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Short Communication

Evaluation of Iinhibition Effect of ZnO Nanoparticles Concentration regarding Seed Germination and Seedling Growth of Fenugreek (*Trigonella foenum-graecum* L.)

Narges Ghasemi Siani[1], SeyfollahFallah[1*]and Ali Rostamnejadi[2]

[1]*Department of Agronomy, Faculty of Agriculture, Shahrekord University, Shahrekord, Iran*
[2]*Electroceram Research Center, Malek Ashtar University of Technology, Shahin Shahr, Isfahan, Iran*

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Abstract

A laboratory trial was conducted to determine whether suspensions of ZnO nanoparticles (ZnO NPs) could interfere with the early growth of fenugreek. This plant species is one of the recommended species by Organization for Economic Cooperation and Development (OECD). Nine concentrations of ZnO NPs (10, 50, 100, 500, 1000, 2000, 3000, 4000, and 5000 mg/L) were prepared in deionized (DI) water (considered as a control). Seed soaking and incubation of seeds in ZnO NPs suspensions were compared. We found that ZnO NPs cannot pass through the seed coat, because neither the seed soaking affect seedling growth nor the germination rate was not affected by ZnO NPs. The root and shoot growth were not affected until 100 and 500 mg/L, respectively, but in concentration more than 100 and 500 mg/L, root and shoot growth negatively were affected. Therefore root growth upon exposure to ZnO NPs was more sensitive than shoot growth.

Keywords: Fenugreek, Nanoparticles, Root, Shoot, ZnO

Introduction

As nanoparticles are increasingly used, the release of nano materials into the environment may pose severe threats for ecological systems and human health [1]. Therefore, a lot of attention is currently paid to the potential risks arising from these materials [2], which have already led to a number of studies that examine their mechanisms of unintentional emission and toxicity [3-5]. NPs closely interact with their surrounding environment and plants are an essential base component of all ecosystems. As a result NPs will inevitably interact with plants. Fenugreek as a medicinal plant was selected because that is one of recommended species by Organization for Economic Cooperation and Development for texicity tests [13].

The phytotoxicity profile of NPs has also been investigated by researchers via seed germination and root elongation tests which evaluate the acute effects of NPs on plant physiology [7]. NPs toxicity is attributed to generation of reactive oxygen species (ROS), which can damage the cell membrane; penetration of nanoparticles into the cell where they interfere with intracellular metabolism, and release of metal ions that hinder enzyme functions [8]. Inhibition of seed germination and root elongation by NPs has been found to be highly dependent on both plant type and NPs properties. For instance, single-wallet carbon nanotube (SWCNTs) significantly affected root elongation of tomato, cabbage, carrot and lettuce but promoted the growth of onion and cucumber in 24 to 48 h after exposure [9]. Tomato showed the highest degree of sensitivity to SWCNTs among the six species tested. In terms of metallic nanoparticles, copper nanoparticles were shown to be toxic to two crop species, Mung bean (*Phaseolus radiatus* L.) and wheat (*Triticum aestivum* L.), as demonstrated by the reduced

[*]Corresponding author: Department of Agronomy, Faculty of Agriculture, Shahrekord University, Shahrekord, Iran
Email Address: Falah1357@yahoo.com

seedling growth rate [10]. Mung bean was more sensitive than wheat and the authors attributed this phenomenon to differences in root anatomy and architecture. Yang and Watts [11] concluded that uncoated alumina particles inhibited root elongation of corn, cucumber, soybean, cabbage and carrot. But, the authors did not identify dissolution of nano- Al_2O_3 in solution, thus, failed to clarify if the phytotoxicity was from nano-Al_2O_3 or aluminum ion in the aqueous solution [12]. Lin and Xing [13] found that between five types of nanoparticles, only Zn and ZnO particles were observed to have significant inhibition on seed germination and root growth of the six plant species. Also authors indicated that the inhibition occurred during the seed incubation process rather than seed soaking stage. Given the extent of application of nanoparticles in different field such as agriculture, one of the most important issues to be addressed before the extensive utilization of nanoparticles is their possible toxicity. This paper explores the impacts of different concentrations of ZnO NPs on seed activities of fenugreek..

Material and Methods

Nanoparticle Synthesis and Structural Ccharacterization

The synthesis process of ZnO nanoparticles was according to Zandi *et al*. [14]. All chemicals (analytical grade reagents) were purchased from Merck Company and used as received without further purification. The starting materials were zinc acetate dehydrate ($Zn(CH_3COO)_2.2H_2O$) and citric acid ($C_6H_8O_7$). Zinc acetate and citric acid powders were mixed in a molar ratio of 1:1. The powders were mixed and ground for 1 hour at room temperature. The milled powder was calcinated at 600˚C for 10 h to obtain ZnO nanoparticles. The sample was characterized by X-ray diffraction (XRD) using Philips X'Pert PRO x-ray diffractometer equipped with a Cu-Kα X-ray source (λ=1.5406 Å) in the scanning angle range of 2θ=20˚-80˚. Figure 1 shows the XRD pattern of the powder sample with the full pattern of the Rietveld analysis result using the FULLPROF program [15]. From Figure1, the sample is found to be single phase without any noticeable trace of impurities. The Reitveld analysis of the pattern shows that the crystal structure of the sample is triclinic with space group p63mc, and from it, the lattice parameters *a*= b= 3.2482 Å, *c*= 5.2069 Å and the unit cell volume, V= 47.57 Å3 are calculated. The broadening of the XRD lines corresponds to the decrease of the particle size. The average particle size *d*, of the particles in the sample is calculated using Scherrer's formula;

$$d = \frac{k^{a}}{\cos^{-}}$$

Where:

d= particle size

k=0.9 is the particle shape factor, considering the spherical shape of the nanoparticles,

λ=1.5405Å is the wavelength of Cu Kα radiation,

β= the full width at half maximum of the XRD peak, and

θ =the diffraction angle of the peak [16].

The mean particle size of ZnO nanoparticles is about 16 nm. The Transmission Electron Microscopy (TEM) micrograph is shown in Figure 2. TEM micrograph shows that the particle size distribution is almost homogenous. The mean particle size is about18 nm which is comparable with the average particle size calculated from XRD line broadening.

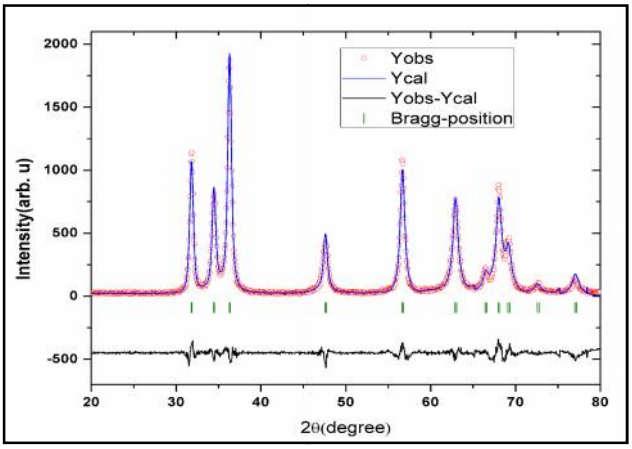

Fig. 1 X-ray diffraction pattern and the corresponding Rietveld refinement of ZnO nanoparticles

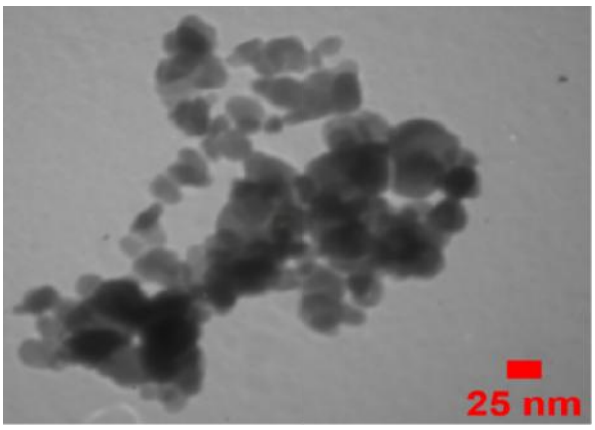

Fig. 2 TEM micrograph of ZnO nanoparticles

Seeds

Seeds of fenugreek (*Trigonella foenum-graceum* L.) were prepared from Isfahan Province, Iran. In according of the standard germination test, the average seed germination percentage of plant seeds was greater than 98% as shown by a preliminary study. Seeds were kept in a dry place in the dark under room temperature before use.

NPs Synthesis and Preparation of Their Suspensions

The nanoparticles were suspended directly in deionized water (DI-water) and dispersed by ultrasonic vibration (ultra schallprozessor up 400s) for 30 min. Small magnetic bars were placed in the suspensions for stirring before use to avoid aggregation of the particles. To measure the concentration of metal ions released from NP suspensions, aliquots of NP suspensions were drawn after the suspensions were incubated at room temperature for 2 hours. The extracts were centrifuged at 19,000 g for 20 min, and supernatants was used to conduct concentration assays of Zn ions by using inductively coupled plasma optical emission spectrometer (ICP-OES). Also concentrations of Zn^{2+} ions were measured 9 days after incubation of NP suspensions to conduct concentration assays of Zn ions over time.

Seed Germination and Seedling Growth Assay

Seeds were first sterilized by soaking them in a 10% sodium hypochlorite solution for 10 min [17]. Then for experiment design, they were soaked in DI-water or different ZnO NPs suspensions (10, 50, 100, 500, 1000, 2000, 3000, 4000, 5000 mg/L) for about 2 h after being rinsed three times with DI-water. All seeds were subsequently transferred into Petri dishes containing one piece of filter paper (90 mm in diameter, Whatman No.1). 10 seeds fenugreek were evenly spaced on top of the filter paper in each petri dish.

Three methods were examined to investigate effect of different concentrations of ZnO NPs:

1. Incubation of seeds in 5cc of different ZnO NPs;
2. Seed soaking in different ZnO NPs for about 2 hours + Incubation of seeds in 5cc of different ZnO NPs
3. Seed soaking in different ZnO NPs for about 2 hours + Incubation of seeds in 5cc DI-water.

Control treatment soaked in DI-water for 2 hours and then moistened with 5 cc of DI-water in Petri dishes. Petri dishes sealed by parafilm tape before being incubated at 20 °C in dark conditions [18].

Data Analysis

After 9 days of incubation, germinated seeds in each petri dishe seed were counted and seed germination percentage was calculated as number of germinated seed*100/total number of seed in each petri dish (10 seeds). Seedling shoot and root length were measured. Shoot tissues were removed from the root tissues, and separately oven dried at 72 ˚C for 24 hours. Tolerance index (TI) of each parameter was calculated as following [19]:

$$TI = \frac{t}{c} \times 100$$

Where:
TI= Tolerance index
t= mean parameters in test sample and
c=mean parameters in control sample.

Statistical analysis

For each condition, experiments were conducted in four replicate, from which standard deviations were calculated. The statistical analysis of experimental data utilized the Fisher test. Statistical significance was accepted when the probability of the result assuming the null hypothesis (p) is less than 0.05. Each of experimental values was compared to its corresponding control with LSD test.

Results

Zinc Concentration of ZnO Suspension

Concentration of Zn ion in ZnO-NPs suspensions ranged 0-2.5 mg/L and 0-3.5 mg/L 2 hours and 9 days after incubation, respectively. Thus we designed an experiment to evaluate the effect of Zn ion in this range in the seedling growth of fenugreek. Concentration–response curves reveal no significant effect of Zn^{2+} on the growth of fenugreek (Fig. 3).

Germination and Seedling Growth

As shown in Table 1, germination percentage of fenugreek was not affected by exposure to ZnO nanoparticles. Analysis of variance showed that root and shoot growth (lenght and dry weight) was significantly affected upon exposure method and concentration of ZnO-NPs (Table 1).

Table 1 Analysis of variance for growth parameters of fenugreek seedling affected upon exposure method and concentration of ZnONPs

Source of variance	df	Seed Germination rate	Root length	Shoot length	Root weight	Shoot weight
Exposure method	2	20	73**	38**	8.4**	30**
Exposure concentration	9	10	28**	20**	2.9**	15**
Method ×concentration	18	36	6**	6**	0.68**	2**
Error	90	28	0.29	0.57	0.023	0.36

** indicate significant effect at 0.01 probability level

Table 2 Tolerance index of growth parameters of fenugreek at different concentrations of ZnO NPs under different exposure method.

ZnO NPs concentration (mg/L)	SI	SI+SS	SS
	TI for root length		
0	100±0a	100±0ab	100±0ab
10	107±2a	114±22a	106±15ab
50	101±26a	101±20ab	100±24ab
100	94±17a	92±20b	100±16ab
500	72±5b	60±9c	93±13ab
1000	31±9cd	23±6d	88±15b
2000	33±5c	20±1d	102±13ab
3000	13±2de	22±4d	102±9ab
4000	13±2e	8±3d	93±12ab
5000	8±2e	6±1d	114±24a
	TI for shoot length		
0	100±0a	100±0a	100±0a
10	102±12a	106±18a	100±13a
50	100±25a	101±21a	99±17a
100	95±11a	100±9a	94±24a
500	89±23ab	94±19a	91±21a
1000	72±16b	63±11b	92±9a
2000	48±22c	50±14bc	96±19a
3000	28±7cd	41±12cd	101±18a
4000	27±7cd	21±2de	102±19a
5000	22±7d	24±5e	104±25a
	TI for root weight		
0	100±0a	100±0ab	100±0ab
10	102±6a	107±5a	97±10ab
50	104±15a	98±6ab	102±11ab
100	101±9a	96±8b	106±7a
500	74±5b	73±8c	98±11ab
1000	50±7c	41±2d	103±16ab
2000	24±3d	29±4e	104±15ab
3000	20±3d	27±6e	104±9ab
4000	20±6d	14±5f	90±4b
5000	21±4d	21±4f	94±2ab
	TI for shoot weight		
0	100±0a	100±0a	100±0a
10	92±7a	100±10a	89±9a
50	90±7a	98±6a	92±14a
100	89±7a	89±10a	98±9a
500	89±10a	89±11a	90±10a
1000	56±7b	60±9b	91±10a
2000	62±6b	61±7b	90±12a
3000	61±15b	55±11bc	95±11a
4000	54±17b	44±7cd	88±11a
5000	55±18b	40±3d	89±9a

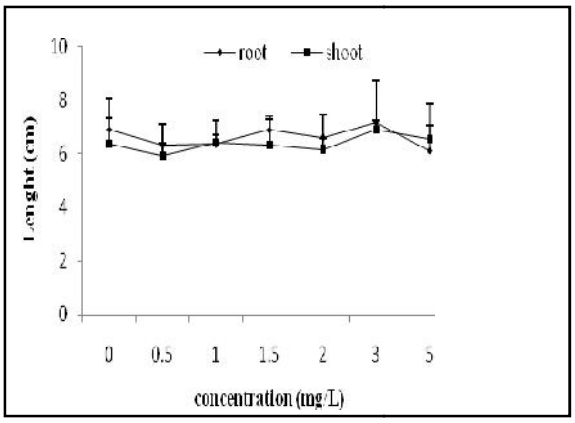

Fig. 3 Concentration–response curves showing effects of Zn^{+2} on seedling growth of fenugreek. Error bars correspond to standard deviation.

Seed soaking in ZnO NPs suspensions had no effect in the root growth (elongation and weight) rather to seed incubation in ZnO NPs suspensions (Figure 4 and 5). It was found that root elongation and root weight were not significantly decreased upon exposure to 100 mg/L under both incubation of seed in ZnO NPs suspensions and seed soaking in ZnO NPs + incubation of seed in ZnO NPs suspensions, but root elongation and root weight inhibition launched upon exposure to 500 mg/L and intensified as concentration increased to excess 5000 mg/L ZnO NPs. TI calculated for root length was 72 and 8 in excess 500 and 5000 mg/L, respectively (Table 2). TI calculated for root weight was 74 and 21 in excess 500 and 5000 mg/L, respectively (Table 2).

Values correspond to average ± standard deviation obtained for each treatment from four replicate.
(SI): incubation of seeds in 5cc of different ZnO NPs;
(SI+SS) Seed soaking in different ZnO-NPs for about 2 hours + incubation of seeds in 5cc of different ZnO-NPs;
(SS) Seed soaking in different ZnO NPs for about 2 hours.
The different letters in parentheses at each column indicate significant differences ($P<0.05$).

As root growth inhibited upon exposure to high concentration ZnO NPs, shoot growth (elongation and weight) inhibited too (Figure 6 and 7). However toxicity launched in higher concentration(1000 mg/L) rather to root growth, as TI for shoot length and shoot weight was 72 and 56 in excess 1000 mg/LZnO NPs, respectively (Table 2).Changes in the seedling phenotype have been illustrated as ZnO NPs concentration increased (Figure 8). As seen, the fenugreek root tip was negatively deleterioused in high concentration of ZnO NPs.

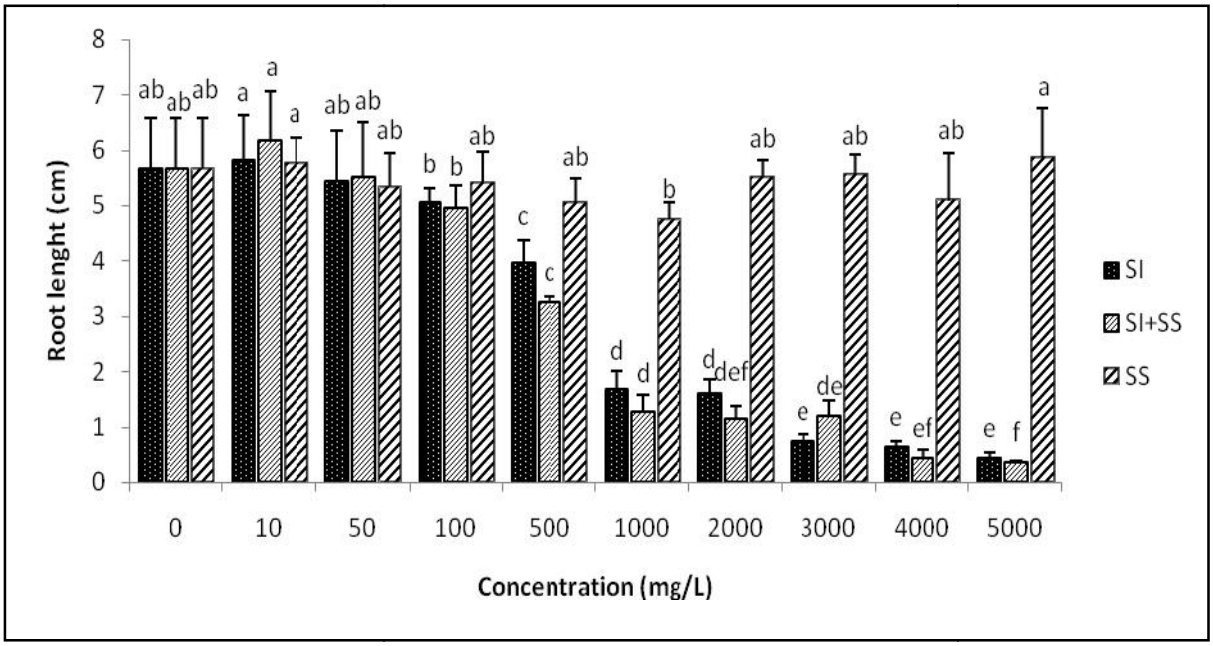

Fig. 4 Effects of ZnO-NPs on root length with different exposure methods. Error bars correspond to standard deviation. (SI): incubation of seeds in 5cc of different ZnO NPs; (SI+SS) Seed soaking in different ZnO-NPs for about 2 hours + incubation of seeds in 5cc of different ZnO-NPs; (SS) Seed soaking in different ZnO NPs for about 2 hours. Different letters in each column series shows significant difference between the treatments ($p<0.05$).

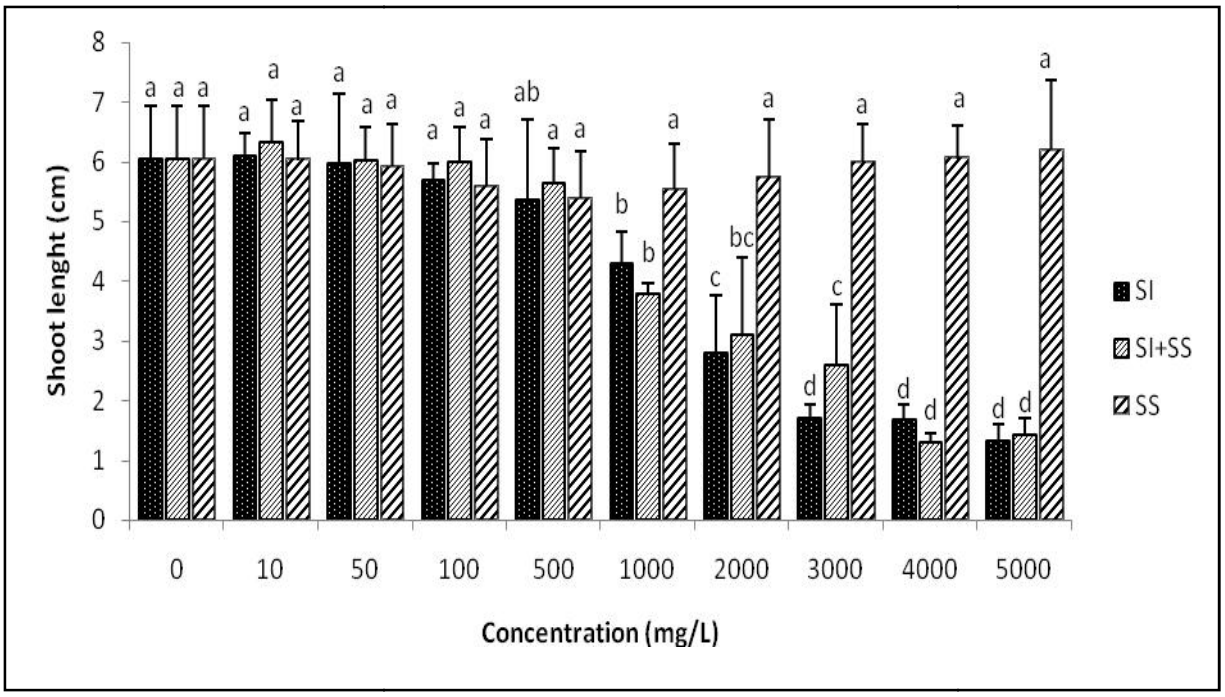

Fig. 5 Effects of ZnO-NPs on shoot length with different exposure methods. Error bars correspond to standard deviation. (SI): incubation of seeds in 5cc of different ZnO NPs; (SI+SS) Seed soaking in different ZnO-NPs for about 2 hours + incubation of seeds in 5cc of different ZnO-NPs; (SS) Seed soaking in different ZnO NPs for about 2 hours. Different letters in each column series shows significant difference between the treatments ($p<0.05$).

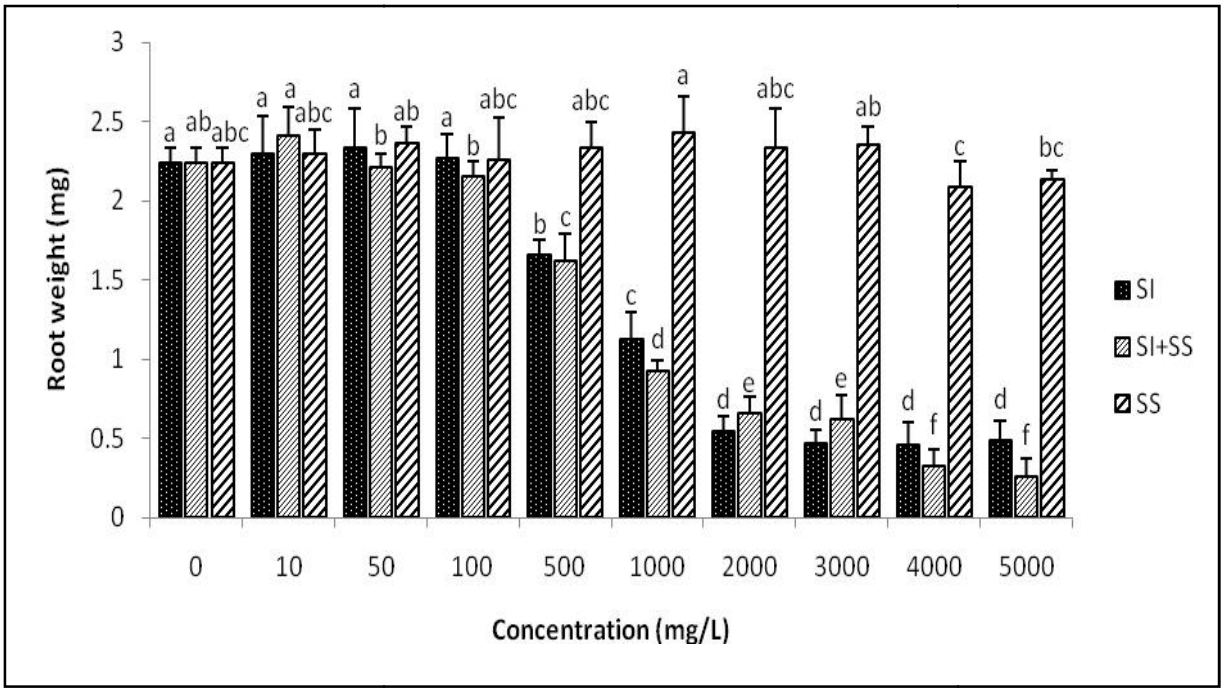

Figure 6. Effects of ZnO-NPs on root weight with different exposure methods. Error bars correspond to standard deviation. (SI): incubation of seeds in 5cc of different ZnO NPs; (SI+SS) Seed soaking in different ZnO-NPs for about 2 hours + incubation of seeds in 5cc of different ZnO-NPs; (SS) Seed soaking in different ZnO NPs for about 2 hours. Different letters in each column series shows significant difference between the treatments ($p<0.05$).

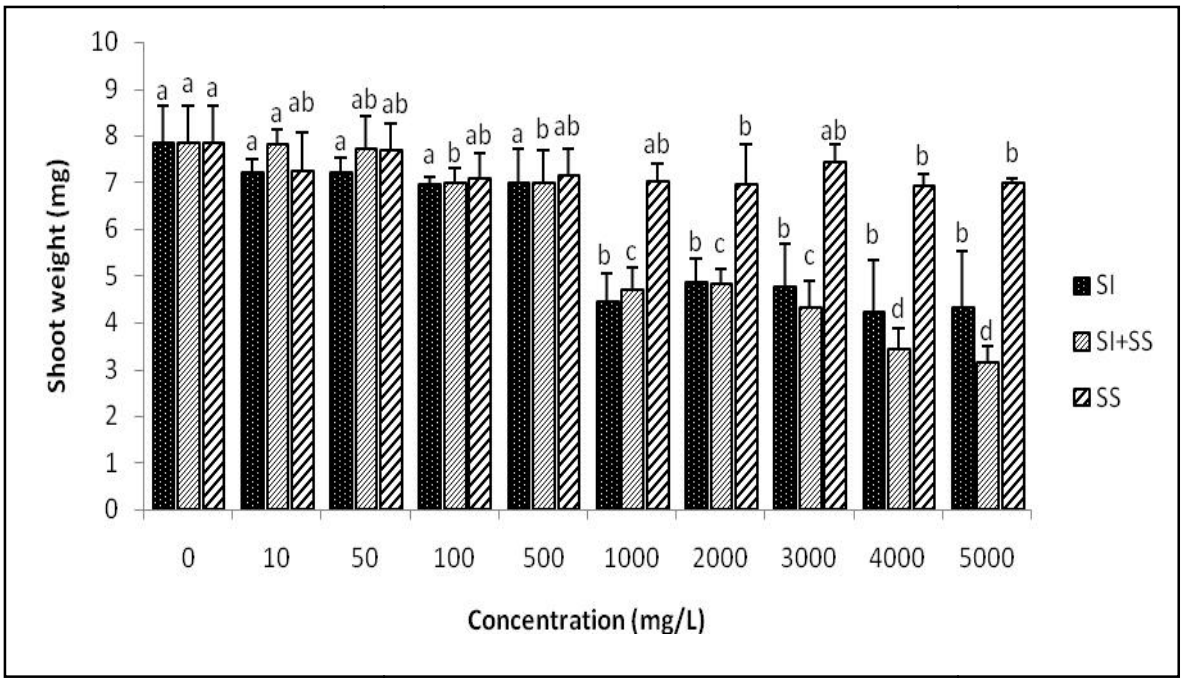

Fig. 7 Effects of ZnO-NPs on shoot weight with different exposure methods. Error bars correspond to standard deviation. (SI): incubation of seeds in 5cc of different ZnO NPs; (SI+SS) Seed soaking in different ZnO-NPs for about 2 hours + incubation of seeds in 5cc of different ZnO-NPs; (SS) Seed soaking in different ZnO NPs for about 2 hours. Different letters in each column series shows significant difference between the treatments ($p<0.05$).

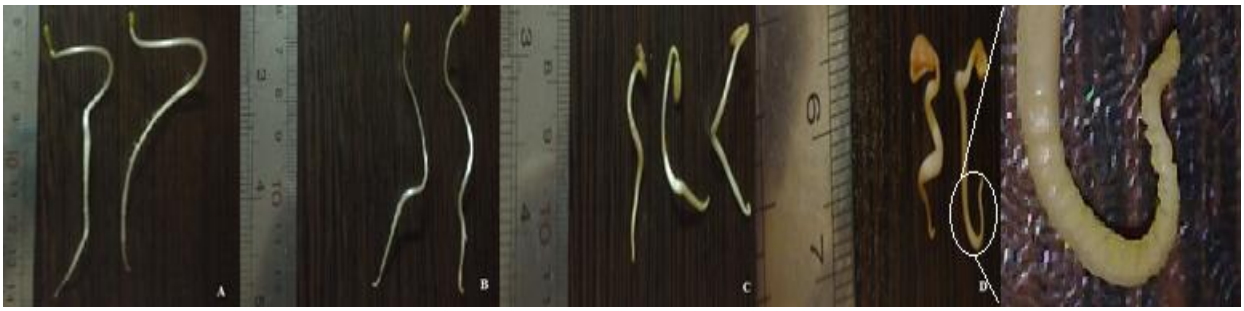

Fig. 8 Phenotypic changes in the seedling growth of fenugreek. A, control: B, 500 mg/LZnO NPs: C, 1000 mg/LZnO NPs; D, 4000 mg/L ZnO NPs.

Discussion

Seed soaking in different concentrations of ZnO NPs suspensions did not any effect in germination percentge. El-temsah and Joner [20] observed that seeds imbibed in a suspension of NPs for 8 h and subsequently germinated in pure water had only a limited ability to demonstrate toxic effects on germination. They reported that when seeds were imbibed in water for 8 h and subsequently germinated in nanoparticles suspensions, a toxicity response was observed. Nevertheless our results demonstrated germination percentage was not affected upon exposure to ZnO NPs in when seeds were incubated in NPs suspensions. Other studies using seed germination tests have also shown that seeds of cucumber and lettuce are not sensitive to metal and oxide nanoparticles, with no detectable inhibitory effects of Ag and Fe_3O_4 nanoparticles at concentrations up to 100 and 116 mg/L, respectively [21]. From the above results we can conclude that ZnO NPs were not allowed to enter through seed coat to influence seed activities. Seed coat plays a very important role in protecting the embryo from harmful external factors. Seed coats can have selective permeability [22]. Pollutants, though having obviously inhibitory effect on root growth, may not affect germination if they cannot pass through seed coats.

However root and shoot growth was highly affected upon exposure to NPs suspensions under seed incubation and seed soaking + seed incubation methods. Root elongation decreased as ZnO-NPs increased. However root elongation was more

sensitive than shoot elongation upon exposure to ZnO NPs. Other researchers have reported inhibition of root elongation of carrot [23], rape, radish and ryegrass [13], rapeseed [24] upon exposure to different concentrations of ZnO-NPs. Since roots are the first to be exposed to the ZnO-NPs suspensions, toxic symptoms seem to appear more in the root rather than in the shoot [25]. Interestingly, seed soaking did not inhibit root elongation even in high concentration of ZnO NPs. Reports revealed that is probably due to dilution of NPs surrounding seed in 5 cc DI-water in Petri dishes. Seed permeability might be related to type of NPs and species plant as Lin and Xing [13] reported root growth of radish and rape incubated in DI-water after being soaked in the nano-Zn suspension was significantly inhibited, but not while being soaked in ZnO NPs suspensions and also significant retardation of ryegrass root was also observed when the seed soaking process was done in either nano-Zn or nano-ZnO suspensions.

The biological effects of NPs in aqueous solutions are closely associated to the concentration of released metal ions [26, 27]. In this research, we measured the concentrations of metal ions released from all ZnO NPs suspensions after 2 hours and 9 days incubation. Zn ion released from ZnO-NPs suspension increased over the time from 0-2.5 ppm to 0-4 ppm. In other studies have been found that Zn^{2+} dissolution from ZnO NPs suspensions measured to be very low [24,28,29]. To compare phytotoxicity between Zn ions and ZnO NPs, we assessed seed activity in range 0-4 mg/L Zn^{2+} made from $ZnSO_4.7H_2O$, But we did not detect toxicity effect in seedling growth of fenugreek in such ranges. Zinc is an essential element for plants, but it is toxic at high levels with effective concentrations (EC_{50}-substrate Zn concentration resulting in 50% biomass reduction) which varied from 43 to 996 mg/L within various plant species [30]. Thus in our research, the phytotoxicity of ZnO nanoparticles could not directly result from their dissolution. This observation was consistence to Lin and Xing [31] concluded that the phytotoxicity of ZnO-NPs could not directly result from its dissolution in the rhizosphere or on the root surface. They revealed ZnO-NPs were able to concentrate in the rhizosphere, enter the root cells, and inhibit seedling growth of ryegrass. Entrance of NPs into the roots may induce ROS production [32], and may have potential damage to proteins and lipids in tissues. Inhibition effect of NPs on the seedling growth of fenugreek also maybe result of the physical interactions between ZnO particles and plant roots [5].

We clearly observed the fenugreek root tip was negatively damaged in high concentration of ZnO NPs. Pokhrel and Dubey [33] revealed that exposure to NPs ZnO caused ‘tunneling-like effect’, characterized by a deep invagination in the primary root tip in maize. Further studies need to be carried out to understand the phytotoxicity mechanisms of NPs.

Conclusion

Our experiments determined the dose-response ZnO NPs on the germination percentage and seedling growth of fenugreek as a plant. Seedling growth was decreased, as NP concentration in the growth media increased. It was discovered that seedling growth of fenugreek was more sensitive to toxic NPs in concentrations more than 500 mg/l. Additionally, this study shows that phytotoxity of ZnO NPs is not due to of releasing of the Zn^{2+} in the NP suspension. They may be due to of uptake of own NPs by the roots and interact with the cells that need to be investigated in next researches.

Acknowledgements

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