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Role of Silver nanoparticles synthesized by *Camellia sinensis* on growth and development of fenugreek plant

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ABSTRACT
A research was conducted to assess the effect of silver nanoparticles synthesized by Camellia sinensis on fenugreek (Trigonella foenum-graecum L.) plant. The aim was to study various growth and molecular parameters such as coleoptiles length, germination percentage, vigour index, fresh weight, dry weight, protein and DNA content. All the experiments were performed in triplicates. Fenugreek seeds were treated with five different concentrations of silver nanoparticles i.e., 1%, 5%, 10%, 20% and 50%. A control plate without any nanoparticles treatment was also maintained. After the seed inoculation on petri plates they were incubated in BOD incubator for 7 days. Growth and molecular parameters were studied after seven days. Results showed the better seed germination and early seedling growth in fenugreek seeds that were treated with lower concentrations of nanoparticles as compared to the seeds that were treated with higher concentration of nanoparticles. Higher concentrations were toxic to the plant. Moreover, the treated seeds indicate positive influence on germination percentage, coleoptile length, fresh and dry weight as compared to those of unexposed seeds. Overall application of silver nanoparticles was beneficial in improving the germination and growth in fenugreek plant.
KEY WORDS: *Fenugreek, germination percentage, growth, nanoparticles, seedling vigour.*

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INTRODUCTION

Excessive use of pesticides and fertilizers causes environmental pollution, emergence of agricultural pests and pathogens, and loss of biodiversity. Nanomaterials have potential agro-biotechnological applications for mitigation of these problems and it also control increasing pathogen and pest resistance and reducing soil biodiversity. Use of nanofertilizer diminishes the negative impact of chemical fertilizers and pesticides as continuous exposure of these contributes to bioaccumulation of pesticides, pollinator decline, and destroys habitat. Moreover, the application of excess volumes of fertilizers adds to the tribulations of the already delicate ecology as run-off [1]. The world demand for fertilizer was increased by 4.8% to 170.4 million metric tons [2]. Therefore, there is a critical need to tackle the excessive practice of fertilizers and pesticides by finding alternatives to existing pesticide and fertilizer deployment, rapidly and locally detecting occurrence of pathogens and pests, as well as pesticides and nutrient levels; and developing procedures for either agrochemical removal or degradation to endorse soil health. Nanomaterials have potential agro-biotechnological applications for mitigation of these troubles.

Currently, diverse forms of chemical fertilizers viz. calcium silicate, sodium silicate, micro silica, and natural silica sources such as rice husk, and plant byproducts are accessible in the market. Unfortunately, these silica sources are not proficiently utilized by the plant system as it is not a direct resource for plants. In fact, the manufacturing cost of synthetic silica fertilizers in microscales is highly expensive as well as the efficiency of silica uptake requires long duration. In addition, the silicon assimilation using metal salts of silicic acid needs their hydrolysis before their uptake, which in terms affects ionic balance of the soil

and plant system [3].

The exploitation of nanomaterials in biotechnology unites the fields of biology and material science. One of the key developments in nanoscience and technology is the creation and application of nanoparticles in biological sciences. The expanding availability of different nanostructures with highly controlled properties in the nanometre size range has sparked widespread interest in their exploitation in biotechnological systems.

A range of nanomaterials hold immense promise regarding their relevance in plant protection and nutrition due to their size-dependent qualities, high surface-to-volume ratio, and unique optical properties. Such as, nanoparticles and nanocapsules offer an efficient ways to allocate pesticides and fertilizers in a controlled manner with high site specificity, thus dropping collateral damage. In terms of plant–pathogen interaction, application of nanoparticles technology and efficient transportation of substances, such as systemic chemicals, to specific sites offer novel solutions for the curing of plants.

The benefits of nanomaterial-based formulations are the enhancement of efficacy, higher solubility, induction of systemic activity due to smaller particle size and higher mobility, and lower toxicity in contrast to conventional fertilizers and pesticides and their formulations [4]. Seeking potential nanomaterials for the benefit of plant growth and yield is increasing in the present scenario. Thus, keeping above view in minds the current project was carried out in fenugreek to analyze effects of nanoparticles on its growth and molecular components.

MATERIAL AND METHODS

Plant Material and treatments

Seeds of fenugreek were purchased from local vendors of Meerut. Seeds were initially washed with a mild detergent solution of Tween-20 and then sterilized with 0.1 % mercuric chloride ($HgCl_2$) solution. Seeds were treated with the nanoparticles solutions of various concentrations ranging from 0% to 50%. Seeds dipped in nanoparticles solutions of each concentration were placed in BOD incubator in dark with continuous shaking. Seeds were inoculated on the petri plates. A control set was also maintained under the same experimental conditions without any nanoparticles exposure. All the petri plates, having 20 seeds each, were incubated in a BOD incubator at 25°C. All the experiment was performed in triplicate. Various growth and molecular parameters were studied to understand the changes in the control and nanoparticles treated seeds.

Methodology

Germination percentage, Coleoptiles lengths and vigour index

Whatmann filter paper dipped in distilled water was placed in petri plates and twenty seeds were inoculated on each plate in three replications at 25°C. Seeds were considered germinated when 1 mm radicle emerged from the seed coat.

$$\textit{Germination percentage} = \frac{\textit{Number of seeds germinated} \times 100}{\textit{Total no. of seeds inoculated}}$$

Coleoptiles lengths were measured in cm. An average of randomly selected 5 seedlings was taken on 7th day of germination. Vigour index was calculated as the product of seedling vigour (coleoptile length) and germination percentage [5].

Vigour Index = Germination percentage x coleoptile length

Seedling Fresh and Dry weights analysis

Fresh weight was calculated by weighing the seedlings and Dry weight was calculated by exposing the seedlings to high constant temperature (70^0C) for 48 hours.

Protein extraction

Germinated seeds (0.2 gm) of fenugreek (control and stress) were separately crushed in mortar and pestle with 2 ml of sodium phosphate buffer (pH 7.0). The crushed solution is then centrifuged at 12,000 rpm for 20 min. The supernatant is then collected in a fresh vial which consists of soluble proteins and was stored at -20 °C for further analysis. Protein concentration was estimated by [6].

DNA extraction

DNA was extracted by [7]. Pre-chilled mortar and pestle was taken and 5g plant tissues were ground to a fine powder in liquid nitrogen. The powder was added to a conical flask containing 50ml of pre warmed CTAB extraction buffer. The mixture was incubated in a circulating water bath at 65°C for 30 mins with gentle swirling occasionally. An equal volume of chloroform-isoamyl alcohol mixture was added, mixed gently and centrifuged in 50 ml oak ridge tubes at 12,000 rpm for 10 min to spin down cell debris. The upper aqueous phase was collected and the previous step was repeated till no white interface was visible. 0.6 volumes of isopropanol were added to the aqueous phase, followed by 500µl of ice cold absolute ethanol. Tubes were slowly inverted several times to precipitate the DNA. After precipitation, the DNA

was pipetted off slowly by rotating a tip in the cold solution. DNA was pelleted at 10,000 rpm and washed with 70% ethanol. The pellet was air dried and then it was dissolved in appropriate quantity of TE buffer.

Purity checking and Quantification of genomic DNA:

DNA quantification was done with UV-visible spectrophotometer and the quality of DNA was observed from the ratio of the OD values noted at 260 and 280nm. The A_{260}/A_{280} ratio around 1.8-1.9 indicates best quality DNA. A conversion factor of 50 was used to convert OD into concentration in µg/ml.

Agarose gel electrophoresis of genomic DNA

DNA extracted by CTAB method was further fractionated on 1% Agarose gel. 50x stock solution of TAE buffer was prepared in 1000ml of distilled water. For this 242 g of Tris base was transferred to a 1000ml beaker. EDTA solution (pH 8.0, 0.5M) was added by weighing 9.31g of EDTA and it was dissolved in 40ml distilled water. pH of the buffer was checked using pH meter. Electrophoresis buffer (1xTAE) was also prepared to fill the electrophoresis tank and to cast the gel. For this 2ml of TAE stock solution was taken and added to 98ml of distilled water to make the volume upto 100ml. The 1x working solution is made by adding 40 mM Tris-acetate/and 1 mM EDTA. Solution of agarose in electrophoresis buffer was prepared at an appropriate concentration. For this 2g agarose was added to 100ml electrophoresis buffer. The neck of flask was loosely plugged and agarose was dissolved by heating. The flask was then transferred into a water bath at 55°C. When the molten gel had cool down, 0.5µg/ml ethidium bromide was added. The gel solution was mixed thoroughly. Then the warm agarose solution was poured into the mold. The gel was allowed to set at room temperature for 30-45 minutes, and then a small amount of electrophoresis buffer was poured on top of the gel, after carefully removal of comb. Electrophoresis buffer was added to cover the gel to a depth of approx. 1mm. DNA samples were mixed with 0.20 volumes of the desired 6X gel-loading buffer. Sample mixtures were slowly loaded into the wells of the gel using a micropipette. The lid of the gel tank was closed and electrical leads were attached so that the DNA would migrate toward the positive anode (red lead). A voltage of 1-5 V/cm (measured as the distance between the positive and negative electrodes) was applied. If the electrodes are 10cm apart then run the gel at 50V. Running was done until the bromophenol blue and xylene cyanol FF have migrated an appropriate distance through the gel. DNA ladder marker was also used with the running sample.

RESULTS

Effect on Growth parameters:

Nanoparticles effects on Germination percentage:

Seeds treated with the different nanoparticles concentrations i.e., 0%, 1%, 5%, 10%, 20% and 50% shows the germination percentage as 100%, 90%, 95%, 100%, 80% and 30% respectively. Maximum germination percentages were observed at control and 10% concentrations (Figure 1 and 2). The detrimental effect of nanoparticles was observed at highest nanoparticles concentration as exhibiting 30% germination value.

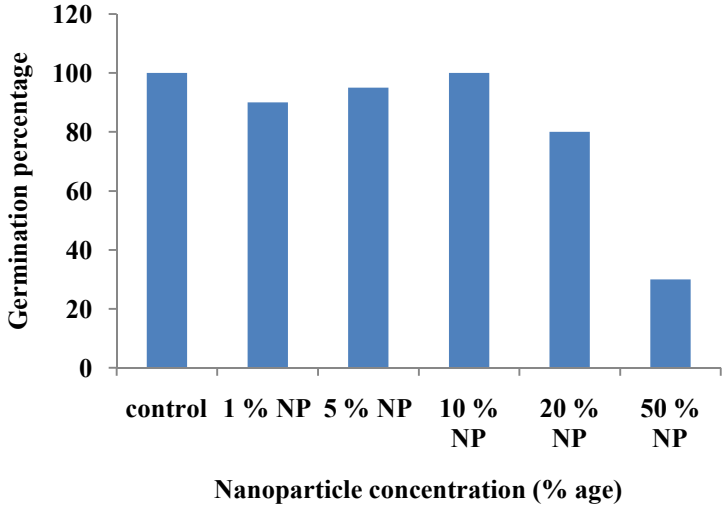

Figure 1: Effects of nanoparticles synthesized from *Camellia sinensis* on germination percentage of fenugreek.

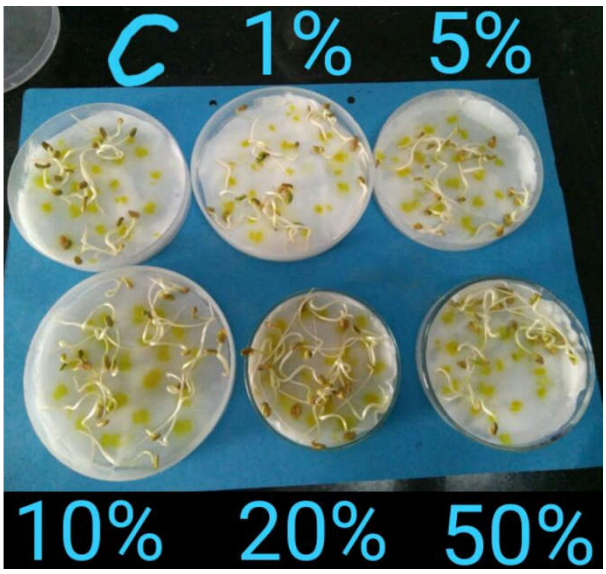

Figure 2: Germinated seeds of fenugreek on exposure to different concentration of nanoparticles synthesized from *Camellia sinensis*.

Nanoparticles effects on coleoptiles length:

Results of the present work shows that the coleoptiles length of the seedlings which were given the nanoparticles treatment of concentrations ranging from 0 to 50% at the time of inoculation showed the difference in their coleoptiles lengths. Coleoptile lengths measured were 11.3cm, 12.1cm, 12.8cm, 16cm, 10.2cm and 4.8cm respectively in the case of seeds treated with nanoparticles solutions of 0%, 1%, 5%, 10%, 20% and 50% respectively. The results showed increase in length as compared to the control upto 10% nanoparticles concentration. Maximum length was observed in case of seeds that were treated with 10% concentration of nanoparticles whereas minimum length was seen in case of seeds treated with 50% solution of nanoparticles (Figure 3).

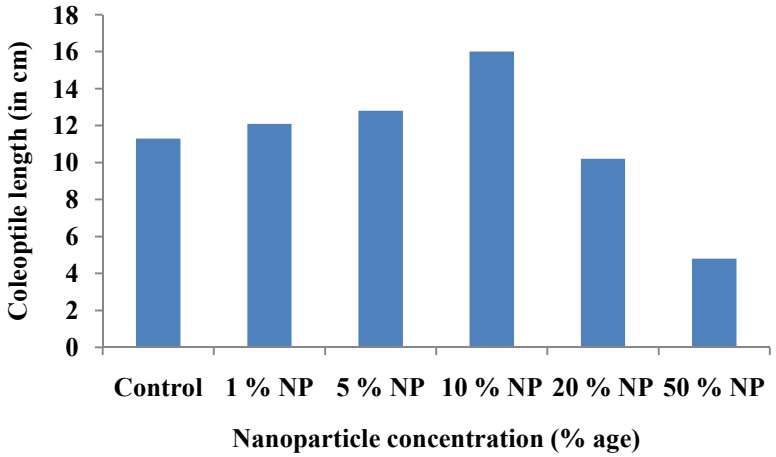

Figure 3: Effect of nanoparticles synthesized from *Camellia sinensis* on coleoptiles lengths of the fenugreek.

Nanoparticles effects on vigour index:

The above results were further supported by analysis of vigour index. Vigour index varied at different nanoparticles concentrations i.e. 0%, 1%, 5%, 10%, 20% and 50% as 1130, 1089, 1216, 1600 and 816 respectively (Figure 4).

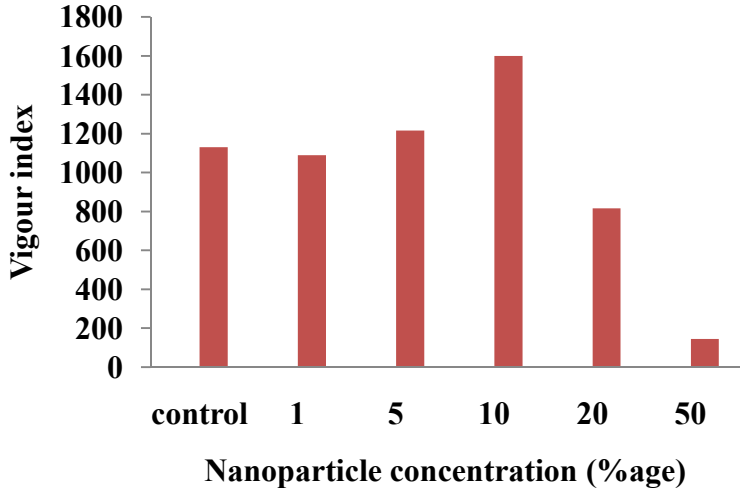

Figure 4: Effects of nanoparticles synthesized from *Camellia sinensis* on vigour index of fenugreek.

Nanoparticles effects on fresh and dry mass:

The fresh mass was quantified through weighing in precision scale. The dry mass was determined through weighing in a precision scale after placing seedlings in an oven with air forced circulation, at a temperature of 70°C upto 48 hours, until constant weight. The average of fresh weight and dry weight of 5 seeds was found to be 0.58g and 0.04g respectively at 10% concentration which was found to have the best growth parameters (Figure 5).

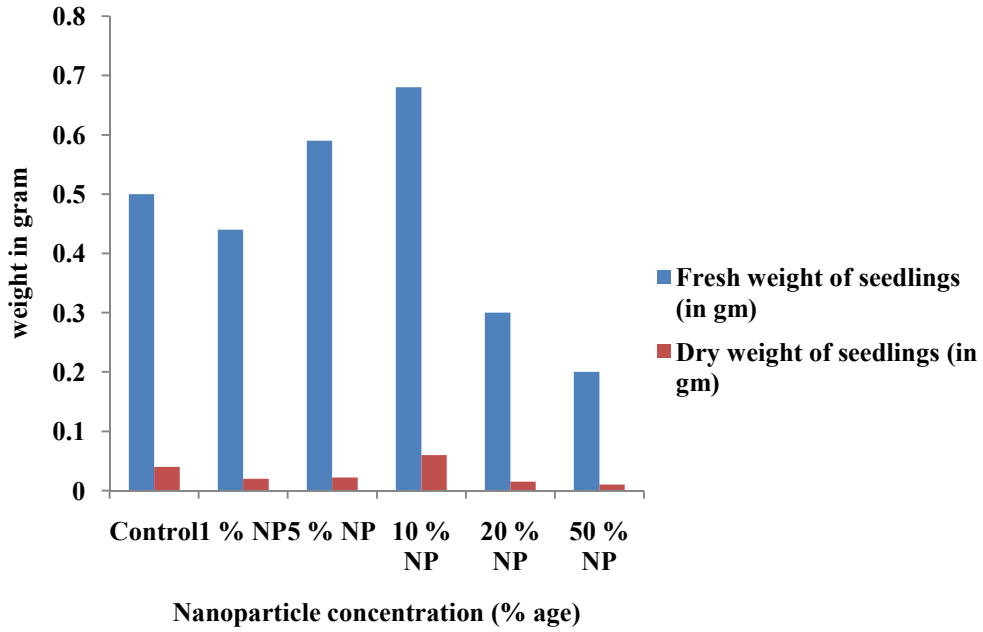

Figure 5: Effects of nanoparticles synthesized from *Camellia sinensis* on fresh and dry weight of fenugreek.

Molecular parameters

Nanoparticles effects on Protein concentration:

In present study it was found that the treatment of different nanoparticles concentrations resulted in differential expression of proteins, as observed decreased and increased protein content at different concentrations. The amount of protein was measured in µg/g fresh weight. Maximum amount of protein was found in seeds treated with 10% concentration of nanoparticles i.e. 0.9 µg/g fresh weight followed by control which was observed 0.66 µg/g fresh weight. Seeds treated with nanoparticles concentrations at 1%, 5%, 20% and 50% gave 0.86, 0.8, 0.5 and 0.4µg/g fresh weight of protein quantity respectively. Least amount of protein i.e. 0.4 µg/g fresh weight was observed at 50% nanoparticles concentration (Figure 6).

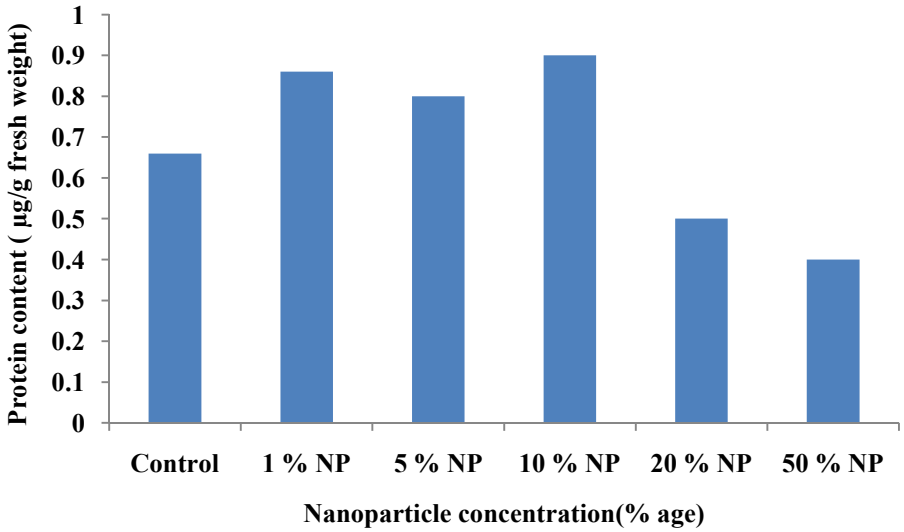

Figure 6: Effects of nanoparticles synthesized from *Camellia sinensis* on protein content of fenugreek.

Nanoparticles effects on DNA concentration:

The quantity of DNA was estimated with UV-visible spectrophotometer. Best DNA quantity was observed at 10% concentration. Amount of DNA observed was 0.88, 0.89, 0.86, 0.9, 0.8 and 0.5µg/g fresh weight at 0, 1, 5, 10, 20 and 50% concentration respectively (figure 7).

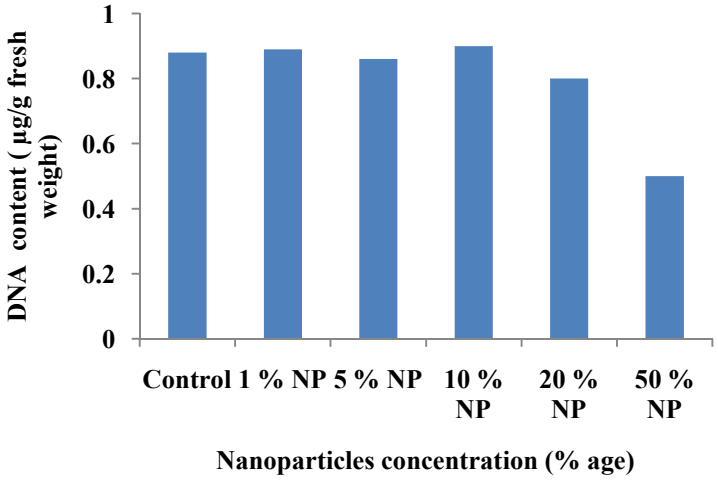

Figure 7: Effects of nanoparticles synthesized from *Camellia sinensis* on DNA content of fenugreek.

Agarose gel electrophoresis of genomic DNA

DNA extracted by CTAB method was further fractionated on 1% Agarose gel. A translucent orange coloured DNA bands were observed. As per the quantative analysis, highest concentration of DNA was observed at 10% nanoparticles treated samples the quality of DNA also exhibit similar results on Agarose gel (Figure 8). The observation of smearing at high concentration resembles that at highest nanoparticles concentration the toxicity of nanoparticles occurs.

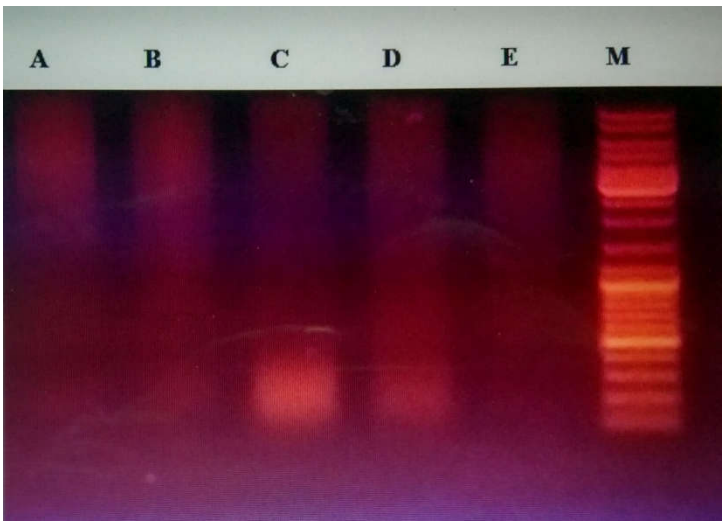

Figure 8: Electrophoresis result showing the quality of DNA obtained by seeds treated with nanoparticles synthesized from *Camellia sinensis* (where M, A, B,C, D and E represents DNA ladder marker, 1%, 5%, 10%, 20% and 50% nanoparticles concentrations on seeds respectively).

DISCUSSION

Seed germination is the foundation for plant growth and development. In present investigation, different concentrations (0, 1, 5, 10, 20 and 50%) of silver nanoparticles were used for the treatment of fenugreek seeds to study their effect on seed germination and early seedling growth. A positive influence on growth and molecular parameters was observed for all the nanoparticles treated seeds as compared to the unexposed seeds. Results indicated that silver nanoparticles at their lower concentrations promoted seed germination and early seedling growth in fenugreek compared to control, however higher concentration showed toxicity. The seedlings growth was observed maximum at 10 % nanoparticles treated seeds. The increased growth rate of the seedlings might be due to the enhanced uptake of water and nutrient by the treated seeds. The present study showed 100% seed germination at 10% concentrations. These results agree with a study conducted by [8] that showed 100% germination of soyabean and chickpea seeds treated with CuO NPs. In another study, fenugreek seeds were treated with silver nanoparticles and results showed increased seed germination and enhanced the seed potential by increasing the characteristics of seed germination [9], this might be due to the seed coat that acts as embryo protector and plays important role in selective permeability. The observations of increased fresh and dry mass in exposure to nanoparticles are similar to findings of [10]. The enhanced growth of fenugreek plants may have been due to significant morphological changes and increased protein content as observed in present study. Biologically synthesized silver nanoparticles induced synthesis of protein content was also reported in *Baopa monnieri* [11].

CONCLUSION

In conclusion, current study reveal that the silver nanoparticles synthesized from *Camellia sinensis* significantly enhanced seed germination potential. Application of silver nanoparticles improved percent seed germination, seed vigour index, seedling fresh weight and dry weight, protein and DNA content. It was found that nanoparticles concentration affects the accumulation and uptake of nanoparticles. Higher concentrations were found to be toxic to seeds with the reduced germination rates and various growth and molecular parameters as compared to the control. Overall, experimental results indicated that the presence of silver nanoparticles affect the growth of fenugreek seedlings at different concentrations. Low concentrations of nanoparticles may act as stimulator because increases in physiological characters were observed.

These results suggest that release of silver NPs synthesized from *Camellia sinensis* into the environment could have only positive effects on plant communities. Enhanced seed germination as well as early plant growth is vital to achieve crop productivity, especially for crops that otherwise show poor germination rates. The profound effect on the early stages of plant growth may be followed by similar enhancements at later stages as well, and by applying nanoparticles we may be able to improve plant productivity too. These results are further in harmony to the protein and DNA content as well as DNA quality.

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