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Research Paper

IMPACT OF IRON STRESS ON OXIDATIVE METABOLISM IN GREEN GRAM PLANTS (*Vigna radiata (*L) Wilczek).

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Abstract

The objective of this work was to evaluate the impact of deficient and toxic concentrations of iron (Fe) on the protective role of antioxidative enzymes as well as antioxidant compounds in green gram (*Vigna radiata* (L) Wilczek) plants. Plants were grown in Hoagland nutrient solution and treated with four Fe concentrations of Fe (10,100,200 and 400 µM Fe) in the form of FeEDTA. Plants showed maximum growth at 100 µM Fe supply (control). Iron deficiency was observed at 10 µM Fe supply and resulted in chlorosis of leaves and decreased concentration of active Fe and chlorophyll content. Apical chlorosis, marginal necrosis and decrease in chlorophyll concentration was also observed in plants treated with 200 and 400 µM Fe. High lipid peroxidation (TBARS) and H_2O_2 content in leaves were detected in Fe deficient and Fe toxic plants as compared to control. The GR increased with increasing Fe concentration at 20 DAT but the GR activity decreased at 40 DAT. The activities of Fe containing enzyme such as CAT, POD and APX also increased with increasing Fe concentration at 20 days but values were lower at 40 DAT. The ASA and NPT contents increased with increasing Fe concentration but decreased at 40 DAT in plants supplied 400 µM Fe. The results indicate that under deficient and toxic levels of Fe, plants suffer increased oxidative stress, which is regulated through change in the activities of antioxidative enzymes and in the contents of the antioxidants ASA and NPT.

Key words: Antioxidant enzymes, green gram, Fe stress, oxidative damage, reactive oxygen species (ROS).

INTRODUCTION

Iron is one of the essential elements for plant growth and reproduction. Iron is a micronutrient and is required by plants in small amounts [1]. Fe in the soil is the fourth abundant element on earth, but its amount is low or not available for the plants and microorganisms needs, due to low solubility of minerals containing Fe in many areas of the world, especially in arid region with alkaline soils. Its solubility and availability are

very changeable and it is dependent on soil pH and Eh. The narrow efficient Fe concentration required for cellular needs is strictly controlled by biological processes acting both at the transport and the storage levels. As a transition element Fe exists in two oxidative states: ferrous (Fe^{2+}) and ferric (Fe^{3+}) and it is required in most of the cellular redox systems such a heme proteins including cytochroms, catalase, peroxidase, leg- hemoglobin and iron sulfur proteins including ferredoxin, acotinase and superoxide dismutase. It is thus one of the major metals involved in the wide range of biological functions like electron-transport chains of plant photosynthesis respiration and nitrogen metabolism. Excess of Fe^{2+} however harms the plant by inhibiting the elongation of plant roots and plant growth. Although most soils are rich in Fe, the ''iron toxicity'' is associated with flooded soils which are high in ferrous Fe levels, particularly in lowland-rice production areas [2] or tea plantations in India[3]. Toxicity of Fe can affect different physiological processes of the plant. It can lead to the enhancement of oxidative stress [4], by increasing the free radical production which irreversibly impairs cellular structure and damages DNA and proteins [5], [6]. Protection strategies against Fe toxicity within the plant may involve enzymatic mechanisms that include the actions of antioxidant enzymes, such as superoxide dismutase, catalase, peroxidase and ascorbate peroxidase and glutathione reductase [7], [8]. Non-enzymatic mechanisms can neutralize oxygen reactive species through organic compounds, such as reduced glutathione (GSH), α-tocopherol, ascorbic acid (AA) and carotenoids [9]. On one hand Fe in the free or in the loosely bound form, acts as a prooxidant factor, catalysing the free radical generation through the Fenton reaction. On the other hand, some of the antioxidant enzymes contain iron, either in heme (POD, CAT) or non-heme form (Fe-SOD). Keeping in mind the dual role of Fe in plant metabolism [10] the present study aims to evaluate the impact of deficient and toxic levels of Fe on the oxidative metabolism in green gram plants at different growth stages.

MATERIALS AND METHODS

Green gram **(***Vigna radiata* L.var. PDM 139) plants were grown under glasshouse conditions

in acid purified silica sand in polyethylene pots with a central drainage hole covered with glass wool under an inverted watch glass that allowed free drainage. The nutrient solution contained 4 mM $Ca(NO_3)_2$, 4 mM KNO_3, 2 mM $MgSO_4$, 1.33 mM NaH_2PO_4, 0.33 mM H_3BO_3, 1.0 µM $ZnSO_4$, 10 µM $MnSO_4$, 1.0 µM $CuSO_4$, 0.1 µM Na_2MoO_4, 0.1 mM NaCl, 0.1 µM $CoSO_4$, 0.1 µM $NiSO_4$, and Fe supplied at four levels (10, 100, 200 and 400 µM Fe) in the form of Fe EDTA. Nutrient solution containing Fe was supplied six days a week after emergence of first leaf. At the week end, pots were flushed with glass distilled water to avoid accumulation of nutrients in the rooting medium. For each treatment, there were three pots, each containing five plants. Twenty days from sowing, when leaves of plants grown with 10 µM and 400 µM Fe displayed initial symptoms of iron deficiency and toxicity respectively and at 40 days of growth when symptoms become more pronounced, plant biomass and Fe concentration in leaves were determined. Simultaneously, the two fully expanded terminal trifoliate were assayed for biomass yield, total active-Fe and leaf tissue concentration of Fe along with chlorophylls (Chl) a and b, carotenoid, hydrogen peroxide (H_2O_2), thiobarbituric acid reactive substances (TBARS), ascorbate (Asc), non protein thiol (NPT), and activity of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), and glutathione reductase (GR).

Plants were separated into roots, stem and leaves, and total biomass was determined by oven drying (70°C) the samples. The tissue Fe concentration determined in roots, stem and fully expanded terminal trifoliate leaves was determined by atomic absorption spectrophotometer (Perkin Elmer A Analyst 300) in wet acid HNO_3 : $HClO_4$ (10 : 1) digests. Chlorophyll (a + b) and Carotenoids were extracted with 80% acetone and measured spectrophotometrically (Perkin Elmer UV/VIS Lambda Bio 20) as described by method of [11]. Total active-Fe content in leaves was measured following the method of [12].

Ascorbate was extracted with 10% trichloroacetic acid (TCA) and assayed according to [13] by following the reduction of $Fe^{3=}$ to Fe^{2+} by ascorbic acid and measuring the color intensity of the Fe^{2+} - α, α bipyrridyl complex at 525 nm. Non-protein thiols were estimated by the method of [14]. Leaves were finally chopped and ground with prechilled mortar and pestle in 5% sulfosalicylic acid. After centrifugation, the reaction was carried out with 10 mM dithiobis 2-nitrobenzoic acid (DTNB) and 0.1 mM glutathione reduced (GSH). After 15 min, the color intensity of the extract was measured with spectrophotometer at 412 nm.

Lipid peroxides were measured in terms of thiobarbituric acid reactive substances (TBARS) as described by [15]. Fresh leaf material was extracted with 1% trichloroacetic acid. The supernatant was centrifuged at 10000 g for 10 min and treated with 0.5% thiobarbituric acid dissolved in TCA. The reaction mixture was incubated in boiling water bath for 30 min and TBARS were measured spectrophotometrically at 532 nm after adjusting for non-specific absorbance at 600 nm. Hydrogen peroxide was estimated by the method of [16]. Freshly chopped leaf tissues were ground in chilled mortar pestle with 100% chilled acetone and centrifuged at 10000 g for 5 min. The supernatant was mixed with water and shaken thoroughly. To the supernatant titanium chloride was added followed by chilled ammonia. The precipitate was solubilized with the help of thin glass rod and centrifuged. The residue was washed repeatedly with acetone to remove chlorophyll. The colorless residue was dissolved in 2N H_2SO_4. The color intensity was read at 415 nm.

Catalase (CAT) and peroxidase (POD) were extracted by homogenization of the fresh leaf tissue in ice cold distilled water (1:10) with a chilled mortar and pestle. The reaction mixture for CAT enzyme assayed by the method of [17] contained 0.005 M hydrogen peroxide in 0.025 mM potassium phosphate buffer (pH 7.0) and was standardized against 0.1 N $KMnO_4$.The reaction was started by adding 1 ml of the suitably diluted enzyme extract. After 5 min, the reaction was stopped by adding 2 ml of 2 N H_2SO_4. Blanks, wherein sulfuric acid was added to the reaction mixture prior to the addition of the enzyme extract were run simultaneously. Unused hydrogen peroxide in the reaction mixture was titrated against 0.1N $KMnO_4$.The reaction mixture for POD assayed by the method of [18] which contained 2 ml of 0.1 M phosphate buffer (pH 6.0), 1 ml of 0.01% H_2O_2 and 1 ml of 0.5% p-phenylenediamine. The reaction was initiated by adding 1 ml of the enzyme extract to the above mixture and was allowed to proceed for 5 min. The reaction was stopped by adding 4N H_2SO_4. Blanks, wherein sulfuric acid was added prior to the addition of the enzyme extract, were run simultaneously.

For assay of SOD**,** APX and GR fresh leaf tissue was ground in 50 mM potassium phosphate buffer (pH 7.0), containing EDTA (1 mM) and PVP (2%) The extracts were centrifuged at 15000 g for 10 min, and the supernatant was assayed for the enzyme activities. Total SOD was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) in 3 ml reaction mixture [19], which containing 50 mM phosphate buffer (pH 7.0), 0.13 mM methionine, 75 µM NBT, 2 µM

riboflavin, 0.1 mM EDTA, and 25 µl of the enzyme extract and read at 560 nm. One unit of enzymes is defined as the amount of enzyme causing 50% inhibition of NBT reduction. For determining APX activity as per the method of [20], the reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, and 0.1 mM H_2O_2.The enzymatic activity was assayed by following oxidation of ascorbate at 290 nm. Estimation of GR was carried out by following oxidation of NADPH and monitoring a decrease in absorbance at 340 nm per min as described by [21] in a reaction mixture containing 100 mM phosphate buffer (pH 7.0), 1 mM oxidized glutathione (GSSG), 1.0 mM EDTA, 0.1 mM NADPH, and 25 to 50µl of the enzyme extract. The reaction mixture was kept in a refrigerator for 20 min and then centrifuged at 4000 g. The color intensity was read at 485 nm. Enzyme activities have been expressed on the basis of total soluble proteins in the enzyme preparations, as determined by the dye binding method of [22].

All measurement was made on samples drawn in triplicate and the data were statistically analysed (ANOVA) for significance (LSD at P= 0.05). The data in figures are presented as bar diagram of mean values ± standard error (SE, n=3).

RESULTS AND DISCUSSION

Increasing concentration of Fe to more than 100 µM in the nutrient solution produced a significant reduction in growth and visual symptoms of Fe toxicity. Fe toxicity caused reduction in leaf size. Plants receiving 100 µM Fe showed optimum growth whereas plants grown with 10 µM, 200 µM and 400 µM Fe showed a decrease in leaf size, which was marked in younger leaves. Plants receiving 200 and 400 µM Fe supply developed toxicity symptoms like bronzing of older leaves after 20 days [6]. The decrease in dry matter production in the roots, stem and leaves increased with increase in Fe concentration and exposure time of the plants to excess Fe. However, decrease in root dry weight was more significant than leaf dry weight (Table 1). The active Fe content in plant leaves increased with increase in Fe supply. An increase in Fe supply from 10 to 400 µM caused an increase in the Fe concentration in leaf tissue from 25.08 to 444.11 µg Fe/g dry wt. Optimum growth was observed in plants containing 97.03 µg Fe/g dry wt. (Table 1).

Excess supply of Fe caused a decrease in concentration of chlorophyll a b and total chlorophyll. Carotenoid concentration was also suppressed due to Fe deficiency and toxicity (Fig.1). Similar results were also reported by other investigators in induced heavy metal toxicity [23], [24]. In the present study, oxidative damage in Fe stressed plant was observed as accumulation of TBARS and H_2O_2 (Fig.1). The level of lipid peroxidation and H_2O_2 increased with increasing Fe concentration and in deficient Fe plants as also with increase in exposure time to Fe treatments. The hydroxyl radicals in the aerobic cells are known to be formed from H_2O_2 in the presence of transition metal ions Cr, Fe, Cu [7]. The accumulation of TBARS is an indication that accumulation and production of reactive oxygen species (ROS) which is induced by toxic concentration of iron. Enhanced lipid peroxidation under excess of Fe has been also reported in different species [25], [26], [27] and has been associated with oxidative stress caused by Fe toxicity.

Besides enzymatic mechanisms, plant can use low molecular weight antioxidants such as glutathione (GSH) and ascorbate (AA) to overcome oxidative stress [28]. Non protein thiols are involved in heavy metal detoxification and play a very important role to overcome oxidative stress. Glutathione is a major component of non-protein thiols and possesses strong antioxidative properties, glutathione consists of the three amino acids; cysteine, glycine and glutamic acid, and is an important part of the Halliwell

Asada Pathway for ROS detoxification. Ascorbate (AA) is a major antioxidant metabolite involved in the detoxification of ROS during oxidative stress in plants [9]. It can directly eliminate hydrogen peroxide and also can be used by the enzyme APX as a reduced substrate. In response to Fe the non protein thiols and AA content in iron treated green gram plants was higher than in controls, indicating an adaptive response of the plants to the oxidative stress caused by the excess of Fe concentration. Increasing the time of exposure (40 days) to Fe treatment gradually reduced the NPT and AA content (Fig.2). An increase in the AA content were also shown in plants of *Bacopa monnieri* treated with Fe [25] and in *Spartina densiflora* [29]. The AA/DHA ratio increased by increasing concentration of Fe relative to the control. The AA/DHA ratio is considered in plant cells as important redox state indicator, which functions as a buffer system to supply antioxidant metabolites during the oxidative stress [28]. This AA/DHA ratio increased due to an increase in AA content and reduction in DHA concentration but after 40 days of treatment the ratio of AA/DHA was decreased (Fig. 2) at toxic Fe supply indicating inability of AA to combat ROS.

The SOD activity was enhanced under Fe toxicity in both the stages but was decreased at deficient Fe supply (Fig. 3). Increasing SOD activity under Fe toxicity has also been observed in other plant species, such as rice [26]. This is contradictory to reports by [30] who observed a decrease in the activity of this enzyme in the leaves of *E.uniflora*. SOD is the first line defence enzyme that dismutates $O_2^{\cdot-}$ to H_2O_2 and O_2 [31], and is one of the most important enzyme in the cell defense against the oxidative stress [8], [7]. The SOD, CAT and POD are important antioxidative enzymes that function in the cells to prevent the buildup of ROS [5], [7]. The H_2O_2 formed by SOD dismutation in turn may be detoxified by CAT and POD. The activity of CAT which are Fe containing enzyme increased with increasing concentration of Fe but the activity of CAT increased up to 200 µM Fe supply. The activity was lower at 40 days, and this may be due to a toxicological effect of Fe. Similar pattern of CAT activity has been also reported under Fe and Cu stress in *Triticum aestivum* [32]. The increase in CAT activity due to high concentration of Fe has been observed in other plant species [26], [27]. The activity of POD and APX also increased with increasing concentration of Fe at both stages but values were lower at 40 days. Due to its high activities in the plant leaves, the enzyme POD and APX seems to have an important role in plant defense against the oxidative stress in green gram. An increase in the activities of these enzymes in response to toxic levels of Fe was observed in rice [26], [33], *Spartina densiflora* [29], and tea [3].

The GR activity decrease in Fe deficient plants but increased with increasing concentration of Fe. The increase was appreciable at 400 µM Fe at 20 DAT (Fig.3). Same result were also reported by [34] who observed an increase in activity of GR. However at 40 DAT the activity of GR was almost half than that at 20 DAT and also showed a decrease than control plants. Glutathione reductase (GR) is a key enzyme of the Ascorbate /Glutathione cycle which is essential to maintain cell homeostasis during the oxidative stress [7]. A decrease in the GR activity at later stages would result in the redox imbalance of the cell, which would not be able to cope with the oxidative damage.

The results of the present study demonstrate that green gram plants suffer strong oxidative stress with increase in lipid peroxidation and H_2O_2 content which is induced by deficient and toxic concentration of Fe. Under the experimental conditions the Fe stressed plant exhibited a complex defense mechanism which is composed of antioxidative enzymes and low molecular weight antioxidants that were capable of ameliorating the impact of the reactive oxygen species production at initial stages of Fe. However after prolonged stress a decrease in AA/DHA ratio and GR activity as well a

decrease relative activity of the other antioxidative enzymes SOD, CAT, POD and APX the plants exhibit oxidative damage as observed by burning of leaves and reduced plants growth.

Table-1: Effect of iron treatment on the leaf area, dry matter yield, tissue iron and total active Fe, in green gram (*Vigna radiata* (L) Wilczek var. PDM 139) plants.

Days after treatment	**Plant part**	**µM iron supply**				**LSD (P=0.05)**
		10	**100**	**200**	**400**	
		Area: cm^2				
20	Leaf	4.70±0.679	20.22±0.308	17.16±0.422	10.39±0.713	1.53
40	Leaf	7.59±0.362	23.67±0.201	19.49±0.115	14.29±0.028	2.22
		Dry matter yield: g $plant^{-1}$				
20	Leaves	0.033±0.002	0.065±0.013	0.076±0.005	0.036±0.004	0.006
	Stem	0.041±0.001	0.139±0.004	0.131±0.046	0.082±0.048	0.005
	Root	0.017±0.001	0.072±0.006	0.050±0.003	0.035±0.002	0.006
	Whole plant	0.092±0.001	0.276±0.082	0.257±0.080	0.153±0.009	0.021
40	Leaves	0.038±0.003	0.156±0.045	0.137±0.051	0.053±0.002	0.003
	Stem	0.045±0.005	0.194±0.052	0.187±0.040	0.091±0.042	0.007
	Root	0.029±0.002	0.085±0.006	0.061±0.005	0.049±0.005	0.006
	Whole plant	0.112±0.091	0.436±0.033	0.385±0.031	0.194±0.054	0.028
		Tissue iron: µg g^{-1}dry wt.				
20	Leaves	25.08±1.201	97.03±3.110	259.29±5.671	444.11±7.235	3.26
	Root	39.66±1.393	123.0±3.010	278.09±5.340	476.22±6.089	4.53
40	Leaves	26.14±1.172	143.2±3.706	281.46±3.011	527.0±8.176	2.94
	Root	57.23±2.076	177.01±4.032	342.11±4.058	586.16±7.087	6.78
		Total active Fe: mg kg^{-1} f. wt.				
20	Leaf	18.3±1.63	83±2.32	135±3.73	317±3.16	3.76
40	Leaf	27.5±2.12	105±3.36	191±3.98	354±4.12	3.23

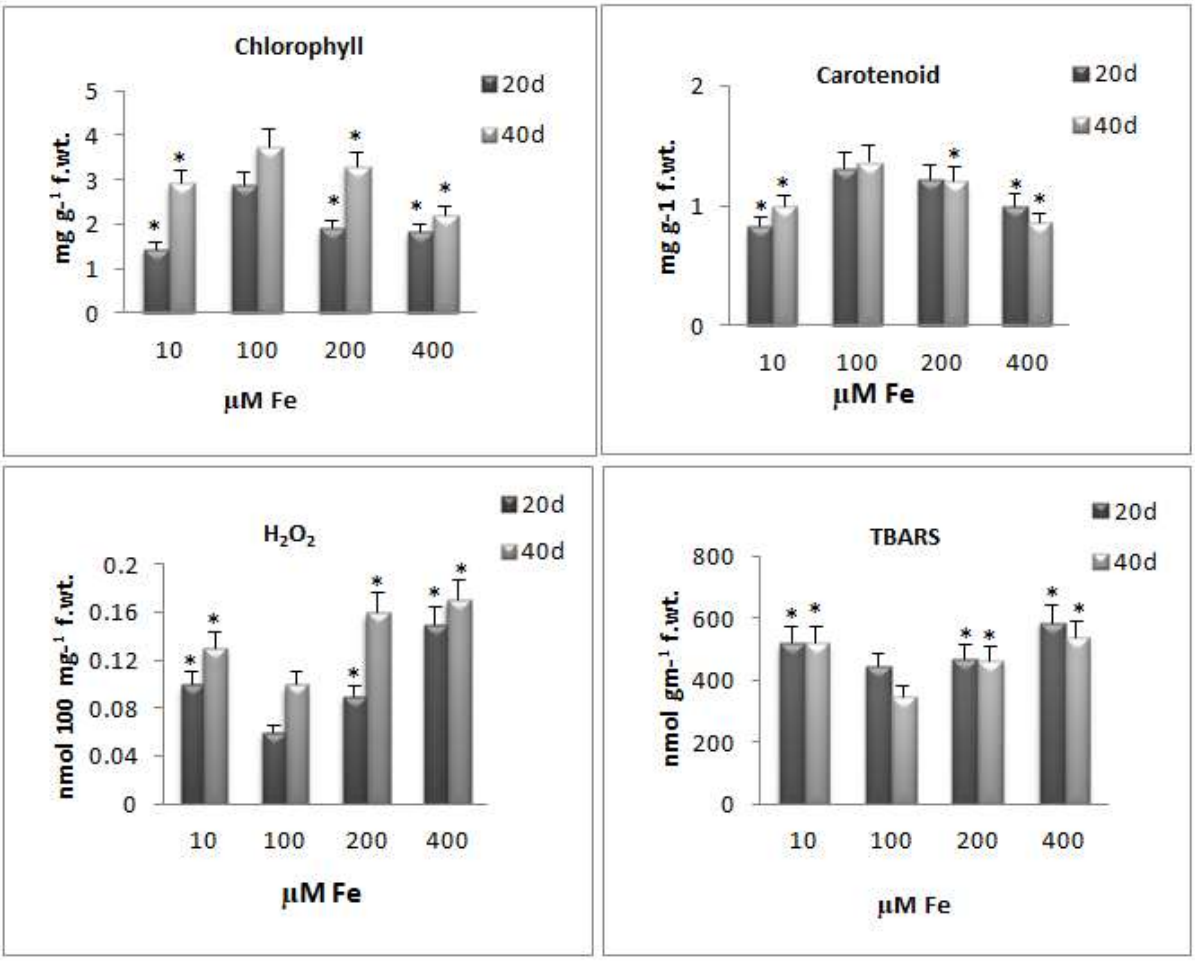

Fig. 1: Effect of iron treatment on concentration of chlorophyll, carotenoids, hydrogen peroxide (H_2O_2), and thiobarbituric acid reactive substances (TBARS) in the leaves of green gram plants. Bars indicates ± S.E. of three independent values. * indicates significant differences compared to control at P=0.05.

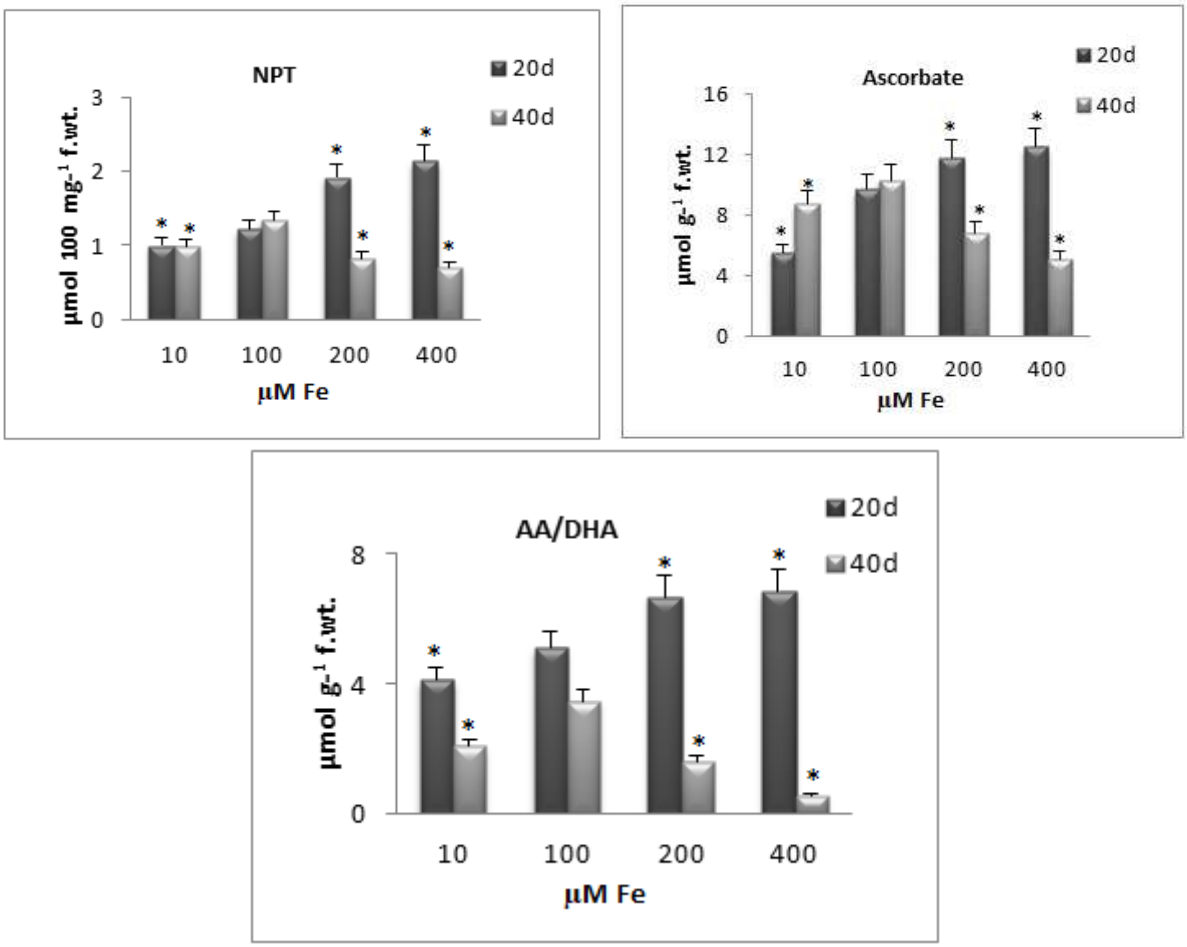

Fig. 2: Effect of iron treatment on concentration of non-protein thiols, ascorbate and ratio of AA/DHA in the leaves of green gram plants. Bars indicates ± S.E. of three independent values. * indicates significant differences compared to control at P=0.05.

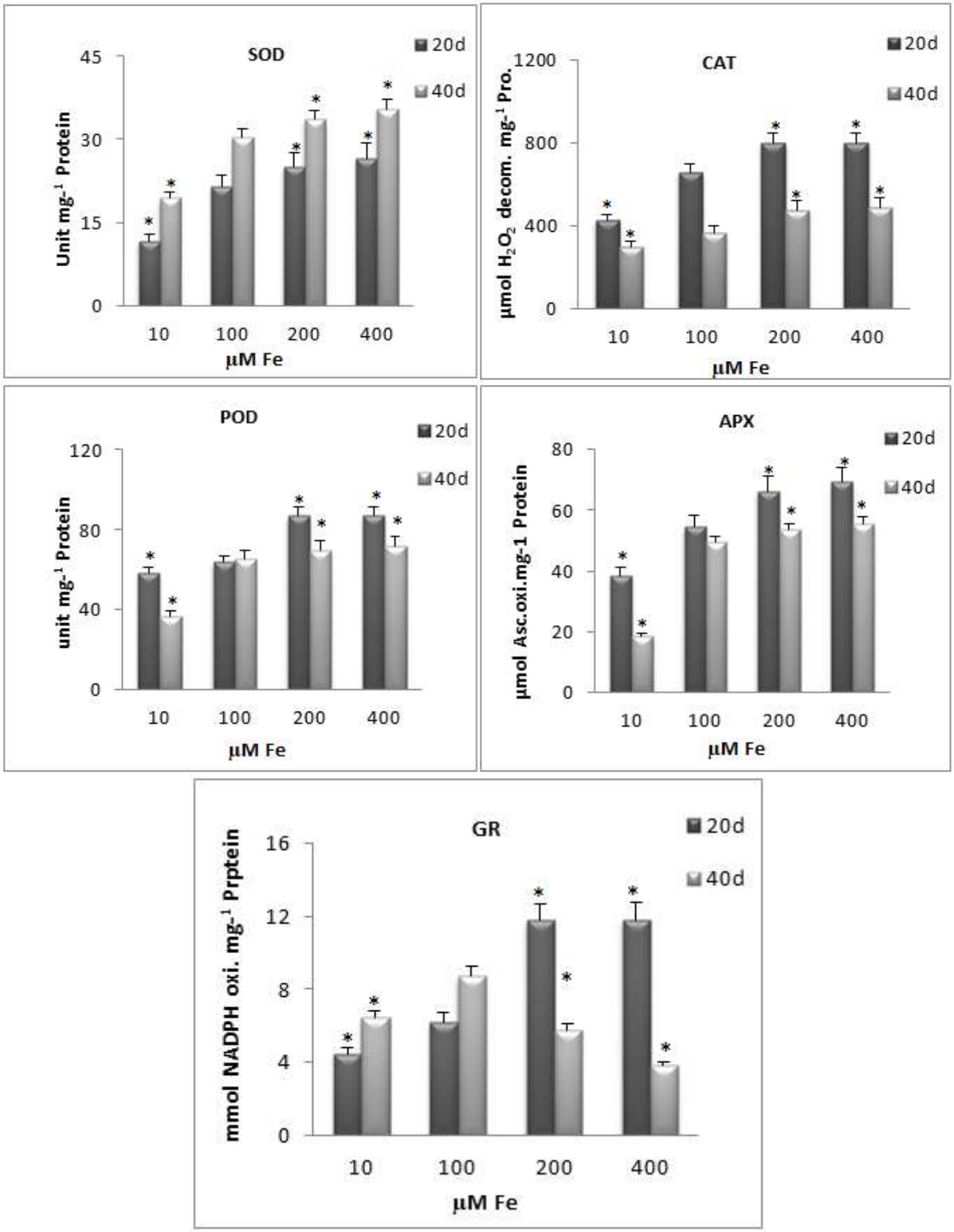

Fig. 3: Effect of iron treatment on activity of SOD (superoxide dismutase), CAT (catalase), POD (peroxidase), APX (ascorbate peroxidase) and GR (glutathione reductase) in leaves of green gram plants. Bars indicates ± S.E. of three independent values. * indicates significant differences compared to control at P=0.05.

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