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Research Article

Keywords: soybean, Fe₃O₄ nanoparticles, salinity stress, oxidative stress, physiological traits, yield

Posted Date: August 7th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10226884/v1>

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Additional Declarations: No competing interests reported.

Iron oxide (Fe₃O₄) nanoparticles improve physiological performance, growth, and yield of soybean under salinity stress

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Abstract

Soybean productivity in saline environments is severely constrained by salt-induced physiological and biochemical disturbances. A pot experiment was conducted from November 2023 to February 2024 at the Department of Agronomy, Gazipur Agricultural University, Bangladesh, to evaluate the effects of foliar-applied Fe_3O_4 nanoparticles (NPs) on soybean under salinity stress. The experiment followed a completely randomized factorial design with two salinity levels (0 and 75 mM NaCl) and six Fe_3O_4 NP concentrations (0-500 ppm). Salinity stress significantly reduced growth, physiological performance, chlorophyll content, and yield-related traits, while increasing stress-associated parameters including malondialdehyde (MDA), proline, hydrogen peroxide (H_2O_2), and water saturation deficit. Foliar application of Fe_3O_4 NPs was associated with partial alleviation of salinity-induced reductions in several measured traits. Among the tested concentrations, 400 ppm generally showed comparatively better performance in terms of biomass accumulation, relative water content, chlorophyll content, membrane stability index, and yield-related parameters under saline conditions. Exploratory correlation and principal component analyses indicated positive associations among yield and several physiological traits, whereas stress-associated traits showed negative relationships with yield performance. The findings suggest that moderate concentrations of Fe_3O_4 NPs may improve soybean performance under salinity stress conditions. However, because soluble iron controls and independent nanoparticle characterization were not included, nanoparticle-specific effects could not be conclusively established.

Keywords: soybean, Fe_3O_4 nanoparticles, salinity stress, oxidative stress, physiological traits, yield.

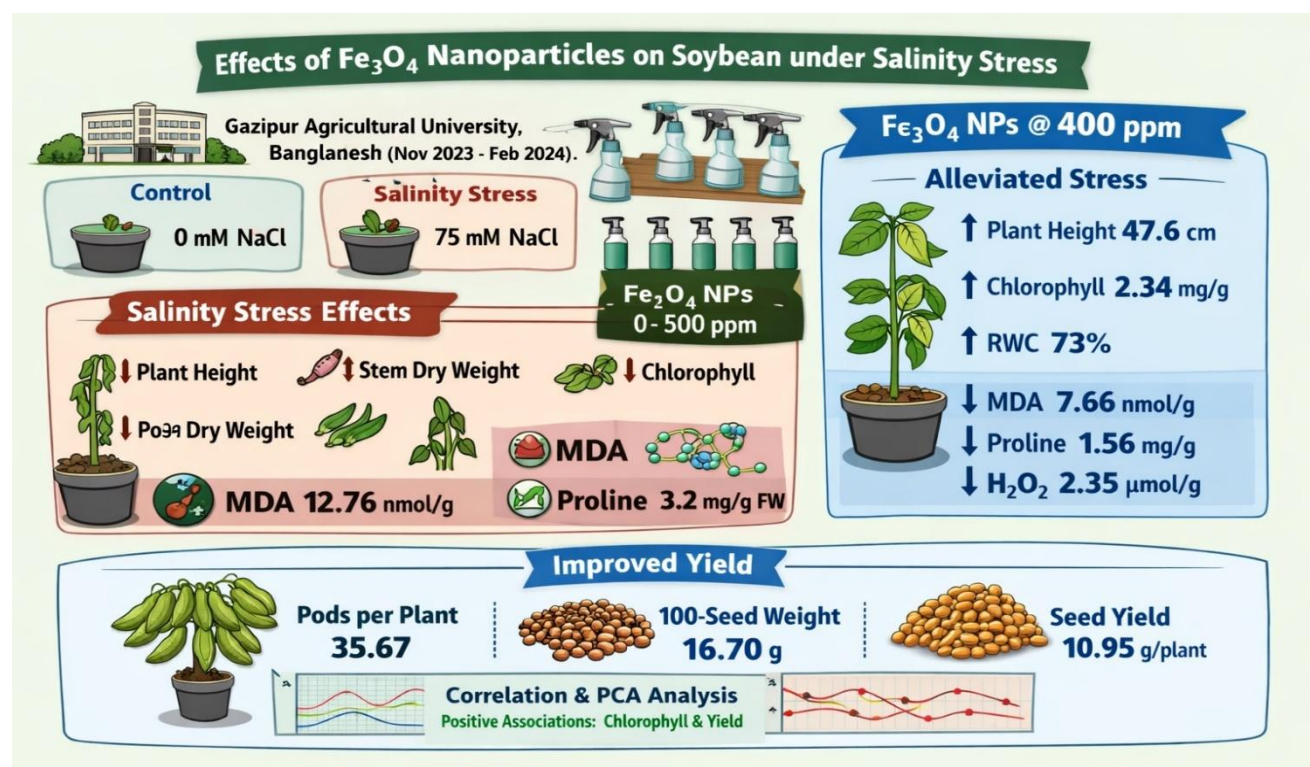


Figure 1. Graphical abstract

1. Introduction

Feeding the rapidly growing global population is an urgent challenge, as agricultural production must increase by 60–100% within the next three decades to meet food demand [1]. The United Nations projects that the world's population will reach 8.5 billion by 2030 and 9.7 billion by 2050 [2], placing unprecedented pressure on agricultural systems. However, food security is increasingly threatened by phytopathogens, excessive use of fertilizers and pesticides, soil degradation, and, most critically, climate change [3-5]. Climate change has intensified abiotic stresses such as drought, salinity, and high temperatures, which severely impair crop productivity, particularly in developing countries [6]. Salinity, in particular, limits plant growth by disrupting water uptake, restricting nutrient absorption, and causing toxic ion accumulation, ultimately reducing photosynthesis, protein synthesis, and yield [7-11].

Bangladesh, a low-lying deltaic country, is highly vulnerable to salinity intrusion. More than 30% of its arable land lies in coastal saline regions, and the 2.86 million hectares of coastal and offshore land, approximately 1.056 million hectares are affected by varying salinity levels [12]. This area continues to expand due to seawater intrusion, cyclonic flooding, and rising groundwater salinity [8]. Increasing agricultural productivity in these regions requires salt-tolerant, high-value crops. Soybean (*Glycine*

79 *max* L.), widely known as the “golden bean” for its high nutritional value and economic importance
80 [13,14], could be an attractive option for coastal cropping systems. Soybean is known to be partially
81 sensitive to salinity. However, high salinity is a big problem that reduces growth and yield of soybean
82 [15,16].

83 To address such yield losses, innovative agronomic solutions that enhance stress tolerance are
84 essential. Nanotechnology offers a promising strategy, with nanoparticles (NPs) such as zinc oxide,
85 silver, cerium oxide, and iron oxide shown to improve plant growth, nutrient uptake, and stress
86 resilience [17,18]. Due to their nanoscale size (1-100 nm) and large surface area, NPs exhibit unique
87 physicochemical properties that allow efficient nutrient delivery and targeted physiological responses
88 [19]. Among these, iron oxide nanoparticles (Fe_3O_4 NPs) are particularly promising because iron is a
89 key micronutrient in chlorophyll biosynthesis, photosynthesis, enzyme activation, and protein
90 metabolism - processes often impaired under salinity stress [20]. At the Department of Agronomy,
91 Gazipur Agriculture University, Bangladesh in earlier experiment it was observed that Iron oxides
92 nanoparticles enhanced the drought tolerance in soybean through modification of physio-biochemical
93 parameters under water deficit conditions [21].

94 Given the increasing extent of salinity-affected soils in coastal Bangladesh and the economic potential
95 of soybean, the scope of this research extends to developing and testing advanced nano fertilizer-
96 based management strategies that can enhance crop performance in saline environments. The
97 justification lies in the potential benefits of Fe_3O_4 nano-fertilizers as an iron source under saline
98 conditions. However, the present study did not directly evaluate iron uptake, nanoparticle
99 localization, or specific physiological mechanisms associated with stress tolerance. Such an approach
100 could not only improve soybean yield and seed quality in marginal lands but also contribute to the
101 sustainable intensification of coastal agriculture. It is hypothesized that Fe_3O_4 nanoparticle
102 application may improve soybean growth, physiological performance, and yield-related traits under
103 salinity stress through responses associated with reduced oxidative damage. Accordingly, the
104 objectives of this study were to investigate the interactive effects of iron oxide (Fe_3O_4) nanoparticles
105 and salinity stress on soybean growth, physiology, biochemical traits, and yield of soybean plants
106 under controlled conditions.

107 **2. Materials and Methods**

108 **2.1. Experimental site, plant material, soil, and experimental design**

109 A pot experiment was conducted at the stress research facility of the Department of Agronomy,
 110 Gazipur Agricultural University (GAU), Gazipur, Bangladesh. The experimental site is located at an
 111 elevation of approximately 8.4 m above mean sea level (24°05'23" N, 90°15'36" E). The study was
 112 carried out inside a polyhouse covered with a portable rain-protective plastic sheet, allowing plants
 113 to grow under near-natural environmental conditions.

114 Certified seeds of soybean (*Glycine max* L.) variety BU Soybean-2, developed by the Department of
 115 Agronomy, Gazipur Agricultural University, were used as planting material. The experiment was
 116 conducted for 110 days from November 2023 to February 2024.

117 Plastic pots were filled with air-dried and sieved soil collected from the experimental farm of GAU.
 118 The experiment followed a completely randomized design with three replications. Each pot was
 119 considered as an experimental unit. The minimum and maximum air temperatures and relative
 120 humidity recorded during the soybean growing period are presented in Figure 2 [22].

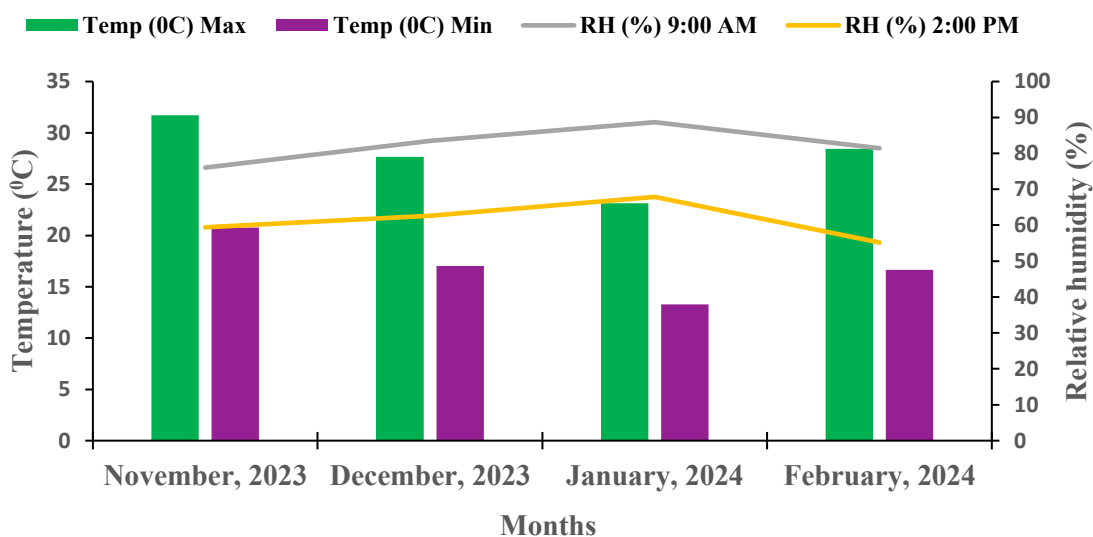


Figure 2. Temperature and relative humidity during experimentation

128 The textural class and physico-chemical characteristics of the experimental soil were determined by
 129 following standard procedures [23,24]. The experimental soil had a pH of 6.31 and a sandy loam
 130 texture, consisting of 52.99% of sand, 33.00% of silt, and 13.21% of clay. Soil organic carbon,
 131 available P, total N, exchangeable K, CEC, and EC values were in that order of 0.55%, 0.06 mg /100
 132 g, 0.07%, 0.73 cmol /kg dry soil, 12.75 cmol / kg dry soil, and 0.02 dS / m. The soil retains around

133 30% of its moisture content at field capacity (FC). Each plastic pot (30 cm in length and 24 cm in
 134 diameter) contained a 4:1 blend of soil and cow excrement. It held eleven kg of blended soil that was
 135 air-dried, 0.933 g of triple superphosphate (TSP), 0.64g of muriate of potash (MOP), and 0.32 g of
 136 urea were evenly applied to each pot following FRG [25], those are equating to 80 kg of urea, 205 kg
 137 of TSP, and 128 kg of MOP per hectare, based on the relevant amounts. Three replications of each
 138 treatment were included in the completely randomized design (CRD) in the experiment.

139 2.2 Treatments

Schematic of Experimental Design and Schedule

Factor A: Salinity levels: 2

i. Control: 0 mM NaCl

ii. Saline :75 mM NaCl

Factor B: Fe₃O₄ NPs doses: 6

i. Control: 0 ppm

ii. 100 ppm

iii. 200 ppm

iv. 300 ppm

v. 400 ppm

vi. 500 ppm

Experimental Schedule

Seed sowing

Salinity & NPs
solution foliar
spray

Physiology & biochemical
measurements

Harvest & yield
analysis

140 Salinity treatment was established using sodium chloride (NaCl) solutions at two levels: 0 mM as
 141 the non-saline control and 75 mM to induce salt stress. Preliminary screening trials indicated that
 142 NaCl concentrations exceeding 75 mM caused severe leaf chlorosis, marked growth inhibition, and
 143 high plant mortality before completion of the reproductive phase. Consequently, 75 mM NaCl was
 144 chosen as an optimal level to impose strong yet quantifiable salinity stress for assessing the
 145 mitigating role of Fe₃O₄ nanoparticles. Comparable salinity levels have previously been reported to
 146 significantly disrupt soybean physiological performance under controlled environments [26 - 28].

The application rates of Fe₃O₄ nanoparticles ranged from 100 to 500 ppm and were determined based on earlier studies demonstrating their effectiveness in modulating plant growth, physiological responses, and tolerance to abiotic stress [29]. It should be noted that the uptake and internal translocation of iron derived from Fe₃O₄ nanoparticles were not directly measured in the present investigation.

2.3 Fe₃O₄ nanoparticles solution preparation

A foliar application solution of iron oxide nanoparticles (Fe₃O₄ NPs) was prepared by adapting procedures previously described by Singh and Kalia [30] and Khan et al. [31]. The Fe₃O₄ nanopowder used in this study appeared black–brown in color, with a reported average particle size of ≤50 nm and a Brunauer – Emmett - Teller (BET) surface area between 60 and 100 m² /g [32]. According to manufacturer specifications, the Fe₃O₄ nanoparticles were characterized by X-ray diffraction (XRD) analysis for phase identification. To prepare the working suspensions, accurately weighed quantities of analytical-grade Fe₃O₄ nanopowder (100, 200, 300, 400, and 500 mg) were used to obtain final concentrations of 100, 200, 300, 400, and 500 ppm, respectively. Each measured amount was initially added to approximately 800 mL of deionized water in separate 1000 mL glass beakers. The mixtures were subjected to continuous magnetic stirring for 30 min to facilitate preliminary dispersion of the nanoparticles. Subsequently, the suspensions were sonicated for 30 min at a frequency of 40 kHz using an ultrasonic bath (Digital Ultrasonic Cleaner, Model XYZ) to minimize particle agglomeration and ensure homogeneous distribution. Following sonication, each suspension was transferred to a 1000 mL volumetric flask, and the volume was adjusted to the mark with deionized water. Independent physicochemical characterization of the Fe₃O₄ nanoparticle suspension, including particle size distribution, zeta potential, morphology, and stability of the working suspension, was not conducted in the present study. The freshly prepared Fe₃O₄ NP solutions were stored in amber glass containers at room temperature (25 ± 2 °C) and applied within 24 h to avoid photodegradation and maintain physicochemical stability. For foliar treatment, the required volume of each nanoparticle suspension was poured into a hand-operated sprayer and uniformly applied to the soybean canopy during the early morning hours to reduce rapid evaporation and enhance leaf surface retention. The experimental design did not include soluble iron or bulk iron treatments; therefore, nanoparticle-specific effects cannot be conclusively distinguished from general iron nutrition effects

2.4 Crop cultivation and treatment application

A total of 200 g of healthy, mature, and uniform seeds of soybean (*Glycine max* L.) variety BU Soybean-2 were surface sterilized using a 1% sodium hypochlorite (NaOCl) solution for 5 min. Following sterilization, the seeds were rinsed several times with distilled water to eliminate residual NaOCl and then air-dried until they returned to their initial moisture level. Ten seeds were sown per pot at equal spacing. Soil moisture was maintained near field capacity through daily watering. Seedlings were thinned at the trifoliate leaf stage, approximately 14 days after sowing, leaving six uniformly growing plants per pot. To minimize positional effects, the pots were repositioned randomly within each block on a weekly basis. Weed growth was controlled manually whenever required. The trifoliate stage, characterized by the development of the third true leaf, represents a critical phase of vegetative development in soybean associated with increased photosynthetic activity and biomass production; therefore, this growth stage was selected for the application of salinity stress [33]. Salinity treatment was imposed by irrigating 18 pots with a 75 mM NaCl solution, while an additional 18 pots were maintained under non-saline conditions and served as controls throughout the growing period. Foliar application of Fe₃O₄ nanoparticle suspensions at concentrations of 0, 100, 200, 300, 400, and 500 ppm was carried out using a hand sprayer on both salt-stressed and non-stressed plants seven days after salinity induction. Each plant received two complete foliar sprays at seven-day intervals.

2.5 Data collection

2.5.1 Measurement of growth-related parameters

Plant height, leaf dry weight, stem dry weight, root dry weight, and total dry weight were assessed after using Fe₃O₄ foliar spray for 15 days on three plants per pot.

2.5.2 Measurements of physio-biochemical parameters

During the pod filling phase, measurements were taken of the relative water content, water saturation deficit, photosynthesis, stomatal conductance, transpiration rate, chlorophyll content, cell membrane stability index, MDA (malondialdehyde), total sugar, proline, H₂O₂ content, carotenoids, flavonoids, and antioxidants on both control and saline-treated leaves.

2.5.2.1. Relative water content and water saturation deficit

Relative water content (RWC) analysis was performed using five randomly selected, fully expanded upper leaves from each treatment. Immediately after sampling, the leaves were sealed in polyethylene bags and transported to the laboratory. Fresh weight (FW) was recorded promptly, after which the leaf samples were submerged in distilled water for 24 h to obtain turgid weight (TW). The samples were then oven-dried at 65 °C for 72 h to determine dry weight (DW). RWC and water saturation deficit (WSD) were calculated following Mannan et al. (2013) [9] using the equations:

$$\text{RWC (\%)} = [(FW - DW) / (TW - DW)] \times 100$$

$$\text{WSD (\%)} = [(TW - FW) / (TW - DW)] \times 100$$

where FW, TW, and DW represent fresh, turgid, and dry weights, respectively.

2.5.2.2. Gas exchange characteristics

Gas exchange parameters, including photosynthetic rate, transpiration rate, and stomatal conductance, were measured using a portable photosynthesis system (LI-6400, Li-COR, Lincoln, NE, USA). Measurements were taken from the uppermost, fully expanded leaves of plants in each pot. Data collection was conducted on clear sunny days between 11:00 and 13:00 h under natural light conditions, with photosynthetically active radiation (PAR) ranging between 1200 and 2000 $\mu\text{mol} / \text{m}^2 / \text{s}$.

2.5.2.3. Chlorophyll content determination

Leaf chlorophyll content was estimated according to the procedure described by Mannan et al. [34]. One fully expanded upper leaf from each replication was used for analysis. Fresh leaf tissue (20 mg) was homogenized in 20 mL of 80% acetone, and the extraction vials were wrapped with aluminum foil and incubated in darkness for 72 h. Absorbance of the extracts was measured at 663 and 645 nm using a double-beam UV–Vis spectrophotometer (Thermo Fisher Scientific, Model 20020). Chlorophyll a, chlorophyll b, and total chlorophyll contents were calculated using standard equations based on absorbance values, extraction volume, and fresh sample weight.

2.5.2.4. Cell membrane stability index

Cell membrane stability index (MSI) was evaluated following the method outlined by Rady et al. [35]. Leaf discs were prepared and divided into two equal sets, each containing ten discs. One set was incubated in a water bath at 40 °C for 30 min, after which electrical conductivity (EC₁) was recorded. The second set was heated at 100 °C for 10 min to release total electrolytes, and electrical conductivity (EC₂) was measured. MSI was calculated using the equation:

$$\text{MSI (\%)} = [1 - (\text{EC}_1 / \text{EC}_2)] \times 100$$

2.5.2.5. Lipid peroxidation assessment

Lipid peroxidation was quantified by estimating malondialdehyde (MDA) content following the method of Rao and Sresty [36]. Fresh leaf tissue (0.5 g) was homogenized in a reaction mixture containing 20% thiobarbituric acid (TBA) and 0.1% trichloroacetic acid (TCA). The absorbance of the resulting supernatant was measured at 530 and 600 nm using a UV–Vis spectrophotometer (Shimadzu UV-1201, Kyoto, Japan), and MDA concentration was calculated accordingly.

2.5.2.6. Total sugar estimation

Total soluble sugar content in leaf tissues was determined following the method described by Somogyi [37]. Fresh leaf samples (0.1 g) were extracted in 80% ethanol by heating in a water bath for 10–15 min. The extraction was repeated at least twice, and the combined extracts were diluted to a final volume of 20 mL with distilled water. An aliquot of 0.5 mL extract was mixed with 0.5 mL of 5% phenol solution, followed by the addition of 3 mL concentrated sulfuric acid. After cooling to room temperature, absorbance was recorded at 490 nm using a UV–Vis spectrophotometer (Systronics UV–VIS 118). Total sugar content was expressed as mg /g fresh weight of leaf.

2.5.2.7. Proline content determination

Free proline concentration was estimated using the method proposed by Bates et al. [38]. Absorbance was measured at 520 nm, and proline content was calculated by comparison with a standard curve prepared using L-proline.

2.5.2.8. Hydrogen peroxide quantification

Hydrogen peroxide (H₂O₂) content was determined following a modified protocol of Velikova et al. [39]. Approximately 300 mg of frozen leaf tissue was homogenized in 2 mL of ice-cold 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 12,000 × g for 15 min at 4 °C. The supernatant (0.5 mL) was mixed with 1 mL of 1 M potassium iodide and 5 mL of 10 mM potassium phosphate buffer (pH 7.0). Absorbance was measured at 390 nm using a Cary 100 Bio spectrophotometer (Varian, Australia). Blanks were prepared using 0–1% TCA, and H₂O₂ standards were treated similarly.

2.5.2.9. Total carotenoid content

Total carotenoid concentration was estimated using the same acetone extract prepared for chlorophyll analysis. Absorbance was recorded at 480 nm, and carotenoid content was calculated using the equations proposed by Kirk and Allen [40]:

$$\text{Total carotenoids (mg g}^{-1} \text{ fresh weight)} = [A_{480} + (0.114 \times A_{663}) - (0.638 \times A_{645})] \times V / (1000 \times W)$$

where V represents extract volume and W denotes fresh sample weight.

2.5.2.10. Flavonoid content estimation

Total flavonoid content was measured spectrophotometrically using the aluminum chloride colorimetric method described by Quettier-Deleu et al. [41]. A diluted leaf extract (1:10) was mixed with an equal volume of 2% aluminum chloride solution prepared in methanol. After incubation at room temperature for 15 min, absorbance was measured at 430 nm. Quercetin was used as the reference standard, and flavonoid content was expressed as mg/ g quercetin equivalent (QE). Each sample was analyzed in triplicate.

2.5.2.11. Total antioxidant activity

Antioxidant capacity was assessed using the DPPH radical scavenging assay according to Okonogi et al. [42]. Methanolic leaf extracts (10 mg /mL) were serially diluted, and 500 µL of each dilution was mixed with an equal volume of 150 µM DPPH solution. The mixtures were incubated in the

dark at room temperature for 30 min, and absorbance was measured at 517 nm. Radical scavenging activity was calculated using the formula:

$$\text{DPPH scavenging (\%)} = [(A_0 - A_1) / A_0] \times 100$$

where A_0 and A_1 correspond to the absorbance of the control and sample, respectively. The IC_{50} value was determined as the extract concentration required to inhibit 50% of DPPH radicals.

2. 5. 3 Estimation of yield and yield contributing parameters

At maturity three plants per pot were harvested and number of pods/plant, number of seeds/pod, 100-seed weight, and seed yield/plant were calculated.

2.6 Statistical analysis

The collected data were analyzed using two-factor factorial analysis of variance (ANOVA), considering salinity level and Fe_3O_4 nanoparticle concentration as fixed factors. The interaction effect between salinity and nanoparticle treatment was also evaluated. Treatment means were separated using the least significant difference (LSD) test at $P \leq 0.05$. [43]. CropStat 7.2 was utilized for the analysis, while visualizations were created using Microsoft Excel 2016. Additionally, principal component analysis (PCA) was conducted using R software (R Core Team, 2019) to explore the relationships among the various physio-biochemical traits and treatments evaluated in the study.

3. Results

3.1 Effects of Fe_3O_4 NPs on growth of soybean plants under salinity stress

3.1.1 Leaf and stem dry weight

Under non-saline conditions, leaf dry weight in untreated plants was 1.50 g. Application of Fe_3O_4 NPs increased leaf dry matter, peaking at 1.68 g under the 400-ppm treatment (Table 1). Under salinity stress, leaf dry weight declined to 1.28 g in the untreated group, indicating a 14.67% reduction. However, under salinity, application of Fe_3O_4 NPs resulted in improved leaf biomass, with the highest value (1.66 g) again observed at 400 ppm, closely matching non-saline values.

Under non-saline conditions, stem dry weight in untreated plants was 0.95 g (Table 1). Application of Fe_3O_4 NPs increased stem dry weight, topping at 1.04 g under 500 ppm treatment indicating a dose-dependent growth promotion. Under salinity stress, stem dry weight declined to 0.76 g in the

untreated group, indicating a reduction of 20%. However, under salinity, application of Fe₃O₄ NPs increased stem dry weight, with the highest value 0.80 g observed at 400 ppm.

Table 1. Effects of Fe₃O₄ NPs on leaf dry weight, stem dry weight of soybean under salinity stress.

Fe ₃ O ₄ NP Concentrations (ppm)	Leaf dry weight (gm)		Stem dry weight (gm)	
	Control	Salinity	Control	Salinity
0	1.50±0.24NS	1.28±0.31	0.95±0.15NS	0.76±0.11
100	1.43±0.25	1.35±0.19	0.82±0.16	0.7±0.14
200	1.51±0.01	1.45±0.06	0.87±0.07	0.68±0.04
300	1.65±0.05	1.50±0.15	0.79±0.17	0.78±0.06
400	1.68±0.14	1.66±0.06	0.85±0.12	0.8±0.04
500	1.62±0.22	1.48±0.04	1.04±0.32	0.71±0.04
CV (%)	22.15		30.4	
Source of variation				
Salinity (S)	NS		NS	
Fe ₃ O ₄ NP (F)	NS		NS	
S x F	NS		NS	

Values are presented as mean ± standard error (SE), *n* = 3. Data were analyzed using two-factor factorial ANOVA considering salinity (S) and Fe₃O₄ nanoparticle concentration (F) as fixed factors. NS = non-significant at *P* ≤ 0.05. CV = coefficient of variation

3.1.2 Root and immature pod dry weight

Under non saline conditions, root dry weight in untreated plants was 0.20 g (Table 2). Application of Fe₃O₄ NPs increased root dry weight, topping at 0.32 g under 500 ppm treatment indicating a dose-dependent growth promotion. Under salinity stress, root dry weight declined to 0.16 g in the untreated group, indicating a reduction 20% (Table 2). However, application of Fe₃O₄ NPs improved root biomass, with the highest value under stress recorded at 400 ppm (0.31 g).

Under non saline conditions, immature pod dry weight in untreated plants was 1.08 g (Table 2). Application of Fe₃O₄ NPs increased immature pod dry weight, reaching at 1.59 g under 400 ppm treatment indicating a dose-dependent growth promotion. Under salinity stress, immature pod dry weight declined to 0.49 g in the untreated group, indicating a reduction 54.62% (Table 2). With Fe₃O₄ NPs application, pod biomass significantly improved, with the highest value of 0.71 g at 400 ppm under saline conditions.

Table 2. Effects of Fe₃O₄ NPs on root dry weight, immature pod dry weight of soybean under salinity stress.

Fe ₃ O ₄ NP Concentrations (ppm)	Root dry wt. (gm)		Immature pod dry wt. (gm)	
	Control	Salinity	Control	Salinity
0	0.20±0.05bc	0.16±0.02c	1.08±0.10d	0.49±0.10e
100	0.24±0.02abc	0.23±0.03abc	1.21±0.07cd	0.56±0.08e
200	0.15±0.02c	0.19±0.03c	1.38±0.10abc	0.64 ± 0.10e
300	0.21±0.03abc	0.26±0.02abc	1.56 ±0.06 ab	0.67 ± 0.01e
400	0.18±0.06c	0.31±0.02ab	1.59 ± 0.06a	0.71 ± 0.02e
500	0.32±0.09a	0.18±0.03c	1.29 ± 0.20bcd	0.64 ± 0.07e
CV (%)	31.2		16.3	
Source of variation				
Salinity (S)	*		***	
Fe ₃ O ₄ NP (F)	*		**	
S × F	*		*	

Values are presented as mean ± standard error (SE), $n = 3$. Means followed by different lowercase letters within a column differ significantly according to LSD test at $P \leq 0.05$. Data were analyzed using two-factor factorial ANOVA considering salinity (S) and Fe₃O₄ nanoparticle concentration (F) as fixed factors. CV = coefficient of variation. *, **, and *** indicate significance at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively.

3.2 Effects of Fe₃O₄ NPs on physio-biochemical parameters

3. 2.1 Effects of Fe₃O₄ NPs on Relative water content

The study showed that salinity substantially decreased relative water content (RWC) in soybean leaves compared to non-saline conditions. This reduction reflects the plant's impaired ability to

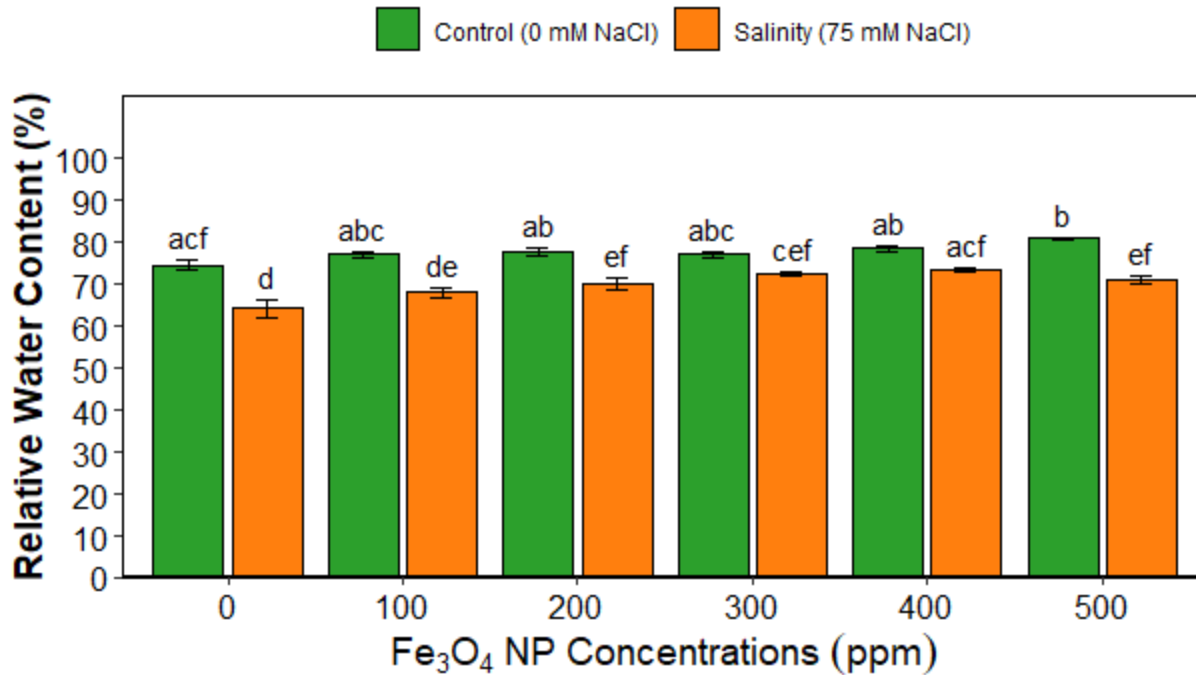


Figure 3. Effect of Fe₃O₄ NPs on relative water content (RWC) of soybean leaf under salinity stress. Values are mean ± SE (n = 3). Different letters indicate significant differences (LSD, P ≤ 0.05).

maintain cellular hydration under salt stress. Application of Fe₃O₄ NPs raised the relative water content in soybean leaves under salinity condition (Figure 3). Under control (non-saline) conditions, relative water content (RWC) was lowest in the 0 ppm Fe₃O₄ NPs treatment (74%), while application of 400 ppm and 500 ppm Fe₃O₄ NPs increased RWC to 78.53% and 81.07%, respectively. The RWC values for 100 ppm, 200 ppm, and 300 ppm Fe₃O₄ NPs were 77.13%, 77.77%, and 77.23%, respectively. Under salinity stress, RWC decreased sharply by 13.01% compared to the control, with the lowest value recorded in the untreated plants. Foliar application of Fe₃O₄ NPs under salinity improved RWC, with values of 68% (100 ppm), 70% (200 ppm), 72% (300 ppm), 73% (400 ppm), and 71% (500 ppm). The highest relative water content found at the dose of 400 ppm Fe₃O₄ NPs under saline condition that is around 73%, indicating partial recovery of plant water status. At 500 ppm, a slight decline in RWC was observed under salinity, although values still exceeded that of the saline control without Fe₃O₄.

3.2.2 Effects of Fe₃O₄ NPs on water saturation deficit

Water saturation deficit (WSD) reflects the degree of water loss and internal water stress experienced by plant tissues, serving as a direct indicator of water balance. In the current study,

salinity significantly elevated WSD in untreated soybean plants, indicating poor water retention and cellular dehydration. Fe₃O₄ NPs were applied to soybean leaves, lowering their water saturation in both saline and control conditions (Figure 4). Compared to the impacts of the Fe₃O₄ NPs at 0 ppm, 100 ppm, 200 ppm, 300 ppm, 400 ppm and 500 ppm, the 400 ppm had more pronounced effects. A water saturation deficit of 25% (0 ppm), 22.87% (100 ppm), 22.23% (200 ppm), 22.77% (300 ppm), 21.47% (400 ppm) and 18.93% (500 ppm) was observed under control condition. Among them 400 ppm (21.47%) dose perform best. When the research soybean leaves were subjected to salt stress, the WSD% increased and that is about 28.90%. The application of Fe₃O₄ nanoparticles significantly reduced WSD under salinity in a concentration-dependent manner, particularly up to 400 ppm.

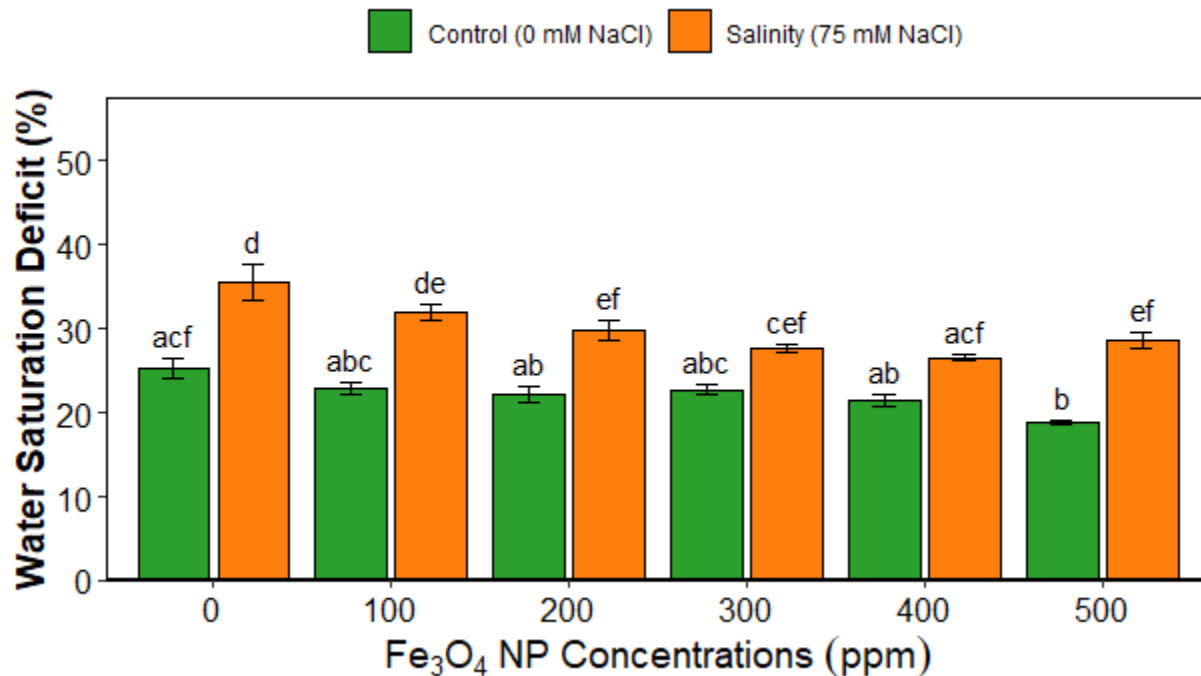


Figure 4. Effect of Fe₃O₄ NPs on water saturation deficit (WSD) of soybean leaves under saline condition. Values are mean $\pm$ SE (n = 3). Different letters indicate significant differences (LSD, $P \leq 0.05$).

After the application of Fe₃O₄ NPs under saline condition water saturation deficits were 31% (100 ppm), 29% (200 ppm), 27% (300 ppm), 26% (400 ppm) and 28% (500 ppm), respectively. Water saturation deficit found higher under saline condition compared to control condition without application of Fe₃O₄ NPs. At the dose of 400 ppm Fe₃O₄ NPs under saline condition, the water saturation deficit is decreased around 25.56%.

3.2.3 Effects of Fe₃O₄ NPs on photosynthetic rate, stomatal conductance, and transpiration rate of soybean leaf under salinity stress

The effects of Fe₃O₄ NPs as a foliar spray on the photosynthetic rate of soybean leaves under control (0 mM NaCl) and salinity (75 mM NaCl) stress are summarized in Table 3. Under control conditions (no salinity), the photosynthetic rate ranged from 49.0 to 50.24 $\mu\text{mol CO}_2 / \text{m}^2 / \text{s}$, showing minor variation among treatments. The highest rate was recorded at 500 ppm Fe₃O₄ NPs (50.24 $\mu\text{mol CO}_2 / \text{m}^2 / \text{s}$). In contrast, salinity stress significantly reduced the photosynthetic rate in untreated plants (41.09 $\mu\text{mol CO}_2 / \text{m}^2 / \text{s}$) compared to the control treatment (49.04 $\mu\text{mol CO}_2 / \text{m}^2 / \text{s}$). The photosynthetic rate was lowered to 16.22% by salinity stress when compared to the control condition. Photosynthetic rates were raised when soybeans were treated with Fe₃O₄ NPs in combination with salt treatment.

Stomatal conductance (gs) values under non-saline control conditions varied widely across treatments, ranging from 0.01 to 0.08 $\text{mol H}_2\text{O} / \text{m}^2 / \text{s}$ (Table 3). The untreated control showed moderate stomatal conductance without treatment (0.01 $\text{mol H}_2\text{O} / \text{m}^2 / \text{s}$). Under salinity stress, stomatal conductance in the untreated control decreased slightly to 0.01 $\text{mol H}_2\text{O} / \text{m}^2 / \text{s}$. The application of Fe₃O₄ nanoparticles altered stomatal conductance in a dose-dependent manner under salinity, increasing significantly from 0.01 $\text{mol H}_2\text{O} / \text{m}^2 / \text{s}$ at 100 ppm to a maximum of 0.08 $\text{mol H}_2\text{O} / \text{m}^2 / \text{s}$ at 300 ppm, indicating enhanced stomatal opening compared to the saline control. However, higher concentrations (400 and 500 ppm) resulted in reduced stomatal conductance of 0.03 and 0.02 $\text{mol H}_2\text{O} / \text{m}^2 / \text{s}$, respectively.

Table 3. Effect of Fe₃O₄ NPs on photosynthetic rate, stomatal conductance, and transpiration rate of soybean leaf under salinity stress.

Fe ₃ O ₄ NP Concentrati ons (ppm)	Photosynthesis rate (μmol CO ₂ /m ² /s)		Stomatal conductance (mol H ₂ O / m ² /s)		Transpiration rate (mol H ₂ O/ m ² /s)	
	Control	Salinity	Control	Salinity	Control	Salinity
0	49.05±1.03abc	41.09±0.8e	0.01±0.0 b	0.01 ± 0.0 b	0.44 ± 0.31b	0.40±0.05b
100	49.58±0.78ab	43.14±0.30e	0.01±0.0ab	0.01±0.0 b	0.89±0.47ab	0.47±0.14b
200	48.35±0.61abcd	44.31±0.44de	0.03±0.01ab	0.01±0.0b	1.44±0.46a	0.69±0.07ab
300	48.07±0.37abcd	48.59±0.53abcd	0.08±0.07a	0.01±0b	0.67±0.38ab	0.63±0.23ab
400	49.02±2.80abc	45.39±0.40bcde	0.01±0 b	0.03±0.01ab	0.81±0.30ab	0.80±0.05ab
500	50.24 ± 4.06a	44.86±0.41cde	0.01±0.01b	0.02±0.01ab	0.75±0.38ab	0.71±0.02ab
CV (%)	5.6		19.34		6.61	
Source of variation						
Salinity (S)	***		*		*	
Fe ₃ O ₄ NP (F)	*		*		NS	
S × F	*		NS		NS	

Values are presented as mean $\pm$ standard error (SE), $n = 3$. Means followed by different lowercase letters within a column differ significantly according to LSD test at $P \leq 0.05$. Data were analyzed using two-factor factorial ANOVA considering salinity (S) and Fe₃O₄ nanoparticle concentration (F) as fixed factors. CV = coefficient of variation. NS = non-significant. *, **, and *** indicate significance at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively.

Transpiration rate reflects the plant's ability to regulate water loss and maintain nutrient transport, both of which are critical under salinity stress. Under non-saline control conditions, transpiration rates varied among treatments from 0.44 to 1.44 mol H₂O /m² /s, with the highest rate observed at 200 ppm Fe₃O₄ NPs (Table 3). Salinity stress slightly reduced transpiration in untreated plants from 0.44 to 0.40 mol H₂O /m² /s. However, foliar application of Fe₃O₄ nanoparticles generally increased transpiration rates under salinity. Transpiration rates increased progressively from 0.47 at 100 ppm to 0.80 mol H₂O /m² /s at 400 ppm, with a slight decline at 500 ppm (0.71 mol H₂O /m² /s).

3.2.4 Effects of Fe₃O₄ NPs foliar spray on chlorophylls in soybean leaf under salinity stress

Table 4 shows the chlorophyll a content in soybean leaves under control and saline conditions with varying concentrations of Fe₃O₄ nanoparticles. Under non-saline (control) conditions, chlorophyll a content remained relatively stable across treatments, ranging from 2.01 mg/g at 0 ppm to a maximum of 2.60 mg/g at 400 ppm Fe₃O₄ NPs. Under salinity stress, chlorophyll a content was significantly reduced in untreated plants (1.04 mg/g). The marked reduction (around 48.25%) in chlorophyll a at 0 ppm Fe₃O₄ NPs under salinity highlights the sensitivity of the photosynthetic apparatus to salt-induced oxidative stress. Under saline conditions, chlorophyll content with Fe₃O₄ NPs treatment was found to be 1.25 mg/g (100 ppm), 1.45 mg/g (200 ppm), 1.55 mg/g (300 ppm), 1.63 mg/g (400 ppm), and 1.50 mg/g (500 ppm). The application of Fe₃O₄ nanoparticles substantially alleviated this effect, with the highest chlorophyll a content observed at 400 ppm Fe₃O₄ NPs (1.63 mg/g).

Table 4. Effects of Fe₃O₄ NPs foliar spray on chlorophyll a, chlorophyll b, and total chlorophyll in soybean leaves under salinity conditions.

Fe ₃ O ₄ NP Concentration (ppm)	Chlorophyll a (mg/g fresh wt. of leaf)		Chlorophyll b (mg/g fresh wt. of leaf)		Total chlorophyll (mg/g fresh wt. of leaf)	
	Control	Salinity	Control	Salinity	Control	Salinity
0	2.01 $\pm$ 0.01d	1.04 $\pm$ 0.04h	0.81 $\pm$ 0.06bc	0.51 $\pm$ 0.05g	2.82 $\pm$ 0.06c	1.55 $\pm$ 0.07h
100	2.02 $\pm$ 0.07d	1.25 $\pm$ 0.04g	0.78 $\pm$ 0.00cd	0.52 $\pm$ 0.00g	2.79 $\pm$ 0.07c	1.76 $\pm$ 0.04g
200	2.34 $\pm$ 0.04b	1.45 $\pm$ 0.04f	0.87 $\pm$ 0.01ab	0.58 $\pm$ 0.01fg	3.21 $\pm$ 0.05b	2.04 $\pm$ 0.03f
300	2.24 $\pm$ 0.04bc	1.55 $\pm$ 0.03ef	0.84 $\pm$ 0.00abc	0.64 $\pm$ 0.02ef	3.08 $\pm$ 0.04b	2.20 $\pm$ 0.05e

400	2.60±0.10a	1.63±0.04e	0.90 ± 0.04a	0.72±0.02de	3.50±0.07a	2.35±0.02d
500	2.15±0.05cd	1.50±0.03ef	0.76±0.00cd	0.61 ± 0.03f	2.91±0.04c	2.11±0.03ef
CV (%)	4.6		7.2		3.4	
Source of variation						
Salinity (S)	***		***		***	
Fe ₃ O ₄ (F)	**		*		**	
S × F	*		*		*	

Values are presented as mean ± standard error (SE), $n = 3$. Means followed by different lowercase letters within a column differ significantly according to LSD test at $P \leq 0.05$. Data were analyzed using two-factor factorial ANOVA considering salinity (S) and Fe₃O₄ nanoparticle concentration (F) as fixed factors. CV = coefficient of variation. *, **, and *** indicate significance at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively.

Table 4 presents the variation in chlorophyll b content in soybean leaves under control and salinity stress conditions as influenced by foliar application of Fe₃O₄ nanoparticles (NPs). Under non-saline (control) conditions, chlorophyll b content found 0.81 mg/g (0 ppm), 0.78 mg/g (100 ppm), 0.87 mg/g (200 ppm), 0.84 mg/g (300 ppm), 0.90 mg/g (400 ppm), 0.76 mg/g (500 ppm) respectively. The application of Fe₃O₄ NPs slightly increased chlorophyll b content compared to the untreated control (0.81 mg/g), with the highest value recorded at 400 ppm. Under salinity stress, a significant reduction in chlorophyll b content was observed in the untreated plants (0.51 mg/g), showing a clear negative impact of salinity on chlorophyll biosynthesis. The application of Fe₃O₄ nanoparticles progressively improved chlorophyll b content under salinity conditions. However, increasing concentrations of Fe₃O₄ NPs gradually alleviated the reduction. Under salinity conditions, with Fe₃O₄ NPs treatment, the following results were found: 0.52 mg/g (100 ppm), 0.58 mg/g (200 ppm), 0.64 mg/g (300 ppm), 0.72 mg/g (400 ppm), and 0.61 mg/g (500 ppm), respectively. The chlorophyll b content increased to 0.72 mg/g at 400 ppm under salinity, marking the peak value, followed by a slight decline at 500 ppm (0.61 mg/g).

Table 4 presents the variation in total chlorophyll content in soybean leaves under control and salinity stress conditions as influenced by foliar application of Fe₃O₄ nanoparticles (NPs). Under control conditions, total chlorophyll content ranges from 2.82 mg/g (0 ppm Fe₃O₄ NPs) to 3.50mg/g (400 ppm Fe₃O₄ NPs). Under saline conditions, total chlorophyll content is reduced by 1.55 mg/g without Fe₃O₄ NPs treatment and the reduction is about 45.03%. However, the foliar application of Fe₃O₄ NPs under salinity stress resulted in a concentration-dependent improvement in total chlorophyll content, reaching a maximum of 400 ppm. At 100 ppm, total chlorophyll increased to 1.76 mg/g, and further enhancement was observed at 200 ppm (2.04 mg/g) and 300 ppm (2.20

mg/g). The highest value was recorded at 400 ppm (2.35 mg/g), representing a 51% increase over the saline control (0 ppm). However, at 500 ppm, the chlorophyll level declined slightly to 2.11 mg/g, suggesting potential phytotoxicity at higher nanoparticle concentrations.

3. 2. 5 Effects of Fe₃O₄ NPs foliar spray on membrane stability index, malondialdehyde, and total sugar in soybean under salinity stress

The data presented in Table 5 reveal the effects of foliar application of Fe₃O₄ nanoparticles (NPs) on the membrane stability index (MSI) of soybean leaves under both control and salinity stress conditions. Under control conditions without Fe₃O₄ NPs treatment, the membrane stability index was found to be 80.92% (0 ppm). After application of Fe₃O₄ NPs MSI% found respectively 81.84% (100 ppm), 82.32% (200 ppm), 82.74% (300 ppm), 83.42% (400 ppm), 81.80% (500 ppm). The highest MSI (83.42%) was observed at 400 ppm Fe₃O₄ NPs, followed closely by 300 ppm (82.74%), indicating a slight enhancement in membrane stability even under non-stress conditions. Under salinity stress, a significant decline in MSI was observed in untreated plants, 57.97% (0 ppm), highlighting the detrimental effect of salinity on membrane integrity and a reduction found 28.36%. However, Fe₃O₄ NPs application mitigated this decline in a dose-dependent manner. The maximum MSI (73.99%) under salinity was recorded at 400 ppm, representing a 27.74% improvement over the untreated salinity-stressed control. Treatments with 300 ppm (71.82%) and 200 ppm (68.52%) also showed marked improvements. Interestingly, the MSI began to decline at 500 ppm (69.17%), suggesting a possible threshold beyond which no additional benefits or even negative effects may occur.

Malondialdehyde (MDA) is a key indicator of lipid peroxidation and oxidative stress in plant cells. The data presented in Table 5 illustrate the changes in malondialdehyde (MDA) levels in soybean leaves under both normal and saline conditions in response to varying concentrations of Fe₃O₄ nanoparticles (NPs). Under control (non-saline) conditions, MDA content ranged from 6.25 to 7.67 nmol/g fresh weight, with a slight increase observed at higher concentrations of Fe₃O₄ NPs.

Table 5. Effects of Fe₃O₄ NPs on membrane stability index, malondialdehyde (MDA), and total sugar in soybean leaf under salinity stress.

Fe ₃ O ₄ NP Concentrations (ppm)	Membrane stability index (%)		MDA (nano mole/g fresh wt. of leaf)		Total sugar (mg/g fresh weight of Leaf)	
	Control	Salinity	Control	Salinity	Control	Salinity

0	80.92±0.89a	57.97±0.05f	6.25±0.04h	12.76±0.0a	1.22 ± 0.02g	2.31±0.0d
100	81.84±0.60a	65.36±0.95e	6.94±0.56fg	11.35±0.04b	1.38±0.02f	2.38±0.02cd
200	82.32±0.34a	68.53±0.50d	6.45±0.05gh	9.46±0.05c	1.48±0.05ef	2.49±0.03abc
300	82.74±0.05a	71.83±1.7bc	6.74±0.04gh	8.62±0.30d	1.52±0.07e	2.53±0.03ab
400	83.42±0.06a	73.99±1.55b	7.67±0.04e	7.66±0.05e	1.55±0.06e	2.55±0.04a
500	81.80±0.10a	69.17±1.7cd	7.45±0.05ef	9.27±0.15c	1.26±0.04g	2.43±0.04bc
CV (%)	2.2		4.0		3.6	
Source of variation						
Salinity (S)	***		***		***	
Fe ₃ O ₄ NP (F)	**		**		**	
S × F	*		*		*	

Values are presented as mean ± standard error (SE), n = 3. Means followed by different lowercase letters within a column differ significantly according to LSD test at $P \leq 0.05$. Data were analyzed using two-factor factorial ANOVA considering salinity (S) and Fe₃O₄ nanoparticle concentration (F) as fixed factors. CV = coefficient of variation. *, **, and *** indicate significance at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively.

The highest MDA level under control was 7.67 nmol/g at 400 ppm, indicating that Fe₃O₄ NPs alone, without salinity, did not drastically affect lipid peroxidation. Under salinity stress, the MDA content in untreated plants (0 ppm) significantly increased to 12.76 nmol/g, indicating severe oxidative damage and membrane lipid peroxidation due to salt stress. After application of Fe₃O₄ NPs MDA content found 11.35 nmol/g (100 ppm), 9.46 nmol/g (200 ppm), 8.62 nmol/g (300 ppm), 7.66 nmol/g (400 ppm), 9.27 nmol/g (500 ppm) respectively. However, foliar application of Fe₃O₄ NPs markedly reduced MDA levels in a dose-dependent manner up to 400 ppm, with the lowest MDA concentration (7.66 nmol/g) observed at 400 ppm Fe₃O₄ NPs representing a ~40% reduction compared to the salinity-stressed control.

As shown in Table 5, total sugar content in soybean leaves varied across treatments under both control and salinity conditions in response to foliar application of Fe₃O₄ nanoparticles (NPs). Under control condition without treatment of Fe₃O₄ NPs total sugar content found 1.22 mg/g (0 ppm). When applying Fe₃O₄ NPs as foliar spray total sugar content found 1.32 mg/g (100 ppm), 1.48 mg/g (200 ppm), 1.52 mg/g (300 ppm), 1.55 mg/g (400 ppm), 1.26 mg/g (500 ppm) respectively. The highest value found in 400 ppm and at 500 ppm, a slight decline to was observed, indicating a potential inhibitory effect at higher concentrations. Under salinity stress, total sugar content was notably higher in all treatments compared to the respective control groups. The untreated salinity-stressed plants recorded 2.31 mg/g (0 ppm). Foliar application of Fe₃O₄ nanoparticles further enhanced total sugar accumulation under salinity. After foliar application of Fe₃O₄ NPs total sugar

content under salinity stress found respectively 2.38 mg/g (100 ppm), 2.49 mg/g (200 ppm), 2.53 mg/g (300 ppm), 2.55 mg/g (400 ppm), 2.43 mg/g (500 ppm). The optimal enhancement was observed at 400 ppm, which likely facilitated better photosynthetic performance and metabolic activity.

3.2.6 Effects of Fe₃O₄ NPs foliar spray on proline content in soybean leaf under salinity stress

Figure 5 shows the influence of different concentrations of Fe₃O₄ nanoparticles on proline accumulation in soybean leaves under both normal and salinity-stressed conditions. Under control condition proline content found 1.11 mg/g without treatment of Fe₃O₄ NPs (0 ppm). Applying Fe₃O₄ NPs under control condition proline content ranges from 1.38 to 1.61 mg/g, under the treatment from 100 ppm to 500 ppm Fe₃O₄ NPs. Under control conditions, proline content gradually increased from 0 ppm to a peak at 300–400 ppm, suggesting that Fe₃O₄ NPs may slightly stimulate proline synthesis even without stress. In contrast, salinity stress significantly increased proline content in the untreated control condition found 3.2 mgg⁻¹, indicating a strong stress response, increased proline content was 34.47%. In contrast, salinity stress significantly increased proline content in the untreated control (≈3.2 mg/g FW), indicating a strong stress response.

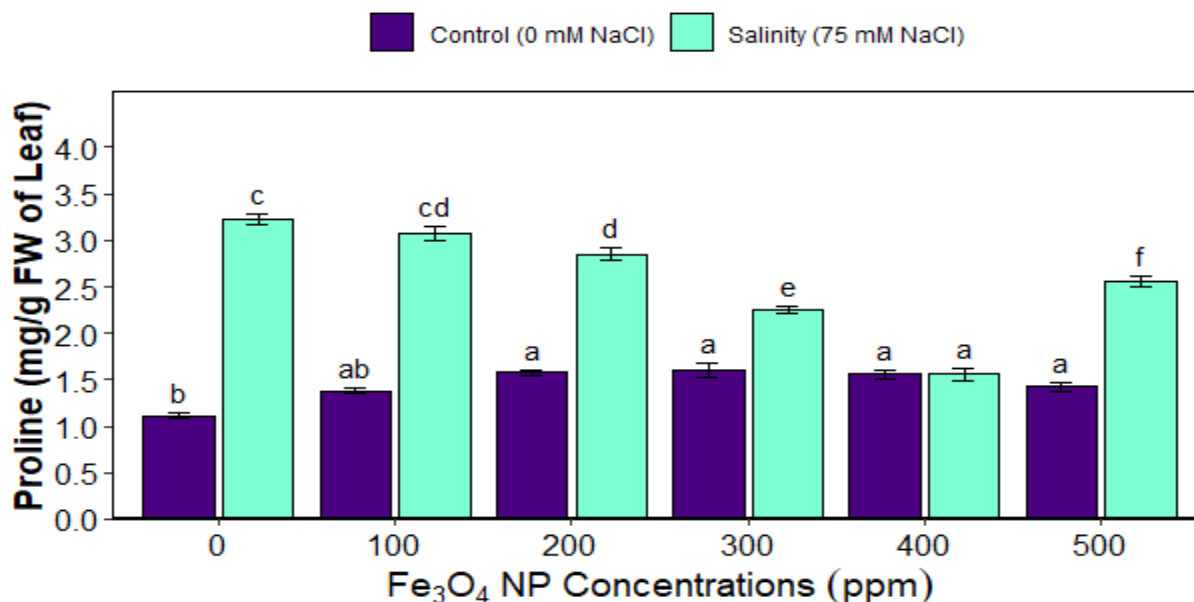


Figure 5. Effect of Fe₃O₄ NPs on proline content of soybean leaf under salinity stress. Values are mean ± SE (n = 3). Different letters indicate significant differences (LSD, P ≤ 0.05).

However, with increasing Fe₃O₄ NPs concentrations, proline content progressively decreased, reaching levels nearly equal to the non-stressed control at 400 ppm and that is around 1.56 mg/g.

Notably, 300 (2.26 mg/g) and 400 (1.56 mg/g) ppm Fe₃O₄ resulted in the greatest reduction in proline accumulation, suggesting improved stress tolerance. A slight increase in proline was again observed at 500 ppm (2.56 mg/g), indicating possible stress induction at high nanoparticle levels.

3. 2. 7 Effects of Fe₃O₄ NPs foliar spray on hydrogen peroxide (H₂O₂) content in soybean leaf under salinity stress

Figure 6 demonstrates the effect of Fe₃O₄ nanoparticles (NPs) on hydrogen peroxide (H₂O₂) content in soybean leaves under control and salinity stress conditions. Under control conditions without Fe₃O₄ NPs treatment, the Hydrogen peroxide content was found to be 2.44 µmol/g. After foliar application of Fe₃O₄ NPs, the hydrogen peroxide content was found to be 2.01 µmol/g (100 ppm), 2.25 µmol/g (200 ppm), 2.48 µmol/g (300 ppm), 2.14 µmol/g (400 ppm), and 2.35 µmol/g (500 ppm). Fe₃O₄ NPs application progressively reduced H₂O₂ accumulation, with the lowest value (2.34

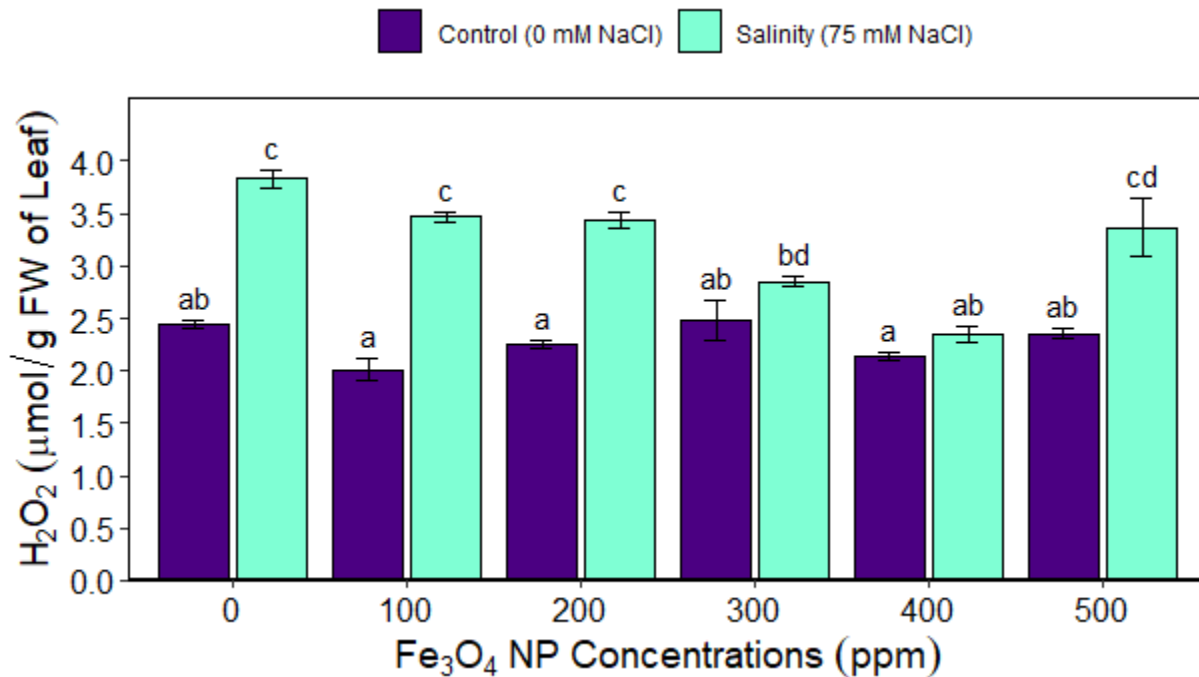


Figure 6. Effect of Fe₃O₄ NPs on hydrogen peroxide (H₂O₂) content of soybean leaf under salinity stress. Values are mean ± SE (n = 3). Different letters indicate significant differences (LSD, P ≤ 0.05).

µmol/g FW) recorded at 400 ppm. Under salinity stress without Fe₃O₄ NPs (0 ppm), H₂O₂ content markedly increased to 3.83 µmol/g FW, compared to the control condition, where H₂O₂ content increased by 36.29%.

After application of Fe₃O₄ NPs, H₂O₂ content was found to be 3.47 µmol/g (100 ppm), 3.44 µmol/g (200 ppm), 2.85 µmol/g (300 ppm), 2.35 µmol/g (400 ppm), and 3.37 µmol/g (500 ppm),

respectively. Application of Fe₃O₄ NPs under salinity conditions progressively reduced H₂O₂ accumulation, with the lowest value (2.35 µmol/g FW) recorded at 400 ppm, closely resembling the control at the same concentration (2.14 µmol/g FW). However, at 500 ppm Fe₃O₄ NPs, H₂O₂ content under salinity slightly rose to 3.37 µmol/g FW. In control plants, Fe₃O₄ NPs caused only minor variations in H₂O₂ levels.

3. 2. 8 Effects of Fe₃O₄ NPs foliar spray on carotenoids, flavonoids and antioxidants contents under salinity stress

Table 6 presents the effect of Fe₃O₄ nanoparticles (NPs) on carotenoid content in soybean leaves under control and salinity stress conditions. In the absence of Fe₃O₄ NPs, carotenoids decreased from 5.38 mg/g FW (control) to 3.46 mg/g FW (salinity). Foliar application of Fe₃O₄ NPs notably improved carotenoid content under both control and saline conditions. Under control conditions, the highest carotenoid content (6.89 mg/g FW) was observed at 200 ppm Fe₃O₄ NPs, followed by 300 ppm (6.08 mg/g FW) and 400 ppm (6.02 mg/g FW). Similarly, under salinity stress, the maximum carotenoid level 4.84 mg/g FW was recorded at 400 ppm Fe₃O₄ NPs, which was 40.29% higher than the salinity control. Beyond the optimal concentration, at 500 ppm, carotenoid content slightly declined under both control and stress conditions

Flavonoid content in soybean leaves decreased significantly under salinity stress compared to the control in the absence of Fe₃O₄ NPs (Table 6). Under control conditions without treatment of Fe₃O₄ NPs, the flavonoid content was found to be 26.94 mg quercetin/g dry wt of leaf. After foliar application of Fe₃O₄ NPs, flavonoid content was found to be 28.18 mg quercetin/g dry wt of leaf (100 ppm), 29.32 mg quercetin/g dry wt of leaf (200 ppm), 30.68 mg quercetin/g dry wt of leaf (300 ppm), 29.48 mg quercetin/g dry wt of leaf (400 ppm), 27.68 mg quercetin/g dry wt of leaf (500 ppm), respectively. Nanoparticles increased flavonoid content at the dose of 300 ppm. Under salinity conditions without treatment of Fe₃O₄ NPs, the flavonoid content was found to be 22.93 mg quercetin/g dry wt of leaf, which is reduced by 14.88%. Under saline conditions after application of Fe₃O₄ NPs, flavonoid content was found to be 25.38 mg quercetin/g dry wt of leaf (100 ppm), 26.74 mg quercetin/g dry wt of leaf (200 ppm), 28.33 mg quercetin/g dry wt of leaf (300 ppm), 29.27 mg quercetin/g dry wt of leaf (400 ppm), 27.67 mg quercetin/g dry wt of leaf (500 ppm), respectively.

Table 6. Effects of Fe₃O₄ NPs on carotenoids, flavonoids, and antioxidants in soybean leaf under salinity condition.

Fe ₃ O ₄ NP Concentrations (ppm)	Carotenoids (mg/fresh wt. of leaf)		Flavonoids (mg quercetin /g dry wt. of leaf)		DPPH radical scavenging activity (IC ₅₀ , mg / mL)	
	Control	Salinity	Control	Salinity	Control	Salinity
0	5.38±0.02c	3.46±0.04g	26.95±0.81de	22.93±0.24g	240.16±1.26a	152.69±1.43f
100	5.45±0.03c	4.14±0.04f	28.18±0.26c	25.38±0.20f	241.47±1.22a	157.53±2.28e
200	6.89±0.05a	4.46±0.04def	29.32±0.20b	26.74±0.16e	242.87±0.67a	162.08±0.71d
300	6.08±0.02b	4.65±0.05de	30.68±0.12a	28.33±0.40c	243.03±1.04a	167.19±1.09c
400	6.02±0.54b	4.84±0.09d	29.84±0.06ab	29.27±0.14b	243.12±1.57a	173.48±1.63b
500	5.74±0.03bc	4.25±0.04ef	27.68±0.04cd	27.68±0.06cd	240.50±0.38a	159.15±1.25de
CV (%)	5.4		1.9		1.1	
Source of variation						
Salinity (S)	***		***		***	
Fe ₃ O ₄ NP (F)	**		**		**	
S × F	*		*		*	

Values are presented as mean ± standard error (SE), *n* = 3. Means followed by different lowercase letters within a column differ significantly according to LSD test at *P* ≤ 0.05. Data were analyzed using two-factor factorial ANOVA considering salinity (S) and Fe₃O₄ nanoparticle concentration (F) as fixed factors. CV = coefficient of variation. Antioxidant activity is expressed as IC₅₀ (mg mL⁻¹), where lower values indicate higher antioxidant capacity. *, **, and *** indicate significance at *P* ≤ 0.05, *P* ≤ 0.01, and *P* ≤ 0.001, respectively.

Under saline conditions, the 400 ppm Fe₃O₄ NPs treatment showed the highest flavonoid content, representing a 27.7% increase compared to the salinity control. Beyond optimal concentrations, such as at 500 ppm, flavonoid levels slightly declined in both conditions.

Antioxidant activity, measured as IC₅₀ (mg/ml), was markedly reduced under salinity stress compared to control conditions (Table 6). Lower IC₅₀ values indicate stronger antioxidant capacity. In the absence of Fe₃O₄ NPs, IC₅₀ decreased from 240.16 mg/ml (control) to 152.69 mg/ml (salinity), suggesting enhanced DPPH radical scavenging activity under salinity stress, likely due to stress-induced accumulation of antioxidant compounds. Foliar application of Fe₃O₄ NPs under control conditions resulted in only minor changes in IC₅₀ values (240.16–243.12 mg/ml), indicating relatively stable antioxidant status under non-saline conditions. Under salinity stress, Fe₃O₄ NP application increased IC₅₀ values compared with the untreated saline control, indicating comparatively lower DPPH radical scavenging activity in NP-treated plants. The untreated saline plants exhibited the strongest antioxidant activity, which may reflect elevated oxidative stress under salinity conditions. However, at 500 ppm Fe₃O₄ NP application, IC₅₀ declined again to 159.15 mg/ml, suggesting reduced effectiveness at excessive nanoparticle concentrations.

3. 3 Effects of Fe₃O₄ NPs foliar spray on yield and yield contributing parameters under salinity stress

The number of pods/ soybean plant was significantly reduced under salinity stress in the untreated control group, decreasing from 40.67 (control condition) to 28.67 per plant (Table 7). Foliar application of Fe₃O₄ nanoparticles alleviated this negative effect of salinity, with the number of pods/ plant increasing significantly in treated plants compared to the salinity control. Under salinity stress, the number of pods increased progressively with increasing Fe₃O₄ NP concentration, reaching the highest values at 200 ppm (35.33) and 400 ppm (35.67) treatments. At 500 ppm, the number of pods slightly decreased but remained significantly higher than the untreated saline control (34.33). Under non-saline conditions, pod numbers remained relatively stable across treatments, with a slight increase at 400 ppm Fe₃O₄ NPs (45.33).

Table 7. Effects of Fe₃O₄ NPs on the number of pods/plants, number of seeds/pods, 100-seed weight, and seed yield of soybean under salinity stress.

Fe ₃ O ₄ NP Concentrati ons	Number of pods / plants		Number of seeds / pods		100-seeds weight (g)	
	Control	Salinity	Control	Salinity	Control	Salinity
0 ppm	40.67±2.33ab	28.67±3.76d	1.84±0.04ab	1.40±0.18b	17.57±0.29ab	11.77±1.58d
100 ppm	39.00±2.31abc	33.67±1.20cd	1.78±0.06ab	1.68±0.01ab	18.33±0.72a	13.87±1.25cd
200 ppm	40.67±2.96ab	35.33±1.20bcd	1.76±0.04ab	1.77±0.14ab	16.77±0.66ab	15.37±0.72bc
300 ppm	42.67±3.18a	32.67±2.91cd	1.79±0.05ab	1.83±0.06ab	17.67±0.52ab	15.57±0.98bc
400 ppm	45.33±1.45a	35.67±0.88bc	1.69±0.19ab	1.84±0.03ab	18.80±0.36a	16.70±1.04ab
500 ppm	40.67±2.03ab	34.33±1.45bcd	1.91±0.47a	1.89±0.11a	17.40±0.78ab	16.37±0.52abc
CV (%)	10.7		16.5		9.2	
Source of variation						
Salinity (S)	***		***		***	
Fe ₃ O ₄ NP (F)	**		*		**	
S × F	*		*		*	

Values are presented as mean ± standard error (SE), *n* = 3. Means followed by different lowercase letters within a column differ significantly according to LSD test at *P* ≤ 0.05. Data were analyzed using two-factor factorial ANOVA considering salinity (S) and Fe₃O₄ nanoparticle concentration (F) as fixed factors. CV = coefficient of variation. *, **, and *** indicate significance at *P* ≤ 0.05, *P* ≤ 0.01, and *P* ≤ 0.001, respectively.

The number of seeds/ pod measured in grams was markedly reduced under salinity stress compared to control conditions (Table 7). Under control conditions without treatment of Fe₃O₄ NPs, the number of seeds / pod was found to be 1.84. After foliar application of Fe₃O₄ NPs number of seeds /pods found 1.78 (100 ppm), 1.76 (200 ppm), 1.79 (300 ppm), 1.69 (400 ppm), 1.91 (500 ppm)

respectively. Salinity stress significantly reduced the number of seeds / pod in untreated soybean plants, dropping from 1.84 seeds/pod under control conditions to 1.40 seeds/pod under salinity. Application of Fe₃O₄ nanoparticles enhanced the number of seeds / pod under saline conditions in a concentration-dependent manner. The number of seeds / pod increased steadily from 1.68 at 100 ppm to 1.89 at 500 ppm Fe₃O₄ NPs, surpassing the saline control and approaching or exceeding values observed in non-saline controls.

Salinity stress significantly reduced the 100-seed weight of soybean from 17.57 g in the control to 11.77 g in untreated saline plants (Table 7). Foliar application of Fe₃O₄ nanoparticles mitigated this reduction, with seed weight increasing progressively with NP concentration under salinity stress. The highest 100-seed weight was observed at 400 ppm Fe₃O₄ NPs (16.70 g), followed closely by the 500ppm treatment (16.37 g). Lower concentrations (100 and 200 ppm) also improved seed weight compared to the saline control, but to a lesser extent. Under non-saline conditions, 100-seed weight remained relatively stable across treatments, with a slight increase at 400 ppm (18.80 g).

Salinity stress significantly reduced the seed yield / plant from 13.13 g in the non-stressed control to 5.21 g in the untreated saline plants (Figure 7). Application of Fe₃O₄ nanoparticles significantly ameliorated this reduction. Under salinity stress, seed yield increased with Fe₃O₄ NP concentration, reaching a maximum of 10.95 g/plant at 400 ppm, followed closely by the 500 ppm treatment (10.64 g/plant). Lower concentrations (100, 200, and 300 ppm) also improved seed yield significantly compared to the saline control. Under non-saline conditions, seed yield remained relatively stable, with the highest yield observed at 400 ppm Fe₃O₄ NPs (14.32 g).

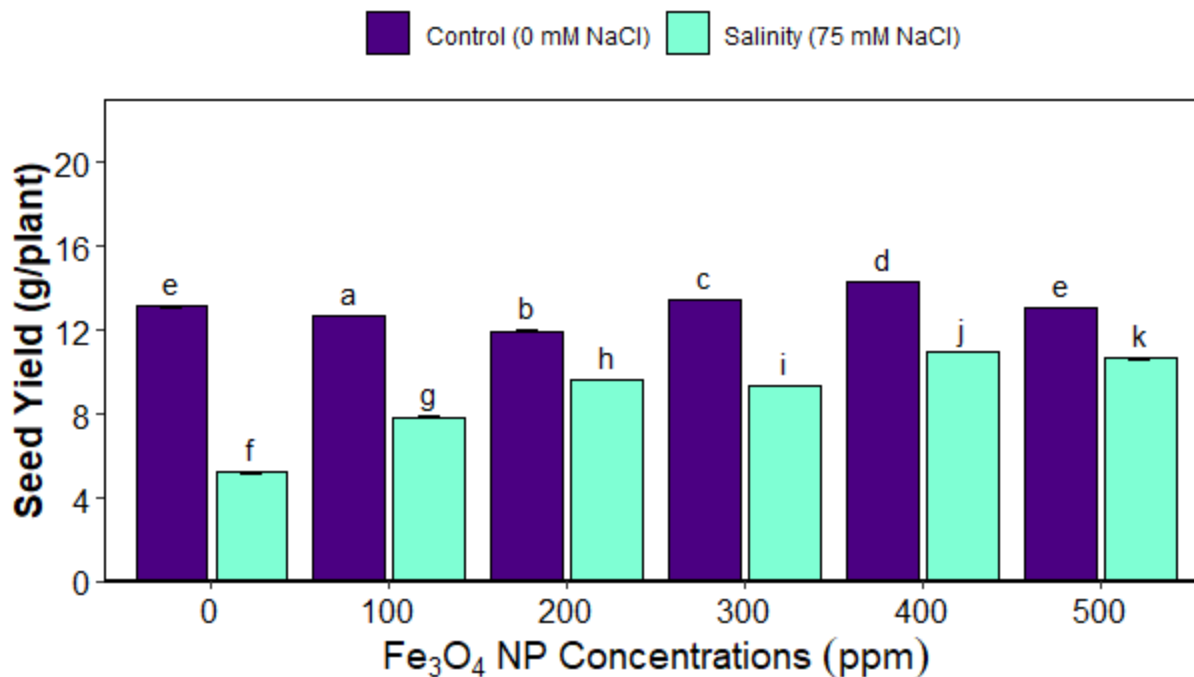


Figure 7. Effect of Fe₃O₄ NPs on the seed yield of soybean leaf under salinity stress. Values are mean $\pm$ SE (n = 3). Different letters indicate significant differences (LSD, $P \leq 0.05$).

Exploratory Pearson Correlation Analysis

Correlation analysis was performed as an exploratory statistical approach to visualize associations among measured traits and treatments and should not be interpreted as evidence of causal relationships. The heat map of Pearson correlation coefficients depicted the association among the 26 measured agronomical, physiological, biochemical, and yield traits. The blue and red squares are negative and positive correlations, respectively (Figure 8). Strong correlations were observed among Yield and Relative water content (0.93), Total chlorophyll (0.92), Phenol (0.73), Flavonoid (0.81), Antioxidant (0.86), Transpiration rate (0.43), Stomatal Conductance (0.30), Carotenoids (0.84), Photosynthetic rate (0.87) and Membrane Stability Index (0.96). But yield is negatively correlated with proline (-0.89), Total sugar (-0.74), MDA (-0.91), water saturation deficit (-0.93), and hydrogen peroxide (-0.87). However, these five traits are positively correlated with each other.

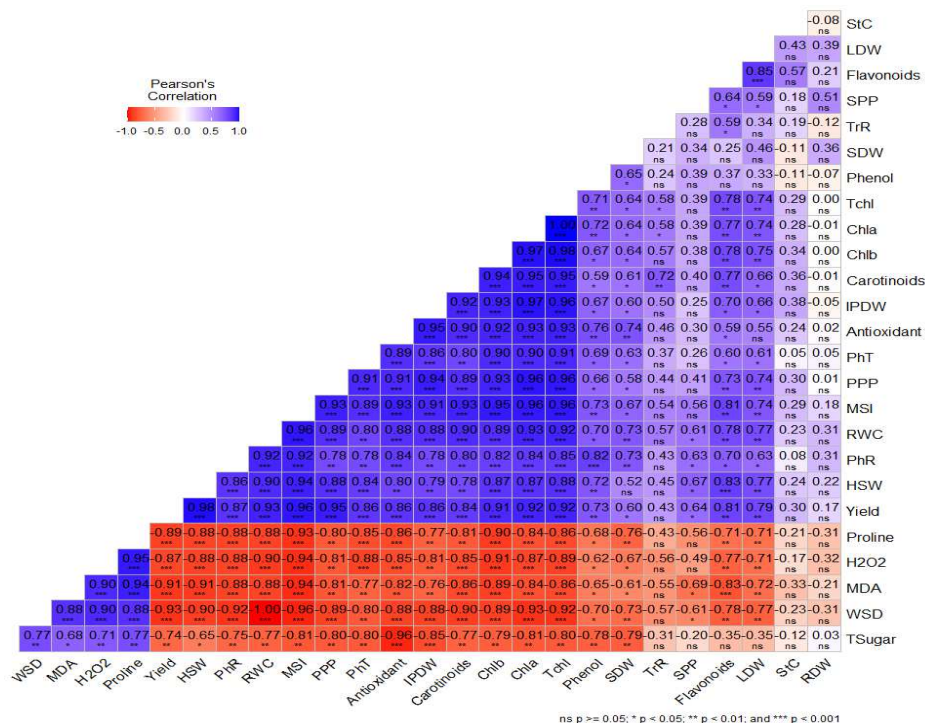


Figure 8. Pearson's correlation coefficients among various traits were assessed under both saline and control conditions in soybean. Correlated marked with ns = non- significant at $P > 0.05$; * = significant at $P \leq 0.05$; ** = highly significant at $P \leq 0.01$; *** = very highly significant at $P \leq 0.001$. The evaluated traits include Relative water content (RWC), Water saturation deficit (WSD), Membrane Stability Index (MSI), Malondialdehyde (MDA), Hydrogen peroxide (H_2O_2), Proline, Flavonoids, Total sugar (TSugar), Antioxidant, Chlorophyll a (Chla), Chlorophyll b (Chlb), Total chlorophyll (Tchl), Carotenoids, Yield, Pods per plant (PPP), Seeds per pod (SPP), 100 seed weight (HSW), Photosynthetic rate (PhR), Stomatal conductance (StC), Transpiration rate (TrR), Plant height (PhT), Leaf dry weight (LDW), Shoot dry weight (SDW), Root dry weight (RDW) and Immature pod dry weight (IPDW)

Exploratory Principal Component Analysis (PCA)

PCA was conducted as an exploratory multivariate approach to visualize treatment patterns and trait associations among the evaluated parameters. The interrelationship among the evaluated treatments across different varieties and traits was assessed using principal component analysis (PCA) (Figure 9). Here, PCA1 (Dim1) explains 71.9% of the total variance, while PCA2 (Dim2) explains 8.4%. Treatments are represented in red, while the blue vectors represent the original traits and their influence on the principal components. The direction and length of the vectors indicate both the contribution and orientation of traits to the observed variability.

The treatments included: 0 mM NaCl (Control), Control + 100 ppm Fe_3O_4 nanoparticles (C+100 ppm Fe_3O_4 NPs), Control + 200 ppm Fe_3O_4 nanoparticles (C+200 ppm Fe_3O_4 NPs), Control + 300 ppm Fe_3O_4 nanoparticles (C+300 ppm Fe_3O_4 NPs), Control + 400 ppm Fe_3O_4 nanoparticles (C+400

ppm Fe₃O₄ NPs), Control + 500 ppm Fe₃O₄ nanoparticles (C+500 ppm Fe₃O₄ NPs), 75 mM NaCl (Saline), Saline + 100 ppm Fe₃O₄ nanoparticles. (S+100 ppm Fe₃O₄ NPs), Saline + 200 ppm Fe₃O₄ nanoparticles (S+200 ppm Fe₃O₄ NPs), Saline + 300 ppm Fe₃O₄ nanoparticles (S+300 ppm Fe₃O₄ NPs), Saline + 400 ppm Fe₃O₄ nanoparticles (S+400 ppm Fe₃O₄ NPs), Saline + 500 ppm Fe₃O₄ nanoparticles (S+500 ppm Fe₃O₄ NPs),

A strong positive correlation was observed among yield-related and physiological traits, including yield and its components (SPP, PPP, HSW, LDW, RDW), photosynthetic rate (PhR), stomatal conductance (StC), transpiration rate (TrR), chlorophyll content (Chl a, Chl b, Tchl), carotenoids, antioxidants, membrane stability index (MSI), and relative water content (RWC).

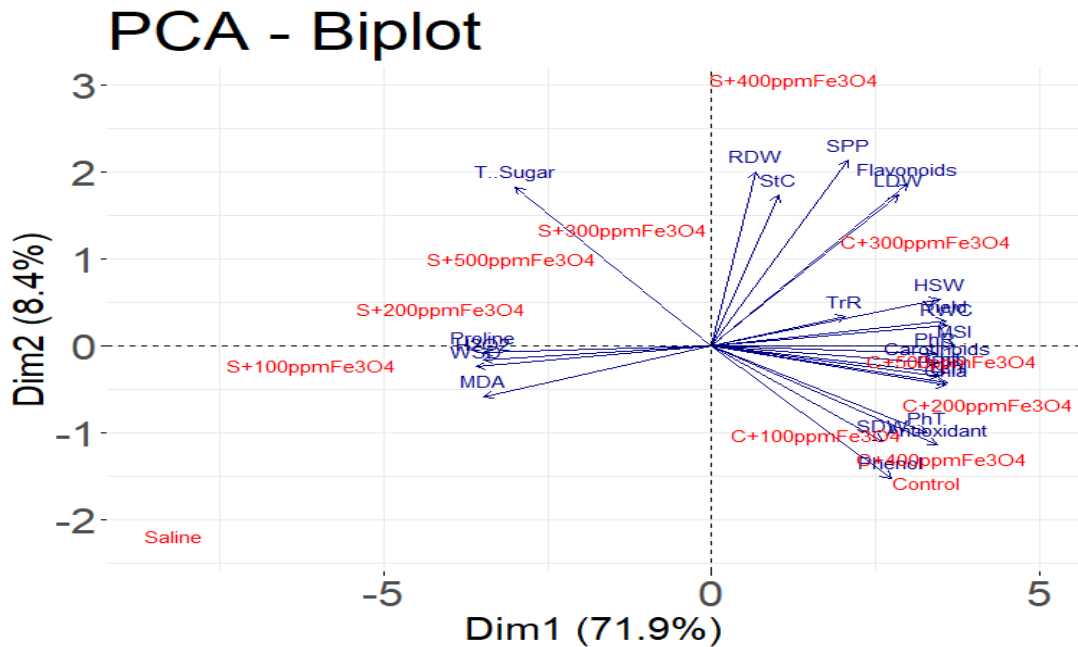


Figure 9. PCA biplot illustrating the effect of nanoparticle treatments on trait variation in soybean under control and saline conditions based on agronomical, physiological, biochemical and yield attributes

These traits clustered together on the positive side of Dim1, suggesting positive associations among physiological performance and yield-related traits. Conversely, stress-related traits, such as water saturation deficit (WSD), malondialdehyde (MDA), proline, and total sugar (T.Sugar), were negatively correlated with yield and physiological parameters. They appeared on the negative side of Dim1, and were strongly associated with saline treatments (Saline and S+100 ppm Fe₃O₄), suggesting elevated oxidative stress and reduced physiological efficiency.

Based on the PCA biplot, C+400 ppm Fe₃O₄ emerged as the most effective treatment under non-saline conditions. It is positioned far along the positive axis of Dim1, in close alignment with the cluster of yield-enhancing and physiological traits, indicating a strong and beneficial effect on plant productivity and stress resilience. Its distance from the origin further highlights its distinct positive contribution compared to other control-group treatments.

Under saline conditions, S+400 ppm Fe₃O₄ appeared to be the most promising treatment. Although not clustering as tightly with the yield-related traits as the non-saline treatments, it is located farther from the negative stress-related traits than the other saline treatments. This positioning indicates that the 400 ppm Fe₃O₄ application helps alleviate stress effects, resulting in improved physiological balance and growth compared to lower (100, 200 ppm) or higher (500 ppm) nanoparticle concentrations under salinity.

In summary, the exploratory PCA suggested that C+400 ppm Fe₃O₄ was associated with superior overall performance under normal conditions, showing positive relationships with yield and favorable physiological traits, whereas S+400 ppm Fe₃O₄ exhibited the strongest mitigation potential under saline conditions.

4. Discussion

Salinity stress markedly constrained soybean growth and biomass accumulation; however, foliar application of Fe₃O₄ nanoparticles (NPs) substantially mitigated these adverse effects. The improvement in leaf and stem biomass under salinity was associated with Fe₃O₄ NP application. However, iron uptake, antioxidant enzyme activity, and chloroplast stability were not directly measured in the present study, although previous studies suggest that these mechanisms may contribute to sustaining photosynthetic capacity under stress [44]. Fe₃O₄ NP application enhanced stem growth under saline conditions, consistent with earlier findings on nano-iron-mediated improvement of Fe nutrition and metabolic activity [45]. Moreover, improved root biomass was observed following Fe₃O₄ NP application under saline conditions [46,47]. Enhanced pod biomass further suggests improved assimilate partitioning, enzymatic activity, and hormonal regulation critical for reproductive development [48,49]. Among all treatments, 400 ppm Fe₃O₄ NPs consistently produced superior growth responses, indicating that moderate Fe₃O₄ NP concentrations were associated with comparatively improved growth responses under salinity stress [50].

Fe₃O₄ NP application significantly improved plant water relations, as evidenced by enhanced relative water content (RWC) and reduced water saturation deficit (WSD) up to 400 ppm under salinity stress. Improved osmotic adjustment, root hydraulic conductivity, and membrane integrity likely contributed to better water uptake and retention [51,52]. Iron's central role in enzymatic and metabolic processes may further support leaf hydration and cellular homeostasis under stress [53]. However, the slight increase in WSD at 500 ppm suggests a threshold beyond which nanoparticle aggregation or oxidative stress may impair water regulation, consistent with reports of nanoparticle-induced phytotoxicity at excessive doses [54].

Fe₃O₄ NP application was associated with improved photosynthetic performance under salinity stress. At 300 ppm, the photosynthetic rate approached that of non-saline control plants, indicating optimal physiological recovery. Iron is an essential component of chlorophyll biosynthesis and the photosynthetic electron transport chain, and Fe₃O₄ NP application was associated with increased chlorophyll content and photosynthetic rate under salinity stress [55,56]. Improved stomatal conductance was observed at moderate NP concentrations under salinity stress, likely due to enhanced plant water status and reduced oxidative stress [57]. In contrast, higher doses (≥ 400 ppm) reduced stomatal conductance, possibly due to reduced effectiveness of higher nanoparticle concentrations or partial stomatal closure, indicating a biphasic response [44,58].

Salinity-induced reductions in chlorophyll a, chlorophyll b, and total chlorophyll were significantly alleviated by Fe₃O₄ NP application, with the most pronounced effects observed at 400 ppm. Iron serves as a cofactor for enzymes involved in chlorophyll biosynthesis and stabilizes photosystem structures under stress [59,60]. Additionally, Fe₃O₄ NP application was associated with lower MDA and H₂O₂ accumulation under salinity stress, thereby protecting chloroplast membranes from oxidative degradation [61]. The slight decline in pigment content at 500 ppm suggests possible reduction in the effectiveness of higher nanoparticle concentrations, emphasizing the importance of dose optimization [47].

Improved membrane stability index (MSI) and reduced malondialdehyde (MDA) content under Fe₃O₄ NP treatments further confirm alleviation of oxidative damage. The strongest reductions in oxidative stress indicators were observed at 300-400 ppm Fe₃O₄ NP treatments, which limit lipid peroxidation and preserve membrane integrity [62,63]. Elevated MDA at 500 ppm indicates that

excessive nanoparticle concentrations may compromise antioxidant efficiency, consistent with earlier reports on nano-induced oxidative stress [64].

Biochemical stress markers further supported the protective role of Fe₃O₄ NPs. Moderate NP doses enhanced soluble sugar accumulation, contributing to osmotic adjustment and stress tolerance, while excessive concentrations reduced sugar content, likely due to metabolic imbalance or nanotoxicity [65]. Similarly, proline accumulation declined with increasing Fe₃O₄ NP concentration up to 400 ppm, reflecting reduced stress severity and improved cellular homeostasis. The slight proline increase at 500 ppm suggests a mild stress response triggered by excessive nanoparticle exposure [47].

The reduction in H₂O₂ content at 200 - 400 ppm Fe₃O₄ NPs highlights reduced H₂O₂ accumulation was observed at moderate Fe₃O₄ NP concentrations under salinity stress [66]. The modest rise in H₂O₂ at 500 ppm further supports the dose-dependent duality of nanoparticle effects. Enhanced carotenoid and flavonoid contents under Fe₃O₄ NPs treatments indicate improved photoprotection and secondary metabolite biosynthesis, which may be associated with improved physiological responses under salinity stress [67,68].

Reproductive performance and yield components responded positively to Fe₃O₄ NP application. Improved number of pod, seed set, seed weight, and seed yield were observed following Fe₃O₄ NP application under salinity stress [59,57]. Seed yield peaked at 400 ppm Fe₃O₄ NPs under salinity, indicating comparatively improved yield performance under salinity stress, while the slight decline at 500 ppm suggests a concentration threshold beyond which benefits diminish. Similar dose-dependent yield improvements have been reported in wheat and maize under abiotic stress [55,58].

Overall, the findings demonstrate that moderate foliar application of Fe₃O₄ nanoparticles (300-400 ppm) effectively mitigates salinity-induced physiological, biochemical, and yield constraints in soybean by enhancing iron nutrition, antioxidant defense, water relations, and photosynthetic efficiency. These results highlight the promise of Fe₃O₄ NPs as a sustainable nano-fertilizer strategy for improving crop productivity in saline environments, provided that application rates are carefully optimized to avoid potential nanotoxicity.

This study has several limitations. Independent characterization of the Fe₃O₄ nanoparticle suspension was not performed, and no soluble or bulk iron control treatment was included. Therefore, the observed responses cannot be conclusively attributed to nanoparticle-specific effects. In addition, iron uptake, antioxidant enzyme activity, and nanoparticle localization in plant tissues were not directly assessed. Future studies incorporating these analyses are necessary to better clarify the mechanisms underlying the observed responses

5. Conclusion

The present study demonstrates that salinity stress severely constrained soybean growth, physiological performance, biochemical attributes, and yield, confirming the high sensitivity of soybean to saline conditions. In contrast, foliar application of iron oxide (Fe₃O₄) nanoparticles was associated with improved plant water relations, chlorophyll content, membrane stability index, physiological traits, and yield-related parameters under saline conditions. These responses were associated with comparatively improved plant performance under saline conditions. Among the tested concentrations, Fe₃O₄ nanoparticles applied at 400 ppm generally showed comparatively better performance among the tested concentrations, resulting in the greatest improvements in yield components, including pods/plant, seeds/pod, hundred-seed weight, and final seed yield. Collectively, these findings suggest that foliar application of Fe₃O₄ nanoparticles, particularly at an optimal concentration of 400 ppm, may have potential for improving soybean performance in saline environments. However, because soluble iron controls and independent nanoparticle characterization were not included, nanoparticle-specific effects could not be conclusively established. Nevertheless, the findings suggest potential for enhancing soybean productivity in salt-affected environments. However, further field-based validation and long-term environmental safety assessments are warranted before large-scale adoption.

Author contributions declaration

“Investigation, methodology, data curation: **Masuma Akter**, Supervision, conceptualization, writing – original draft: **Md. A. Mannan**, Supervision, review & editing: **Nurunnaher Akter**, Review & editing: **Md. Motahar Hossain**, Writing-review & editing: **Most. Tanjina Akter**, Formal analysis, writing – review & editing: **Dipanjoli Baral Dola**, Data acquisition, review & editing: **Jay Karan Sah**, Review & editing: **Ummay Habiba**, Visualization, writing-review &

editing: **Usman Zulfikar**, Writing-review & editing: **Mohammed S. Alotaibi**, Writing- review & editing: **Mohamed M. El-Mogy**, Review & editing: **Mayank Anand Gururani**. All authors reviewed the manuscript.”

Funding declaration

This research was financially supported by the Ministry of Science and Technology (MoST), Government of the People's Republic of Bangladesh, through the National Science and Technology (NST) Fellowship awarded to the first author for the fiscal year 2023–2024. The publication of this article was supported by the Deanship of Scientific Research, Vice Presidency for Graduate Studies and Scientific Research, King Faisal University, Saudi Arabia (Project No. KFU262600).

Acknowledgement

The authors gratefully acknowledge the Ministry of Science and Technology (MoST), Government of the People’s Republic of Bangladesh, for providing financial support through the National Science and Technology (NST) Fellowship for conducting this research work. We also thankful to Prof Dr. Ziban Chandra Das, GAU for providing Sonicating facilities of nanoparticle preparation. This work was supported by the Deanship of Scientific Research, Vice Presidency for Graduate Studies and Scientific Research, King Faisal University, Saudi Arabia (KFU262600).

Data availability statement

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval:

This study did not involve human participants or animals requiring ethical approval.

Consent to participate:

Not applicable.

Consent to publish:

Not applicable.

Competing Interest:

The authors declare no competing interests.

Use of AI tools for language editing

The only application of ChatGPT (OpenAI GPT-5) was for language editing, which included readability, grammar, and clarity enhancements. The authors conducted all scientific content, study design, data analysis, results interpretation, and conclusions.

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