3.1 Equipments

Different equipments used for the synthesis and characterization of nanoparticles are Electronic weighing balance (Metler Toledo, New classic), Centrifuge and homogenizer (Spinwin, tarson), Vortex shaker (Yorco, Japan), Sonicator (MSW-347, MAC), UV-visible spectrophotometer (ELICO), Transmission electron microscope (Hitachi FEI Philips Morgagni 268D TEM), Magnetic stirrer (Spinot), Particle size analyser - Zeta sizer (Nanoseries ZS-90, Malvern Instruments Ltd. India), Fourier transform infrared spectroscope (Affinity-FTIR, Shimadzu), Laminar air flow ( NSW 201 Horizontal laminar, India), CO\textsubscript{2} incubator (ACMAS Technocracy (P) Ltd, India), Shaker incubator (Orbitek, Sciengenics biotech), Microwave (Samsung), Water purifier (Ultra 370 series, ULTRA aqua Rions, India), Oven (NSW) and Vertical autoclave (Torco, Japan).

3.2 Materials

3.2.1 Chemicals

Silver nitrate (AgNO\textsubscript{3}), 5-bromo-2-deoxyuridine, colchicine, hoechst 33258 was purchased from Sigma Aldrich, India. Fetal bovine serums, phytohaemagglutinin, penicillin, streptomycin, Geimsa stain, RPMI-1640 culture medium were purchased from Hi-media. Nutrient agar, nutrient broth, potato dextrose agar, potato dextrose broth were purchased from Hi-media.

3.2.2 Glassware

All the required glassware (Borosil) was immersed in liquid detergent for few hours. Cleaned and washed thoroughly and then rinsed with distilled water. Cleaned glassware were allowed to dry in hot air oven and stored for further use. Before usage all glassware were sterilized in autoclave at 15 psi pressure for 20 minutes.

3.2.3 Microbial cultures

The test strains i.e. Enterobacter sp. (MTCC 5112), Staphylococcus aureus (MTCC 3160), Klebsiella pneumonia (ATCC 700603) and Pseudomonas florescence (MTCC 2421)
were procured from IMTECH, Chandigarh. Pathogenic Bacillus strain was kindly provided by CPB, HAU Campus, Hisar, Haryana. Fungal strains i.e. Alternaria solani (ITCC 4632), Thanatephorus cucumeris (ITCC 5332), Botryodiplodia theobromae (ITCC 6741) were purchased from IARI, New Delhi.

3.3 Methods

3.3.1 Biogenesis of nanoparticles

NPs in the present work were synthesized by biological methods [bacteria, fungi and fruit peel (Citrus limetta and kinnow) extract]. Detail methodology is given in further subsections:

3.3.1.1 Isolation and screening of bacterial and fungal cultures from soil

Five gm soil sample was collected from the roots of three different tomato plants. Soil was homogeneously crushed and stored. Bacterial cultures were isolated from soil by serial dilution method. Firstly, 1 gm soil was mixed in 9 ml of distilled water to make the solution of concentration $10^{-1}$ and then made serial dilution up to the concentration of $10^{-7}$. This suspension was shaken for 5 minutes at vortex and 0.1 ml of soil suspension was plated on NA and PDA plates containing 2 mM AgNO$_3$ for isolation of bacteria and fungi, respectively. These NA plates were incubated at 37°C for 24h and PDA plates at 25°C for 72h, respectively and observed for growth of bacterial and fungal colonies. Random samples of colonies were streaked several times on plates for isolation of pure cultures of bacteria and fungi using the spread plate method (Wise, 2006). For screening, isolated bacterial and fungal cultures which showed good growth were again streaked on NA and PDA plates containing higher concentration of AgNO$_3$ (5 mM) to obtain microbes having high AgNO$_3$ tolerance.

3.3.1.2 Characterization of microbial cultures

Based on initial screening, the bacteria which showed good growth on 3mM AgNO$_3$ and fungus isolates which showed growth on 5mM AgNO$_3$ were characterized. Barnett and Hunter scheme (Bernet & Hunter, 1972) was followed for initial identification of the fungal isolates which involve morphological and microscopic observations (such as color, texture, spore formation etc.). For microscopic observation slides were prepared using dye lactophenol cotton blue. Identification of fungal strains was further confirmed from ITCC, IARI, New Delhi. Characterization of bacteria was done using 16 S rRNA sequencing technique from Institute of Microbial Technology (IMTECH), Chandigarh.
3.3.1.3 Biogenesis of nanoparticles using bacterial culture

Biogenesis of NPs was done according to Nithya et al. (2012) with some modifications. Bacteria culture was allowed to grow in nutrient broth at 35°C under continuous shaking conditions (200 rpm) for 24 hours. Broth was centrifuged at 10,000 rpm in refrigerated centrifuge at 4°C for 15 minutes for separating bacteria cells from rest of the broth. Pellet was washed twice with distilled water and resuspended in 100 ml of distilled water for intracellular biogenesis. Supernatant, thus obtained was further used for extracellular biogenesis. Silver nitrate solution was added to the supernatant and biomass so that final concentration of solution becomes 3mM. It was kept in complete dark conditions for 24 hours at 37°C and 200 rpm. Controls containing cultures (without AgNO₃) as positive and AgNO₃ (without culture) as negative controls were also run simultaneously along with the experimental flasks. Flasks were observed for any color change.

3.3.1.4 Biogenesis of nanoparticles using fungal culture

Fungal biomass was obtained by incubating the fungal strains in potato dextrose broth (PDB) at 28°C for 3 days under shaking culture conditions (180 rpm). Biomass was filtered using Whatmann filter paper No.1. Biomass thus obtained was washed twice with distilled water and re suspended in 100 ml of distilled water in separate flask. Silver nitrate was added in flask containing biomass and supernatant separately for intra and extracellular NP synthesis respectively, so that the final concentration becomes 5 mM.

3.3.1.5 Biogenesis of nanoparticles using fruit peel extract

NPs were synthesized from the fruit peels of *Citrus limetta* (sweet lime, locally called as Mosambi) and locally available citrus fruit kinnow (hybrid of *Citrus nobilis* and *Citrus deliciosa*) using Kahrilas et al. (2013) procedure with some modifications.

Fresh and healthy fruits were rinsed thoroughly with distilled water. Colored peel segments were cut away from the fruit and cut into smaller pieces. Peels were immediately transferred into a beaker containing 50 ml of distilled water. The mixture was heated in a commercial food grade microwave for approximately one minute. The mixture was filtered through Whatman No.1 filter paper. Extract obtained was allowed to cool down at room temperature and stored at 4°C for further use.

To the peel extracts, silver nitrate solution (5 mM) was added in small lots with continuous shaking. Mixtures were microwaved in conventional microwave for 1 minute.
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Mixtures were kept at room temperature to cool down and reactions were allowed to continue and observed for any color change.

3.3.2 Recovery and drying of synthesized NPs

Biosynthesized AgNPs were recovered from filtrate by using cryogenic centrifuge at 15,000 rpm for 15 minutes. Recovered NPs were washed twice with distilled water and oven dried at 45°C for 24 hours.

3.3.3 Characterisation of nanoparticles

The resultant nanoparticles were characterized with conventional instruments based analysis i.e. UV-visible spectroscopy, TEM, PSA and FTIR.

3.3.3.1 UV-visible spectral analysis

UV visible spectroscopy is useful and powerful tool for characterization of absorption, transmission and reflectivity of a variety of important materials. It is based on the principle of Lambert Beer’s law which states that “The intensity of monochromatic radiation passing through a homogenous sample solely depends upon the concentration of sample and the path length”. It involves interaction of the material with radiations. Some particular wavelengths of radiations are absorbed depending upon type of the material. In a conventional spectrophotometer electromagnetic radiation is passed through sample and reference cell having only solvent. Spectrophotometer records the difference in intensity of radiation. “Blue shift” in the absorption spectra is seen when material lies in nanorange as compared to bulk matter. This happens due to increase in band gap (Bhui, 2009).

Nanoparticles have unique optical properties which are measure of their shape, size, type and agglomeration state. For spectroscopic characterization, samples were subjected to UV–visible spectroscopy (ELICO). This is non destructive, real time and most widely used method of analysis (Beitia et al., 2000).

3.3.3.2 Transmission electron microscope (TEM) analysis

TEM is the microscopy technique used to obtain greater magnification and resolution of matter to get detailed information about matter. In TEM accelerated electrons (few hundred KeV) are allowed to pass through ultra thin specimen. Electron beam and matter interact to produce different radiations including X-rays, scattered, back scattered and transmitted electrons. All these signals are studied to obtain details of the specimen to be
studied. The basic principle of TEM lies behind “Dual nature of electron” which enables them to have higher resolution capabilities (Brundle & Evans, 1992).

TEM analysis was done for revealing morphological details of the synthesized nanoparticles. Grids loaded with nanoparticles were studied under transmission electron microscope (Hitachi FEI Philips Morgani 268D TEM) at an accelerating voltage of 100 KeV. Sample preparation for TEM is an important aspect. TEM samples were prepared in distilled water and ultrasonication was done for 5 minutes for complete dispersion of nanoparticles. One or two drops of the suspension were placed onto carbon-coated copper grids and mounting done after air drying. For intracellular studies sample fixation was done by using 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 8 hours at low temperature.

3.3.3.3 Particle size analyzer (PSA)

Zetasizer is based on the principle of dynamic light scattering. It measures Brownian motion and correlates this with the particle size. The smaller is particle size, more will be Brownian motion and vice versa. It uses ‘Mie theory’ of light scattering. Optical properties of dispersant such as refractive index (RI), viscosity and temperature etc. affect the readings. Thus, refractive index, viscosity and dielectric constant were kept constant for all determinations. Water was used as dispersant. Sizes of NPs were studied out by using zetasizer (Nanoserries Nano ZS90, Malvern). Size of samples was measured at 25°C using disposable zetasizer cuvette. Z-average value and polydispersity index (PDI) values were also recorded.

3.3.3.4 FTIR spectral analysis

FTIR spectroscopy is the non invasive technique used to obtain an infrared spectrum of sample. It is an easy way to find the presence of different functional groups in the sample. FTIR is used for chemical analysis of materials. FTIR is work as finger print for chemical structure of materials. Chemical analysis of structure is based on IR spectrum which can be obtained by calculating the Fourier transform of the interferogram. Chemical bonds present in chemical structure are analysed in the form of spectra.

The nanoformulations were analyzed with Fourier transform infra-red spectroscopy (FTIR). Bonding between different groups was studied by FTIR spectra. For FTIR
measurements, individual samples were grounded with KBr pellet and then analyzed using a FTIR spectrophotometer in the range of 400–4000 cm\(^{-1}\).

3.3.4 Assessment of antimicrobial properties

Pathogenic cultures were freshly incubated on NA for 24 h for bacteria and on PDA for 72 h in case of fungi. Nanoparticles solution was prepared in deionised water. Sonication for 2 minutes was done for complete dispersal of NPs.

3.3.4.1 Antibacterial assay

3.3.4.1.1 Agar well diffusion assay

Nutrient agar plates were prepared by pouring 20 ml freshly prepared media in well sterilized petri plates. One ml of freshly prepared bacterial suspension was spread uniformly with the help of sterile swab. Five equidistant wells were made by cutting agar media. Four wells were filled with different concentrations of NPs (10µl, 20µl, 40µl, 60µl) and one well with 60µl of antibiotic (Penicillin) solution. The plates were allowed to incubate at 37°C for 24 hours. After that diameter of zone of inhibition was measured using zone measuring scale.

3.3.4.2 Antifungal assay

3.3.4.2.1 Mycelium growth inhibition assay

Percentage inhibition of fungal mycelium was studied by mycelium growth inhibition assay. For this purpose, 20 ml of freshly prepared PDA was poured in properly autoclaved petri plates and allowed to solidify. After solidification, 100 µl NP dispersion having concentration 100 µg/ml was spread uniformly by using sterilized glass rod. Five mm disc of 7 days old cultured test fungi was cut and placed at the center of petri plate. One petri plate with no nanoparticle having disc of test fungi was also placed. Plates were allowed to incubate at 27±2°C for 7 days. Mycelium inhibition was calculated by formula already discussed by Satish et al. (2007) and Kaur et al. (2014) which is described as

\[
\text{Mycelium inhibition} \% = \frac{C - A}{C} \times 100
\]

where C is mycelium inhibition of control plate while A is mycelium inhibition of treated plates

3.3.5 Cytotoxicity

Cytotoxicity of synthesised silver NPs was evaluated by sister chromatid exchange (SCE) frequency.
3.3.5.1 Sample Collection

Five mL of human venous blood was taken from healthy individuals in sodium heparin coated vacutainer tubes for lymphocyte culture set up.

3.3.5.2 Culture set up

The cultures were set up from peripheral blood lymphocytes (PBL) according to method of Moorhead et al. (1960) with minor modifications. During culture set up, 500 µl of heparinized whole blood was added to a culture tube having 5 ml of RPMI-1640 culture medium with L glutamine (1%). The supplement such as fetal bovine serum (20%) phytohaemagglutinin (2 %) and antibiotic [penicillin (100UI/ml) and streptomycin (100µg/ml)] were also added.

3.3.5.3 Sister chromatid exchange (SCE)

For sister chromatid exchange (SCE) analysis, the culture was treated with silver nanoparticles in varying concentrations (25 – 125 µg/ml). The cultures were then incubated for 72 h at 37°C and ±5% CO₂. For differential staining of chromosomes, 5-bromo-2-deoxyuridine (1 mg/ml) was added after 24 h of culture set up. Colchicine was added 45 min prior to the harvesting at final concentration of 0.2 µg/mL to arrest chromosomes at metaphase stage. The harvesting was performed using treatment with hypotonic solution (0.075 M KCl) kept at 37°C and repeated washing of colchicine treated cells with fixative (methanol: acetic acid in 3:1 ratio). The slides were prepared by dropping the cells on pre-cleaned slides and allow to air dried. It was followed by differential staining with Hoechst 33258 and 4% Giemsa stain. Then slide analysis was done to determine the frequency of SCE per cell, by analyzing well spread 25 metaphase plates/per slide.

3.3.6 Data analysis

All treatments were carried out in duplicates. One way ANOVA was applied for comparison of SCE frequency level among control and treated groups using SPSS 16.0. The level of significance was set at \( P < 0.05 \) and results were expressed as Mean±S.D.

3.3.7  \textit{In vivo} control of \textit{Alternaria solani} in tomato plants

3.3.7.1 Coating of seeds with nanoparticles

Seeds were surface sterilized by immersion in 5% hypochlorite solution for 10 min then thoroughly rinsed with distilled water several times (Farghaly and Nafady, 2015).
Approximately 100 sterilized seeds were treated with CMC (carboxymethylcellulose) which was used as binder. CMC solution was prepared by dissolving 4 gm carboxymethylcellulose in 100 ml of distilled water and dissolved completely. The flask was shaken vigorously for about one minute until the seeds were uniformly coated. Nanoparticles at concentration 100µg/ml was added in to conical flask containing seeds and shaken gently for about one minute and allowed to soak for half an hour. After proper coating, the seeds were spread on butter paper and allowed them to dry at room temperature. Nanoparticle treated seeds become darker in colour than the untreated seeds. Seeds treated with distilled water were used as control.

3.3.7.2 Pot study

Pots were filled with autoclaved field soil. Pots were made sick with pathogenic culture of *Alternaria solani* (1x10^5cfu/gm) one week prior to sowing of seeds except one pot which acted as control. Pathogenic culture was obtained from 6 day old culture grown on sterile Petri plate at 28°C (Bhajbhuje, 2013). Five seeds per pot were sown. Pots were kept in continuous observation for 20 days after sowing. After fifteen days, foliar spray of pathogenic suspension was done to ensure outbreak of disease and plants were covered with poly bags to maintain moisture. Plants were observed for any symptoms of disease every day after foliar spray.

3.3.8 Effect of nanoparticles on growth parameters of tomato plant

*In vivo* study of effect of nanoparticles on growth parameters of tomato plant was carried out. Concentration of silver nanoparticle to be applied for *in vivo* study was decided on the basis of cytotoxicity assay. Experiment was performed in replica. Test plants were kept in local green house in Sirsa, Haryana.

3.3.8.1 Collection of seeds

Tomato seed variety “Lalit Hisar” susceptible to *Alternaria solani* was kindly provided by vegetable department, HAU, Hisar, Haryana.

3.3.8.2 Sowing of seeds in soil

Soil for experiment was collected from local field in Sirsa, Haryana. Non sterilized soil was used as such and filled in pots. Control and NP treated seeds were sown and allowed to germinate under suitable conditions (10 seeds per pot). Pots were maintained in green
house at 25°C. After thirty days, the experiment was terminated. Plants and soil sample were collected for data collection. Soil sample was used for studying effect of NPs on microflora of soil on plant growth parameters such as root-shoot ratio and effect on biomass etc.

3.3.8.3 Impact of nanoparticles on seed germination

Effect of NPs on seed germination was studied under in vivo conditions. Number of plants sprouting out after day 10 was considered.

3.3.8.4 Impact on root-shoot ratio (length)

To determine root shoot ratio plants from each pots were uprooted randomly and washed with tap water to remove any soil traces. Plants were blot dried on filter paper to remove excess surface moisture. Roots were separated from shoots from soil line with the help of sharp surgical blade. Length of each root and shoot were noted separately. Shoot length was measured from end of soil line to tip of leaf using a meter scale. Root- shoot lengths of NP treated seeds were compared with control. The root/shoot ratio can be calculated for each treatment (Zimmer and Grose, 1958).

Root-shoot ratio = the ratio of the length of the roots to the length of shoot.

3.3.8.5 Impact on biomass

Ten plants from each batch were randomly uprooted and washed with distilled water to remove any loose soil. Plants were tapped dry on paper towel. Plants were allowed to dry in oven at 100°F overnight to remove the moisture content. Dry weight of individual plants were taken and compared with control (Wood and Roper, 2000).

3.3.8.6 Effect of AgNPs on soil microflora

Soil samples were collected from rhizosphere region of the experimental plants, uprooted randomly. Total viable cell count of bacteria and fungi was determined using spread plate method (Salle, 1973). For that 1 gm of soil sample was suspended in 9 ml of distilled water, vortexed and serially diluted. Bacterial and fungal colonies counting were done on by spreading 100 µl of suspension on NA and PDA respectively, after incubation period of 24 h at 30°C in case of bacteria and at 28°C for 7 days in case of fungi. The number of viable cells/ml was calculated by using the formula (Harley, 2004):

No. of viable cells/ml = (number of colonies) x (dilution factor) x (volume of inoculums in ml)
3.3.9 Data analysis

Analysis of data related with effect of nanoparticles on growth parameters of tomato plants were done using one way analysis of variance (ANOVA) to check significance of NP treatment. It was applied to know if there is any significant difference due to the treatment with nanoparticle or not as compared to control. Level of significance was set at P < 0.05 and results were expressed as Mean ± Standard deviation.