



Response in chickpea (*Cicer arietinum* L.) seedling growth to seed priming with iron oxide nanoparticles

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Abstract

The increasing application of nanoparticles (NPs) in various fields has made it crucial to study its impact on environment. Considering the positive and negative effect, study of nanoparticles is of concern in crops. It is known to directly influence crop growth and also enter food chain affecting living beings. Therefore, the present study was undertaken with the aim to evaluate the effect of iron oxide nanoparticles on seedling growth in Chickpea (*Cicer arietinum* L.) variety *Digvijay*. Seed priming was carried out using different concentrations of iron oxide (Fe_2O_3) NPs with an increment of 4 $\mu\text{g}/\text{ml}$ ranging from 4 to 16 $\mu\text{g}/\text{ml}$. Starch (10%) was used as a coating agent. The primed seeds were air dried and further used to study growth of seedling by paper towel method for germination *in vitro* and *in vivo* study as well. The grown seedlings were evaluated for growth parameters. Significant observations were noticed for seed germination and growth parameters *viz.* shoot length, root length, root to shoot ratio, fresh and dry weight that were considered as crucial indicators. These indicators summarises cumulative growth enhanced in seeds primed with lower concentrations up to 12 $\mu\text{g}/\text{ml}$ Fe_2O_3 NPs and inhibits further growth at higher concentrations. Hence, from the results it demonstrates that optimised dose levels of Fe_2O_3 NPs can be used as co-fertilizer to improve growth in chickpea at lower concentrations.

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Introduction

Nanotechnology is a rapidly growing field with broad applications in industrial sector *viz.* pharmaceuticals, food, cosmetics, electronics, textiles, energy, environmental bioremediation etc. (Shah *et al.*, 2014; Fan *et al.*, 2014; Moll *et al.*, 2016). Presently, nanotechnological application has provoked interest in agricultural sector as well (Banik and Perez-de-Luque, 2017). Extensive study on use of nanoparticles for enhancing yield and protecting crops against various biotic and abiotic stresses has been undertaken by various researchers (Li *et al.*, 2016). Nanoparticles are considered most remarkable due to their unique physicochemical properties such as nanosize, surface to volume ratio, easy absorption, less sedimentation etc. compared to the bulk materials (Sheikhabaglu *et al.*, 2014). Excess use of these nanomaterials may escalate and accumulate in environment, with a prerequisite to study its impact on plants. Plants are the major player enabling entry of NPs in the food chain. Numerous studies have been carried out to understand the interaction between NPs and plants. However, diverse outcomes are observed owing to association of NPs varying from species to species (Thunugunta *et al.*, 2018). Besides this, the response of NPs varies particularly based on size, shape, method of synthesis, chemicals used etc. (Rastogi *et al.*, 2017). NPs penetrate the cell wall, results in various morphological and physiological changes thereby altering the growth of plants.

Iron is an important micronutrient required for plant growth involved in various physiological processes *viz.* photosynthesis, respiration, redox reaction etc. while deficiency leads to extreme yield losses (Li *et al.*, 2016). Iron is present abundantly in soil, despite that there are reports suggesting 30% of soil in the world are iron limiting.

The anticipated reason being presence of iron in insoluble (Fe^{3+}) form mostly in alkaline and aerobic soils, rather than available (Fe^{2+}) form which is used by plants. This deficiency in soil not only affects plants' overall growth but also leads to deficiency in humans causing anaemia (Rui *et al.*, 2016). Chickpea

is the third most important pulse crop in India grown in semi-arid regions (Thaware *et al.*, 2017). It is a valuable source of carbohydrates, rich in proteins, minerals, cholesterol free fats, vitamins and soluble and insoluble fibres, supporting in providing nutrition to animals and humans in developing countries (Arab *et al.*, 2010). Micronutrient deficiencies are the major constraint in chickpea production, of which iron has severe effect on plant growth. Hence, revealing need of Fe based fertilizer to cope up with iron deficiency. Due to varied properties of iron oxide nanoparticles, it may be used as promising co-fertilizer for providing iron in deficient soils. Priming seeds with iron oxide nanoparticles can be a better option to cope up with the issue of iron availability in mostly alkaline soils. It may lead to various physiological and biochemical changes in seeds thereby enhancing the growth (Pawar and Laware, 2018).

Previous studies on iron oxide nanoparticles have shown stimulatory as well as inhibitory effect on crops. Positive effects were observed in wheat (Khaghani and Ghanbari, 2016), watermelon (Wang *et al.*, 2015), Ginger (Siva and Benita, 2016) etc. whereas negative effect was seen in *Capsicum annum* plants (Yuan *et al.*, 2018), bacterial system in soil etc. (Rashid *et al.*, 2017). Iron oxide nanoparticles have also been reported to show improved physiological mechanism in plants and tolerance against abiotic stresses (Zia-ur-Rehman *et al.*, 2018).

Due to the varied responses seen in plants, the present study was undertaken for systematic investigation of various iron oxide nanoparticles' concentrations on chickpea seed germination and seedling growth during *in vitro* and *in vivo* study.

Materials and methods

Seeds and Fe_2O_3 NPs

Chickpea seeds of variety 'Digvijay' were procured from Mahatma Phule Krishi Vidyapeeth, Rahuri, Maharashtra and uniform seeds were used in the study. Iron oxide nanoparticles with an average size

of 8-10 nm were synthesized by hydrothermal method and globular morphology of NP was determined by Field emission scanning electron microscopy (Hitachi, S-4800) (Fig.1).

Seed nanoprimering with Fe₂O₃ NPs

Seed nano priming is the process in which seeds are pre-treated with nanoparticles before sowing or planting. In current experiments, Fe₂O₃ NPs are used. Iron oxide NPs of different concentrations with an increment of 4µg/ml (4 to 16µg/ml) were prepared in 10% starch solution from a stock of 10 mg/ml (prepared in sterile distilled water). Starch was used as a coating agent solubilised by boiling in distilled water. NP solution was dispersed before use on magnetic stirrer to prevent agglomeration (Alharby *et al.*, 2016).

Germination experiments

Seeds were disinfected with 0.1% (w/v) HgCl₂ solution for 2-3 min and washed with sterile distilled water thoroughly (Zafar *et al.*, 2016). Seeds were coated with different concentrations of Fe₂O₃ NPs separately for 4-5 mins, then dried overnight and used for experiments. Untreated seeds were used as control. *In vitro* study involved seed germination on germination paper as per paper towel method (Phaneendranath, 1980; modified from Masangwa *et al.*, 2017).

They were watered as required and germinated seeds were recorded after every 2 days. After 10 days of germination, seedlings were harvested and radicle length (RL), plumule length (PL), seedling length (SL), root to shoot ratio, fresh weight (FW) and dry weight (DW) was recorded. Radicle is the first part of the seedling grown from the seed during the process of germination. Radicle is the embryonic root whereas plumule is the embryonic shoot giving rise to the complete seedling. DW was obtained by drying seedlings in hot air oven at 60°C for 48 hr and measured (Panwar *et al.*, 2012).

Pot culture: In vivo experiments

Primed and control seeds after overnight drying were

sown in pots containing uniform mixture of soil and coco peat in 2:1 ratio. The plants were watered regularly for 10 days and then uprooted carefully, followed through cleaning with water to remove attached soil. The plants were used to measure morphological parameters namely shoot length (SL), root length (RL), plant height (PH), root to shoot ratio, fresh weight (FW) and dry weight (DW).

Data analysis

Germination indices such as Promptness index (PI), Germination stress tolerance index (GSI), Plant height stress tolerance index (PHSI), Root length stress tolerance index (RLSI) and Dry matter stress tolerance index (DMSI) were calculated from the data collected (Laware and Raskar, 2014; Prasad *et al.*, 2012). Percent germination was calculated from data generated by observing number of seeds germinated among the total number of seeds used for germination after 10 days. All the experiments were conducted in three different sets. Each parameter was evaluated through average readings obtained from these three different sets. To study the influence of NPs on plant growth and to understand statistical significance among treatments, one way analysis of variance (ANOVA) extended with Kruskal-Wallis test was performed using software PAST-3. Comparing data of control, percent increase or decrease over control (PI/DOC), standard error of mean (SEM) and critical difference at significance level of 0.05 ($p < 0.05$) was calculated.

Results and discussion

The effect of Fe₂O₃ NPs on seedling growth in chickpea during *in vitro* study and *in vivo* study are seen in Fig. 2.

Seedling growth

The influence of Fe₂O₃ NPs during *in vitro* study is evaluated in Table 1. Seeds were germinated using germination paper under controlled conditions.

The radicle length and plumule length was significantly enhanced at lower concentrations whereas decreased at higher concentrations

compared to the control (Fig.3). Specimen treated with concentration (STWC) 8µg/ml showed maximum seedling length of 44 cm, followed by 40.67 cm with 4µg/ml whereas decreased at 12µg/ml (35.33 cm) compared to lower concentrations. Remarkably,

a significant decrease of plumule and radicle length at 16µg/ml was observed compared to control.

STWC 16µg/ml decreased seedling growth to 26.67 cm compared to the control seedling length 30.67 cm.

Table 1. Effect of Fe₂O₃NPs on seedling growth grown on germination paper *in vitro* study.

Fe ₂ O ₃ NPs (µg/ml)	RL (cm)	PI/DOC	PL (cm)	PI/DOC	SL (cm)	PI/DOC	Root/Shoot ratio	FW (g)	PI/DOC	DW (g)	PI/DOC
0	16.33	0.00	14.33	0.00	30.67	0.00	1.15	4.52	0.00	1.34	0.00
4	21.67	32.65	19.00	32.56	40.67	32.61	1.15	5.41	19.73	1.50	12.34
8	23.33	42.86	20.67	44.19	44.00	43.48	1.13	5.49	21.43	1.57	17.60
12	17.67	8.16	17.67	23.26	35.33	15.22	1.00	4.96	9.74	1.44	7.89
16	13.00	-20.41	13.67	-4.65	26.67	-13.04	0.96	4.75	5.09	1.40	4.89
SEM ±	0.63		0.77		1.00		0.07	0.33		0.21	
CD 5%	1.90		2.33		3.04		0.20	1.01		0.65	

Radicle length (RL), Plumule length (PL), Seedling length (SL), Fresh weight (FW), Dry weight (DW).

CD= critical difference; PI/DOC= percent increase or decrease over control.

Similarly, on studying influence of Fe₂O₃ NPs on seedling growth during *in vivo* study considering Table 2, root and shoot length was significantly accelerated in seeds primed with lower concentrations of NPs (Fig. 4). STWC 12 µg/ml (45.00 cm) showed maximum seedling length followed by 8µg/ml (42.67 cm), 4µg/ml (40.67cm), 16µg/ml (37.00 cm) compared to the control (32.33 cm).

Root to shoot ratio

Root to shoot ratio is an indicator explaining growth of roots to shoots based on the ratio value. Ratio of root to shoot was above 1 in all the treatments during *in vitro* (Table 1) and *in vivo* experiments (Table 2), specifying enhanced effect on root length compared to shoot length. In case of germination experiments, at higher concentration (16µg/ml), root length was slightly less; ultimately reducing root to shoot ratio.

Table 2. Effect of Fe₂O₃ NPs on seedling growth during *in vivo* study.

Fe ₂ O ₃ NPs (µg/ml)	RL (cm)	PI/DOC	SL (cm)	PI/DOC	PH (cm)	PI/DOC	Root/Shoot ratio	FW (g)	PI/DOC	DW (g)	PI/DOC
0	18.33	0.00	14.00	0.00	32.33	0.00	1.32	5.19	0.00	1.62	0.00
4	22.67	23.64	18.00	28.57	40.67	25.77	1.26	5.70	9.78	2.21	13.29
8	23.33	27.27	19.33	38.10	42.67	31.96	1.22	5.79	11.66	2.45	25.36
12	24.67	34.55	20.33	45.24	45.00	39.18	1.23	5.87	13.10	2.61	34.04
16	21.00	14.55	16.00	14.29	37.00	14.43	1.32	5.34	2.85	2.03	4.19
SEM ±	0.64		1.18		0.99		0.10	0.15		0.13	
CD 5%	1.94		3.59		3.00		0.30	0.45		0.40	

Root length (RL), Shoot length (SL), Plant height (PH), Fresh weight (FW), Dry weight (DW).

CD= critical difference; PI/DOC= percent increase or decrease over control.

Enhanced seedling growth at lower concentrations might be the effect of Fe₂O₃ NPs, where its penetration through seed coat leads to enhanced uptake of O₂ and water.

and ultimately seedling growth. Increase in growth might be a result of enhanced hydrolytic enzymes making available the food reserve by converting polysaccharides to monosaccharides (Mahakham *et al.*, 2017).

This would have boosted the process of germination

Table 3. Effect of Fe₂O₃ NPs on germination indices of seedling grown *in vitro* study.

Fe ₂ O ₃ NPs (µg/ml)	PI	GSI	PHSI	RLSI	DMSI
0	79.33	100.00	100.00	100.00	100.00
4	83.00	104.66	132.72	132.72	118.09
8	87.67	110.52	143.58	142.89	121.05
12	91.33	115.15	115.30	108.21	108.94
16	84.00	105.90	87.06	79.41	100.95
SEM ±	0.75	0.96	3.33	4.10	15.38
CD 5%	2.27	2.90	10.09	12.43	46.65

On the contrary, at higher concentrations, NPs might have accumulated in the cell wall causing oxidative stress, which might have caused production of

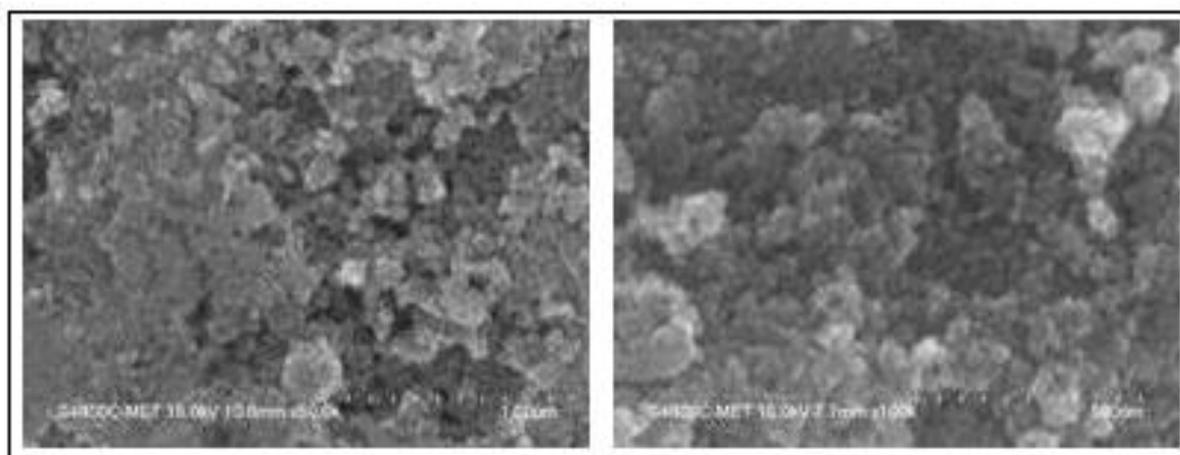
reactive oxygen species (ROS). ROS affects the growth by damaging the cell membranes and may also lead to DNA damage.

Table 4. Effect of Fe₂O₃ NPs on germination indices of seedling grown *in vivo* experiments.

Fe ₂ O ₃ NPs (µg/ml)	PHSI	RLSI	DMSI
0	100.00	100.00	100.00
4	125.82	123.78	140.05
8	131.98	127.49	155.33
12	139.11	134.70	164.76
16	114.43	114.72	128.41
SEM ±	3.03	3.63	9.59
CD 5%	9.18	11.00	29.09

Similar observations were in accordance with Yuan *et al.* (2018), observing positive effect on *Capsicum annum* growth at lower concentrations. However, adverse effects were seen at higher concentrations

due to accumulation of Fe NPs in the cell walls, causing Fe to be clogged in the apoplast and limiting its presence in central cylinder cells.

**Fig. 1.** SEM micrograph of iron oxide NPs.

This condition blocked iron transport leading to iron deficiency and rendering structural damages in the plants at higher concentration. Similarly, Siva and Benita (2016) showed positive effect of iron oxide nanoparticles on ginger with increase in plant height,

number of leaves, chlorophyll and iron content. They gave a better solution to Fe deficiency that caused chlorosis. Likewise Korishettar *et al.* (2016) also found increased field emergence, seed germination, seedling growth and vigour when pigeon pea seeds

were primed with Zn and Fe NPs at 750 ppm and 500 ppm respectively. These parameters were decreased at higher concentration (1000 ppm). The above results gave an insight that use of iron oxide nanoparticles largely depends on plant responses,

which varies with concentration of NPs. It narrates adverse consequences at higher doses while augment various morphological and physiological processes at lower concentrations.

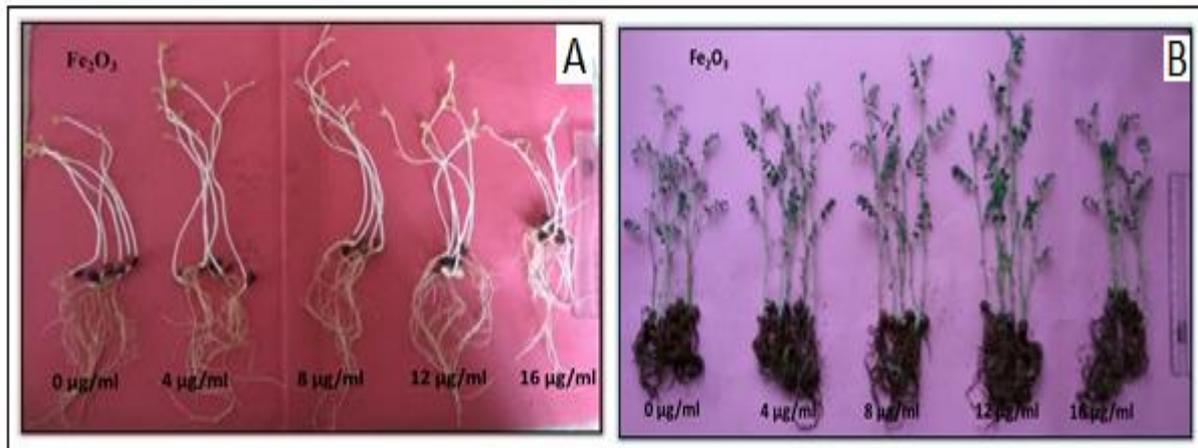


Fig. 2. Effect of Fe_2O_3 NPs on seedling growth A) *in vitro* study and B) *in vivo* study.

Fresh weight and Dry weight (g)

In case of fresh weight (FW) and dry weight (DW) of chickpea seedlings grown on germination paper, there was increase at 4 and 8 $\mu\text{g/ml}$ but decreased at 12 and 16 $\mu\text{g/ml}$ compared to lower concentrations (Table 1, Fig. 5a). Furthermore, in case of pot

experiments, there was constant increase in fresh weight and dry weight of seedlings from 4 $\mu\text{g/ml}$ to 12 $\mu\text{g/ml}$ and slight decrease at higher concentration namely 16 $\mu\text{g/ml}$ (Table 2, Fig. 5b). Comparing FW and DW with control, weight was increased throughout all the treatments.

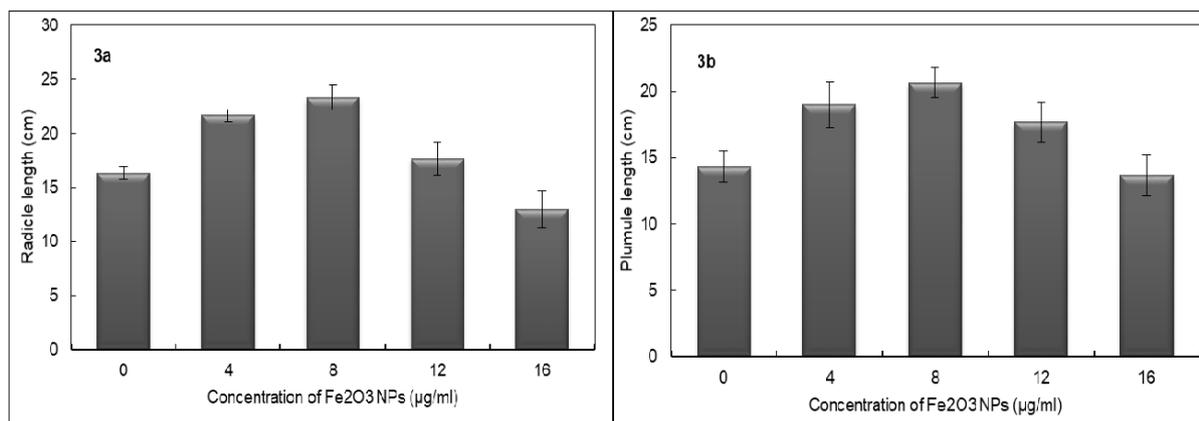


Fig. 3. *In vitro* study on effect of Fe_2O_3 NPs expressed as mean with standard deviation: 3a. Radicle length and 3b. Plumule length.

The enhanced growth led to improved weight at lower concentrations, proving plants to be healthy. It might be the effect of iron absorption from iron oxide nanoparticles thereby stimulating various physiological processes, enhancing photosynthesis and increasing food production.

Germination indices in vitro study

Germination indices are an indicator of phytotoxicity, aiding in studying effect of different treatments on seeds. Data on germination indices related to *in vitro* trials as shown in Table 3 and Fig. 6, indicated increased promptness index at lower concentrations

from 4 to 12 $\mu\text{g/ml}$ whereas reduced at 16 $\mu\text{g/ml}$ compared to 4 to 12 $\mu\text{g/ml}$. Promptness index (PI) is calculated based on seed germination after every 2 days till 10th day. In case of GSI, which is an indicator of rate of seed germination, was maximum when STWC 12 $\mu\text{g/ml}$ followed by 8 $\mu\text{g/ml}$ and 4 $\mu\text{g/ml}$ and decreased when STWC 16 $\mu\text{g/ml}$ compared to lower

concentration of Fe_2O_3 NPs. PI and GSI was enhanced at all treatments compared to control. Higher PHSI (143.58) was observed when STWC 8 $\mu\text{g/ml}$ followed by 4 $\mu\text{g/ml}$ (132.72) and diminished at 12 $\mu\text{g/ml}$ (115.30) and 16 $\mu\text{g/ml}$ (87.06) compared to lower concentrations.

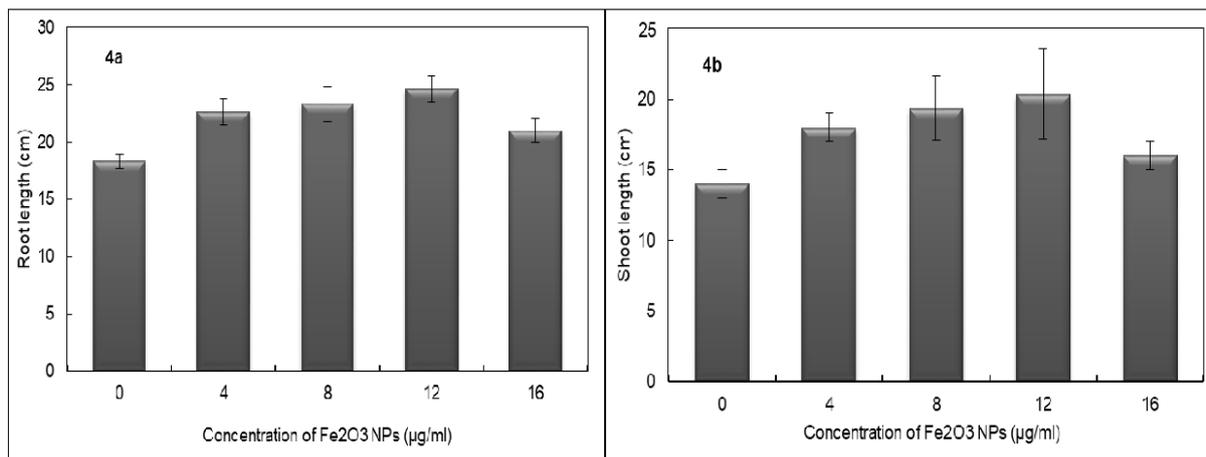


Fig. 4. *In vivo* study of Fe_2O_3 NPs on growth expressed as mean with standard deviation: 4a. Root length and 4b. Shoot length.

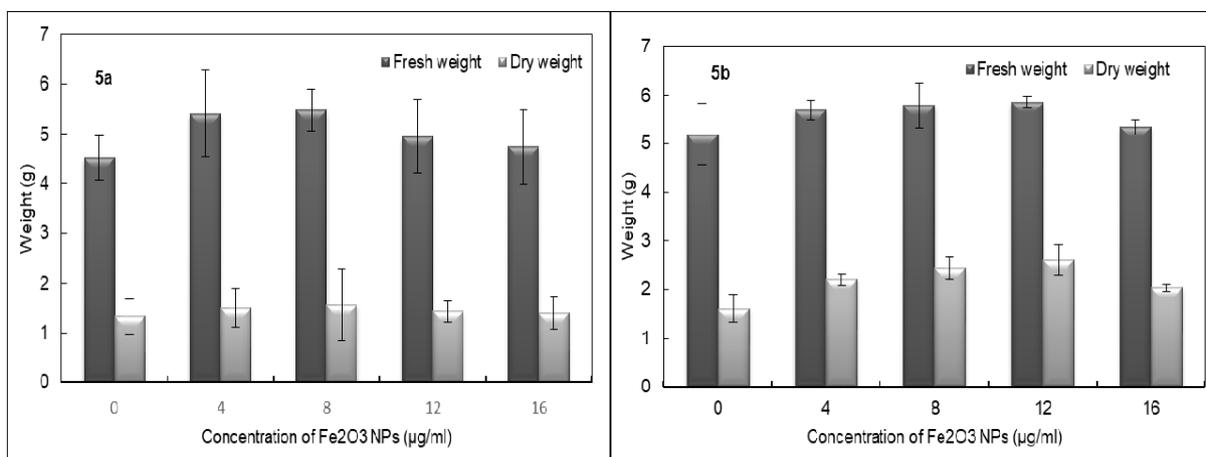


Fig. 5. Effect of Fe_2O_3 NPs on fresh and dry weight expressed as mean with standard deviation 5a) *In vitro* study 5b) *In vivo* study.

The decreased PHSI value when STWC 16 $\mu\text{g/ml}$ indicates the phytotoxic effect. Also similar trend was seen in RLSI, where RLSI values were increased when STWC 4 and 8 $\mu\text{g/ml}$ and decreased when treated with 12 and 16 $\mu\text{g/ml}$. STWC 12 and 16 $\mu\text{g/ml}$ decreased PHSI and RLSI values, indicating toxic effect of NPs on plant growth. However in case of DMSI, there was increase in all treated seedlings compared to control. Similar trend was observed by Raskar and Laware

(2014) in onion seedlings treated with different concentration of ZnO NPs. Likewise, Rui *et al.* (2016) studied effect of Fe_2O_3 NPs and EDTA-Fe on peanut. They observed enhanced growth in peanut due to application of NPs. Also, positive effect on seed quality was observed on foliar spraying of iron oxide nanoparticles on maternal plants (Sheikhbaglu *et al.*, 2014). Over all, proving 8 $\mu\text{g/ml}$ of iron oxide nanoparticles to be better dose to be applied for

chickpea seed priming to enhance its growth through *in vitro* study.

Stress indices in pot culture

Considering data on stress indices related to *in vivo* study as shown in Table 4, PHSI, RLSI and DMSI, these values were significantly increase when STWC 4, 8 and 12 μ g/ml whereas slightly decreased when STWC 16 μ g/ml compared to lower concentrations, though being more than control.

In nutshell, study exhibits iron oxide nanoparticles to enhance growth of all the treated plants stimulating from lower to higher concentrations and decreasing after critical point. *In vitro* experiments, 8 μ g/ml showed positive effect on seedlings; however growth decreased above that level.

Whereas *in vivo* experiments growth reduces above 12 μ g/ml this concentration level was found to be optimum concentration for the study.

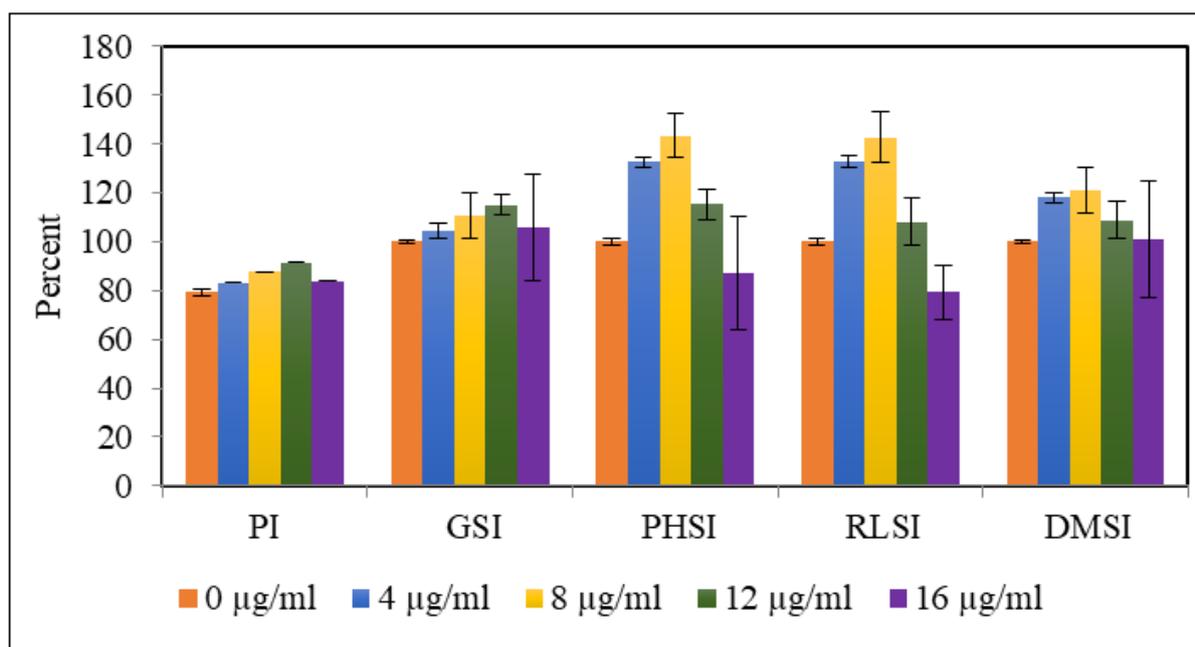


Fig. 6. Effect of Fe₂O₃ NPs on germination indices *in vitro* study expressed as mean with standard deviation.

Conclusion

From the above results, it can be concluded that Fe₂O₃ NP priming at lower concentrations enhances seedling growth whereas inhibits growth as the concentration of NPs increases. NPs might be absorbed by the seeds through imbibition and may lead to synthesis of hydrolytic and antioxidant enzymes, causing mobilization of food reserves. This enhances germination rate and seedling growth with neutralizing oxidative stress by quenching free radicals. Decreased plant growth observed at higher concentrations, which might be due to the accumulation of these NPs in the cell walls hampering the transport of nutrients and developing oxidative stress. It can be concluded that Fe₂O₃ NPs can be used as a potential co-fertilizer providing iron for

plant growth at lower concentrations. It is also established that plants respond diversely to nanoparticles based on its concentration and doses. Thus, gives an insight to study NPs based on different aspects in field to avoid any detrimental effect on environment.

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