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Vermiwash Assisted Synthesis of Iron Oxide Nanoparticles and Their Efficacy for Seed Nanopriming of Black Chickpea (*Cicer arietinum* L.)

Rajwinder Kaur[1], Neeraj Rani[1] and Nitish Dhingra[2]*

[1]Department of Soil Science, Punjab Agricultural University, Ludhiana, Punjab, India - 141004
[2]Electron Microscopy & Nanoscience Laboratory, Punjab Agricultural University, Ludhiana, Punjab, India – 141004
*Correspondence for materials should be addressed to ND (email: nitishdhingra@pau.edu)

Abstract

The priming of seeds with nanoparticles (NPs) has the potential to aid crop production and agricultural sustainability. Nevertheless, it depends on the crop type being investigated and NPs dosage. The present study reports the impact of seed nano-priming with varied concentrations of iron oxide (Fe_2O_3) NPs on the seedling growth of black chickpeas. Iron oxide NPs were synthesized via a chemical route using $FeCl_3$ and $Fe(NO_3)_3$ as precursors, along with vermiwash (earthworm-based extract) and NaOH for adjusting the pH of the resulting solution. Seed nano-priming was carried out using different concentrations of iron oxide NPs (0, 10, 50, 100, 150, 200 ppm). The primed seeds were air-dried and evaluated for growth using a wet filter paper method. The grown seedlings were evaluated based on various growth parameters such as germination rate, root length, shoot length, root-to-shoot length ratio, fresh and dry weights, and seedling vigour indices (I and II). The study revealed that nano-priming with iron oxide NPs improved the germination percentage at the end of the seventh day and also led to enhancement in various growth indicators, such as root length, shoot length, biomass accumulation, and seedling vigour indices, compared to the control at all concentrations up to 150 ppm. Further increase in NPs concentration inhibited the seedling growth. The correlations among growth parameters were examined using Pearson correlation and Principal Component Analysis (PCA) methods. This study demonstrated the potential of optimized doses of iron oxide NPs as co-fertilizer to aid early-stage growth in black chickpea.

Keywords: Chick-pea; Iron oxide; Nanoparticles; Seed priming; Vermiwash

Introduction

The chickpea (*Cicer arietinum L.*) is a protein-rich legume crop abundant in vitamins, carbs, and minerals (Madurapperumage et al., 2021). Like other leguminous crops, chickpeas fix nitrogen biologically to maintain soil productivity (Kebede, 2021). To support the global rise in population, the improved production of chickpeas is given a prime focus. However, numerous biotic and abiotic elements significantly impact chickpea growth and productivity (Zhang et al., 2024). Cold temperatures can increase oxidative stress and impact the antioxidant potential of chickpeas (Karami-Moalem et al., 2018). Similarly, salt stress can affect the morphology and germination of chickpeas (Mergeb, 2021). Consequently, there is a need to identify and characterize novel fertilizers that can influence crop sustainability without affecting the ecosystem. In recent years, nanotechnology has emerged as one of the remarkable fields of science that finds applications in various areas such as wastewater treatment, agriculture, the food industry, and electronics (Neme et al., 2021). Nanoparticles (NPs) are being widely explored for their broad potential role in various

fields, including the pharmaceutical industry, disease diagnostics, biosensing, agriculture, food industry, environmental monitoring, and energy storage (Altammar, 2023 and Goyal et al., 2023).

Iron is a crucial micronutrient essential for plant development and plays a key role in various physiological processes such as photosynthesis, respiration, and redox reactions. The iron deficiency can cause significant yield losses (Li et al., 2016). Although iron is abundantly present in soil, approximately 30% of global soils are limited in iron availability (Zuluaga et al., 2023). This limitation is primarily attributed to the predominance of iron in its insoluble (Fe^{3+}) form, particularly in alkaline and aerobic soils, as opposed to the soluble (Fe^{2+}) form accessible to plants. Such deficiencies hinder overall plant growth and contribute to human health issues, including anemia (Rui et al., 2016). Chickpea, recognized as the third-largest pulse crop produced worldwide, is cultivated in semi-arid regions of India (Koul et al., 2022). It serves as a vital source of carbohydrates and is rich in proteins, minerals, cholesterol-free fats, vitamins, and both soluble and insoluble fibers, thereby providing essential nutrition to both humans and animals in developing countries (Begum et al., 2023). Micronutrient deficiencies, particularly iron, pose a major challenge for chickpea production, significantly impacting plant growth. This underscores the necessity for iron-based fertilizers to address iron deficiency. Given the diverse properties of iron oxide NPs, they may serve as a promising co-fertilizer to supply iron in deficient soils. Additionally, priming seeds with iron oxide NPs could also present an effective strategy to enhance iron availability in predominantly alkaline soils, potentially leading to various physiological and biochemical changes in seeds that promote growth (Pawar and Laware, 2018).

Previous studies performed using iron oxide NPs have revealed both positive and negative effects on crops depending upon the concentrations of the NPs. Beneficial effects have been noticed in wheat (Singh et al., 2022) and cherry tomato (Shahzad et al., 2024), while adverse effects have been observed at higher concentrations in *Capsicum annuum* (Yuan et al., 2018). Studies have revealed that at low concentrations, iron oxide NPs promote plant growth by altering the leaf organization, increasing the chloroplast number and grana stacking, and regulating the development of vascular bundles. Iron oxide NPs could be absorbed in the roots and then transported to the central cylinder in bio-available forms, where these NPs are translocated and utilized by the leaves and stems. In contrast, iron oxide NPs can be harmful to plants at high concentrations, as the majority of iron oxide NPs are aggregated into cell walls and transported via the apoplastic pathway in the roots, which may potentially block the transfer of iron nutrients. Therefore, the present research was undertaken for systematic investigation of the impact of varied concentrations of iron oxide NPs on chickpea seed germination and seedling growth through in-vitro studies.

Material and methods

The black chickpea seeds (variety PBG-7) were procured from the Director (Seeds), Punjab Agricultural University (PAU), Ludhiana. The raw materials necessary to prepare vermiwash were collected from various locations within PAU, Ludhiana. Paddy straw and wheat straw were collected from the integrated farming system unit. Neem (*Azadirachta indica*) leaves were collected from the adjoining areas of the school of organic farming. Kitchen waste was collected from the mess of hostels at PAU, Ludhiana. However, onion, garlic, and acidic peels of fruits and vegetables were avoided due to their odour, potential to create unfavourable conditions, and the likelihood of irritating the sensitive skin of earthworms, causing them to move away or perish. Cow dung was gathered from the dairy farm unit at Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana. Cow dung had an initial moisture content of 70–75%. Figures 1(a)-(d) show various raw materials used for the vermiwash synthesis. The chemicals (AR/ACS grade) required for iron NPs synthesis, namely, Ferric chloride, Ferric nitrate and NaOH, were procured from Central Drug House Private Limited, New Delhi.

Vermiwash production

Preparation of vermiwash (v/w) was done at Vermicompost Unit, PAU Ludhiana, as per the method standardized by Ismail (1997). An indigenous vermiwash production assembly (Figure 1(e)) was set up, which consisted of fifteen earthen pots kept on iron stands, with the lowermost pots fitted with plastic gate-valves to facilitate the drainage of liquid extract. The middle pots were filled with gravel (1- 2" size), above which a layer of cow dung was placed. Above the cow dung, a layer of different organic wastes (Table 1) was added pot-by-pot, followed by another layer of cow dung. Around 10-15 adult earthworms (E. foetida) were released into it. The necks of the middle pots were gently cracked down to provide better aeration for earthworms. The unit was moistened every day (60-70% moisture) using a set of top pots that facilitated drop-by-drop water supply through a drip system. These pots were kept for decomposition for 15 days with frequent turnings.

Eisenia foetida, an epigeic, compost-dwelling, non-burrowing worm, lives at the intersection of soil and litter and possesses a variety of preferences such as feeding on a wide range of compostable organic wastes, enduring in a wide range of weather conditions, living indoors, and multiplying rapidly. As a result, it is an ideal option for vermicomposting residential and industrial organic waste. After fifteen days, elutes were collected daily from the third pot arranged at the bottom of the assembly. The water slowly percolated through the compost and drilospheres, carrying nutrients from freshly formed castings and washings from the drilospheres through the filter unit. Collected vermiwash was stored at 4 ºC and used for analyzing the physicochemical nutrient composition and other parameters.

Fig. 1. (a) Neem leaves (b) Paddy straw (c) Wheat straw (d) Cow dung, and (e) Indigenous vermiwash production assembly.

Table 1: Details of material required for preparation of different vermiwash samples.

Sr. No.	Vermiwash	Material used
1.	Paddy straw	Paddy straw chopped + cow dung (1:1) + 15- 20 adult earthworms
2.	Wheat straw	Wheat straw chopped + cow dung (1:1) + 15- 20 adult earthworms
3.	Kitchen waste	Kitchen waste + cow dung (1:1) + 15- 20 adult earthworms
4.	Neem leaves	Neem leaves + cow dung (1:1) + 15- 20 adult earthworms
5.	Cow dung	100 % Cow dung (1:1) + 15- 20 adult earthworms

Synthesis of Iron Oxide NPs

Kitchen waste-based vermiwash was found to be the best in terms of nutritional value and was used to synthesize iron oxide NPs. The work pertaining to the synthesis of iron oxide NPs was carried out at the Electron Microscopy & Nanoscience Laboratory, PAU Ludhiana. The vermiwash was subjected to filtration through a funnel with filter paper to remove the solids. Equal volumes (200 ml) of 1 mM ferric chloride, 1 mM ferric nitrate and NaOH (to adjust the pH to around 8) were mixed with an equal volume of kitchen waste-based vermiwash. The mixture was subjected to magnetic

stirring at 80 °C for about 2 hours until the colour of the mixture changed from dark yellow to black (Figures 2(a)-(c)). This colour change indicated the formation of iron oxide NPs. The resulting precipitate was separated by centrifugation (Hettich Mikro 22R) at 6,000 rpm for 5 mins. The black-coloured precipitate was first rinsed with distilled water and then ethanol to remove the impurities. After drying in an oven at 80 °C, these NPs were characterized using spectroscopic techniques (UV-visible spectroscopy and Scanning Electron Microscopy).

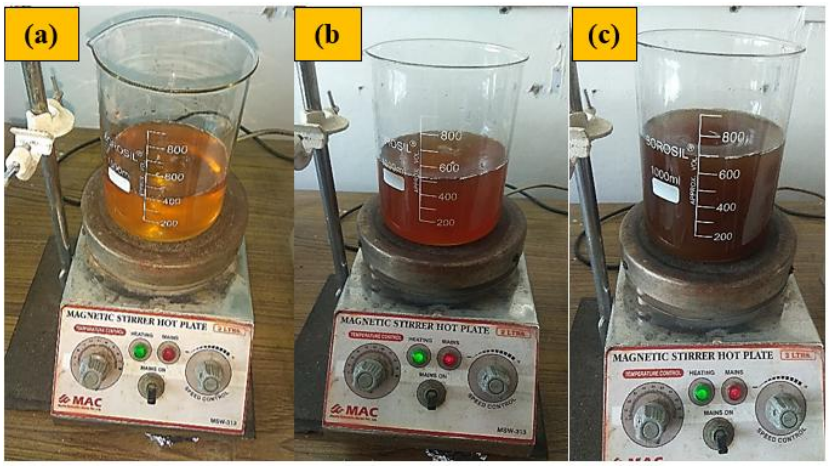

Fig. 2. (a) $FeCl_3$ and $Fe(NO_3)_3$ solution (b) After NaOH addition (c) After vermiwash addition.

Characterization of iron oxide NPs

UV-visible spectroscopy for absorbance analysis

A UV-visible spectrophotometer measures the extinction (scattering and absorption) of light passing through a sample. NPs have unique optical properties sensitive to the size, shape, concentration, agglomeration state and refractive index near the NP surface, making UV-visible spectroscopy a valuable tool for identifying, characterizing and studying nanomaterials. Iron oxide NPs were characterized using a UV-visible spectrophotometer (Shimadzu, UV-1800, Japan). The iron oxide NPs in aqueous solutions were filled up to 3/4th of the quartz cuvette having path length of 1 cm and deionized water was used as a reference. The absorbance of the sample was recorded in the wavelength range between 200 - 1100 nm using a double-beam spectrophotometer. The wavelength corresponding to maximum absorption (λ_{max}) was obtained from the recorded spectrum.

Scanning Electron Microscopy analysis

The scanning electron microscopy (SEM) analysis of the iron oxide NPs was performed using a scanning electron microscope (JSM-IT500, JEOL Limited, Japan). The aluminium stub (~1 cm diameter) was employed on the sample holder and cleaned to remove surface oils or dirt by using acetone and blowing with compressed gas. The double-coated conductive carbon tape was used as adhesive and pasted on the stub. The thin film of the sample (~0.1 ml) was dropped on the adhesive surface and allowed to dry by putting it under a mercury lamp for 5 minutes. A sample holder containing the dried sample was placed in the vacuum chamber. The magnification was carried out to get a clear morphology of synthesized iron oxide nanoparticles at the accelerating voltage of 10 kV with a working distance of 11.1 mm.

Nanopriming of black chickpea seeds with iron oxide NPs

To perform the efficacy studies of synthesized NPs for seed priming, the black chickpea seeds were first screened for uniformity. Healthy seeds with a known weight (15-20 gms) were taken and washed with 1% sodium hypochlorite solution for 10 mins for surface sterilization and then with deionized water. The nano-priming of chickpea seeds was done by soaking the seeds (10-15 seeds per NPs concentration) in varied concentrations of iron oxide NPs (0, 10, 50, 100, 150, 200 ppm) and shaking over the magnetic stirrer for 1 hour to ensure uniform coating of seeds. Table 2 summarizes various treatments of iron oxide NPs used for the study. Subsequently, to perform the in-vitro germination studies, the seeds were transferred onto wet filter papers (5 ml water for control and 5 ml NPs solution for treatments) and kept in petri dishes. The primed seeds were dried under shade and finally placed in the growth chamber at 25 °C. The evaluation of seedlings were done based on various growth parameters such as root length, shoot length, root-to-shoot length ratio, fresh and dry weights, and seedling vigour indices.

Table 2. Details of the treatments using iron oxide NPs.

Sr. No.	Treatments	Concentration of iron oxide NPs (ppm)
1	T_1	0 (Control)
2	T_2	10
3	T_3	50
4	T_4	100
5	T_5	150
6	T_6	200

Where T_1 = Control (without application of NPs), T_2 to T_6 = Different concentration levels of iron oxide NPs.

Seed germination

The germination test was conducted in three replicates of 10-15 seeds each by following the wet paper method and incubation at 25 ± 2 °C temperature and 90 ± 5 % RH. The number of normal seedlings in each replication was counted on the seventh day and the mean germination was calculated and expressed in percentage (ISTA, 2024a).

Seed germination % = (Number of normal seedlings/Total number of seeds) ×100

Root length

From the samples of germination test, five normal seedlings were randomly selected in each treatment from all the replications on the seventh day. The root length was measured from the tip of primary root to the base of hypocotyls and the mean root length was expressed in centimetres (ISTA, 2024b).

Shoot length

From the samples of the germination test, five normal seedlings randomly selected for root length measurement were also used for the measurement of shoot length. The shoot length was measured from the base of the primary leaf to the base of hypocotyls and the mean shoot length was expressed in centimetres (ISTA, 2024b).

Seedling fresh and dry weights

The five randomly selected seedlings were taken in a butter paper and dried in a hot air oven at 70 ^{0}C for 24 h. Then, the seedlings were removed and allowed to cool in desiccators for 30 min before weighing in an electronic balance. The average weight was calculated and expressed in milligrams (ISTA, 2024b).

Seedling Vigour Index – I

The seedling vigour index – I was determined using the germination percentage and the total seedling length (Abdul-Baki and Anderson, 1973) as:
Seedling vigour index – I = Seed germination percentage x Mean seedling length (cm),
where, seedling length (cm) = root length (cm) + shoot length (cm).

Seedling Vigour Index – II

The seedling vigour index – II was determined using the germination percentage and the seedling dry weight (Abdul-Baki and Anderson, 1973) as:
Seedling vigour index – II = Seed germination percentage x Mean seedling dry weight (mg).

Statistical Analysis

The data represents mean ± standard deviation of three replicates. Statistical analysis was done using one-way analysis of variance (ANOVA) technique and Tukey's range test. The Pearson correlation analysis was performed between NP treatments and the growth parameters of black chickpea ($p < 0.05$). Principal Component Analysis (PCA) was also employed to ascertain correlations between the altered growth parameters of black chickpea and concentrations of iron oxide NPs.

Results & Discussion

Synthesis of iron oxide NPs from kitchen waste vermiwash and their characterization

UV-visible spectrophotometry for absorbance analysis

The UV-visible absorbance spectra were recorded to analyze the optical properties of vermiwash and iron oxide NPs. The absorbance measured as a function of wavelength is depicted in Figure 3. The black curve represents the absorbance spectrum of vermiwash, whereas the red curve

corresponds to the iron oxide NPs. The absorbance spectrum of iron oxide NPs shows a prominent absorption peak at 293 nm, which indicates the nanoscale nature of the particles and confirms their strong optical absorption in the UV region. In contrast, the absorbance spectrum of vermiwash exhibits a peak near 350 nm, albeit with much lower intensity, indicating the presence of biomolecules or organic compounds that can absorb UV light. Both absorbance curves display a decreasing absorbance trend with increasing wavelength, indicating the dominant absorption in the UV region and minimal absorbance in the visible and near-infrared (NIR) regions. The iron oxide NPs exhibit a higher overall absorbance than vermiwash, which may be attributed to the strong optical properties of iron oxide at the nano-scale. These results confirm the synthesis and optical activity of iron oxide NPs while also highlighting the absorbance characteristics of vermiwash.

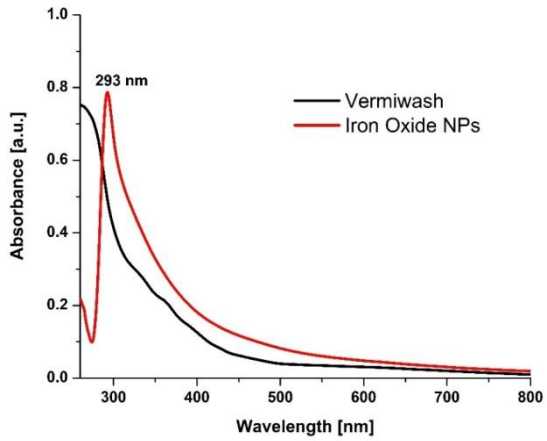

Fig. 3. Absorbance as a function of wavelength of the light used.

Scanning Electron Microscopy for surface morphology analysis

The surface morphology of the synthesized iron oxide NPs embedded with vermiwash nutrients was examined using Scanning Electron Microscopy. The SEM micrograph (Figure 4), depicts iron oxide NPs in the form of well-defined nanorods, which exhibit an elongated rod-like structure with a high aspect ratio. These nanorods are uniformly distributed, with some degree of aggregation. In addition, irregularly shaped smaller particles adhering to the nanorods are vermiwash-derived nutrient contents. This suggests a strong interaction between the iron oxide nanorods and biomolecules in the vermiwash, potentially facilitating surface functionalization.

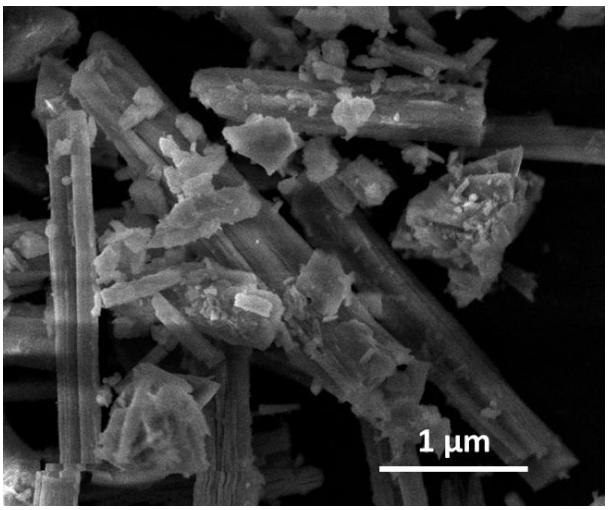

Fig. 4. SEM micrograph of iron oxide NPs embedded in vermiwash particles.

Effect of iron oxide NPs on quality parameters of chickpea seed

The impact of different concentrations of iron oxide NPs on the chickpea growth was assessed by studying various growth parameters over a period of 7 days. Figure 5 shows the seedling growth for control and two concentrations (50 ppm and 150 ppm) of NPs at the end of seventh day.

Seed germination percentage

The effect of different concentrations of iron oxide NPs on seed germination percentage displayed a notable variation over seven days (Figure 6). The highest germination percentage was observed at 150 ppm concentration, where 70% of seeds germinated by the third day, and the germination

percentage remained stable at 80% on the fifth and seventh days. The second-highest germination percentage occurred at 100 ppm concentration, with 60% germination on the third day and the subsequent rise to 80% by the fifth and seventh days. At 50 ppm concentration, the germination percentage was 60% on the third day and increased to 80% by the fifth and seventh days, showing similar results to 100 ppm concentration. In contrast, the control group (0 ppm) showed the lowest germination at 40% on day three, which increased to 70% by day seven.

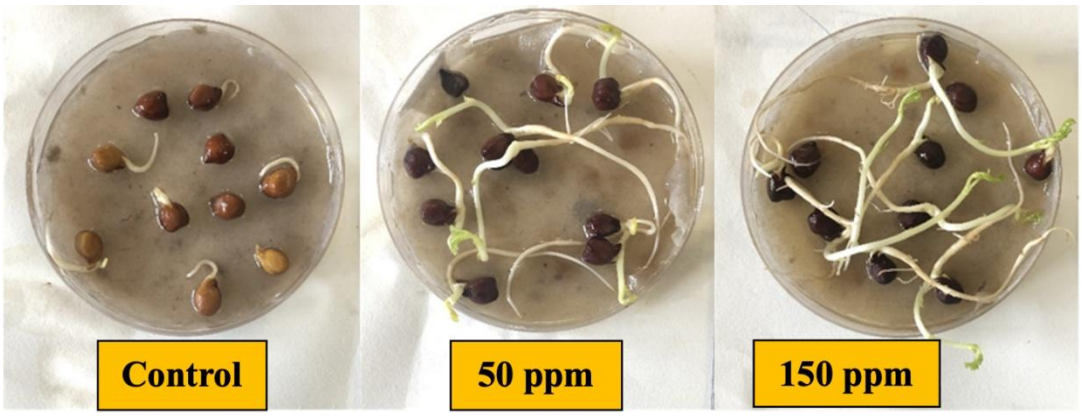

Figure 5: Seedling growth after 7 days of sowing the nano-primed chickpea seeds using different concentrations of iron oxide NPs.

Interestingly, germination was initially higher than the control at 10 ppm concentration but reached the same final value (70%) by day seven. The lowest germination occurred at a concentration of 200 ppm, which reached only 50% on the seventh day. These results are in agreement with those of Tamindžić G et al. (2024), who reported a positive effect (5-10%) of various NPs (Ca, Si, and Fe) on the germination rate of pea under both optimal and drought-stressed conditions. Similar findings were reported by Naskar et al. (2020), who observed significant improvement in the chickpea germination following the application of iron oxide NPs. The iron oxide NPs enhance the antioxidant enzymatic activity resulting in improved seed germination (Yoon et al., 2019).

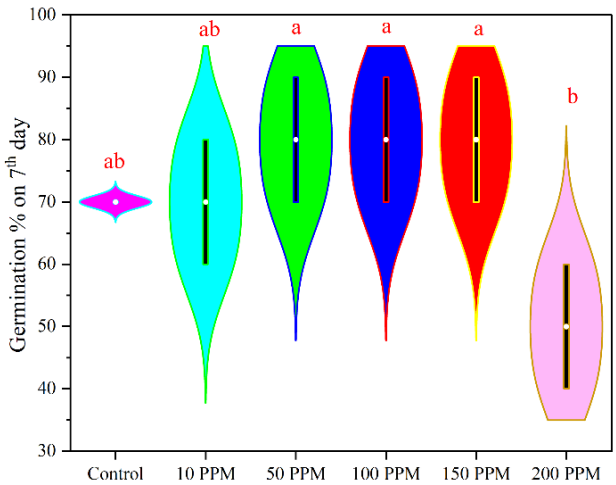

Fig. 6. Comparison of seed germination percentage among various treatments of iron oxide NPs.

Root length

The root length observed with different concentrations of iron oxide NPs showed a clear trend in growth over the seven days (Figure 7(a)). The highest root length was recorded at 150 ppm concentration, with 1.30 cm on the third day, 3.16 cm on the fifth day, and 6.58 cm on the seventh day. However, at 100 ppm and 50 ppm concentrations, the root length reached 5.94 cm and 5.68 cm, respectively, by day seven. In contrast, the control (0 ppm) exhibited the lowest root length, starting at 0.15 cm on day three and reaching 1.68 cm on day seven. At 10 ppm concentration, the root length was higher than the control but lower than the other concentrations, reaching 1.70 cm on the seventh day. At 200 ppm concentration, the root growth was initially absent on the third day but increased to 0.83 cm by the fifth day and 1.49 cm by the seventh day, which was significantly lower than all other treatments. These results align with the findings of Mazhar et al. (2023), who investigated the application of iron oxide NPs on garden pea under drought conditions and observed a 38% increase in the root length and 24% increase in the number of leaves. The present findings are further supported by Maswada et al. (2018), who demonstrated the effectiveness of

iron oxide NPs as a pre-sowing treatment to enhance the germination and seedling growth in sorghum. Iron oxide NPs enhance seed coat permeability, facilitating greater uptake of water and oxygen, which in turn promotes faster germination and improved seedling growth (Gupta et al., 2022).

Shoot length

Significant variations were observed in the shoot length measurements with different concentrations of iron oxide NPs (Figure 7(b)). The highest shoot length was observed at the 150 ppm concentration, where no growth was seen on the third day, but it reached 1.66 cm on the fifth day and 3.97 cm on the seventh day. The 100 ppm concentration displayed a similar pattern, with growth starting at 1.20 cm on the fifth day and reaching 3.89 cm on the seventh day. At 50 ppm concentration, the shoot length reached 0.82 cm on the fifth day and 2.99 cm on the seventh day, showing results comparable to 100 ppm. The control (0 ppm) showed no shoot length on the third day, with a minimal growth of 0.50 cm on the seventh day. At 10 ppm concentration, the shoot length was 0.47 cm on the fifth day and 1.30 cm on the seventh day, which was better than the control but lower than higher concentrations. At 200 ppm concentration, a significantly lower growth was observed, with 0.35 cm on the fifth day and 1.02 cm on the seventh day. The finding are in concordance with the observations of Das and Dutta (2022), who reported positive effects of zinc oxide and silver NPs on the growth and yield-attributing parameters of chickpea.

(a)

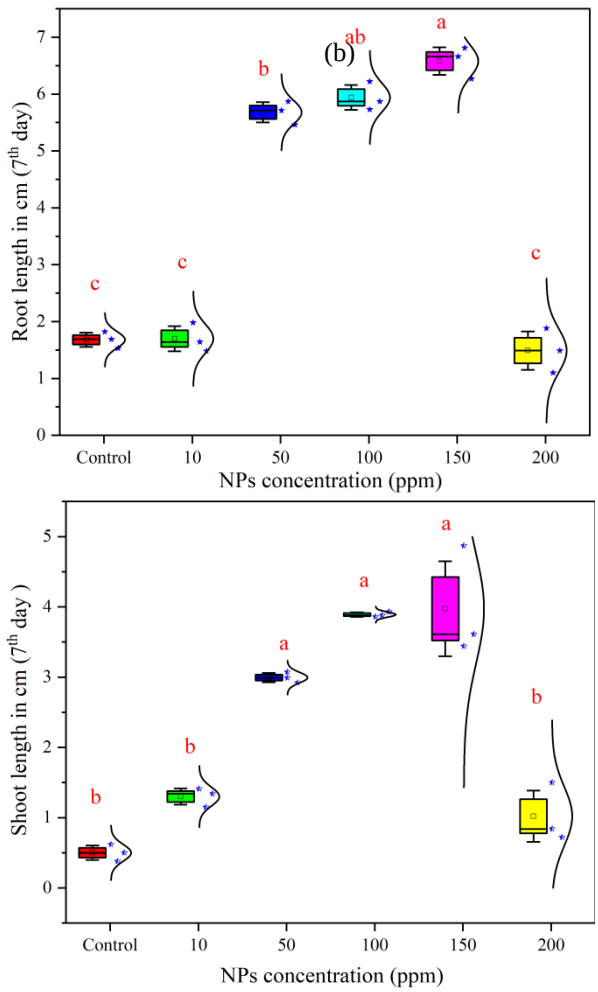

Fig. 7. Comparison of seedling (a) root length (b) shoot length among various treatments of iron oxide NPs.

Fresh weight

The measurements on fresh weight (Figure 8(a)) demonstrated a clear trend of increasing weight with higher concentrations of iron oxide NPs. The highest fresh weight was observed at 150 ppm, starting at 0.3790 gm on the third day, increasing to 0.4507 gm on the fifth day, and reaching 0.5865 gm on the seventh day. Close behind was 100 ppm concentration, which showed 0.3788 gm on the third day, 0.4346 gm on the fifth day, and 0.5674 gm on the seventh day. At 50 ppm concentration, the fresh weight increased from 0.3751 gm on the third day to 0.5153 gm by the seventh day. The 10 ppm concentration exhibited consistent growth, with 0.3428 gm on the third day, 0.3894 gm on the fifth day, and 0.4329 gm on the seventh day. The control (0 ppm) had the lowest fresh weight,

starting at 0.3287 gm on the third day and reaching 0.4058 gm by the seventh day. The 200 ppm concentration showed minimal variation, with fresh weight increasing from 0.3529 gm on day three to 0.4537 gm on day seven, slightly lower than other concentrations. These observations are in agreement with the findings of Kaningini et al. (2023), who reported a significant enhancement in biomass accumulation (up to 1.5-fold) following the application of iron oxide NPs in chickpea.

Dry weight

The dry weight measurements (Figure 8(b)) revealed a clear increase with higher concentrations of iron oxide NPs. The highest dry weight was observed at 150 ppm, starting at 0.3140 gm on the third day, rising to 0.3511 gm on the fifth day, and reaching 0.5020 gm by the seventh day. The 100 ppm concentration showed similar growth, with 0.3139 gm on day three, 0.3459 gm on day five, and 0.4814 gm on day seven. The 50 ppm concentration had a steady increase, starting at 0.3008 gm on day three and reaching 0.4242 gm by day seven. The 10 ppm concentration showed a consistent increase from 0.2661 gm on day three to 0.3472 gm on day seven. The control (0 ppm) had the lowest dry weight, with 0.2579 gm on day three and 0.3263 gm on day seven. The 200 ppm concentration showed minimal increase, starting at 0.2847 gm on the third day and reaching 0.3618 gm by the seventh day, which was lower compared to other concentrations. The enhanced growth observed at lower concentrations resulted in increased biomass, indicating improved plant health. This effect may be attributed to the increased iron uptake from iron oxide NPs, which likely stimulated key physiological processes, enhanced photosynthetic efficiency, and ultimately led to greater biomass accumulation. These findings are in good agreement with the observations of Pawar et al. (2019).

(a)

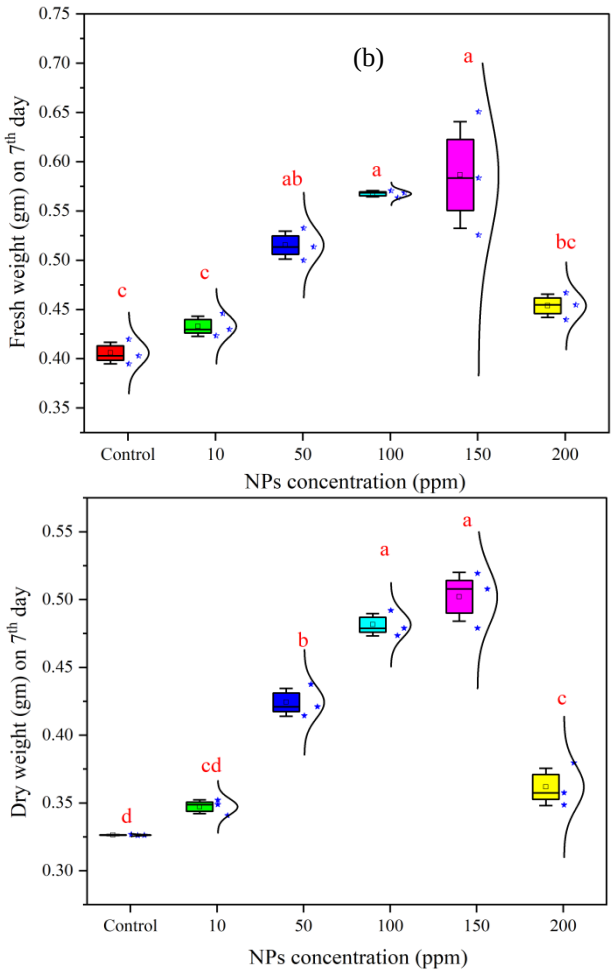

Fig. 8. Comparison of seedling (a) fresh weight (b) dry weight among various treatments of iron oxide NPs.

Root-to-Shoot length ratio

The root-to-shoot length ratio exhibited variation across different concentrations of iron oxide NPs (Figure 9). The highest ratio was observed at 200 ppm concentration on the fifth day, with a value of 6.09, before reducing to 1.54 by the seventh day. For the control (0 ppm), the ratio was 4.18 on the fifth day and decreased to 3.48 by the seventh day. The 10 ppm concentration also displayed a decreasing trend, with a ratio of 2.90 on day five and 1.31 on day seven. Similarly, the 50 ppm concentration had a ratio of 2.91 on day five and 1.90 on day seven. The 100 ppm concentration

exhibited a ratio of 2.00 on day five, which slightly reduced to 1.52 by day seven, showing similar results as the 150 ppm concentration, where the ratio was 1.99 on the fifth day and 1.71 on the seventh day.

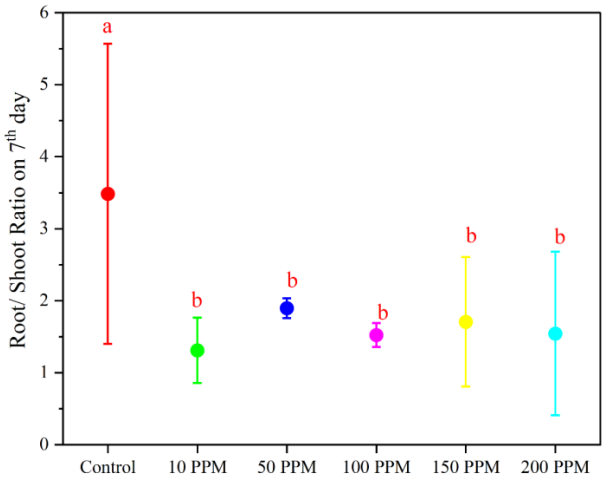

Fig. 9. Comparison of seedling root-to-shoot lengths ratio among various treatments of iron oxide NPs.

Seedling Vigour Indices (I and II)

Seedling vigour indices (SVI-I and SVI-II) demonstrated a clear enhancement with different concentrations of iron oxide NPs (Figures 10(a)-(b)) up to 150 ppm concentration of NPs. The maximum SVI-I and SVI-II were observed at 100 ppm and 150 ppm concentrations, with no significant difference between them. A moderate increase was seen at 50 ppm which was significantly higher than 10 ppm and control. This indicates that iron oxide NPs at moderate concentrations (100–150 ppm) enhance seedling vigour, possibly by improving nutrient availability and root elongation. However, the lowest SVI-I and SVI-II values observed at 200 ppm, indicated poor seedling vigour, likely due to NP-induced oxidative stress.

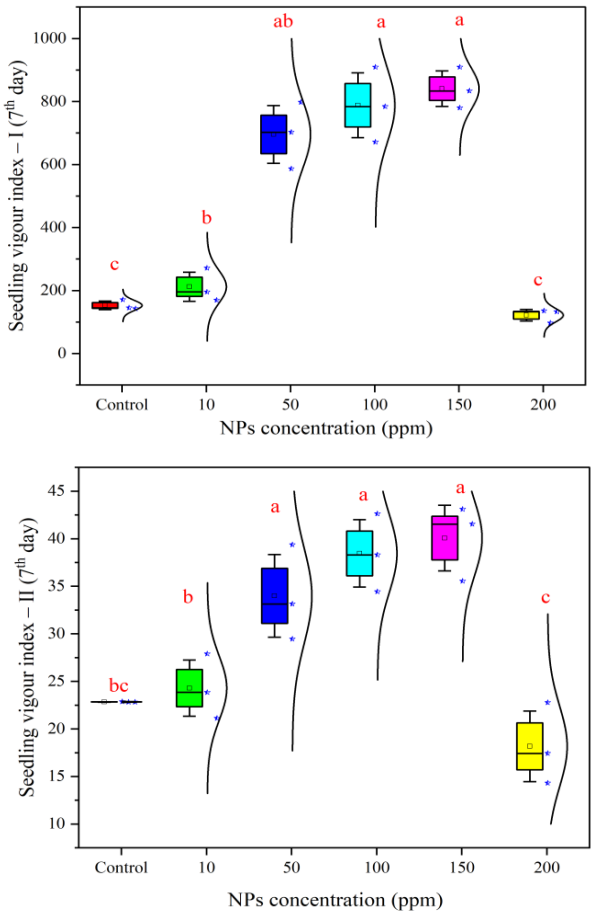

Fig. 10. Comparison of seedling (a) vigour index-I (b) vigour index-II among various treatments of iron oxide NPs.

Pearson Correlation Analysis

The Pearson correlation coefficient was determined to ascertain statistically significant correlations between the altered growth parameters of black chickpea and iron oxide NPs (Table 3). The correlation coefficient above 0.79 was treated as significant. The NPs treatment showed positive relationship with the germination percentage, root length, shoot length, fresh and dry weights, and seedling vigour indices, whereas negative correlation was observed with root-to-shoot lengths ratio. However, the NPs treatments didn't show a significantly positive or negative correlation with the growth parameters. Germination percentage showed significant positive correlation with the root length and seedling vigour indices. Likewise, root length showed significant positive correlation with shoot length, fresh and dry weights, and seedling vigour indices. Shoot length had positive correlation with fresh and dry weights, and seedling vigour indices. A significant positive correlation was observed between fresh and dry weights. Seedling vigour indices showed positive correlations with all the growth parameters.

Principal Component Analysis

Principal Component Analysis (PCA) was also carried out to investigate the possible correlations between the NPs concentrations and various chickpea seed growth parameters. PCA is a multivariate technique (Abdi and Williams, 2010) that transforms the original data into a new set of uncorrelated variables known as principal components (PCs). These principal components are linear combinations of the original parameters, where generally the first two principal components, PC1 and PC2, are taken that capture the largest and second largest variance in the data, respectively. In the present analysis, PC1 (82% variance) was driven by the growth parameters, germination percentage, root length, and seedling vigour indices, whereas, PC2 (15.3% variance) was driven strongly by root-to-shoot length ratio. The PCA biplot (Figure 11) revealed a clear differentiation of treatments based on NPs concentrations. The 100 ppm and 150 ppm treatments clustered on the right-hand side of the plot displayed strong positive correlations with the germination percentage, seed vigour indices, and root length. These parameters had dominant contribution towards PC1, indicating that moderate NPs concentrations (100, 150 ppm) had positive influence on the seedling growth. In contrast, control (0 ppm), 10 ppm and 200 ppm treatments clustered on the left-hand side indicated limited or adverse impact on the chickpea seedling growth. The root-to-shoot length ratio contributed predominantly to PC2, displaying a unique directional vector. This indicated that its variation was independent from the other parameters, potentially reflecting a stress response due to NPs exposure.

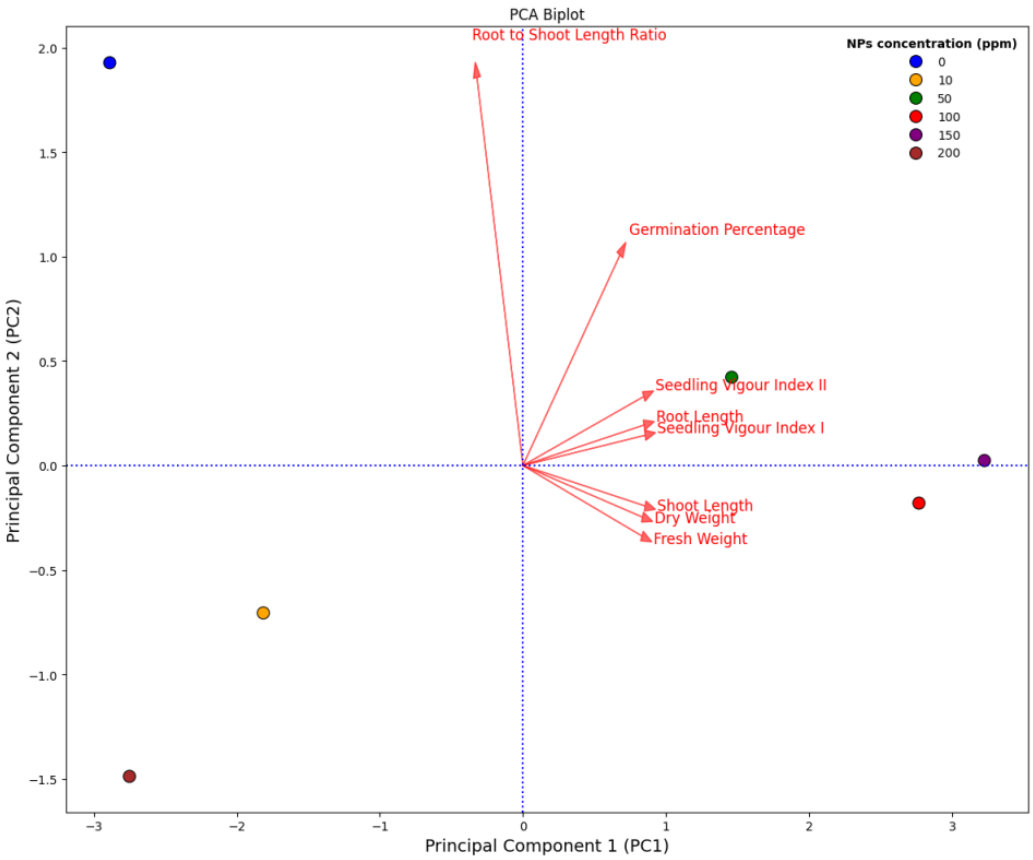

Fig. 11. PCA biplot with eight traits evaluated for six concentrations of iron oxide NPs.

Table 3. Pearson correlation coefficients for various growth parameters of black chickpea under iron oxide NPs nano-priming treatments.

Parameter	NPC	GP	RL	SL	FW	DW	SVI-I	SVI-II	RTSLR
NPC	1								
GP	0.40	1							
RL	0.22	**0.79**	1						
SL	0.31	0.74	**0.97**	1					
FW	0.47	0.62	**0.95**	**0.98**	1				
DW	0.44	0.64	**0.95**	**0.99**	**0.99**	1			
SVI-I	0.20	**0.81**	**0.99**	**0.98**	**0.95**	**0.96**	1		
SVI-II	0.0875	**0.88**	**0.97**	**0.96**	**0.92**	**0.93**	**0.98**	1	
RTSLR	-0.47	0.03	-0.27	-0.41	-0.45	-0.42	-0.31	-0.24	1

Highly significant positive/negative correlations are highlighted in bold. NPC: NPs concentration, GP: germination percentage, RL: root length, SL: shoot length, FW: fresh weight, DW: dry weight, SVI-I: seedling vigour index – I, SVI-II: seedling vigour index – II, RTSLR: root-to-shoot lengths ratio.

Conclusion

This study investigated the potential of nano-priming with iron oxide nanoparticles (NPs) to promote growth in black chickpea. Seed nano-priming was carried out using varied concentrations of iron oxide NPs (0, 10, 50, 100, 150, 200 ppm). The seedling growth under in-vitro conditions was assessed using various growth parameters such as germination percentage, root length, shoot length, root-to-shoot length ratio, fresh and dry weights, and seedling vigour indices. Iron oxide NPs treatments up to 150 ppm concentration enhanced the germination percentage, seedling root and shoot lengths, biomass accumulation, and seedling vigour indices. However, higher concentrations (200 ppm) inhibited the seedling growth. While most growth parameters showed a positive, albeit statistically non-significant, correlation with NPs concentration (as determined using Pearson correlation analysis), root-to-shoot lengths ratio did not follow this trend. Principal Component Analysis (PCA) indicated that moderate NP concentrations (100–150 ppm) enhanced multiple growth parameters, while both low (0–10 ppm) and high (200 ppm) concentrations were less effective or inhibitory to early seedling development. Overall, the findings highlight the potential of iron oxide NPs as a co-fertilizer to support early-stage growth in black chickpea, with optimal effects observed at concentrations up to 150 ppm.

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Author Contributions

NR and ND conceived the concept and supervised the study. RK performed the experiment and collected data. RK, ND and NR designed the analysis tools and performed analysis. ND and NR wrote the manuscript with input from all the authors. RK, ND and NR reviewed and approved the manuscript.

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Competing interest

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Ethics approval

Not applicable.

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