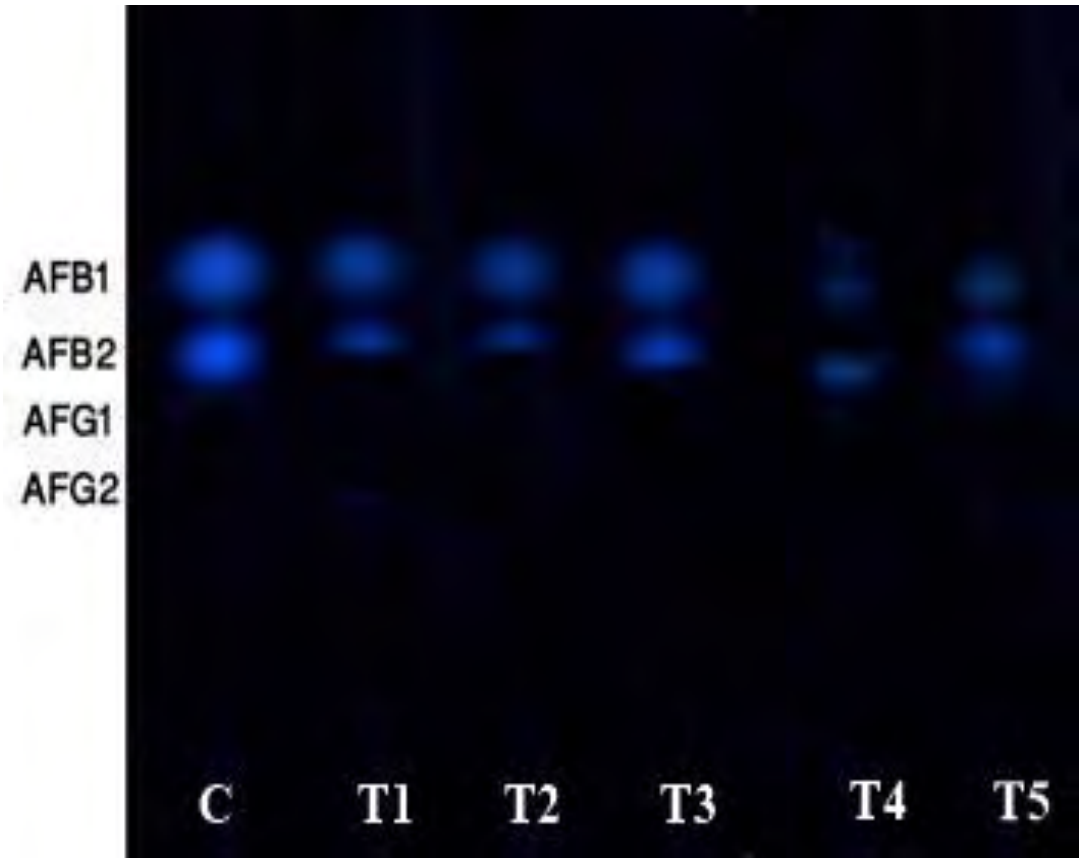

Figure 3.15 Thin layer chromatography profile of aflatoxins B and G series in peach fruits after their treatment with different concentration of Fe_2O_3 NPs. Vertical columns represent diseased fruit (C), fungal inoculated fruit treated with different concentration of NPs including 0.1 mg/ml (T1), 0.25 mg/ml (T2), 0.5 mg/ml (T3), 1.0 mg/ml (T4), 1.5 mg/ml (T5).

. 3.2.7.2. ***Enzyme-linked immunosorbent assay (ELISA)***

ELIZA successfully detected aflatoxins (B_1 and B_2) in peach fruit. The highest amount of aflatoxin was detected in control fruit. Treatment of tomato fruit with Fe_2O_3 NPs inhibited disease and reduced the production of aflatoxins in fruit. The lowest aflatoxin concentration was observed in fruit treated with 1.0 mg/ml concentration of Fe_2O_3 NPs. Moreover, the concentration of aflatoxin B_1 was found to be higher than aflatoxin B_2 (Fig. 3.16).

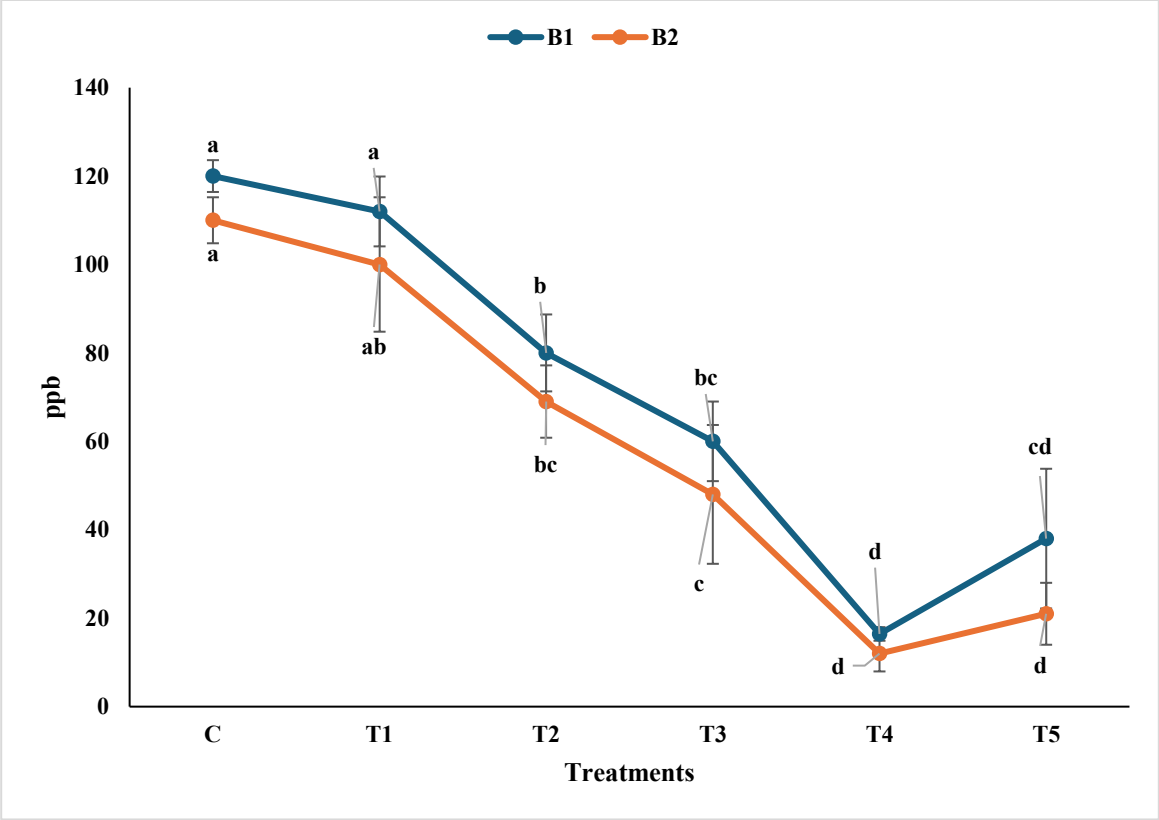

Figure 3.16 Quantitative estimation of aflatoxin in infected peach fruits under variable concentration of Fe_2O_3 NPs by ELISA technique. C = control, T1 = 0.1 mg/ml, T2 = 0.25 mg/ml, T3 = 0.5 mg/ml, T4 = 1.0 mg/ml, and T5 = 1.5 mg/ml concentration of *Fe_2O_3* NPs.

3.2.7.3 High Performance Liquid Chromatography (HPLC)

HPLC analyses of fungal inoculated control fruit and the diseased fruit treated with different concentrations of Fe_2O_3 NPs showed the presence of variable concentrations of aflatoxins. A calibration curve was established to estimate the concentration of B_1 and B_2 and a standard regression equation was used to estimate aflatoxin content in samples. Findings of this analysis revealed that the control peach fruit and all Fe_2O_3 NPs treated fruit were contaminated with variable concentration of aflatoxin B_1 and of aflatoxin B_2 (Table 3.5). Control fruit exhibited maximum presence of aflatoxins while the treatment of fruit with 1.0 mg/ml concentration of Fe_2O_3 NPs reduced the production of aflatoxins in infected fruit.

Table 3.5 Quantity of aflatoxin B_1 and B_2 present in peach fruit after their treatment with different concentrations of Fe_2O_3 NPs.

Conc of NPs (mg/ml)	Aflatoxin B1 (µg/kg-1)	Aflatoxin B2 (µg/kg-1)
0.1	11.52	1.57
0.25	10.36	1.12
0.5	7.22	1.08
1	5.88	0.78
1.5	8.11	1.01
Control	18.67	2.21

3.3 Experiment 3 Fe_2O_3 nanoparticles modulate titratable acidity, ascorbic acid contents, and firmness of Strawberries to enhance their shelf-life at room temperature.

3.3.1 Characterization of Fe_2O_3 NPs

3.3.1.1 UV-Vis Spectroscopy

Analysis of the UV-Visible spectrum showed that the Surface-Plasmon-Resonanace of the nanoparticles was at 420 nm (Figure 3.17). The colour of the cell-free filtrate of *T. harzianum* culture which was initially thick and yellowish changed to creamy whitish. This colour change indicates the bio-reduction of iron acetate by the fungal metabolites and the formation of Fe_2O_3 NPs (Munis et al., 2022).

3.3.1.2 Structural analysis by XRD

XRD analysis confirmed the formation of Fe_2O_3 NPs from iron acetate (Figure 3.18). The diffraction peaks of Fe_2O_3 NPs were compared with the standard JCPDS data. The peaks at 2θ value of 31, 34, 36, 47, 56, 62 and 67 are attributed to the hexagonal structure of NPs and the reflections correspond to (100), (002), (101), (102), (110), (103) and (200) planes respectively (Kumar et al., 2013). Debye Scherrer's formula determined the average particle size (12nm). High peaks indicated high purity and crystallinity of Fe_2O_3 NPs (Khan et al., 2019). These results support the use of fungal filtrate in the reduction of Iron acetate to the pure oxides, as postulated by Aarts et al., (2020).

3.3.1.3 Functional Groups Analysis by FTIR

The active biomolecules that can reduce Fe ions and do the capping of Fe_2O_3 NPs were determined by FTIR spectra (Figure 3.19). This spectrum showed absorption bands at 3390 cm^{-1} (related to –NH groups of the primary aliphatic amines), 1056 cm^{-1} (related to –C-O groups of alcohols), and 577 cm^{-1} (related to C–Cl groups of halogens). The broad peak around 3300 cm^{-1} indicated O–H stretching from alcohols. Other peaks at 1113 cm^{-1} and 541 cm^{-1} attributed to C-O stretching of alcohols and Fe-O stretching of FeO (hematite). The proteins present in the biomass are also considered to be good capping agents for the nanoparticles because of their amide groups (Farhana et al., 2022).

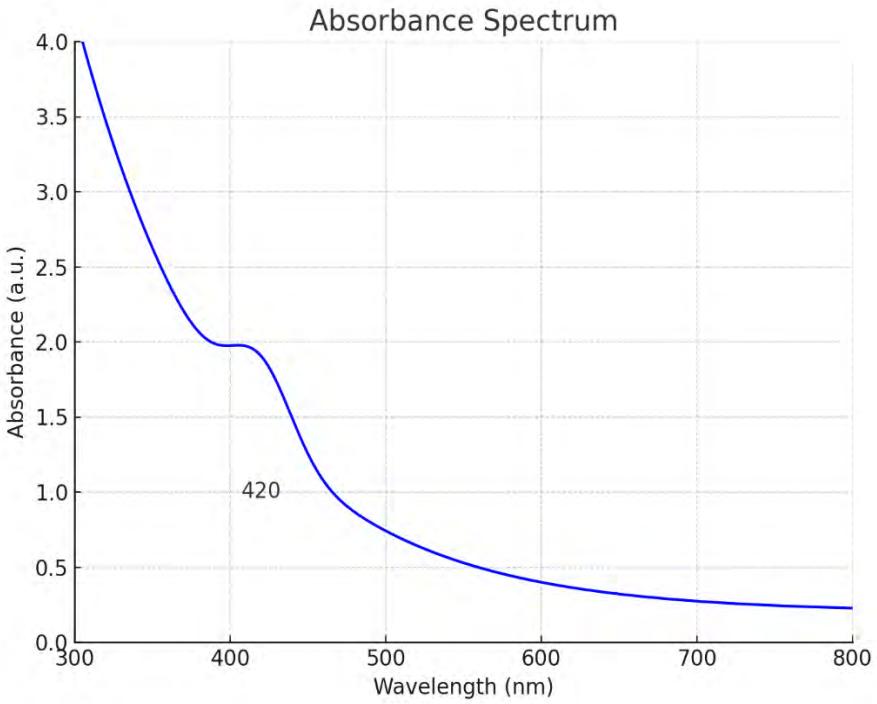

Figure 3.17 UV-Vis spectroscopy of Fe_2O_3 NPs.

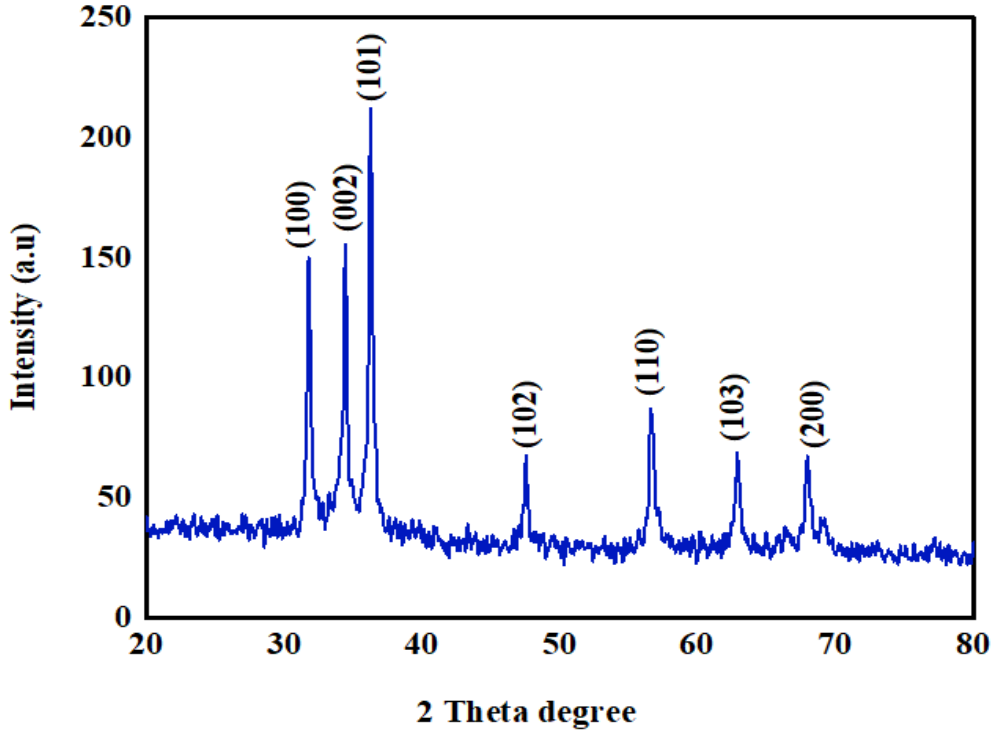

Figure 3.18 XRD spectrum of Fe_2O_3 NPs.

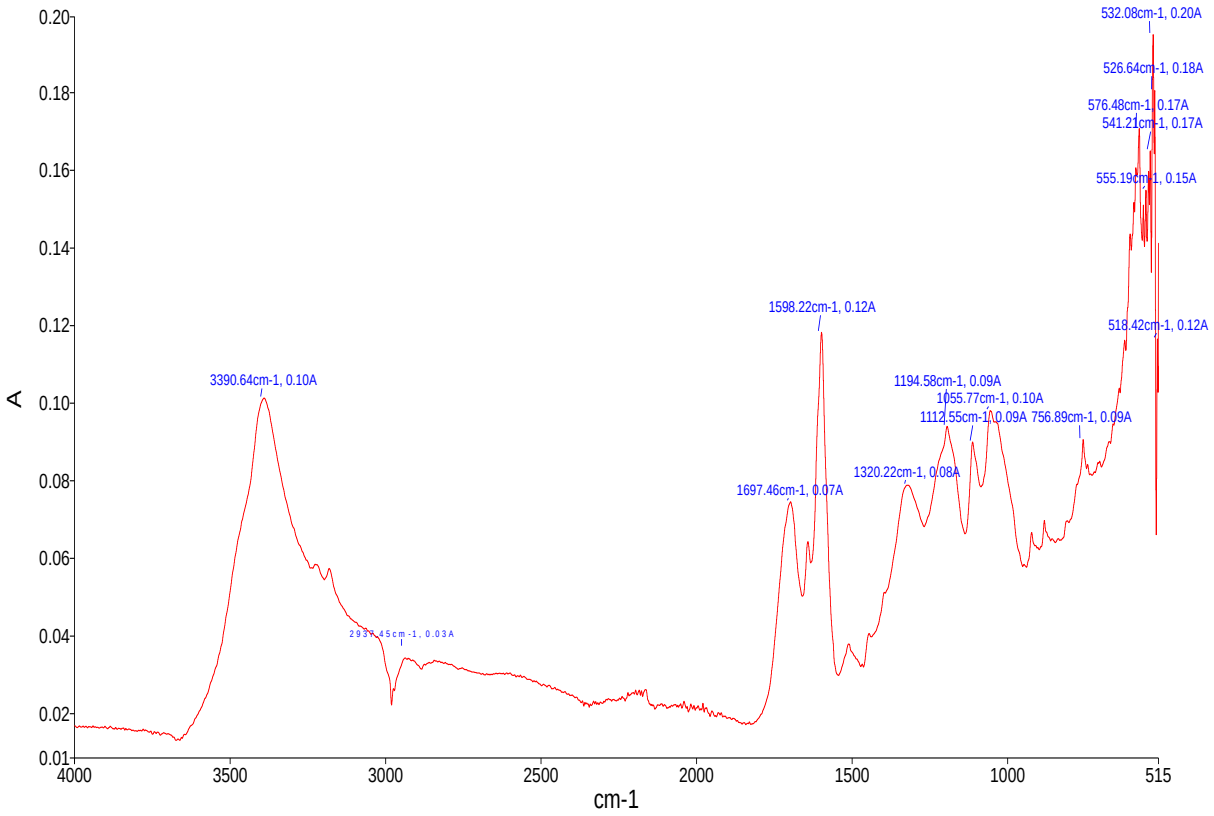

Figure 3.19 FTIR Analysis of Fe_2O_3 NPs.

3.3.1.4 SEM and EDX micrograph analysis

Morphological studies of Fe_2O_3 NPs, using SEM indicated spherical shape of the the particles (Figure 3.20). The SEM pictures revealed almost spherical formations of clustered FeO nanoparticles in agglomerations, with dimensions of around 1 nm. The chemical nature of the reducing agents present in the fungal filtrate controls the nanoparticles' size and morphology. As are consistent with our findings, similar spherical or flower-like morphologies of the fungal-derived Fe_2O_3 NPs have been earlier (Kumar et al., 2013). EDX micrograph revealed the purity and composition of Fe_2O_3 NPs (Figure 3.21). EDX spectrum displayed the highest percentage of Fe (68%) and Cl (21.5 %), with minor presence of oxygen (3.8 %) and Mn (6.7%).

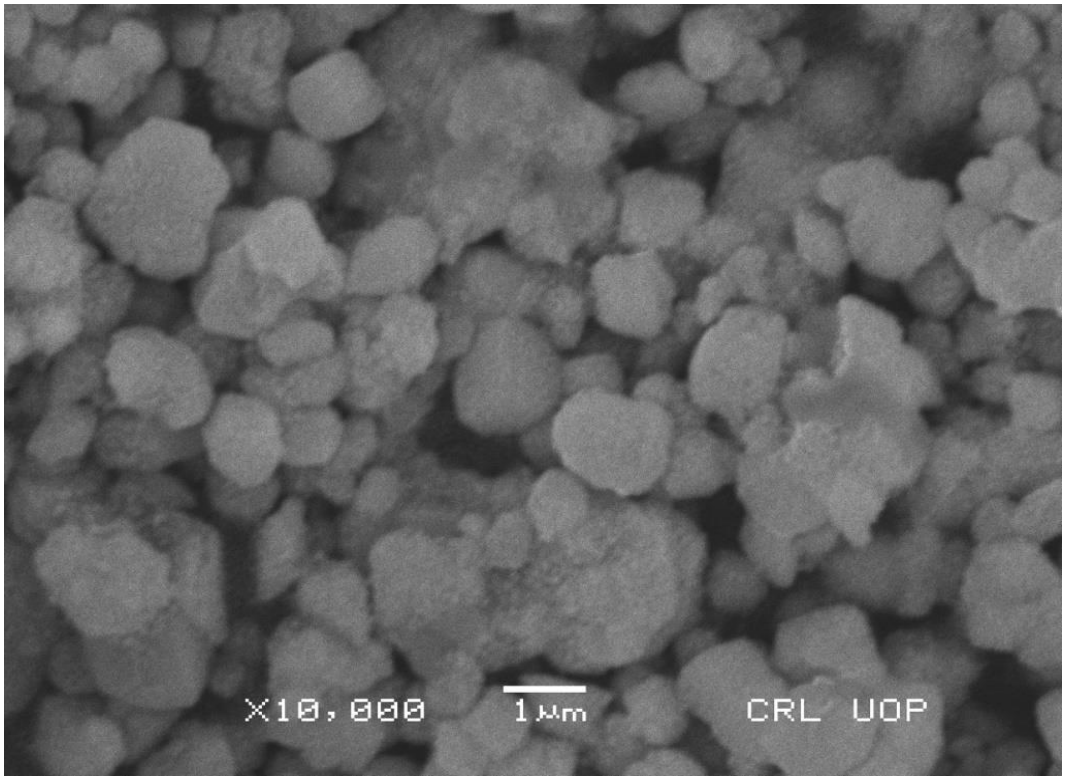

Figure 3.20 SEM Micrograph of Myco-fabricated Fe_2O_3 NPs, Scale bar = 5 microns

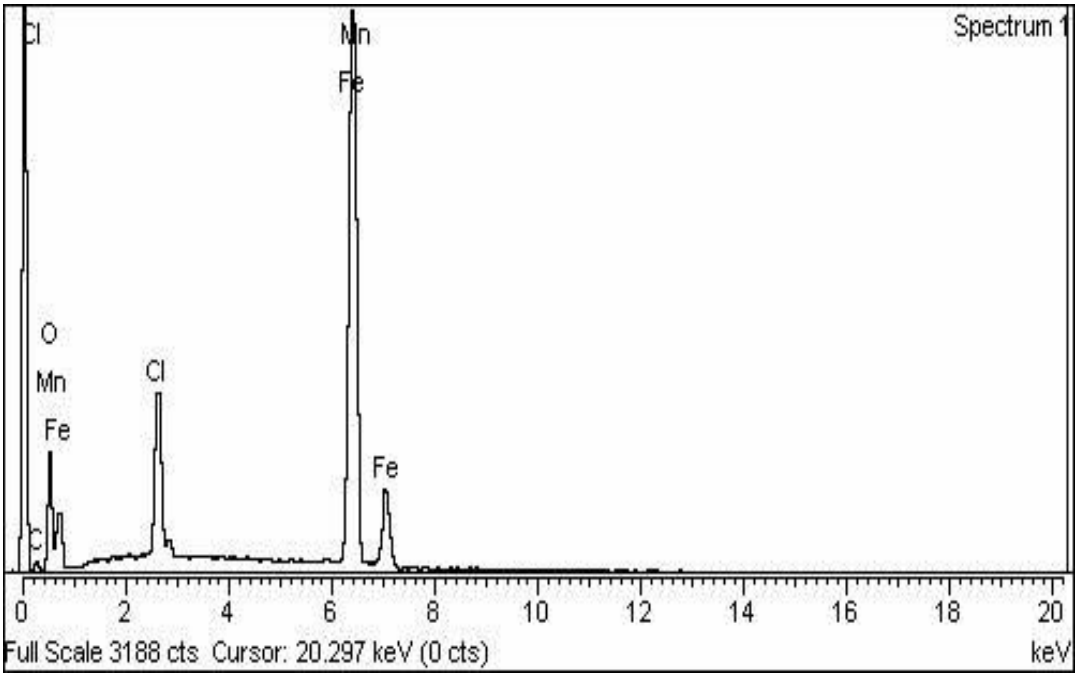

Figure 3.21 EDX spectrum showing the elemental composition of Fe_2O_3 NPs

3.3.2 Postharvest physiochemical qualities of strawberry

3.3.2 .1 Firmness

The decline of fruit firmness of strawberries at room temperature was effectively suppressed by post-storage treatments of all concentrations of Fe_2O_3 NPs (Figure 3.22 a). The least loss in fruit firmness was observed at 1mg/ml concentration of Fe_2O_3 NPs. Untreated strawberry fruit displayed maximum loss of fruit firmness.

3.3.2.2 Weight loss of strawberry

Fruit treated with Fe_2O_3 NPs displayed less weight loss during storage at room temperature (Figure 3.22 b). The maximum weight loss was observed in control. Minimum weight loss was observed at 1mg/ml concentration of Fe_2O_3 NPs, followed by 0.25 mg/ml concentration of Fe_2O_3 NPs.

3.3.2.2 Titratable acidity

High titratable acidity was maintained in all treatments of Fe_2O_3 NPs (Figure 3.22 c). During storage for six days at room temperature, the highest TA was observed on the 3rd day and a gradual decrease of TA on the 6th day, in all treatments. After one-week, high TA was maintained at 1 mg/ml and 0.25 mg/ml concentrations of Fe_2O_3 NPs. Consumption of melic acid during respiration could be the reason for reducing TA. During storage, control fruit showed the highest reduction of TA, as compared to Fe_2O_3 NPs treated strawberry.

3.3.2.3 Soluble solids

Applying Fe_2O_3 NPs promoted the preservation of soluble solids in strawberries during storage at room temperature (Figure 3.22 d). Though all the concentrations of NPs helped strawberry fruit to store higher soluble solids, as compared to control, 1.0 mg/ml concentration performed best in maintaining the highest concentration of soluble solids. The decline of soluble solids in control fruit depicted water loss in storage.

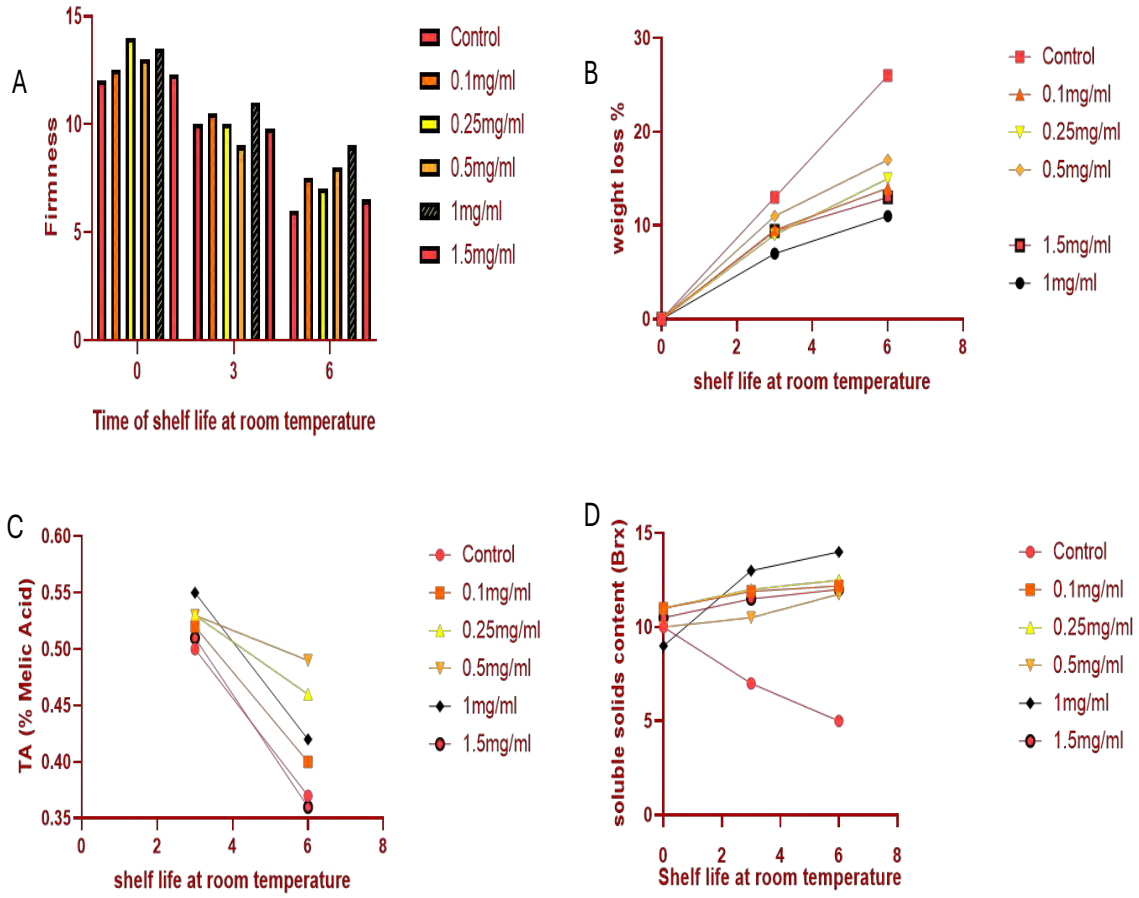

Figure 3.22 Effect of Fe_2O_3 NPs on quality attributes of strawberries including firmness (A), weigh loss (B), titratable acidity (C), and soluble solids (D).

3.3.2.4 Ascorbic acid analysis

Ascorbic acid contents were decreased in all treatment groups during storage. The application of Fe_2O_3 NPs slowed down this decrease (Figure 3.23 A). These results indicate that Fe_2O_3 NPs might have retarded oxygen permeability of the stored fruit. Though all Fe_2O_3 NPs treatments showed efficient results, 1.0 mg/ml concentration performed best after six days of storage.

3.3.2.5 Carotenoid content analysis

This study revealed an increase in carotenoid contents during storage in all the treatments (3.23 B). When stored at room temperature, the authors found that strawberries that were not

treated in any way contained the highest level of carotenoid (16 mg 100^{-1} g) after six days of storage. The treatments of Fe_2O_3 NPs demonstrated a profound influence on the regulation of carotenoid levels at the lower concentration than the control. In all four treatments, only 0. 1 mg/ml and 0.50 mg/ml of Fe_2O_3 NPs were the most effective.

3.3.2.5 Iron oxide level

Iron is an essential component in the human bones (Du et al., 2006). The strawberry fruit treated with 1.0 mg/ml and 0.75 mg/ml concentrations has the highest amount Fe, followed by 0. 50 mg/ml and 0.25 mg/ml concentrations of Fe_2O_3 NPs (Figure 3.23 C). A very low amount of iron oxide was detectable in the untreated strawberry fruit.

3.3.2.6 Sensory assessment

The sensory assessment was conducted based on overall acceptability (OAA) and odor of the control and Fe_2O_3 NPs treated strawberry fruit, with the help of hedoic scale (Figure 3.23 D). Overall, at all concentrations of Fe_2O_3 NPs, fruit had good sensory perception (> 5) than the control. Among these treatments, 0.25 mg/ml and 1.0 mg/ml concentrations of Fe_2O_3 NPs showed slightly better sensory score, while control fruits showed the lowest OAA and odor, after six-day storage at room temperature.

3.3.3 Shelf-life of strawberry

The application of Fe_2O_3 NPs increased the shelf life of strawberry fruits at room temperature, effectively (Figure 3.24). Two concentrations of Fe_2O_3 NPs (0.25 mg/ml and 1 mg/ml) efficiently increased the shelf life of strawberry fruit and displayed their acceptable color, skin, pulp level and aroma. Control fruit showed maximum loss of acceptable attributes of strawberry fruit.

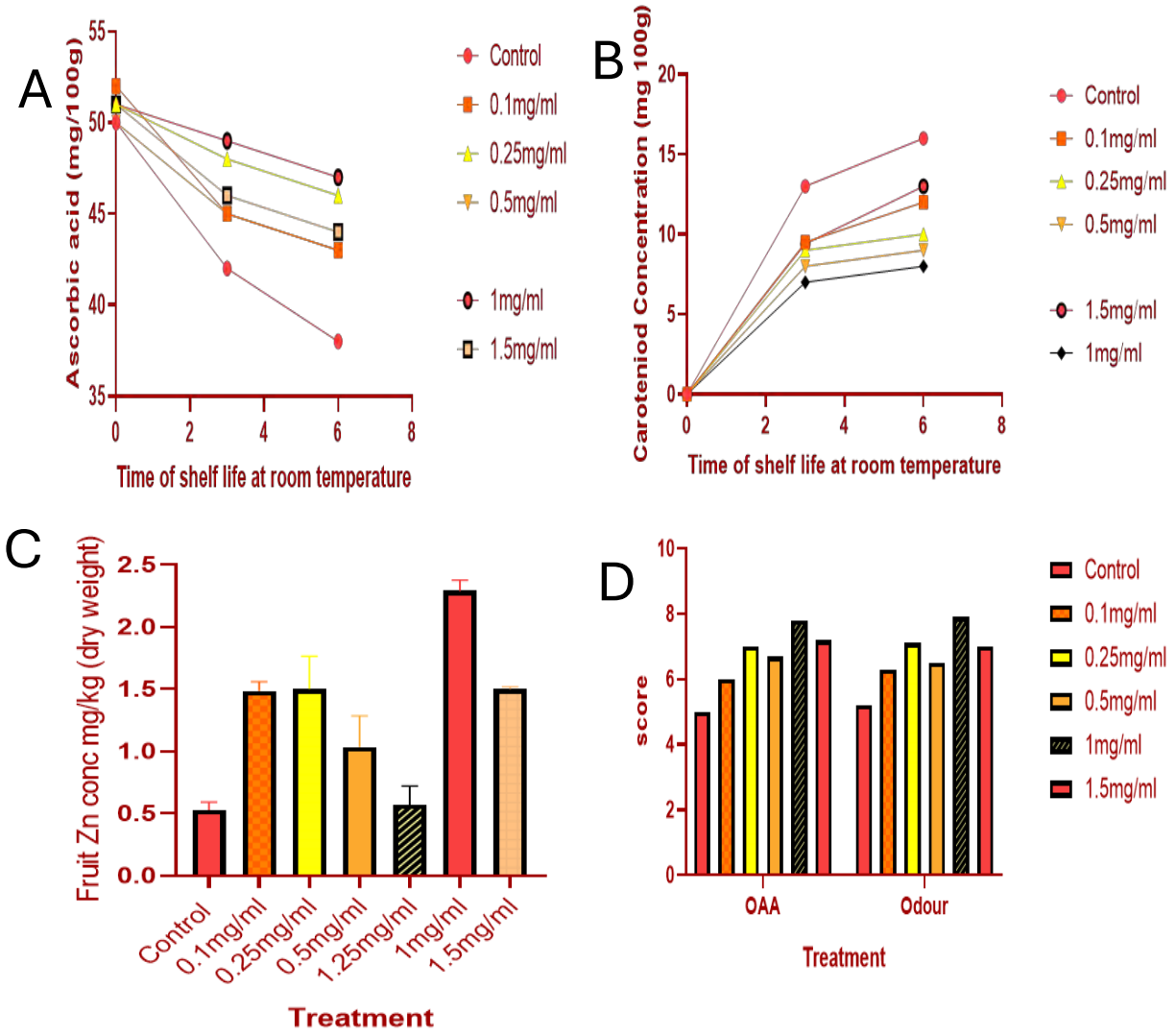

Figure 3.23 Effect of Fe_2O_3 NPs on quality attributes of strawberry including ascorbic acid (A), carotenoid content (B), Zn concentration (C) and sensory assessment (D).

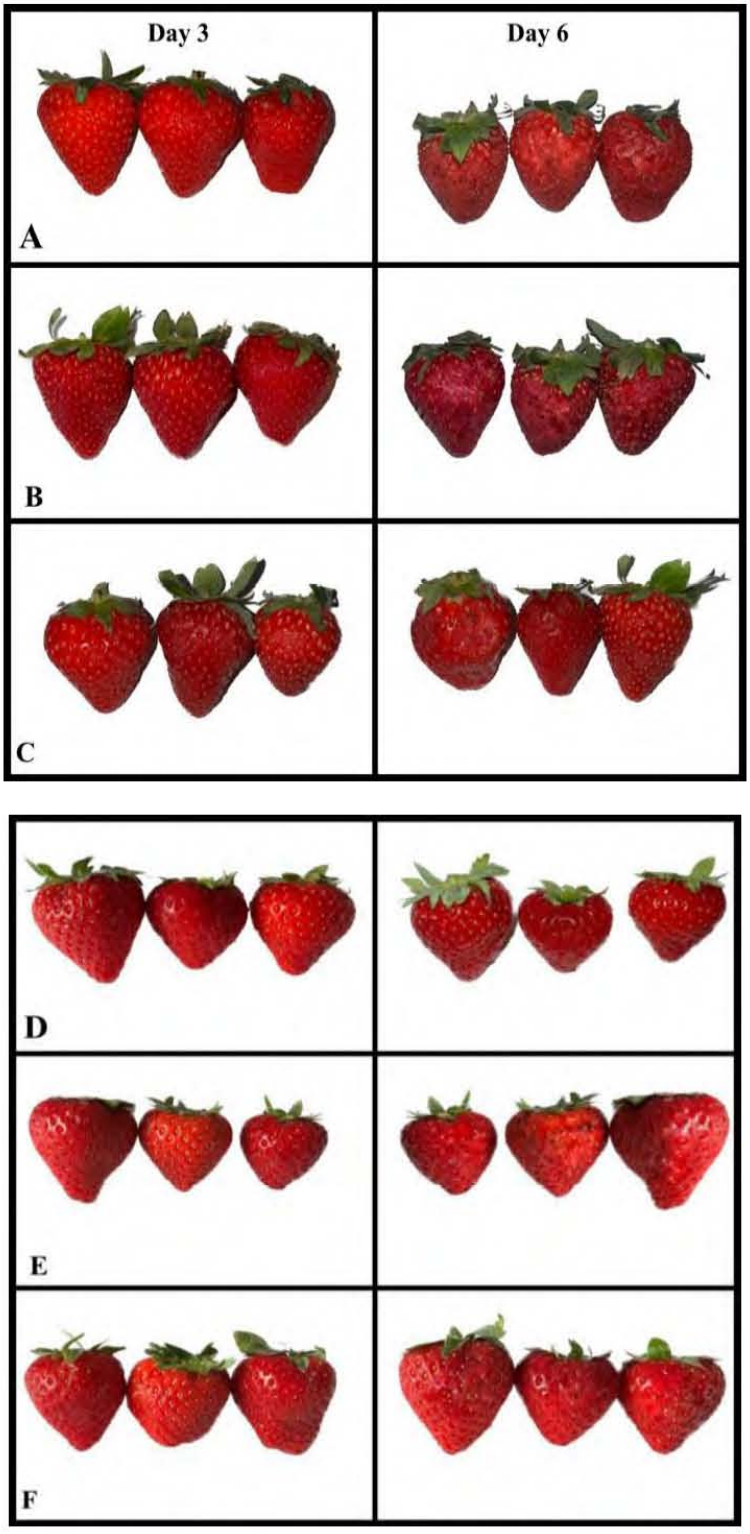

Figure 3.24 Visual appearance of strawberry fruit, elaborating their shelf life at different concentrations of Fe_2O_3 NPs, control (A), 0.1 mg/ml (B), 0.25 mg/ml (C), 0.5 mg/ml (D), 1 mg/ml (E), and 1.5 mg/ml (F).

4. DISCUSSION

The key objective of this study was to explore the potential of Fe_2O_3 nanoparticles, synthesized through mycogenic processes to control fungal pathogens in apples, peaches and strawberries, and improve their quality and extent shelf-life. This investigation is framed within the context of increasing global concerns regarding food safety, post-harvest losses, and the need for sustainable alternatives to chemical preservatives (Villarreal et al., 2010).

Fe_2O_3 NPs not only controlled controlling disease incidence but also preserved the sensory qualities of fruits, which are crucial for consumer acceptance (van Gorkom et al., 2019). The implications of this research extend beyond the immediate context of fruit preservation, as the principles and methods developed could be adapted for use in other areas of agriculture and food processing. This study revealed the underlying mechanisms by which these particles stop the infection of *F. oxysporum* and *A. flavus*. The study demonstrated that Fe_2O_3 nanoparticles exhibit potent antifungal properties by significantly reducing fruit rot disease in apples, peaches and strawberries. Understanding these mechanisms is essential for using NPs in the field of agriculture (Bin-Jumah et al., 2021).

Generation of reactive oxygen species (ROS) is the primary mechanisms by which Fe_2O_3 NPs employ their antifungal activities. This oxidative stress disrupts cellular structures and functions of fungal cells. ROS can damage proteins, lipids, and nucleic acids within fungal cells. The oxidative stress disrupts the integrity of fungal cell membrane integrity and causes its lysis. This mechanism is particularly effective against fungi due to their relatively simple cellular structures compared to higher organisms, making them more susceptible to oxidative damage (Shin et al., 2019).

Another key mechanism is the direct interaction of Fe_2O_3 NPs with the cell wall of the fungus. These small size NPs can pass through the cell wall and interact with various cellular components. The nanoparticles can disrupt the cell wall's structural integrity by interfering with the synthesis of key polysaccharides, such as chitin and glucan, which are essential for maintaining the rigidity and strength of cell wall. In this way, Fe_2O_3 nanoparticles weaken the fungus's defense mechanisms, making it more vulnerable to environmental stresses and other antimicrobial agents Bibi et al., 2020).

Previous studies describe that, upon interaction with the fungal cells, Fe_2O_3 nanoparticles may undergo partial dissolution, releasing iron ions into the surrounding environment (Gupta et

al., 2019). These free iron ions can further enhance the generation of ROS through Fenton-like reactions. Additionally, iron ions can interfere with the fungal cell's metabolic processes by displacing essential metal cofactors in enzymes, leading to the inhibition of critical biochemical pathways. This disruption of metabolic processes contributes to the overall antifungal effect, as the fungus is unable to carry out essential functions such as energy production and DNA replication (Azmath et al., 2016). Fe_2O_3 nanoparticles interact with fungal DNA, causing strand breaks and other forms of genetic damage (Musacchi & Serra, 2018). This genetic damage causes apoptosis and effectively kills fungal cells. This mechanism is particularly significant as it suggests that Fe_2O_3 nanoparticles not only inhibit fungal growth but also prevent the reproduction and spread of the pathogen, thereby reducing the likelihood of reinfection (Saravanakumar et al., 2020a).

In addition to these direct mechanisms, the study observed that Fe_2O_3 nanoparticles could induce systemic resistance in the treated fruits. In systemic resistance, a local infection triggers a broad-spectrum resistance throughout the plant and enhances resistance against the stimulus. Moreover, these NPs stimulate the transcription of defense-related proteins in the plant tissues, such as pathogenesis-related proteins. This not only helps in controlling the primary infection but also provides long-term protection to the fruit, consequently, their shelf life is increased. The effectiveness of Fe_2O_3 nanoparticles is also influenced by their respective size, particular shape, surface charge, and crystalline structure. The nanoparticles used in this study were small and crystalline. These properties are famous for antimicrobial activity. The crystalline structure of Fe_2O_3 nanoparticles contributes to their stability and durability, ensuring that they remain active for extended periods and continue to exert their antifungal effects.

Surface modifications of Fe_2O_3 nanoparticles can further enhance their antifungal activity. In this study, the biomolecules of fungal filtrate did the capping and reduction of nanoparticles. These biomolecules, including proteins, enzymes, and secondary metabolites, provide additional functionality to the nanoparticles. For instance, capping agents attach nanoparticles on the surface of fungus, increase their local concentration and enhance their antifungal efficacy. Moreover, these biomolecules may themselves possess antimicrobial properties, contributing to the overall antifungal effect. The environmental implications of using Fe_2O_3 nanoparticles as antifungal agents are also noteworthy. The study's findings suggest that these nanoparticles offer a sustainable alternative to chemical fungicides, which are toxic in

nature. The use of Fe_2O_3 nanoparticles, particularly those synthesized through mycogenic processes, minimizes the use of hazardous substances. However, the potential negative effect of nanoparticles must also be considered before their application (Zubair et al., 2022). Fe_2O_3 nanoparticles demonstrate a multifaceted approach to pathogen control, combining oxidative stress induction, cell wall disruption, metabolic interference, genetic damage, and systemic resistance induction. These mechanisms work synergistically to provide a robust defense against fungal pathogens.

The antifungal activity of Fe_2O_3 nanoparticles is a consequence of the activation of complex interactions (Niazi et al., 2023). By targeting multiple pathways within the fungal cells, these nanoparticles can inhibit their growth and protect fruits (Loi et al., 2020). These findings suggest Fe_2O_3 nanoparticles as a sustainable and effective alternative to traditional fungicides, offering a new avenue for enhancing crop protection and food security (Consolo et al., 2020). The preservation of fruit composition and organoleptic properties during storage and transportation is extremely important. Application of Fe_2O_3 nanoparticles offers a promising solution by not only protecting fruits from pathogenic fungi but also maintaining their nutritional and sensory qualities. This section explores the effectiveness of Fe_2O_3 nanoparticles in preserving the biochemical composition and organoleptic properties of fruits such as peaches and strawberries, focusing on factors like texture, color, taste, aroma, and nutritional content.

Fruit quality is described by the presence of essential nutrients like vitamins, minerals, sugars, and organic acids. During storage, these components can deteriorate due to enzymatic activities, microbial growth, and oxidative processes. The application of Fe_2O_3 nanoparticles was found to significantly inhibit the degradation of these nutrients, primarily by preventing fungal infections that would otherwise accelerate spoilage. By reducing fungal load, the nanoparticles help in maintaining the integrity of cellular structures within the fruit, thereby preserving its nutritional content. The study demonstrated that fruits treated with Fe_2O_3 nanoparticles retained higher levels of vitamin C, which is a critical antioxidant that contributes to the fruit's nutritional value and is also maintained the firmness of the fruit. The oxidative stress generated by fungal infections typically leads to the breakdown of vitamin C, resulting in a loss of firmness and an increase in susceptibility to mechanical damage. Fe_2O_3 nanoparticles, by inhibiting fungal growth, help in preserving the vitamin C content. Another important aspect of fruit composition is the preservation of sugars and organic acids. These compounds are very important in proving

taste and sweetness to the fruit. The study found that fruits treated with Fe_2O_3 nanoparticles exhibited a slower rate of sugar degradation during storage. This is likely due to the nanoparticles' ability to suppress the activity of fungal enzymes that metabolize sugars into less desirable compounds. By preserving the sugar content, Fe_2O_3 nanoparticles help in maintaining the natural sweetness of the fruit, which is a key factor in consumer acceptance and marketability.

The preservation of organic acids is important for fruit's acidity and overall flavor profile. Among these, citric and malic acids are the key organic acids. The study observed that Fe_2O_3 nanoparticles helped in maintaining the levels of these acids, which are often reduced during fungal infections. Organic acids have been reported to inhibit microbial growth and enhance the shelf-life of the fruit. These organic acids, preserved by the application of Fe_2O_3 nanoparticles, help in preserving the fruit's natural flavor, making it more appealing to consumers. The organoleptic properties of fruits, such as texture, color, taste, and aroma, are critical determinants of their market value and consumer preference. The study found that Fe_2O_3 nanoparticles effectively preserved these properties during storage. The texture of fruits was better maintained in treated fruits. The nanoparticles' ability to inhibit fungal enzymes helps in maintaining the fruit's firmness and crispness.

Color is another important organoleptic property that affects the visual appeal of fruits. During storage, some piments (anthocyanins and carotenoids) degrade, leading to a loss of color intensity and overall attractiveness of the fruit. The study showed that Fe_2O_3 nanoparticles helped in preserving the color of the fruits by protecting these pigments from oxidative damage. The nanoparticles' ability to generate ROS in a controlled manner preserves these pigments, thereby maintaining the fruit's vibrant color throughout the storage period. Taste and aroma are the most direct indicators of fruit quality and are closely linked to consumer satisfaction. The study found that Fe_2O_3 nanoparticles had a positive impact on the preservation of these properties. Fe_2O_3 nanoparticles help in reducing waste and improving the profitability of fruit production. The use of Fe_2O_3 nanoparticles in preserving fruit quality also offers potential benefits for the food processing industry. Fruits that maintain their composition and organoleptic properties during storage are useful for jams, juices, and preserved as dried fruits. Furthermore, the preservation of nutritional content, particularly antioxidants like vitamin C, enhances the

health benefits of these processed products, making them more attractive to health-conscious consumers.

The application of Fe_2O_3 nanoparticles is not without challenges. The potential for nanoparticle residues to remain on the fruit surface and affect the human gut needs to be carefully assessed. Additionally, the long-term effects of consuming fruits treated with nanoparticles need to be studied. Environmental considerations are also important when evaluating the application of NPs in agriculture. These nanoparticles offer a sustainable alternative to chemical fungicides, their potential negative impacts need to be thoroughly investigated. The nanoparticles that may persist in the soil and accumulate in the food chain are areas of concern that require further research. Developing environmentally friendly synthesis methods and ensuring proper disposal and recycling of nanoparticle-treated materials are essential steps in minimizing the ecological impact of this technology.

The application of Fe_2O_3 nanoparticles in preserving fruit composition and organoleptic properties represents a promising approach to enhancing fruit quality and extending shelf life. By protecting fruits from fungal infections and oxidative degradation, these nanoparticles help in maintaining the nutritional content, texture, color, taste, and aroma of the fruit. The findings from this study suggest the significance of the application of Fe_2O_3 nanoparticles. However, the deployment of these nanoparticles is not without its challenges and limitations. One of the primary technical challenges in using Fe_2O_3 nanoparticles is achieving a consistent and effective application on a commercial scale. Ensuring uniformity in these parameters is essential for maintaining the nanoparticles' antifungal properties and minimizing variability in results. However, producing nanoparticles with consistent characteristics at a large scale can be technically demanding and costly. Additionally, the stability of nanoparticles during storage and application must be ensured to prevent aggregation or changes in their properties that could reduce their effectiveness. The mode of application of Fe_2O_3 nanoparticles is another technical challenge. Inconsistent coverage can lead to untreated areas that remain susceptible to fungal infections. Moreover, the method of application must be compatible with existing agricultural practices and equipment to facilitate adoption by farmers. Developing efficient and cost-effective methods for applying nanoparticles in the field or during post-harvest processing remains a significant challenge.

Environmental concerns are also a major limitation in the application of Fe_2O_3 nanoparticles in agriculture. There is still a need to study the influence of nanoparticles on soil health, water resources, and non-target organisms. Once applied, nanoparticles may accumulate in the soil, potentially affecting soil microbial communities and plant health. There is also a need to comprehensively study the long-term effects of nanoparticle accumulation in the environment, raising concerns about their potential to disrupt ecosystems. Additionally, nanoparticles could leach into water bodies, posing risks to aquatic life and water quality. While the study ensured that nanoparticles were washed off the fruit surface before consumption, there is a risk that residues may remain, leading to human exposure. The effect of long-term exposure of nanoparticles is still not well understood. The potential for Fe_2O_3 nanoparticles to cause oxidative stress, inflammation, or toxicity in humans is a concern that requires thorough investigation. Moreover, the safety of consuming fruits treated with nanoparticles must be established through rigorous toxicological studies.

Economic considerations are also a limitation in the adoption of Fe_2O_3 nanoparticles in agriculture. The cost of producing high-quality nanoparticles can be prohibitive. The initial investment in nanoparticle synthesis equipment, as well as the ongoing costs of production, storage, and application, could limit the accessibility of this technology. Additionally, the potential need for specialized equipment for nanoparticle applications may further increase costs. While the use of nanoparticles could lead to savings by reducing losses due to fruit rot and extending shelf life, these benefits must be weighed against the upfront costs. The economic feasibility of using Fe_2O_3 nanoparticles in different agricultural contexts needs to be carefully evaluated, considering factors such as farm size, crop type, and market demand. The introduction of any new technology in the food industry requires thorough regulatory scrutiny to ensure safety for consumers and the environment. The absence of standardized testing methods and safety assessments makes it difficult to apply them at commercial levels.

Moreover, the public perception and growing awareness and concern among consumers about nanotechnology must also be addressed. Negative perceptions and fears about the safety of consuming nanoparticle-treated fruits could lead to resistance from consumers, even if the technology is proven to be safe. Public education in agriculture is crucial for gaining consumer trust and acceptance. Additionally, labeling requirements and clear information on food products may be necessary to address consumer concerns and ensure informed choices.

The scalability of nanoparticles is another limitation that must be addressed. The conversion from small-scale laboratory synthesis to large-scale production requires careful optimization of processes to ensure that the nanoparticles maintain their desired properties and effectiveness. Additionally, the infrastructure required for large-scale nanoparticle production, including facilities for synthesis, storage, and quality control, may be lacking in many regions. Addressing these scalability issues is critical for making Fe_2O_3 nanoparticles a viable option for their widespread use. The introduction of new technologies often raises ethical questions about their impact on society and future generations. The application of Fe_2O_3 nanoparticles in food production may raise concerns about food safety, environmental sustainability, and the potential for unintended consequences. Ethical considerations should be integrated, and the technology must be used responsibly and equitably. This may involve engaging with stakeholders, including farmers, consumers, policymakers, and environmental organizations, to address concerns and build consensus on their appropriate use.

The application of Fe_2O_3 nanoparticles is a rapidly evolving field with significant potential. As the technology continues to advance, several future prospects and research directions emerge that could further enhance the utility of Fe_2O_3 nanoparticles in agricultural practices. This section explores these future directions, focusing on improving nanoparticle synthesis and functionality, exploring novel applications, assessing long-term environmental and health impacts, and developing regulatory frameworks and public engagement strategies. Current methods for nanoparticle synthesis often involve complex processes. Future research could include exploring alternative synthesis pathways, such as green chemistry approaches that use natural precursors and energy-efficient processes. Additionally, research into the functionalization of Fe_2O_3 nanoparticles could enhance their properties, such as increasing their stability, solubility, and specificity in targeting fungal pathogens.

Another key area of future research is the exploration of novel applications for Fe_2O_3 nanoparticles beyond their current use in controlling fruit rot diseases. Fe_2O_3 nanoparticles can be used for pest control, soil enhancement, and nutrient delivery. For instance, Fe_2O_3 nanoparticles could be explored as carriers for slow-release fertilizers. Additionally, Fe_2O_3 nanoparticles could be investigated for their potential to enhance plant growth. There is also a need to study the degradation of nanoparticles over time and their potential to bioaccumulate in food chains. Additionally, research into the potential toxicity of Fe_2O_3 nanoparticles to humans,

particularly through chronic exposure, is essential for ensuring the safety of this technology. Long-term toxicological studies could provide valuable data to inform safety guidelines and regulations.

By incorporating data on nanoparticle properties, environmental conditions, and biological interactions, predictive models could provide insights into the behavior and impact of nanoparticles in different agricultural contexts. These tools could also be used to optimize nanoparticle design and application methods, minimizing risks while maximizing benefits. There is a pressing need for the development of comprehensive regulatory frameworks. Future research could contribute to the establishment of standardized testing methods and safety assessments for Fe_2O_3 nanoparticles. Collaboration between scientists, regulatory agencies, and industry stakeholders is crucial for creating regulations that protect public health and the environment while fostering innovation. Additionally, research could explore the harmonization of regulations across different countries to facilitate the global trade of nanoparticle-based agricultural products.

Public engagement and education are also critical components of the future of Fe_2O_3 nanoparticle use in agriculture. As technology advances, it is important to address public concerns and misconceptions about nanotechnology. Future research could explore effective strategies for communicating the benefits and risks of using Fe_2O_3 nanoparticles in food production to consumers. This could include developing educational materials, conducting public awareness campaigns, and engaging with stakeholders through participatory approaches. By fostering a transparent dialogue between scientists, policymakers, farmers, and consumers, it is possible to build trust and support for the responsible use of nanotechnology in agriculture. Public engagement efforts should also consider the ethical implications of nanotechnology and address concerns related to food safety and social equity. The convergence of nanotechnology with other advanced technologies could lead to the development of innovative solutions for sustainable agriculture.

Another promising research direction is the exploration of the synergistic use of NPs with other technologies. Combining Fe_2O_3 nanoparticles with other antifungal agents could enhance their efficacy in controlling fruit rot diseases. Future research could investigate the potential of these combinations to provide broad-spectrum protection against phytopathogens while minimizing the risk of resistance. The development of synergistic nanoparticle-based

formulations could be very effective in agriculture. The exploration of alternative sources for the synthesis of Fe_2O_3 nanoparticles is another area for future research. Currently, the synthesis of nanoparticles often relies on chemical precursors that may not be sustainable or environmentally friendly (Saravanakumar et al., 2020). These green synthesis approaches could reduce the environmental footprint of nanoparticle production and make the technology more accessible to farmers in developing countries. Additionally, research could investigate the potential of using waste materials from other industries, such as iron-rich byproducts from mining or steel manufacturing, as raw materials for nanoparticle synthesis.

The prospects for Fe_2O_3 nanoparticles in agriculture are vast. The continued advancement of synthesis methods requires exploration of novel applications, assessment of long-term impacts, and development of regulatory frameworks. Additionally, public engagement and the integration of Fe_2O_3 nanoparticles with other emerging technologies offer exciting possibilities for enhancing agricultural productivity and sustainability. The successful adoption of Fe_2O_3 nanoparticles in agriculture will depend on continued collaboration between scientists, policymakers, industry stakeholders, and the public, working together to create a sustainable and resilient agricultural future. The use of Fe_2O_3 NPs in controlling fruit rot in peaches, particularly caused by *A. flavus*, has shown promising results. The pathogen *A. flavus* is known for its ability to produce aflatoxins, which are toxic and carcinogenic. In this study, the application of Fe_2O_3 NPs effectively controlled the incidence of rot in infected peach fruits. The data revealed a remarkable 72.5% decrease in mycelial growth of *A. flavus* when treated with these nanoparticles, highlighting their potent antifungal properties. This significant reduction is attributed to the nanoparticles' ability to break the cell membrane of the pathogen and diminish infection rates.

Characterization of Fe_2O_3 NPs was conducted using various analytical techniques to understand their properties and the presence of functional groups associated with the stabilization and capping of nanoparticles. These functional groups enhance the interaction between pathogen and NPs, leading to effective antifungal activity. XRD analysis confirmed the size range that contributes to their strong antimicrobial properties. Due to their small size, nanoparticles can penetrate fungal cells, disrupting their growth and activity. In addition to controlling the pathogen, Fe_2O_3 NPs also positively impacted the quality and composition of apples. The treated apples retained higher levels of soluble solids, which are essential for the fruit's sweetness and

overall flavor. The presence of ascorbic acid, an important antioxidant, was also maintained in the treated apples, further enhancing their nutritional value. The firmness of apples, an important quality attribute, was preserved with the use of Fe_2O_3 NPs. By extending the shelf life of apples, Fe_2O_3 NPs contribute to reduced post-harvest losses and improved marketability.

Overall, the effectiveness of Fe_2O_3 NPs in inhibiting *F. oxysporum* growth, combined with their impact on preserving the fruit's composition and sensory attributes, makes them a valuable tool in post-harvest management. The application of Fe_2O_3 NPs provides an environmentally friendly and efficient alternative to traditional fruit preservation methods, benefiting both producers and consumers. The application of Fe_2O_3 NPs to strawberries demonstrated significant improvements in fruit preservation, including reduced rot incidence and maintained sensory attributes. Fe_2O_3 NPs were applied to strawberries to evaluate their effectiveness in prolonging shelf life and preserving fruit quality. Nanoparticles helped reduce the incidence of rot and spoilage, which are common issues in strawberries. The data indicated that the nanoparticles could inhibit the growth of spoilage organisms and maintaining the fruit's freshness. The treated strawberries retained their firmness, which is a critical factor for consumer acceptance. Firmness is influenced by enzymatic activities and moisture loss, both of which were effectively managed by the nanoparticles. The treated strawberries exhibited reduced moisture loss, preventing weight loss and maintaining their visual appeal. In addition to firmness, the Fe_2O_3 NPs helped preserve the fruit's soluble solids and ascorbic acid content. Soluble solids are important for the fruit's flavor, while ascorbic acid contributes to its nutritional value. The maintenance of these attributes indicates significance of the use of Fe_2O_3 NPs. Overall, Fe_2O_3 nanoparticles can extend the shelf life of strawberries while preserving their quality.

In conclusion, Fe_2O_3 nanoparticles have demonstrated significant potential in managing fruit rot and preserving quality in peaches, apples, and strawberries. Their effectiveness in controlling pathogens, maintaining fruit composition, and extending shelf life highlights their value as a post-harvest treatment. The positive impact of Fe_2O_3 NPs on fruit quality and sensory attributes, combined with their environmental and practical advantages, underscores their potential for widespread application in fruit preservation. The findings of this study provide a foundation for future research in this area. By continuing to explore and optimize the use of Fe_2O_3 NPs, the agricultural industry can advance towards more sustainable and effective solutions for managing fruit diseases and enhancing post-harvest quality.

Conclusion And Further Prospective

The study on the mycosynthesis and application of Fe_2O_3 NPs has revealed substantial advancements in managing fruit rot diseases while preserving the quality and sensory attributes of peaches, apples, and strawberries. This research has showcased the effectiveness of Fe_2O_3 NPs as an innovative and eco-friendly solution for controlling fruit rot and extending shelf life, presenting a reliable alternative to traditional chemical treatments.

The benefits of Fe_2O_3 NPs extend beyond their effectiveness in controlling fruit rot. Their low toxicity and environmental friendliness make them a safer alternative to traditional chemical treatments, aligning with the growing emphasis on sustainable agricultural practices (Chauhan et al., 2018). Easy application and cost-effectiveness of Fe_2O_3 NPs offer practical benefits for fruit producers, potentially reducing post-harvest damages and increasing their availability (Arshad et al., 2020). The positive impact of Fe_2O_3 NPs on fruit quality, including higher levels of soluble solids, ascorbic acid, and firmness, underscores their potential to enhance consumer satisfaction and market value.

In conclusion, the study on Fe_2O_3 nanoparticles has provided valuable insights into their potential as a novel approach for managing fruit rot and preserving quality in peaches, apples, and strawberries. The demonstrated effectiveness of Fe_2O_3 NPs in controlling pathogens, maintaining fruit composition, and extending shelf life highlights their promise as a sustainable and efficient alternative to traditional treatments (Bin-Jumah et al., 2021). The prospects for Fe_2O_3 NPs in agriculture are promising. Addressing these future research directions and practical considerations will be essential for advancing post-harvest technology and promoting sustainable agricultural practices (Abdelbasset et al., 2021).

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ORIGINAL ARTICLE

Mycosynthesized Fe_2O_3 nanoparticles diminish brown rot of apple whilst maintaining composition and pertinent organoleptic properties

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Abstract

Aims: Iron oxide nanoparticles (Fe_2O_3 NPs) were mycosynthesized using *Trichoderma harzianum* and applied to control brown rot of apple. The influence of Fe_2O_3 NPs on the quality of fruit was also studied.

Methods and Results: Diseased apple fruits with brown rot symptoms were collected, and the disease-causing pathogen was isolated and identified as *Fusarium oxysporum*. To control this disease, mycosynthesis of Fe_2O_3 NPs was executed using *T. harzianum*. FTIR spectroscopy revealed the occurrence of stabilizing and reducing agents on NPs. X-ray diffraction (XRD) analysis determined their average size (17.78 nm) and crystalline nature. Energy-dispersive X-ray (EDX) showed strong signals of iron, and scanning electron microscopy (SEM) displayed a high degree of polydispersity of synthesized NPs. Foliar application of NPs significantly reduced brown rot and helped fruits to maintain biochemical and organoleptic properties. Firmness and higher percentage of soluble solids, sugars and ascorbic acid depicted its good quality.

Conclusion: Environment-friendly mycosynthesized Fe_2O_3 NPs can be effectively used to control brown rot of apple.

Significance and Impact of the Study: *Trichoderma harzianum* is a famous biocontrol agent, and the synthesis of NPs in its extract is an exciting avenue to control fungal diseases. Due to its nontoxic nature to human gut, it can be applied on all edible fruits.

KEYWORDS

ascorbic acid, brown rot, Fe_2O_3 NPs, *Fusarium oxysporum*, SEM, *Trichoderma harzianum*

INTRODUCTION

Apple belongs to rose family (Rosaceae), and it is the most widely grown species of genus *Malus*. Due to its sweet taste and great nutritional value, apple is considered as one of the favourite fruits of the world. Apple juice is considered as a rehydration source and is frequently consumed around the world. China, USA and Turkey are the major producers of apple. In Pakistan, apple is the major consumed fruit and apple cider is also used extensively. In temperate regions of Pakistan, a large quantity of apple fruit is produced (Wiehle et al., 2021).

Perishable fruits like apple are affected by various biotic and abiotic factors. Amongst these factors, fungi are the most devastating pathogens to cause yield losses of fruits and vegetables (Chohan et al., 2015). Studies have

described the involvement of fungi alone in more than half of biotic losses in fruit crops (Carmona-Hernandez et al., 2019). In addition, fungi affect all the members of our ecosystem (Fisher et al., 2012). Due to ultimate health benefits, there is a growing tendency towards planting more apple trees and control their diseases. A variety of fungi has been reported to cause apple diseases. *Penicillium expansum* has been described to cause blue mould disease of apple, whilst *Diplodia seriata* causes black rot of apple fruit (Crespo et al., 2018). Few opportunistic fungi like *Alternaria alternata* have been reported to cause postharvest decay on apple fruit (Jurick et al., 2014). Fungi have also been reported to cause black, brown and bitter rots of fruit, rust, fruit spot, core rot and powdery mildew of leaves and fruits (Casadevall, 2017).

According to several studies, the majority of the pathogens that cause these diseases are rapidly gaining resistance to currently available synthetic fungicides (Villa et al., 2017). Managing diseases of cultivated crops is comparatively easier than that of fruit trees. Fungicides application on tress is not cost-effective and needs more expensive equipment and labour. Moreover, application of fungicides results in the loss of predaceous mites. Fungicides are not health friendly and their application on fruits might cause different health ailments. These synthetic fungicides are hazardous and nonbiodegradable, and they quickly accumulate in soil, water and different plant parts and disturb the food chain (Villa et al., 2017). Scientists are working on the development of new antifungal agents, derived from biological agents, that are safe, environmentally friendly, cost-effective and easily degradable (Liaquat et al., 2021). Currently, many fungi are being used as biological control agents (BCAs). *Trichoderma* is the most used biocontrol organism (Saravanakumar et al., 2021), and it is famous for its efficient antimicrobial activity. More than 60% of bio-fungicides have been reported to be obtained from different strains of *Trichoderma* (Sood et al., 2020).

Nanotechnology is an emerging field and many phytopathologists have started working on using this technology to control plant diseases. Nanoparticles (NPs) have been observed to possess great antimicrobial potential and they are being used to control various plant diseases (Kato, 2011). Due to their very small size, NPs possess antifungal and antibacterial activities, and this ability helps scientists to use them as nontoxic fungicides. For the control of fruit diseases, scientists are mainly focusing on the synthesis of metal oxide NPs. These NPs are considered safe for humans. Different plant extracts and microbes such as algae, bacteria and fungi have been used to synthesize NPs, both intracellularly and extracellularly. In the past decade, different metals including silver, platinum, gold, zirconia, silica, zinc, iron and titanium have been used to synthesize NPs (Li et al., 2011). Nanofungicides are relatively new to the farmers and their application in the field is still evolving. Many studies have focused on the production of NPs in powder form. In this way, NPs can be easily carried to the field and applied on plants after mixing in water solvent (Ali et al., 2021).

The use of fungi for the biosynthesis of NPs is an emerging advancement in the field of plant pathology. It is termed as mycosynthesis of NPs, and it is resulting in considerable control of different plant diseases (Tyagi, 2016). Due to their nano-size, these NPs can penetrate the cells of pathogens and inhibit their growth (Alghuthaymi et al., 2015). For the mycosynthesis of NPs, fungi are subjected to mild stress and translated fungal proteins are used for the capping and reduction of NPs. As no toxic chemicals are used for the synthesis of these NPs, they are more suitable for biomedical and medicinal applications (Kanagasubbulakshmi & Kadirvelu, 2017). Mycosynthesis is even easier than bacterial NP synthesis, as it does not require specialized apparatus to achieve a clear filtrate from colloidal broth (Sastry et al., 2003).

Current study is based on the diagnosis and biocontrol of brown rot of apple. For the control of this disease, mycosynthesis of NPs was performed using *T. harzianum*. Before the analyses of their antifungal activity, synthesized iron oxide NPs were adjudged using scanning electron microscopy (SEM), Energy-dispersive X-ray (EDX), FTIR and X-ray diffraction (XRD). After the incidence of disease, fruit quality parameters were studied to see the effect of foliar application of biosynthesized Fe_2O_3 NPs on biochemical composition and organoleptic properties of apple.

MATERIALS AND METHODS

Sample collection

During apple fruiting season (February 2021 to May 2021), two surveys were carried out in apple orchards of District Mansehra (34°20′23.0″N 73°11′53.7″E). During both surveys, 30 diseased apple fruits with brown rot symptoms were collected, altogether. The diseased samples were then brought to the laboratory, for further testing.

Growth and examination of pathogen

For the isolation of pathogen, diseased fruits were surface sterilized using 70% ethanol and the diseased fruit areas (2–3 mm) were excised and placed in Petri plates containing potato dextrose agar (PDA) media. These plates were placed in an incubator at 25°C for 5–6 days. Petri plates

were then examined to observe the growth and morphology of isolated fungus.

Pathogenicity test

Koch's postulates were followed to confirm the pathogenicity of the isolated pathogen. For this purpose, the protocol of Ali et al. (2021) was adopted. Briefly, 10 healthy apple fruits were wounded with sterilized needle. Five of them were inoculated with 5 μl fungal conidial suspension (1×10^6 conidia per ml) and the remaining five healthy fruits were inoculated with 5 μl distilled water (control). After 4–5 days of infection, symptoms were observed, and the disease-causing pathogen was re-isolated on PDA. Fungus was allowed to grow for 5–6 days, and its morphology was compared with inoculated fungus. To ensure the accuracy of these results, the experiment was performed in triplicate.

Characterization of the isolated pathogen

For the microscopic identification of the isolated pathogen, a standard slide culture protocol was followed (Liaquat et al., 2021). Fungus mycelia were observed under the light microscope. For molecular characterization of the isolated pathogen, rDNA sequence was amplified and analysed. Using CTAB method, fungal DNA was isolated (Umesha et al., 2016). Forward ITS1 primer (TCCGTAGGTGAACCTGCGG) and reverse ITS4 primer (TCCTCCGCTTATTGATATGC) were used (White et al., 1990) to amplify rDNA sequences in polymerase chain reaction (PCR). A 25 μl reaction mixture comprised 0.5 μl of 2.5 mM dNTP, 0.25 μl of 5 U/μl *Taq* DNA polymerase enzyme, 1 μl of 50 ng/ml genomic DNA, 2.5 μl of 10× buffer and 1 μl of each primer of 10 pmoles/μl concentration. PCR was carried out at 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 57°C for 1 min and 72°C for 1 min. The PCR product was sequenced, and the obtained sequence was aligned with the NCBI database, using the BLASTn search program (http://www.ncbi.nlm.nih.gov). To see the evolutionary relationship, the resultant sequence was aligned with 15 related sequences using MUSCLE (Edgar, 2004) and a phylogenetic analysis was performed using MEGA 7 (Kumar et al., 2016).

Preparation of mycological Fe_2O_3 NPs

Trichoderma harzianum was obtained from the 'First Fungal Culture Bank of Pakistan' (FCBP), Punjab University, Lahore. Fungus was grown in potato dextrose broth media for 7 days at 28°C and filtered. Obtained fungal biomass was washed three times with distilled water. To get mycotoxins, fungus was subjected to mild stress conditions. For this purpose, 35 g of mycelial biomass was added in 150 ml of sterilized water and placed in a shaker incubator at 150 rpm and 40°C for 10 days. The biomass was then sonicated for 30 min and filtered. The pH of filtrate was maintained at 7.2 and it was used further for the synthesis of NPs.

In a beaker, a mixture of cell-free filtrate and 5 mM solution of iron chloride (1:1 ratio) was prepared for the synthesis of Fe_2O_3 NPs. The mixture was placed in a shaking incubator at 150 rpm and 40°C for 24 to 48 h. The colour change in the solution confirmed the reduction of iron oxide NPs. Mycosynthesized samples were centrifuged at 5000*g* for 20 min and the pellet was washed and placed in an oven at 40°C overnight. The NPs were calcined in a furnace for 2 h at 500°C.

Characterization of iron oxide nanoparticles

Before antifungal activity analyses, three replicates of mycosynthesized NP sample were characterized by the following methods:

Fourier transform infrared (FTIR) spectroscopy

Following a standard protocol of Ashokkumar and Ramaswamy (2014), FTIR spectroscopy was performed using a PerkinElmer Fourier transform infrared spectrometer. For FTIR analysis, scan range of 400–4000 cm^{-1} was used. Air-dried samples were produced in the powder form. Dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS90 was used to determine the nature of synthesized Fe_2O_3 NPs.

X-ray diffraction (XRD)

XRD was performed to estimate the size and shape of mycosynthesized NPs. X'Pert HighScore software was used, and the particle sizes of prepared iron oxide NPs were calculated by the following formula:

$$D = K\lambda / \beta\cos\theta,$$

where: D denotes the average crystalline size; K is constant (0.9 for crystal structure); λ is X-ray wavelength; β is the full width at the half maximum (FWHM) and θ is the diffraction angle.

Scanning electron microscopy (SEM) and energy-dispersive X-ray (EDX) analyses

To study the elemental composition and shape of NPs, SEM and EDX analyses were performed. For this purpose, the NP suspension was sonicated in distilled water for 5 min. A small amount of suspension was placed on conductive tape with a double carbon coating and dried under a lamp. A thermionic emission SEM system (VEGA3 TESCAN) was used for these analyses.

Mycelial growth inhibition assay, in vitro

Antifungal activity of Fe_2O_3 NPs was assessed using 'poisoned food method' (Liaquat et al., 2021). Fungus growth media (PDA) was amended with various concentrations of Fe_2O_3 NPs (2, 1.5, 1, 0.75, 0.5, 0.25 and 0.1 mg/ml). PDA media with no NPs served as control. Fungal discs (5 mm) of 5-day-old fungal culture were placed on NP-amended PDA media and incubated at 25°C for 1 week. The growth of fungus on control and NP-amended PDA media was measured in millimetres (Ali et al., 2021). The mycelial growth inhibition percentage was observed by the following equation:

$$\text{Growth Inhibition}\,\% = (C - T)\,/\,C \times 100,$$

where: C = average mycelial growth on control PDA media, T = mycelial growth on treated (NP-amended) media.

Disease control assay on fruit, in vivo

Based on in vitro assay, NPs with 1 mg/ml concentration and below were carried forward for in vivo antifungal activity analysis.

For in vivo antifungal activity analysis of mycosynthesized Fe_2O_3 NPs, 'wound inoculation method' was adopted (Ali et al., 2021). To follow this method, 18 healthy apple fruits were wounded with sterilized needle and inoculated with 5 µl fungal conidial suspension (1×10^6 conidia per ml). Inoculated fruits were placed in an incubator at 25°C for 2 days to let fungus penetrate and cause infection on apple fruit. Two days post inoculation; different concentrations of Fe_2O_3 NPs (1, 0.75, 0.5, 0.25 and 0.1 mg/ml) were sprayed (till run off) on randomly selected fruits. Three fruits were sprayed with each concentration of NPs and three fruits were sprayed with distilled water (control). All fruits were again placed in an incubator at 25°C. Seven days post inoculation, diseased area of each fruit was measured.

Study of biochemical and organoleptic properties

After 10 days of inoculation with fungus, the quality of NP-treated fruit, inoculated control fruit and untreated fruit was determined by the assessment of their biochemical and organoleptic properties. A digital refractometer (Pam Abbe model PA203X, MISCO Refractometer, Solon, OH) was used to quantify soluble solids of diseased apple fruits of all treatments. Other quality-related biochemical compounds including sucrose and total sugars were measured using a standard protocol of Helrich (1990a). To measure ascorbic acid in all fruit samples, the titration was carefully performed using 2,6-dichlorophenolindophenol sodium salt solution (Helrich, 1990b).

The effects of fungus inoculation on the firmness of apple in all treatments were determined using TA.XTplus Texture Analyser (Stable Micro Systems Ltd., Godalming, Surrey, UK). Firmness was tested by touching the surface and entering the probe into the sample to a depth of 10 mm by applying a force of 2 g (de Jesús Ornelas-Paz et al., 2018).

Statistical analysis

All the experiments were performed in three replicates and the data were analysed using the Microsoft Office Excel 121 (Microsoft). Univariate analysis of variance (ANOVA) was performed, followed by Tukey's least significant differences (LSD) method using Statistix 8.1 at $p < 0.05$.

RESULTS

Isolation and identification of fungus

In the field, typical brown rot symptoms (tan-brown and circular spots) were observed on apple fruit (Figure 1a). Disease-causing fungus was successfully isolated, and its rapid growth was observed on PDA media. In 5–7 days, whitish colonies covered the entire Petri plate (Figure 1b). Mycelia were observed to be entangled and woolly to cottony in texture.

Pathogenicity test successfully described the virulence of the isolated pathogen. On wounded control fruit, no symptoms were observed after 2 days (Figure 1c) and 5 days (Figure 1d) of wounding. On inoculated fruit, typical symptoms could be observed after 2 days of inoculation (Figure 1e), and these symptoms progressed further after 5 days (Figure 1f). These symptoms were similar to

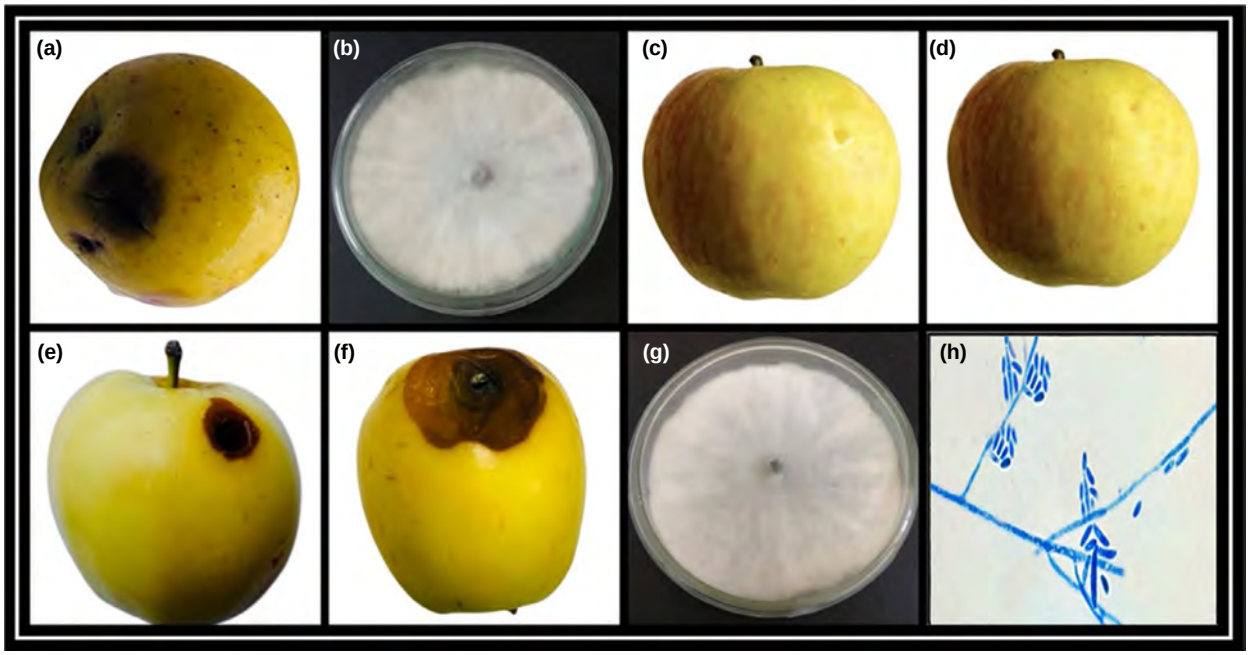

FIGURE 1 Apples with brown rot symptoms were collected from the field (a). The pathogen was isolated and grown on PDA media (b). Control apple fruit showed no disease symptoms after 2 (c) and 5 days (d), whilst inoculated fruit exhibited disease symptoms after 2 (e) and 5 days (f) of inoculation. The pathogen was re-isolated on PDA (g). Microsopic study efficiently helped to observe mycelial morphology (h). Scale bar: (h) = 10 μm

the field symptoms. Fungus was isolated from these self-inoculated apple fruit, and it displayed similar morphology with the inoculated fungus (Figure 1g). These findings fulfilled Koch's postulates and suggested the involvement of isolated and re-inoculated fungus in brown rot of apple. Microscopic studies of this fungus revealed macroconidial septation (Figure 1h).

rDNA sequence analysis and phylogenetic tree

BLAST alignment of resultant PCR sequence demonstrated 100% similarity with *Fusarium oxysporum* strain FO_11 (Accession no. MT447552.1). This result verified our morphological and microscopic observations, which also described disease-causing pathogen to be *F. oxysporum*. Phylogenetic tree also confirmed these results, and the isolated pathogen was present in the same clade with *F. oxysporum* (Figure 2).

Characterization of mycosynthesized iron nanoparticles

FTIR analysis

FTIR spectrum showed specific peaks to represent different functional groups. Amongst these, peak at 3251.25 represented N-H stretching (secondary amine group), 3080.71 depicted O-H stretching (alcohol), 2181.60 demonstrated C=C bending (alkyne), 2166.69 showed N=N=N stretching (azide), 1609.64 represented C-H bending (aromatic compounds) and 854.52 cm^{-1} presented C-Cl stretching (alkene) (Supplementary Figure S1). All these compounds act as capping and reducing agents for the synthesis of NPs.

XRD analysis

XRD pattern of synthesized NPs showed distinct diffraction peaks at 2θ 22.97^0, 30.48^0, 58.66^0, 65.06^0 and 67.70^0, corresponding to (013), (216), (2011), (034) and (221) planes of hexagonal with P3 space group, indicating iron oxide (Supplementary Figure S2). The planes of XRD pattern are in good agreement with JCPDS (Joint Committee on Powder Diffraction Standards) number 01-076-1821. JCPDS provides database to characterize powder. The average size of mycosynthesized iron oxide NPs was calculated to be 17.78 nm and they were crystalline in nature.

SEM and EDX analyses

Iron salts were biologically reduced to form Fe_2O_3 NPs by *T. harzianum*. SEM of Fe_2O_3 NPs revealed high

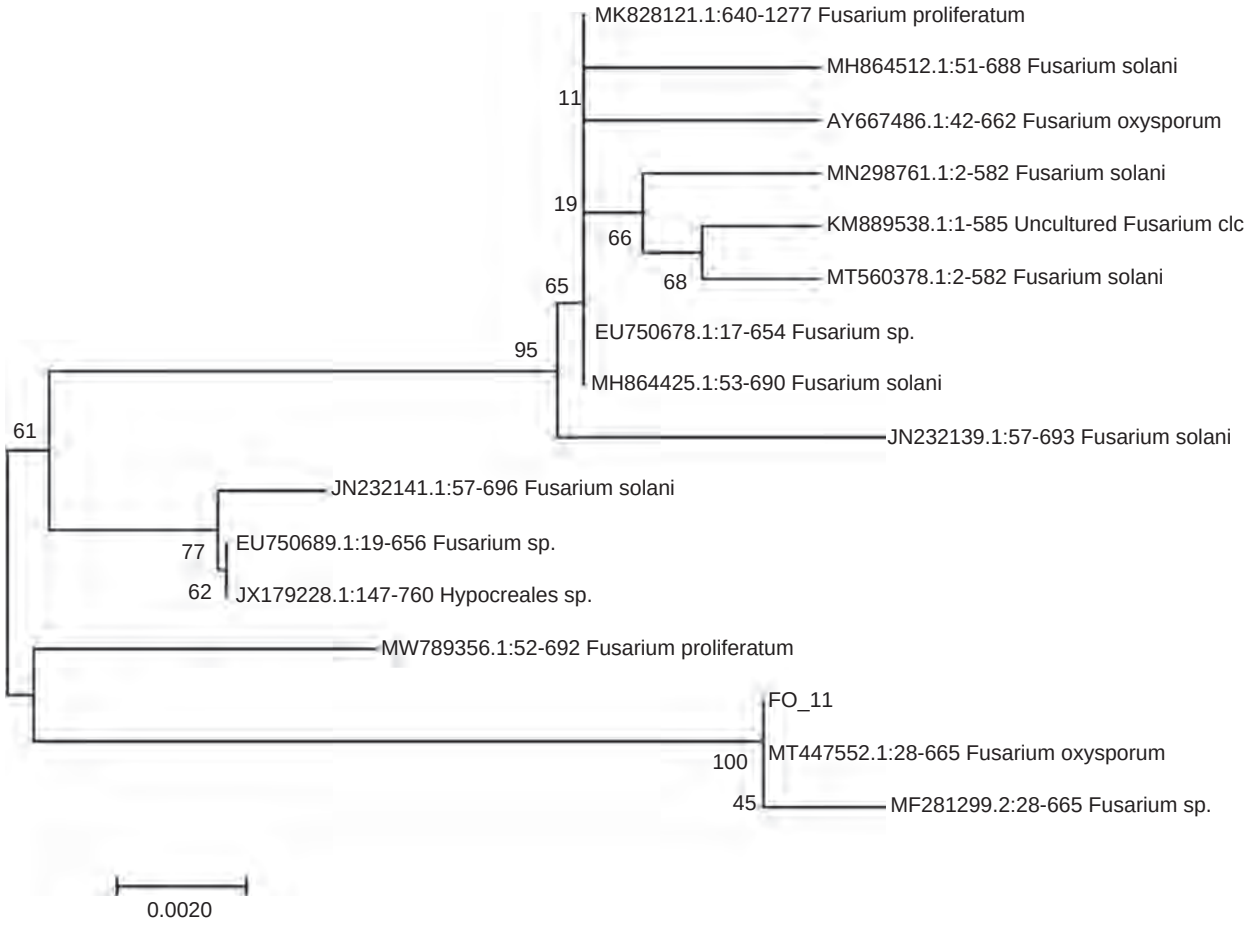

FIGURE 2 Evolutionary relationaship of isolated *Fusarium oxysporum* strain FO_11, using 15 related GenBank sequences

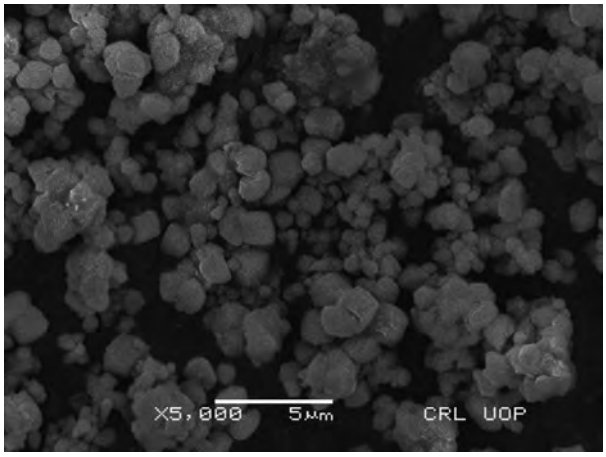

FIGURE 3 Scanning electron micrograph of mycosynthesized Fe_2O_3 nanoparticles. Scale bar = 5 microns

degree of polydispersity, and they were crystalline and spherical in shape (Figure 3). The EDX spectrum of NPs significantly showed strong signals of iron (74.38%) and low signal of C (3.20%), O (15.39%) and Cl (7.03%) (Supplementary Figure S3).

Antifungal activity of iron oxide nanoparticles, in vitro

Different concentrations of mycosynthesized Fe_2O_3 NPs showed variable growth inhibition of fungal mycelia (Figure 4). The highest growth reduction was exhibited by 1 mg/ml concentration (65.4%). A reduction in mycelial growth inhibition was observed at lower concentrations, that is, 0.75 mg/ml concentration (56.6%), 0.5 mg/ml concentration (12.4%), 0.25 mg/ml concentration (7.84%) and 0.1 mg/ml concentration (6.62%) (Table 1). Interestingly, very high concentration (1.5 mg/ml and 2 mg/ml) of NPs was also not effective for disease control. Previous studies have also described the similar behaviour of NPs against *F. oxysporum* where higher concentrations of NPs were not effective in mycelial growth inhibition (Akpinar et al., 2021). Very high concentrations of NPs activate diverse stress compensation pathways and lower the oxidative stress on fungi, and they can partially survive against nanofungicides (Kotzybik et al., 2014).

Based on these results, NPs with 1 mg/ml concentration and below were carried forward for in vivo antifungal activity analysis.

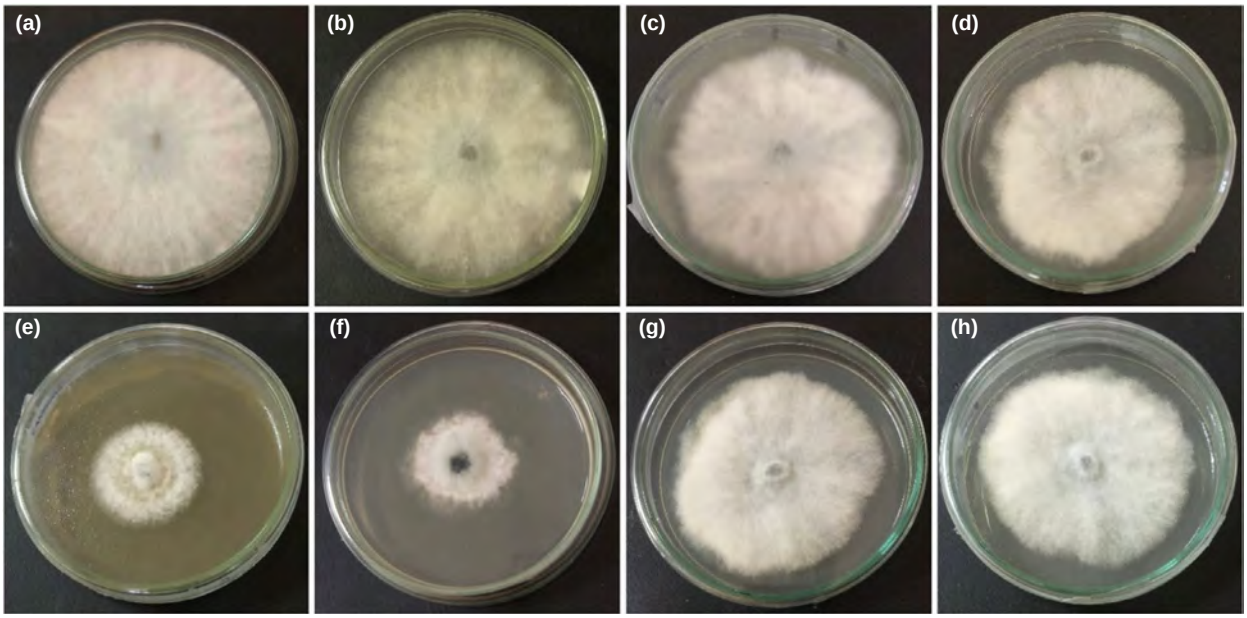

FIGURE 4 Effect of nanoparticles on the growth of *Fusarium oxysporum* strain FO_11. Seven different concentrations of NPs were compared with control (a). Fungus was grown on Petri plates amended with NP concentrations of 0.1 mg/ml (b), 0.25 mg/ml (c), 0.5 mg/ml (d), 0.75 mg/ml (e), 1 mg/ml (f), 1.5 mg/ml (g) and 2 mg/ml (h)

TABLE 1 Mycelial growth inhibition, in vitro, at different concentrations of mycosynthesized NPs

Concentration (mg/ml)	Growth inhibition (%)
0.1	6.6 ± 1.3^{e}
0.25	7.8 ± 1.1^{de}
0.5	11.2 ± 1.3^{c}
0.75	56.6 ± 2.9^{b}
1.0	65.7 ± 3.6^{a}
1.5	9.2 ± 1.2^{cd}
2.0	8.5 ± 1.1^{d}

Values are described as mean and ±denotes standard error. Dissimilar alphabets represent significantly different values from each other.

Antifungal activity of iron oxide nanoparticles, in vivo

Inoculation of *F. oxysporum* resulted in typical brown rot symptoms. Application of Fe_2O_3 NPs successfully controlled brown rot disease of apple and the disease area was decreased (Figure 5). Increasing concentration of NPs displayed reduced diseased area (Table 2). The maximum disease control was exhibited at 1.0 mg/ml concentrations of Fe_2O_3 and 91.5% less diseased area was observed than control (Table 2).

Biochemical and organoleptic changes

The inoculation of fungus severely affected the quality of fruit. Assessment of their biochemical and organoleptic characteristics helped us to understand the useful role of Fe_2O_3 NPs in mycelial growth inhibition and ultimately influencing the quality of inoculated fruit (Table 2). Application of Fe_2O_3 NPs helped to maintain higher soluble solids of diseased apple fruit. These higher amounts of soluble solids resulted in increased firmness of fruit. Higher soluble solids are related with the firmness and good quality of fruit (Peck et al., 2006). Analysis of diseased fruit also revealed decreased sucrose and total sugar contents in control fruit. Application of NPs helped plants to accumulate higher sucrose and total sugar contents. Sucrose is an important sugar which describes the sweet taste of apple and depicts its good quality (Cichowska et al., 2020). Many studies have described that the higher levels of sugars and lower percentage of acids indicate good quality of apple fruit (Sudheeran et al., 2018). In this study, application of NPs also influenced diseased fruits to maintain higher contents of ascorbic acid. In apple, ascorbic acid exhibits strong antioxidant properties to protect DNA and proteins from the damage of free radicals (Gorkom et al., 2019).

DISCUSSION

Brown rot is a common disease of many fruits, and it can destroy half or more of the produce. This disease has been reported to affect different plant parts including fruits, shoots and flowers (Umesha et al., 2016). In this study, brown rot disease of apple has been diagnosed and *F. oxysporum* has been identified as the disease-causing pathogen.

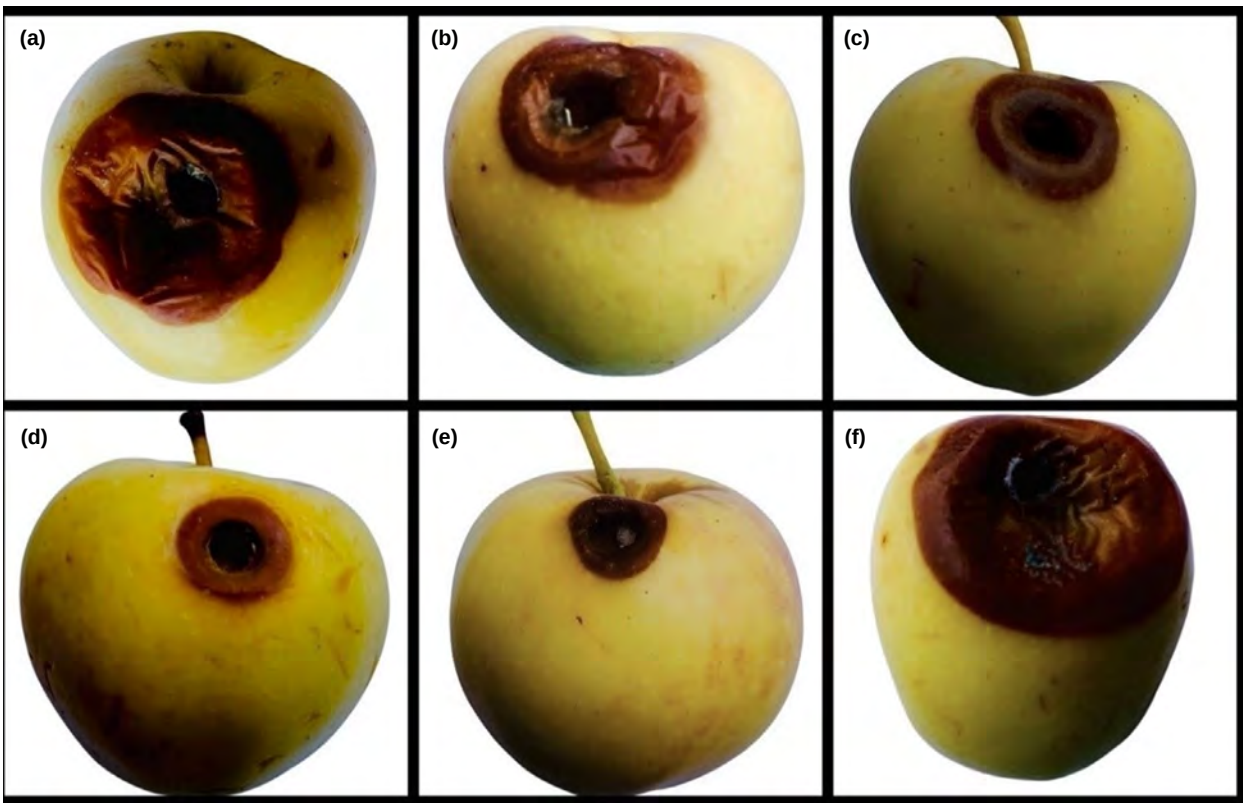

FIGURE 5 Apple fruits were infected and treated with 0.1 mg/ml (a), 0.25 mg/ml (b), 0.5 mg/ml (c), 0.75 mg/ml (d) and 1 mg/ml (e) concentration of NPs. No NPs were applied on control fruit (f)

TABLE 2 Biochemical and organoleptic changes in diseased fruit at different NP concentrations

NP conc. (mg/ml)	Diseased area (mm^2)	Soluble solids (%)	Sucrose (%)	Total sugars (%)	Ascorbic acid (mg/100 g)	Firmness (N/cm^2)
0.1	1406 ± 0.7^{b}	13.1 ± 1.5^{c}	2.2 ± 0.9^{c}	10.7 ± 0.8^{b}	7.2 ± 1.3^{c}	179.1 ± 15.5^{c}
0.25	780 ± 0.9^{c}	13.2 ± 1.1^{c}	2.2 ± 1.2^{c}	11.8 ± 1.1^{ab}	7.2 ± 1.1^{c}	189.2 ± 17.7^{bc}
0.5	399 ± 1.5^{d}	14.4 ± 0.8^{bc}	2.4 ± 0.7^{ab}	11.7 ± 1.3^{ab}	8.2 ± 2.3^{bc}	209.5 ± 14.2^{b}
0.75	225 ± 1.6^{e}	15.4 ± 2.1^{ab}	3.4 ± 0.8^{b}	12.2 ± 1.7^{a}	9.4 ± 1.3^{bc}	219.3 ± 18.7^{b}
1.0	132 ± 2.1^{f}	15.1 ± 1.3^{ab}	3.5 ± 1.1^{b}	12.5 ± 0.9^{a}	10.2 ± 1.4^{ab}	288.4 ± 22.1^{a}
Control	1554 ± 1.9^{a}	13.2 ± 0.9^{c}	2.0 ± 1.2^{c}	10.8 ± 1.2^{b}	7.1 ± 1.4^{c}	145.6 ± 14.3^{d}
Untreated fruit	0	15.2 ± 0.9^{ab}	3.4 ± 0.8^{b}	12.4 ± 1.1^{a}	10.1 ± 0.8^{ab}	292.1 ± 18.1^{a}

Values are described as mean and ±denotes standard error. Dissimilar alphabets represent significantly different values from each other.

Biological control is an eco-friendly strategy to control plant diseases (Cook, 2007). Various plant growth-promoting fungi (PGPF) have been successfully used for the control of plant diseases (Hossain et al., 2014). These biological agents adopt single as well as multiple action mechanisms to destroy phytopathogens. These mechanisms include the secretion of hydrolytic enzymes (Benizri et al., 2001), synthesis of hydrogen cyanide (Compant et al., 2005) and induced systematic resistance (Siddiqui & Shaukat, 2002). *T. harzianum* is known as an important biocontrol agent to inhibit the growth of fungi, oomycetes and bacteria through different mechanisms, which are activated in pathogens. *T. harzianum* irritates phytopathogens by challenging nutrients and space, releasing antibiotics and inducing systemic resistance of plants (Gupta et al., 2019).

For the synthesis of NPs, different organisms such as bacteria, fungi and plants are being used to reduce metal salts and form NPs (Azmath et al., 2016). Biological reduction of metals is a clean, nontoxic and environmentally friendly approach (Banu & Balasubramanian, 2014). Extracts of the PGPF play important role in surface coating of NPs with different enzymes, proteins, hormones and other metabolites. These characteristics impart different

properties to them. Capping of NPs also stops agglomeration of the NPs and play a major role in controlling their size and shape (Ahmad et al., 2003).

In this study, FTIR helped us to recognize capping and reducing agents. FTIR is a useful technique for the recognition of chemical bonds (Vahabi et al., 2011; Bilesky-José et al., 2021). XRD analysis of this study revealed the small size (17.78 nm) and crystalline nature of mycosynthesized NPs. Previous studies have proved that the small size and crystalline nature of NPs provide them strong antimicrobial properties (Santos et al., 2019). Nanoparticles with small size (5–20 nm) work as excellent antimicrobial agents (Hoag et al., 2009). In EDX spectrum of this study, high peaks of iron (Fe) and oxygen (O) were observed, which characterize the oxide form of iron oxide NPs (Shahwan et al., 2011).

In this study, mycosynthesized Fe_2O_3 NPs controlled the growth of *F. oxysporum*, both in vitro and in vivo. NPs display antibacterial (Shin et al., 2019), antifungal (Ali et al., 2021) and antioxidant activities (Saravanakumar et al., 2020a). They are very effective in mycelial growth inhibition as they disrupt the sheath of microbes and produce reactive oxygen species (ROS). The NPs after rupturing of microbial membrane move through nanometre pores, without any opposition (Jadhav et al., 2011). Similarly, iron oxide NPs display biocidal activities by disrupting cell membrane of fungus and enhancing the penetrability potential of the microbial membrane (Sawai et al., 1998). Due to the production of hydrogen peroxide and superoxide radicals, NPs efficiently display antimicrobial activity (Touati, 2000) and increase shelf life of fruits and vegetables (Saravanakumar et al., 2020b). Production of ROS impose stress, which causes disruption in electronic and ionic transport chains due to higher affinity of iron NPs with the membrane of microbes. The hydrogen peroxide reacts with ferrous ion to produce hydroxyl radicals. The assembly of free radicals creates an important intracellular stress, including necrosis of microbial cell (Plachtová et al., 2018).

Application of NPs inhibited fungal growth and helped apple fruit to maintain fruit quality. Higher percentage of soluble solids and sugars give good taste to apple and depict its food quality (Cichowska et al., 2020). Due to its antioxidant nature, presence of ascorbic acid also indicated the positive influence of NP on apple fruit (Gorkom et al., 2019). NP-treated fruits were firmer and showed less disease symptoms. Due to its perishable nature, good organoleptic and physical properties define the market value of apple fruit. Its good taste and nutritional values are dependent on balanced composition of phenolic compounds, sugars, organic acids and triterpene compound (Musacchi & Serra, 2018).

Mycosynthesis and application of NPs is a simple, reliable and eco-friendly way to control fruit rot diseases. Mycotoxins of *T. harzianum* can effectively do the coating and stabilization of NPs. Synthesized Fe_2O_3 NPs exhibit excellent antifungal activity and control the deterioration of apple fruit. The results confirmed that the optimum concentration (1.0 mg/ml) of Fe_2O_3 NPs can be effectively used to control brown rot in apple.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this article.

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Spherical Fe_2O_3 nanoparticles inhibit the production of aflatoxins (B_1 and B_2) and regulate total soluble solids and titratable acidity of peach fruit

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ABSTRACT

Aflatoxin is a group I carcinogen and causes significant public health and food safety risks, throughout the world. This study was carried out to assess the levels of aflatoxin contamination in diseased peach (*Prunus persica* L.) fruit and their control using myco-synthesized iron oxide nanoparticles (Fe_2O_3 NPs). Diseased peach fruit were diagnosed to be infected with *Aspergillus flavus*. The isolated pathogen was cultured under UV light (365 nm) and exposed to ammonium hydroxide (31 %) vapors, which confirmed its ability to produce aflatoxin. For the control of this disease, Fe_2O_3 NPs were synthesized in the filtrate of a biocontrol fungus (*Trichoderma harzianum*) and characterized before analyzing their potential in disease control. FTIR spectrum described the presence of capping and reducing agents (secondary amines, alcohol, alkyne and aromatic compounds) on the surface of Fe_2O_3 NPs. X-ray Diffraction (XRD) described the crystalline size (7.78), while the spherical shape of Fe_2O_3 NPs was described by the SEM analysis. The EDX spectrum indicated the successful formation of Fe_2O_3 NPs by showing strong signals of iron (74.38 %). All concentrations displayed mycelial growth inhibition, in vitro and the greatest growth reduction (65.4 %) was observed at 1 mg/ml concentration of NPs. At the same concentration of Fe_2O_3 NPs, significant control of fruit rot of peach was also observed, in vivo. Treatment of Fe_2O_3 NPs maintained higher soluble solids, sucrose, total sugar, ascorbic acid, titratable acidity and firmness of peach fruit. Diseased fruit were further investigated for the presence and detection of aflatoxins. All three methods viz. thin layer chromatography (TLC), enzyme-linked immunosorbent assay (ELISA) and high-performance liquid chromatography (HPLC) confirmed a higher production of aflatoxins in control plants, while this production was significantly reduced in Fe_2O_3 NPs-treated peach fruit.

1. Introduction

Filamentous fungi have been reported to produce different types of secondary metabolites. Among these, mycotoxins are famous for causing diseases in vertebrate animals (Zain, 2011). Mycotoxins are ingested by human and they can also be absorbed through skin. Major mycotoxin producing fungal species include Aspergillus, Penicillium and Fusarium. These fungi are present in the field and infect foods during storage (Sweeney and Dobson, 1998). Later, these contaminated food commodities are consumed by human and cause a variety of diseases (Reddy et al., 2011). Though hundreds of mycotoxins have been identified in different studies, only few of them have been reported harmful for human beings. The most dangerous mycotoxins include aflatoxins, zearalenone, nivalenol/deoxynivalenol (DON), ochratoxin, patulin and fumonisins (Cinar and Onbaşı, 2019). At different pre-harvest and postharvest stages, different molds have been reported to produce mycotoxins. These mycotoxins might cause digestive problems in humans and other livestock. Quickly after ingestion, food-borne mycotoxins may cause severe illness. For last many years, hundreds of mycotoxins have been identified, and about 10–12 mycotoxins have been exclusively

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studied due to their significant impact on human health and their frequent occurrences in the food (Omotayo et al., 2019).

Aflatoxins are carcinogenic in nature and they are mostly produced by Aspergillus species (Gourama and Bullerman, 1995). *Aspergillus flavus* and *A. parasiticus* are the most commonly reported Aflatoxin producing species, which produce this mycotoxin in dry fruits, spices, corn and rice (Niessen et al., 2018). Aflatoxins have also been reported to be produced in grains (Manjula et al., 2009), milk and dairy products (Prandini et al., 2009). Aspergillus species produce aflatoxin B_1 (AFB_1) and aflatoxin B_2 (AFB_2) in contaminated food items. Few studies have also described the production of aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2) by *A. parasiticus*, though *A. flavus* has not been reported to produce AFG_1 and AFG_2 (Ting et al., 2020). All these four types of aflatoxins are harmful and cause different human ailments. Among these, AFB_1 has been reported to be the most harmful and it is responsible for more than 75 % of all aflatoxins related contaminations (Benkerroum, 2020). AFB_1 is extremely carcinogenic and listed as "Group I carcinogen" (Wild and Gong, 2010). Due to its presence in food items, more than 4.5 billion people have been reported to be exposed to AFB_1 (Rushing and Selim, 2019). Annually, 155,000 cases of AFB_1-induced human hepatocellular carcinoma (HCC) are being reported (Kew, 2013). In food and agricultural products, *A. flavus* is the major aflatoxin producing fungi. Majority of *A. flavus* strains are highly aerobic and cause diseases on different economically important crops. *A. flavus* also attacks harvested crops and produce aflatoxins in the post-harvest period (Yang et al., 2022). Aflatoxin B_1 causes hepatocellular carcinoma and immunosuppression in humans and livestock. Once produced, these aflatoxins cannot be eliminated from the food items (Langeswaran et al., 2012).

For the last few decades, nanotechnology is being frequently employed for the control of crop diseases. For the synthesis and reduction of nanoparticles, different biotic materials (plant extracts, fungi, bacteria) are used (Malaikozhundan et al., 2022a; Vinothini et al., 2023). Several studies have described the low-cost and efficient production of nanoparticles and their production in controlling pre-harvest and postharvest diseases (Malaikozhundan et al., 2022b; Farhana et al., 2023). Along with plant extracts, several useful fungi and bacteria are being used for the synthesis of nanoparticles. Different analyses including FTIR, XRD, SEM, EDX and TEM etc. assist the characterization of nanoparticles and determine their size and shape (Zubair et al., 2022). Though all livings factors are efficient for the capping and reduction of nanoparticles, fungi are considered best, in this regard. Along with a variety of proteins and polysaccharides, NADH-dependent reductase enzyme of fungi has also been reported to reduce metals, for the formation of nanoparticles (Martín et al., 2005; Talekar et al., 2016).

Present study was designed to identify and control fruit rot pathogen of peach. For better understanding of fruit rotting, different fruit quality parameters and the production of aflatoxins was studied. This is the first study in Pakistan which has described the production of Aflatoxins in fruit and provided its management strategies. To advise environment-friendly control of this disease, nano-fungicides were synthesized in the filtrate of a famous biocontrol fungus (*Trichoderma harzianum*) and used for the control of fruit rot of peach. This study will open new avenues for environment-friendly control of biotic and abiotic diseases of different fruits and vegetables.

2. Materials and methods

2.1. Sample collection and isolation of pathogen

Diseased samples of peach fruit showing fruit-rot symptoms were collected from Swat District (35.2227° N, 72.4258° E). Two different surveys were conducted and 24 diseased fruit samples were collected in sterilized bags and brought to the laboratory. For the growth of fungus, Sabouraud dextrose agar (SDA) media was prepared. SDA (39 g) was mixed in 1000 ml water and autoclaved. After sterilizing peach fruit with 70 % ethanol, the infected part was aseptically transferred to Petri plates containing SDA and incubated at 28 °C. Pure isolates were obtained after 3–4 days and mycelial morphology was observed, microscopically and macroscopically. Morphological and microscopic traits of isolated pathogen were compared with the compendium of phytopathogenic fungi (Barnett and Hunter, 1972). For DNA sequence analysis of isolated pathogen, internal transcribed spacer (ITS) regions and intervening 5.8S rDNA was amplified and sequenced (White et al., 1990). Further, the evolutionary and phylogenetic analyses were carried out by using MEGA (version 7). By applying the Neighbor-joining method, the evolutionary history was inferred of all closely related sequences of fungal strains. To check the pathogenicity of isolated strain, Koch's postulates were followed (Fredericks and Relman, 1996).

2.2. Determination of aflatoxins production by cultural methods

Isolated pathogen was identified as *Aspergillus flavus*, which is one of the most important aflatoxin producing pathogens. As all the strains of *A. flavus* do not produce aflatoxins, two cultural methods (UV test and exposure to NH_4OH) were carried out for preliminary detection of aflatoxins production (Saito and Machida, 1999).

In UV test, the SDA Petri plate containing a single colony of pure *A. flavus* strain, was inversely placed and observed under UV light (365 nm). The gray or black boundary around colony margins provided a rough idea about aflatoxigenic nature of the strain; in contrast, the white boundary depicted the non-aflatoxigenic properties of mold.

In ammonium hydroxide (NH_4OH) vapor test, 2–3 drops of 31 % NH_4OH were placed in the lid of Petri plate and the lower portion of the plate (containing fungal colonies) was inverted on its lid. Results were drawn, based on the fact that the changes in color from yellow to pink or plum-red indicates aflatoxin synthesizing ability of the strain (Abbas et al., 2004).

2.3. Preparation of mycological Fe_2O_3 NPs

Trichoderma harzianum was grown in potato dextrose agar (PDA) broth media at 28 °C. After 7 days of shaking, fungal biomass was obtained. For the secretion of proteins and secondary metabolites from *T. harzianum*, obtained biomass (35 g) was deliberately subjected to mild stress conditions. The fungal biomass was transferred to water (150 ml) and incubated at 40 °C. After 10 days of shaking at 150 rpm, the biomass was filtered and its pH was maintained at 7.2. This filtrate was mixed (1:1 ratio) with the solution of $FeCl_3$ to reduce iron salt and synthesize Fe_2O_3 NPs. After 24–48 h of shaking, the change in color was observed, which indicated the reduction of Fe_2O_3 NPs. Then the mixture was centrifuged and the resultant pellet was placed at 40 °C, overnight. The calcination of died pallet was performed at 500 °C for 2 h.

2.4. Characterization of Fe_2O_3 NPs

Successful synthesis of Fe_2O_3 NPs was determined by studying their following characteristics:

2.4.1. Fourier transform infrared (FTIR) spectroscopy

Following the protocol of Griffiths (1983), the FTIR spectroscopy was performed. A PerkinElmer spectrometer was used to determine the nature of functional groups, present on the surface of NPs (Malaikozhundan et al., 2017a).

2.4.2. X-ray diffraction (XRD)

Size and shape of Fe_2O_3 NPs was determined, using XRD and X'Pert HighScore software described the size of NPs (Malaikozhundan et al., 2017b).

2.4.3. Scanning electron microscopy (SEM) and energy-dispersive X-ray (EDX) analyses

The shape and elemental composition was determined using SEM and EDX analyses (Malaikozhundan et al., 2020). Calcined Fe_2O_3 NPs were suspended in water and positioned on the double carbon coated conductive tape. The samples were dried and analyzed using a standard SEM system (VEGA3 TESCAN).

2.5. Fungal growth assay, in vitro

Performance of different concentrations of Fe_2O_3 NPs in reducing mycelial growth was determined with 'poisoned food method' (Ferhout et al., 1999). In an in vitro experiment, 15 ml PDA media was poured in Petri plates with variable amounts of Fe_2O_3 NPs (0.1 mg/ ml, 0.25 mg/ ml, 0.5 mg/ ml, 1 mg/ ml, and 1.5 mg/ ml). In control treatment, no NPs were added in the PDA media. Once solidified, all Petri plates were inoculated with fungal discs of 5 mm diameter. These fungal discs were placed in the center of each Petri plate and allowed to grow at 25 °C. After a week, mycelial growth in NPs treated (T) and control (C) Petri plates was measured and mycelial growth inhibition was determined in percentage.

2.6. Disease assay on peach fruit

Different amounts of Fe_2O_3 NPs (0.1 mg/ ml, 0.25 mg/ ml, 0.5 mg/ ml, 1 mg/ ml, and 1.5 mg/ ml) were sprayed to control fruit rot of peach. Initially, 18 healthy peach fruit of equal size were obtained and 5 μL of conidial suspension (1×10^6 conidia ml^{-1}) was injected. To assure infection, these fruit samples were placed at room temperature. After 48 h, three infected fruit were sprayed, till run-off, with each above mentioned amount of Fe_2O_3 NPs. Three fruit (control) were not treated with NPs and sprayed with distilled water, only. All treated and control fruit were incubated and the diseased area was measured after one week of incubation.

2.7. Detection of aflatoxin in diseased fruit

2.7.1. Thin layer chromatography (TLC)

For the qualitative analysis of aflatoxin, TLC technique was used (Hoeltz et al., 2010). From each treatment, 50 g of fruit sample was blended in distilled water (50 ml) and acetone (200 ml). The mixture was filtered and 150 ml of filtrate was taken as a representative sample for analysis. The filtrate was then spotted (two spots of 1 uL and 5 uL) on TLC plate. The lower 2 cm part of silica plate was scratched and plate was developed in CH_3Cl - acetone (85:15) solution. After development, TLC plate was examined under long wave UV light (150–350 nm).

2.7.2. Enzyme-linked immunosorbent assay (ELISA)

Veratox 8030® aflatoxin test kit was used for ELISA. Peach fruit sample (50 g) was shaken in 70 % methanol (250 ml) for 1 h at 200 rpm. The filtrate was obtained and its pH was maintained (6–8). Following kit instructions, the conjugate was taken in the red-colored well and mixed with the calibration standard solution and the extract. The mixture was incubated in the antibody coated well and rinsed with deionized water. The substrate solution was incubated in the antibody coated well for 3 min, and after adding stop solution, the reading of the solution was obtained.

2.7.3. High performance liquid chromatography (HPLC)

For HPLC, grinded fruit samples (50 g) were mixed in 80 % methanol (100 ml). The sample mixture was blended for 2 min, filtered through Whatman No. 4 filter paper and placed in air-tight amber vials. Immunoaffinity columns (IACs) were used for sample cleaning. For this purpose, filtered extracts (2 ml) were mixed with PBS (14 ml) and dispensed in IACs. For standard cleaning, a flow rate of 1 drop sec^{-1} was maintained. For the washing of columns, 20 ml of PBS (pH 7.4) was passed through at 5 ml min^{-1} flow rate and air dried, rapidly. Aflatoxins were eluted from vials with methanol (Daradimos et al., 2000).

2.8. Fruit quality attributes

Quality of infected peach fruits was compared in different treatments. Fruit pulp (300 g) from each treatment was homogenized with a blender, filtered through Whattman Filter paper No. 1, and centrifuged at 3000 rpm for 15 min. Obtained clear juice was used to measure the following quality parameters:

2.8.1. Total soluble solids (TSS)

For the measurement of total soluble solids, a standard protocol was followed (Ying et al., 2005). From each treatment, 100 ml of juice filtrate(s) were used to determine the percentage of TSS, by using a hand refractometer.

2.8.2. Titratable acidity

Following the protocol of Lobit et al. (2002), a clear filtrate of inoculated and un-inoculated peach fruit was used to determine total titratable acidity (TTA). After titration with NaOH (0.1 N), phenolphthaline was used as an indicator. The percentage of acidity was calculated as mg citric acid per 100 g fresh weight of peach fruit, by using the following equation:

$$\text{Acidity}\% = \text{ml of NaOH used} \times \text{N of NaOH (0.1)} \times 0.064/\text{Sample volume of peach (ml)}$$

2.8.3. Ascorbic acid

Ascorbic acid contents were determined by following the protocol of Matias et al. (2016). Filtered juice was diluted with 3 % metaphosphoric acid and titrated against 2, 6-dichlorophenol indophenol (until a steady light pink color developed), and ascorbic acid contents were determined in mol kg^{-1}.

2.8.4. Firmness

Following a standard protocol (Wang et al., 2013), firmness of each fruit was determined using TA.XTplus texture analyzer (Stable Micro Systems Ltd., Godalming, Surrey, UK).

2.9. Statistical analysis

Each experiment was executed in triplicate and the experimental data was statistically analyzed by following one-way ANOVA. The significance of difference between treatments was tested by least significance difference (LSD) post hoc test ($\alpha = 0.05$). All data was expressed as mean ± standard error.

3. Results and discussion

3.1. Isolation of disease-causing pathogen

Diseased peach fruit were brought to the laboratory (Fig. 1A) and disease-causing pathogen was isolated in Petri plates. After one-week, greenish mycelia with white edges were observed on PDA media (Fig. 1B). Light microscopy revealed clear conidial heads on hyaline hyphae. Moreover, flask-shaped or elliptical phialides were also observed (Fig. 1H). All these features indicated this pathogen to be *Aspergillus flavus* (Zubair et al., 2022). Koch's postulates successfully confirmed the pathogenicity of the isolated pathogen (Fig. 1D–F). Sequence alignment displayed 100 % similarity with the isolate of *A. flavus* (Accession No. OP218632.1). Phylogenetic analysis also confirmed the close relationship of the sequence of isolated pathogen with *A. flavus* (Fig. 2).

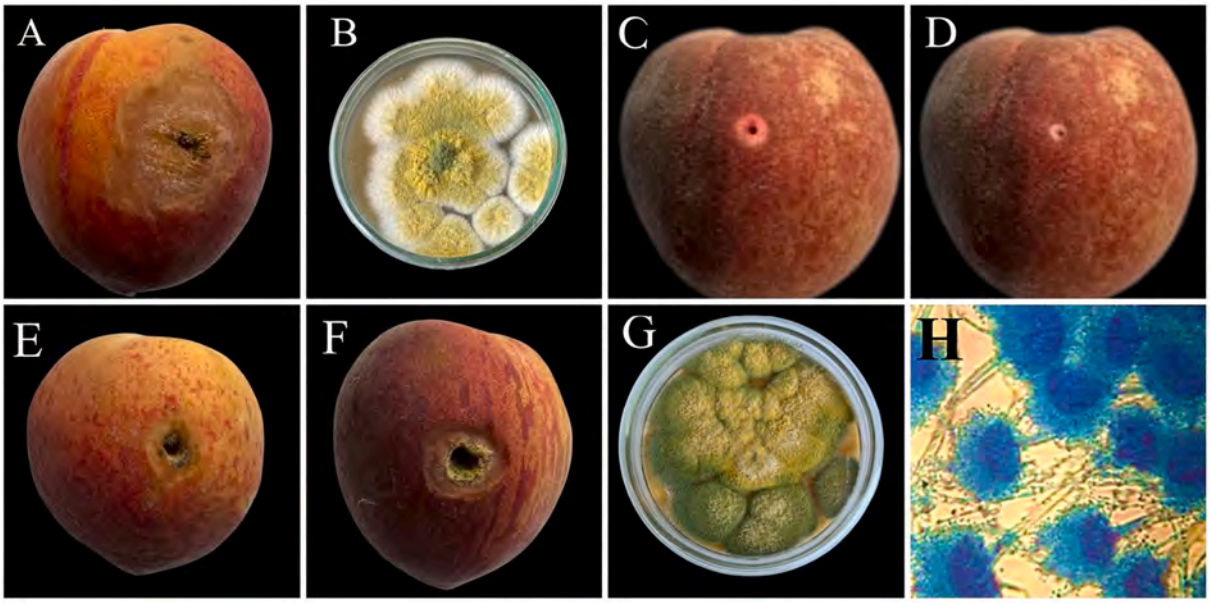

Fig. 1. Peach fruit displayed fruit rot symptoms in the field (A). The morphology of pathogen was observed in Petri plates (B). Control fruit displayed no symptoms after 2 days (C) and 5 days (D) of inoculation. After fungal inoculation, distinct symptoms could be observed after 2 days (E) and 5 days (F). Similar morphology of re-isolated pathogen was observed on PDA (G) and observed under microscope (H).

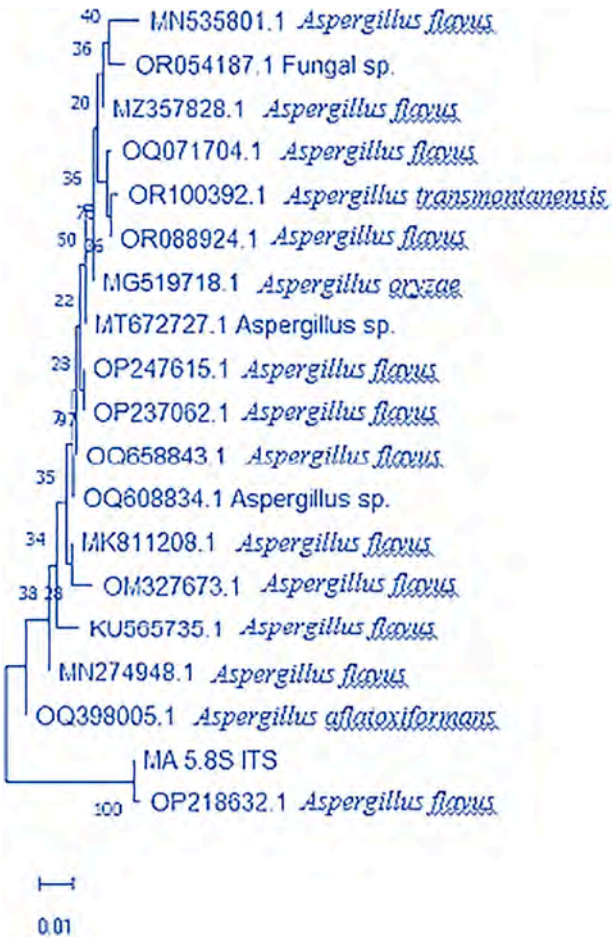

Fig. 2. Phylogenetic tree depicting the taxonomic relationship of isolated fungus with 18 closely related fungal strains.

3.2. Determination of aflatoxins production by cultural methods

Isolated pathogen (*A. flavus*) was successfully grown on PDA media and observed from front and backside of Petri plates (Fig. 3A, B). These plates were observed under UV light (365 nm), which displayed greyish black boundaries around colony margin (Fig. 3C, D) and provided preliminary evidence of aflatoxins production. By the application of 2–3 drops of ammonium hydroxide (NH_4OH) vapor, the underside color of the colony changed from yellow (Fig. 3E) to pink / plum-red (Fig. 3F), indicating the production of aflatoxins. Later, by the addition of 2–3 drops of glacial acetic, the colonies retained their original color, partially (Fig. 3G, H).

3.3. Characterization of Fe_2O_3 NPs

3.3.1. Fourier transform infrared (FTIR) spectroscopy

Obtained FTIR spectra displayed peaks of specific functional groups (Fig. 4). A sharp peak at 3391.83 cm^{-1} showed N—H stretching of secondary amine group. Peaks at 3179.90 cm^{-1} determined C—H stretching of alkene, 2168.75 cm^{-1} described N=N=N stretching of azide, 1594.02 cm^{-1} displayed N—O stretching of nitro compound, and 1404.53 cm^{-1} indicated C—F stretching of floro compound. Other peaks (in the range of 500–580 cm^{-1}) presented C—Br stretching of halo compound. These functional groups indicated the capping and reduction of synthesized NPs.

3.3.2. X-ray diffraction (XRD)

XRD analysis described noticeable pattern of Fe_2O_3 NPs peaks at 22.82, 56.18, 59.63, 59.99, and 67.55 degrees of 2θ, corresponding to Hexagonal planes (Fig. 5). These results indicated the formation of magnetite iron oxide. The planes of XRD patterns corresponded to JSCPD number 01076-1821. The average nanoparticle size was determined as 7.78 nm (Table 1).

3.3.3. SEM and EDX analysis

SEM analysis displayed spherical shape and high polydispersity of biologically reduced Fe_2O_3 NPs (Fig. 6). In EDX spectrum, strong signals of iron were observed (Fig. 7), indicating the successful formation of Fe_2O_3 NPs.

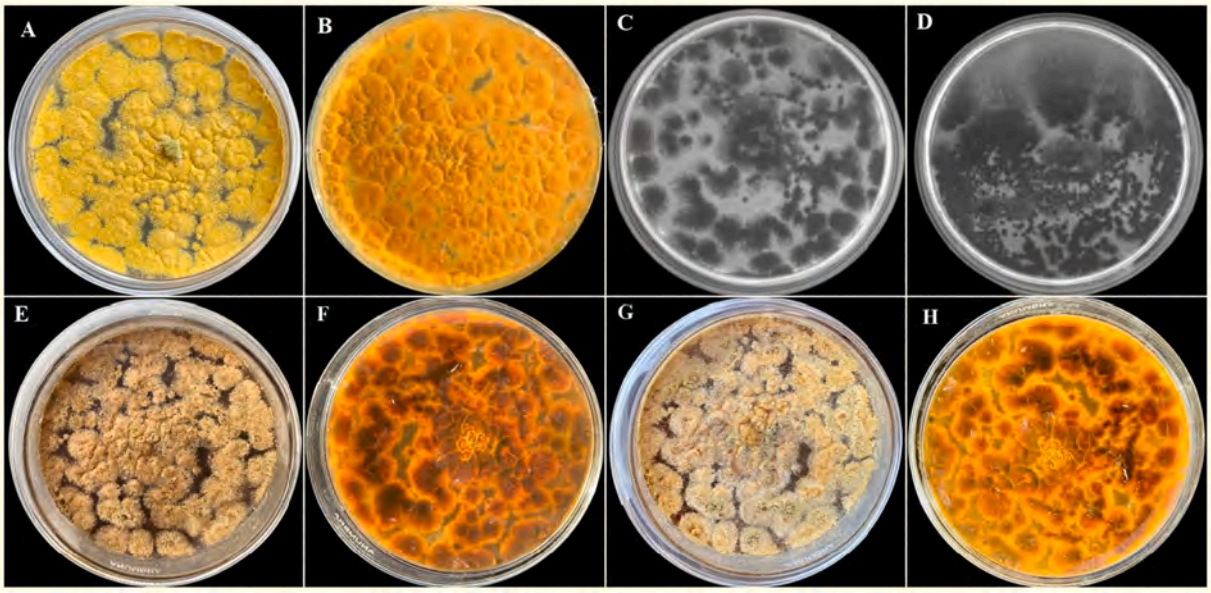

Fig. 3. Appearance of pure fungal colonies of *A. flavus* on PDA media from front side (A) and back side (B) of Petri plates. Plates were observed under UV (C, D). Addition of NH_4OH changed colony color (E, F), which was restored, partially by the addition of glacial acetic acid (G, H).

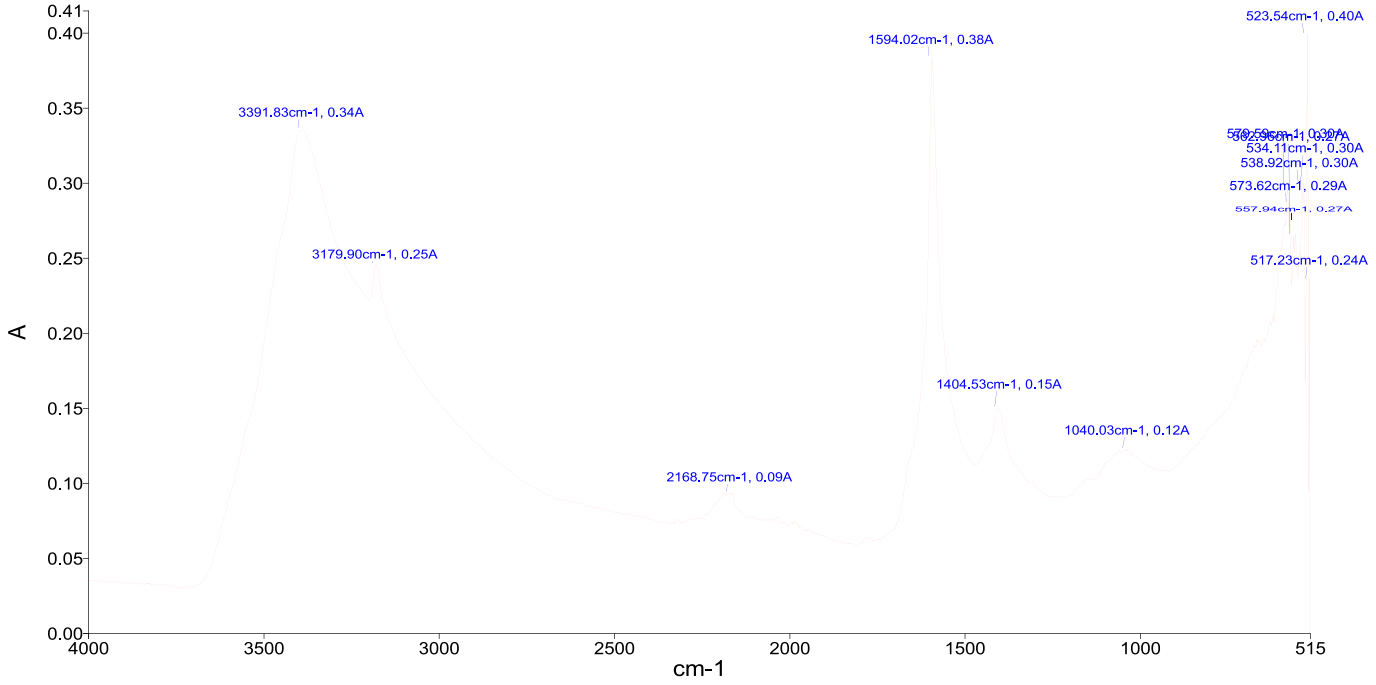

Fig. 4. FTIR spectra of mycosynthesized Fe_2O_3 NPs showing the peaks of specific functional groups.

3.4. Fungal growth assay, in vitro

T. harzianum mediated Fe_2O_3 NPs showed variable mycelial growth inhibition, in vitro (Fig. 8). Though all concentrations of NPs inhibited mycelial growth, the maximum growth inhibition was observed at 1 mg/ml concentration of NPs (72.5 %). A reduced inhibition was noticed at 0.1 mg/ ml (12.2 %), 0.25 mg/ml (26.5 %), 0.5 mg/ ml (49.1 %), and 1.5 mg/ml (64.2 %) concentrations. It has been described earlier that the efficiency of NPs decreases at very high concentrations due to the activation of diverse stress-compensation-pathways (Molina-Hernández et al., 2022).

3.5. Disease control of peach fruit, in vivo

Fe_2O_3 NPs efficiently inhibited fruit rot of peach fruit (Fig. 9). Increasing concentration of Fe_2O_3 NPs controlled disease and the least disease incidence was observed at 1.0 mg/ml concentrations.

3.6. Fruit quality attributes

Fruit quality was severely affected by fungus inoculation. Different biochemical and organoleptic characteristics of fruit were preserved by the application of Fe_2O_3 NPs (Table 2). Along with soluble solids,

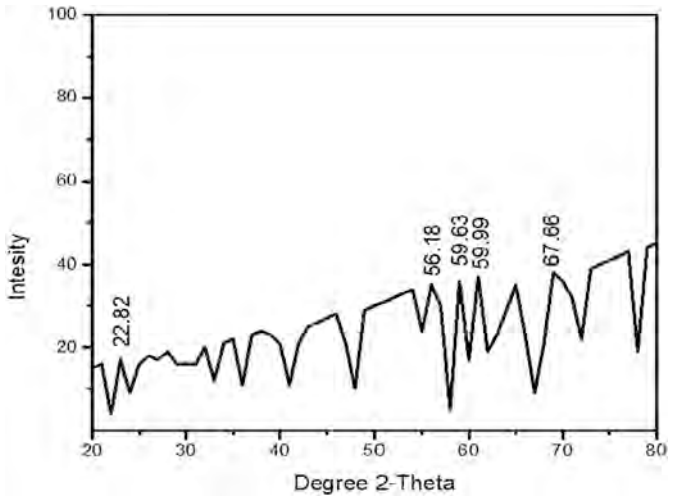

Fig. 5. XRD spectra depicting noticeable pattern of Fe_2O_3 NPs peaks.

Table 1
XRD analysis to determine the size of Fe_2O_3 NPs.

No. peaks	Peak position 2 theta	FWHM	Crystallite size D (nm)	D nm (Average)
1	22.82	3.52	7.71	**7.78**
2	56.18	3.68	6.68	
3	59.63	3.60	6.72	
4	59.99	3.70	6.53	
5	67.55	2.06	11.25	

treatment of Fe_2O_3 NPs enabled peach fruit to maintain higher titratable acidity, ascorbic acid contents and fruit firmness. Previous studies have described them as key fruit quality parameters (dos Santos et al., 2022). Ascorbic acid has been reported to play key role in protecting cellular DNA and proteins (van Gorkom et al., 2019).

3.7. Detection of aflatoxin in diseased fruit

3.7.1. Thin layer chromatography (TLC)

The preliminary qualitative detection of aflatoxins (B_1, B_2, G_1 and G_2) was performed using TLC (Fig. 10). The results depicted that aflatoxins B_1 and B_2 were the major compounds in peach fruit. Light bands were observed in diseased fruits treated with 1.0 mg/ml concentration of Fe_2O_3 NPs (T4).

3.7.2. Enzyme-linked immunosorbent assay (ELISA)

ELIZA successfully detected aflatoxins (B_1 and B_2) in peach fruit. The highest amount of aflatoxin was detected in control fruit. Treatment of tomato fruit with Fe_2O_3 NPs inhibited disease and reduced the production of aflatoxins in fruit. Lowest aflatoxin concentration was observed in fruit treated with 1.0 mg/ml concentration of Fe_2O_3 NPs. Moreover, the concentration of aflatoxin B_1 was found to be higher than aflatoxin B_2 (Fig. 11).

3.7.3. High performance liquid chromatography (HPLC)

HPLC analyses of fungal inoculated control fruit and the diseased fruit treated with different concentration of Fe_2O_3 NPs showed the presence of variable concentrations of aflatoxins. A calibration curve was established to estimate the concentration of B_1 and B_2 and a standard regression equation was used for the estimation of aflatoxin content in samples. Findings of this analysis revealed that the control peach fruit and all Fe_2O_3 NPs treated fruit were contaminated with variable concentration of aflatoxin B_1 and of aflatoxin B_2 (Table 3). Control fruit exhibited maximum presence of aflatoxins while the treatment of fruit with 1.0 mg/ml concentration of Fe_2O_3 NPs reduced the production of aflatoxins in infected fruit.

4. Discussion

Fruits are important commodities with rich nutritional values. Fruits are affected by a variety of stresses and fungi are the most important biotic factors which produce a variety of mycotoxins in fruits, vegetable, crops, fruit juices, stored commodities and dried fruits (Sanzani et al., 2016). Along with microbes, different abiotic stresses and non-technical

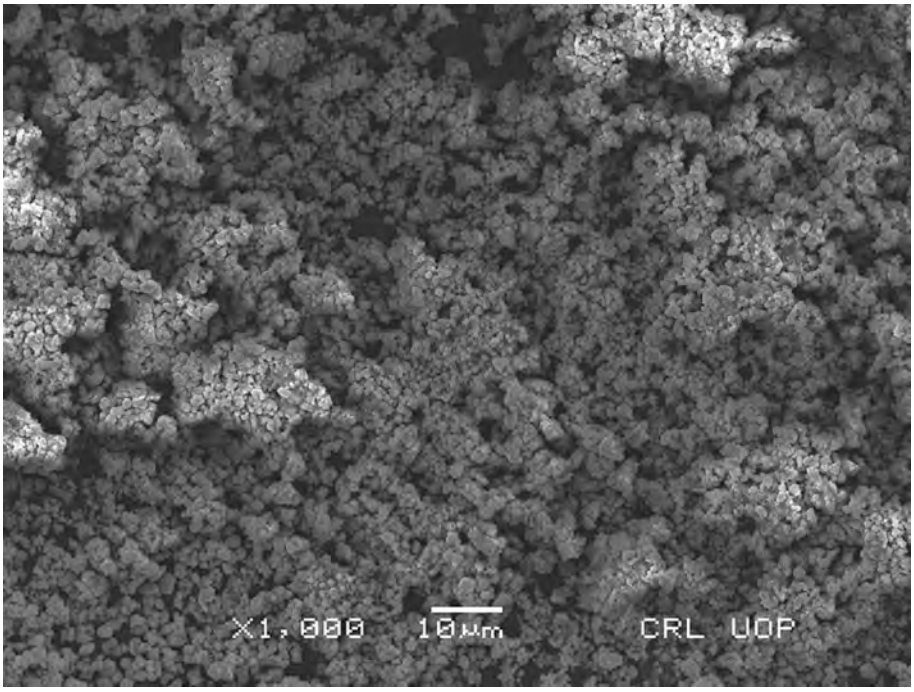

Fig. 6. Scanning electron microscopy displayed spherical shape and high polydispersity of biologically reduced Fe_2O_3 NPs. Scale bar = 10 µm.

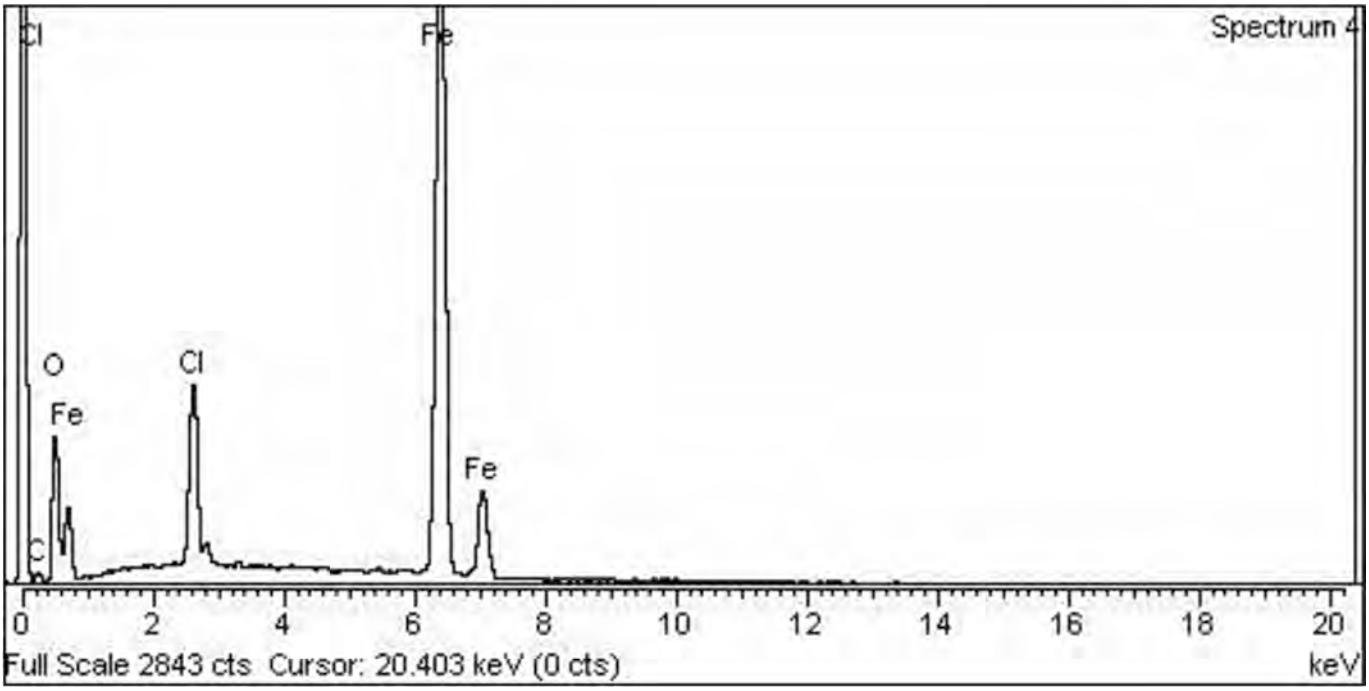

Fig. 7. The EDX spectrum of mycosynthesized Fe_2O_3 NPs, indicating strong signals of Fe.

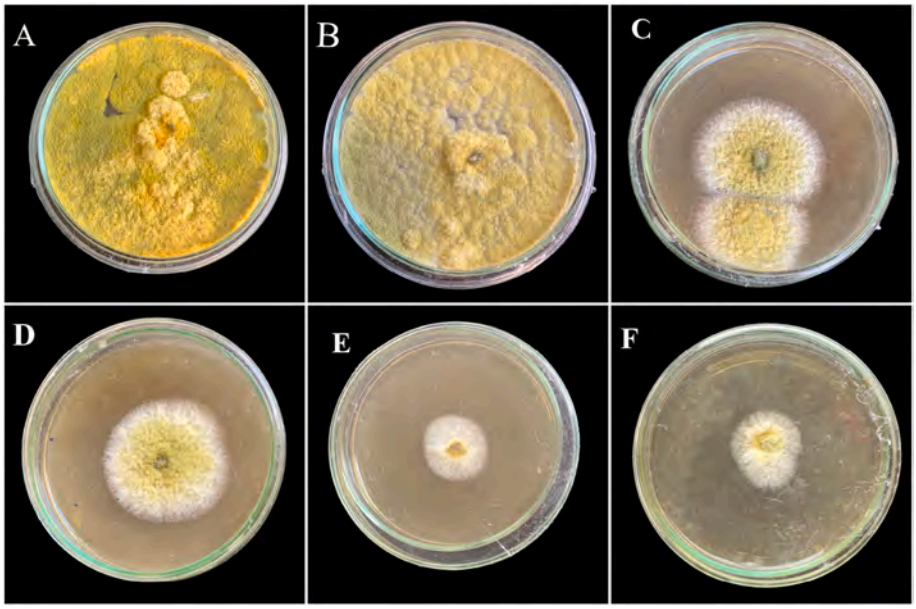

Fig. 8. Fungus was grown on simple PDA (control) (A) and at different concentrations of NPs including 0.1 mg/ ml (B), 0.25 mg/ ml (C), 0.5 mg/ ml (D), 1 mg/ ml (E), and 1.5 mg/ ml (F).

post-harvest handling also affects the quality of harvested fruit, worldwide.

In this study, the infected peach fruit were diagnosed and *A. flavus* was recognized as fruit rot pathogen. *A. flavus* is a plant pathogen and it is famous for producing aflatoxins (Ting et al., 2020). This study has described the production of aflatoxins B_1 and B_2 in infected peach fruit and suggested an environmentally friendly way to inhibit their production in fruits. Although previous studies have also reported the production of aflatoxins in fruits (Saleem, 2017), but they have not described any methodology to control disease infestation and stop the production of aflatoxins. *A. flavus* is known for contaminating several agricultural products. It is a plant pathogen and attacks a variety of crops, fruits and vegetable. Though different strains of *A. flavus* have been reported to infect many fruits, to our knowledge, this is the first report of *A. flavus* causing fruit-rot of Peach in Pakistan and around the world. Controlling the infestation of *A. flavus* is very important for human health as this pathogen produces mycotoxin, contaminates harvested crops and reduces their shelf life during storage. Aflatoxin is one

Fig. 9. Inoculation of fungus displayed maximum disease in untreated peach fruit (A). Disease infestation was controlled with different concentrations of Fe_2O_3 NPs including 0.1 mg/ ml (B), 0.25 mg/ ml (C), 0.5 mg/ ml (D), 1 mg/ ml (E), and 1.5 mg/ ml (F).

Table 2
Fruit quality attributes after treatment with Fe_2O_3 NPs.

Conc of NPs (mg/ml)	Diseased area (mm^2)	Soluble solids (%)	TTA (%)	Ascorbic acid (mg/ 100 g)	Firmness (N cm^{-2})
0.1	1101 ± 10.2 ^{ab}	12.1 ± 1.3 ^{d}	2.1 ± 0.5 ^{c}	7.1 ± 0.8 ^{c}	232.7 ± 18.2 ^{c}
0.25	552 ± 6.8 ^{c}	14.2 ± 2.1 ^{c}	2.7 ± 1.0 ^{b}	7.7 ± 1.1 ^{c}	218.6 ± 13.3 ^{d}
0.5	232 ± 3.4 ^{de}	15.3 ± 0.7 ^{bc}	3.0 ± 0.5 ^{a}	9.2 ± 1.4 ^{ab}	246.3 ± 18.0 ^{c}
1	124 ± 2.1 ^{f}	18.1 ± 1.6 ^{a}	3.3 ± 0.7 ^{a}	10.2 ± 1.2 ^{a}	302.2 ± 16.8 ^{a}
1.5	258 ± 4.2 ^{d}	16.3 ± 1.1 ^{b}	2.9 ± 0.5 ^{ab}	9.1 ± 1.0 ^{ab}	266.7 ± 14.2 ^{b}
Control	1204 ± 9.5 ^{a}	11.3 ± 0.8 ^{de}	1.8 ± 0.7 ^{d}	6.6 ± 1.1 ^{d}	188.3 ± 12.9 ^{e}
Untreated fruit	0	18.6 ± 1.2 ^{a}	3.4 ± 0.5 ^{a}	10.8 ± 0.8 ^{a}	311.5 ± 12.2 ^{a}

Note: Values with same digit (a, b, c, d, e or f) are not significantly different from each other.

of the most studied mycotoxins and B_1 is considered as the most toxic compound (McKean et al., 2006). Aflatoxins are carcinogenic and they are notorious for their hepatotoxic abilities. They also have mutagenic properties and cause economic losses by contaminating staple food commodities (Loi et al., 2020). Though in this research, fruit rot of peach has been controlled by using biodegradable Fe_2O_3 NPs, which were manufactured in the cultural extract *T. harzianum*. For more than 40 years, *T. harzianum* is considered as one of the most effective biocontrol agents (Elad et al., 1980). Many studies have described the synthesis of a variety of nanoparticles from *T. harzianum* and their successful application to inhibit the growth of pathogenic fungi (Consolo et al., 2020). In this study, a remarkable mycelial growth inhibition (72.5 %) was observed, which signifies the efficiency of synthesized NPs. Characterization of prepared Fe_2O_3 NPs with FTIR spectroscopy confirmed the presence of different molecules as predominant capping and stabilizing biomolecules, on the surface of NPs. FTIR spectrum analysis assured the presence of organic compounds and several bio-micromolecules in all samples, which indicates successful stabilization and reduction of Fe_2O_3 NPs (Niazi et al., 2023). XRD analysis described noticeable peaks pattern of Fe_2O_3 NPs at 22.82, 56.18, 59.63, 59.99, and 67.55 degrees of 2θ,

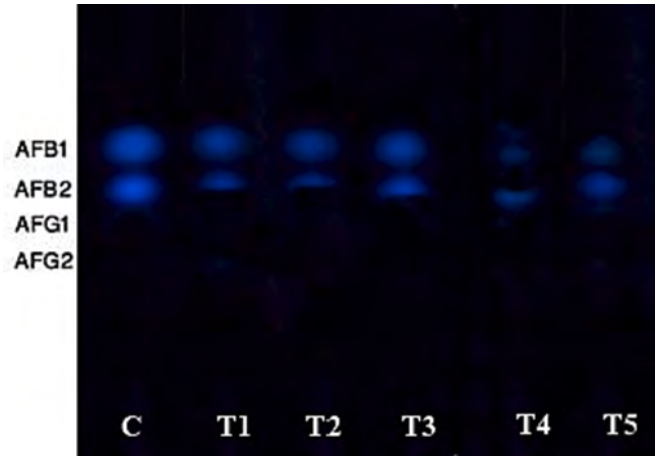

Fig. 10. Thin layer chromatography profile of aflatoxins B and G series in peach fruits after their treatment with different concentration of Fe_2O_3 NPs. Vertical columns represent diseased fruit (C), fungal inoculated fruit treated with different concentration of NPs including 0.1 mg/ml (T1), 0.25 mg/ml (T2), 0.5 mg/ml (T3), 1.0 mg/ml (T4), 1.5 mg/ml (T5).

corresponding to the planes of Hexagonal with P3 space group. XRD results showed nano-size (less than 100 nm) of synthesized Fe_2O_3 NPs, which suggest their antimicrobial potential that enormously depends upon the size and crystal-like nature of NPs (Zubair et al., 2022). Crystalline nature of NPs provides them antimicrobial properties and helps them to damage fungal cell wall (Oussou-Azo et al., 2020). SEM analysis displayed nearly spherical shape of NPs, which is coherent with previous results (Niazi et al., 2023). Spherical shape of synthesized nanoparticles provided them antimicrobial and wound healing properties and made them a very useful fungicide for disease control and plant protection (Surendra and Roopan, 2016).

Findings of this study revealed that different concentrations of Fe_2O_3 NPs inhibit mycelial growth and maintain higher number of soluble solids in the infected peach fruit. These remarkable results indicated the importance of Fe_2O_3 NPs in increasing the shelf life of Peach fruit. Better shelf life also ensures the trade and storage of fruits for a longer period of times (Li et al., 2016). Fruits with higher percentage of ascorbic acid and soluble solids have better taste and have higher nutritional properties (Matias et al., 2016). In the present study, ascorbic acid contents were severely decreased in infected fruit, while the application of Fe_2O_3 NPs helped to maintain it. Ascorbic acid is an antioxidant micronutrient which plays a key role in health promotion. Under stress conditions, the antioxidant capacity of fruits and vegetables is reduced rapidly (Szeto et al., 2002). Firmness is one of the most crucial physical factors to assess the quality of fruits and increase its acceptability for the consumer. In this study, Fe_2O_3 NPs helped to retain firmness of peach fruit, which may be attributed to the selective permeability of NPs to gas and water transfer, which lowers the respiration ratio, enzyme activities, and the most metabolic changes while delaying fruit ripening and over-softening (Pasquariello et al., 2013). Maintenance of pertinent organoleptic properties of peach fruit indicates the usefulness of Fe_2O_3 NPs.

5. Conclusion

This study has provided information about the production of aflatoxins in peach fruit. This is first report of rot disease of peach fruit and this disease was managed by the application of Fe_2O_3 NPs, synthesized in the filtrate of *T. harzianum*. Used iron salt has very modest price (2–3 USD kg^{-1}) and it is easily available in every part of the world. The outcomes demonstrated that the fruit rot of peach can be effectively controlled by using the optimal concentration (1.0 mg/ml) of Fe_2O_3 NPs. These NPs has great potential to replace chemically hazardous fungicides to manage the diseases of fruits and vegetables.

Table 3
Quantity of aflatoxin B_1 and B_2 present in peach fruit after their treatment with different concentrations of Fe_2O_3 NPs.

Conc of NPs (mg/ml)	Aflatoxin B1 ($\mu g/kg^{-1}$)	Aflatoxin B2 ($\mu g/kg^{-1}$)
0.1	11.52	1.57
0.25	10.36	1.12
0.5	7.22	1.08
1	5.88	0.78
1.5	8.11	1.01
Control	18.67	2.21

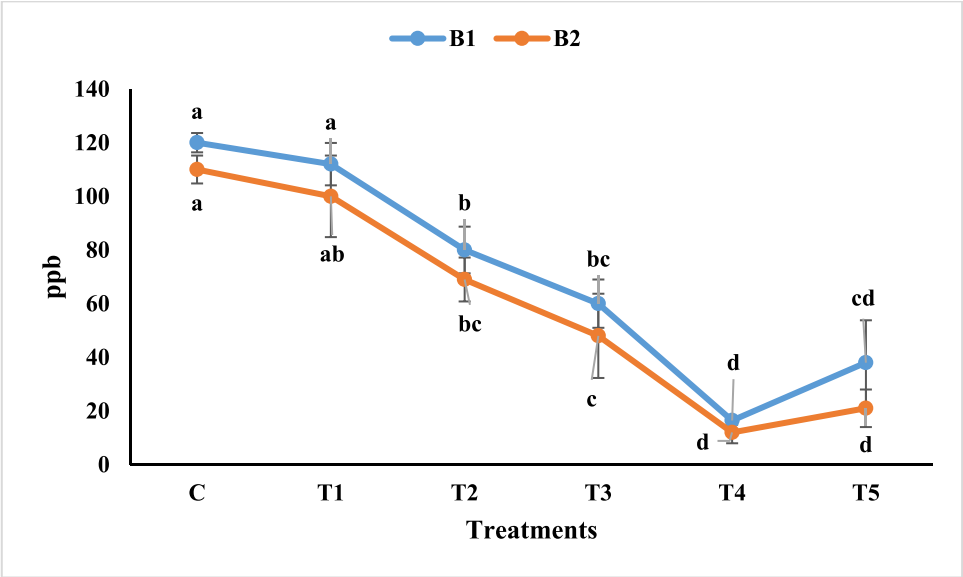

Fig. 11. Quantitative estimation of aflatoxin in infected peach fruits under variable concentration of Fe_2O_3 NPs by ELISA technique. C = control, T1 = 0.1 mg/ml, T2 = 0.25 mg/ml, T3 = 0.5 mg/ml, T4 = 1.0 mg/ml, and T5 = 1.5 mg/ml concentration of Fe_2O_3 NPs.

CRediT authorship contribution statement

Mahnoor Akbar: Conceptualization; methodology; formal analysis; investigation; writing – original draft. **Naeem Ali:** Methodology; writing – review and editing. **Muhammad Imran:** Methodology; writing – review and proof reading. **Arshad Hussain:** formal analysis. **Syed Waqas Hassan:** analysis; investigation, **Urooj Haroon** and **Asif Kamal:** Methodology; writing – review and editing. **Farhana:** methodology. **Hassan Javed Chaudhary:** Formal analysis; manuscript editing. **Muhammad Farooq Hussain Munis:** Supervision; writing – review and editing, resources; funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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