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Application of ferrous sulfate alleviates negative impact of cadmium in rice (*Oryza sativa* L.)

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Abstract: Soil contamination with toxic heavy metals [such as cadmium (Cd)] is becoming a serious global problem due to rapid development of social economy. Iron (Fe), being an important element, has been found effective in enhancing plant tolerance against biotic and abiotic stresses. The present study investigated the extent to which different levels of Ferrous sulphate ($FeSO_4$) modulated the Cd tolerance of rice (*Oryza sativa* L.), when maintained in artificially Cd spiked regimes. A pot experiment was conducted under controlled conditions for 146 days, by using natural soil, mixed with different levels of $CdCl_2$ [0 (no Cd), 0.5 and 1 mg/kg] together with the exogenous application of $FeSO_4$ at [0 (no Fe), 1.5 and 3 mg/kg] levels to monitor different growth, gaseous exchange characteristics, oxidative stress, antioxidative responses, minerals accumulation, organic acid exudation patterns of *O. sativa*. Our results depicted that addition of Cd to the soil significantly ($P < 0.05$) decreased plant growth and biomass, gaseous exchange parameters, mineral uptake by the plants, sugars (soluble, reducing, and non-reducing sugar) and altered the ultrastructure of chloroplasts, plastoglobuli, mitochondria, and many other cellular organelles in Cd-stressed *O. sativa* compared to those plants which were grown without the addition of Cd in the soil. However, Cd toxicity boosted the production of reactive oxygen species (ROS) by increasing the contents of malondialdehyde (MDA), which is the indication of oxidative stress in *O. sativa* and was also manifested by hydrogen peroxide (H_2O_2) contents and electrolyte leakage to the membrane bounded organelles. Although, activities of various antioxidative enzymes like superoxidase dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) and non-enzymatic antioxidants like phenolics, flavonoid, ascorbic acid, anthocyanin and proline contents increased up to a Cd level of 0.5 mg/kg in the soil but were significantly diminished at the highest Cd level of 1 mg/kg in the soil compared to those plants which were grown without the addition of Cd in the soil. The negative impacts of Cd injury were reduced by the application of $FeSO_4$ which increased plant growth and biomass, improved photosynthetic apparatus, antioxidant enzymes,

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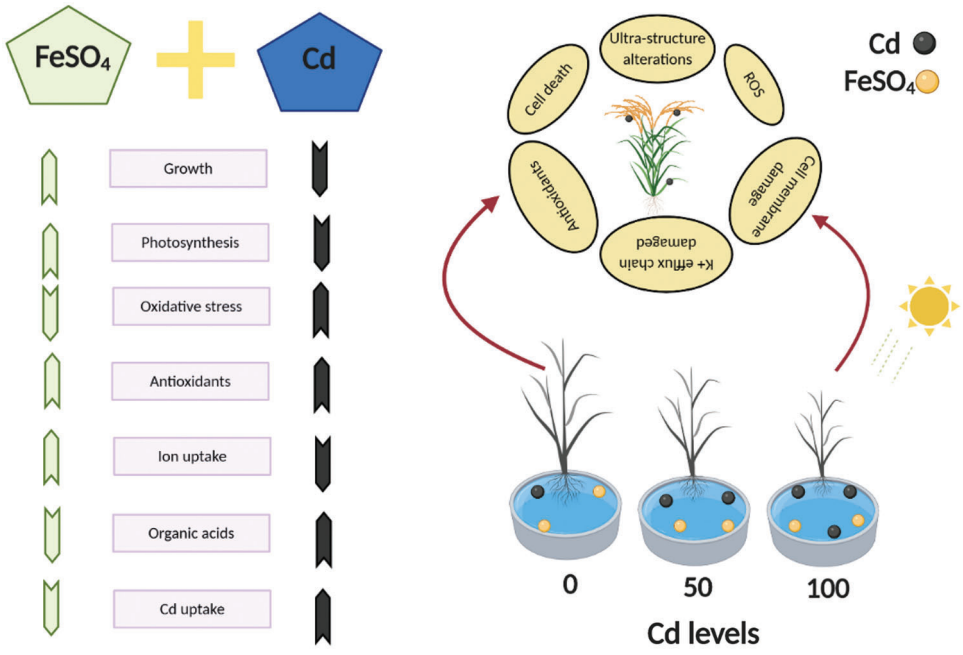
minerals uptake together with diminished exudation of organic acids as well as oxidative stress indicators in roots and shoots of *O. sativa* by decreasing Cd retention in different plant parts. These results shed light on the effectiveness of $FeSO_4$ in improving the growth and upregulation of antioxidant enzyme activities of *O. sativa* in response to Cd stress. However, further studies at field levels are required to explore the mechanisms of $FeSO_4$-mediated reduction of the toxicity of not only Cd, but possibly also other heavy metals in plants.



Introduction

Soils may become contaminated by the accumulation of heavy metals and metalloids through emissions from the rapidly expanding industrial areas, mine tailings, disposal of high metal wastes, leaded gasoline and paints, land application of fertilizers, animal manures, sewage sludge, pesticides, wastewater irrigation, coal combustion residues, spillage of petrochemicals, and atmospheric deposition (Imran *et al.*, 2020a; Riaz *et al.*, 2020; Saleem *et al.*, 2020a; Zaheer *et al.*, 2020b). Soils are the major sink for heavy metals released into the environment by aforementioned anthropogenic activities and unlike organic contaminants which are oxidized to carbon (IV) oxide by microbial action, most metals do not undergo microbial or chemical degradation (Rehman *et al.*, 2019b; Saleem *et al.*, 2020b), and their total concentration in soils persists for a long time after their introduction (Khan *et al.*, 2015; Nagajyoti *et al.*, 2010). Heavy metal contamination of soil may pose risks and hazards to humans and the ecosystem through: direct ingestion or contact with contaminated soil, the food chain (soil-plant-human or soil-plant-animal human), drinking of contaminated ground water, reduction in food quality (safety and marketability) via phytotoxicity, reduction in land usability for agricultural production causing food insecurity, and land tenure problems (Imran *et al.*, 2020b; Kamran *et al.*, 2020; Rehman *et al.*, 2020a). Contamination of agricultural soils with cadmium (Cd) has become one of the most toxic and widespread environmental problems (El-Esawi *et al.*, 2020; Javed *et al.*, 2020). In plants, excess Cd typically causes direct or indirect inhibition of various physiological processes, such as respiration, transpiration, photosynthesis, oxidative stress, cell elongation, nitrogen metabolism and uptake of mineral nutrition, finally resulting in growth retardation, leaf chlorosis and reduced biomass (Rizwan *et al.*, 2016a; Rizwan *et al.*, 2016b). In the case of Cd stress, the plant has involved several strategies that can resort to a number of defense systems, such as: (1) immobilization; (2) exclusion; (3) synthesis of phytochelatins; (4) compartmentalization; (5) synthesis of metallothioneins; (6) synthesis of stress proteins; (7) production of stress ethylene (Abbas *et al.*, 2020; Adrees *et al.*, 2020). Due to its persistent nature, Cd is ranked as the seventh most toxic heavy metal out of 20 metals, and acceptable concentrations in crops range from 0.013 to 0.22 mg/kg for cereal crops, 0.07–0.27 mg/kg for fodder and 0.08–0.28 mg/kg for leguminous plants (Javed *et al.*, 2017; Madhu and Sadagopan, 2020). Moreover, Cd influx in plant cells occurs via ion specific channels and other proteins that mediate transport of ions at the plasmalemma (Kong *et al.*, 2020; Wen *et al.*, 2020). Cd reduces the photosynthetic capacity of plants by devastating the enzymes of Calvin cycle and carbohydrate metabolism and, also, modulates the plant's antioxidant machinery (Ji *et al.*, 2017; Shahid *et al.*, 2019; Shahid *et al.*, 2020; Wang *et al.*, 2019). All these physiological changes result in decreased plant yield. In addition, Cd is indirectly involved in the biological redox reaction, the oxidative burst is produced by increasing the activity of NADPH oxidases, which results in production of extracellular super oxide, peroxide, and intracellular lipid

peroxidation (Javed *et al.*, 2019; Saleemi *et al.*, 2019). Higher metal concentration in plants cause ultra–structural alterations (Parveen *et al.*, 2020; Saleem *et al.*, 2020c; Saleem *et al.*, 2020e), oxidative stress in plants and increased electrolyte leakage (EL), malondialdehyde (MDA) concentrations, whereas induced alterations in antioxidant enzyme activities such as superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) and ascorbate peroxidase (APX) (Saleem *et al.*, 2020g; Saleem *et al.*, 2020h; Saleem *et al.*, 2020j). The uptake of mineral nutrients is changed by Cd exposure which in turn may disturb biochemical structures. However, some nutrients (Ca, Fe, Zn, Si, etc.) have been found to counteract the toxic effects of Cd stress in plants through the production of phytochelatins (Shahid *et al.*, 2019; Tanwir *et al.*, 2015; Ullah *et al.*, 2016). Hence, it is immensely required to safeguard plant from Cd toxicity to counter the phytotoxicity and oxidative stress triggered by the uptake of Cd in plant.

Different practices have been used for the management of Cd contaminated soils and its reduction in crop plants (Anwar *et al.*, 2017; Bashir *et al.*, 2018). Iron (Fe) plays an important role in the physiological processes of plants, and it is necessary for metabolic reactions in organelles, such as pigment synthesis, respiratory, and chains of electron transport in photosynthesis (Ghasemi *et al.*, 2014; Zaheer *et al.*, 2020b). Diverse approaches have been used to reduce Cd uptake and toxicity in crops, including screening for cultivars, irrigation, application of soil amendments, and fertilizers (Abbas *et al.*, 2020; Ali *et al.*, 2015; Anwar, 2019). The application of Fe provides a direct and effective approach for reducing Cd uptake and toxicity in crops (Ghasemi *et al.*, 2014; Zaheer *et al.*, 2020c). Fe and Cd are closely correlated in crops because of their similar chemical properties and entry route. Many studies have characterized the effects of Fe in crops under Cd stress, especially on photosynthetic capacity and the antioxidative system (Li *et al.*, 2019; Wen *et al.*, 2020). For instance, the application of Fe enhanced the yield, photosynthetic rate, and antioxidant enzyme activities of plants under Cd toxicity (Bashir *et al.*, 2018; Hussain *et al.*, 2019). Previous studies showed that Fe can efficiently be used as an effective amendment in reducing Cd and other metal toxicity in different plant species (Bashir *et al.*, 2018; Rizwan *et al.*, 2016b; Zaheer *et al.*, 2020c). A report suggested by Bashir *et al.* (Bashir *et al.*, 2018) that foliar application of Fe significantly increased plant growth and biomass, biochemical and physiological attributes in *O. Sativa* grown in Cr stress environment. However, the underlying mechanisms of Cd reduction by different Fe fertilizers are poorly understood.

Rice (*Oryza sativa* L.) is a cereal grain, it is the most widely consumed staple food for a large part of the world's human population, especially in Asia and Africa. It is the agricultural commodity with the third-highest worldwide production (*O. sativa*, 741.5 million ton in 2014), after *Saccharum officinarum* (1.9 billion ton) and *Zea mays* (1.0 billion ton) (Faostat, 2018). *O. sativa* cultivation is well-suited to countries and regions with low labor costs and high rainfall, as it is labor-intensive to cultivate and requires ample water. However, rice can be grown practically anywhere, even on a steep hill or mountain area with the use of water-controlling terrace systems. Although its parent species are native to Asia and certain parts of Africa, centuries of trade and exportation have made it commonplace in many cultures worldwide (Afzal *et al.*, 2018; Bashir *et al.*, 2018). Archaeologists focusing on East and Southeast Asia argue that *O. sativa* farming began in south-central China along the Yangtze River and China is one of the leading countries for rice production (Afzal *et al.*, 2018; Vaughan *et al.*, 2008). Cd is one of the main pollutants in paddy fields, and its accumulation in *O. sativa* and subsequent transfer to food chain is a global environmental issue (Bashir *et al.*, 2018; Zhao *et al.*, 2019). Although, it was also reported that Cd can be readily taken up by *O. sativa* and translocated to shoot and then to grains (Rizwan *et al.*, 2016b). Thus, Cd can enter into the food chain through *O. sativa* consumption, even at low Cd concentrations in the soils, and cause toxicities to humans. During recent years, certain heavy metals have received considerable attention on plant morphology and physiology owing to increasing environmental exposure which also likely to have negative impact on cereal crops including *O. sativa*. Previously, few studies on *O. sativa* were executed to investigate its morphology and physiology under metal stress (Afzal *et al.*, 2018; Chen *et al.*, 2019; Imran *et al.*, 2020a; Zhao *et al.*, 2019), but synergistic application of Fe on various morpho-physiological characteristics, ionomics and organic acid exudation potential of *O. sativa* was rarely investigated under Cd stressed soil. It was hypothesized that Fe application will confer growth enhancement to Cd stressed *O. sativa* by maintaining optimum levels of organic acid production and exudation. Therefore, the present study was conducted to study (i) the effect of different levels of $FeSO_4$ on plant growth, biomass, and gaseous exchange parameters of *O. sativa* under Cd stress, (ii) oxidative stress and response of different antioxidative enzymes, (iii) essential minerals uptake and organic acids exudation and their relationship with Cd accumulation in different organs of *O. sativa* under Cd stress.

Materials and Methods

Experimental designed and growth conditions

Our earlier work described that, from ten genotypes of rice (*Oryza sativa* L.) under same levels of Cd toxicity, 'Lu 9803' was more resistant to Cd toxicity in hydroponic culture experiment (Afzal *et al.*, 2018). The seeds of Lu 9803 were collected from Hubei Tianmen Di Long Seed Industry Co., Ltd., Hubei, China and the seeds were surface-sterilized with 10% (v/v) commercial bleach for 15 min followed by a thorough washing in distilled water. The seeds were germinated in a small box and placed in growth chamber under white lights (100 W, Guangdong PHILIPS Co., Guangdong, China) with a day/night temperature of 25 ± 2°C and day/night humidity of 80%. After 14 days of seed sowing, uniform sized seedlings were shifted to the pots (6 kg/pot). Uncontaminated soil, obtained from the research fields at Huazhong Agricultural University, was air dried and passed through a 5-mm sieve. The physicochemical properties of the soil used for the pot experiment were as follows: pH 7.12, available potassium 190 mg/kg, organic

matter 13.72 g/kg, olsen-P, 17.07 mg/kg, alkaline hydrolysis nitrogen 54.13 mg/kg and total Cd, 0.072 mg/kg. The soil was artificially treated with Cd at various concentrations, i. e., 0 (no Cd), 0.5, and 1 mg/kg using $CdCl_2$. For iron (Fe) application, stock solution was made from 15 g of $FeSO_4.7H_2O$ and 1 L of distilled water and Fe was supplied at 0 (no Fe), 1.5 and 3 mg/kg which were defined as low, moderate, and high Fe doses, respectively and distilled water was supplied on the control plants. After adding the Cd, the pots were equilibrated for 2 months with four cycles of saturation with distilled water and air drying. A complete randomized design (CRD) with three replications was used. This experiment was conducted in a glass house, under controlled conditions at the experimental station of Huazhong Agricultural University, Wuhan, China (114.20°E, 30.28°N; 50 m above sea level). The plants received natural light with day/night temperatures of 35/30°C and day/night humidity of 70/80%. All pots were monitored daily and weeding and metal-free water were provided as needed and every pot had one hole at the bottom to assimilate moisture. All plants in the glass house territory received natural light, with day/night temperature of 25/30°C and day/night humidity of 60/70%. The total time for plant growth was 146 days (ripening phase). Although, this experiment was conducted in pots, but for the collection of organic acids, two seedlings were transferred to the rhizoboxes which consist of plastic sheet, nylon net and wet soil (Javed *et al.*, 2013). After 48 h, plants were taken from the rhizoboxes and the roots were washed with redistilled water to collect the exudates from root surface. The samples were filtered through a 0.45 μm filter (MillexHA, Millipore) and collected in Eppendorf tubes (Greger and Landberg, 2008). The collected samples were mixed with NaOH (0.01 M) in order to analyze the organic acids. However, the samples used for analysis of oxalic acid were not treated with NaOH (Javed *et al.*, 2013). All chemicals used were of analytical grade and procured from Sinopharm Chemical Reagent Co., Ltd., China.

Morphological traits and data collection

The plants were harvested in November 2018 (146 days after seed transfer) for the analysis of various morphological parameters. Leaves from each treatment group were picked for chlorophyll, carotenoid, and antioxidant analysis. A fully functional leaf was harvested for the various enzymatic and pigment studies. The leaves were washed with distilled water, placed in liquid nitrogen, and stored at −80°C for further analysis. The plants from each treatment were washed with tap water to remove debris and waste, and then with distilled water. The morphological measurements such as plant height, root length, plant fresh weight and plant dry weight were noticed at three different growth stages i.e., tillering stage (38 days old), vegetative stage (90 days old), and maturity stage (146 days old). Plant height was measured from the top of the leaf tips to the bottom of the roots and root length was also measured in the same way. Plant fresh weight was determined by measuring the weight of plant with a digital weighting balance. The plant samples were oven-dehydrated at 65°C for 72 h for Cd and ions concentration determination and the total plant dry weight was also measured. Yield parameters such as biological yield, 1,000 grain weight, straw yield, grain yield, relative growth rate and harvest index were also measured after the harvesting all the sampled plants at maturity stage (146 days old). Before oven dried, roots immersed in 20 mM Na_2EDTA for 15–20 min to remove Cd adhered to the surface of roots. Then, roots were washed thrice with distilled water and finally once with de-ionized water and dried for further analysis.

Determination of photosynthetic pigments and gas exchange parameters

Leaves were collected for determination of chlorophyll and carotenoid contents. For chlorophylls, 0.1 g of fresh leaf sample was extracted with 8 mL of 95% acetone for 24 h at 4°C in the dark. The absorbance was measured by a spectrophotometer (UV-2550; Shimadzu, Kyoto, Japan) at 646.6, 663.6, and 450 nm. Chlorophyll content was calculated by the standard method of Arnon (1949).

Gas exchange parameters were also measured during the same days. Net photosynthesis (*Pn*), leaf stomatal conductance *(Gs)*, transpiration rate (*Ts*), and intercellular carbon dioxide concentration (*Ci*) were measured from three different plants in each treatment group. Measurements were conducted between 11:30 and 13:30 on days with clear sky. Rates of leaf *Pn*, *Gs, Ts*, and *Ci* were measured with a LI-COR gas-exchange system (LI-6400; LI-COR Biosciences, Lincoln, NE, USA) with a red-blue LED light source on the leaf chamber. In the LI-COR cuvette, CO_2 concentration was set as 380 mmol/mol and LED light intensity was set at 1000 mmol/m^2 s, which is the average saturation intensity for photosynthesis in *O. sativa* (Austin, 1990).

Determination of oxidative stress indicators

The degree of lipid peroxidation was evaluated as malondialdehyde (MDA) contents. Briefly, 0.1 g of frozen leaves were ground at 4°C in a mortar with 25 mL of 50 mM phosphate buffer solution (pH 7.8) containing 1% polyethene pyrrole. The homogenate was centrifuged at 10,000 × *g* at 4°C for 15 min. The mixtures were heated at 100°C for 15–30 min and then quickly cooled in an ice bath. The absorbance of the supernatant was recorded by using a spectrophotometer (xMark™ Microplate Absorbance Spectrophotometer; Bio-Rad, USA) at wavelengths of 532, 600, and 450 nm. Lipid peroxidation was expressed as l mol/g by using the formula: 6.45 (A532-A600)-0.56 A450. Lipid peroxidation was measured by using a method previously published by Heath and Packer (1968).

To estimate H_2O_2 content of plant tissues (root and leaf), 3 mL of sample extract was mixed with 1 mL of 0.1% titanium sulfate in 20% (v/v) H_2SO_4 and centrifuged at 6000 × g for 15 min. The yellow color intensity was evaluated at 410 nm. The H_2O_2 level was computed by extinction coefficient of 0.28/mmol cm. The contents of H_2O_2 were measured by the method presented by Jana and Choudhuri (1981).

Stress-induced electrolyte leakage (EL) of uppermost stretched leaves was determined by using methodology of Dionisio-Sese and Tobita (1998). The leaves were cut into minor slices (5 mm length) and placed in test tubes having 8 mL distilled water. These tubes were incubated and

transferred into water bath for 2 h prior to measuring the initial electrical conductivity (EC_1). The samples were autoclaved at 121°C for 20 min, and then cooled down to 25°C before measuring the final electrical conductivity (EC_2). Electrolyte leakage was calculated as by the following formula:

$$EL = (EC_1/EC_2) \times 100$$

Determination of antioxidant enzyme activities

To evaluate enzyme activities, fresh leaves (0.5 g) were homogenized in liquid nitrogen and 5 mL of 50 mmol sodium phosphate buffer (pH 7.0) including 0.5 mmol EDTA and 0.15 mol NaCl. The homogenate was centrifuged at 12,000 × *g* for 10 min at 4°C, and the supernatant was used for measurement of superoxidase dismutase (SOD) and peroxidase (POD) activities. SOD activity was assayed in 3 mL reaction mixture containing 50 mM sodium phosphate buffer (pH 7), 56 mM nitro blue tetrazolium, 1.17 mM riboflavin, 10 mM methionine, and 100 µL enzyme extract. Finally, the sample was measured by using a spectrophotometer (xMark™ Microplate Absorbance Spectrophotometer; Bio-Rad). Enzyme activity was measured by using a method by Chen and Pan (1996) and expressed as U/g FW.

POD activity in the leaves was estimated by using the method of Sakharov and Ardila (1999) by using guaiacol as the substrate. A reaction mixture (3 mL) containing 0.05 mL of enzyme extract, 2.75 mL of 50 mM phosphate buffer (pH 7.0), 0.1 mL of 1% H_2O_2, and 0.1 mL of 4% guaiacol solution was prepared. Increases in the absorbance at 470 nm because of guaiacol oxidation was recorded for 2 min. One unit of enzyme activity was defined as the amount of the enzyme.

Catalase (CAT) activity was analyzed according to Aebi (1984). The assay mixture (3.0 mL) was comprised of 100 µL enzyme extract, 100 µL H_2O_2 (300 mM) and 2.8 mL 50 mM phosphate buffer with 2 mM ETDA (pH 7.0). The CAT activity was measured from the decline in absorbance at 240 nm as a result of H_2O_2 loss (ε = 39.4 mM/cm).

Ascorbate peroxidase (APX) activity was measured according to Nakano and Asada (1981). The mixture containing 100 µL enzyme extract, 100 µL ascorbate (7.5 mM), 100 µL H_2O_2 (300 mM), and 2.7 mL 25 mM potassium phosphate buffer with 2 mM EDTA (pH 7.0) was used for measuring APX activity. The oxidation pattern of ascorbate was estimated from the variations in wavelength at 290 nm (ε = 2.8 mM/cm).

Determination of non-enzymatic antioxidants, sugars, and proline contents

Plant ethanol extracts were prepared for the determination of non-enzymatic antioxidants and some key osmolytes. For this purpose, 50 mg of plant dry material was homogenized with 10 mL ethanol (80%) and filtered through Whatman No. 41 filter paper. The residue was re-extracted with ethanol and the two extracts were pooled together to a final volume of 20 mL. The determination of flavonoids (Pękal and Pyrzynska, 2014), phenolics (Bray and Thorpe, 1954), ascorbic acid (Azuma *et al.*, 1999), anthocyanin (Lewis *et al.*, 1998) and total sugars (Dubois *et al.*, 1956) was performed from the extracts.

Fresh leaf material (0.1 g) was mixed thoroughly in 5 mL aqueous sulfosalicylic acid (3%). The mixture was centrifuged at 10000 × g for 15 min and aliquot (1 mL) was poured into a test tube having 1 mL acidic ninhydrin and 1 mL glacial acetic acid. The reaction mixture was first heated at 100°C for 10 min and then cooled in an ice bath. The reaction mixture was extracted with 4 mL toluene and test tubes are vortexed for 20 s and cooled. Thereafter, the light absorbance at 520 nm was measured by using UV–VIS spectrophotometer (Hitachi U-2910, Tokyo, Japan). The free proline content was determined on the basis of standard curve at 520 nm absorbance and expressed as µmol/g FW (Bates *et al.*, 1973).

Determination of nutrient contents

For nutrient analysis, plant roots and shoots were washed twice in redistilled water, dipped in 20 mM EDTA for 3 s and then, again washed with deionized water twice for the removal of adsorbed metal on plant surface. The washed samples were then oven dried for 24 h at 105°C. The dried roots and shoots were digested by using wet digestion method in HNO_3:$HClO_4$ (7:3 V/V) until clear samples were obtained. Each sample was filtered and diluted with redistilled water up to 50 mL. The root and shoot contents of Fe, Mg, and K and were analyzed by using Atomic Absorption Spectrophotometer (AAS) model Agilent 240FS-AA.

Root exudates analysis and Cd contents

In order to determine the concentration of organic acids, freeze dried exudates were mixed with ethanol (80%) and 20 µL of the solutions was injected into C18 column (Brownlee Analytical C-183 µm; length 150 mm × 4.6 mm^2, USA). Quantitative analysis of organic acids in root exudates was executed with high performance liquid chromatography (HPLC) having a Flexer FX-10 UHPLC isocratic pump (PerkinElmer, MA, USA). The mobile phase used in HPLC was comprised of acidic solution of aceto-nitrile containing aceto-nitrile: H_2SO_4:acetic acid in ratios of 15:4:1 respectively, and pH of 4.9. The samples were analyzed at a flow rate of 1.0 mL/min for a time period of 10 min. The inner temperature of the column was fixed at 45°C and quantification of organic acids was carried out at 214 nm wavelength with the help of a detector (UV–VIS Series 200, USA) as described by Uddin *et al.* (2015). Freeze-dried samples were dissolved in redistilled water and the pH of the exudates was recorded with LL micro-pH glass electrode by using a pH meter (ISTEK Model 4005–08007 Seoul, South Korea).

Finely ground samples were digested with pure HNO_3 at 190°C for 45 min (10 min pre-heating, 15 min heating, 20 min cooling) in a microwave oven (Mars 6, CEM Corporation, USA) with the settings described in details by Jezek *et al.* (2015). Samples were diluted with 2% HNO_3 and determined by atomic absorption spectrophotometer (AAS) model Agilent 240FS-AA.

Transmission electron microscopy (TEM)

For TEM, leaf samples were collected and placed in liquid nitrogen. Small sections of the leaves (1–3 mm in length) were fixed in 4% glutaraldehyde (*v/v*) in 0.2-mol/L SPB (sodium phosphate buffer, pH 7.2) for 6–8 h and post-fixed in 1% OsO_4 for 1 h, then in 0.2-mol/L SPB (pH 7.2) for 1–2 h. Samples were dehydrated in a graded ethanol series (50%, 60%, 70%, 80%, 90%, 95% and 100%) followed by acetone,

filtered and embedded in Spurr resin. Ultra-thin sections (80 nm) were prepared and mounted on copper grids for observation under a transmission electron microscope (JEOL TEM-1200EX) at an accelerating voltage of 60.0 kV or 80.0 kV.

Statistical analysis

Statistical analysis of data was performed with analysis of variance (ANOVA) by using a statistical program Co-Stat version 6.2, Cohorts Software, 2003, Monterey, CA, USA. All the data obtained was tested by two-way analysis of variance (ANOVA). Thus, the differences between treatments were determined by using ANOVA, and the least significant difference test ($P < 0.05$) was used for multiple comparisons between treatment means. Logarithmic or inverse transformations were performed for data normalization, where necessary, prior to analysis. Pearson's correlation analysis was performed to quantify relationships between various analyzed variables. The graphical presentation was carried out by using Origin-Pro 2017. The Pearson correlation (heat-map) coefficients between the measured variables of *O. sativa* were also calculated using the RStudio software.

Results and Discussion

Effect of toxic levels of Cd stress on plant growth and biomass under various application levels of $FeSO_4$

Heavy metal contamination of the environment through anthropogenic activities and/or natural processes is a widespread and serious problem (Saleem *et al.*, 2020d; Saleem *et al.*, 2019a; Saleem *et al.*, 2020f). Heavy metals occur in various forms in soil, which differ greatly with respect to their solubility/bioavailability. The geochemical behavior of heavy metals in soil, their uptake by plants, and effect on crop productivity is affected by various physicochemical properties of soil (Hashem *et al.*, 2020; Saleem *et al.*, 2020i; Saleem *et al.*, 2020k). Although, the plants grown in soil containing high levels of Cd show visible symptoms of injury reflected in terms of chlorosis, growth inhibition, browning of root tips and finally death (Afzal *et al.*, 2018; Imran *et al.*, 2020a). The inhibiting effect of Cd on fresh and dry mass accumulation, height, root length, leaf area, and other biometric parameters of plants are reported in almost all investigations. Differences in the degree of expressed phytotoxicity due to various Cd-concentrations applied to the root medium, the duration of treatment, as well as the characteristics of species and cultivars were established (Madhu and Sadagopan, 2020; He *et al.*, 2017). Cd inhibited growth of most plant species (El-Esawi *et al.*, 2020; Imran *et al.*, 2020a; Javed *et al.*, 2020). In the present study, we also noticed the same pattern, i.e., plant height, root length, plant fresh weight, plant dry weight at different growth stages (Fig. 1) (tillering, vegetative and maturity stage) and various yield parameters i.e., biological yield, 1,000 grain weight, straw yield, grain yield, relative growth rate and harvest index (Fig. 2) were

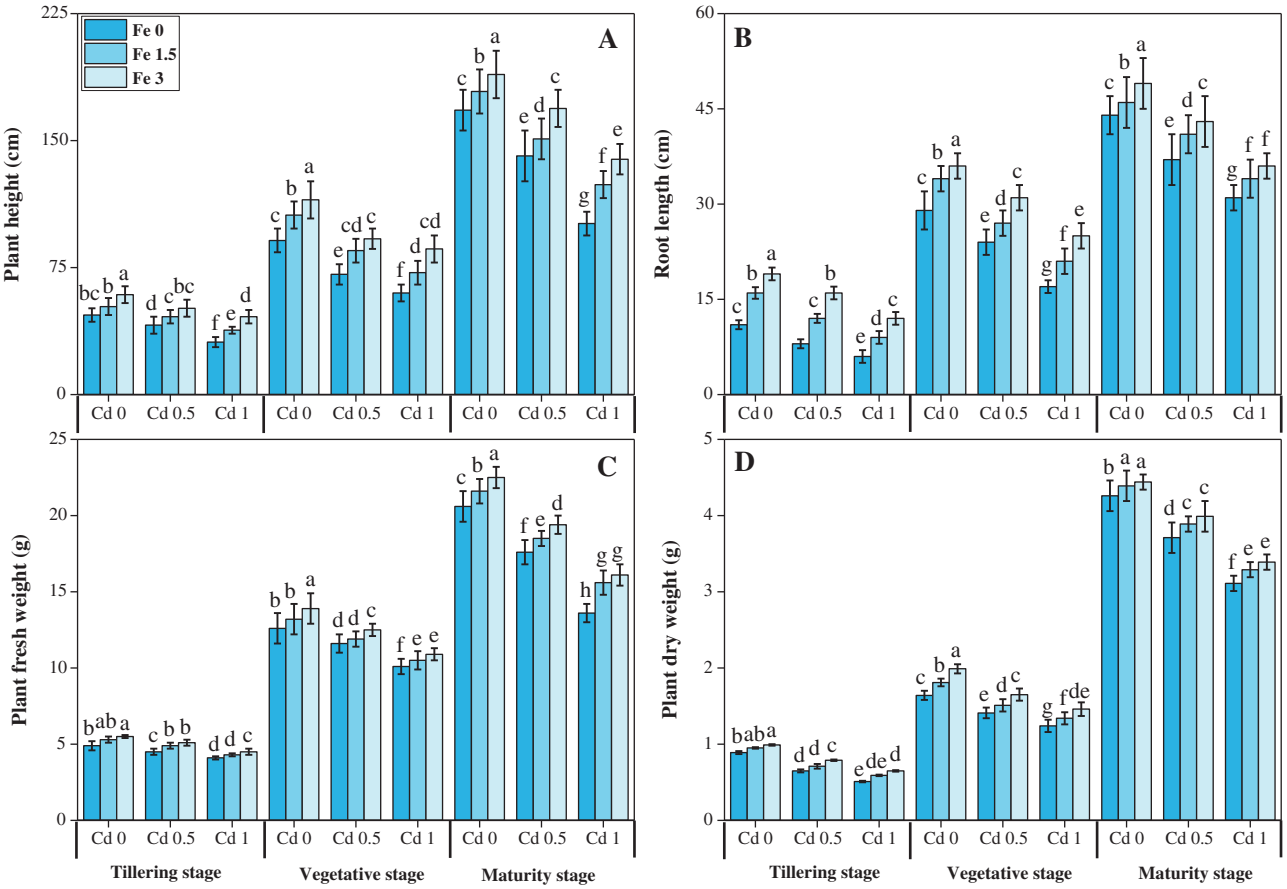


FIGURE 1. Effect of different concentrations of exogenous application of $FeSO_4$ (0, 1.5 and 3 mg/kg) on morphological attributes, i.e., total plant height (A), root length (B), plant fresh weight (C) and plant dry weight (D) of *Oryza sativa* grown under different levels of Cd stress (0, 0.5 and 1 mg/kg). Bars sharing different letter(s) for each parameter are significantly different from each other according to Duncan's multiple range test ($P < 0.05$). All the data represented is the average of three replications (n = 4). Error bars represent standard deviation (SD) of three replicates.

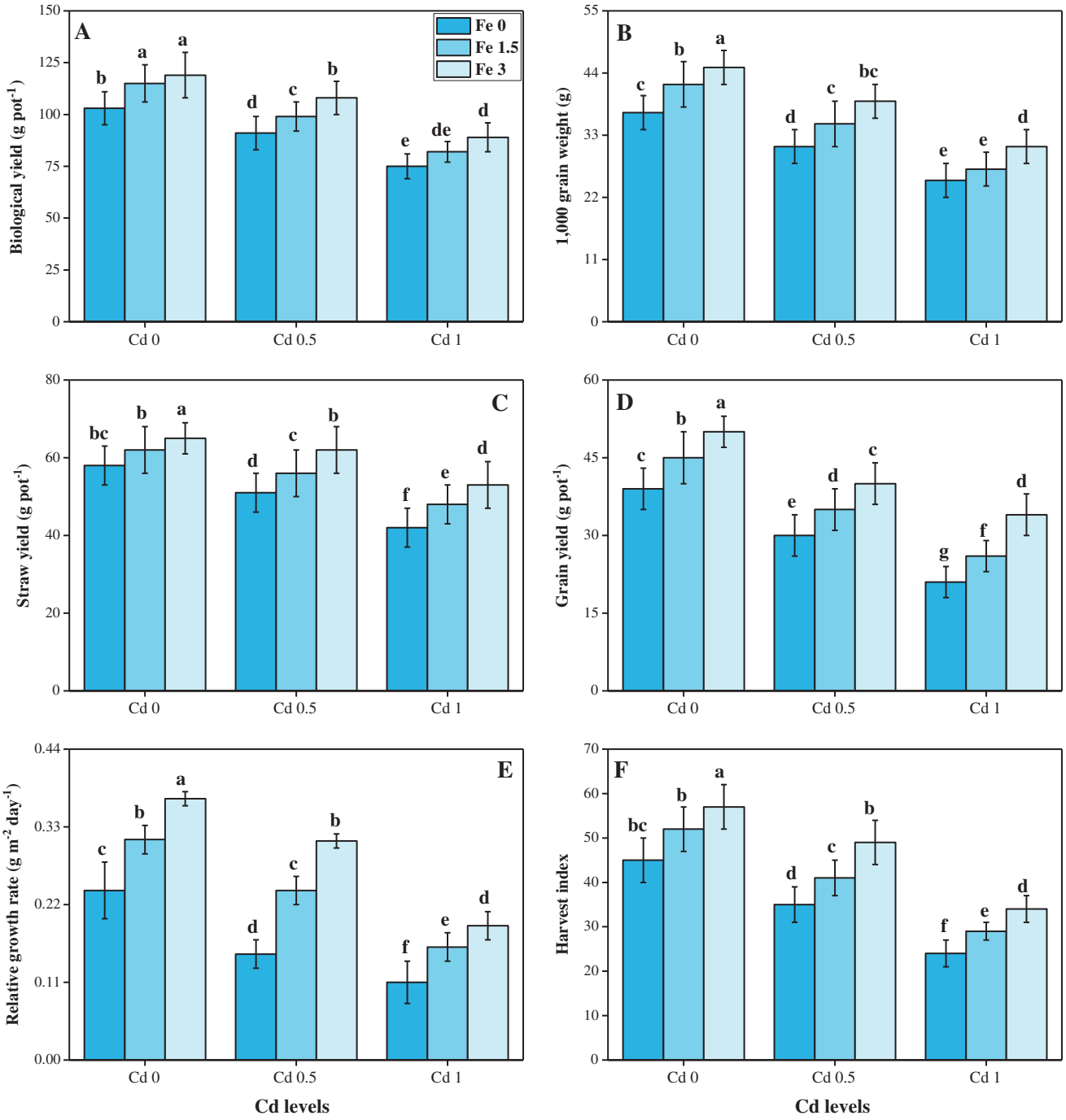


FIGURE 2. Effect of different concentrations of exogenous application of $FeSO_4$ (0, 1.5 and 3 mg/kg) on yield attributes, i.e., biological yield (A), 1,000 grain weight (B), straw yield (C), grain yield (D), relative growth rate (E) and harvest index (F) of *Oryza sativa* grown under different levels of Cd stress (0, 0.5 and 1 mg/kg). Bars sharing different letter(s) for each parameter are significantly different from each other according to Duncan's multiple range test ($P < 0.05$). All the data represented is the average of three replications (n = 4). Error bars represent standard deviation (SD) of three replicates.

also decreased with the addition of Cd in the soil. The maximum growth and yield parameters were observed in the plants which were grown in the control treatment, while increasing Cd concentration in the soil, induced a significant decreased in various growth, biomass, and yield parameters. Although, growth and yield-related attributes in *O. sativa* can be increased the exogenous application of $FeSO_4$, which non-significantly ($P < 0.05$) increased all these parameters, compared to those plants which were grown without the application of $FeSO_4$ (Figs. 1 and 2). This is because of Fe is required by plants for their normal growth parameters and it has been found beneficial regarding its role as reducing heavy metals toxicity in various plants (Danish *et al.*, 2019a; Kobayashi *et al.*, 2019; Zaheer *et al.*, 2020b). This is also because Fe enhances protein properties (metabolic function and stock of amino acid function in plants), increases the photosynthetic processes for creating a healthy plant and provides a substantial growth in a short time in the stress condition or even in the plants grown in a normal soil (Ghasemi *et al.*, 2014; Zaheer *et al.*, 2020c).

Effect of toxic levels of Cd stress on photosynthetic pigments and gaseous exchange attributes under various application levels of $FeSO_4$

Photochemical efficiency, chlorophyll content, and photosynthetic intensity are considered as sensitive indicators for the growth status of plants under Cd stress (Abbas *et al.*, 2020; Rizwan *et al.*, 2016a). Studies have shown that Cd is an effective inhibitor of photosynthesis (Bashir *et al.*, 2018; He *et al.*, 2017). Cd, in fact, strongly inhibits the synthesis of chlorophylls and their stable binding to proteins, thereby damaging the photosynthetic apparatus, in particular decreasing light-harvesting complex II and photosystems I (PSI) and II (PSII), and inhibiting some of the enzymes of the Calvin cycle (Hoseini and Zargari, 2013; Rizwan *et al.*, 2019b). Cd can bind competitively to the essential Ca-binding sites in PSII during the photoactivation of the water-splitting system, and direct inhibition of oxygen evolution is also possible (Ali *et al.*, 2015; Chen *et al.*, 2011). Ferrous sulfate ($FeSO_4$) also known as copperas or black alum, common iron fertilizer for flowers and trees, it is easy to dissolve in water, can promote the chlorophyll formation, make color Nonglv and full of luster, effectively prevent the plant yellows due to iron deficiency, but also to regulate the soil acidity regulator, long-term use can change soil acidity and salinity, ensure that keep the soil acidity (Di Palma *et al.*, 2015). In the present study, we have noticed that chlorophyll contents, carotenoid contents, Net photosynthesis (*Pn*), leaf stomatal conductance *(Gs)* and transpiration rate (*Ts*) were decreased with the increasing levels of Cd concentration in the soil, compared to the control treatment. Although, intercellular carbon dioxide concentration (*Ci*) showed non-significant results at all levels of Cd and $FeSO_4$ (Fig. 3). The photosynthetic pigments and gas exchange attributes in Cd-stressed can be increased by the application of $FeSO_4$, which non-non-significantly ($P < 0.05$) increased photosynthetic pigments and gas exchange attributes compared to those plants which were grown without the application of $FeSO_4$ (Fig. 3). These findings might be explained by the fact that Fe plays a critical role in metabolic reactions in organelles, such as pigment synthesis and chains of electron transport in photosynthesis (Wang *et al.*, 2020). In addition, these findings revealed that photosynthetic capacity in response to Fe fertilizers depended on Fe types. This difference may be explained by the fact that Fe may hamper leaf penetration and movement in the apoplast because of the negatively charged plant cuticles and cell walls (Ghasemi *et al.*, 2014; Wang *et al.*, 2020; Zaheer *et al.*, 2020b). A previous study has demonstrated that Fe can increase photosynthetic pigments and gas exchange attributes under Cd stress (Bashir *et al.*, 2018).

Effect of toxic levels of Cd stress on oxidative stress and antioxidant response under various application levels of $FeSO_4$

In normal plant growth, reactive oxygen species (ROS) are produced during respiration and photosynthesis as a by-product and act as a signaling molecule that controls various processes in plants such as programmed cell death (Alam *et al.*, 2020; Rana *et al.*, 2020a). Although, stress conditions can disturb the dynamic equilibrium of production and elimination under normal growth in plants (Rana *et al.*, 2020c; Saleem *et al.*, 2019b), which promotes ROS accumulation, membrane lipid peroxidation, and disrupt the structure and function of cell membrane system (Ali *et al.*, 2020a; Kamran *et al.*, 2019; Mohamed *et al.*, 2020; Rehman *et al.*, 2019a). Numerous reports demonstrated that Cd stress increased the ROS generation in plants, which may cause oxidative damage in plants (Adrees *et al.*, 2020; Madhu and Sadagopan, 2020; Wen *et al.*, 2020). Moreover, Cd also caused cellular damage in plants which can be examined by estimating the level of lipid peroxidation in plants by disturbing the stability of membranes (Kong *et al.*, 2020; Shahid *et al.*, 2019). Environmental stress can trigger ROS production in plants, which results in oxidative damage. However, plants possess an efficient antioxidative defense system to detoxify the ROS generation (Ali *et al.*, 2020b; Rana *et al.*, 2020b; Rehman *et al.*, 2020b). Antioxidants are involved in ROS metabolism by dismutation of superoxide anion to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) thus maintains superoxide radicals in a steady state (Yaseen *et al.*, 2020; Zaheer *et al.*, 2020a). Our results reported an increase in lipid peroxidation and oxidative stress in the roots and leaves of *O. sativa* (Fig. 4), This increase in lipid peroxidation and oxidative stress might be due to the progressive increase in soil Cd concentration, which is a stress factor triggering physiological responses in plants (Rizwan *et al.*, 2016b; He *et al.*, 2017). In fact, oxidative stress indicators showed a dose-dependent increase with the concentration of Cd in the soil (Fig. 4). In this study, enzymatic and non-enzymatic antioxidants were increased up to the level of 0.5 mg Cd/kg soil, which were decreased with further increase in Cd stress, i.e., 1 mg Cd/kg soil, compared to those plants which were grown in 0 mg Cd/kg soil (Figs. 5 and 6). The variable antioxidant response under different Cd stress conditions might be due to changes in gene expression and function of the protein in various plant tissues (El-Esawi *et al.*, 2020; Hoseini and Zargari, 2013; He *et al.*, 2017). In the present study, we also noticed the contents of sugar also decrease due to Cd exposure in the soil while proline contents were increased with the increase in Cd concentration in the soil (Fig. 6). This is because of proline accumulation in plant tissue/organs is a response to Cd toxicity, which might be associated with signal transduction and prevents membrane distortion (Abbas *et al.*, 2020; Javed *et al.*, 2020). Although, oxidative stress indicators, i.e., MDA contents, H_2O_2 initiation and EL (%) decreased in Cd-stressed *O. sativa* by increasing enzymatic (SOD, POD, CAT and APX) and non-enzymatic (phenolic, flavonoid, ascorbic acid, and anthocyanin) contents under the application of $FeSO_4$ compared to those plants which were grown without the application of $FeSO_4$. Increasing the activities of various antioxidants suggests that the plant has a better defense system, which can scavenge ROS generation (Rizwan *et al.*, 2019b; Zafar-Ul-Hye *et al.*, 2020). It is well known that the exogenous supplementation of Fe decreases oxidative stress in plants (Bashir *et al.*, 2018; Ghasemi *et al.*, 2014).

Effect of toxic levels of Cd stress on nutritional status and Cd contents under various application levels of $FeSO_4$

Essential nutrients are required for normal growth of plants. Numerous reports demonstrated that the uptake and

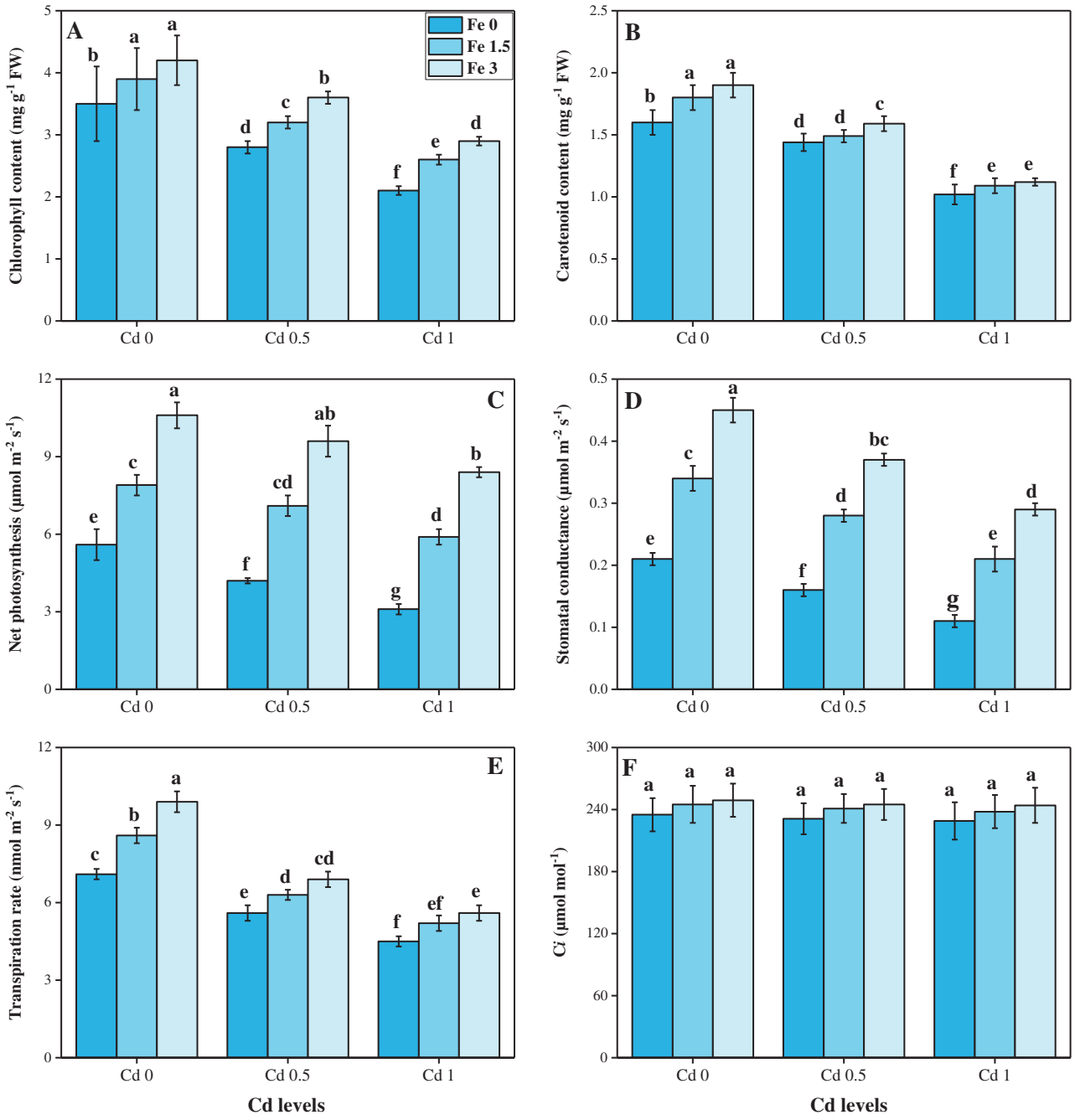


FIGURE 3. Effect of different concentrations of exogenous application of $FeSO_4$ (0, 1.5 and 3 mg/kg) on photosynthetic pigments and gaseous exchange attributes, i.e., total chlorophyll contents (A), carotenoid contents (B), net photosynthesis (C) stomatal conductance (D), transpiration rate (E) and intercellular CO_2 (F) of *Oryza sativa* grown under different levels of Cd stress (0, 0.5 and 1 mg/kg). Bars sharing different letter(s) for each parameter are significantly different from each other according to Duncan's multiple range test ($P < 0.05$). All the data represented is the average of three replications (n = 4). Error bars represent standard deviation (SD) of three replicates.

translocation of essential elements in plants were restricted under Cd stress (Rizwan *et al.*, 2016b; Rizwan *et al.*, 2019a; Rizwan *et al.*, 2019b). Excess Cd decreased the Fe, Mg and P contents in numerous plant species which may cause ions deficiency in plants. With increasing concentrations of Cd (0.5 and 1 mg/kg) in growth medium, the contents of Fe, Mg and P in the roots and shoots of *O. sativa* were decreased significantly ($P < 0.05$) when compared to those plants grown without Cd addition (Fig. 7). Similarly, Cd concentration in the roots and shoots of plants increased significantly ($P < 0.05$) by increasing the Cd concentration in the soil, compared to those plants which were grown in the without Cd addition (Fig. 8). According to the results, the contents of essential minerals (Fe, Mg and P) were slightly higher in shoots as compared to roots (Fig. 7). This might be attributed to small acropetal translocation of these elements possibly due to reduction in mineral ions accumulation as Cd and these ions compete for ZIP transporters at uptake sites (Abbas *et al.*, 2020; Javed *et al.*, 2017). Moreover, the decrease in essential ions accumulation in different organs of *O. sativa* seedlings under varying Cd concentrations might also be due to the alteration in the physiological processes such as the failure of biosynthesis of chlorophyll and carotenoid contents (Afzal *et al.*, 2018; Imran *et al.*, 2020a; Madhu and Sadagopan, 2020). The contents of

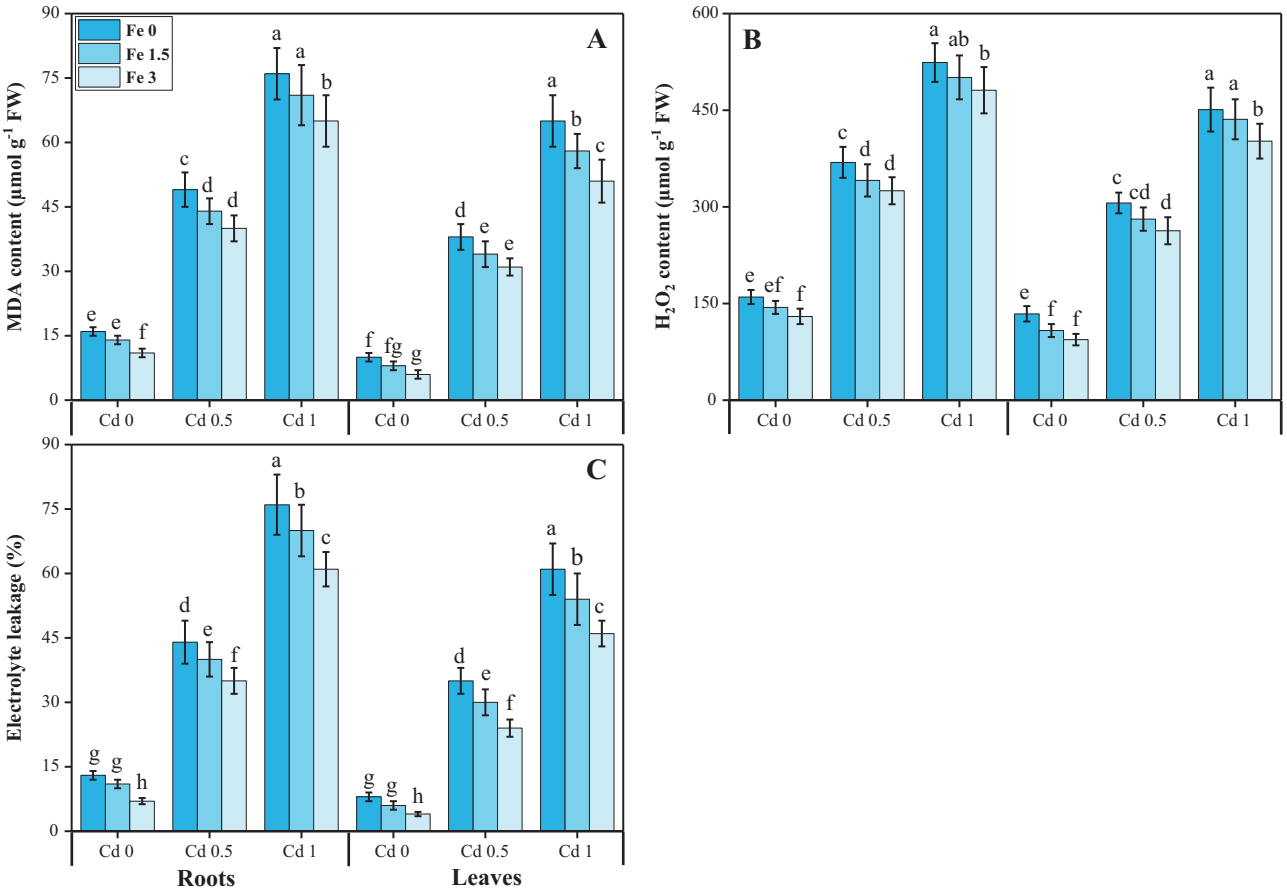


FIGURE 4. Effect of different concentrations of exogenous application of $FeSO_4$ (0, 1.5 and 3 mg/kg) on oxidative stress indicators, i.e., MDA contents (A), H_2O_2 contents (B) and EL percentage (C) in the roots and leaves of *Oryza sativa* grown under different levels of Cd stress (0, 0.5 and 1 mg/kg). Bars sharing different letter(s) for each parameter are significantly different from each other according to Duncan's multiple range test ($P < 0.05$). All the data represented is the average of three replications (n = 4). Error bars represent standard deviation (SD) of three replicates.

Fe, Mg and P in the roots and shoots of *O. sativa* can be increased by the application of $FeSO_4$, which significantly ($P < 0.05$) increased the contents of Fe, Mg and P in the roots and shoots of *O. sativa*, compared to those plants which were grown without the application of $FeSO_4$ (Fig. 7). However, application of $FeSO_4$ decreased the contents of Cd in the roots and shoots of *O. sativa* compared to those plants which were grown without the application of $FeSO_4$ (Fig. 8). The reduced Cd concentration in all organs of *O. sativa* might be due to the higher Fe contents and other nutrients in the plants, as Cd and some other nutrients showed antagonistic effects reported previously by Danish *et al.* (Danish *et al.*, 2019b). Excess Cd reduced the Fe and other nutrients concentration and its root to shoot translocation in *O. sativa* (Rizwan *et al.*, 2016b) while exogenous Fe supply reduced the Cd concentrations in *O. sativa* compared to the plants without Fe supply (Bashir *et al.*, 2018). It was also reported that both Fe and Cd share the same entry routs within *O. sativa* and the expression of Fe transport system may be induced under Cd stress (Afzal *et al.*, 2018; Bashir *et al.*, 2018).

Effect of toxic levels of Cd stress on organic acids exudation pattern under various application levels of $FeSO_4$

Since Fe supply reduced Cd accumulation in *O. sativa* in this study without affecting tolerance mechanism and Cd acropetal translocation, we speculated that an avoidance mechanism plays a pivotal role in Fe-mediated reduction of Cd uptake (Fig. 8). Root exudates, especially fumaric acid, formic acid, acetic acid, citric acid, malic acid, and oxalic acid are suggested to be efficient ligands to alleviate Cd toxicity in plants as they can reduce the bioavailability of Cd (Greger and Landberg, 2008; Konate *et al.*, 2017; Ullah *et al.*, 2016). Organic acids are exuded as anions by anion channels, and their release should be balanced by cations/proton efflux as reported for poplar roots under metal stress (Javed *et al.*, 2019; Shahid *et al.*, 2019). *O. sativa*, metal stress impaired the pumping activities of H^+ ATPase, which caused a decreased efflux of protons. This could explain the application of $FeSO_4$ in the present study at low Cd treatment, besides a direct binding of Cd to organic anions (Fig. 8). Addition of organic acids to soil promotes ammonification which produces protons and leads to a pH decrease. Therefore, acidosis of root exudates might also depend on nitrogen conversion triggered by decomposition of the exuded organic acids (Abbas *et al.*, 2020; Javed *et al.*, 2020).

Effect of toxic levels of Cd stress on transmission electron microscopy under various application levels of $FeSO_4$

Addition of Cd can change the anatomic and structural features of cells, which is considered as the worst effect of Cd. Shah and Dubey (1995) observed low mitotic index, cell division, cell proliferation, and chromosomal aberration in various plant species under Cd stress. Moreover, increased nucleolus and vacuole numbers, condensed cytoplasm,

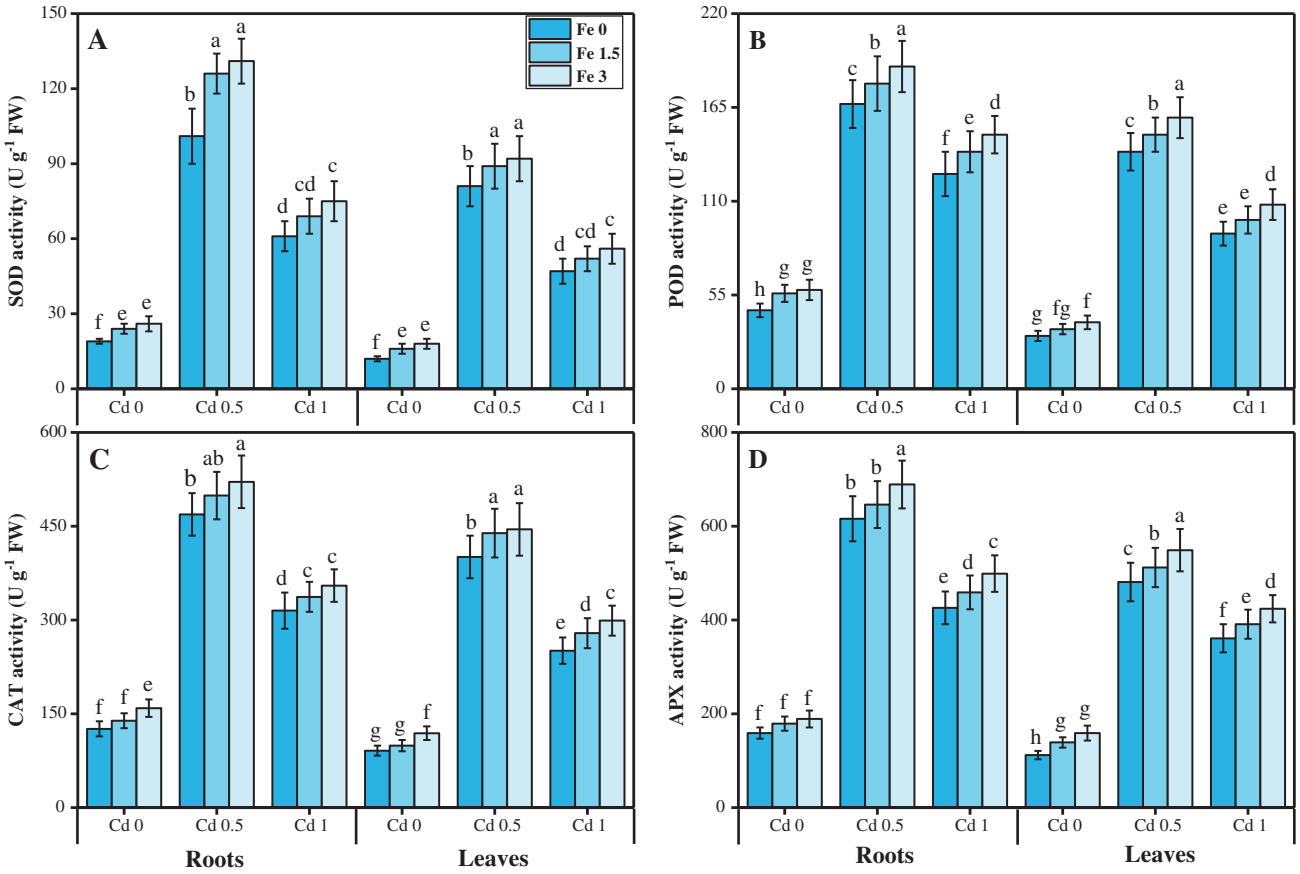


FIGURE 5. Effect of different concentrations of exogenous application of $FeSO_4$ (0, 1.5 and 3 mg/kg) on enzymatic antioxidants, i.e., SOD activity (A), POD activity (B), CAT activity (C) and APX activity (D) in the roots and leaves of *Oryza sativa* grown under different levels of Cd stress (0, 0.5 and 1 mg/kg). Bars sharing different letter(s) for each parameter are significantly different from each other according to Duncan's multiple range test ($P < 0.05$). All the data represented is the average of three replications (n = 4). Error bars represent standard deviation (SD) of three replicates.

reduced mitochondria number, plasmolysis, enlarged vacuoles, disorganized chloroplasts, and ruptured nuclear envelope were found in the root and leaf cells of different crops under Cd stress. Ali *et al.* (2013) observed cracked cell walls, undeveloped mitochondria, plasmolysis, and endoplasmic reticulum absence in the root tip cells of *Brassica napus* grown under a high concentration of Cd (500 mmol/L). The ability to detect significant genetic alterations in plants at different Cd exposure levels, prior to the onset of physiological effects, might serve as a useful molecular biomarker for the early detection of Cd exposure and indicator of related biological effects (Ali *et al.*, 2013; He *et al.*, 2017). Similar results, we also observed in the present study, that addition of Cd in the soil affected most of the cellular organelles of the cell especially ultra-structure of chloroplast (Fig. 9). Although, the ultra-structure of double membranous bounded organelles can be improved by the application of $FeSO_4$, and little improvement in chloroplast structure occurred with the application of different levels of Fe in the soil (Fig. 9). The concentration of Fe in the cells of the plants is crucial because it can regulate many important amino acids, cell division, and cell construction (Ghasemi *et al.*, 2014).

Correlation between Cd uptake, growth, physiological traits, and ions uptake in O. sativa

A heatmap-histogram analysis was also constructed to explore the relationship between the different growth, nutrient uptake and organic acids exudation and Cd uptake attributes (Fig. 10). The significant differences were observed in the plant growth parameters, photosynthetic pigments, gas exchange parameters, nutrient uptake and sugar contents in the treatments which were not spiked artificially with Cd (comprised with application of $FeSO_4$). While rest of the heat-map showing non-significant results with all other parameters with the treatments of Cd in the nutrient solution. Although, blue color is showing non-significant differences within the treatments, while black color is depicting a significant different in the histogram study. This histogram study is showing a clear differences of Cd toxicity on ecophysiology of *O. sativa* under the application of $FeSO_4$ in the soil.

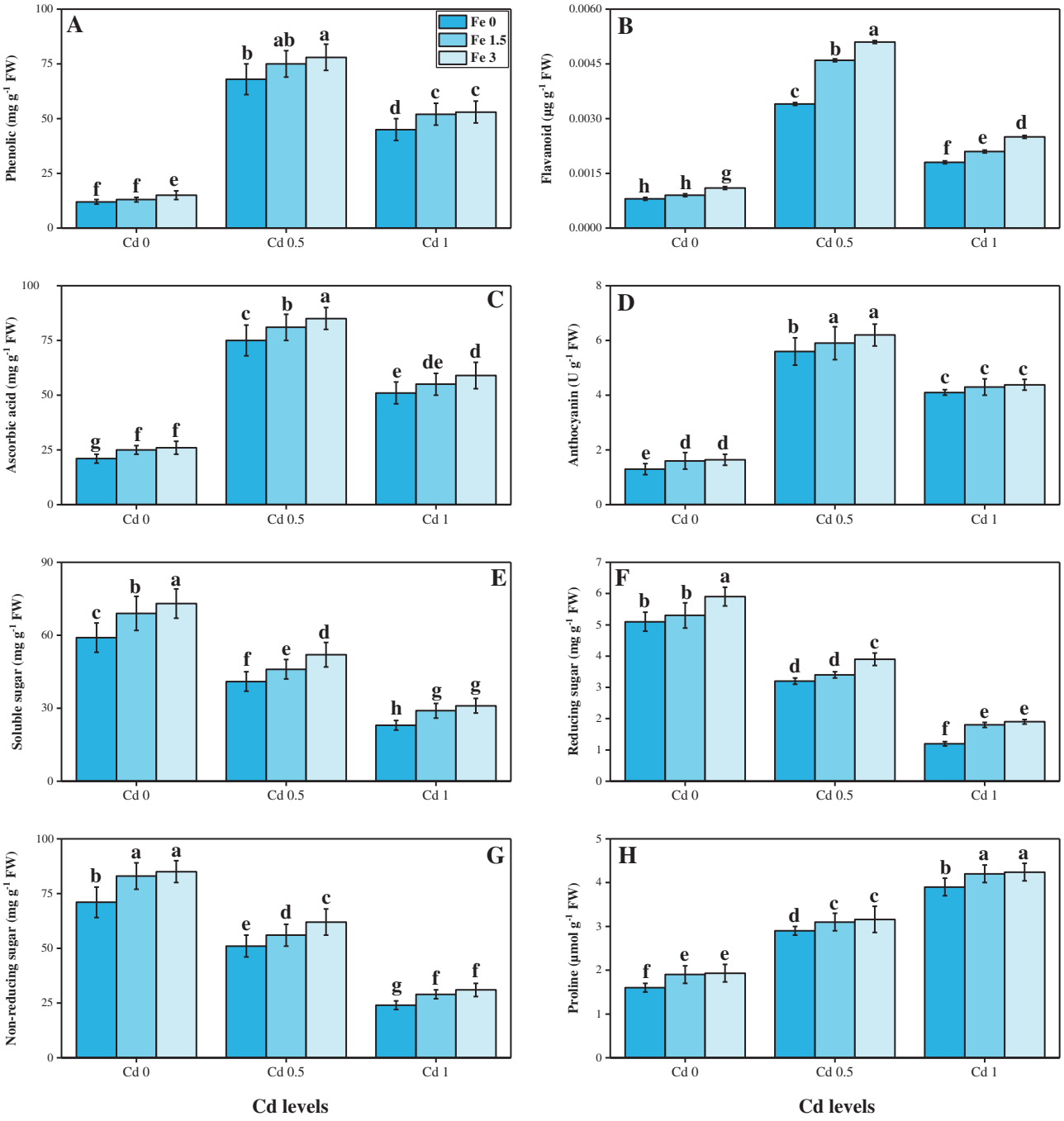


FIGURE 6. Effect of different concentrations of exogenous application of $FeSO_4$ (0, 1.5 and 3 mg/kg) on non-enzymatic antioxidants, i.e., phenolic contents (A), flavonoid contents (B), ascorbic acid contents (C), anthocyanin contents (D), soluble sugar contents (E), reducing sugar contents (F), non-reducing sugar contents (G) and proline contents (H) in the leaves of *Oryza sativa* grown under different levels of Cd stress (0, 0.5 and 1 mg/kg). Bars sharing different letter(s) for each parameter are significantly different from each other according to Duncan's multiple range test ($P < 0.05$). All the data represented is the average of three replications (n = 4). Error bars represent standard deviation (SD) of three replicates.

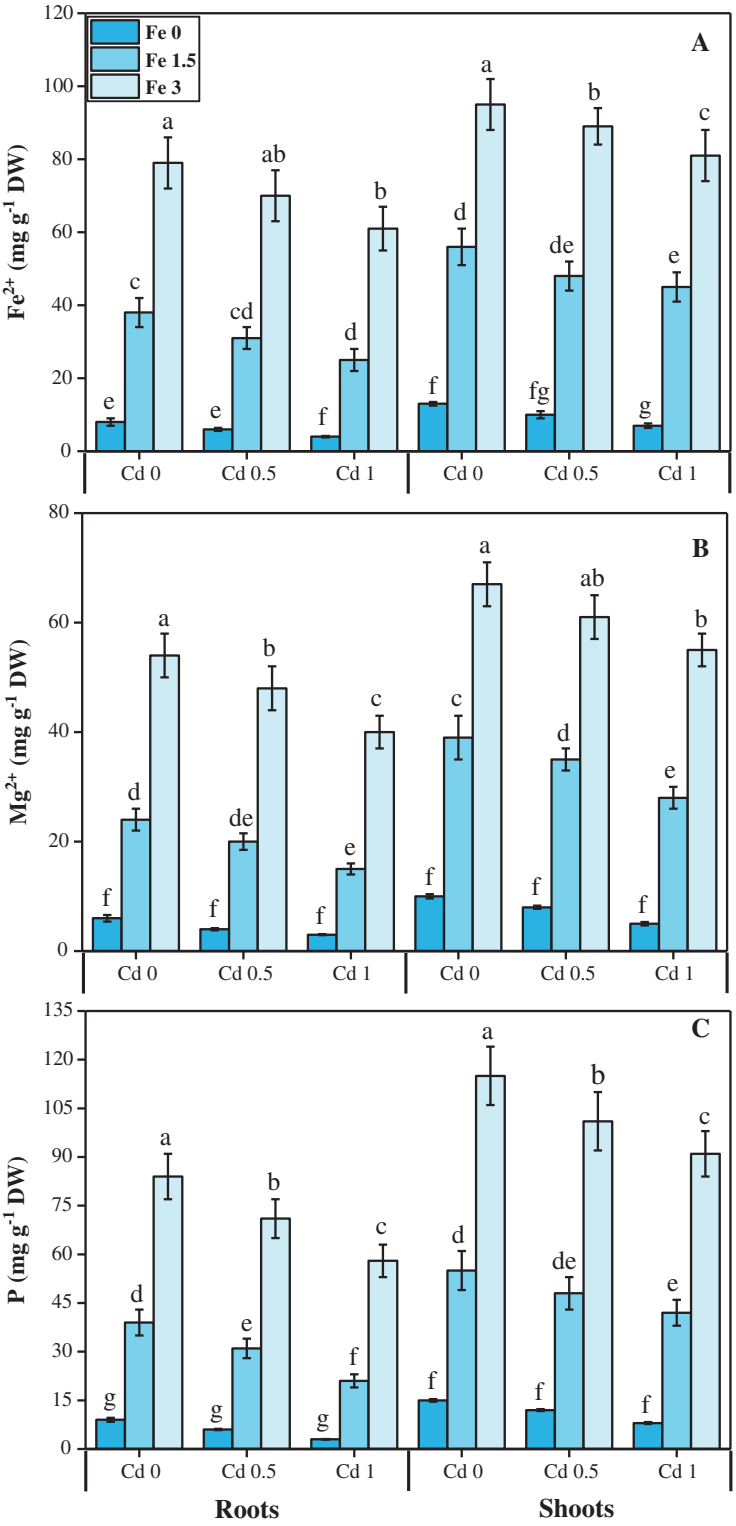


FIGURE 7. Effect of different concentrations of exogenous application of $FeSO_4$ (0, 1.5 and 3 mg/kg) on nutritional status, i.e., iron contents (A), magnesium contents (B) and phosphorus contents (C) in the roots and shoots of *Oryza sativa* grown under different levels of Cd stress (0, 0.5 and 1 mg/kg). Bars sharing different letter(s) for each parameter are significantly different from each other according to Duncan's multiple range test ($P < 0.05$). All the data represented is the average of three replications (n = 4). Error bars represent standard deviation (SD) of three replicates.

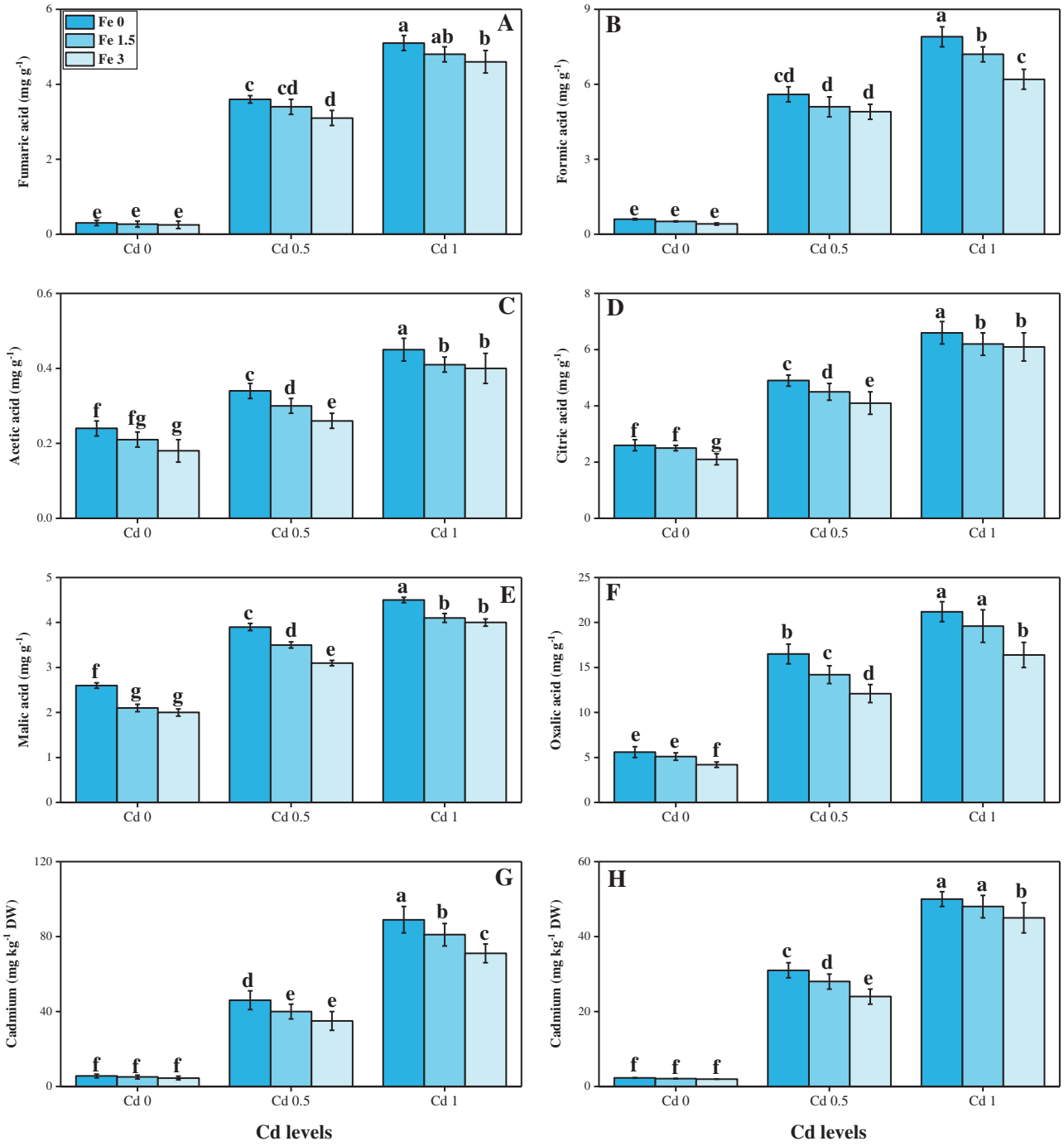


FIGURE 8. Effect of different concentrations of exogenous application of $FeSO_4$ (0, 1.5 and 3 mg/kg) on organic acids exudation pattern and Cd uptake/accumulation, i.e., fumaric acid contents (A), formic acid contents (B), acetic acid contents (C), citric acid contents (D), malic acid contents (E), oxalic acid contents (F) in the roots and Cd concentration in the roots (G) and Cd concentration in the shoots (H) *Oryza sativa* grown under different levels of Cd stress (0, 0.5 and 1 mg/kg). Bars sharing different letter(s) for each parameter are significantly different from each other according to Duncan's multiple range test ($P < 0.05$). All the data represented is the average of three replications (n = 4). Error bars represent standard deviation (SD) of three replicates.

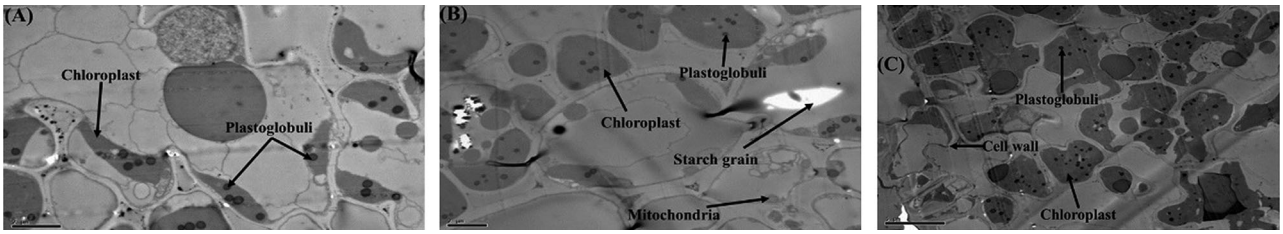


FIGURE 9. Transmission electron microscopy (TEM) analysis of *Oryza sativa* leaf structure after treated with Cd and Fe concentration in the nutrient solution. (A) *O. sativa* (10,000) with Cd 1 + Fe 0, (B) *O. sativa* (10,000) with Cd 1 + Fe 1.5, (C) *O. sativa* (5,000) with Cd 1 + Fe 3.

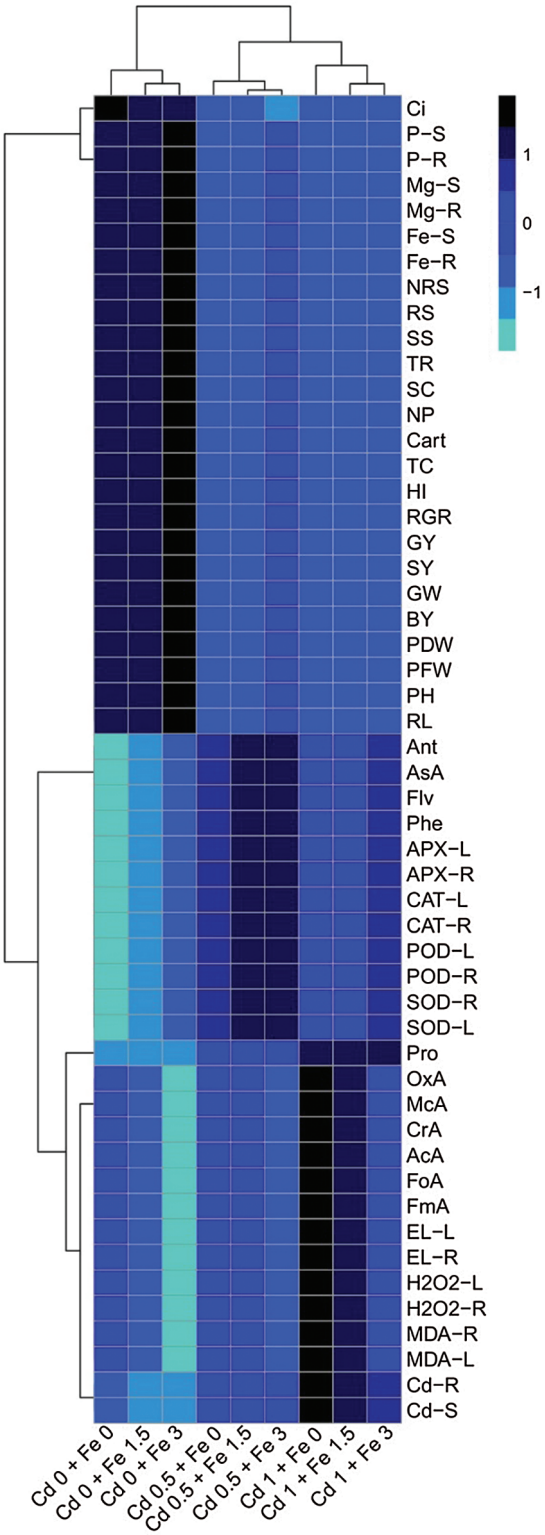


FIGURE 10. Heatmap histogram correlation between Cd uptake/accumulation with different morpho-physio-biochemical attributes of *Oryza sativa* grown under different levels of Cd stress (0, 0.5 and 1 mg/kg) with different concentrations of exogenous application of $FeSO_4$ (0, 1.5 and 3 mg/kg).
Different abbreviations used are as follow: Ci (intercellular CO_2), P-S (phosphorus contents in the shoots), P-R (phosphorus contents in the roots), Mg-S (magnesium contents in the shoots), Mg-R (magnesium contents in the roots), Ca-S (calcium contents in the shoots), Ca-R (calcium contents in the roots), Fe-S (iron contents in the shoots), Fe-R (iron contents in the roots), NRS (non-reducing sugars), RS (reducing sugars), SS (soluble sugars), TR (transpiration rate), SC (stomatal conductance), NP (net photosynthesis), Carot (carotenoid contents), TC (total chlorophyll), RDW (root dry weight), SDW (shoot dry weight), RFW (root fresh weight), SFW (shoot fresh weight), TPL (total plant length), RL (root length), Ant (anthocyanin contents), AsA (ascorbic acid contents), Flv (flavonoid contents), Phe (phenolic contents), APX-L (ascorbate peroxidase activity in the leaves), APX-R (ascorbate peroxidase activity in the roots), CAT-L (catalase activity in the leaves), CAT-R (catalase activity in the roots), POD-R (peroxidase activity in the roots), POD-L (peroxidase activity in the leaves), SOD-R (superoxidase dismutase activity in the roots), SOD-L (superoxidase dismutase activity in the leaves), Pro (proline contents), OxA (oxalic acid contents), McA (melic acid contents), CrA (citric acid contents), AcA (acetic acid contents), FoA (formic acid contents), FmA (fumaric acid contents), EL-L (electrolyte leakage in the leaves), EL-R (electrolyte leakage in the roots), H_2O_2-L (hydrogen peroxide initiation in the leaves), H_2O_2-R (hydrogen peroxide initiation in the roots), MDA-R (malondialdehyde contents in the roots), MDA-L (malondialdehyde contents in the leaves), Cd-R (Cd concentration in the roots) and Cd-S (Cd concentration in the shoots).

Conclusion

Outcomes of current study revealed that toxic level of Cd significantly affected plant growth and biomass, photosynthetic pigments, gaseous exchange traits, antioxidative machinery and minerals uptake by *O. sativa*. Furthermore, Cd toxicity increased the oxidative stress indicators, organic acids exudations and Cd contents in plant organs. However, application of $FeSO_4$ improved plant growth and biomass, decreased ROS production, maintain essential minerals, and decreased the Cd contents of plant organs. Furthermore, balanced exudation of organic acids after the application of $FeSO_4$ further confers the normal metabolic activities of *O. sativa* even under Cd stress. Therefore, field studies should be executed to draw parallels amongst plants/crops root exudations, metal stress, $FeSO_4$ fertigation regimes, nutrients mobility patterns and plant growth in order to gain insights into underlying mechanisms.

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Availability of Data and Materials: The data will be provided on demand.

Author Contributions: Conceptualization, Shahid Hussain, Imran Khan and Omar Aziz; Data curation, Javaria Afzal, Muhammad Shoaib Rana and Samrah Afzal Awan; Formal analysis, Chengxiao Hu; Funding acquisition, Javaria Afzal, Sajid Fiaz and Xiukang Wang; Investigation, Muhammad Shoaib Rana, Sajid Fiaz, Samrah Afzal Awan, Kashif Kubar and Chengxiao Hu; Methodology, Muhammad Hamzah Saleem, Xuecheng Sun, Imran Khan, Muhammad Shoaib Rana and Omar Aziz; Project administration, Xuecheng Sun and Chengxiao Hu; Resources, Shafaqat Ali, Muhammad Hamzah Saleem, Xiukang Wang, Samrah Afzal Awan, Kashif Kubar and Chengxiao Hu; Software, Shafaqat Ali, Shakeel Ahmed, Shahid Hussain, Imran Khan, Omar Aziz, Kashif Kubar and Chengxiao Hu; Supervision, Chengxiao Hu; Validation, Samrah Afzal Awan; Visualization, Shahid Hussain, Sajid Fiaz, Muhammad Shoaib Rana, Omar Aziz and Kashif Kubar; Writing–original draft, Shakeel Ahmed, Shafaqat Ali, Javaria Afzal, Xuecheng Sun, Muhammad Hamzah Saleem and Kashif Kubar; Writing–review & editing, Shakeel Ahmed, Shafaqat Ali, Muhammad Hamzah Saleem, Sajid Fiaz and Xiukang Wang.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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