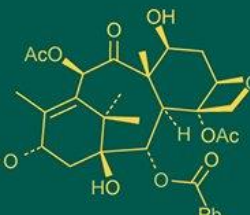
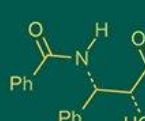


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Impact of green engineered AgNPs on plant physiological properties of Khasi Mandarin (*Citrus reticulata* Blanco)

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Abstract

The use of silver nanoparticles, or AgNPs, in biological systems is one of the major developments in nanobiotechnology. AgNPs have been used on plants to promote seed germination, increase agricultural yields, and speed up growth. However, both positive and negative effects of NPs on the internal structure of the plant have been documented. Citrus leaves were used as a reducing agent and silver nitrate (AgNO_3) as a precursor to produce biogenic AgNPs. A visual observation a change in color from yellow to reddish brown confirmed the development of AgNPs. A rolled towel method was used to investigate the phytotoxicity of AgNPs at various concentrations (10, 20, 50, 100, 200, 500, 1000, 1500, 2000, 2500, 3000, and 3500 ppm). Both plant growth and the percentage of seeds that germinated were found to have increased by up to 1700 ppm. Higher concentrations, starting at 1800 ppm, were found to have an adverse effect. Relative water content (RWC) and photosynthetic pigments were shown to rise when AgNP concentrations increased, but to fall at higher concentrations (2000-3500 ppm).

Keywords: Nanobiotechnology, phytotoxicity, seed germination, silver nanoparticles

Introduction

Khasi Mandarin (*Citrus reticulata*) that belongs to the family Rutaceae is one of the most economically important fruit crop and is considered to be the third most important fruit crop after mango and banana (Sekhar *et al.*, 2024) [21]. In addition to being incredibly delicious qualities, it has been utilized extensively as a natural remedy (Zhang *et al.*, 2012) [30]. Recently, attention is focused on novel approaches that could help plants support their fundamental processes.

One significant aspect of the advancement of nanobiotechnology, which is a viable solution to issues pertaining to food crop production, is the application of silver nanoparticles (AgNPs) in biological systems. Plant mediated synthesis is one of the broadly acknowledged and inexpensive method for producing AgNPs. AgNPs are utilized as biofertilizers in the agricultural industry, which enable NPs to directly contact plants. They have been utilized to boost crop yields, accelerate growth, and accelerate seed germination in plants (Dutta *et al.*, 2023) [5]. Although nanoparticles exhibited a great potential in increasing the productivity of a crop, there is limited knowledge about the impact of various NPs releases on plant improvement. It was reported that the plants treated with nanoparticles could absorb and circulate the materials to different parts of the plants (Iqbal *et al.*, 2018) [9]. At this point, there have been reports of both NPs' beneficial and detrimental impacts on the plant's internal structure (Hong *et al.*, 2005) [8]. According to Miao *et al.* (2009) [16], Ag NPs have also been shown to both increase and decrease the development of plants, photosynthesis, and chlorophyll synthesis. According to Musante and White (2012) [17], the subspecies *Ovifera* of *Cucurbita pepo* exhibits notable differences in phytotoxicity, biomass, decrease in growth rate when exposed to Ag and Cu NPs. In plants like *Zea mays*, *Glycine max*, *Brassica oleracea*, *Cucumis sativus*, and *Daucus carota*, Al_3O_2 NPs prevent the elongation of their roots (Yang and Watts 2005) [28]. However, the size, chemical composition, surface structure, concentration, aggregation, and solubility of engineered nanoparticles, as well as their impacts on various plant species, might vary significantly depending on the plant growth stage, contact manner, and time period (Real *et al.*, 2017). According to Davies (2009) [4, 18],

AgNPs are the most widely used engineered nanoparticles in a variety of commercial goods. According to a number of reports, proper concentrations of AgNPs are crucial for improving plant growth (Sharma *et al.*, 2012) [23], seed germination (Shelar and Chavan 2015) [24] and increased the amount of physiological efficiency. Thus, the purpose of this research is to investigate how employing AgNPs can improve the growth of *Khasi* mandarin plants as well as to study the impact of AgNPs on plant growth and health.

Materials and Methods

Preparation of bioextracts

Fresh leaves of wild citrus (10g) collected from College of Post Graduate Studies in Agricultural Science, Central Agricultural University (Imphal), Umiam, Meghalaya were extracted using methanol (10%) and were centrifuged at 5000 rpm. The supernatant collected were used for biosynthesis of silver nanoparticles.

Biosynthesis of silver nanoparticles (AgNPs)

Citrus leaves extract were added to 1mM AgNO₃ in 1:9 in a 100ml conical flask under constant stirring in a dark condition till a change in color was observed. The temperature as well as the time of synthesis were standardized.

Characterization of AgNPs

Zetasizer (Zetasizer-Nanoseries, Malvern equipment ZEN 3600) was used to determine the average hydrodynamic diameter and zeta potential of produced AgNPs utilizing DLS. In order to investigate the velocity distribution of particle movement, the analysis was carried out at Bioscience and Bioengineering Department, IIT Guwahati to quantify continuous variations in light scattering intensity caused by the particle's Brownian motion. AgNPs surface morphology was ascertained using SEM at a magnification of 2000X and an accelerating voltage of 20 KV at the Sophisticated Analytical Instrumentation Facility (SAIF), North-Eastern Hill University (NEHU), Shillong, Meghalaya. To determine the crystalline nature of the material, the powdered AgNPs were also subjected to XRD (Rigaku X-ray diffractometer, IIT, Guwahati, Assam) operated at 40 kV and 100 mA. The X-ray diffraction was performed at 2-Theta between 38.12° and 134.94°. The pattern was recorded by CuKα1 radiation with wavelength of 1.5406 Å and nickel monochromator. The size of the nanoparticles was calculated through the Scherer's equation; $D = \frac{\kappa\lambda}{b\cos\theta}$ $D = \kappa\lambda/\beta\cos\theta$, where D is the average crystal size, K is the Scherer coefficient (0.89), λ is the X-ray wavelength (λ= 1.5406Å), θ is Bragg's angle (2θ), θ the full width at half maximum (FWHM) in radians

The size and concentration of AgNPs in Brownian motion were measured using NTA (Nanoparticle Tracking Analysis; Model NanoSight300 Make: Malvern Analytical, Netherlands) at IIT, Delhi. AFM (Atomic Force Microscope) or Scanning Probe Microscope (SPM) from Asylum Research of Oxford Instruments, i.e., Asylum Research MFP-3D-BIO, gives a 3-D surface topography and structure at an atomic level. The analysis was conducted at North East Centre for Biological Sciences and Healthcare Engineering (NECBH), IIT Guwahati. Particle size analysis was performed by a laser diffraction particle size analyzer (PSA 1190) at IIT, Dhanbad, Jharkhand in the wet method. The detectors measured the energy dispersed and absorbed

at angles after the samples were exposed to incoming light. The particles size was analysed with the software Kalliope™.

Collection of biological material and culture

The local variety of *Khasi* mandarin were purchased from Police Bazaar, Shillong, Meghalaya. *Escherichia coli* (*E. coli*- MTCC-723) was purchased from MTCC, Chandigarh.

Inoculation of *E. coli* on *Khasi* Mandarin plants

Although a human pathogen *E. coli* does not caused diseases in plants, they are known to trigger plant defense by contaminating, colonizing, disrupting and survive in plant tissue through stomatal entry (Seo and Matthews, 2012) [22]. *E. coli* (1 x 10⁶ CFU/ml) was inoculated on disease free seedlings of *Khasi* Mandarin plants.

Treatment of seeds with AgNPs: The phytotoxicity of the biosynthesized AgNPs was assessed at different concentrations (0, 10, 20, 50, 100, 200, 300, 500, 1000, 1500, 2000, 2500, 3000, 3500 ppm) by rolled paper method using the guidelines given by International Seed Testing Association (ISTA, 2013) [10]. The collected seeds were sterilized for 10 min in 4% sodium hypochlorite solution and then subsequent rinsing with distilled water thrice. The seeds were soaked with different concentrations of AgNPs for 24 hrs in Petri dish kept in dark at room temperature. Ten seeds that were washed with distilled water were transferred and rolled with germination paper and 0.5 ml of AgNPs were added per day for 7 days. The seed germination percentage were observed everyday upto 7 days. The germination percentage was calculated by the formula:

$$\text{Seed germination (\%)} = \left[\frac{\text{No. of seeds germinated}}{\text{Total no. of seeds}} \right] \times 100$$

The length of the root and shoot were also determined after transplanting the seeds into trays. The length of the root and shoot were measured every week for three consecutive weeks. All the experiments were replicated thrice.

Physiological parameters

Chlorophyll content: To determine the chlorophyll content, fresh leaves were collected from the AgNPs treated plants as well as untreated control plants according to Kshetrimayum *et al.*, (2018) [14]. About 0.5 g leaves samples were cut into small pieces and transferred to 10 mL acetone (80%) and crushed with the help of mortar and pestle. The homogenate was centrifuged at 5000 rpm for 25 min. at 4 °C and the supernatant collected in a test tube and 4.5 mL of acetone (80%) solvent was added to 0.5 mL of it. The amount of Chl a and Chl b can be calculated by the formulae:

- Chl a (µg/ml)=13.36 (A_{664.2})- 5.19 (A_{646.6})
- Chl b(µg/ml)= 27.46 (A_{648.6})- 8.12 (A_{664.2})
- Total chlorophyll (µg/ml)= Chl a + Chl b

Where Chl a and Chl b are chlorophyll a and b respectively and A is the absorbance of the extracts.

Carotenoid content: The carotenoid content of the AgNPs treated and untreated plants were determined according to Lichtenthaler and Welburn (1983) [15]. The total carotenoid content was calculated by the formulae:

Total carotenoid=[1000 A_{470} - (2.270 Chl a - 81.4 Chl b)]/227

Relative Water Content (RWC): The RWC of leaves was determined according to Barrs and Weatherley (1962) ^[1]. Around 1.5 cm in diameter leaf disc were cut from the topmost leaves of treated Khasi Mandarin plants. The weight of the leaf discs were immediately taken after which they were hydrated with deionized water to full turgidity under room temperature and light for four hours in a closed Petri plate (Plate 6). The leaf disc was taken out after 4 hours and were dried to remove excess water with tissue paper. The discs were then weighed to obtained fully turgid weight (TW) after which they were subjected to 80°C for 24 hrs. The weight of the dried discs were determined after cooled down and the relative water content were determined using the formula:

$$\text{RWC (\%)} = [(W-DW)/(TW-DW)] \times 100$$

Where, W-Fresh weight (g)

- TW-Turgid weight (g)
- DW-Dry weight (g)

Statistical analysis: All the experiments were performed in triplicate and each treatment and experimental result will be presented as arithmetic mean $\pm$ SE (Standard Error).

Results and discussion

During the synthesis of AgNPs from plant extracts, a change in color was observed which indicates the formation of AgNPs (Plate1). The change in color from light yellow to reddish brown was observed on citrus leaves mediated AgNPs due Surface Plasmon Resonance (SPR) of the forming AgNPs (Ag^0) (Jaast and Grewal, 2021) ^[11]. The suspension's particle aggregation, hydrodynamic diameters, and polydispersity were assessed using DLS and it was observed that the average size of AgNPs synthesised at 60°C was 17 nm with a polydispersity index (PDI) of 0.219, indicating the biosynthesised AgNPs were polydisperse in nature (Plate 2a). Our results are in agreement with Yang *et al.* (2016) ^[29] who used *Lonicera Japonica* Thunb combo for synthesizing silver nanoparticles. According to Tripathi *et al.* (2010) ^[25], PDI value less than 0.10 are regarded as monodispersed, whereas values more than 0.10 are regarded as polydispersed. The nanoparticles of the present study have different sizes, approximately ~10-125 nm (Plate 2b), and are mostly spherical and uniformly distributed using SEM studies. The structure and morphology of our green-synthesized AgNPs were similar to Cicek *et al.* (2015) ^[3] who determined the shape and size of Euphorbia-mediated silver nanoparticles and observed that the AgNPs were well dispersed and mostly spherical in shape, with a size ranging from 7 to 20 nm.

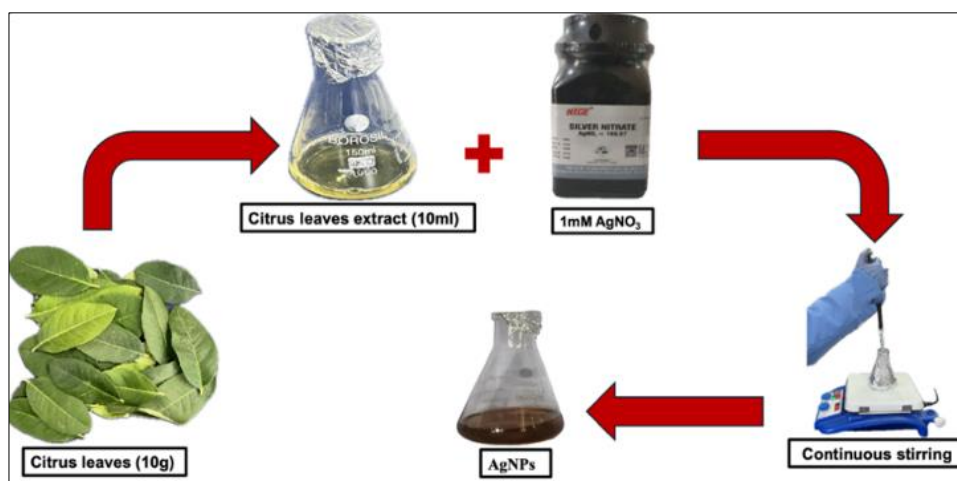


Plate 1: Green synthesis of Silver Nanoparticles (AgNPs) from citrus leaves extracts

The XRD pattern of biosynthesized AgNPs was recorded at a range of 38.12 to 134.94 at 2-theta angles (Plate 2c). The biosynthesized AgNPs showed Bragg's diffraction peaks at 2-theta angles of 38.12°, 44.31°, 64.46°, and 77.41°, corresponding to the (111), (200), (220), and (311) facets, respectively. The AgNP's XRD pattern revealed a face-centered cubic (fcc) which is crystalline structure similar to the usual diffraction lines of cubic Ag. Our results are consistent with the findings of Tyagi *et al.* (2021) ^[26], whose XRD peaks confirm the presence of monocyclic crystals in the solution. According to NTA measurements made using Ag-NPs' Brownian motion, the mean size of the synthesized silver nanoparticles was 63.8 nm with a concentration of 3.10×10^{10} particles per ml of suspension (Plate 2 d & e). The concentration of the AgNPs was mostly found within the size of 100 nm, extending up to 400 nm (Plate 2 f). Our results are consistent with the findings of Gupta *et al.* (2014) ^[6] on the magnitude of AgNPs mediated by Lawsonia,

which measures 6-72 nm. The topographic image of AgNPs have been displayed in Plate 2g. The image showed that the AgNPs are spherical in shape, well dispersed and uniformly spread. Urnukhsaikhani *et al.* (2021) ^[27] determined the characteristics of *Carduus crispus*-mediated silver nanoparticles (AgNPs) using AFM and size of the AgNPs obtained from the whole plant was 70 nm, from the flower was 33 nm, and the size of the AgNPs obtained from the stem was 131 nm. Plate 2h displayed data of laser diffraction by using instrument PSA 1190 LD showing a particle size of 90.292 nm. Notably, our findings are consistent with the particle sizes determined by scanning electron microscopy (SEM). Unlike physical or chemical synthesis which can produce highly monodispersed (Gaussian) particles, green synthesis involves the use of natural extracts of plants that often result in a wider, more complex range of particle sizes (Edo *et al.*, 2025).

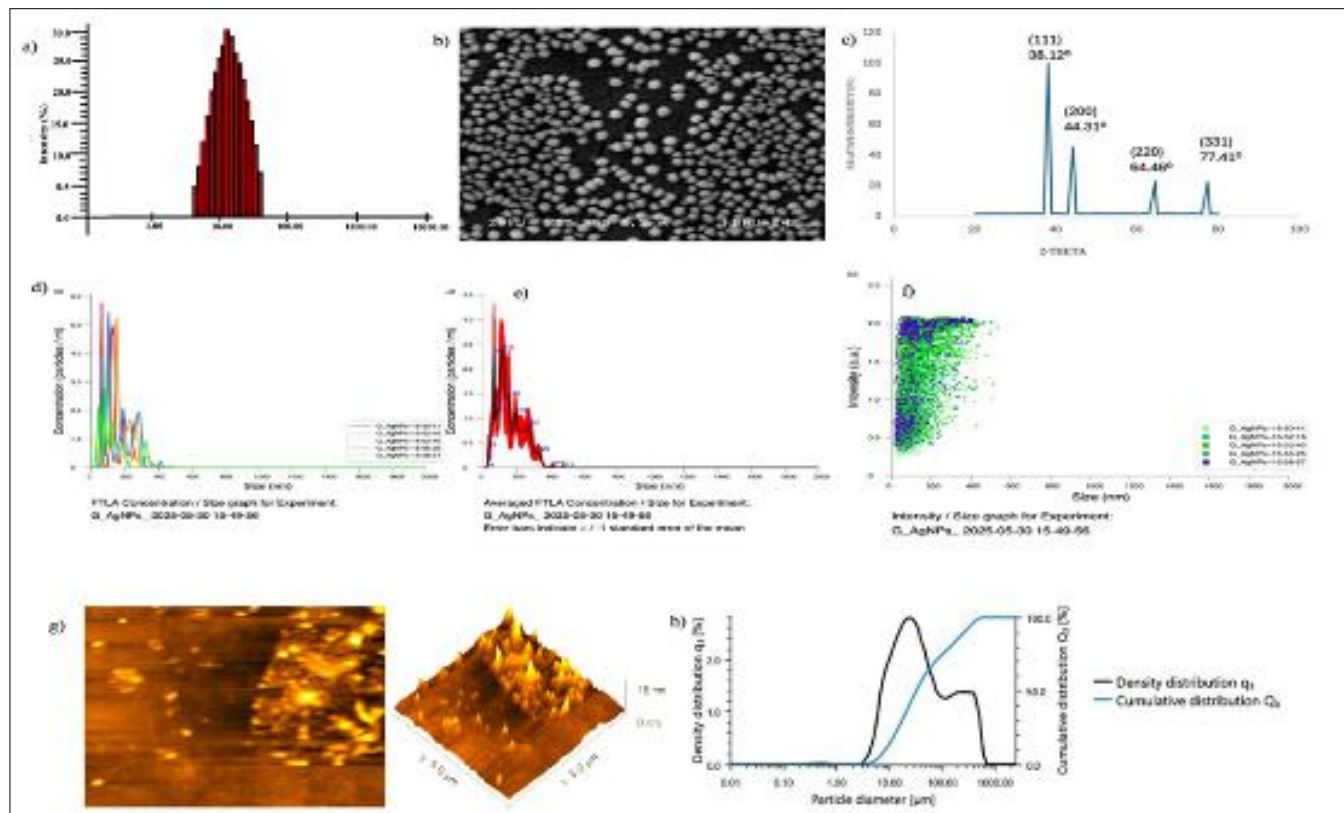


Plate 2: Characterization of biosynthesized silver nanoparticles

Plate 2: a) The DLS analysis of the biosynthesized AgNPs showed hydrodynamic diameter b) SEM analysis of biosynthesized AgNPs c) Analysis of biosynthesized AgNPs by XRD (Bragg's diffraction peaks at 2-theta angles of 38.12°, 44.31°, 64.46°, and 77.41°, corresponding to the (111), (200), (220), and (311) facets, respectively). NTA measurements made using Ag-NPs Brownian motion, the mean size of the biosynthesized AgNPs was d) 63.8 nm with a concentration of e) 3.10×10^{10} particles per ml of suspension. f) The concentration of the AgNPs was mostly found within the size of 100 nm extending upto 400 nm g) The topographical image of biosynthesized AgNPs under AFM h) Laser diffraction by liquid particle size analyzer showed that the biosynthesized AgNPs showed a particle size of 92.92 nm.

Seed germination and plant growth

Phytotoxicity of the biosynthesized AgNPs were determined on seed germination by rolled paper method. The germination of 10 seeds per paper was recorded everyday upto 7 days. Seed germination started from the 2nd day, and it was observed that the germination percentage of AgNPs treated seeds were higher in lower concentration of AgNPs compared to the higher concentrations. It was also noticed that overall seed germination was enhanced compared to the control. After 7 days of treatment, the germination percentage of the treated seeds from 10 ppm to 500 ppm of AgNPs was 96.67%, and 1000 ppm to 1500 ppm of AgNPs was found to be 100% but in higher concentration *i.e.* from 2000 ppm to 3500 ppm the germination percentage decreases to 76.66 - 60.00% (Plate 3a, Fig 1a). Overall, the results showed that seed germination percentage of AgNPs treated Khasi Mandarin seeds increased with the increased

concentrations of AgNPs up to 1500 ppm while declined in higher concentration from 2000 - 3500 ppm.

An extent experiment was conducted to ascertain the phytotoxic concentration of AgNPs between 1500-2000 ppm (Fig 1b). Results from table 4 revealed that after 7 days, AgNPs treated seeds showed 100% germination at 1500 -1700 ppm while 1800 - 2000ppm of AgNPs declined the germination percentage from 86.667- 76.667%.

Plant height like the length of the root and shoot were measured after 1st, 2nd, 3rd week of transplanting into trays. Fig 1 shows that the length of the root and shoot of AgNPs treated seedlings increased upto a certain concentrations and gradually decreased. Higher concentrations of AgNPs reduced the plant growth and development (Plate 3b) Measurement of plant height in each week showed that maximum height was found with the plants treated at 1700 ppm AgNPs. The length of the root and shoot increases with the increase in concentrations of AgNPs but decrease at higher concentrations *i.e* from 1800 ppm to 3500 ppm (Plate 3b).

Seeds treatment with different concentration of AgNPs increased the growth parameters compared to control plants. These findings bolster the beneficial impact of reduced concentrations. According to a different study, adding Ag NPs impacted on the stevia plant's branch length. Shoot length and yield per transplant were highest at lower Ag NP concentrations (Castro-González *et al.* 2019) [2]. Similar results were obtained by Sadak (2019) [20] who noticed that lower concentrations of the biosynthesized AgNPs stimulate the growth of fenugreek plant while higher concentrations hindered the growth of the plants. The ability of AgNPs to suppress ethylene signaling in fenugreek plants may be the reason of the induced growth increases brought on by varying AgNP concentrations (Rezvani *et al.* 2012) [19].



Plate 3: a) Phytotoxicity of AgNPs on seed germination of Khasi mandarin by rolled paper method b) Phytotoxicity of AgNPs on plant growth (determination length of root and shoot)

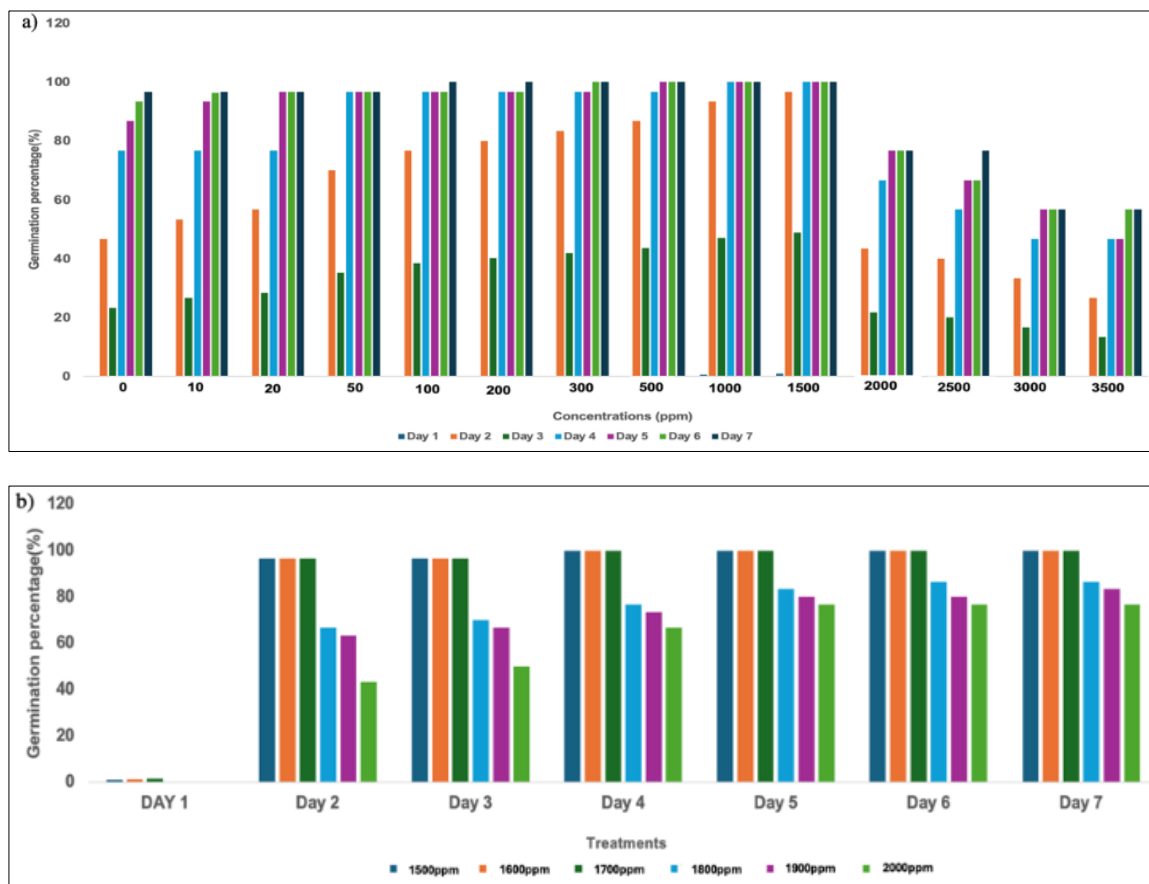
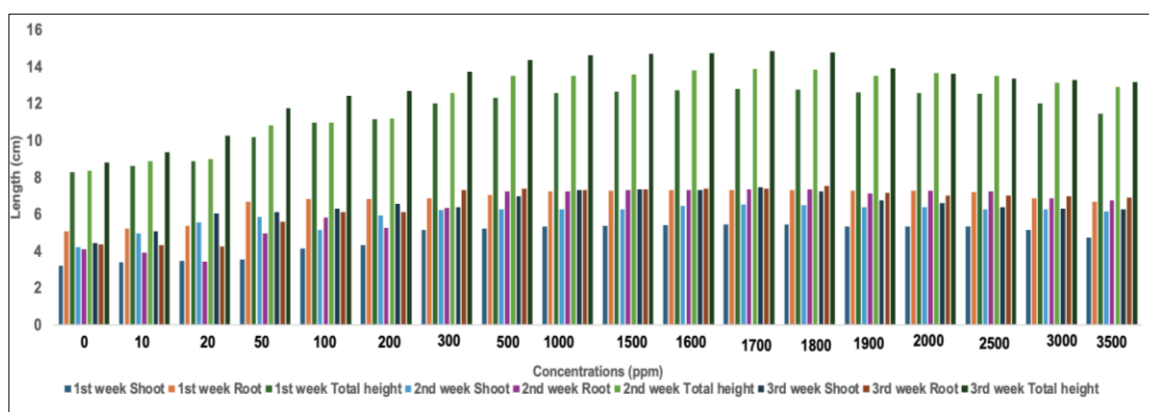


Fig 1: Phytotoxicity of different concentrations of AgNPs on seed germination by Rolled paper method at a) 0-3500ppm b) 1500-2000ppm



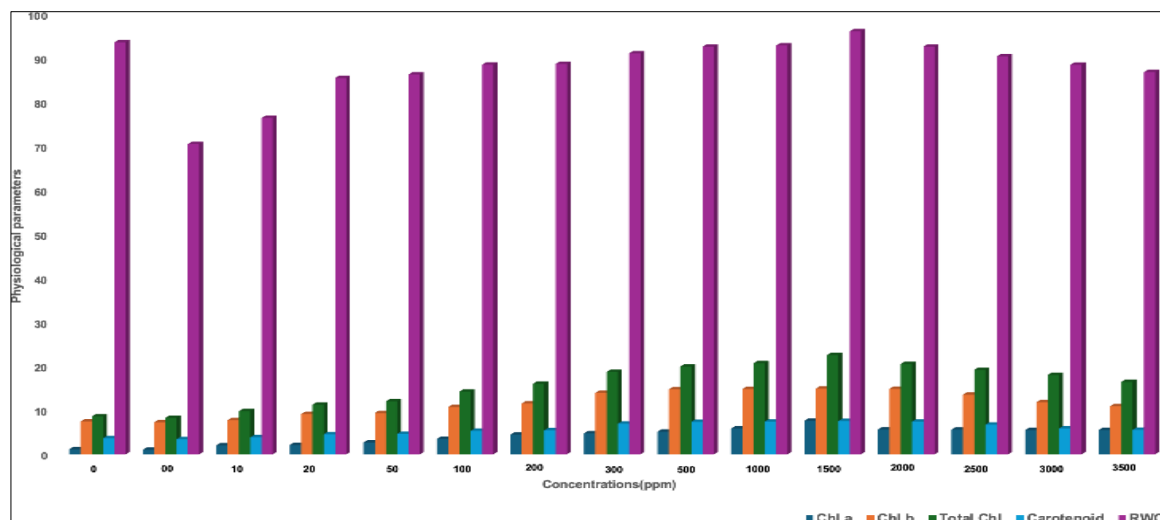


Fig 2: Phytotoxicity of different concentrations of AgNPs on plant growth

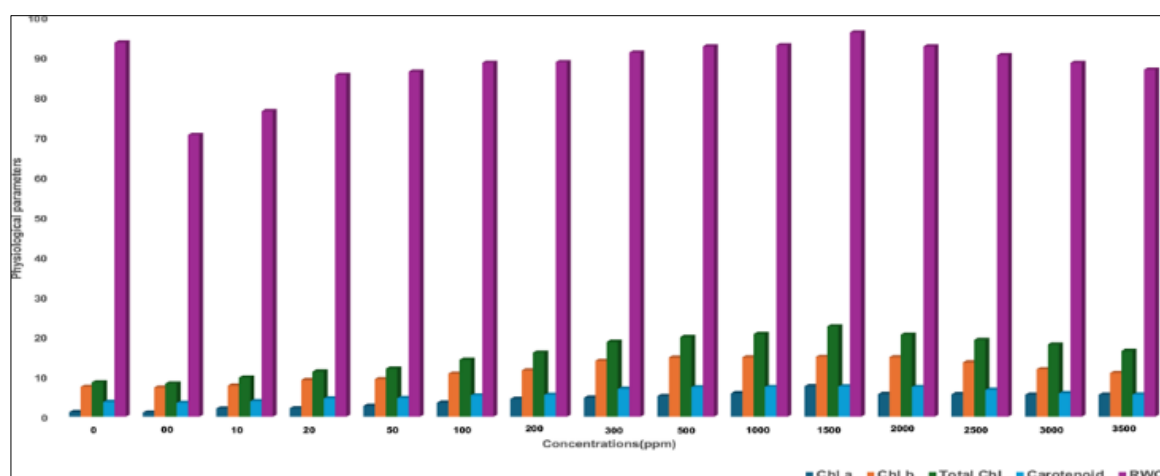


Fig 3: Effect of biosynthesized AgNPs on the physiological parameters of Khasi mandarin

Physiological parameters: The effect of *E. coli* inoculated seedlings treated with AgNPs at different concentrations were assessed for their physiological changes in their chlorophyll pigments and their relative water content (RWC).

Estimation of Chlorophyll pigments and Relative Water Content (RWC): The amount chlorophyll pigments in plants control the rate of photosynthesis. The photosynthetic capacity of the plants may be hampered by biological stresses like plant pathogenic bacteria causing necrosis and finally reduced the photosynthetic pigments. Fig 3 shows the effect of AgNPs sprayed on the leaves surface of Khasi mandarin on photosynthetic pigments like the chlorophyll content and relative water content (RWC). The effect of AgNPs on *E. coli* inoculated seedling were studied and compared with the uninoculated seedlings (T_0) and untreated *E. coli* inoculated seedlings (T_{00}) based on their photosynthetic pigments and RWC.

The photosynthetic pigments were greatly reduced in the *E. coli* inoculated plants (T_{00}) comparing to the healthy control plants (T_0). Results showed the significant increase in photosynthetic pigments in response to treatments with different concentrations of AgNPs compared to the healthy control plants as well as the *E. coli* inoculated plants. Fig 3 showed that the photosynthetic pigments increased with the increase in concentrations of AgNPs upto 1500 ppm and

gradually decreased in higher concentrations (2000 ppm - 3500 ppm). The most effective treatment was 1500 ppm as it induced the highest chlorophyll and carotenoid pigment.

The RWC of *E. coli* inoculated seedlings was greatly reduced by 28.14% compared to the healthy control plants. The highest RWC was found with AgNPs treatment at 1500 ppm with 96.08% RWC, followed by the healthy control plants. As the concentration of AgNPs increases from 10 to 1500 ppm the RWC (%) was improved in the plants. However the amount of the relative water content decreases in higher concentrations as it reached 3500 ppm. The decreased in RWC was observed from the concentration 2000 ppm and above.

The present study on phytotoxicity of the biosynthesized AgNPs revealed that lower concentrations of AgNPs are beneficial for seed germination, plant growth, and phytochemicals like photosynthetic pigments (Chl a, Chl b, total chlorophyll, and carotenoid) and relative water content (RWC). This is because AgNPs act as a hermetic agent, stimulating beneficial physiological responses at low doses while causing toxicity at higher doses (Khan *et al.*, 2023) [13]. By improving water absorption, boosting antioxidant activity, and functioning as a moderate stressor that activates defense systems without compromising cellular integrity, AgNPs stimulate growth at lower doses (El-Saadony *et al.* 2022) [6]. Conversely, higher concentrations of AgNPs cause severe oxidative stress by producing

excessive amounts of ROS (reactive oxygen species), which damages cellular structure and results in reduced relative water content, stunted growth, inhibited germination, and decreased chlorophyll content (Kannaujia *et al.*, 2022)^[12].

Conclusions: The use of wild citrus leaves mediated AgNPs is non-toxic, ecofriendly and cost effective approach. Our studies showed that the biosynthesized AgNPs could be a useful agent for enhancing seed germination, plant growth and increasing the plant physiological activities at a certain lower dose. However, showed a detrimental effect to the plant at higher dose.

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