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Research Paper

EFFECT OF FUNGAL GENERATED N- TiO_2 ON FEGUNGREEK (*Trigonella foenumgraecum*) SEED GERMINATION AND SEEDLING GROWTH

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Abstract

The present study was undertaken to investigate the effect of biologically synthesised Titanium dioxide nanopartices (N-TiO_2) on the germination of fenugreek (*Trigonella foenumgraecum* L.) seeds. The fungus *Aspergillus niger* was used for the biosynthesis of titanium dioxide nanoparticles. Nanoparticles were characterized by UV visible spectroscopy, scanning electron miscroscopy (SEM), and X-ray diffraction (XRD) pattern. Further, fenugreek seeds were soaked in two different concentrations (25 µg and 50µg) of N-TiO_2. Seeds soaked in water alone served as control for this study. The germination characteristics such as shoot length, root length, germination percentage, and seed vigor index were calculated after treating with N-TiO_2. The seeds soaked in 50µg titanium dioxide nanoparticles were found to germinate faster and grow when compared to the growth of control seedlings and 25µg treatment. Biochemical parameters including protein, carbohydrates, reducing sugars, chlorophyll were estimated in the treated seedlings. There was a significant increase in the biochemical profile of the seedlings treated with titanium dioxide nanoparticle. Root length, shoot length, germination rate and seed vigor were significantly enhanced upon the treatment of 50µg TiO_2 when compared to 25µg treatment. The observed results indicate that N-TiO_2 is a good promoter of fenugreek seed germination and seedling growth.

Key words: *Titanium dioxide nanoparticles, Aspergillus niger, Fenugreek seeds, shoot length, chlorophyll*.

INTRODUCTION

The recent development in nanobiotechnology has expanded the wide applications of nanomaterials in the field of agriculture to enhance seed germination and plant growth. The beneficial effects of nanomaterials are due to their unique characteristics including their size and greater surface area when compared to their respective bulk form [1]. Plant biotechnology and agricultural research are being harnessed with the emerging new approach in nanotechnology. Sufficient research works on exploitation of growth promoting effect of nanoparticles have been achieved in the last decade and developed new strategies in the field of Nano-agriculture [2]. For instance, SiO_2 and TiO_2 nanoparticles have enhanced the nitrate reductase activity in seeds leading to germination and growth in soybean [3]. TiO_2 nanoparticles exhibit their ability to promote plant photosynthesis and nitrogen metabolism by which plant growth was increased to greater extent [4]. Nanoparticles interact with living cells at molecular level so that they impart beneficial effect in the crops [5].

Fenugreek (*Trigonella foenumgraecum* L.) is a flowering annual plant having autogamous flowers. Though this crop is native to regions spanning from Iran to northern India, it is widely cultivated in China, India, Egypt, Ethiopia, Morocco, Ukraine, Greece, Turkey, etc. [6]. Fenugreek normally grows in winter seasons and possesses several therapeutic effects; antioxidant, anticancer, antidiabetic effect, and insulin sensitizing activity etc., [7-8]. Seed priming is a successful method that has been proved to improve seed germination and emergence of seedlings. It is a controlled hydration treatment at low water potential that induce pre-germinative metabolism and prevents radicle emergence [9]. We can implement nanobiotechnology to develop effective strategies to improve the germination of seeds of economically important crops such as fenugreek. In view of this context, the present study was aimed to investigate the fungal synthesized Titanium dioxide nanoparticles for their growth promoting effect on fenugreek seeds and further to evaluate the biochemical parameters in the developed seedlings.

MATERIALS AND METHODS

Biomass production

Aspergillus niger was grown in 250ml Erlenmeyer flask containing 100ml malt extract peptone (MGYP) medium. After adjusting the pH of medium to 6.8, the culture was grown with continuous shaking on a rotary shaker (150 rpm) at 28oC for 72hrs. After 72hrs, fungal balls of mycelia were separated from the culture broth by centrifugation (4000 rpm) at 4oC for 10min and then fungal mycelia were washed with sterile distilled water. The harvested fungal biomass (15 g wet weight) was re-suspended in100ml sterile Milli-Q-Water in 250ml Erlenmeyer flask and again kept on shaker (150 rpm) at 28oC for 62hrs.

Synthesis of Titanium dioxide nanoparticles

After incubation the cell free suspension was obtained which was added to TiO2 salt in concentration of 0.1 M (optimum salt concentration from our preliminary experiment). The entire mixture was put into shaker (150 rpm) at 28oC and the reaction allowed for a period of 48hrs. The biologically transformed particles were collected periodically and monitored for further characterization [10].

Characterisation of synthesized nanoparticles

UV-Vis spectrophotometric analysis

Optical properties of TiO2 NPs were measured by subjecting the sample to UV-Visible spectrophotometer within the range 200 to 800nm and absorbance was plotted on a graph.

Scanning electron microscopic analysis

The morphology and size of the synthesised nanaoparticles were determined using scanning electron microscope. Scanning Electron Microscopic (SEM) analysis was done using Joel Model No. 6390 SEM machine. Thin films of the sample were prepared on a carbon coated copper grid by just dropping a very small amount of the sample on the grid. Extra powder was removed and was subjected for SEM analysis.

X-Ray Diffraction analysis

Crystal structure, phase composition, phase purity and mean size of the nanoparticles are analyzed by X-Ray diffraction spectroscopy. The X-Ray diffraction pattern of the synthesized nanoparticles was recorded between the ranges 10^o to 90^o.

Plant material and Seed germination

The experiment was conducted to determine the growth promoting effect of nano titanium dioxide on fenugreek seed germination. The different concentrations of TiO_2 (25μg, and 50μg) were chosen to soak the seeds along with control (water). The fenugreek seeds were surface sterilized with 0.1% mercuric chloride, and washed thrice with distilled water. These seeds were allowed to germinate in petridishes containing cotton. Each petridish contained 20 seeds impregnated on cotton. Three replicates were used for each concentration of TiO_2 in this study. Seeds in petridishes were irrigated with distilled water and allowed to germinate and grow for 14 days. At the end of 14 days, the percentage of germination, seed vigor index, shoots length and root lengths were estimated.

Biochemical estimations

Estimation of Protein estimation

500mg of the sample was weighed and ground with pestle and mortar in 5 to 10 ml of the buffer and further protein was isolated through centrifugation. Protein was estimated as described in the method of Lowry *et al* (1951).

Estimation of Total carbohydrate

Anthrone method was adopted to measure the carbohydrate content in the seedling tissues. According to this method, carbohydrates are first hydrolyzed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This compound forms with anthrone a green coloured product with absorption maximum at 630nm when added to anthrone reagent [11].

Estimation of reducing sugars

Reducing sugars are estimated as described by Nelson and Somogyi. The reducing sugars when heated with alkaline copper tartarate reduce the copper from the cupric to cuprous state and thus cuprous oxide is formed. When cuprous oxide is treated with arsenomolybdic acid, the reduction of molybdic acid to molybdenum blue occurs. The blue colour thus developed was compared with a set of standards in a colorimeter at 620nm [12-13]

Estimation of chlorophyll

Chlorophyll was extracted in 80% acetone and the absorption at 663 and 645nm were measured using spectrophotometer. Absorption coefficient was taken into consideration for calculating the amount of chlorophyll [14]

RESULTS

TiO_2 nanoparticles were synthesized using *Aspergillus niger* in biological route and characterized with UV-Visible spectroscopy. Absorption spectra were obtained in the range between 202 nm and 752 nm which ascertained the existence of nanomaterials (Figure 1). The scanning electron microscope images also confirmed the presence of nanoparticles whose size was found to be in the range of 73.58 to 106.9 nm. SEM analysis clearly reveals the morphology and size of the synthesised nanoparticles (Figure 2). In XRD method, Crystal structure and crystallite size were determined with Debye Scherrer equation where scattering angle was considered. Broadening of a particular peak in the XRD diffraction pattern along with particular planar reflection was observed in the crystal unit cell. The X-Ray diffraction pattern reveals three characteristic peaks cantered at 25^{o}, 47^{o}, 53^{o} (Figure 3). Broader peaks indicate the smaller size of crystallite and vice versa due to random arrangement of crystallites. Tall cum narrow peaks reveal individual crystallite structure. XRD pattern clearly indicates that anatase form of TiO_2 synthesized by *Aspergillus niger*.

Exposure to TiO_2 NPs significantly increased seed germination rates upto 91 and 98% when treated with 25µg and 50µg respectively. 72.5% seed germination was observed in the control seedling (Figure 4). The positive effect of TiO_2 NP treatment on seed germination was confirmed with the measures observed in the root length (2.58 cm and 2.81cm), shoot length (16.32 and 19.77cm) and seed vigor index (1724.63 and 2229.7) upon 25 and 50µg NPs treatment respectively. These measures of germination rate and seedling growth index in treated groups were comparable with the control group (Table 1).

The protein content of the seedlings was estimated after treatment with TiO_2 nanoparticles. 25µg and 50µg N-TiO_2 was found to significantly enhance the protein level in the shoot by 29% and 38% and that of the roots by 24.3% and 37.8% respectively (Table 2). The increase was greater upon treatment with 50µg nanoparticles compared to 25µg nanoparticle concentration. The carbohydrate content of the seedlings was quantified in the treated and control seedlings. 25µg and 50µg titanium dioxide nanoparticles enhanced the carbohydrate level in the shoot by 47.9% and 71% and that of the roots by 56.9% and 99% respectively (Table 3). Both protein and carbohydrate contents were found to increase upon N-TiO_2 treatment in dose dependant manner. 50µg nanoparticles exhibited more influence than that of 25µg concentration.

The reducing sugar content in the seedling was measured after treatment with TiO_2 nanoparticles. 25µg and 50µg titanium dioxide nanoparticles were found to enhance the reducing sugars in the shoot by 40.2 % and 60.7 % and that of the roots by 65.5 % and 79.9% respectively (Table 4). The reducing sugar content was found to increase upon nanoparticle treatment in dose dependant manner. The increase was greater in treatment with 50µg nanoparticles compared to 25µg nanoparticle concentration. The increase in chlorophyll content of fenugreek leaves treated with different concentrations of Titanium dioxide nanoparticles was evaluated (Table 5). The amount of chlorophyll significantly increased from 0.63 mg/g (control) upto 1.20 and 1.33 mg/g tissue when treated with 25µg and 50µg N-TiO_2. The chlorophyll content was observed to be almost doubled in TiO_2 nanoparticles treated seeds.

DISCUSSION

Characterization of biologically synthesized TiO_2 nanoparticles ascertains that *Aspergillus niger* releases extra cellar enzymes capable of producing nanosized TiO_2. The absorption peaks of UV visible spectrum substantiated the formation of nanoparticle in the fungal culture, which is due to the surface Plasmon resonance /vibration in the reaction mixture [15]. Scanning electron microscopy (SEM) results also proved that nanoparticles are almost spherical, with high homogeneity and narrow size distribution [16]. XRD pattern clearly indicated that anatase form of TiO_2 nanoparticles and this reduction in crystallite size was attributed to broadening of peak in the case of TiO_2 produced by fungal biosynthesis [17]. In biological study, TiO_2 nanoparticles enhanced the germination of fenugreek seeds, which might be due to the promoting effect of N-TiO_2 on photosynthesis and nitrogen metabolism. This was supported by a previous report in which spinach seeds were found to germinate consistently faster when treated with TiO_2 NP [4]. Similarly, seeds soaked in suspension of TiO_2 NPs at concentration of 100 mg/l positively influenced seed germination and root growth in previous research reports [18]. Protein, carbohydrate, reducing sugar and chlorophyll contents in the seedlings were enhanced by 50µg TiO_2 nanoparticles when compared to25µg concentration and control samples. Priming effect of nanomaterials on seeds has been reported to induce the de-novo synthesis of peptides in a study conducted in sunflower seeds [19].

Results reveal that translation process might have influenced by the nanoparticles thereby increasing protein level in the seedlings. A significant increase in protein content of *Phaseolus vulgaris* seeds when treated with silver nanoparticles under magnetic field was previously reported by other researchers [20].Titanium dioxide nanoparticles might have improved electron transport, photosynthetic efficiency and RUBISCO activity leading to the carbohydrate synthesis and consistent growth in the present study. Studies with TiO_2 nanoparticles in *Spinacia oleracea* had recorded positive interactions of nanoparticles with photosystems especially enhancement of RUBISCO activity responsible for enhanced growth and seed germination [20, 4, 21]

Exposure of seeds to N-TiO_2 at both 25 and 50µg significantly enhanced Chlorophyll content in the leaves. N-TiO_2 treatment might be involved in photoreduction activity of photosystem II and electron transport chain leading to increas pigments [21]. The chlorophyll was observed to be almost doubled in treated seedling when compared to untreated control. Previous studies have examined the changes of photosynthetic pigments in maize sprayed with TiO_2 at various concentrations. Nano TiO_2 significantly enhanced chlorophyll content (a and b), total chlorophyll, chlorophyll a/b, carotenoids and anthocyanins contents of maize flowers [22-23].

CONCLUSION

According to this investigation, we conclude that *Aspergillus niger* releases extra cellular enzymes capable of synthesizing Titanium dioxide nanoparticles. Characterization of studies using UV visible spectroscopy, scanning electron microscopy and X-ray diffraction methods confirmed the presence of nano-sized TiO_2. These N- TiO_2 improved fenugreek seed germination and growth of seedling. Further, there was significant increase in the levels of protein, carbohydrates, reducing sugars and chlorophyll content of fenugreek seedlings. The possible contribution of TiO_2NPs facilitated the seed coats to allow water penetration and nutrients and enhance the seed germination rate, lengths of shoot and root, and essential biomolecules in seedling. Hence, titanium dioxide nanoparticles can be used as an effective growth promoter to enhance seed germination.

Table1: The effect of TiO_2 nanoparticles on germination and growth.

TiO_2 concentration	Root length (cm)	Shoot length (cm)	Germination percentage	Seed vigor Index
Control	1.31± 0.62	6.8± 0.79	72.5± 0.61	587.98± 0.46
25µg	2.58± 0.57	16.32± 0.62	91.25± 0.54	1724.63± 0.56
50µg	2.81± 0.42	19.77± 0.54	98.75± 0.43	2229.7± 0.71

*Values are mean ± SD of three samples in each group

Table 2: Effect of TiO_2 on the protein content of Fenugreek seedlings

TiO2 concentration	Shoot (mg/g)	Root (mg/g)
Control	38.10 ± 0.69	37.03±0.58
25 µg	49.63±0.64a**	46.33±0.64a**
50 µg	52.57±1.27b**c**	51.10±0.00b**c**

*Values are mean ± SD of three samples in each group
Group comparison : a – Control vs 25 µg N-TiO_2; b – Control vs 50 µg N-TiO_2;
c – 25 µg N-TiO2 vs 50 µg N-TiO_2; [**- Significant at 1% ($p<0.01$)]

Table 3: Effect of TiO_2 on the carbohydrate content of Fenugreek seedlings

N-TiO2	Shoot (mg/g)	Root (mg/g)
control	13.27.±0.31	8.57±0.40
25 µg	19.63±0.55a**	13.45±0.31a**
50 µg	22.72±0.31b**	17.08±0.32b**

*Values are mean ± SD of three samples in each group
Group comparison : a – Control vs 25 µg N-TiO_2 ; b – Control vs 50 µg N-TiO_2 ;
c – 25 µg N-TiO_2 vs 50 µg N-TiO_2; [**- Significant at 1% ($p<0.01$)]

Table 4: Effect of TiO_2 on the reducing sugar content of fenugreek seedlings

N-TiO2	Shoot (mg/g)	Root (mg/g)
0(control)	12.91±0.32	10.54±0.31
25 µg	18.18±0.31a**	17.48±0.55a**
50 µg	20.75±0.50b**c**	18.97±0.22b**c**

*Values are mean ± SD of three samples in each group
Group comparison : a – Control vs 25 µg N-TiO_2; b – Control vs 50 µg N-TiO_2;
c – 25 µg N-TiO_2 vs 50 µg N-TiO_2. [**- Significant at 1% ($p<0.01$)]

Table 5: Effect of TiO2 on the chlorophyll content of fenugreek leaves

N-TiO2	Chlorophyll (mg/g)
Control	0.63±0.06
25 µg	1.20±0.10a**
50 µg	1.33±0.15b**

*Values are mean ± SD of three samples in each group
Group comparison: a – Control vs 25 µg N-TiO_2; b – Control vs 50 µg N-TiO_2;
c – 25 µg N-TiO_2 vs 50 µg N-TiO_2. [**- Significant at 1% ($p<0.01$)]

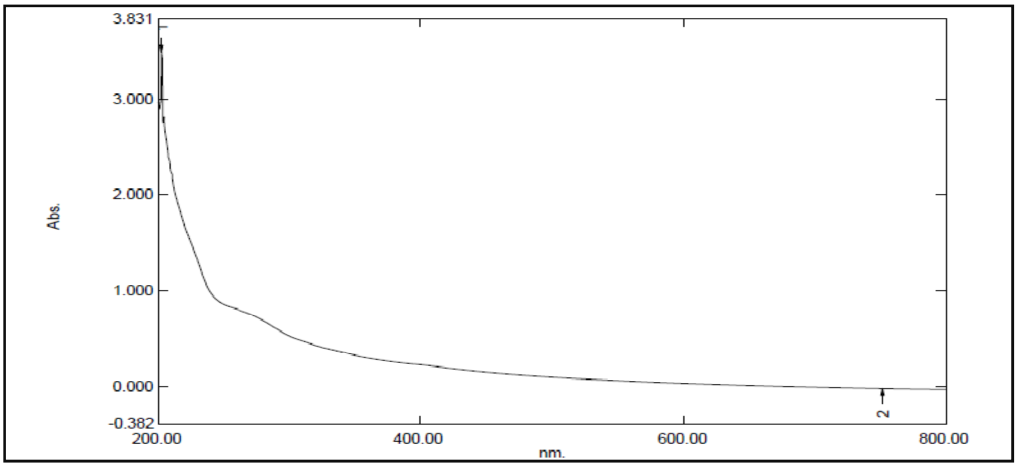

Figure 1: UV-Visible absorption spectrum in the range of 202 and 752 nm.

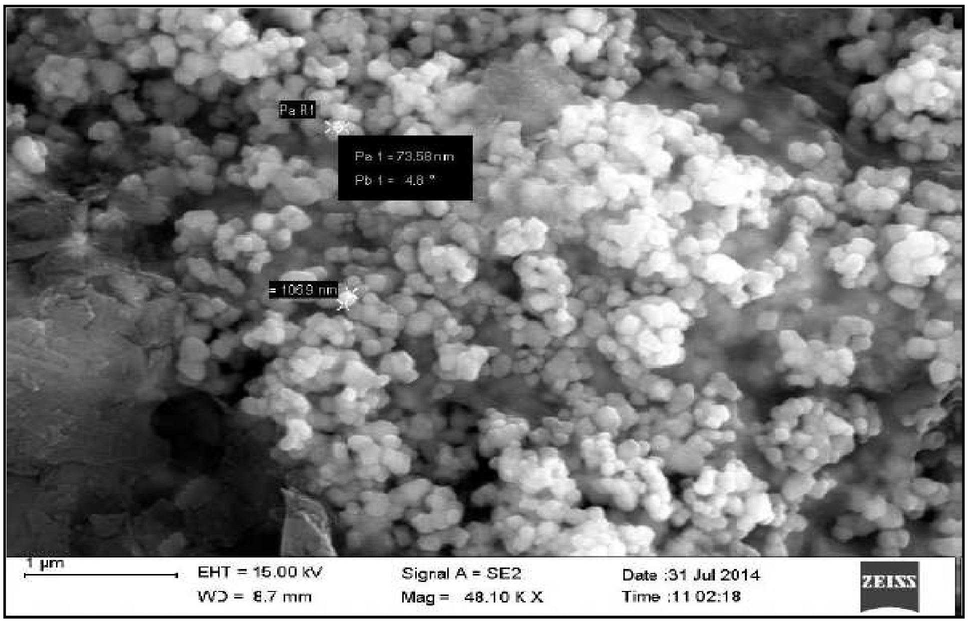

Figure 2: SEM image of TiO_2 nanoparticles in size of 73.58 to 106.9 nm.

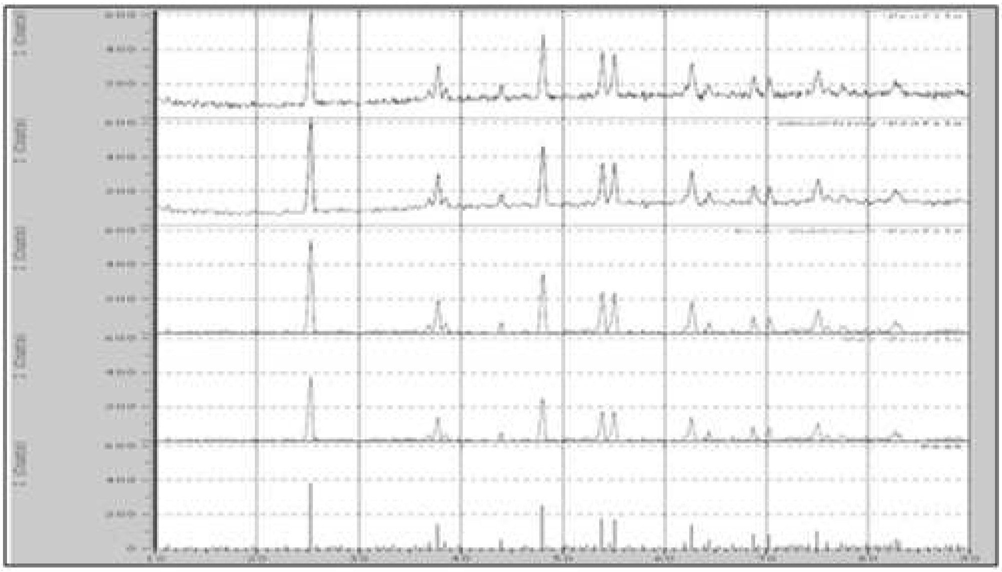

Figure 3: XRD pattern indicating anatase for TiO_2 nanoparticles.

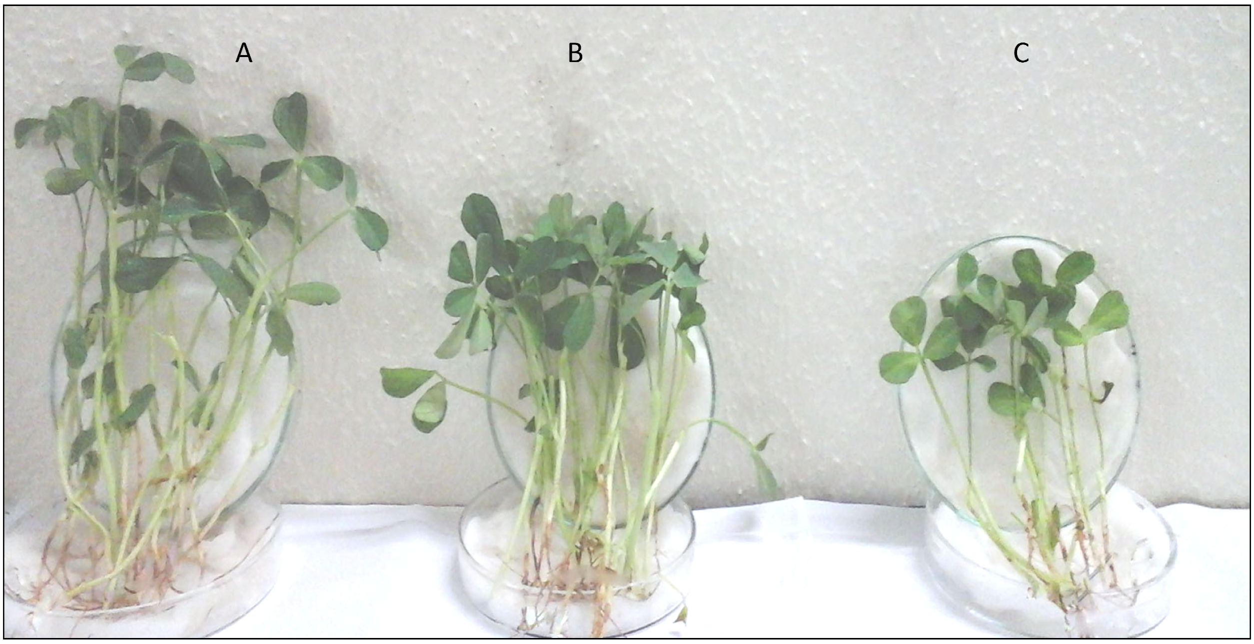

Figure 4: Influence of TiO_2 nanoparticles on seed growth index and length of shoot/root (A- 50µg; B- 25µg; C- control)

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