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Impact of iron sulfate ($FeSO_4$) foliar application on growth, metabolites and antioxidative defense of *Luffa cylindrica* (Sponge gourd) under salt stress

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Salt stress is becoming a major issue for the world's environment and agriculture economy. Different iron [Fe] sources can give an environmentally friendly alternative for salt-affected soil remediation. In this study the effects of Iron sulfate on *Luffa cylindrica* (Sponge gourd) cultivated in normal and saline water irrigated soil were examined. When $FeSO_4$ (0.01, 0.025, 0.05, 0.1 ppm) were applied to salt affected soil, the length, fresh and dry biomass of sponge gourd plant roots and shoots inclined by an average of 33, 28, 11, 21, 18 and 22%, respectively. In plants irrigated with saline water, leaf count was raised successively (23–115%) with increasing concentration of $FeSO_4$ (0.025-0.1 ppm) compared to stress only plants. The use of $FeSO_4$ boosted sponge gourd growth characteristics in both normal and salt-affected soils compared to respective controls. The application of Iron sulfate under salt stress boosted photosynthetic indices such as chlorophyll *a* (22%), chlorophyll *b* (34%), carotenoids (16%), and total chlorophyll levels (22%). Iron sulfate application also exhibited incline in primary (total free amino acids, 50%; total soluble proteins, 46%) and secondary (total phenolics, 9%; flavonoid content, 51%) metabolites in salt-affected soils. Oxidative enzymatic activities such as catalase (CAT), peroxidase (POD), polyphenol oxidase (PPO) and DPPH scavenging activity (36%) were also increased by foliar spray of $FeSO_4$ in control and salt stressed *L. cylindrica* plants. $FeSO_4$ had a considerable impact on the growth and development of *Luffa cylindrica* in normal and salt-affected soils. It is concluded that $FeSO_4$ application can effectively remediate salt affected soil and improve the production of crop plants.

Salt stress is a major component in halting plant growth and output[1]. Salinity levels influence around 33% of irrigated agriculture to varying degrees, and this figure may surpass 50% by 2050[2]. It is estimated that global annual agricultural land losses due to salt surpass 12 billion dollars[3]. In general, the susceptibility of plants to excessive salt can limit growth and interrupt developmental processes in a variety of ways, including osmoregulation, cytostatic produced by high Na+ and Cl−, and nutritional insufficiency[4]. It induces physiological and biochemical changes in plants, resulting in a reduction in growth, photosynthesis, respiration, protein synthesis, and eventually nucleic acid metabolism disturbance[1]. In plants, salt stress causes ion toxicity, water regulation stress, and notably oxidative stress[5]. A high degree of salinity influences enzyme conditioning, stomatal opening, and photosynthesis[6]. The accumulation of salts reduces the quantities of photosynthetic pigments like chlorophyll and carotenoids due to the inhibition of ribulose-1,5 bisphosphate, which consequently stops the photosynthetic mechanism. As a result, larger levels of reactive oxygen species (ROS) are produced, increasing the scavenging rate[7]. Because phosphate ions precipitate with Ca+ ions, soil salinity greatly inhibits phosphorus [P] absorption[8]. Salinity stress ranging from 0.6 to 3.2 dSm^{-1} directly disrupts crop production and electrical conduction of salt[9].

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Salinity has also induced alterations in the structure of the root system and a decrease in the total root length of the plant[10]. It has the capacity to change osmotic potential, resulting in a water deficit situation in the soil[11]. High concentrations of Na+ and Cl− ions in soil solution can inhibit nutrient ion activity and result in high ratios of $Na+/Ca^{2}+$, Na+/K+, $Ca^{2}+/Mg^{2}+$, and $Cl-/NO^{3-12}$.

Iron deficiency in vegetables and plants is a well-known issue that affects crop quality and nutritional value[13]. The capacity of an iron-containing formulation to perforate the cuticle and stomata, allowing it to travel past the cell membrane of leaf cells to reach the cytoplasm and ultimately the chloroplast, is critical to the efficacy of foliar administration[14]. In plants, iron participates in the oxidation process that releases energy from carbohydrates and starch and also converts nitrate to ammonia. It has a vital role in the rate of nucleic acid metabolism[15]. Plants absorb iron from the soil in the form of $Fe^{2}+$, which is suitable for evolution and improvement[16]. Iron deficiency and toxicity harm 30% and 18% of global soil, respectively[17].

Sulfur shortage also has a deleterious impact on crop growth and development at maturity. Inorganic sulfur is essential for plant growth and anabolism of protein and chlorophyll[18]. Because this important nutrient is available to plants in sulfate form, most fertilizers contain sulfur in sulfate form. Sulfur requirements for optimal plant growth range from 0.1 to 0.5% dry biomass weight[19]. Photosynthesis is slowed by deficiency of sulfur[20]. Foliar spray of iron sulfate improves the transport of modest amounts of Fe to plants and may be a less expensive and more environmentally friendly technique for addressing iron shortage in crops[21]. The regulation of sulfur metabolism in plant life might aid in decreasing the detrimental effects of salinity because its isoenzyme controls a wide range of plant development. The formation of S-containing mixtures via S breakdown is linked to the antioxidant system in vegetation under salt stress[22]. Foliar treatment of $FeSO_4$ in vegetable plants can modulate photosynthetic mechanisms, improving sponge gourd output and development.

A restricted investigation on cucurbitaceous plants with the administration of iron sulfate under saline circumstances has been published in the literature. Still the effect of $FeSO_4$ on *L. cylindrica* L. plant growth and development in salt affected soil is not explored. *L. cylindrica*, a vital cucurbit crop, plays a crucial role in ensuring food security and dietary diversity for impoverished communities. As a diploid species with 26 chromosomes, Luffa is cross-pollinated and produces cylindrical, green fruits[23]. These fruits can be used as sponges when mature or consumed as food in various forms, including curries and fresh or dried preparations[24]. Luffa is a versatile crop with numerous applications. It serves as a source of edible oil, food, fodder, and industrial oil/biodiesel. The crop is rich in minerals, tannins, oxalates, and phytic compounds, making it a valuable source of vegetable protein for both humans and animals[25]. Luffa seed oil, a semi-drying oil, has potential applications in surface coatings, soap manufacturing, and biodiesel production[26]. This versatile crop contributes to both food security and economic growth.

The purpose of this study is to look into the influence of iron sulfate foliar spray on the growth and physio-biochemical characteristics of sponge gourd under salt stress. The amount of micronutrients they can absorb has been connected to abiotic stressors[27]. People who cannot afford to purchase additional micronutrient-rich goods for a balanced diet are more vulnerable to micronutrient deficiencies[28]. To address this issue, numerous studies provide specific recommendations that could aid in the management of micronutrient application optimization in modern crop production[29,30]. Plants are the primary source of iron, either directly as staple crops or indirectly as animal feed. Biofortification is a long-term treatment to iron insufficiency that involves increasing the iron content of edible plant parts and is recommended due to its sustainability and cost efficiency[31,32].

Materials and methods

Experimental setup and layout

In this study, a two-way completely randomized design pot experiment with three replicates was conducted at The University of Lahore, Lahore, Pakistan. Two factors under examination were salinity stress, maintained artificially using NaCl and Fe supplements applied in the form of $FeSO_4$ foliar spray. The experiment was performed during spring 2023 in an open field under natural conditions. The average temperature and the relative humidity during the experimental period were 29 °C and 67.9%, respectively. The *L. cylindrica* seeds were obtained from the Amir nursery, Wapda Town, Lahore. Soil used in this study was sandy clay loamy in texture. The physiochemical characteristics of soil were analyzed by Soil Fertility Research Institute, Lahore (Table 1). Uniform and healthy seeds (7–8) were sown in pots filled with 7 Kg of soil. After germination, 5 seedlings were maintained at equal distance from each other in every pot. Various treatments were applied on 4th week of germination. For salinity stress, 60 mM NaCl solution was used for irrigation purposes, in place of simple water (control). The salinity stress was maintained throughout the experiment by using 60 mM NaCl solution as irrigation water at regular intervals. $FeSO_4$ solutions of varying concentrations (0, 0.01, 0.025, 0.05, 0.1 ppm) were prepared in 1% Tween20 and applied on *L. cylindrica* plants via foliar spray until the plants get fully wet. $FeSO_4$ foliar spray was applied two times during the whole experimental period at two weeks interval. One month after the first foliar application, the plants were uprooted to measure growth attributes. Photosynthetic pigments, primary and secondary metabolites and antioxidant enzymes activities were also assessed, the detail of protocols used is listed below.

Photosynthetic pigments determination

Fresh leaves (0.5 g) were taken from each replicate and homogenized in 80% acetone (10 mL). This homogenate was filtered, and filtrate was stored at 4 °C for a day. Next day, the absorbance for filtrate was read at 663, 645 and 480 nm against 80% acetone as control. The methods of Arnon[33] and Lichtenthaler[34] were employed to determine the content of chlorophyll and carotenoids, respectively.

Physio-chemical characteristics of Soil	
Soil texture	Sandy clay loam
pH	7.21
Moisture contents	43.28%
Electrical conductivity	2.67 dS m^{-1}
Organic matter	2.61%
Organic carbon	1.52%
Nitrogen	6.24 mg Kg^{-1}
Phosphorus	32.52 mg Kg^{-1}
Sodium	0.78 mg Kg^{-1}
Zinc	18.16 mg Kg^{-1}
Potassium	94.77 mg Kg^{-1}

Table 1. Physiochemical properties of soil used in this experiments for pots filling.

Determination of primary metabolites (total free amino acids and total soluble proteins)

The free amino acid content was quantified using a modified method outlined by Hamilton et al.[35]. Fresh leaves (0.1 g) were homogenized in a pH 7.0 buffer solution. The homogenate was then centrifuged (8,000 rpm) for ten minutes to isolate the supernatant. A reaction mixture was prepared by combining the supernatant (1.0 mL) with 10% pyridine (1.0 mL) and 2% ninhydrin (1.0 mL). This mixture was heated in a boiling water bath for 30 min, resulting in a violet-colored solution. After dilution with distilled water, the absorbance at 570 nm was measured spectrophotometrically.

The Bradford method[36] was employed to determine the soluble protein content in the leaves. Bovine serum albumin served as the standard, and Coomassie Brilliant Blue G-250 was used as the dye. Frozen leaf samples (0.5 g) were ground in liquid nitrogen and homogenized in 10 mL of 50 mM sodium phosphate buffer. The homogenate was centrifuged (11,000 rpm, 10 min, 4 °C), and the supernatant (0.1 mL) was mixed with Bradford reagent (3 mL). The absorbance at 595 nm was measured to quantify the total protein content, with a standard curve of bovine serum albumin used for concentration determination.

Determination of secondary metabolites (total flavonoids and total phenolics)

The flavonoid content was assessed using a modified method described by Beketov et al.[37]. The plant extract (0.2 mL) was mixed with 90%ethanol (4.5 mL), 2% aluminum chloride (0.2 mL and 33%acetic acid (0.1 mL). The mixture was incubated in the dark for 30 min, and the absorbance at 414 nm was subsequently measured spectrophotometrically.

The total phenolic content was quantified following the method of Zhang & Quantick[38]. Leaf samples (1 g) were extracted with hydrochloric acid-methanol (1%, 5 mL). The extract was filtered and diluted with hydrochloric acid-methanol up to 10 mL. The absorbance of the diluted extract was measured at 280 nm to determine the total phenolic content. A standard curve constructed using gallic acid was used for quantification.

Determination of oxidative stress

The malondialdehyde (MDA) contents were assessed using the protocol of Heath & Packer[39], with minor modifications. A leaf sample (0.5 g) was crushed in trichloroacetic acid (TCA; 0.1%, 5 mL) to prepare a homogenate mixture. This homogenate was subjected to centrifugation for 5 min at 10,000 rpm. In 1 mL of supernatant obtained after centrifugation, 20%TCA prepared in 0.5%thiobarbituric acid (4 mL) was added. The mixture was heated at 95 °C for 30 min on shaking hot water bath. After that the mixture was quickly cooled in an ice bath and again centrifuged at 10,000×g for 10 min. Following that absorbance of the supernatant at 532 nm was checked and nonspecific absorption at 600 nm was subtracted. The MDA content was calculated by using an extinction coefficient of 155 mM^{-1} cm^{-1}.

To extract hydrogen peroxide (H_2O_2) from leaf tissues, 50 mg of the tissue was homogenized in 3 mL of phosphate buffer (50 mM, pH 6.5). The homogenate was then centrifuged at 6000 x g for 25 min. The supernatant was mixed with 1 mL of 0.1% titanium sulfate in 20% (v/v) sulfuric acid (H_2SO_4) and centrifuged again at 6000 x g for 15 min. The intensity of the yellow color of the supernatant was measured at 410 nm using a spectrophotometer. The concentration of H_2O_2 in the sample was calculated using the extinction coefficient of 0.28 μmol^{-1} cm^{-1}[40].

Determination of antioxidant enzymes activities

The plant extract used for total soluble protein determination was also analyzed for antioxidant enzyme activities. Catalase and peroxidase activities were measured following the method of Chance & Maehly[41]. For catalase reaction mixture was prepared as; 25 mM potassium phosphate buffer (pH 6.8), 10 mM H_2O_2, and enzyme extract were mixed to make total volume of 1 mL. The decrease in absorbance at 240 nm was monitored to measure hydrogen peroxide decomposition. For peroxidase reaction mixture contained potassium phosphate buffer (25 mM, pH 6.8), 10 mM H_2O_2, guaiacol (0.05%), and diluted enzyme extract. To assess peroxidase activity, oxidation of guaiacol was mounted at 470 nm. For PPO activity, reaction mixture comprised of 2.85 mL of potassium phosphate buffer (50 mM, pH 7.0), 50 µL of 60 mM catechol, and 0.1 mL of supernatant. The absorbance was measured at 420 nm[42].

The antioxidant activity of the extracts was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. 50 µL of plant extract from each replicate was placed in test tubes, followed by the addition of 14.50 mL of methanol and 5.0 mL of DPPH solution. The mixtures were incubated in the dark at room temperature for 30 min. Butylated hydroxytoluene (BHT) (1 mM) was used as a positive control. After the incubation period, the absorbance was measured at 517 nm against a blank. The percentage inhibition of the DPPH free radical by each extract was calculated using the following formula:

$$Radical\ scavenger\ effect\ (\%) = \{(A_0 - A_1)/A_0\} \times 100$$

where A_0 represents the absorbance of the DPPH solution and A_1 represents the absorbance of the extract with DPPH[43].

Statistical evaluation

For the statistical analysis of the data, Statistix 8.0 was utilized, and the analysis of variance (ANOVA) was employed for multiple treatment comparisons, along with least significant difference test ($p \leq 0.05$) for mean comparison. The graphical presentation was created using Microsoft Excel 2016, graphs bars represented average value of three replicates ± standard errors.

Results

Effect of $FeSO_4$ foliar spray on growth attributes of *L. cylindrica* plants grown in salinity stress

Statistical analysis showed significant differences in plant height as well as root and shoot fresh and dry biomass (Table 2). Salinity reduced the root length by 33%, shoot length by 28.47%, root fresh and dry biomass by 11 and 18% while shoot fresh and dry biomass displayed a significant drop by 21 and 22% respectively. Salinity also reduced the number of leaves by 7% as compared to the plants grown in normal soil. Iron Sulfate application under salinity displayed positive effect on the growth and biomass of *L. cylindrica* plants at all applied concentrations. *L. cylindrica* plants exhibited a significant incline in root length by 28% at 0.01 ppm, 29% at 0.025 ppm, 32.83% at 0.05 ppm and 38.27% at 0.1 ppm concentration of iron sulfate as compared to stressed only plants. Similarly, *L. cylindrica* shoot displayed improved growth by 23, 26.31, 28 and 33% at 0.01, 0.025, 0.05 and 0.1 of Iron Sulfate respectively, as compared to salt treated plants. Plant fresh and dry biomass also followed the similar trend and maximum increase in root fresh biomass was calculated at 0.1 ppm iron sulfate by 20 to 24% while shoot fresh and dry biomass was improved by 20 and 29%, respectively, as compared to only salt treated plants. Moreover, leaf count was reduced to 13% under saline soil which further enhanced to 7, 18, 27 and 53% when plants were foliar sprayed with 0.01, 0.025, 0.05 and 0.1 ppm of $FeSO_4$ under salt stress.

Effect of $FeSO_4$ foliar spray on photosynthetic pigments of *L. cylindrica* plants grown in salinity stress

Chlorophyll *a*, *b*, total chlorophyll and carotenoids showed positive response with an increase in the concentration of iron sulfate foliar spray (Fig. 1). Salinity reduced the chlorophyll *a* by 18%, chlorophyll *b* by 25%, total chlorophyll by 19%, and carotenoid by 13%, as compared to control plants. Foliar application of Iron Sulfate under salinity displayed positive influence on *L. cylindrica* photosynthetic pigments at all applied concentrations. These plants exhibited a significant incline in chlorophyll *a* by 11% at 0.01 ppm, 12% at 0.025

Condition	Foliar ppm	Root length (cm)	Shoot Length (cm)	Root fresh weight(g)	Shoot fresh weight(g)	Root dry weight(g)	Shoot dry weight(g)	Leaf Count (*n*)
Control	0	5.125^{e}	32.25^{d}	3.625^{h}	7.875^{de}	0.1525^{e}	1.3075^{f}	3.75^{cd}
	0.01	8.375^{b}	37.25^{c}	5.000^{ef}	8.375^{d}	0.355^{cd}	1.3125^{f}	4.00^{cd}
	0.025	10.125^{a}	39.75^{bc}	5.250^{de}	10.875^{b}	0.365^{cd}	2.0675^{c}	4.75^{bcd}
	0.05	10.750^{a}	40.25^{ab}	6.125^{bc}	13.250^{a}	0.515^{b}	2.365^{b}	5.25^{bc}
	0.1	11.125^{a}	42.75^{a}	6.875^{a}	13.375^{a}	0.6575^{a}	2.6475^{a}	6.00^{ab}
Salinity	0	3.635^{f}	21.25^{g}	2.875^{i}	6.125^{f}	0.1375^{e}	1.180^{f}	3.25^{d}
	0.01	$5.625d^{e}$	25.00^{f}	4.250^{gh}	7.125^{e}	0.310^{d}	1.2125^{f}	3.50^{d}
	0.025	6.250^{d}	28.75^{e}	4.500^{fg}	8.625^{d}	0.325^{d}	1.595^{e}	4.00^{cd}
	0.05	7.625^{c}	31.00^{de}	5.875^{cd}	9.875^{c}	0.400^{c}	1.7325^{de}	4.50^{bcd}
	0.1	8^{c}	31.50^{de}	6.625^{ab}	10.875^{b}	0.512^{b}	1.870^{cd}	7.00^{a}
ANOVA (F Value)								
Salinity(S)		***	***	***	***	***	***	*
Foliar(F)		***	***	***	***	***	***	***
Salinity*Foliar		***	*	*	***	***	***	**

Table 2. Effect of foliar application of Iron Sulfate on growth indices of *L. cylindrica* under salinity stress. Each value is a mean of three replicates; different alphabetic letters indicate significant differences ($P \leq 0.05$) among treatments, *, ** and *** indicated significance at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ respectively; ns indicated non-significant differences.

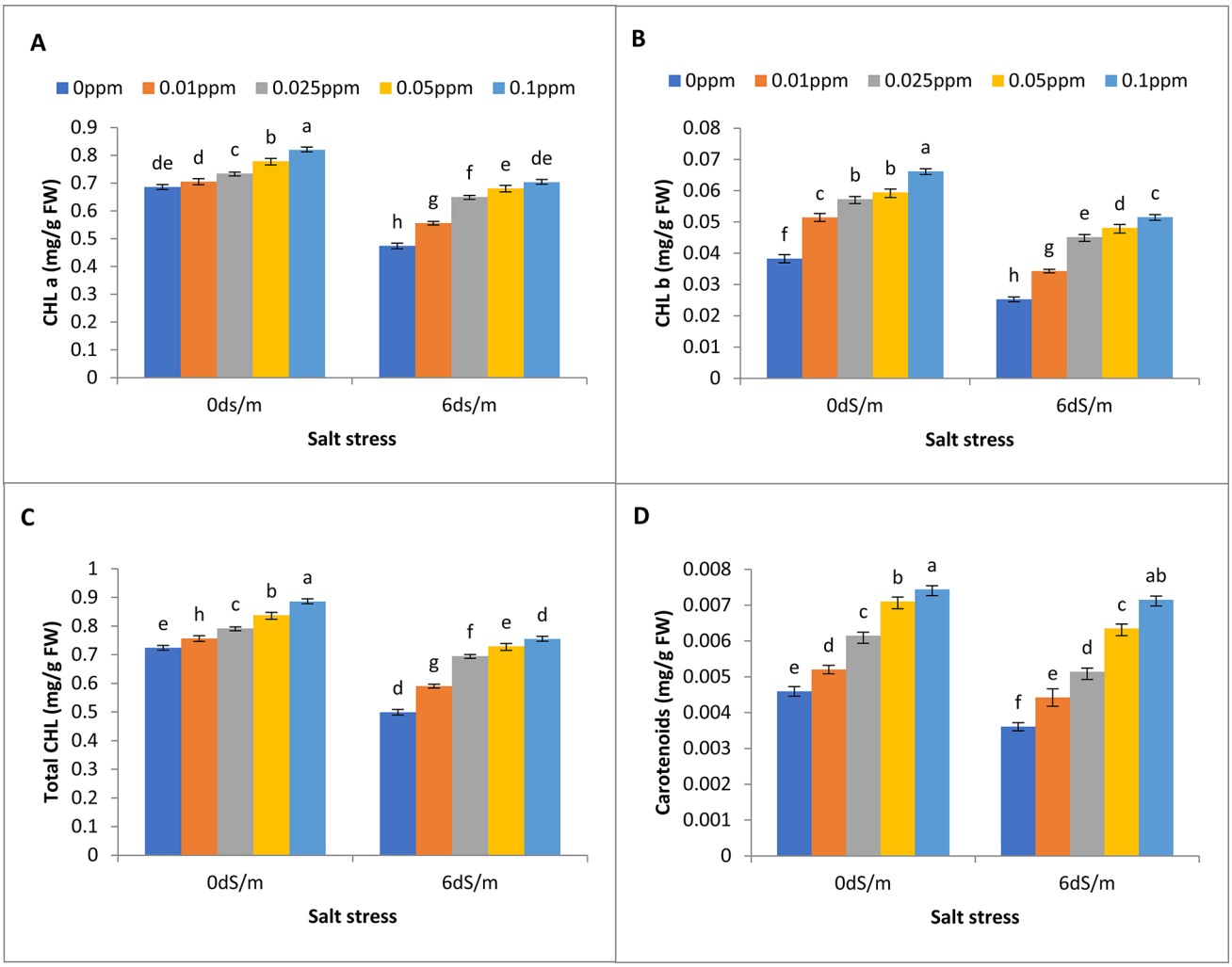

Fig. 1. The effect of different concentrations of foliar application of Iron Sulfate on photosynthetic pigment analysis of sponge gourd (*Luffa cylindrica)* under salt stress. (**A**) chlorophyll *a* (CHL *a*), (**B**) chlorophyll *b* (CHL *b*), (**C**) total chlorophyll (CHL) and (**D**) carotenoids. Mean values are the average of four replicates. The upper case alphabetic letters indicate significant differences ($p \leqq 0.05$) among treatments using Tukey's HSD test.

ppm, 14% at 0.05 ppm and 22% at 0.1 ppm iron sulfate concentration as compared to only salinity treated plants. Similarly, *L. cylindrica* displayed improvement in chlorophyll *b* by 18, 21, 23 and 34% at 0.01, 0.025, 0.05 and 0.1 ppm of iron sulfate, respectively, as compared to salinity treated plants. Total chlorophyll also followed the similar trend, and maximum increase was calculated at 0.1 ppm iron sulfate by 22% while maximum incline of 16% in carotenoids was calculated at 0.1 ppm concentration of iron sulfate compared to the plants grown under salt stress.

Effect of $FeSO_4$ foliar spray on primary metabolites synthesis in*L. cylindrica* plants grown in salinity stress.

Primary metabolites (total free amino acid and total soluble protein) also showed gradual increase in their values with increasing concentrations of iron sulfate (Fig. 2). Statistical analysis showed significant differences in total free amino acids and total soluble protein. Salinity reduced the total free amino acids by 22% and total soluble protein by 5% respectively, as compared to control plants. Iron Sulfate foliar application under salinity displayed positive effect on primary metabolites at all applied concentrations. Plants exhibited a significant and successive increase in total free amino acids with increasing concentration of iron sulfate as compared to the stress only plants. Similarly, *L. cylindrica* total soluble protein was successively improved by 3.2, 3.8, 4.3 and 6% at 0.01, 0.025, 0.05 and 0.1 ppm of iron sulfate respectively, as compared to salinity treated plants.

Effect of $FeSO_4$ foliar spray on secondary metabolites synthesis inL. cylindrica plants grown in salinity stress

Secondary metabolites showed gradual increase with increasing concentrations of iron sulfate in *L. cylindrica* plants grown in salinity as well as under normal condition (Fig. 3). Salinity increased the total phenolics by 45%, and total flavonoids by 29% as compared to plants grown under normal conditions. These plants exhibited a significant incline in total phenols by 33% at 0.01 ppm, 26% at 0.025 ppm, 27% at 0.05ppm and 30% at 0.1 ppm iron sulfate concentration as compared to the plants under salinity. Similarly, *L. cylindrica* under saline

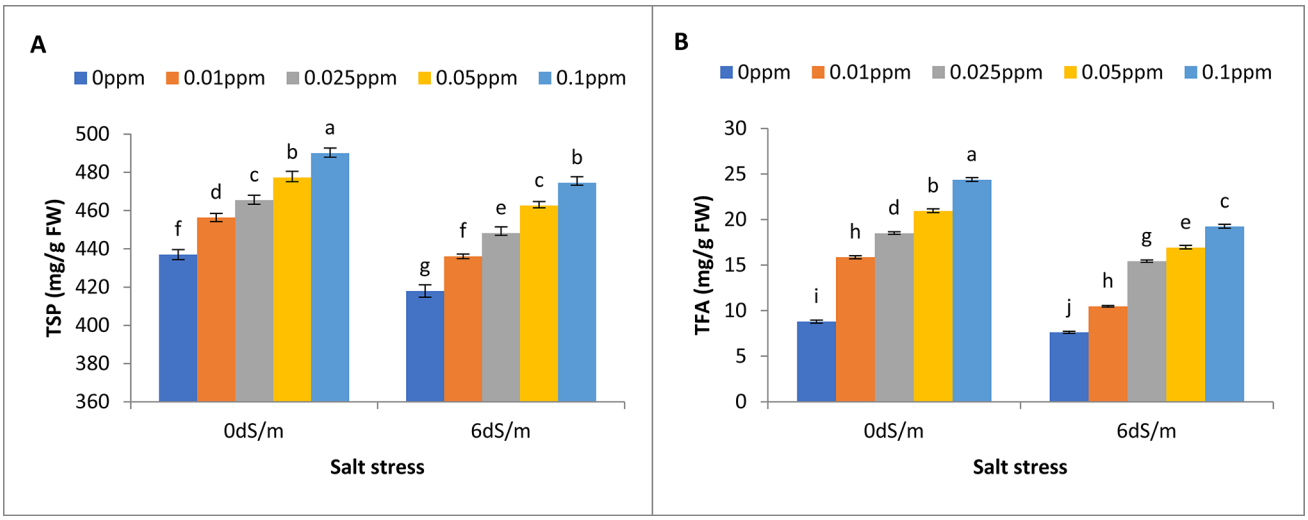

Fig. 2. The effect of different concentrations of foliar application of Iron Sulfate on primary metabolites of sponge gourd (*Luffa cylindrica)* under salt stress. (**A**) total soluble proteins (TSP), (**B**) total free amino acids (TFA). Mean values are the average of four replicates. The upper case alphabetic letters indicate significant differences ($p \leqq 0.05$) among treatments using Tukey's HSD test.

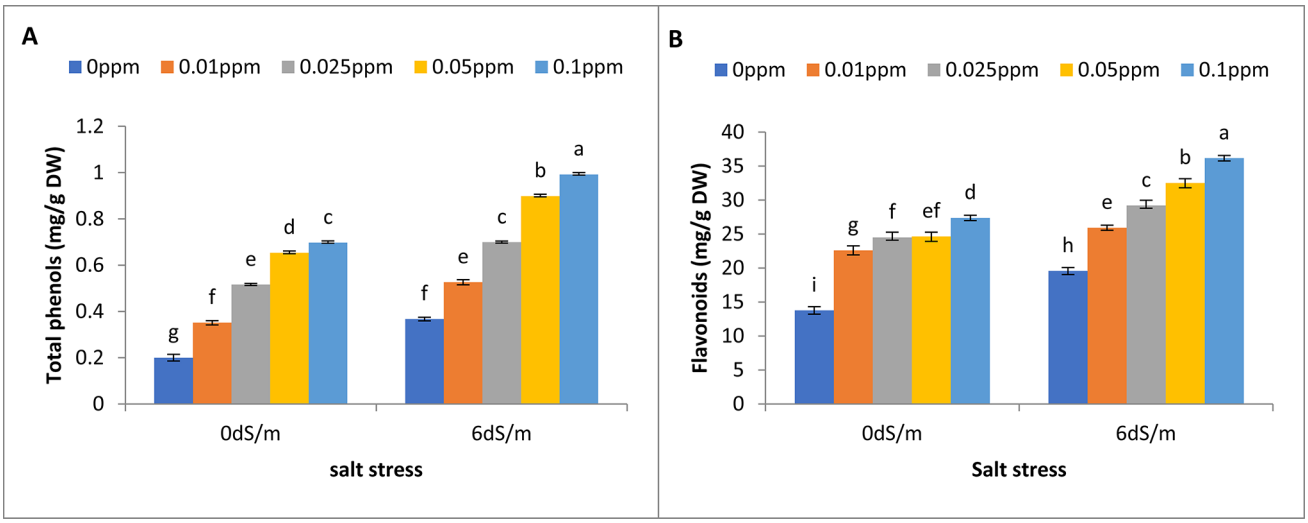

Fig. 3. The effect of different concentrations of foliar application of Iron Sulfate on secondary metabolites of sponge gourd (*Luffa cylindrica)* under salt stress. (**A**) total phenols, (**B**) flavonoids. Mean values are the average of four replicates. The upper case alphabetic letters indicate significant differences ($p \leqq 0.05$) among treatments using Tukey's HSD test.

environment exhibited more total flavonoids contents by 13, 17, 25 and 27.32% at 0.01, 0.025, 0.05 and 0.1 ppm concentrations of iron sulfate respectively, as compared to control plants.

Effect of $FeSO_4$ foliar spray on malondialdehyde and hydrogen peroxide contents in *L. cylindrica* plants grown in salinity stress

Oxidative stress markers showed a significant increase in *L. cylindrica* plants under saline conditions as compared to control (Table 3). Lipid peroxidation in the form of inclined level of MDA contents and uncontrolled release of reactive oxygen species (H_2O_2) was observed. However, the foliar spray of $FeSO_4$ effectively reduced lipid peroxidation and scavenged ROS generated during saline stress, as indicated by lowered levels of MDA and H_2O_2 under the effect of $FeSO_4$ foliar spray when applied in saline and control conditions.

Effect of $FeSO_4$ foliar spray on enzymatic antioxidants activities in*L. cylindrica* plants grown in salinity stress

Catalase, peroxidase, polyphenol oxidase and DPPH scavenging assays exhibited an increase in these activities with the increasing concentration of iron sulfate under salt stress as compared to control plants (Fig. 4). Salinity increase the catalase activity by 18%, peroxidase activity by 20%, polyphenol oxidase activity by 28%

Condition	Foliar (ppm)	MDA (µmol g−1 FW)	H_2O_2 (µmol g−1 FW)
Control	0	7.12^{c}	12.25^{c}
	0.01	6.37^{c}	10.25^{cd}
	0.025	5.12^{cd}	10.75^{cd}
	0.05	5.75^{cd}	8.25^{d}
	0.1	5.12^{cd}	7.75^{d}
Salinity	0	12.63^{a}	20.25^{a}
	0.01	9.62d^{b}	15.00^{b}
	0.025	8.25^{bc}	12.75^{c}
	0.05	8.62c^{c}	11.00^{cd}
	0.1	7.54^{c}	10.50^{cd}
ANOVA (F Value)			
Salinity(S)		***	***
Foliar(F)		***	***
Salinity*Foliar		ns	ns

Table 3. Effect of foliar application of Iron Sulfate on stress indicators of *L. cylindrica* under salinity stress. Each value is a mean of three replicates; different alphabetic letters indicate significant differences ($P \leq 0.05$) among treatments, *, ** and *** indicated significance at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ respectively; ns indicated non-significant differences.

and DPPH assay by 26% as compared to control plants. Iron Sulfate application under salinity displayed an incline in catalase activity by 15% at 0.01 ppm, 18% at 0.025 ppm, 19.25 at 0.05 ppm and 22% at 0.1 ppm iron sulfate concentration as compared to control plants. Similarly, peroxidase activity displayed an increase of 13, 21, 24 and 25.87% at 0.01, 0.025, 0.05 and 0.1 ppm concentrations of iron sulfate, respectively, as compared to control plants. Polyphenol oxidase and DPPH assay also followed the similar trend and maximum activities were calculated at 0.1 ppm iron sulfate by 33 and 36%, respectively, as compared to plants grown under normal conditions.

Discussion

Salt stress produces numerous variations and changes in different cellular organelles in plants, because of the overproduction of reactive oxygen species (ROS) and lowering photosynthetic performance[44,45]. Plants, on the other hand, create diverse enzymatic and non-enzymatic antioxidants in response to salt stress[46]. Iron (Fe) is a microelement that has a considerable impact on plant health, yield quality, and by-product generation in many crops[21]. Because Fe is required for a range of physiological operations in plants, including DNA formation, respiration, photosynthesis, and protein synthesis. A lack of Fe, on the other hand, is highly associated with a number of soil properties such as high pH, high salinity, low organic matter, and free calcium carbonate in soils[47].

The current study found that plants under salinity stress had reduced growth. The shoot and root biomass of *L. cylindrica* was greatly declined at 60 mM NaCl stress. These findings aligned with previous literature. For example, soybean plants had significantly reduced growth when exposed to saline stress[48]. However, *L. cylindrica* plants were treated with iron sulfate had higher values of vegetative growth measurements such as plant length, shoot and root fresh weight, shoot and root dry weight, and leaf count when compared to non-treated plants in both normal and stress conditions (Table 2). These findings were congruent with the findings of Li et al.[49] who found that increasing the concentrations of Fe_3O_4 NPs (1, 2, and 3 mg/L) enhanced total plant mass, root length, number of leaves, and biomass of *Ocimum basilicum* L. Another study stated that root elongation improved in broad bean plants when treated with different sources of iron[21]. Furthermore, some researchers claimed that spraying nano-iron significantly improves plant-growth parameters and fruit quality of over-chelated and conventional iron treatments by increasing photosynthesis pigment concentration (chlorophyll and carotenoid) and nutrient absorption[50,51]. Chlorophyll is a key pigment in photosynthesis that absorbs and transfers light energy[21]. The amount of leaf chlorophyll and carotenoid is the primary indicator used to define the physiological performance of plant photosynthetic tissues, and it has a significant impact on plant photosynthesis[52]. Carotenoids are antioxidant bioactive pigments that protect chlorophyll from photodegradation and oxidation[53]. The current study found that salinity stress significantly reduced photosynthetic pigments in *L. cylindrica* plants. In contrast, spraying iron sulfate considerably raised the level of leaf chlorophyll and leaf carotenoid when compared to untreated plants. The superiority of leaf chlorophyll and carotenoid contents in broad beans treated with FeNPs was observed[54]. One reason for this was that FeNPs increased the activity of iron oxygen reductase, which indirectly increased porphyrin metabolism to create aminolaevulinic acid, a precursor to chlorophyll. According to Mahmoud et al.[21] FeNPs and ZnNPs considerably increase the leaf carotenoid concentration in red radish. It has also been reported that FeNPs boosted chlorophyll levels in *Eruca sativa* leaves relative to the control group[49]. Furthermore, increasing chlorophyll concentration in plants is crucial for biotechnological applications[55]. The increase in photosynthetic pigments is linked to incline in growth related attributes, as observed in this study. This might be due to enhanced photosynthesis rate that resulted in high

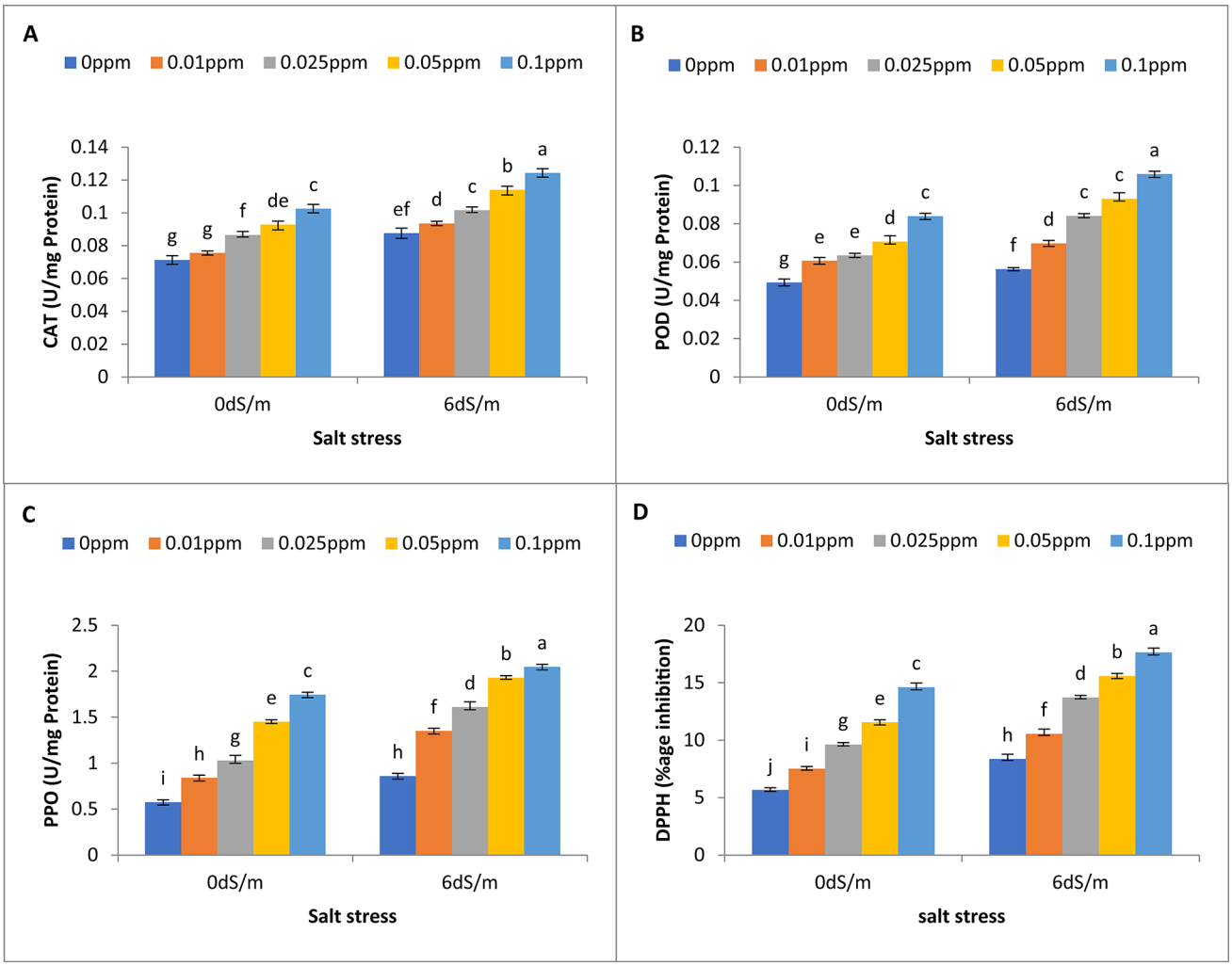

Fig. 4. The effect of different concentrations of foliar application of Iron Sulfate on enzymatic antioxidant activites of sponge gourd (*Luffa cylindrica)* under salt stress. (**A**) catalases (CAT), (**B**) peroxidases (POD), (**C**) polyphenol oxidases (PPO) and (**D**) 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. Mean values are the average of four replicates. The upper case alphabetic letters indicate significant differences ($p \leqq 0.05$) among treatments using Tukey's HSD test.

energy production and its efficient utilization under stress conditions[56]. Fe is a cofactor for various enzymes utilized in the synthesis of photosynthetic pigments especially chlorophylls. Fe also plays crucial role in electron transport chain, as part of Fe-S proteins. Thus, this is conceivable that foliar spray of $FeSO_4$ increased chlorophyll synthesis and photosynthesis of *L. cylindrica* plants. This ultimately improved growth of plants even under saline conditions.

The current study found that salinity affects total soluble protein and total free amino acids. It was much lower than in control plants. Salinity stress also had significant influence on biochemical parameters in other crops like *D. carota*[57] and *C. annuum*[58]. Iron sulfate was engaged in cell membrane function and several enzymatic systems in plants, which is why the biochemical parameters were improved[59]. As stated earlier, Fe is part of various metabolic pathways being co factor for various enzymes. For example, it is cofactor for ribonucleotide reductase enzymes involved in the synthesis of DNA. This indirectly supports the synthesis of proteins. This might be the reason for incline in total soluble proteins and total free amino acids in *L. cylindrica* plants under the effect of $FeSO_4$.

The current study found that salt stress increased the total phenolics and flavonoids content of leaves when compared to control plants. Ahmed et al.[60] reported that with increasing concentration of Fe, the flavonoid (rutin and quercetin), essential oil components, biochemical and medicinal qualities of the treated plants were remarkably promoted. In another study, Lingyun et al.[61] stated that foliar application of iron boosted the total phenolics and flavonoids levels in pea plants. Similarly, Shi et al.[62] reported that foliar spraying iron sulfate boosted total flavonoids under salt conditions compared to control plants. It has also been reported that foliar application of green-synthesized calcium and iron nanoparticles increased antioxidative chemicals, mineral content (Ca, Fe, N, P, K, Mg, and Zn), phenolics, and flavonoids in *Botrytis cinerea*-infected strawberry plants[63]. The synthesis of phenolic compounds including total phenolics and flavonoids is dependent on phenylpropanoid pathway. Fe is a

co-factor for various enzymes involved in this pathway. $FeSO_4$ foliar spray boosted the activity of these enzymes and thus the synthesis of secondary metabolites in *L. cylindrica* plants. Plants have also adaptability to enhance the synthesis of secondary metabolites as a defense strategy to overcome oxidative stress. The inclined level of total phenolics and flavonoids in this study can be attributed to stress response changes in plants. Interestingly, the level of flavonoids increased with increasing concentration and frequency of foliar application. This suggests that iron supplements can be utilized as a safe and healthy fertilizer to promote the medicinal qualities of this plant. Another reported investigation explained that the effects of iron nanoparticles on secondary metabolite biosynthesis may be similar to stress signals and may promote their biosynthesis[64].

Salinity stress imposed oxidative stress to *L. cylindrica* plants as indicated by elevated levels of lipid peroxidation in the form of elevated malondialdehyde (MDA) and H_2O_2 contents. This increment in oxidative stress markers especially MDA and H_2O_2 due to salinity stress have been observed in various crops including *L. esculentum*[65], *C. annuum*[58] and *D. carota*[57]. The uncontrolled release of reactive oxygen species (ROS) damages the cell membrane and other membrane bounded organelles such as chloroplasts. ROS also affected the activity of ribulose 1, 5 bisphosphate the enzyme of Calvin cycle. In this way salinity stress reduced photosynthetic pigmentation and rate of photosynthesis which can be observed in the form of reduced plant growth under saline conditions. However, the application of $FeSO_4$ effectively reduced lipid peroxidation and membrane damage through reducing the level of MDA and H_2O_2 in *L. cylindrica* plants under both normal and saline conditions.

The current study found that salinity increased many antioxidant activities (CAT, POD, and PPO) in comparison to control plants. These findings are consistent with those of Mogazy et al.[63] who discovered a similar rise in antioxidant species (CAT, POD, PPO) in chili pepper plants sprayed with Ca and Fe nanoparticles under both salt free and salt stress conditions. Abdoli et al.[64] discovered that foliar application of salicylic acid (SA) alone or in combination with iron nanoparticles alleviated salt toxicity by improving antioxidant enzyme activities including CAT, POD, APX and SOD. While salinity increased the contents of total phenol, anthocyanin, and flavonoid, as well as the activities of ascorbate peroxidase, glutathione reductase, catalase, and guaiacol peroxidase, according to another report that foliar application of iron oxide nanoparticles increased them even more, and this can improve the antioxidant defense system in the plant[66]. Haydar and cowerkers (2022) evaluated the effects of iron oxide nanoparticles and EDTA on mulberry growth and development in order to substitute traditional Fe-fertilizer with nano-micronutrient fertilizer. They investigated soil and foliar spray treatments[59]. They reported increased morpho-physiochemical features such as sprouting percentage, number of leaves, plant biomass, root length, and faster first leaf appearance period, as well as increased chlorophyll and sugar content and higher antioxidant enzyme activities such as catalase, peroxidase, and NADPH oxidase. Fe is a key part of super oxide dismutase (Fe-SOD), besides this Fe is also part of heme group in catalase (CAT) enzymes. On other hand, sulfur is essential for the synthesis of sulfur containing amino acids like cysteine and methionine which are important for structure and function of antioxidants enzymes. For this reason, $FeSO_4$ foliar spray in this study boosted antioxidant enzymes (CAT, POD and PPO) activities in *L. cylindrica* plants. This is also evident from the increase in DPPH scavenging activities of the plant extract as observed in this study. This inclined in antioxidant enzymes activities helped *L. cylindrica* plants to overcome salinity through scavenging reactive oxygen species. The scavenging of ROS also resulted in improved photosynthesis and related attributes. These changes ultimately improved plant growth and development under saline conditions.

Use of $FeSO_4$ as exogenous supplements potentially mitigated salinity stress by improving photosynthesis related attributes, primary and secondary metabolites and antioxidant enzyme activities in *L. cylindrica* plants in a pot experiment. This is a cost effective, ecofriendly and easily accessible approach to remediate saline affected soil. However, its implication in field could be an open challenge for researchers. Cost effectiveness, adaptability and acceptance of this technique for the farmers depend on several factors. For instance, the ease of integration into existing farming practices and the perceived benefits in terms of increased yield, or enhanced crop quality on saline affected soil.

Conclusion

In this study the effects of Iron sulfate on *Luffa cylindrica* (Sponge gourd) cultivated in normal and saline water treated soils were examined. Salt stress significantly hampered the growth of *L. cylindrica* reducing its shoot length, root length, fresh and dry biomass. Salt stress also had negative effects on photosynthetic pigments as it caused oxidative stress in *L. cylindrica* plants which is evident from increased lipid peroxidation in the form of higher MDA and H_2O_2 contents. In contrast, $FeSO_4$ foliar spray mitigated the adverse effects of salinity stress through modulations in physiological responses of *L. cylindrica* plant and improving the synthesis of primary and secondary metabolites. $FeSO_4$ foliar spray also increased the antioxidant enzymes activities thus scavenged excessive reactive oxygen species and lowered lipid peroxidation. These effects of $FeSO_4$ foliar spray ultimately resulted in improved growth of *L. cylindrica* plants in salt stress conditions. It is concluded that $FeSO_4$ application can effectively remediate salt stress and improve the production of crop plants.

Future recommendations

The results of this study showed that $FeSO_4$ can potentially ameliorate the salinity stress in crop plants. It could be a cost effective and ecofriendly approach to remediate saline soils and to grow healthy crops on saline affected soils. However, its implication in big fields could be a major challenge for researchers. There is need of extensive research to get real time results of using $FeSO_4$ for saline stress amelioration.

Data availability

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

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Author contributions

MW; Experimentation and Methodology, NA; Supervision and Validation, ZuN & MYA; writing-original draft preparation and Statistical analysis, SU & AAS; Conceptualization, Data curation and Formal analysis, VR & MAE; Resource acquisition and writing-revised draft preparation. All authors read and approved the final manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Ethical approval and consent to participate

We declare that the manuscript reporting studies do not involve any human participants, human data or human tissues. So, it is not applicable.
Our experiment follows with the relevant institutional, national, and international guidelines and legislation.

Additional information

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