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AYESHA NAZ

SU92-MSBTW-F22-005

FOS

**Stress Impact of Biogenically Synthesized Silver Nanoparticles on Orange
(*Citrus Sinensis*) Microplants**



SUPERIOR UNIVERSITY

Thesis Submitted to

The Superior University Lahore

In Partial Fulfillment of the

Requirement for the Degree of

MS Biotechnology

By

AYESHA NAZ

Roll No. SU92-MSBTW-F22-005

Session: 2022-2024

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DEDICATION

*Every word of mine is dedicated to my ALLAH
ALMIGHTY for HIS fruitful help and
Grandparents*

Mr. Muhammad Shafi & Mrs. Rani Bibi

My beloved parents

Mr. Manzoor Hussain and Mrs. Anwari begum

And my respectable Supervisors

Dr. Ghulam Zahara Jahangir & Dr. Aisha Younas

Whose Love is softer than anything else

Who always pray for my success

Who is my greatest strength in weakness

Who sacrifice their longing for upholding my Studies.

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LIST OF ABBREVIATION

AgNO ₃	Silver nitrate
AgNPs	Silver nanoparticles
BAP	Benzylaminopurine
2, 4 D	2,4-Dichlorophenoxyacetic acid
FTIR	Fourier transmission Infrared
EDX	Energy dispersive x-ray
Kin	Kinetin
HgCl ₂	Mercuric chloride
NAA	Naphthalene Acetic Acid
NaOCl	Sodium hypochlorite
SNPs	Silver nanoparticles
SEM	Scanning electron microscopy
PDI	Polydispersity Index

ABSTRACT

Citrus sinensis (Orange) is a member of the *Rutaceae* family has a large amount of polymethoxylated, citric acid and flavanones. Orange (*Citrus sinensis*) tissue culture techniques have been used all over the world to investigate the mechanisms behind plants development, cell division and biochemical process in tissue culture lab. However, microbial contaminants inhibit their growth, leading to reduced output. The goal of the dissertation is to create biologically produced silver nanoparticles from orange juice at a concentration that is safe to use as anti-contaminant agents from micropropagation media. The ratio 1:4 of orange juice extract to 1% AgNO₃ produced the most effective nanoparticles in the context of stability, quality and reducing property when analyzed through surface plasmon resonance. The Zeta sizer potential analysis was showed -23.8mV which revealed the stability of SNPs. The synthesized AgNPs size was 37.42nm measured by using image J software on micrograph of Scanning electron microscopy (SEM) at 500nm. Energy dispersive x-rays (DTX) revealed 33.06 percent elemental silver from orange juice extracts. *Citrus sinensis* explants were grown in MS media supplemented with 1mg/l or 2mg/l BAP for micropropagation of orange. The 2ppm, 3ppm, 5ppm biogenically synthesized silver nanoparticles were added in MS media supplemented with 2mg/l BAP. 3ppm concentration of silver nanoparticles concentration was more effective in term of shoots, roots length, number of leaves and chlorophyll content, while compare to other treatment with control plant. This research showed that the low concentration of biogenically synthesized silver nanoparticles in micropropagation media can be used as anti contaminant agents in *Citrus sinensis* tissue culture without affecting it growth.

CHAPTER 1

INTRODUCTION

Orange belongs to sub-genus citrus, family *Rutaceae* and sub-family *Aurantioideae*, which includes three different sub-genera: *Citrus*, *Fortunella* and *Poncirus trifoliata* [1]. A small evergreen plant, Sweet oranges (*Citrus sinensis* L. Osbeck) is named after it to differentiate it from closely similar species such as orange, *C. reticulata*, *C. aurantium* and mandarin orange. It is most frequently planted fruit tree in the world, Originating in southern China where it was cultivated for many years. Now days, it is native to commercial cultivation in tropical, semi-tropical, and some similarly temperate regions of the world [2, 3]. Citrus is the world's second-most productive fruit crop, producing almost 108 million tons (FAO Statistics 2006).

Numerous phytochemicals, including naringenin and hesperetin, are found in sweet orange. Naringenin functions as immune system modulators, antioxidants, scavenger of free radicals and anti-inflammatory in humans. It is estimated that an orange contains about 60 flavonoids which are well-known for their anti-tumor and anti-blood clotting abilities as well as about 170 phytonutrients. All of these characteristics work together to improve health [4].

Atoms grouped together with diameter ranging from 1nm to 100nm are called silver nanoparticles (AgNPs) [5]. The scientific community is very interested in biosynthesis of silver nanoparticles due to its many applications. Detection and treatment of cancer are also being effectively carried out by using these silver nanoparticles [6, 7]. Typically, a variety of chemical and physical techniques are used to prepare nanoparticles, which can be costly and environmentally hazardous as they offer the use of hazardous chemicals that cause a risk to human health and environment [8].

Instead of using chemicals, Plant synthesized silver nanoparticles are used because plant extracts are inexpensive and energy efficient, which help to sustain human health and the environment. Plant synthesized silver nanoparticles help to create safer products by reducing waste, improving work place and community health.

Unmatched uses of green synthesized silver nanoparticles have key characteristics of nanotechnology. Utilizing plants for nanoparticles synthesis can be advantageous over other biological entities since it can prevent the laborious process of using microorganism and maintaining their culture, which can cause the bacteria to lose their ability for synthesis of nanoparticles. Therefore, in the upcoming decades, the utilization of plant extracts for AgNPs could have a significant impact [9].

Orange tissue culturing have a significant importance due to its role in overcoming challenges in citrus breeding, such as genetic diversity, long juvenile periods, and susceptibility to diseases. Overall, orange tissue culturing not only aids in the production of disease-free plants but also contributes to the development of new cultivars, seedless fruit production, and germplasm conservation, emphasizing its crucial role in the citrus industry [10].

In tissue culture, microbial infection (both bacterial and fungal) is a significant issue. Utilization of nanoparticles (NPs) in plant tissue culture has effectively eliminated the microbial infection from explants. It exhibited positive effects on the establishment of organogenesis, callus, somatic embryos, and cell suspension cultures etc. Nanoparticles just minimized or finished the infection but did not affect the health of explants at its optimum conditions [11, 12].

The goal of the study to synthesized silver nanoparticles by using orange extracts and the silver nanoparticles has been characterized by UV visible spectrophotometer. The optimization of different ratios has been prepared. The molecular characterization is carried out by scanning electron microscopy (SEM), Energy Dispersive X-rays (EDX) and Zeta size potential analysis for identified nanoparticles size, morphology and percentage of pure elemental silver and stability of silver nanoparticles, respectively. The orange has been grown on culture medium containing MS media enriched with various ratio of hormones for shoot, root propagation and callus induction. To avoid contaminants that invade tissue culture plants, silver nanoparticles have been tested as plant preservative. Three different concentrations (2ppm, 3ppm, 5ppm) have been

applied to Orange cultures that added to micro propagation media. Treatment and control media are maintained at $25\pm 2^{\circ}\text{C}$ with 16 hours of artificial light and 8 hours of darkness, using fluorescent lamps with 2500 and 3000 lux intensity. Physical parameters like number of leaves/shoot and root length, chlorophyll content has been analyzed.

AIM AND OBJECTIVES

The objectives of present study are

- Molecular identification of germplasm.
- Establishment of in-vitro culture of *Citrus sinensis*.
- Bio-synthesized silver nanoparticles from silver nitrate using juice extract of oranges.
- Biological synthesized silver nanoparticles were analyzed through the techniques like UV-Visible Spectrophotometer, Energy dispersive X-ray (EDX), Zeta sizer potential and Scanning Electron Microscopy (SEM).
- Biological synthesized AgNPs has been used in plant tissue culture medium as anti-microbial agents.
- Stress impact of bio-synthesized silver nanoparticles were analyzed for physical parameter (Number of leaves, shoot/root length and chlorophyll content) of *in-vitro* culture of *Citrus sinensis* and compared with untreated orange plant.

CHAPTER 2

LITERATURE REVIEW

The purpose of this review of literature is to investigate the biogenic production of silver nanoparticles using plant extracts and analyze antimicrobial effects. Through a comprehensive analysis of extent research and academic publications, this study aim to integrate existing knowledge and identify knowledge gaps about synthesis methods, characterizations and anti-contaminant effectiveness, physical impact of silver nanoparticles derived from extract of plants on tissue culture of *Citrus sinensis*.

2.1 *In Vitro* Culture of orange Explants

Yusuf, Z. *et al.*, (2024) investigation focused on optimizing the hormonal composition of the media with regards to plant growth regulators for the *in vitro* multiplication of sweet orange cultivars obtained from nodal segment explants. The nodal segment explants were sourced from three citrus cultivars, namely Washington Navel, Valencia, and Tangelo. Utilizing Murashige and Skoog (MS) medium supplemented with sucrose and varying concentrations of growth regulators, the researchers conducted shoot proliferation and root induction experiments to determine the most effective medium composition. By utilizing nodal segments from citrus cultivars, micro shoots were induced from the roots, with findings indicating that NAA exhibited affective hormonal activity compared to IAA [13].

Jaffal, S. M. *et al.*, (2022) used *Citrus sinensis* plant tissues, including zygotic embryos, leaves, Stems and cotyledons in their evaluations. When compared to other tissue types, they discovered that zygotic embryos had the highest rate of callus induction. In this particular group, the combination of 3 mg/L 6-BAP and 3mg/L 2, 4 D produced the best callus development. Similarly callus development from cotyledons and leaves was successfully induced by dose of 3 mg/L, 2, 4 D and 1 mg/LBAP. The best integration, however, for stems was 2mg/L, 2.4 D and 1 mg/L BAP. The callus was loose, friable and colour anywhere from white to yellow, regardless of the type of tissue [14].

Hasan, M. N. *et al* (2019) developed a method for *In vitro* regeneration for *Citrus*

sinensis, using various plant growth regulators (PGRs) and basal media, including MS, CHU (N6), and Gamborg (B5) media. Different concentration of 2, 4 d and NAA and combination of both was used. The results revealed that *Citrus sinensis* exhibited the highest callus induction on MS medium supplemented with 3mg/l 2, 4 d, compared to lower frequency observed on N6 and B5 media supplemented with same hormone. The study found that MS medium supplemented with 3mg/l 6-benzylaminopurine (BAP) had the highest regeneration potential, with a frequency of $89.3\% \pm 8.8\%$ from embryogenic calli, while non-embryogenic calli showed no regeneration. The regenerated plantlets were successfully rooted on MS medium with the addition of 5 mg/L NAA. These findings offer valuable insights for the genetic improvement of *Citrus sinensis* with desired traits [15].

Pandey, A. & Tamta, S. (2016) focused on *In-vitro* regeneration of sweet orange through direct and indirect organogenesis. For indirect organogenesis, the callus formation was started from leaf sample of *in vitro* seedlings on MS media supplemented with 2, 4 dichlorophenoxyacetic acid (2, 4 d) alone and combined with benzyladenine (BA) and α -naphthalene acetic acid (NAA). Direct organogenesis included placing sample on MS media containing BA alone and combined with NAA and gibberelic acid. Notably, a combination of $4.53\mu\text{M}$ 2, 4 d and $5.37\mu\text{M}$ NAA include callus in 93.33% and 87.67% of the callus into average of 6.93 shoots per explants. The addition of $4.44\mu\text{M}$ BA with $1.54\mu\text{M}$ GA_3 enhanced the shoot multiplication rate (17.73 ± 1.69 shoots per explants) as compared to the control (0.00 ± 0.00 shoots per explants). The best rooting was observed when micro-shoots were treated with $100\mu\text{M}$ NAA for 48 hours. The rooted plantlets were subsequently acclimatized and transferred to soil, where they matured in the polyhouse [16].

Fazle, Azam *et al.*, (2011) used apical shoots and internodes as explants to study the development shoot and induction of callus. The hormone used in this study to induce callus was 2, 4 D di-chlorophenoxy-acetic acid (2, 4 D). When 2, 4 D at a dosage 2.0mg/L was added to MS basal media, the greatest rate of callus induction 68% was seen. Seeds enriched with 6-benzyladamine (BA) and kinetin (KIN) were treated in MS medium, both in the presence and absence of seed coats. The removal of seed coat

caused the shoot development to begin earlier. The seeds treated with MS basal media supplemented with 1.0mg/L of BA showed the greatest amount of shoot development (70%) in seeds without seed covering. Nevertheless, all employed concentrations, kinetin failed to elicit any sort of response for the production of shoots [17].

Khan, E. U *et al.*, (2009) investigated the culture of leaf explants derived from in vitro seedling of two different cultivars of Oranges (*Citrus sinensis*). Different shoot regeneration results were obtained when explants were cultivated on shoot-inducing media (SIM) that included MT salts in combination with different plant growth regulators. The most successful formulation for directly generating shoot growth from leaf explants was M1 (MT + 0.5 mg/L⁻¹ Kinetin + 0.5 mg/L⁻¹ BA + 0.1 mg/L⁻¹ NAA + 0.8% agar + 3% sucrose, pH 5.8). Between the two cultivars, a notable variation in the responsiveness of regeneration of shoot bud was noted. Culturing explants derived from fully matured leaves exhibited a superior capacity for shoot regeneration compared to those obtained from under developed leaves. The regenerated plants from both genotypes were found to be identical to their original donor plants, including that there was no discernible genetic variation in them, according to phenotypic assessment and analysis using randomly amplified polymorphic DNA. The data provided in this research demonstrate the successful direct initiation of plants from leaf explants [18].

2.2 Bio-Synthesis of Silver Nanoparticles

There are various benefits of using plants extracts to produce silver nanoparticles. This approach shows to be affordable, cost effective and energy efficient. Additionally, it lessens waste and produces safer products by protecting the environment and human health. Silver nanoparticles made using green synthesis demonstrate amazing features of nanotechnology with wide range of uses. Using plants to synthesize nanoparticles has several advantages over other biological agents since it eliminates the need for microbes and the time it takes to maintain their cultures. This strategy helps to avoid the possible loss of efficacy during nanoparticle production [19].

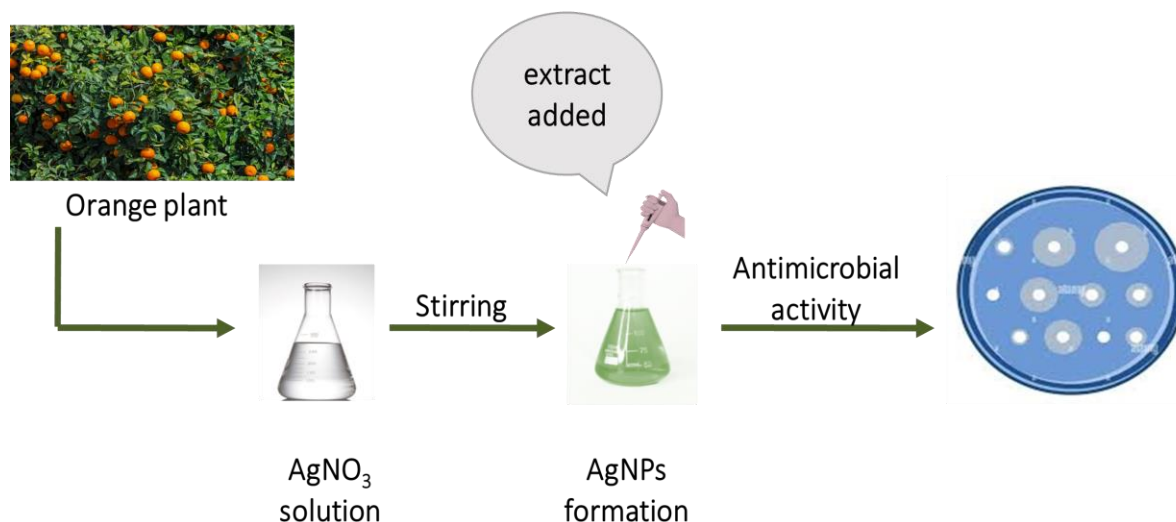


Figure 2.1: Biogenic synthesis of silver nanoparticles and their antimicrobial activity.

Ghiuta, I. *et al.*, (2023) reported the biosynthesis of silver nanoparticles, employing orange peel extract as a stabilizer and reducer and AgNO_3 as a precursor. In preparation for the plant extract, the samples were made in a 9:1 ratio. After around half an hour, the first indication of a successful synthesis were noticed when the color of the nanoparticles changed from pale yellow to brown. UV-Vis spectrophotometry was used to characterize the samples that were obtained after the synthesis processes. The analysis revealed an absorbance peak with the highest intensity around a wavelength of 420 nm. Particles that are meant to be employed in medicine and water treatment, followed by the testing of antibacterial and photocatalysis, can now be utilized more freely when harmful ingredients are eliminated from the synthesis stages [20].

Alaallah *et al.*, (2023) demonstrated the lemon juice (*Citrus limon*) use in the biosynthesis of silver nanoparticles (AgNPs). Surface Plasmon Resonance (SPR) analysis was used to analysis the production of silver nanoparticles, demonstration their production as indicating by a measured $\lambda_{\text{max}} = 430$ nm. Further investigation into putative biomolecules in the charge that cause to reduce of silver ions was done by using Fourier Transform Infrared (FTIR) spectroscopy. Different peak was found at (111, 200, 220 and 311) according to X-ray Diffraction (XRT) test, which demonstrated the face-centered cubic crystal shape silver nanoparticles in solution. Transmission electron

microscopy (TEM) revealed the spherical shape of silver nanoparticles and size between 10 to 50 nm. Test assessing the trapping of 2, 2-diphenyl-1-picrylhydrazyl radicals, total reducing agents and antioxidants capacity demonstrated the remarkable antioxidants qualities of the produced AgNPs. Additionally, utilizing the well diffusion method the antibacterial property of silver nanoparticles was evaluated and result showed that the AgNPs were sensitive against gram-positive bacteria' and gram negative bacteria' ones. These result have a potential implications in the biological and morphological domains based on Nanobiotechnology [21].

Chen *et al.*, (2022) used an aqueous extract of oranges as a non-toxic green reductants and capping agents to produce silver nanoparticles (AgNPs). The characteristics of these nanoparticles were analyzed through Fourier Transform infra-red spectroscopy (FTIR), Ultra-Visible Spectroscopy (UV-Vis) and Field emission scanning electron microscopy (FESEM) to determine their physiochemical properties. They were investigated for possible biological use in antioxidant and anticancer test. In the most current cellular and molecular study, AgNPs- treated cells were subjected to MIT assay for 48 hours in order to assess the cyto-toxicity and anti-human lung carcinoma effect on lung carcinoma and normal (HUVEC) cell lines, such as PC-14, NCI-H1299, HLC-1, NCI-H1661 and LC-2/ad. When exposed to AgNPs, viability of malignant lung cells' declined in a dose- dependent manner. According to the result, chemotherapy medication or dietary AgNPs made from the aqueous extract of *citrus sinensis* leaves can be used to create chemotherapy medication and dietary supplement to treat different kind of lung adenocarcinoma in humans [22].

Kumar, A. *et al.*, (2021) examined the medicinal properties of citrus fruits and their capability to synthesize silver nanoparticles. The medical benefits of citrus fruits and their capacity to produce silver nanoparticles were the main topics of Kumar, A. (2021). The modern world needs to identify new and effective natural antibiotic agents because resistance to existing antibiotics is growing. Likewise, the biosynthesis of nanoparticles is being promoted for its eco-friendly benefits.. Citrus fruit juice is utilized to reduce silver ions for the green synthesis of silver nanoparticles (AgNPs), due to the outstanding medical and industrial applications of AgNPs. Citrus fruits have

antibacterial properties due to a variety of ingredients, as. These ingredients also provide citrus fruit juice its radical scavenging properties. In addition, the AgNPs made from citrus fruits were examined for size and shape using transmission electron microscopy (TEM), Fourier transform infrared (FTIR) spectroscopy, and Ultra-Violet Visible (UV-VIS) spectroscopy. AgNPs' anticancer efficacy was assessed using Colo-205 cell lines, and at a dose of 10 μ g/ml, it was discovered to inhibit 37.9% of the cell lines' proliferation. Therefore, when combined with other anti-cancer medications, fabricated AgNPs can be employed to efficiently combat cancer cell lines [23].

Skiba *et al.*, (2019) research was to use sweet orange peel to create environmentally friendly silver nanoparticles (AgNPs). The orange peel was ground into fine powder during the procedure. Then 40 milliliters of distilled water were combined with one gram of dry orange peel powder and stirred. After five minutes of CNP discharge, the resultant combination was filtered and the newly obtained orange peel water extract was used immediately. This extract ensured the long-term stability of the produced AgNPs by being used in bioreduction of silver nitrate. UV-vis spectroscopy, Dynamic light scattering and Scanning Electron Microscopy (SEM) were used in the AgNPs characterization. The roughly spherical shape of the silver nanoparticles was shown in SEM picture. The crystal structure of the silver nanoparticles was cubic and face-centered, according to analysis powder X-ray diffraction (XRD). The product AgNPs zeta potential value of -21.7 mV indicates a considerable degree of stability for the nanoparticles that were developed [24].

Jackson *et al.*, (2018) made nanoparticles using green synthesis techniques, Citrus fruit juices (*Citrus limetta*, *Citrus paradisi*, *Citrus sinensis* and *Citrus aurantifolia*) were utilized like both reducing and stabilizing agents while silver nitrate used as silver precursor. The synthesis of nanoparticles was confirmed by a discernible colour shift from colourless to characteristic reddish-brown tint. The surface plasmon resonance peak' of the AgNPs was observed at 451 and 452nm. When antibacterial properties were evaluated against *Pseudomonas*, *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus*, the result showed that the MIC values varied between 25 and 100 mg/ml for each of the examined pathogens. Notably, *Bacillus subtilis* and *staphylococcus aureus*

showed the least susceptibility to the silver nanoparticles, whereas *Pseudomonas aeruginosa* showed the maximum sensitivity [25].

Omran, *et al.*, (2018) examined the use of warm water extract from *Citrus sinensis* (sweet oranges) peels, the single-step environment friendly production of silver nanoparticles (AgNPs). When silver nitrate was reduced with peel extract, there was a discernible colour change that indicated the synthesis and stability of AgNPs. From pale yellow to brown, the hue altered. Silver nanoparticles (AgNPs) unique absorption peak at λ_{max} 422 nm was confirmed by UV-Vis Spectrophotometry analysis. Dynamic Light Scattering revealed that the average particle size was 23.81nm. Energy-dispersive X-ray (EDX) confirmed the presence of the elemental silver with strong indications. This indicates their crystalline structure. The average size of the biosynthesized AgNPs was confirmed by X-ray to be 15 nm in size. Using Fourier transformation infrared (FTIR) spectroscopy, possible functional groups involved in stabilizing and capping the synthesized AgNPs were identified. The biogenic synthesis of spherical-shaped AgNPs with average size 3 to 12nm was recognized by this study as a viable and environmentally sustainable method [26].

Hungund, B. S.*et al.*, (2015) described a quick and eco-friendly approach for synthesizing silver nanoparticles and evaluated their antibacterial properties. Silver nitrate was reduced and capped using a variety of fruit juices, including orange, lime, and sweet lime, in an environmentally friendly synthetic process. Silver nanoparticles were created using two distinct thermal reduction techniques: a hot plate and a microwave oven. In a few of minutes, the process reduced the silver ions into silver nanoparticles. Using a scanning electron microscope and UV-visible spectrophotometer, the produced silver nanoparticles were examined. Using UV-visible spectra and the Mie Scattering procedure, an effort was also made to estimate the size of the silver nanoparticles. The morphology of silver nanoparticles was usefully showed by the scanning electron microscopy (SEM). When tested against *Salmonella typhimurium*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Staphylococcus aureus*, the produced silver nanoparticles had antibacterial properties. The antibacterial properties of orange fruit juice-biosynthesized silver nanoparticles were more effective. According to the

study, silver nanoparticles have a greater antibacterial effect on gram negative bacteria. These investigations are essential to proving the medicinal value of silver nanoparticles [27].

Albahadly Z. K. *et al.*, (2009) fabricated silver nanoparticles using *Citrus sinensis* L. and *Citrus aurantium* L. leaves. A wide range of methods including the particles analyzers, zeta potential, FTIR (Fourier transform infrared spectroscopy), XRD and UV-Vis spectroscopy used to characterize the produced AgNPs. These techniques had used to examine the aspect of optical spectroscopy, structure characteristics and particle size. *Citrus sinensis* L. and *Citrus aurantium* L. leaves showed AgNPs diameters of 60.5 nm and 31.72 nm respectively, with a crystallized face-centered cubic shape. Furthermore, synthesis of silver nanoparticles (AgNPs) exhibited significant antifungal inhibitory effect against *Candida albicans* [28].

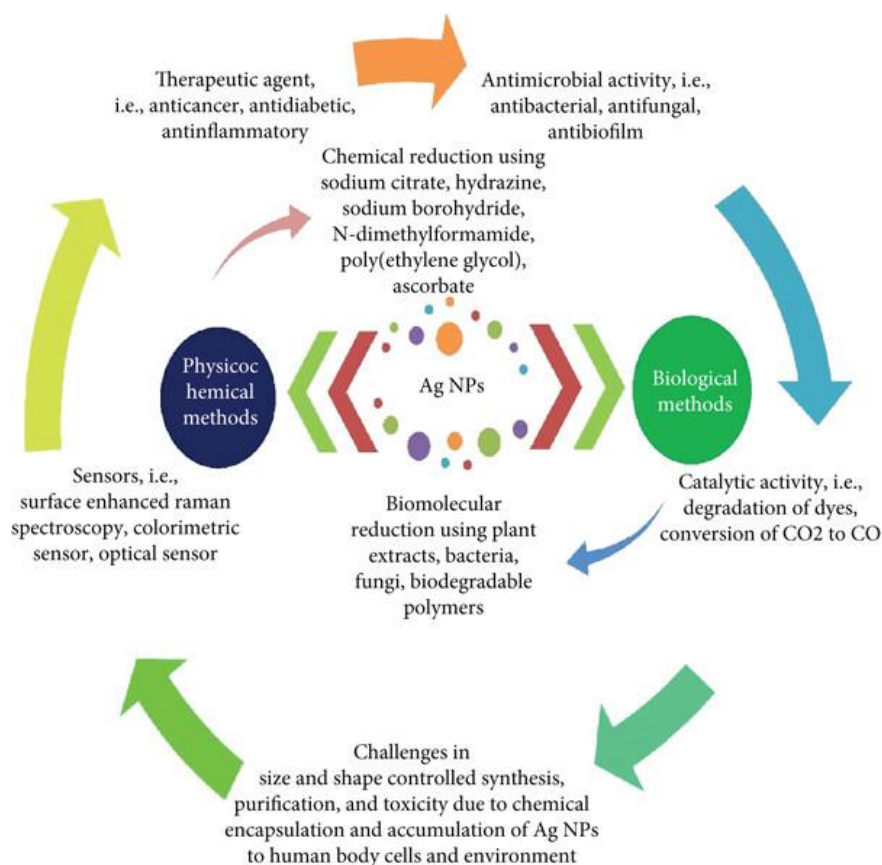


Figure 2. 2: Flow chart of manufacturing, challenges and application of AgNPs [29].

2.3 Silver nanoparticles as Plant Preservative

Zhang, J., *et al.*, (2017) presented a novel method for producing AgNPs from leaf extracts of *Fatsia japonica*. It was discovered that the amount of AgNO₃, NaCl and leaf extracts had significant effect on the yield and the size of AgNPs. These nanoparticles demonstrated a potent preservation effect against *P. italicum*-induced rot in citrus fruits when used under ideal circumstances. Furthermore, TEM examination and inhibition tests demonstrated that the produced AgNPs induced *P. italicum* cytoplasmic leakage, cell deformation, and ultimately cell death. Additionally, the AgNPs showed strong antibacterial qualities which may be useful for citrus fruit preservation. Overall, this work suggests a unique method for citrus fruit preservation in addition to developing an efficient way for synthesizing AgNPs [30].

Silver nanoparticles were used in this study to address Pb toxicity in two genotype G1 and G2 of Mung bean (*Vigna radiata*). AgNPs were used to target different Pb concentrations (0, 25, 50µM) in order to improve plant properties like plant height, fresh biomass, and total chlorophyll content. AgNPs improved the growth of *Vigna* genotype and allowed for the balanced absorption of minerals. When compared to G1 and AgNPs treatment, the G2 genotype showed a significant tolerance and fewer negative impacts on the examined features. These outcomes show that AgNPs affect plant responses to lead stress, significantly enhancing plants ability to tolerate heavy metals [31].

Barbasz, *et al.*, (2016) investigated AgNPs have a variety of roles as stimulants for plant growth and anti-microbial agents. These nanoparticles have special qualities that can affect a plant's morphology and physiology because of their physical and chemical composition. It is important to select the right concentration of nanoparticles that will stimulate plant development in the desired way without causing chronic cytotoxicity, as extensive application of AgNPs may be hazardous to plants. It's also crucial to comprehend the process underlying their cellular effects. The culture of callus in vitro offers an ideal environment for investigating important interactions between nanoparticles and plant cell processes [32].

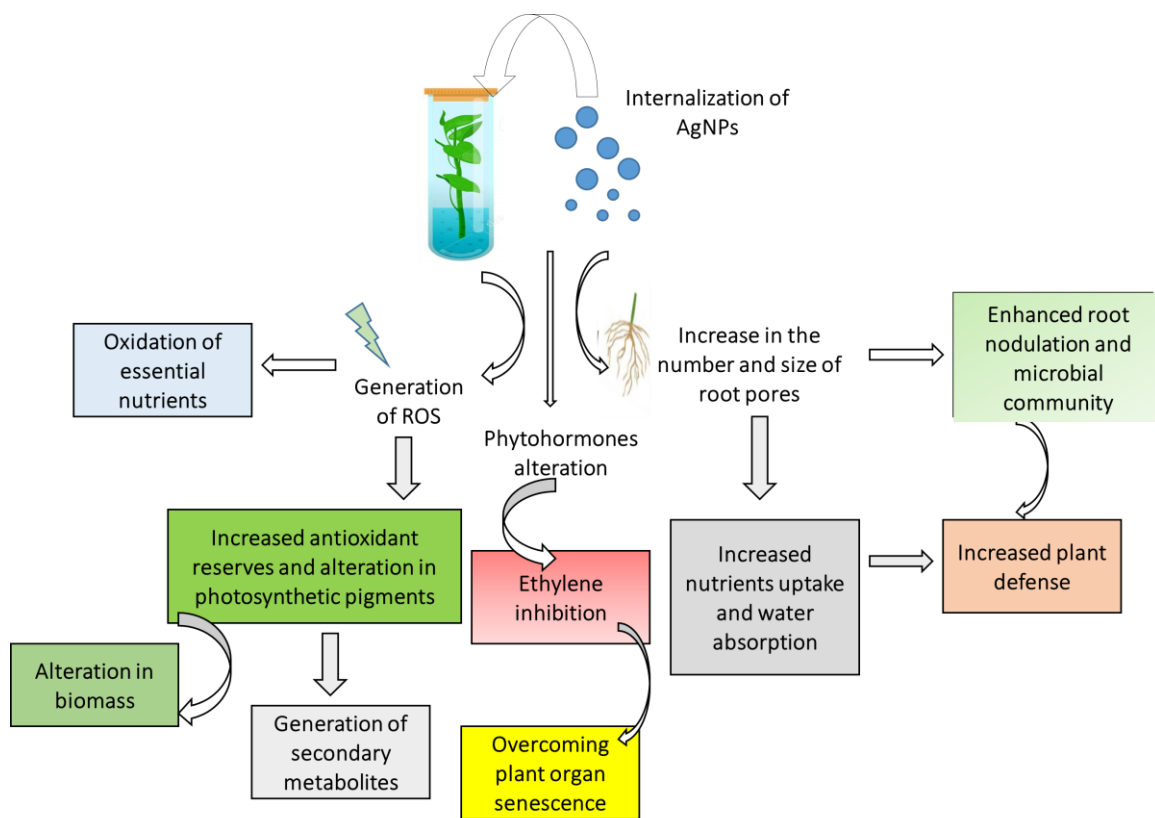


Figure 2. 3 : A brief overview of the way in which AgNPs work on plants [33].

CHAPTER 3

MATERIALS AND METHODS

This series of experiments was executed at the Plant Molecular Biology and Biotechnology department of Applied Molecular Biology (CAMB), University of the Punjab, based in Lahore. The aim was to examine stress effect caused by biogenically produced silver nanoparticles in the plant tissue culture medium of orange.

3.1 Collection of samples

Orange fruit were purchased from local market of Lahore. Mature seeds were used as sample to be cultured in plant tissue culture lab. Orange plant sample were collected from field of the Centre for Applied Molecular Biology (CAMB) for research purpose. Young apical shoots and nodal section were used as samples for callus induction.



Figure 3. 1: Plants of orange (A, B) planted at CAMB field.

3.2 Surface sterilization of orange explants

Various experiments were tested to lower the maximum amount of contamination in orange seeds, apical shoots and nodal segments whether from bacteria or fungi. These protocols are discussed below:

3.2.1 Surface Sterilization protocol I

The surface sterilization method described by Fazle, Azam *et al.* (2011) was utilized with certain modifications. Orange seeds and apical shoots and nodal segments were first rinsed with running tap water to remove dust particles separately. Orange plant parts were then disinfected with a detergent solution for 15 minutes. After rinsing the seeds, apical shoots and nodal sections with distilled water, they were transferred to a sterilized laminar air flow hood where they were soaked in 4% sodium hypochlorite (Appendix II) for 15 minutes. Following this treatment to eliminate fungal and bacterial contaminants, the orange plants parts were washed three times with distilled water. Finally, the seeds were placed on blotting paper to remove surface moisture and allowed to air dry in a sterile environment for 10-15 minutes.

3.2.2 Surface sterilization protocol II

Mature orange seeds, apical shoots and nodal sections were washed with tap water. After 10 to 20 minutes of detergent disinfection, the seeds, nodal section and apical shoots were cleaned with distilled water. These orange plant parts were soaked for 10 minutes in a 2% mercuric chloride (HgCl_2) solution (Appendix II). To reduce fungal attack, the seeds, nodal section and apical shoots were cleaned with distilled water while in a sterilized and laminar air flow environment to eliminate any possible contamination.

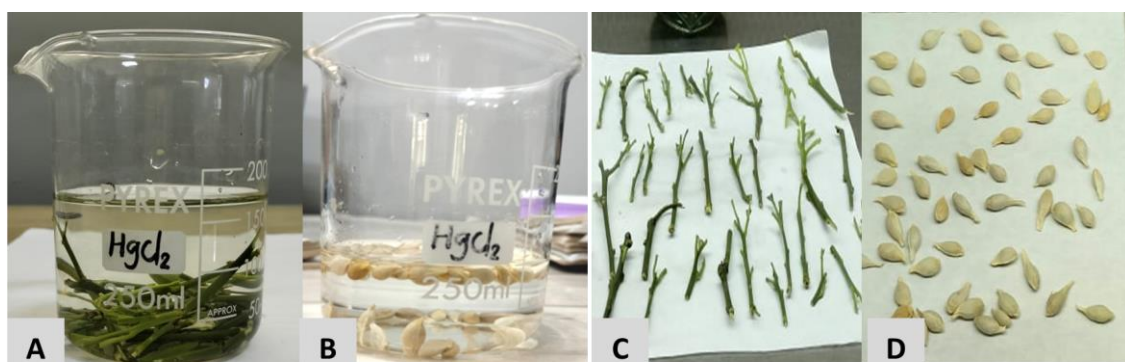
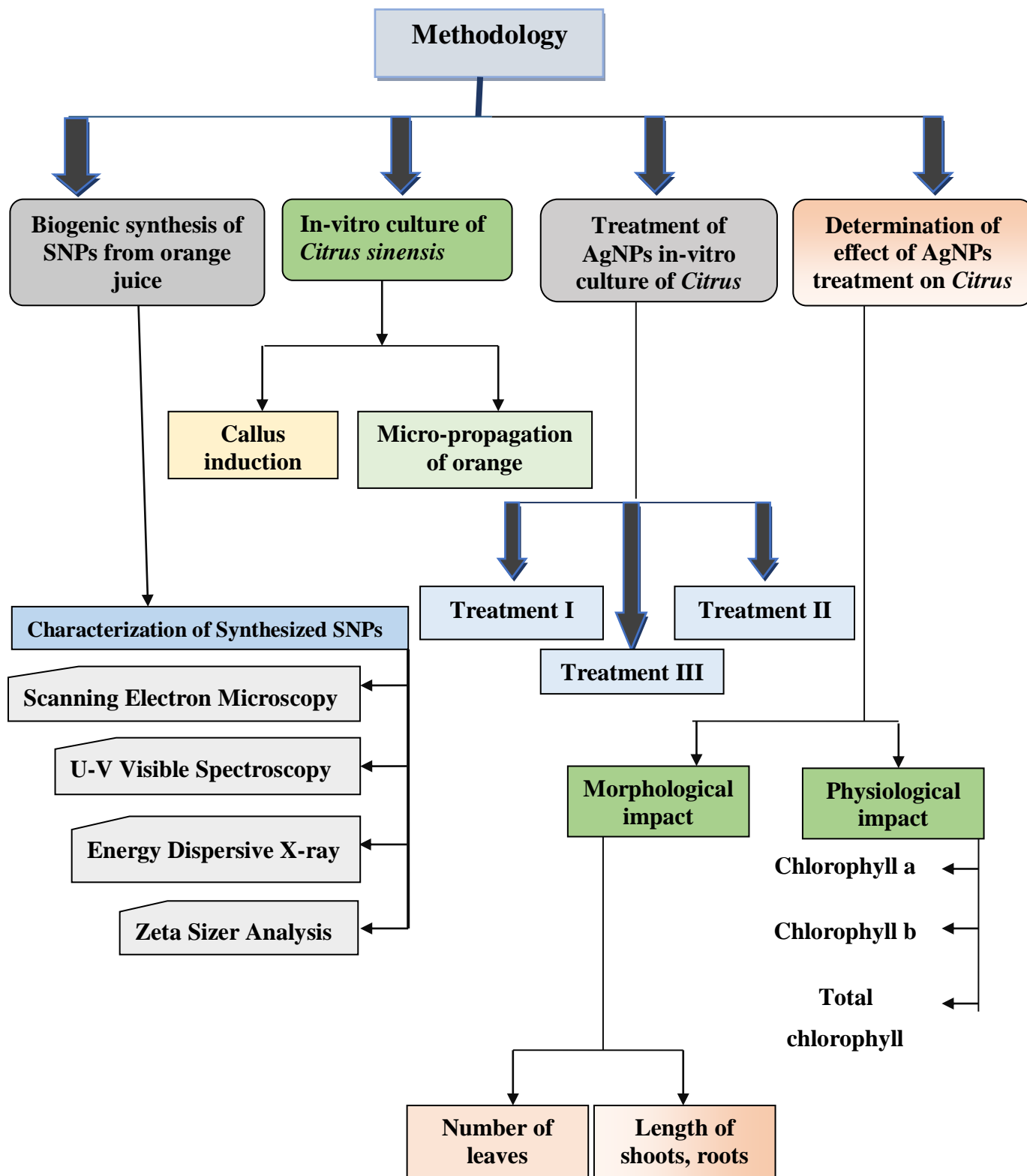


Figure 3. 2: Surface sterilization protocol: (A, B) Surface sterilization of explants section, (C, D) Air dry on filter paper in Laminar flow hood.



Flow chart of methodology: Biogenic synthesis of silver nanoparticles and determine the stress impact of biogenic AgNPs on *Citrus sinensis* micro-plants.

3.3 Preparation of BAP solution

BAP solution was prepared by dissolving BAP (Sigma Aldrich, CAS No. 1214-39-7) in autoclaved distilled water.

3.4 Preparation of plant growth media

The following plant growth media were prepared under sterile conditions for the micropropagation of orange plants.

3.4.1 Plant propagation media I

For the micropropagation of orange seeds, a 1 mg/L BAP medium was prepared to support the seeds' growth. For 1L Muraishige and Skoog basal media preparation, 4.43g MS (Sigma Aldrich M5519-10L) and 30g sucrose (Phytotech CAS-No. 57-50-1) were mixed in autoclaved distilled water through magnetic stirrer. 1000µl of BAP 1mg/L solution were also added, volume raise to 1L after and the pH was maintained in between 5.5 to 5.7. Then 2.5g phytagel was added. The media was transferred to autoclave tubes then capped with plug and packed in test tubes container for autoclave at 121°C for 15 minutes.

3.4.2 Plant propagation media II

For the micropropagation of orange seeds, a 2 mg/L BAP medium was prepared to support the seeds' growth. For 1L Muraishige and Skoog basal media preparation, 4.43g MS (Sigma Aldrich M5519-10L) and 30g sucrose (Phytotech CAS-No. 57-50-1) were mixed in autoclaved distilled water through magnetic stirrer. 2000µl of BAP 2mg/L solution were also added, volume raise to 1L after and the pH was maintained in between 5.5 to 5.7. Then 2.5g phytagel was added. The media was transferred to autoclave tubes then capped with plug and packed in test tubes container for autoclave at 121°C for 15 minutes.

3.4.3 Plant propagation media III

For the micropropagation of orange seeds, a 2 mg/L BAP medium was prepared to support the seeds' growth. For 1L Muraishige and Skoog basal media preparation, 4.43g MS (Sigma Aldrich M5519-10L) and 30g sucrose (Phytotech CAS-No. 57-50-1) were

mixed in autoclaved distilled water through magnetic stirrer. 2000µl of BAP 2mg/L solution and 2000µl of 2, 4-d 2mg/l solutions were also added, volume raised to 1L after and the pH was maintained in between 5.5 to 5.7. Then 2.5g phytigel was added. The media was transferred to autoclave tubes then capped with plug and packed in test tubes container for autoclave at 121°C for 15 minutes.

3.4.4 Preparation of plant callus induction media

The following plant growth media for callus induction of orange explants were prepared under sterile conditions.

3.4.4.1 Callus Induction Media I

For the preparation of 500ml Muraishige and Skoog basal media, 2.251g MS Sigma Aldrich M5519-10L) and 15g sucrose (Phytotech CAS-No. 57-50-1) were added in autoclaved distilled water. Then it was mixed thoroughly by constant stirring and raised the volume 500ml. 1000µl of 2, 4-d 1mg/l solution was added for plant growth regulator. The pH was maintained in between 5.5-5.7. Then 7g agar was added and sealed the flask with aluminum foil to autoclave it at 121°C for 15 minutes. After autoclave, the media was poured into petri dishes.

3.4.4.2 Callus Induction Media II

For the preparation of 500ml Muraishige and Skoog basal media, 2.251g MS Sigma Aldrich M5519-10L) and 15g sucrose (Phytotech CAS-No. 57-50-1) were added in autoclaved distilled water. Then it was mixed thoroughly by constant stirring and raised the volume 500ml. 2000µl of 2, 4-d 2mg/l solution was added. The pH was maintained in between 5.5-5.7. Then 7g agar was added and sealed the flask with aluminum foil to autoclave it at 121°C for 15 minutes. After autoclave, the media was poured into petri dishes.

3.4.5 Inoculation of orange explants

Callus induction media was used to inoculate nodes and shoot tips. Seeds were inoculated without their seed coats in autoclave test tubes in order to induce shoot and root development. The cultures were kept at 25±2°C with 16 h of photoperiod and 8 h of

darkness with the light intensity of 3000 lux under -white fluorescent lamps.

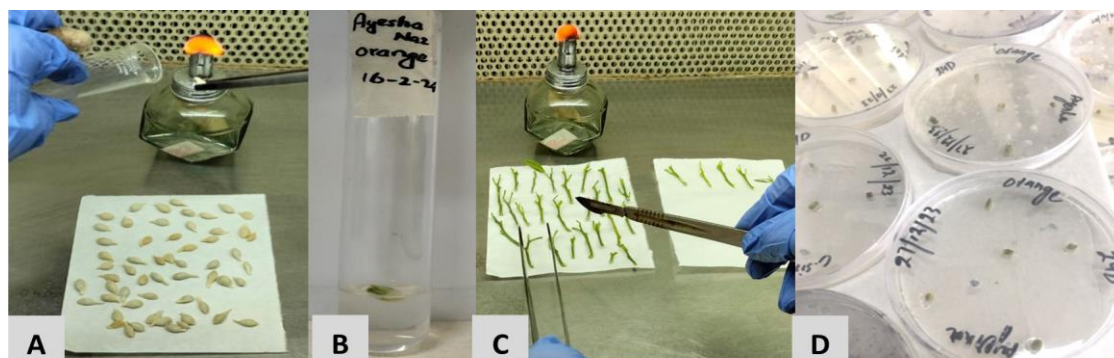


Figure 3. 3: Inoculation of seeds in test tubes: (A, B), inoculation of nodal section in petri plates (C, D).

3.4.6 Organogenesis

The process for producing shoots began with calli. Pieces of the callus that were in good condition were cut out and put on the shoot and root initiation medium. MS medium supplemented with 2mg/l BAP for shoot induction and 2mg/l 2, 4 D for root induction. Every 7 days, visual observations were made.

3.5 DNA extraction for Molecular identification of germplasm

0.1 g leaves of *Citrus sinensis* was grinding with the help of pestle and mortar. The ground leaves were transferred into separate Eppendorf tubes. To each sample tubes, 1000µl CTAB was added, mixed well by inverting the tubes. The Eppendorf tubes were then heated at 65°C for 25 mints. After heating, 500µl chloroform: isoamyl alcohol was added to each tube. The sample were mixed and centrifuged at 10000 rpm for 10 mints. After centrifugation, 500µl of the supernatant was carefully transferred to a new tube, and then 500µl pre chilled iso-propanol was added to the supernatant. The mixture was incubated on ice (-20°C) for 20 minutes, centrifuged at 14000 rpm at 10 minutes. After that supernatant was removed, leaving DNA pellet behind. The pellet was washed with 500µl of ethanol, and the sample centrifuged again 14000 rpm for 10 minutes. The supernatant was removed and the pallet was air-dried. The DNA pallet was resuspended in 50µl of distilled water (H₂O).

3.5.1 Gel Electrophoresis

1% Agarose gel was prepared in 1X TAE buffer for DNA visibility. 1g of agarose was taken in empty dried clean flask and added 100ml of 1X TAE buffer. It was mixed by shaking and heated in microwave for 10 minutes until it turns to be transparent. 10 μ l of ethidium bromide (1mg/ml) were added carefully. Gel was transferred to casing tray. 5 μ l extracted DNA was mixed with 2 μ l of 6X loading dye in each well. The gel was run on major science mini 300gel electrophoresis systems with 100 voltage and 400 amperes current for 35-40 minutes for better result. The gel was visualized on UV Trans illuminator.

3.5.2 Molecular identification of germplasm

To ensure the precise molecular identification of germplasm sample (*Citrus sinensis*) collected during this research, DNA sample were sent to Center for Applied Molecular Biology (CAMB) sequence faculty.

3.6 Green synthesis of Silver nanoparticles

Silver nanoparticles have been synthesized using orange juice extract as a reducing and capping agent to convert the silver nitrate (metal salt) to silver nanoparticles. The concentration of salt and plant extract mainly contribute to the AgNPs synthesis. In order to optimize the experiment, metal salt (1% silver nitrate solution, Appendix IV) has been mixed in various ratios with orange juice extract in different ratios to reduce the metal salt into silver nanoparticles.

Different ratios of silver nitrates were mixed with constant ratios of orange juice extract. For this reason, 7 different ratios v/v 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:20 were prepared in beaker and covered with aluminum foil. More the presence of phytochemicals in extract, more and quick synthesis of silver nanoparticles will take place. Later, UV-Visible spectrophotometer was used to monitor synthesized silver nanoparticles.

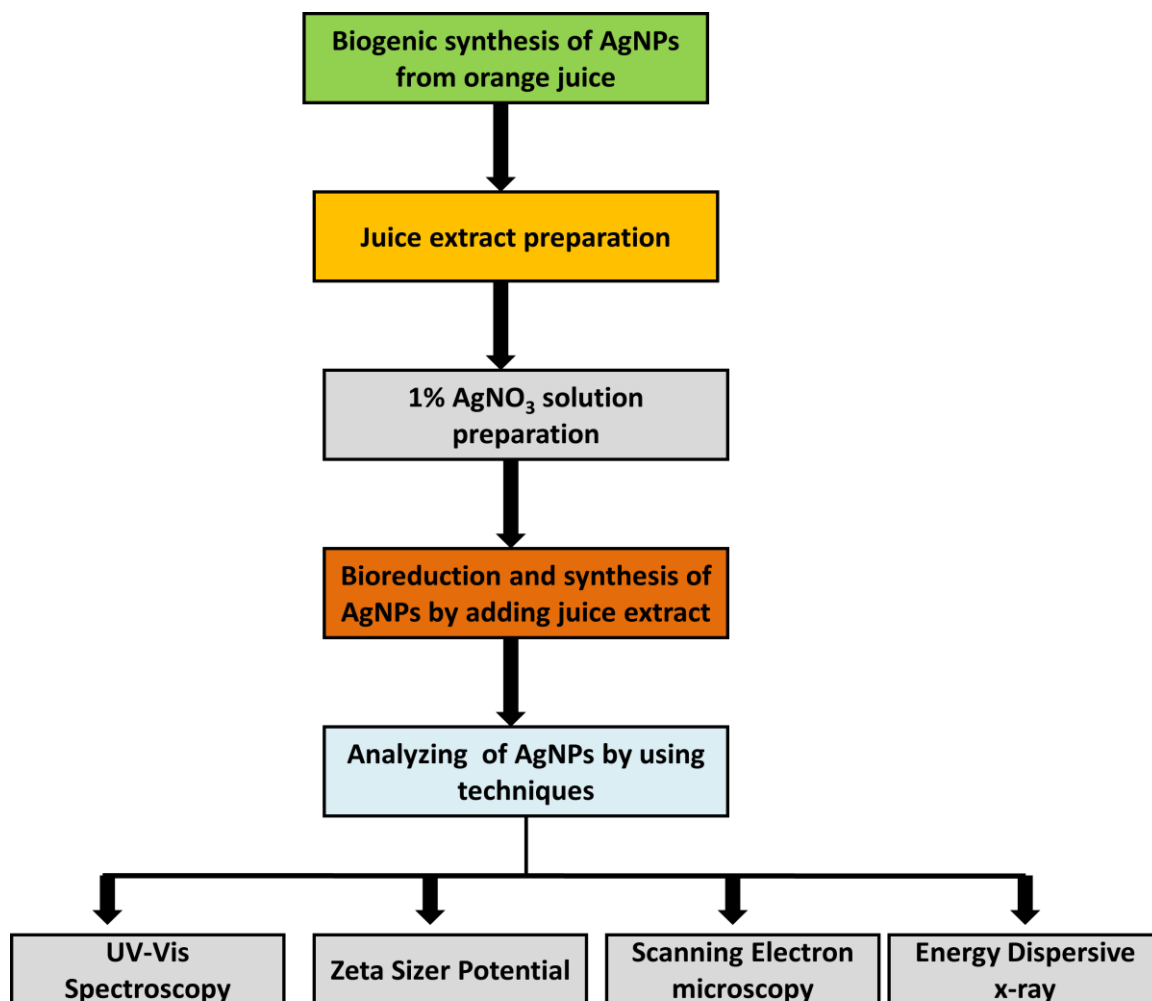


Figure 3. 4: Flow chart of biogenic synthesis of AgNPs by using citrus juice extract.

3.6.1 Preparation of orange juice extract for green synthesis of silver nanoparticles

The green synthesis of silver nanoparticles was performed by utilizing juice extract of orange. The experiment for extract preparation and filtration is described as:

3.6.1.1 Preparation of orange extract (juice)

The fresh orange juice was obtained by removing the fruit peel and seeds. First, water was used to wash them in order to remove any dust that cling to their peels. The oranges were thoroughly cleaned and dried with clean cloth. After that, oranges cut in equal half and squeezed carefully and the juice was collected in beaker. The extract was filtered through a clean Whatman filter paper. The filtrate was collected and kept frozen

at -10°C until used in the experiment for the synthesis of silver nanoparticles.

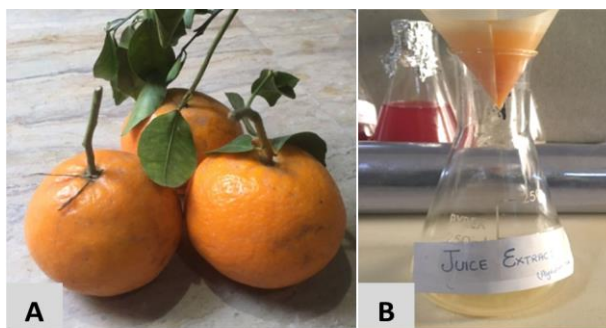


Figure 3. 5: Preparation of orange juice: Orange fruits for juice extract (A), Filtration of orange fruit extract (B).

3.7 Characterization of Silver nanoparticles through UV-Visible Spectrophotometer

The silver nanoparticles synthesized from orange juice were examined for the stability of the strong absorption peak associated with surface plasmon resonance (SPR) using a UV-Visible Spectrophotometer (BMS, UV-2800) over a wavelength range of 300-600nm. Sharp peaks formed within the 400-450 nm absorption range were recorded to compare data from different experimental ratios, aiming to identify the most stable reaction mixture for bulk preparation and further characterization. Absorbance was measured for each wavelength from 300-600 nm for the respective samples.

3.8 Preparation of pellet for Molecular Characterization of biogenically synthesized AgNPs

The reaction mixture of biogenically synthesized silver nanoparticles sample was transferred to 1.5ml Eppendorf tube for centrifugation at 14000 rpm for 15 minutes. The supernatant was eliminated and the pellet was treated thrice with autoclaved distilled water to avoid impurities and dried the silver nanoparticles pellet. The wet pellets were allowed to air dry at room temperature. Green synthesized silver nanoparticles were prepared for molecular characterization from dried pellets and then collected in 2ml Eppendorf. The dried pellets sample was shipped to the Department of Physics' commercial facility, LUMS, for silver nanoparticle molecular analysis as follow:

3.8.1 Zeta sizer Analysis

The sample was sent for zeta sizer analysis that determined the stability of the AgNPs.

3.8.2 Scanning electron microscopy (SEM)

Biogenically synthesized silver nanoparticles analyzed through SEM and determined the morphology (size, shape) of AgNPs.

3.8.3 Energy Dispersive x-ray (EDX)

For the determination of elemental composition of biogenically synthesized AgNPs, the sample was sent for Energy dispersive x-ray analysis.

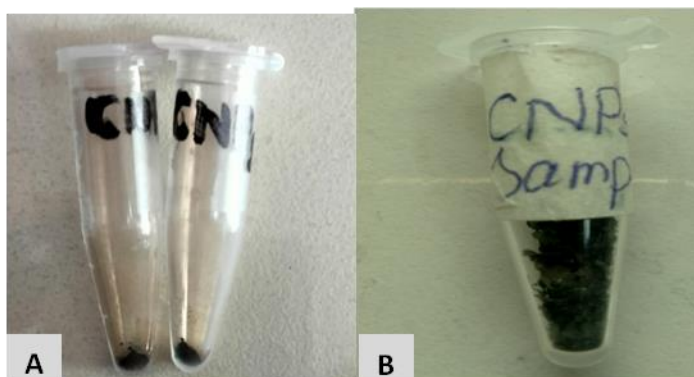


Figure 3. 6: Pallets of Biogenically synthesized silver nanoparticles (A), Collection of dried pallets in 2ml Eppendorf (B).

3.9 AgNPs used as Antimicrobial agents in orange plant tissue culture

In order to evaluate AgNPs antibacterial action, different concentrations were added to plant micropropagation media.

3.9.1 Treatment I

2ppm or 0.002g/l of AgNPs was used in 2mg/l of BAP plant growth regulator in MS media whose recipe has been described in section 3.4.2 plant propagation media II.

3.9.2 Treatment II

3ppm or 0.003g/l of AgNPs was used in 2mg/l of BAP plant growth regulator in MS media whose recipe has been described in section 3.4.2 plant propagation media II.

3.9.3 Treatment III

5ppm or 0.005g/l of AgNPs was used in 2mg/l of BAP plant growth regulator in MS media whose recipe has been described in section 3.4.2 plant propagation media II.

3.10 Transfer of healthy cultures to AgNPs treatments

Healthy and green orange cultures were transferred from AgNPs treated test tubes media in sterile laminar air flow hood without touching their wall in front of flame to avoid any kind of contamination. The control and treated cultures have been kept at $25\pm 2^\circ\text{C}$ with 16 hours of artificial light and 8 hours of dark cycle using fluorescent lamps with 2500 and 3000 lux intensity.

3.11 Measurement of growth parameter

The physical parameter is the primary the indicator of cytotoxicity of silver nanoparticles which was observed in term of number of leaves/roots, length of shoots and chlorophyll content as shown in figure 3.7:

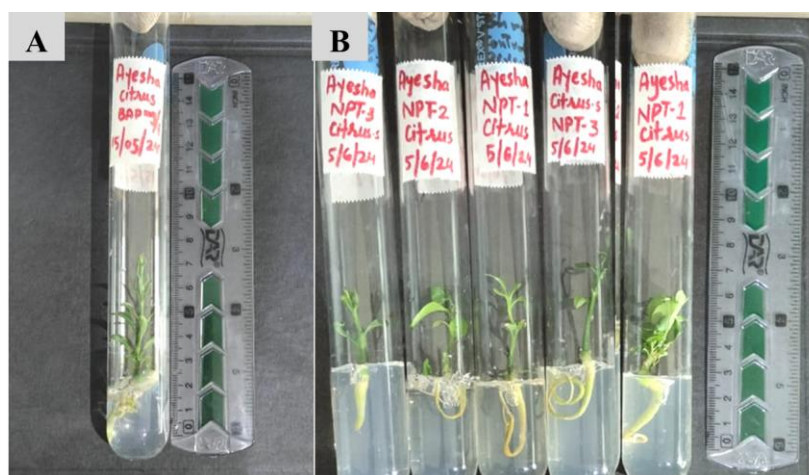


Figure 3. 7: Physical parameter evaluation: Shoot and root length was measured with the help of scale in centimeters (A), Comparison of different concentration of AgNPs treatment with control plant (B).

3.11.1 Shoot length

Shoot length of all tissue culture plants was measured in centimeters.

3.11.2 Number of leaves

Numbers of leaves of tissue cultured plants treated with AgNPs were counted and recorded in data.

3.11.3 Root length

Root length of all tissue culture plants was measured in centimeters.

3.11.4 Physical appearance

Physical appearance of orange tissue cultures was observed in term of color, shape and size of leaf.

3.12 Biochemical analysis

3.12.1 Chlorophyll content estimation

For the purpose of chloroplast estimation, orange sample from tissue culture plant and plant treated with AgNPs was used. Each sample was weighed 1g and grinded in pestle and mortar. Then these grinded samples were soaked in 10ml 80% acetone in test tubes that were covered and labeled. The tubes were kept in darkness at 25°C for 1 day. The absorbance of each sample at 663nm and 645nm wavelength was noted using UV-Visible spectrophotometer after the incubation period. The experiment was carried out in three replicates and results were recorded. The chlorophyll a and b level were calculated by using following formula:

$$\text{Chlorophyll 'a'} = [(12.21 * \text{OD at 663}) - (2.81 * \text{OD at 665})]$$

$$\text{Chlorophyll 'b'} = [(20.13 * \text{OD at 663}) - (5.03 * \text{OD at 665})]$$

$$\text{Total chlorophyll content} = \text{chl 'a'} + \text{chl 'b'}$$

CHAPTER 4

RESULTS

In this dissertation, the biosynthesis of silver nanoparticles was achieved using orange juice extract. Cultures were established by carefully selecting and preparing plant tissues under sterile conditions to ensure optimal growth and development. These biosynthesized silver nanoparticles were then tested for their efficacy as plant preservative materials in plant tissue culture medium.

4.1 DNA Extraction result for molecular identification of *Citrus sinensis*

DNA extracted from orange samples were visualized on 1% agarose gel, where M represents the Lambda HindIII and orange samples from C1-C9. 5 μ of DNA sample with 2X loading dye was used to evaluate the samples. All the samples produced bands after DNA extraction as shown in Figure 4.1.

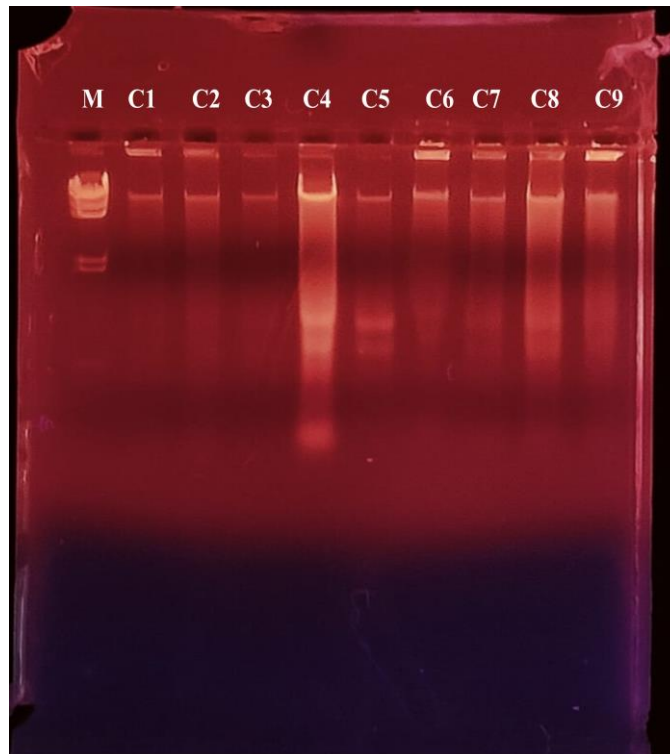
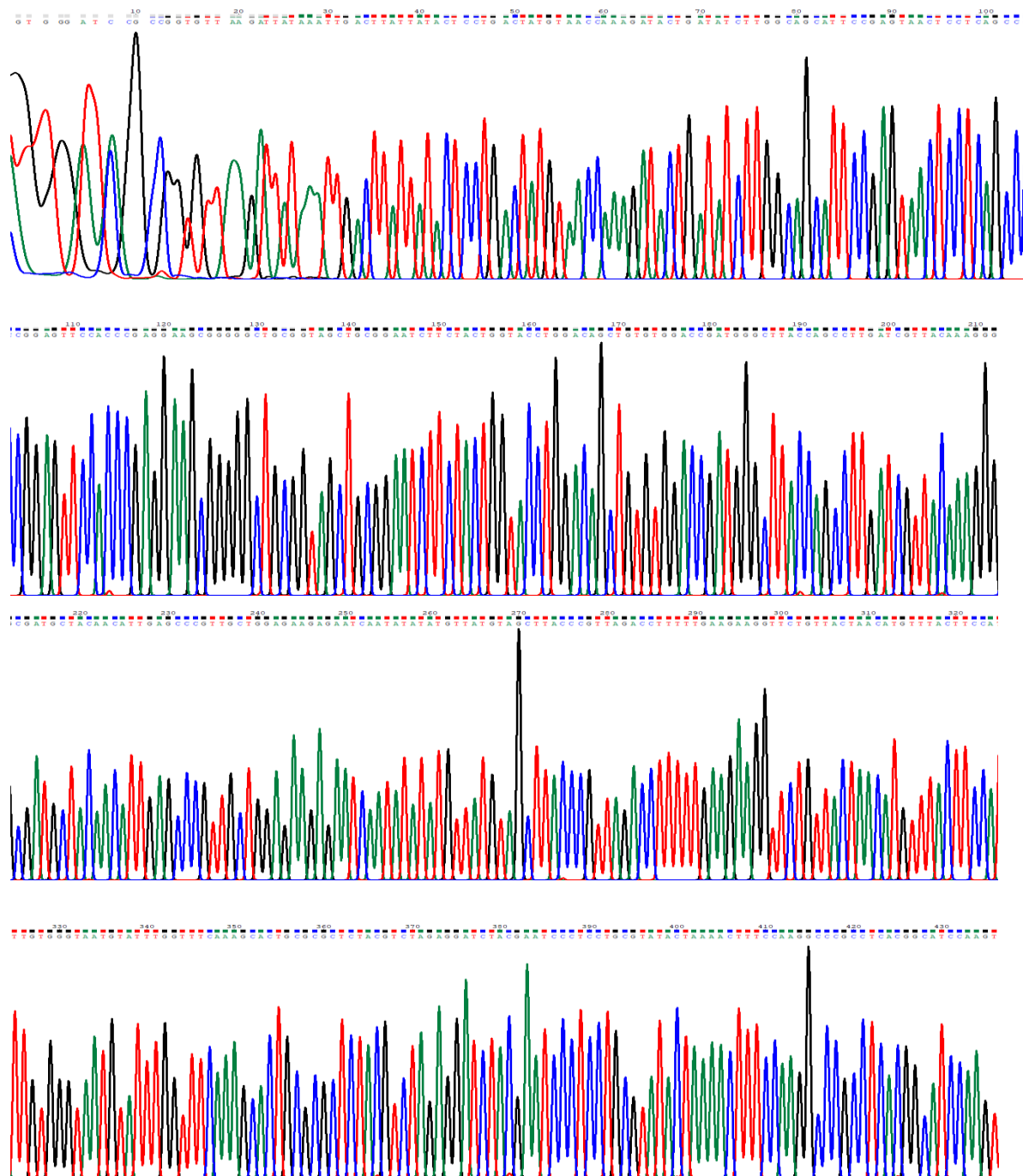


Figure 4. 1: DNA extraction, M is Lambda HindIII and orange sample from C1-C9.

4.2 Molecular identification of *Citrus sinensis*

The sequence results of *Citrus sinensis* are presented below in fig-4.2. The FASTA format DNA sequence has been taken from chromatogram and then translated BLASTn analysis reveals 99.85% homology of this sequence with published *Citrus sinensis* DNA sequencing.



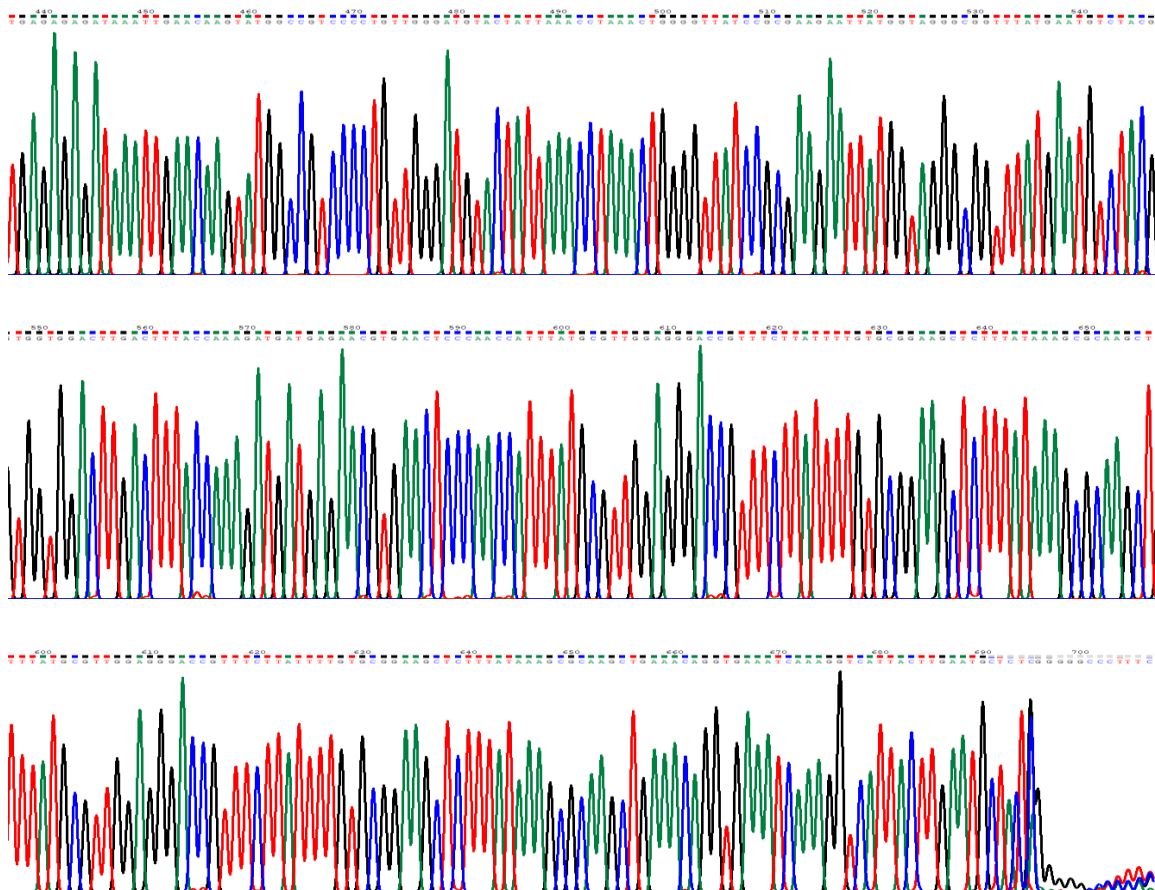


Figure 4. 2: Molecular identification of *Citrus sinensis*: Photo of chromatogram files from DNA sequencer's file.

Sequences producing significant alignments Download ▾ Select columns ▾ Show 100 ▾ ?

select all 100 sequences selected [GenBank](#) [Graphics](#) [Distance tree of results](#) [MSA Viewer](#)

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
<input checked="" type="checkbox"/> Citrus x aurantium chloroplast complete genome	Citrus x aurantium	1256	1256	96%	0.0	99.85%	160140	MT106672.1
<input checked="" type="checkbox"/> Citrus sinensis mitochondrion complete genome	Citrus sinensis	1256	1804	96%	0.0	99.85%	640906	NC_037463.1
<input checked="" type="checkbox"/> Citrus depressa chloroplast DNA complete genome isolate: Oku	Citrus depressa	1256	1256	96%	0.0	99.85%	160090	LC218435.1
<input checked="" type="checkbox"/> Citrus reshni isolate CTR ribulose-1,5-bisphosphate carboxylase/oxygenase large (rbcl) gene partial cds:...	Citrus reshni	1256	1256	96%	0.0	99.85%	705	MG025934.1
<input checked="" type="checkbox"/> Citrus x changshan-huyou chloroplast complete genome	Citrus x changs...	1256	1256	96%	0.0	99.85%	160186	PP905390.1
<input checked="" type="checkbox"/> Citrus limon plastid complete genome	Citrus x limon	1256	1256	96%	0.0	99.85%	160101	NC_034690.1
<input checked="" type="checkbox"/> Citrus maxima plastid complete genome	Citrus maxima	1256	1256	96%	0.0	99.85%	160133	NC_034290.1
<input checked="" type="checkbox"/> Clymenia polyantra 8280 chloroplast DNA complete genome	Clymenia polya...	1256	1256	96%	0.0	99.85%	160597	LC794896.1

Figure 4. 3: 99.85% homology shown the sample (*Citrus sinensis*) with published data.

4.3 Establishment of orange callus culture

In the experiment, 1mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2mg/l of 2,4-D were introduced into Murashige and Skoog (MS) medium to initiate callus formation through the utilization of apical shoots and nodal sections. It was determined that MS media containing 2mg/l 2,4 D was found to be the more effective for inducing callus and promoting callus growth when explants (such as apical shoots and nodal sections) were inoculated. The examination revealed that the texture of the callus varied, being friable in apical shoots and compact in nodal sections.

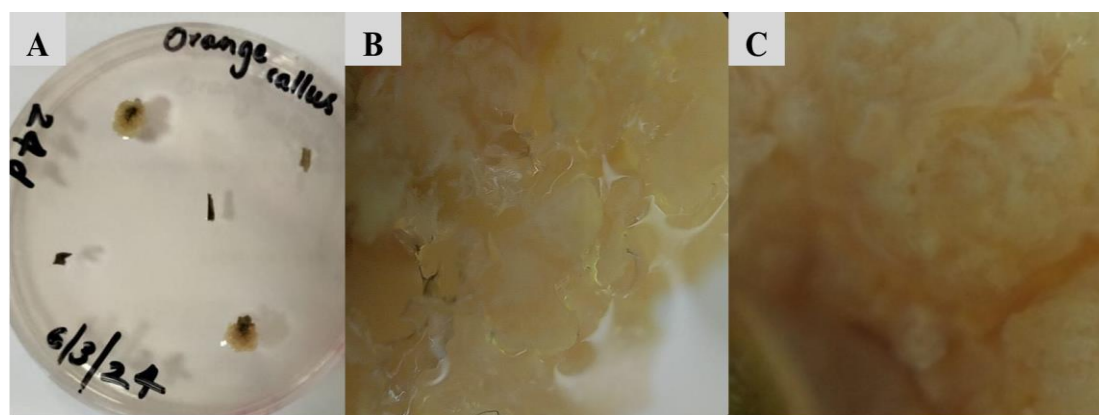


Figure 4. 4:Callus formation in nodes (A), Magnified sections of friable callus in figure (B), (C).

4.4 Establishment of orange plant micropropagation

Orange was effectively cultivated in a tissue culture laboratory at the Center for Applied Molecular Biology, Punjab University Lahore, under aseptic conditions. Plant propagation media II demonstrated a higher level of shoot growth with branching in comparison to plant propagation media I and plant propagation media III. The addition of BAP to MS media was utilized for the purpose of promoting root and shoot propagation to achieve the desired growth. The culture was developed using a combination of 1mg/l BAP, 2mg/l BAP, and 2mg/l BAP in combination with 2mg/l 2, 4 D. Among these, the utilization of 2mg/l BAP proved to be more effective when compared to alternative plant propagation media.

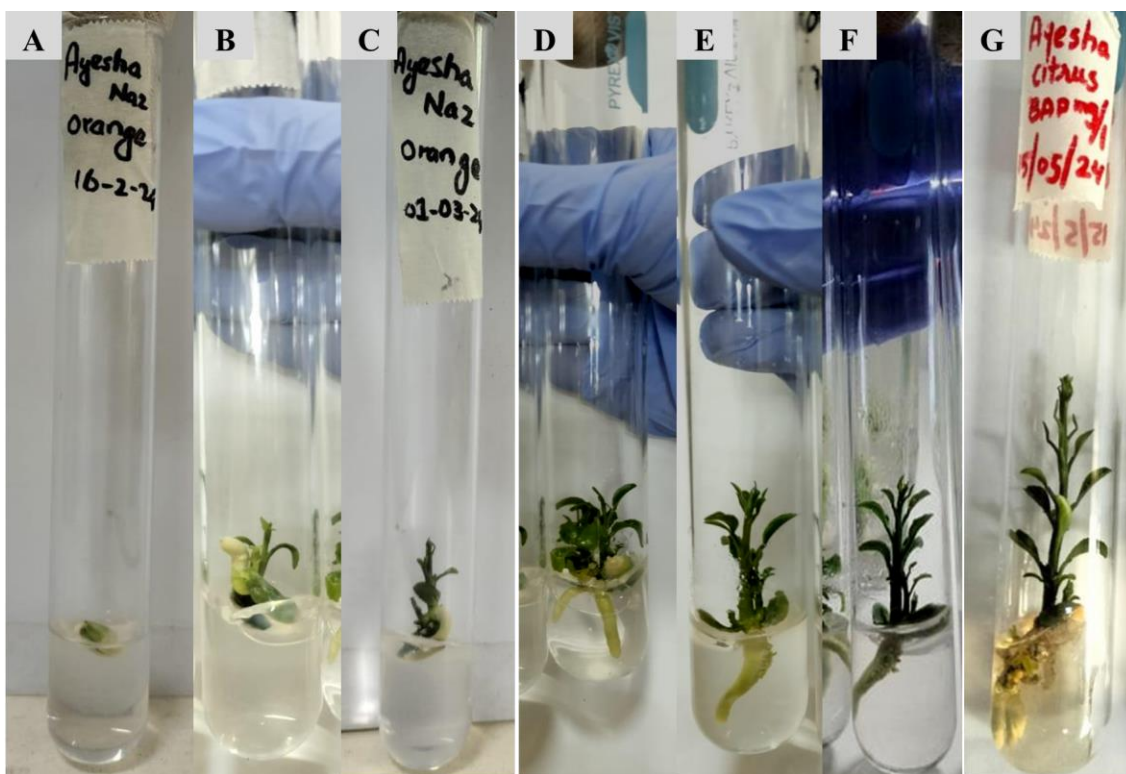


Figure 4. 5: Growth stage of orange plant during micropropagation from left to right in 2mg/l BAP media: Inoculation of seed in test tube (A), growth shown in 7 days of inoculation (B), growth shown after 14 days (C), Multiple shooting stage was observed after 20, 24 days and 28 days (D, E, F, G).
Microplants ready for AgNPs treatment.



Figure 4. 6: Microplants of *Citrus sinensis* cultured in CAMB lab.

4.5 Induction of roots and shoots from the calli

After 14 days of inoculation, shoot developed. Within 21 days of the inoculation, roots developed. It took the roots 28 days to mature.

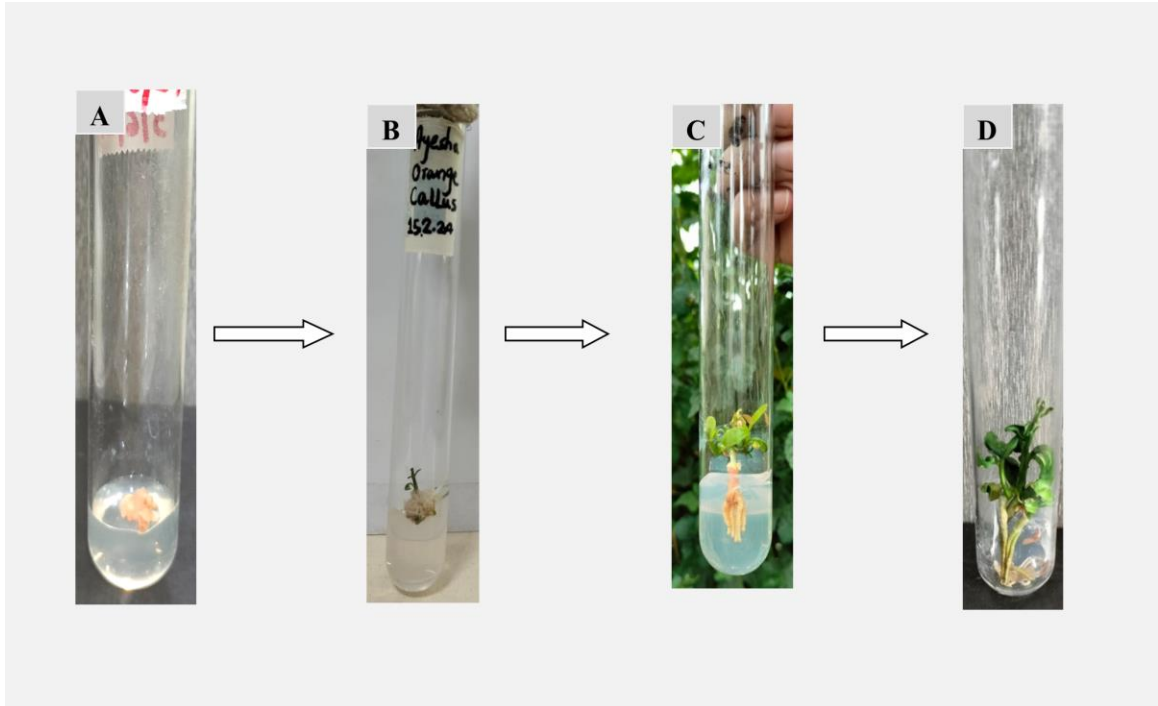


Figure 4. 7: Inoculation of callus (A), Shoots growth start after 7 days of inoculation (B), Roots and shoots develop after 15 days of inoculation (C), orange plants fully develop after 21 days (D).

4.6 Optimization of Synthesized Silver Nanoparticles using Orange Juice Extract

The evaluation of AgNPs produced using orange juice extract can be effectively conducted through visual examination of the change in color within the reaction mixture. The color of the seven prepared ratios v/v, such as 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, and 1:20, has changed since the appropriate volume of silver salt was added to each ratio. In contrast to other ratios, the most immediate reddish-brown color was only seen in 1:4, indicating earlier stability and capping of silver nanoparticles. All of these ratios' stability and surface plasmon resonance bands were measured using a UV-visible spectrophotometer between 300 and 600 nm in wavelength.



Figure 4. 8: Indication of colour change in different ratios v/v (JE/SNS): The reddish-brown colour is visible in 1:4 ratio.

4.6.1 Optimization of the UV-Visible spectra of silver nanoparticles through the utilization of orange juice extract

The surface plasmon resonance bands of all seven reaction mixtures were recorded. These prepared ratios showed different absorbance at 450 nm wavelength. The SPR bands have been recorded from time to time and it is evident that 1:4 has provided desirable absorbance of 2.592 at 450nm whereas 1:1, 1:2, 1:3, 1:5, 1:20 showed

absorbance range between 1.3-1.8. 1:7 has provided distorted peak.

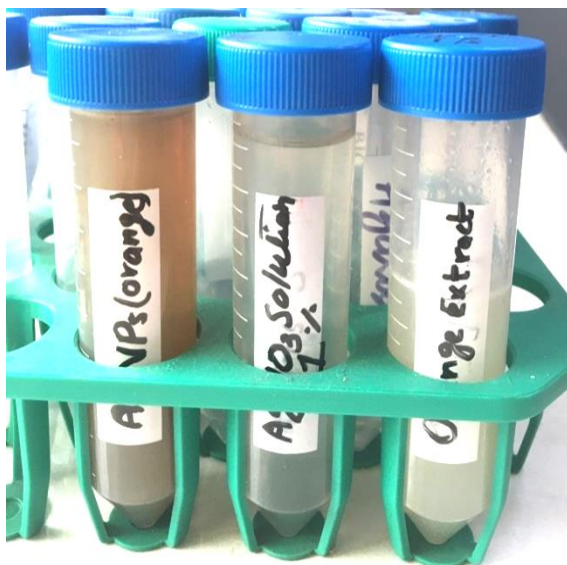


Figure 4. 9:Indication of reddish-brown colour change in orange juice extract + Ag Nitrate solution.

4.7 Synthesis of AgNPs using Orange juice extract

The production of silver nanoparticles through the utilization of orange juice extract was conducted by combining 5ml of the extract with 20ml of silver nitrate salt in order to monitor the transformation of color to a reddish brown hue.

4.7.1 Analysis of UV-Visible spectroscopy using extracts of orange juice

In order to determine the stability of the synthesized silver nanoparticles, the reaction solutions including orange juice extract at a ratio of 1:4 were prepared and then analyzed at 10, 30, 60, and 120 minutes. After 120 minutes, the juice extract reaction mixture showed distinct peaks and attained its maximum stability. The juice reaction mixture's UV-visible spectra, obtained after 10, 30, 60, and 120 minutes. The UV-Visible spectra of the reaction mixture of juice recorded after 10, 30, 60 and 120 minutes.

4.8 Molecular characterization of AgNPs

4.8.1 Zeta Sizer Potential Analysis

Particle size allocation and zeta sizer potential values of the silver nanoparticles

synthesized from orange juice have a zeta average mean size 217.2 nm and polydispersity index (PDI) of 0.336. The PDI value shows the average uniformity of silver nanoparticles, illustrating their agglomeration and homogeneity across the sample. A wide size distribution is showing by a PDI value greater than 0.7, whereas a mono-dispersed distribution is indicating by a value less than 0.1. The electric charge of the silver nanoparticles is showed by its Zeta potential. This value gives information about the stability of the particles. The graph of the Zeta potential distribution is presented in Figure 4.10. The Zeta sizer Potential of orange juice, silver nanoparticles was revealed to be -23.8 mV. The negative zeta potential in my results represented repulsion between particles and the stability of the colloidal state.

Table 1: Size distribution of synthesized Silver nanoparticles using orange juice extract.

Name	Value
Z-Average (nm)	217.2
PDI	0.336
Count rate (keps)	520.8
Intercept	0.819

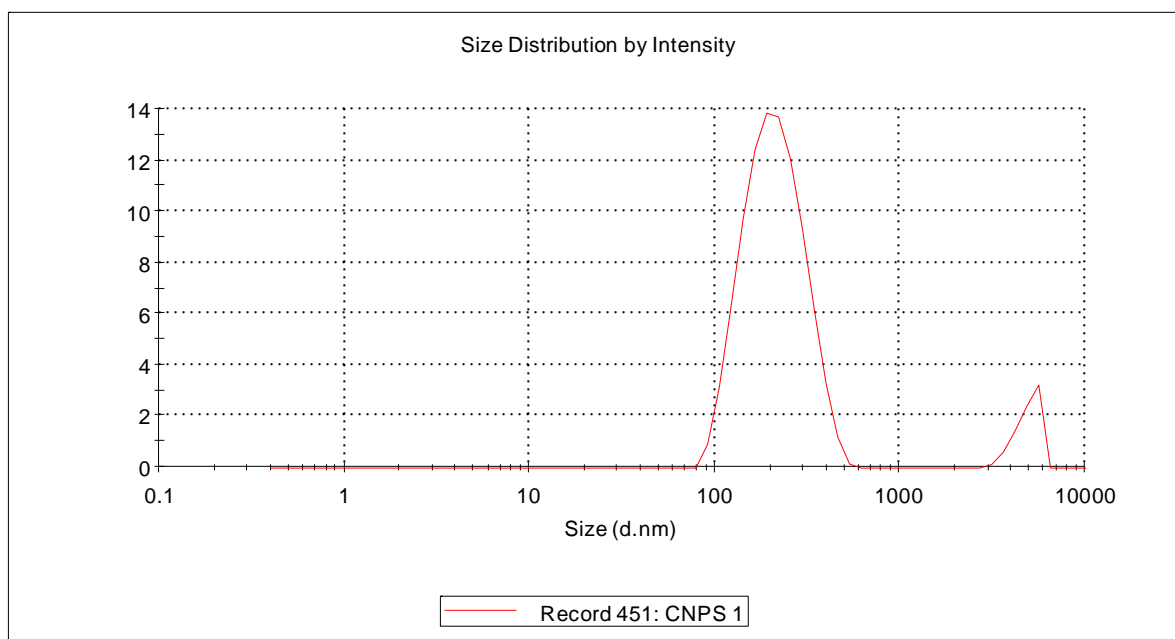


Figure 4. 10: Zeta average size potential by intensity of synthesized AgNPs using orange juice extract.

Table 2: Zeta sizer potential of AgNPs from orange juice extracts showed the stability of AgNPs.

Name	Value	Means (mV)	Area (%)	SD
Zeta Potential (mV)	-23.8	-23.8	100.0	5.14
Zeta Deviation (mV)	5.14	0.00	0.0	0.00
Conductivity (mS/cm)	0.0181	0.00	0.0	0.00

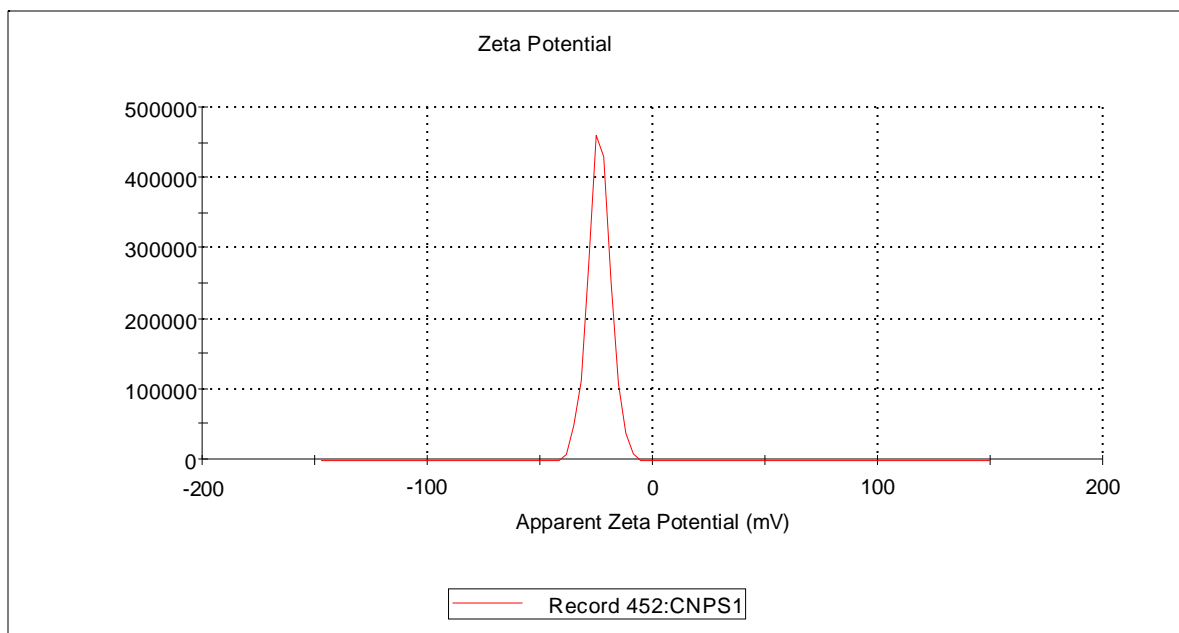
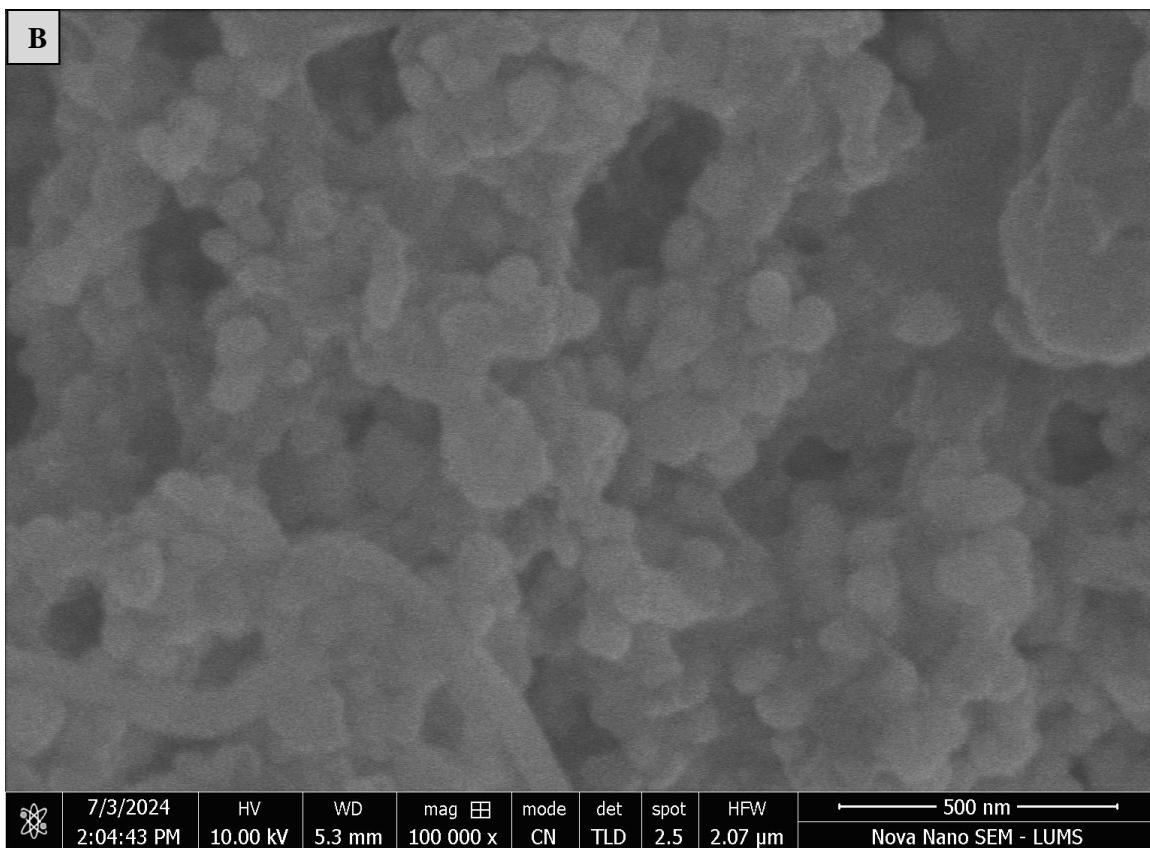
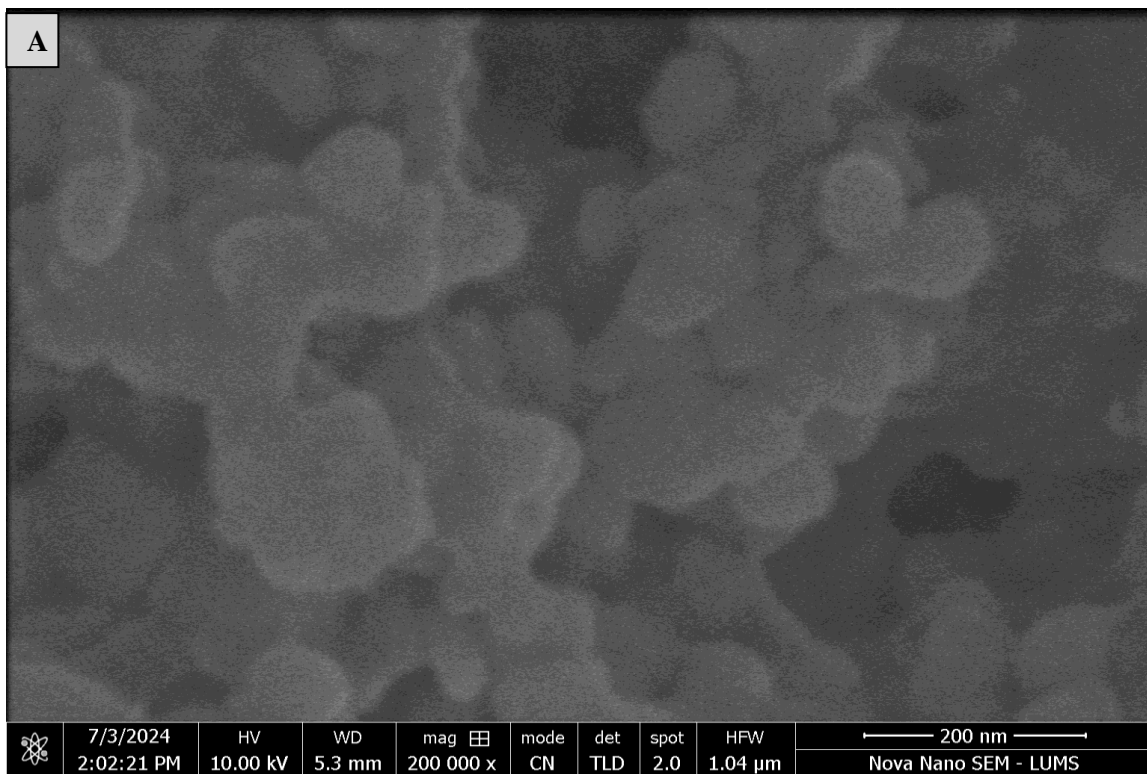


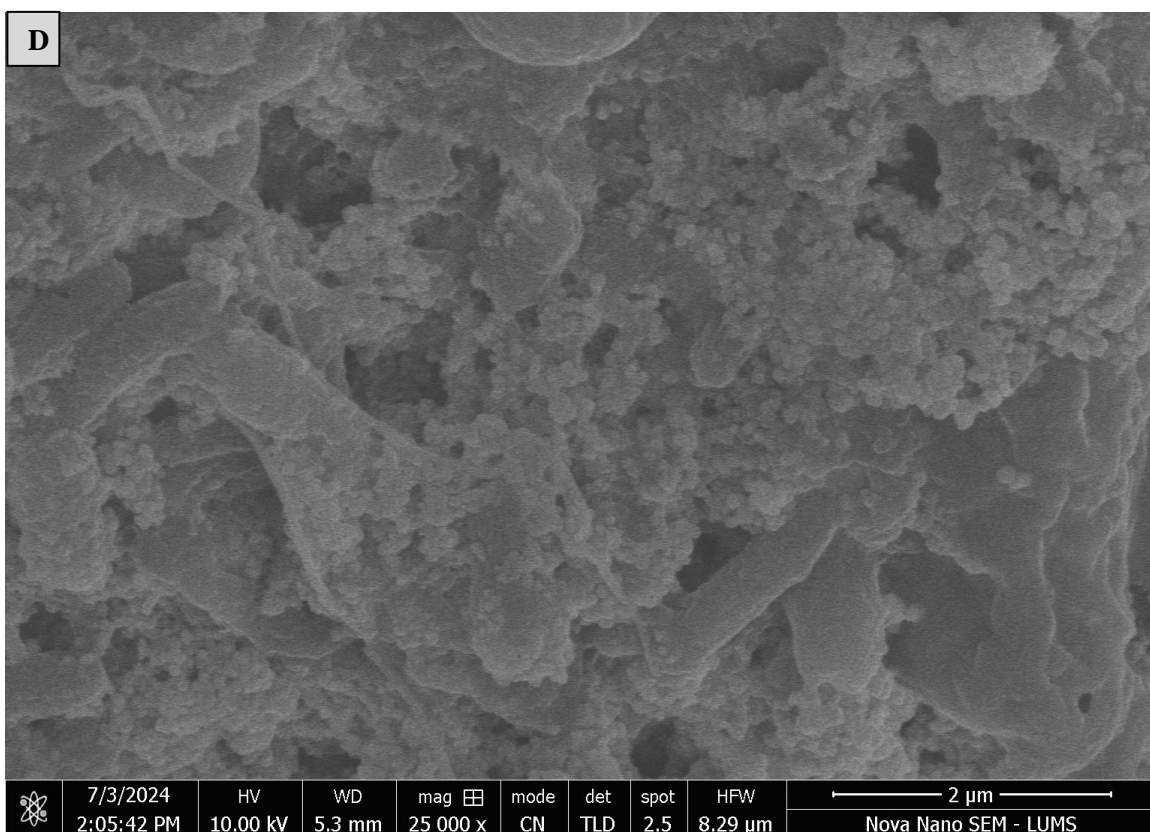
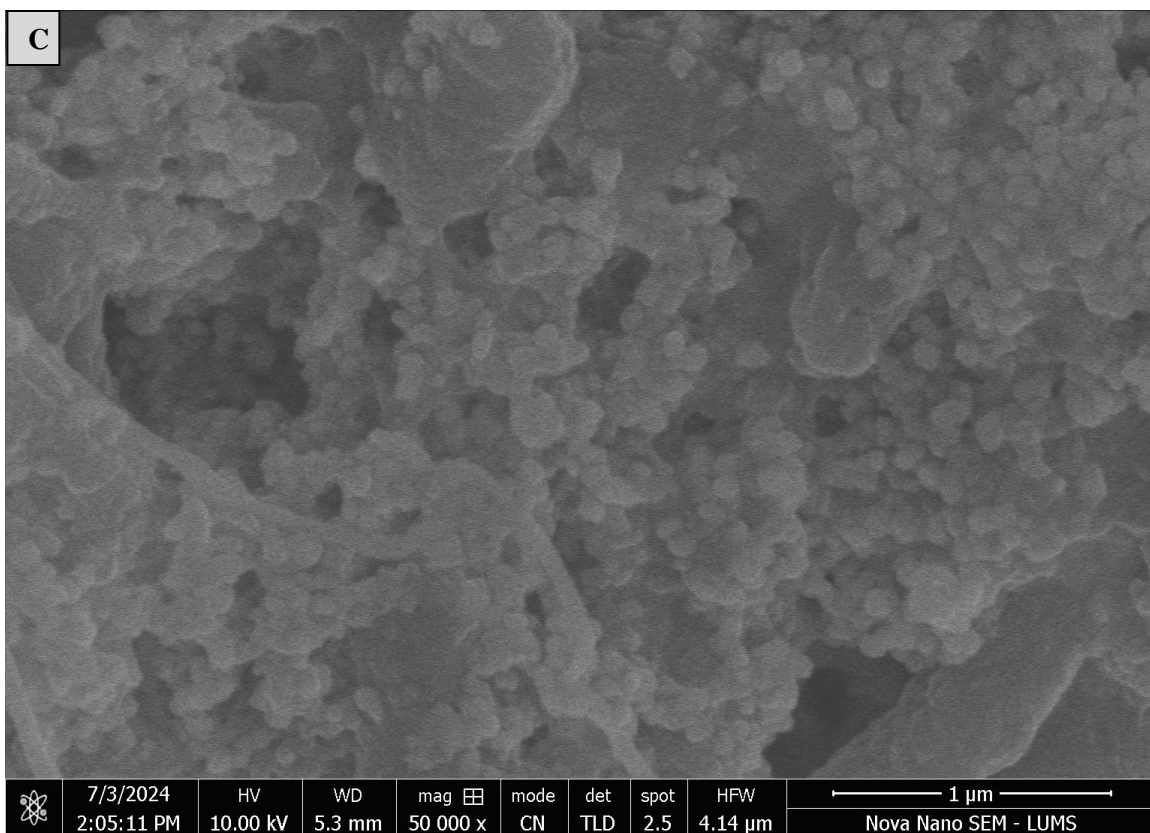
Figure 4. 11: Zeta sizer potential of AgNPs from orange juice extract shown negative value which indicated the stability of SNPs.

4.8.2 Scanning Electron microscopy

The morphology and the size of the synthesized silver nanoparticles using orange juice extract have been characterized by a scanning electron microscope. The SEM micrograph morphology of synthesized AgNPs at five different resolutions has been showed in Figure 4.12. The findings of molecular characterization of AgNPs through SEM micrograph exposed the size of silver nanoparticles of 37.42nm have been synthesizing with circular shape. The SEM micrograph revealed that the synthesized silver nanoparticles are homogenous in nature. The findings support my findings of the UV-Vis spectra, as AgNPs are reported to have UV-Vis spectra in the 400-450nm.

The SEM micrograph has studied using Image J software (Java 1.8.0_345, 64-bit Java 8) to determine the average possible size of synthesized silver nanoparticles by inserting the micrograph of 500nm resolution in the software. The acquired data has been analyzed through origin software (Version Origin pro 2024b) and represented in histogram distribution.





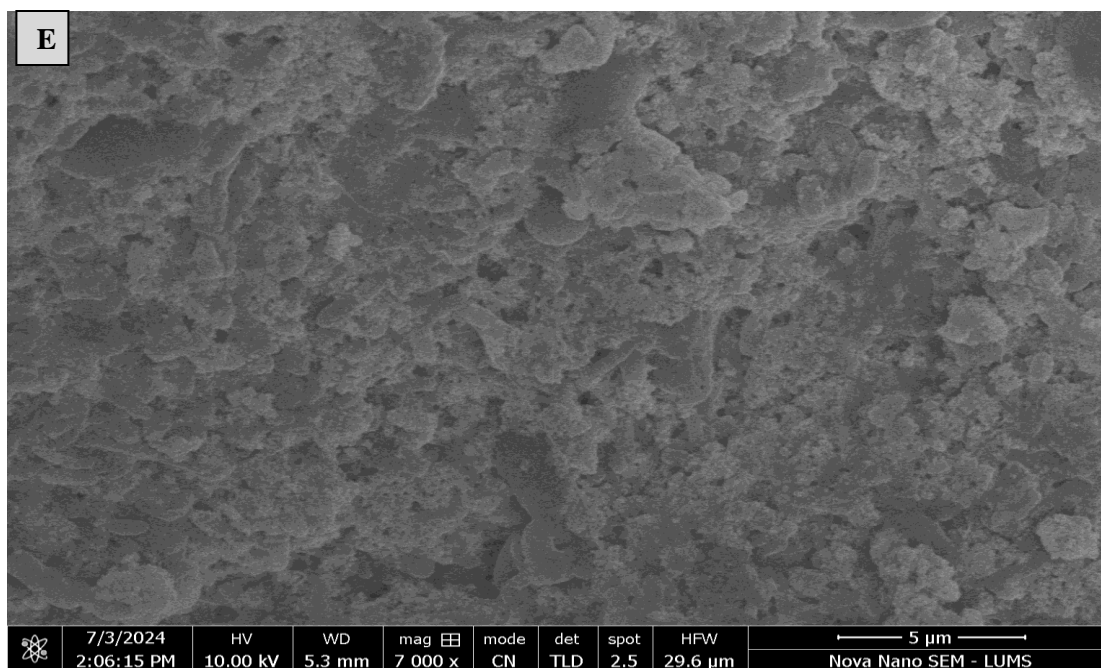


Figure 4. 12:SEM micrograph of synthesized silver nano particles using orange juice extract at 200nm (A), SEM micrograph of synthesized silver nano particles using orange juice extract at 500nm (B), SEM micrograph of synthesized silver nano particles using orange juice extract at 1 μ m (C), SEM micrograph of synthesized silver nano particles using orange juice extract at 2 μ m (D), SEM micrograph of synthesized silver nano particles using orange juice extract at 5 μ m (E).

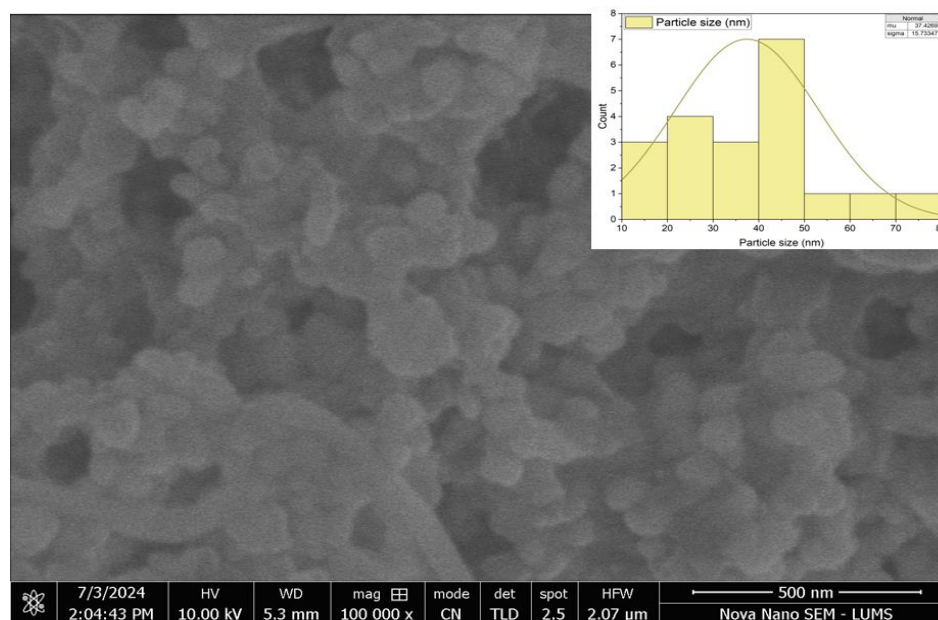


Figure 4. 13: Average size distribution of synthesized AgNPs from orange juice SEM micrograph at 500nm resolution have 37.42nm size.

4.8.3 Energy Dispersive X-Rays

In order to confirm the formation of pure elemental silver (Ag) in the synthesized from orange juice extract, an energy dispersive x-ray has been employed at 2.5 KeV. The spectral data has been analyzed that the weight percentage of silver element (L series) in the synthesized silver nanoparticles from orange juice extract is 33.06 % in figure 4.12. The EDX spectrum and table showing the percentage of elemental content found in sample of AgNPs and table no 4.7.

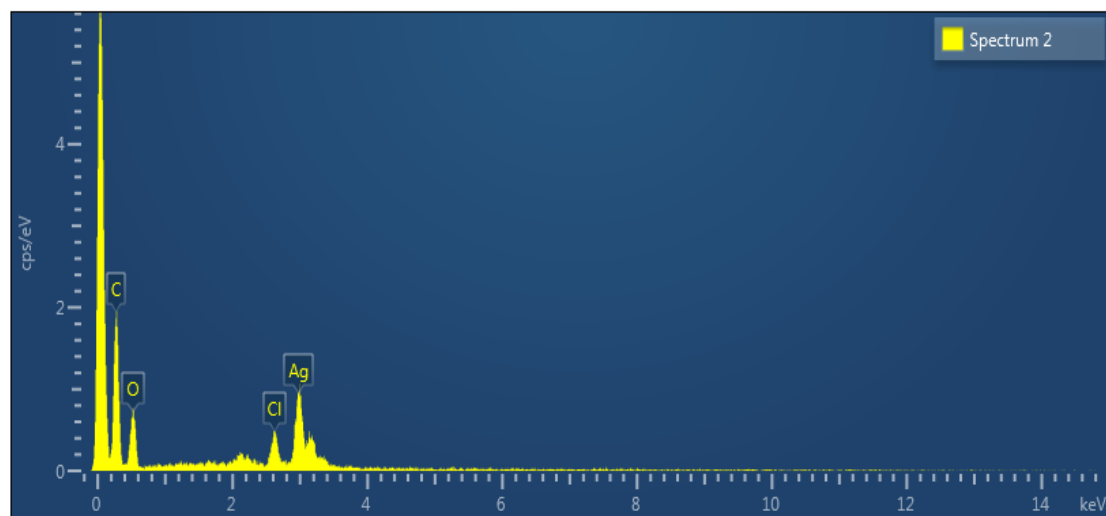


Figure 4. 14: EDX showing a Peak of pure silver element among other identifiable element in the sample of AgNPs.

Table 3: Percentage and apparent concentration of recognizable elements in the sample of AgNPs from orange juice extract.

Element	Line type	Apparent concentration	K Ratio	Wt%	Wt% sigma	Standard label	Factory standard
C	K Series	10.32	0.10323	39.05	1.81	C Vit	Yes
O	K	7.06	0.02376	23.82	1.77	SiO ₂	Yes

	Series						
Cl	K Series	2.17	0.13498	4.07	0.34	NaCl	Yes
Ag	L Series	13.5	0.13498	33.06	1.48	Ag	Yes
Total				100			

4.9 Silver nanoparticles treatments in micropropagation media

Three appropriate concentrations of silver nanoparticles 0.002 g/l, or 2 ppm, 0.003 g/l, or 3 ppm, and 0.005 g/l, or 5 ppm were given to orange plants in micropropagation media after the bulk production of biosynthesized silver nanoparticles using orange juice extract, established as treatments I, II, and III, respectively. In orange tissue culture, all of these treatments demonstrated the antibacterial and antifungal action of silver nanoparticles. They assault the cell wall or membrane of microorganisms, causing oxidative stress. When compared to other treatments, Treatment II exhibits the most beneficial response.

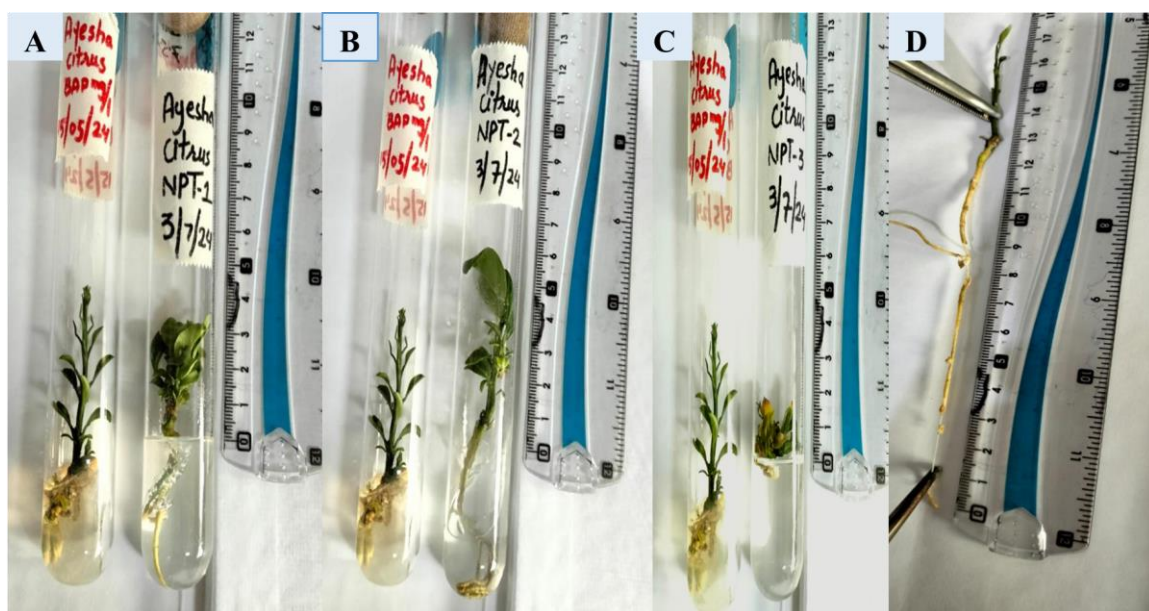


Figure 4. 15: Physical parameter evaluation: Shoot length was being measured with the help of scale in

centimeter in T1 (A), T2 (B), T3(C), Root length was being measured in 3ppm dose of AgNPs (D).

4.10 Evaluation of silver nanoparticles impact on orange plant culture

Silver nanoparticles can enter cells through the cell membrane and wall because of their diminutive size. Several factors were examined in order to assess the potential cytotoxicity of silver nanoparticles at different concentrations. These parameters include the following:

4.10.1 Growth parameter

Shoot length, number of leaves, and fresh weight were among the growth parameters that were recorded in the data. Microsoft Excel was used to apply total and averages.

4.10.2 Shoot length

Using a centimeter scale, the shoot length of three orange plant replicates was measured. Due to the interaction of silver nanoparticles with orange plants cultivated in test tubes, treatment II showed an improvement in shoot length, with shoot length surpassing that of the control plants, as indicated in table:

Table 4: Comparison of length of shoots in treatment I, II, III along with control.

	Shoot length (cm)			Mean length
Control	5.1	5.3	4.8	5.0
T ₁	2.9	3	3.3	3.0
T ₂	5.3	5.5	5.2	5.3
T ₃	2.0	2.2	1.5	1.9

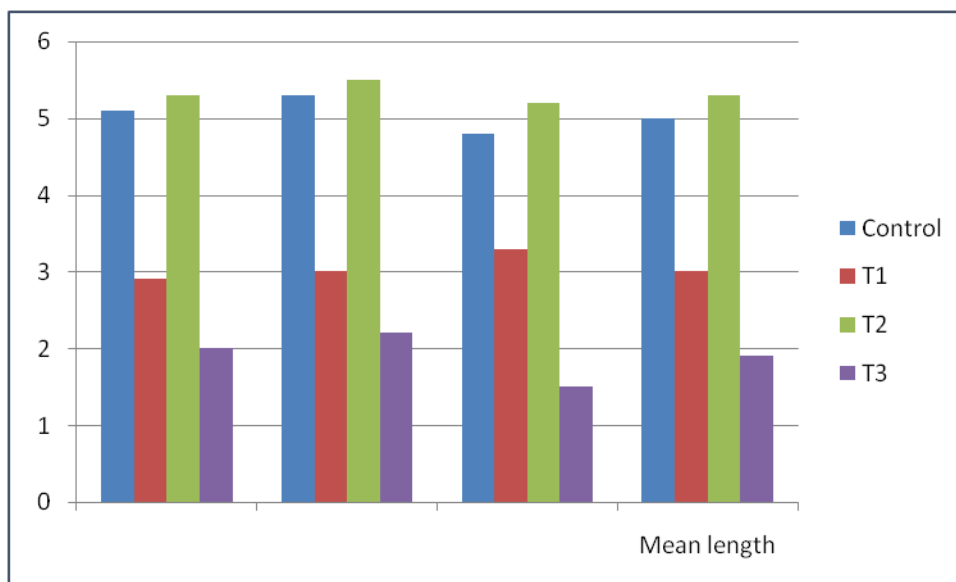


Figure 4. 16: Comparison of length of shoots in treatment I, II, III along with control.

4.10.3 Numbers of leaves

The number of leaves on all treated cultured plant was carefully recorded. This data provided insight into the effects of silver nanoparticles on leaf production and overall plant health. Treatment II showed an improvement in number of leaves as compared to other treated plants, as indicated in table:

Table 5: Comparison of number of leaves in treatment I, II, III along with control.

	Number of leaves			Mean
Control	28	25	29	27.3
T ₁	25	23	26	24.6
T ₂	29	31	28	29.6
T ₃	15	9	8	10.6

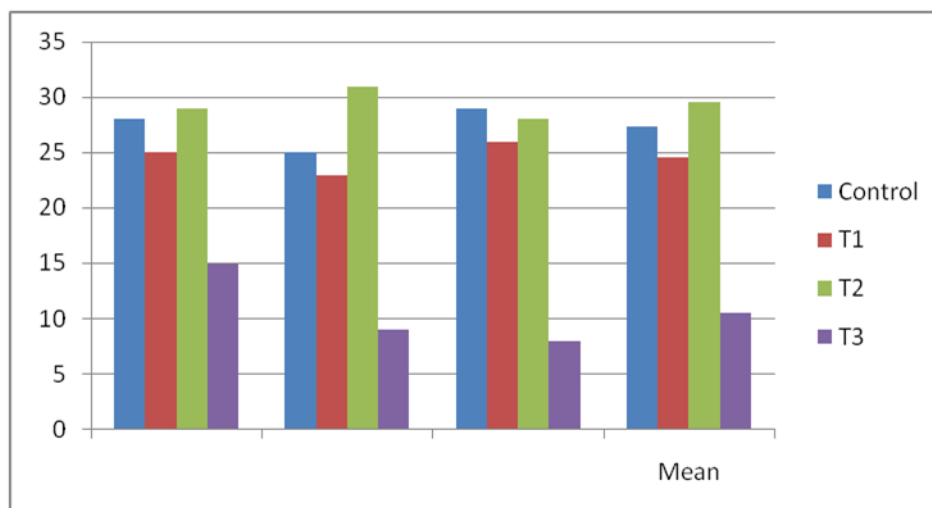


Figure 4. 17: Comparison of number of leaves in treatment I, II, III along with control.

4.10.4 Root length

The root lengths of three orange plant replicates were measured using a centimeter scale. Due to the interaction of silver nanoparticles with the orange plants cultivated in test tubes, treatment II demonstrated an improvement in root length, surpassing the root lengths of the control plants, as indicated in the table:

Table 6: Root length of orange plants in treatment I, II, III along with control.

	Root length (cm)			Mean length
Control	14	15	13.5	14.16
T ₁	12.5	11.7	10.5	11.05
T ₂	15	14.5	16.5	15.3
T ₃	8.5	5.5	7.5	7.16

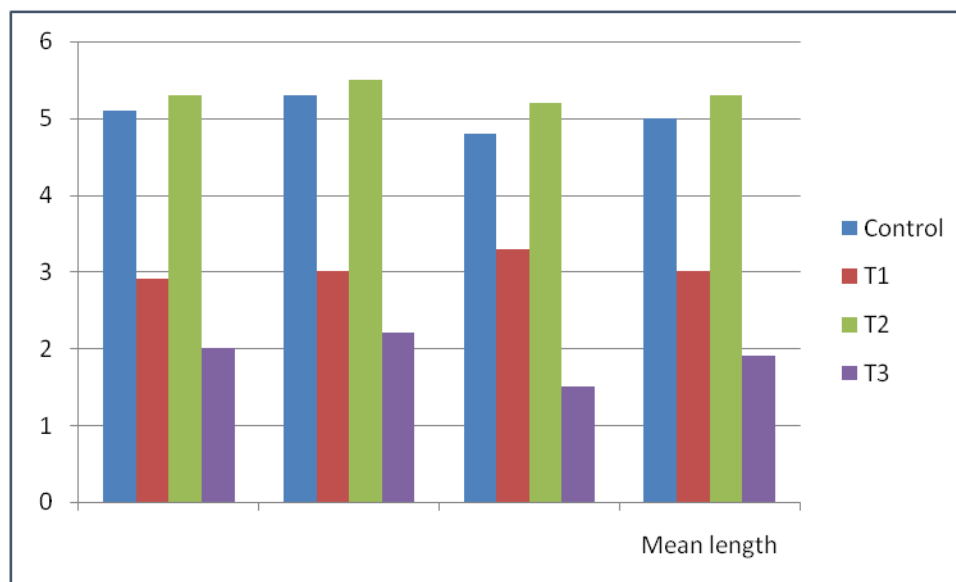


Figure 4. 18: Comparison of root length in treatment I, II, III along with control.

4.10.5 Chlorophyll content

The chlorophyll content of each of the three treatments was measured in replicates, reported in the table below and evaluated using statistical analysis. To determine the impact of silver nanoparticles on the photosynthetic activity and metabolism by measuring the amount of chlorophyll.

Table 7: Chlorophyll estimation (a, b and total chlorophyll content)

Parameters	Pot plant	Control plant	Treatment I	Treatment II	Treatment III
Chlorophyll a	17.5	5.0	4.2	6.41	0.84
	17.51	4.8	4.23	6.45	0.5
	18.2	4.5	4.21	6.32	0.2
Avg	17.75	4.7	4.3	6.39	0.51

Chlorophyll b	22.6	8.15	6.78	12.5	1.3
	22.65	8.04	6.56	12.52	1.35
	22.5	8.14	6.12	12.56	1.32
Avg	22.5	8.11	6.48	12.52	1.32
Total chlorophyll content	40.1	13.1	10.9	18.91	2.14
	40.16	12.8	10.7	18.97	1.85
	40.7	12.6	10.3	18.88	1.52
Avg	40.32	12.8	10.6	18.92	1.83

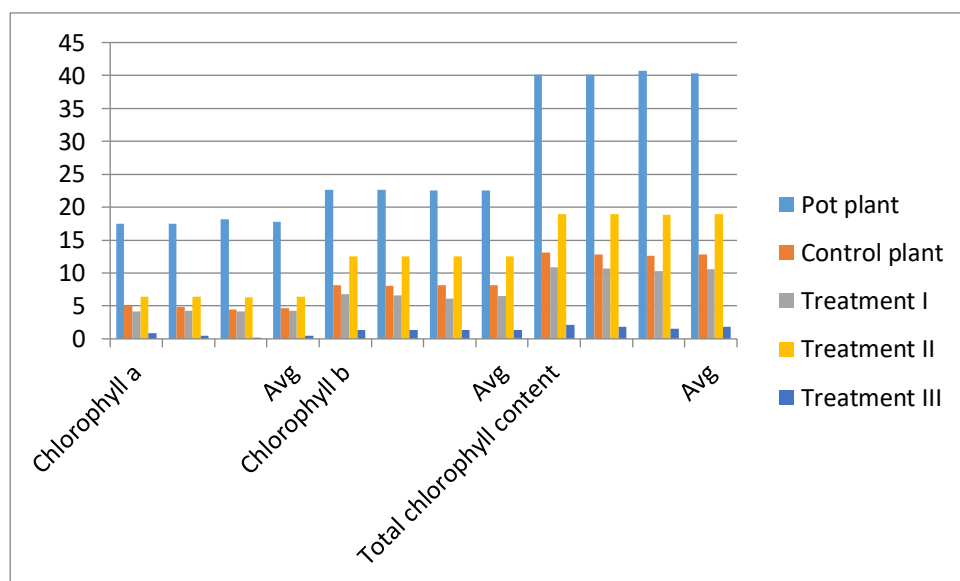


Figure 4. 19: comparison of chlorophyll content along with pot and control plant and treated plant.

4.11 Estimation of contamination with SNPs and control

Microbial contamination in tissue culturing is a significant issue, resulting in the loss of resources and germplasm. When contamination occurs in culture with SNPs, the microplants may survive, but without AgNPs, the plants typically die as shown in figure 5.9. The survival rate of cultures with AgNPs is higher as compared to control as described in table 4.8:

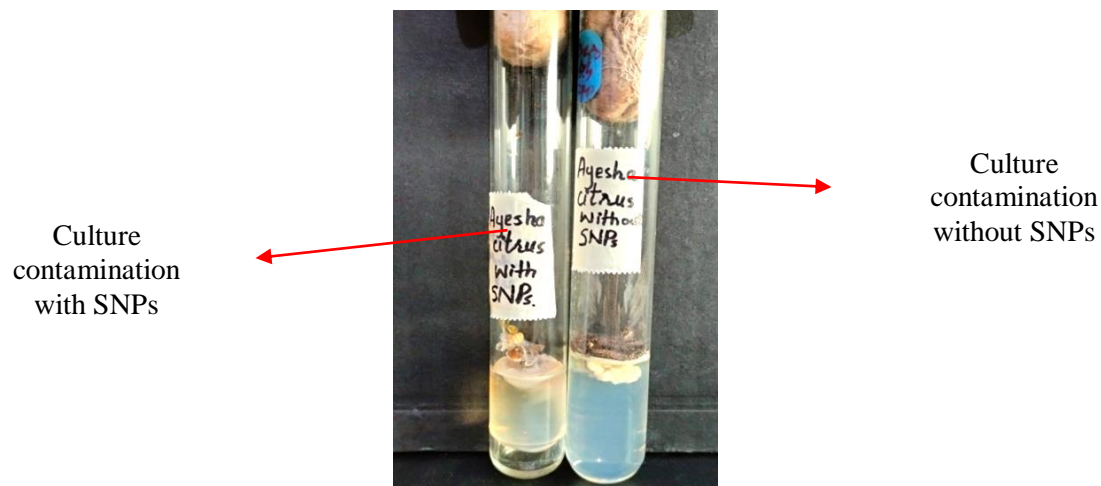


Figure 4. 20: Culture contamination with SNPs and without SNPs

Table 8: Determination of antimicrobial potential of AgNPs treatments on *in vitro* cultures of *Citrus sinensis*: Percentage calculated after 60 days of incubation in MS medium supplemented with 2mg/l BAP having AgNPs in treated plant and compared with control.

	Total no. of plants studied	Total no. of plants survived	Survival percentage
Exposure to treatment-1	50	42.5	85%
Exposure to treatment-2	50	48	96%
Exposure to treatment-3	50	35	70%
Control plant group	80	42.4	53%

CHAPTER 5

DISCUSSION

5.1 Methodology for synthesis of silver nanoparticles by using orange plant material

Methodology detail and reaction mixture of metal salt and the reducing agents of the relevant plant of the silver nanoparticles information are available. A particular plant has particular phytochemicals of specific nature; which contribute in synthesis of SNPs. Various ratios have been prepared in this research to evaluate the biogenic synthesis of silver nanoparticles. The primary methodologies are explained in studies published by Jackson *et al.*, (2018); Jackson *et al.*, (2018) has been followed in this dissertation and various ratios have been prepared. In these findings little modified the methodology of Jackson *et al.*, (2018) as seven different ratios of silver nitrate (1% silver nitrate solution) and juice extract of plant have been prepared. In this study, the extract concentration has been kept constant and by varying concentration of silver nitrate solution from 1 to 20ml to investigate the most suitable ratio that can be served to grassroots approach [25]. The findings of studies provided that successful results in 1:4 out of seven ratios prepared reaction mixture of orange juice extract and 1% silver nitrate salt which is close align with the results Arooj N, *et al.*, (2014) [34].

The appearance of reddish brown colour in the reaction mixture of silver nanoparticles and fruit juice has been reported by Jackson *et al.*, (2018), Khane Y, *et al* (2022) [35], [32]. In the presented work, 1:4, 1:5 and 1:6 have shown reddish brown colour, hence, these findings are closely related to Jackson *et al.*, (2018) [25] and Arooj N, *et al.*, (2014) [34], contrary to it remaining reaction mixture of 1:2,1:3 and 1;1 of the presented work have shown the grayish colour.

5.2 Characterization of silver nanoparticles

The silver nanoparticles synthesis from orange juice extract has been characterized through UV-Visible spectroscopy and the surface plasmon resonance bands in the presented work are recorded at 450nm which has also been described by Jackson *et al.*,

(2018) [25].

The zeta sizer potential of silver nanoparticles from orange juice has shown negative value which indicated the stability of SNPs. The zeta potential of SNPs from orange juice has been -23.8 and zeta deviation has been 5.14 that are proximity to the work described by Khane Y, *et al* (2022) [35]. The data reported by Khane Y, *et al* (2022) on the stability of SNPs has demonstrated that the PDI index has been 0.542 which is closely align with my findings. The SEM micrographs at various resolutions in μm and nm of AgNPs synthesized from orange juice that served as a better substrate to bring change in the oxidation state of silver nanoparticles as observed in results that it produced defined morphology of circular shape and homogeneity of silver nanoparticles. Moreover the EDX spectrum has shown the peak of pure silver element in the L-series and other elements as C, O and Cl in K-series.

5.3 Establishment of orange cultures

The research work's objective was to improve the culture condition for *Citrus sinensis* micropropagation and callus induction techniques. The findings of this study indicate that shoot and root productions have been achieved on MS medium supplemented with different concentration of BAP. 2mg/l BAP has shown the most effective supplement as compared to 1mg/L BAP. The study outcome is close to the findings of Fazle, Azam *et al.*, (2011) who conducted the micropropagation of orange form seeds at different concentration of BAP and Kin with MS media.

The findings of this research works indicated that the callus induction have been achieved on MS media supplemented with 1mg/l 2, 4 D and 2mg/l 2, 4 D. The rapid and more callus induction have been occurred in MS media supplemented with 2mg/l. This finding closely aligned with Fazle, Azam *et al.*, (2011). MS medium supplemented with 2mg/l BAP for shoot induction and 2mg/l 2, 4 D for root induction from callus which inoculated in test tubes. After 28 days, mature microplants have been developed [17].

5.4 Establishment of orange culture supplemented with silver nanoparticles

The anti-contaminant potential of AgNPs on the growth of orange microplants and growth medium were purposed to be reported. Silver nanoparticles with three different concentrations were added to MS media containing 2mg/l BAP for this purpose. It can be determined that all three concentration (2ppm, 3ppm, 5ppm) proved their antimicrobial and antifungal activity as they controlled all contamination in plant tissue culture laboratory. Tung *et al.*, (2021) determined silver nanoparticles to be antimicrobial agent in MS media when Chrysanthemum apical meristems on MS media supplemented with 4.0 ppm of silver nanoparticles [36]. Abdi G, *et al.*, (2008) proved silver nano (NS) to be used in MS media when valerian (*Valeriana officinalis* L.) explants were cultured on MS medium supplemented with 5 mg l⁻¹ Kin and 0.1 mg l⁻¹ NAA and using 100 mg l⁻¹ of NS solution, it was concluded that NS had a good potential for removing of the bacterial contaminants in plant tissue culture procedures [37].

4.5 Impact of AgNPs on growth parameter of orange

Improvement of shoot and root length of orange plants when grown in medium containing silver nanoparticles was observed. The improvement in shoot length was closely related to the result of Hegazi ES, *et al.*, (2021) cleared that silver nanoparticles (AgNPs) at 5 mg L⁻¹ recorded the highest percentage of bud sprouting, shoot length, number of shoots/explant and number of leaves of in vitro propagation of Picual Olive [38]. El-Mahdy, *et al.*, (2019) evaluated that the banana explants were cultured in MS medium containing various concentrations of AgNPs or AgNO₃ (0, 1, 5, 10, 50, 100, and 200 mg/l). The results appeared that lesser concentrations of both AgNPs and AgNO₃ had an invigorating impact on the growth of the plantlets as well as pigments content, whereas the greater concentrations initiated an inhibitory effect which is closely aligned with our research work and high concentration has been considered the lethal effect on orange microplants [39]. Aghdaei M, *et al.*, (2012) evaluated the effects of silver nanoparticles (SNPs) at concentrations ranging from 5 to 80 mg/l on MS media on growth properties of *T. undulata* in aseptic condition. Adding of SNPs in MS medium

increased the mean number of fresh shoots per explants (MNFS/E), the percentage of explants producing shoots (PEPS) and also plant survival, due to its action on ethylene blockage [40]. The results of this dissertation for chlorophyll content are similar to published by Casto-Gonzalez *et al.*, (2019), where they reported that total chlorophyll considerably increased at dose of 25, 50 and 100mg/l of AgNPs in wheat established in vitro conditions [41].

CONCLUSION

This research work was found that silver nanoparticles successfully synthesized by using orange juice extract. The silver nanoparticles exhibited surface plasmon resonance within the range of 300 to 600 nm, indicating successful synthesis. Zeta sizer analysis confirmed the stability of the nanoparticles. Silver nanoparticle size synthesized from orange juice possessed 37.42nm and pure elemental silver 33.06% as confirmed by SEM analysis and EDX analysis respectively. Additionally, MS media supplemented with 2 mg/L BAP proved to be an effective growth medium for orange propagation and 2mg/l 2, 4 D for callus induction. The synthesized silver nanoparticles at 2ppm, 3ppm and 5ppm concentration proved demonstrated potential as an anti contaminant agent in plant growth media, providing a promising approach for enhancing plant growth and protection. 2ppm and 3ppm did not affect the plant growth and chlorophyll content. 5ppm concentration proved to be lethal dose which lead necrosis. Biosynthesized silver nanoparticles can be used as plant protective material in plant tissue culture medium.

FUTURE PERSPECTIVE

Synthesized biogenic AgNPs (Silver Nanoparticles) have the potential to revolutionize germplasm preservation by enhancing longevity of plant tissues. Incorporating AgNPs into plant tissue culture protocols can significantly reduce the risk of microbial contamination. Identifying the optimal concentration of AgNPs is critical to maximizing their beneficial effects on plant growth and health while minimizing any potential cytotoxicity. By integrating AgNPs into plant tissue culture media, the risk of contamination can be significantly reduced, leading to higher success rates in tissue culture experiments.

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APPENDICES

Appendix I

Surface sterilization of culture room, hood bench and working bench:

75% Ethanol	Prepared 125ml 75% solution by adding 98.684ml of 75% ethanol solution and 26.316ml of water to make volume up to 125ml
4% NaOCl	Prepared 4% of sodium hypochlorite solution by adding 40ml NaOCl in 960ml autoclaved water to get volume 1000ml

Appendix II

Surface sterilization of explants:

4% NaOCl	Prepared 4% of sodium hypochlorite solution by adding 40ml NaOCl in 960ml autoclaved water to get volume 1000ml
2% MgCl ₂	Prepared 2% mercuric chloride solution by adding 2g HgCl ₂ (Sigma CAS-No. 7487-94-7) in 100ml autoclaved distilled water

Appendix III

In-Vitro culture of explants:

0.0001g/ml BAP	Prepared 1mg/l stock solution of BAP by dissolving 0.0001g BAP (Sigma Aldrich CAS-No. 1214-39-7) in 100ml autoclaved distilled water.
0.0002g/ml BAP	Prepared 2mg/l stock solution of BAP by dissolving 0.0002g BAP (Sigma Aldrich CAS-No. 1214-39-7) in 100ml autoclaved distilled

	water.
0.0001g/ml 2,4 D	Prepared 1mg/l stock solution of 2, 4 D by dissolving 0.0001g 2,4 D(Sigma Aldrich CAS-No. 94-75-7) in 100ml autoclaved distilled water.
0.0002g/ml 2,4 D	Prepared 2mg/l stock solution of 2, 4 D by dissolving 0.0001g 2,4 D(Sigma Aldrich CAS-No. 94-75-7) in 100ml autoclaved distilled water.

Appendix IV

Preparation of silver nitrate solution:

1% AgNO ₃	Prepared 1% AgNO ₃ solution by dissolving 0.01AgNO ₃ in 10ml distilled water
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