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A study to evaluate the effect of silver nanoparticles synthesized by *Sonchus asper* on fenugreek plant

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Abstract
The present study was conducted on fenugreek (*Trigonella foenum-graecum* L.) with an objective to study the effect of nanoparticles synthesized from *Sonchus asper* on several growth parameters viz. germination percentage, coleoptiles length, vigour index, fresh weight and dry weight, as well as on protein and DNA content. The research was carried out in a randomized block design with three replications. Silver nanoparticles treatment was given in five concentrations having 1%, 5%, 10%, 20% and 50%. A control plate was also maintained in which no nanoparticles was added. Seeds were inoculated on petri plates and incubated in BOD incubator for 7 days. After seven days seeds were studied for analyzing effect of nanoparticles on fenugreek growth. Seed germination results indicated that lower concentration of silver nanoparticles resulted in significant increase in seed germination as well as early seedling growth in fenugreek however, elevated concentrations (above 20%) exhibited toxicity to fenugreek plant. The positive influence on germination percentage, coleoptile length, fresh weight and dry weight was correlated with protein and DNA content which was also recorded to be highest at 20% nanoparticles concentration. The results of the current study concluded that the use of silver nanoparticles at low concentration exhibit a positive effect on germination and growth in fenugreek.

Keywords: Germination percentage, fenugreek, nanoparticles, seedling vigour

Introduction
Nanotechnology triggers a wide array of opportunities in the current decade and is projected to give major impulses to scientific and technological innovations in variety of industrial sectors in the future. Various materials are used to synthesize nanoparticles using bottom-up or top-down approaches for wide applications. They include magnetic materials, metal oxides, semiconductor quantum dots, ceramics, lipids, dendrimers, polymers and emulsions (Puoci *et al.*, 2008) [12].

Presently, many carbon-based materials and metal oxide nanoparticles are being used for agriculture applications to investigate the phytotoxicity and environmental toxicity (Dimkpa *et al.*, 2013) [3]. However, the consequence of nanoparticles on plants can be either positive or negative (Arora *et al.*, 2012; Pallavi *et al.*, 2016) [2, 11]. Enhancement of plant growth is one of the main concerns for exploration of nanomaterials in seed germination. The intensity may depend on the nature of nanomaterial, its dosage and its potential applications. For this reason, the understanding of biological processes at nanoscale level is essential for development in the field of nanotechnology (Ndeh *et al.*, 2017) [10].

New technologies in nano sciences can be a resolution for existing problems associated to agrochemicals, fertilizers, herbicides, pesticides regulation and excellent utilization. Transport of nanoparticles is implicated in a potential plant pathway for valuable outcome (Joseph and Morrison, 2006; Singh *et al.*, 2016) [5, 14]. A wide range of nanomaterials hold promising applications in plant growth, protection and nutrition because of their size-dependent qualities, high surface-to-volume ratio and distinctive optical properties (Singh *et al.*, 2016) [14].

Wide applications of nanotechnology in the area of agriculture includes slow discharge of nanomaterial-assisted fertilizers, biofertilizers, and micronutrients for efficient utilization, delivery of nanocides–pesticides encapsulated in nanomaterials for controlled release; field applications of agrochemicals and nanomaterials assisted delivery of genetic material for crop enhancement. Other areas in agriculture that can benefit from nanotechnology are as follows: nano sensors for plant pathogen and pesticide detection and nanoparticles for soil conservation or remediation (Kim *et al.*, 2006) [6].

Nanomaterials have potential applications in the field of agro biotechnology for the alleviation of problems such as indiscriminate application of pesticides and chemical fertilizers that causes environmental pollution, emergence of agricultural pests and pathogens and more severely loss of biodiversity. Similarly, it increases pathogen and pest resistance, reduces soil biodiversity, diminishes nitrogen fixation, contributes to bioaccumulation of pesticides, pollinator fall off as well as destruction in habitat for birds. In addition, the application of surplus volumes of fertilizers adds to the tribulations of the already delicate ecology as run-off (Tilman *et al.,* 2002) [15].

The world demand for fertilizer was rising exponentially (Heffer and Prudhomme, 2010) [4]. Hence, there is an critical need to tackle the excessive usage of fertilizers and pesticides by i) searching alternatives to current fertilizer and pesticide deployment, ii) speedily and locally detecting presence of pathogens and pests, as well as nutrient levels and pesticides; and iii) developing procedures for either agrochemical removal or degradation to support soil health. Nanomaterials have prospective agro-biotechnological applications for alleviation of these alarming problems.

The benefits of nanomaterial-based formulations are smaller particle size, higher solubility, higher mobility, the improvement of efficacy, induction of systemic activity and lower toxicity in comparison to conventional fertilizers (Sasson *et al.,* 2007) [13]. Therefore, seeking potential nanomaterials for the promotion of plant growth and yield is increasing in the current scenario. Thus, keeping above views the current work is designed to investigate effects of nanoparticles synthesized from *Sonchus asper* on various parameters of fenugreek (*Trigonella foenum-graecum* L.).

Material and Methods

Material

Seeds of fenugreek (*Trigonella foenum-graecum* L.) were purchased from local market of Meerut.

Sterilization

Seeds of fenugreek were initially washed with a mild detergent solution of Tween-20 and then sterilized with 0.1 % mercuric chloride ($HgCl_2$) solution.

Treatment

Seeds were treated with the nanoparticle solutions of various concentrations ranging from 0% to 50%. Seeds dipped in nanoparticle solutions of each concentration were placed in BOD incubator in dark. Seeds were inoculated on the petri plates. A control set was also maintained under the same experimental conditions without any nanoparticle exposure. All the petri plates, having 20 seeds each, were incubated in a BOD incubator at 25 °C. All the experiment were performed in triplicate. Various growth parameters were studied to understand the changes in the control and nanoparticle treated seeds.

Methodology

Germination percentage

Twenty seeds in three replication of each were placed on Whatmann filter paper dipped in distilled water in petriplate at 25°C. Only seeds with coleoptile longer than 2 mm were considered germinated in current study.

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

Coleoptiles length measurement

Coleoptiles length was measured in cm as an average of randomly selected 5 seedlings on 7th day of germination.

Vigour Index

Vigour index was calculated as the product of seedling vigour (coleoptile length) and germination percentage (Abdul Baki and Anderson, 1973) [1].

Vigour Index = Germination percentage x coleoptile length

Fresh and dry weight analysis

Fresh weight was calculated by weighing the seedlings and dry weight was tested by exposing the seedlings to high constant temperature oven method, at 70 ^{0}C 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). After proper crushing the 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. Protein concentration was estimated by Lowry *et al.* (1951) [8].

DNA extraction using CTAB method

DNA was extracted by Murray and Thompson (1980) [9]. The steps are as follows:

- Plant tissues (5 g) were ground in liquid nitrogen to fine powder using pre-chilled mortar and pestle.
- The powder was transferred to a 250ml conical flask containing 50ml of pre warmed CTAB extraction buffer.
- The mixture was incubated at 65°C for 30 min in a circulating water bath with occasional mixing by gentle swirling.
- An equal volume of chloroform-isoamyl alcohol mixture was added and then the contents were gently mixed 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.
- To the aqueous phase 0.6 volume of isopropanol was added and mixed gently. The 500µl of ice cold absolute ethanol was added and tubes were slowly inverted several times to precipitate the DNA.
- Following precipitation, the DNA was pipetted off by slowly rotating/spinning a tip in the cold solution.
- The DNA was pelleted at 10,000 rpm and then it was washed with 70% ethanol.
- The pellet was air dried and dissolved in appropriate quantity of TE.

Purity checking and Quantification of genomic DNA:

The quantity of DNA was estimated with UV-visible spectrophotometer. The quality of DNA was observed from the ratio of the OD values recorded 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.

Protocol

1. **Preparation of 50x stock solution of TAE buffer in 1000ml of distilled H_2O:** For preparing stock solution 242 g of Tris base was weighed and transferred to a 1000ml beaker. EDTA solution (pH 8.0, 0.5M) was prepared by adding 9.31g of EDTA and it was dissolved in 40ml distilled water. Both solutions were mixed and final volume was made 1000ml.
2. **Preparation of 1xTAE electrophoresis buffer (40 mM Tris-acetate/1 mM EDTA):** For this 2ml of TAE stock solutions was taken in an Erlenmeyer flask and volume was made upto 100ml by adding 98ml of distilled water.
3. **Preparation of 2% agarose gel:** For preparing gel 2 grams of agarose was added to 100ml electrophoresis buffer.

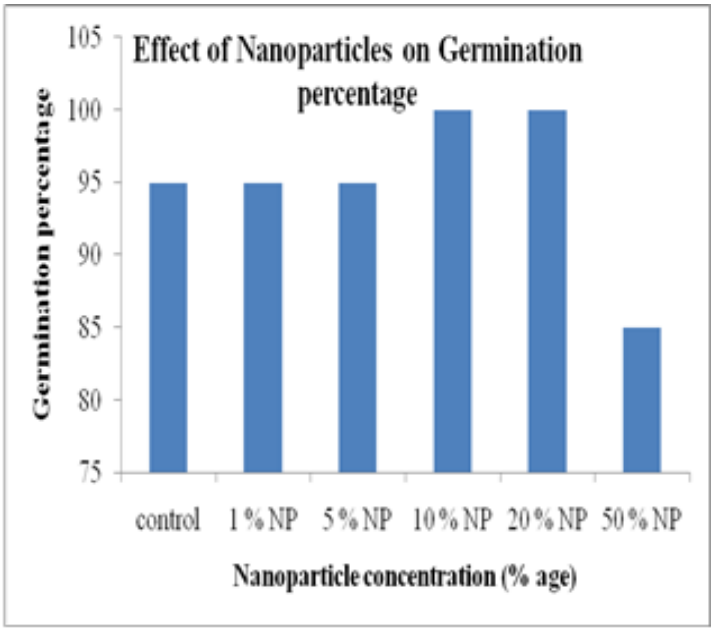
- The neck of flask was loosely plugged and agarose was dissolved in microwave.
- In the molten gel 0.5µg/ml of ethidium bromide was added. The gel solution was mixed thoroughly by gentle swirling.
- The luke warm agarose solution was poured into the electrophoresis assembly.
- After pouring, the gel was allowed to set completely (30-45 minutes at room temperature), then comb was removed carefully Electrophoresis buffer was added to cover the gel to a depth of approximately 1mm.

4. **Preparation of sample:** The samples of DNA were mixed with 0.20 volumes of the 6X gel-loading buffer.
5. The DNA sample mixtures were slowly loaded into the slots of the submerged gel using a micropipette.
6. A voltage of 1-5 V/cm (measured as the distance between the positive and negative electrodes) was applied to run samples.
7. Running was done until the bromophenol blue and xylene cyanol FF have migrated an appropriate distance through the gel.
8. DNA ladder marker was also used with the running sample.

Results and Discussion

Effect of nanoparticles treatment on germination percentage

In the life cycle of plants percentage germination represents a dynamic phase as the seed makes the shift from a metabolically quiescent to a vigorous and growing entity. Germination percentage of fenugreek (*Trigonella foenum-graecum* L.) was observed to be 95%, 95%, 95%, 100%, 100% and 85% with the increase in nanoparticles concentration from 0%, 1%, 5%, 10%, 20% and 50% respectively. Optimum germination percentage was found at 10% and 20% nanoparticles concentration (Figure 1 and 2). Singh *et al.* (2016) [14] also reported increase in germination percentage of *Solanum lycopersicum* treated with ZnO nanoparticles synthesized by *Elaeagnus angustifolia* (Russian olive). Contrary to our results Ndeh *et al.* (2017) [10] reported phytotoxic effect of green synthesized gold nanoparticles on rice germination.



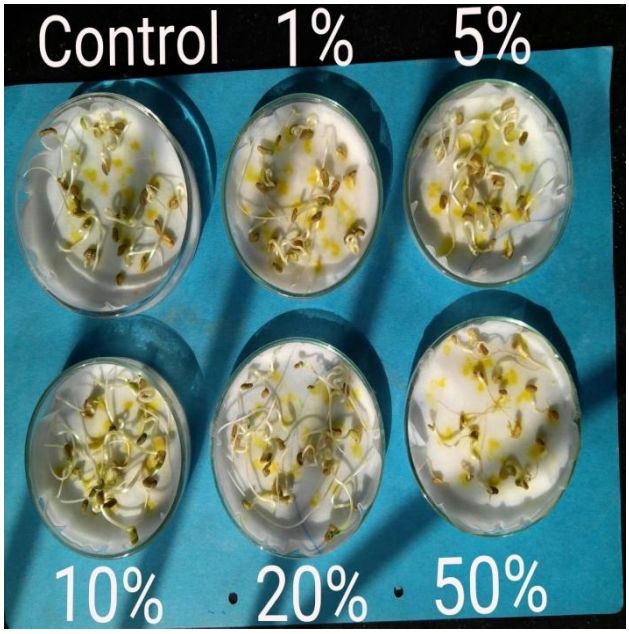


Fig 1, 2: Effects of nanoparticles synthesized from *Sonchus asper* on germination percentage of fenugreek.

Effect of nanoparticles concentration on coleoptile lengths

The results of the present work shows that the coleoptile length of the seedlings which were given nanoparticles treatment at the time of inoculation is increases than those which were not given nanoparticles treatment. Coleoptile length of the seeds varied from minimum 7.8cm to maximum 10.5cm. Maximum length was observed in case of seeds that were treated with 20% concentration of nanoparticles whereas minimum length was seen in case of seeds treated with 1% solution of nanoparticles (Figure 3). The increased growth rate of the seedlings as observed might be due to the enhanced uptake of water and nutrient by the treated seeds. Arora *et al.* (2012) [2] studied effect of gold nanoparticles on *Brassica* seedlings and suggested that the changes in the growth profile on exposure to gold nanoparticles might be because of the interference in plant hormone action. The increase in length is probably linked to the bio-compatible nature of green synthesized nanoparticles (Ndeh *et al.*, 2017) [10].

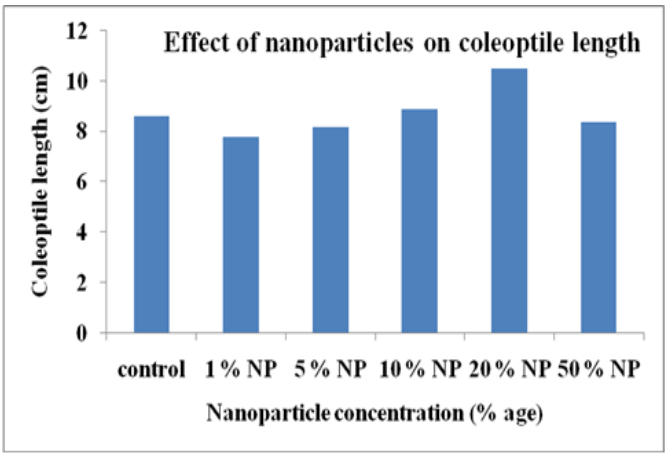


Fig 3: Effects of nanoparticles synthesized from *Sonchus asper* on coleoptiles length of fenugreek.

Effect of nanoparticles concentration on vigour index

The above results were further supported by analysis of vigour index. Data shown in figure 4 revealed that vigour index was highest in case of 10% and 20% nanoparticles treated seeds. Control showed higher vigour index as compared to the highest nanoparticles concentration treated seeds. The value of vigour index increased ranging from 1 to 20% but decreased in higher concentration i.e. 50% as 741, 779, 890, 1050 and 714 respectively. The vigour index calculated in case of control was found to be 817. In harmony to our result Singh *et al.* (2016) [14] also reported increase in seedling vigour of *Solanum lycopersicum* at low concentration of *Elaeagnus angustifolia* (Russian olive) synthesized ZnO nanoparticles. They also reported that improvement in plant growth and development by ZnO nanoparticles is due to increase in chlorophyll content, sugar content and antioxidative enzyme activity.

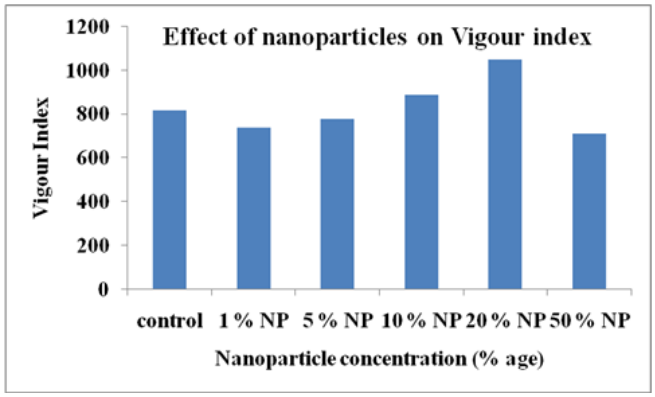


Fig 4: Effects of nanoparticles synthesized from *Sonchus asper* on vigour index of fenugreek.

Effect of nanoparticles concentration on Fresh weight and Dry weight

The average of fresh weight and dry weight of five seeds was found to be 0.43g and 0.05g respectively at 20% concentration which was found to be the representative of best growth parameters. Whereas, the control had the maximum fresh weight i.e. 0.59g followed by seedlings treated with 20% nanoparticles i.e. 0.58g. Minimum fresh weight was observed in 1% nanoparticles treated seedling which is 0.32g. Similarly dry weight was observed to be 0.04, 0.02, 0.04, 0.045, 0.05 and 0.031 respectively (Figure 5). Similar to our findings, Pallavi *et al.* (2016) [11] also studied effect of silver nanoparticles on three crops namely wheat (*Triticum aestivum*, var. UP2338), *Brassica* (*Brassica juncea*, var. Pusa Jai Kisan) and cowpea (*Vigna sinensis*, var. Pusa Komal) and reported that on applying AgNPs to soil, plant biomass increased significantly.

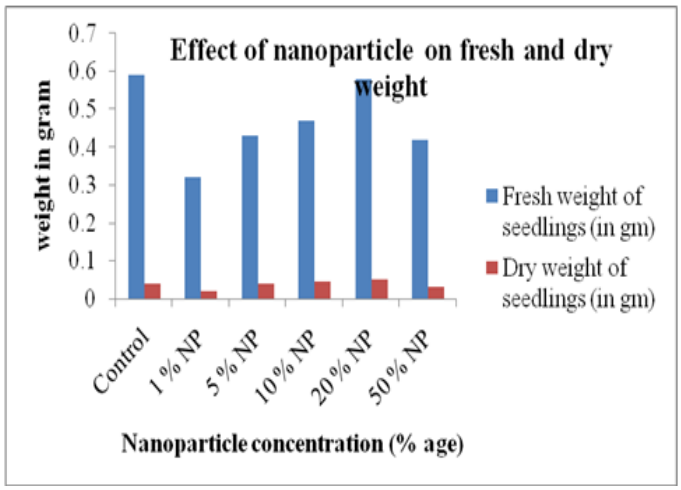


Fig 5: Effects of nanoparticles synthesized from *Sonchus asper* on fresh and dry weight of fenugreek.

Effect of nanoparticles concentration on protein content

The amount of protein was measured in µg/g fresh weight. The amount of protein was measured maximum in 20% concentration treated seeds which was 0.029 µg/g fresh weight followed by control with 0.025 µg/g fresh weight. Seeds treated with concentrations at 1%, 5%, 10% and 50% gave 0.01, 0.015, 0.02 and 0.008 µg/g fresh weight of protein quantity respectively. 50% nanoparticles treated seeds were found to have the least amount of protein i.e. 0.008 µg/g fresh weight (Figure 6). Krishnaraj *et al.* (2012) [7] also reported that biologically synthesized silver nanoparticles induced synthesis of protein and carbohydrate content in *Baopa monnieri.* Similarly, Singh *et al.* (2016) [14] also reported increase in protein content of *Solanum lycopersicum* at low concentration of *Elaeagnus angustifolia* (Russian olive) synthesized ZnO nanoparticles.

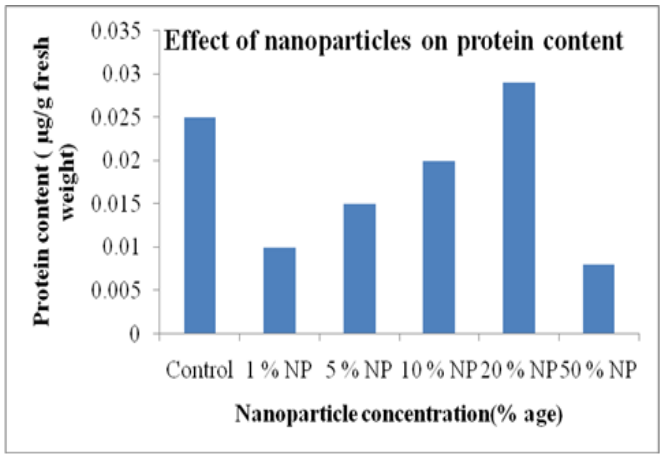


Fig 6: Effects of nanoparticles synthesized from *Sonchus asper* on protein content of fenugreek

Effect of nanoparticles concentration on DNA content

The concentration of DNA varied with the difference in concentrations of nanoparticles. Best results were seen at 20% concentration. Amount of DNA observed was 0.25, 0.3, 0.33, 0.4, 0.45 and 0.2µg/g fresh weight at 0, 1, 5, 10, 20 and 50% concentration respectively (Figure 7).

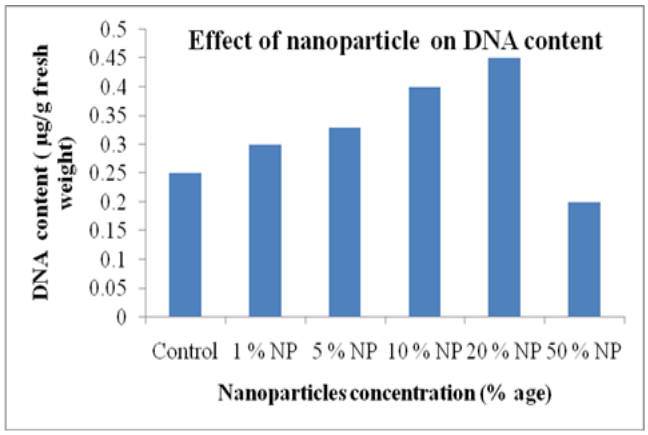


Fig 7: Effects of nanoparticles synthesized from *Sonchus asper* on DNA content of fenugreek.

Effect of nanoparticles concentration on quality of DNA

A translucent orange coloured DNA bands were observed. The result of quantitative analysis of DNA exhibited increase in DNA content as well as highest concentration of DNA at 20% nanoparticles treated samples. The quality of DNA also exhibit similar results on Agarose gel (Figure 8). The observation of smearing at highest concentration resembles that at high nanoparticles concentration caused the toxicity to the plant.

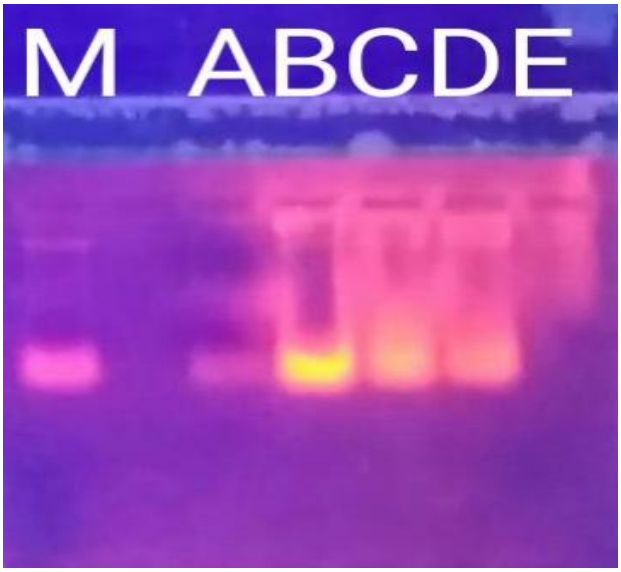


Fig 8: Electrophoresis result showing the quality of DNA obtained by seeds treated with nanoparticle synthesized from *Sonchus asper* (where M, A, B, C, D and E represents DNA ladder marker, 1%, 5%, 10%, 20% and 50% nanoparticle concentrations on seeds respectively).

Conclusion

Nanoparticles are projected to be the resources for the new millennium. Nanoparticles of size below 100nm drop in the transition zone between individual molecules and the related bulk materials, which generate positive and/or negative biological effects in living cell. Briefly, we investigated effect of silver nanoparticles synthesized by a green method using plant leaf extracts of *Sonchus asper* on fenugreek. Several growth parameters such as germination percentage, coleoptiles length, fresh and dry weight, vigour index and protein and DNA content were observed for examining effect of nanoparticles.

Fenugreek responded positively to silver nanoparticles synthesized from *Sonchus asper* as exhibited by higher seeds germination percentage compared to control. The seedlings growth was also observed maximum at 20% nanoparticles treated seeds. These results recommend that release of silver nanoparticles synthesized from *Sonchus asper* into the environment could have only positive influences on plant communities. Improved seed germination and early plant growth is vital to attain crop productivity, especially for crops that otherwise exhibit poor germination rates. The profound consequence on the initial stages of plant growth may be followed by similar enhancements at later stages as well. Thus, it may be possible to improve plant productivity too by applying nanoparticles. These results are further in harmony to the protein and DNA content as well as the quality of genomic DNA.

In conclusion, these results of the present research reveals that the application of silver nanoparticles synthesized from *Sonchus asper* resulted in significant enhancement of seed germination potential. Application of silver nanoparticles triggers improved seed germination percentage, seed vigour index, seedling fresh weight and dry weight. It was found that the accumulation and uptake of nanoparticles was dependent on the exposure concentration. Higher concentrations were found to be toxic to seeds with the reduced germination rates as compared to the control. From the present study, it can be concluded that plant and green synthesized silver nanoparticles interaction is very complex and, by optimizing the silver nanoparticles concentration, growth promotion of plant can be achieved without causing harm to the environment.

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