

Effect of Iron and Phytohormones Application on Antioxidant Enzymes Activity, Chlorophyll and Grain Yield of Maize in Iron-deficient Soil

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Authors' contributions

This work was carried out in collaboration between both the authors. Author Kavita designed the study, managed the literature searches, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author VK managed experimental trial. Both authors read and approved the final manuscript.

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ABSTRACT

The study investigated the effect of foliar application of gibberellic acid (GA₃) and cytokinin on antioxidative enzymes, chlorophyll content and grain yield of maize. Three factor randomized block design (RBD) was used to carry out the investigation in experimental farm of Dr. Rajendra Prasad Central Agricultural University, Pusa, Bihar during 2012-13.

The field experiment was laid out in Fe-deficient soil (Fe < 0.5 ppm) with contrasting cultivars of maize. The factors were: i) Fe-efficient (Suwan) vs. inefficient cultivar (Shaktiman-3); ii) 'control' (no Fe-spray) vs. one soil application of Fe (20 kg ha⁻¹) + two foliar spray of Fe as 0.5% ferrous sulphate at pre-flowering and 7-days after flowering; and iii) foliar application of phytohormones viz., GA₃ at 10 and 20 ppm; cytokinin at 10 and 20 ppm and their combinations.

Application of Fe and phytohormones significantly increased enzymes activity like catalase,

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peroxidase and superoxide dismutase. The highest activity was observed with combined application of cytokinin + GA₃ at 10 ppm. For example, the value of catalase increased from 196.0 to 217.0 $\mu\text{mol}/\text{min}/\text{g}$ fresh protein, and peroxidase from 90.0 to 103.0 Units mg^{-1} fresh protein. There were significant increases in chlorophyll content of leaves, and grain yield with combined application of cytokinin + GA₃ at 10 ppm. The maximum chlorophyll content was recorded in 'Suwan' (32.7 SPAD value) having treatment Fe application plus GA₃ + cytokinin at 10 ppm each. Grain yield increased significantly from 58.6 to 64.6 q ha^{-1} in 'Suwan' provided with Fe application and GA₃ + cytokinin at 10 ppm each compared to control (50.3 q ha^{-1}). Results indicated that phytohormones were involved in regulation of nutrient availability and conversely mineral nutrients influenced hormone biosynthesis suggesting a relationship between hormones and nutritional homeostasis.

Conclusion: Exogenous application of phytohormones could alleviate Fe deficiency stresses in maize and application of Fe and phytohormones acted synergistically. Hence, application of GA₃ + cytokinin at 10 ppm may be done to alleviate Fe stress and to improve grain yield of maize.

Keywords: Antioxidant enzymes; iron; maize; phytohormones; yield.

1. INTRODUCTION

Iron (Fe) deficiency is a widespread agricultural problem in many crops including maize grown in alkaline, calcareous soils. It plays a vital role as a significant co-factor for several enzymes that are involved in mitochondrial respiration, photosynthesis, nucleic acids synthesis and repair, metal homeostasis, and in maintaining the structural and functional integrity of proteins and chlorophyll [1,2,3,4]. Therefore, understanding the impact of higher or lower availability of Fe on the healthy growth and development of rice plants is necessary. The soil is the primary source of Fe for plants and its optimum availability in the form of Fe²⁺ is essential for their healthy growth and development. Both a deficiency and an excess of Fe in the soil hinder several physiological functions in the rhizosphere [5,6,7]. About 30% and 18% of the global soil is Fe deficient and Fe toxic, respectively [8,9]. Altered soil redox potential, soil pH, soil fertility status, and intensity of Fe deficiency and Fe toxicity can cause significant grain yield reductions under Fe-deficient and Fe-toxic soil [10]. Phytohormones are involved in regulation of nutrient availability. Conversely, mineral nutrients influence hormone biosynthesis, suggesting a relationship between hormones and nutritional homeostasis [11]. Exogenous application of phytohormones may alleviate Fe deficiency stress. There are also several investigations indicating that gibberellic acid (GA) is involved in Fe nutrition in plants [12]. Cytokinins control the root iron uptake machinery through a root growth dependent pathway in order to adapt nutrient uptake to the demand of the plant [13]. Reactive oxygen species (ROS) such as anion radical (O₂⁻), singlet oxygen (¹O₂), hydrogen peroxide (H₂O₂) and hydroxide radical (OH) are formed at

higher rates under several stress condition including inadequate mineral nutrition. Recent research has shown that a small amount of nutrients, particularly Zn and Fe applied by foliar spraying can affect the susceptibility of plants to stress [14,15]. Such conditions lead to impairment of various physiological and biochemical processes, damaging many cellular components including protein, membrane lipids, nucleic acids etc. A better understanding of maize physiological responses may help improving grain yield in iron-deficient soil. Therefore, the aim of this study was to evaluate the effects of iron and phytohormones on the physiological response viz., activity of various antioxidant enzymes, chlorophyll content, and grain yield of maize in iron-deficient soil.

2. MATERIALS AND METHODS

2.1 Experimental Details

A field experiment was laid out in 3-factor randomized block design (RBD) with three replications in Fe-deficient soil (Fe < 0.5 ppm). The three factors were: i) Fe-efficient (Suwan) vs. inefficient cultivar (Shaktiman-3); ii) 'control' (no Fe-spray) vs. one soil application of Fe (20 kg ha^{-1}) + two foliar spray of Fe as 0.5% ferrous sulphate at pre-flowering and 7-days after flowering; and iii) foliar application of phytohormones viz., GA₃ at 10 and 20 ppm; cytokinin at 10 and 20 ppm and their combinations. The plot size was 5×3 m. Spacing between rows was 75 cm while between plants it was 20 cm. Number of rows per plot was four while number of plants per plot was 100. Sampling for assay of various physiological and biochemical parameters were done at flowering stage.

2.2 Enzyme Extraction and Assay

Leaf extracts were assayed quantitatively for activity of enzymes viz., catalase, peroxidase and superoxide dismutase activity. The leaf samples, weighing about 100 mg, were homogenized with 5 ml of phosphate buffer pH 6.8 (0.1 M). This was then centrifuged at 2°C for 15 min at 17,000 g in a refrigerated centrifuge. The clear supernatant was taken as the enzyme source.

2.2.1 Catalase

The activity of catalase as well as peroxidase was assayed after the method of Chance and Maehly [16] with the following modifications: Five ml of the assay mixture for the catalase activity comprised: 300 μ moles of phosphate buffer, pH 6.8, 100 μ moles of H_2O_2 , and 1 ml of the twice diluted enzyme extracted. After incubation at 25°C for 1 min, the reaction was stopped by adding 10 ml of 2% (v/v) H_2SO_4 and the residual H_2O_2 was titrated against 0.01 N $KMnO_4$ until a faint purple colour persisted for at least 15 sec. A control was run at the same time in which the enzyme activity was stopped at "zero" time. One unit of catalase activity is defined as that amount of enzyme which breaks down 1 μ mol of H_2O_2 in one minute under the assay conditions described.

2.2.2 Peroxidase

For this, 5 ml of the assay mixture comprised of 125 μ moles of phosphate buffer, pH 6.8, 50 μ moles of pyrogallol, 50 μ moles of H_2O_2 and 1 ml of the 20 times-diluted enzyme extract. This was incubated for 5 minute at 25°C after which the reaction was stopped by adding 0.5 ml of 5% (v/v) H_2SO_4 . The amount of purpurogallin formed was determined by taking the absorbancy at 420 nm.

2.2.3 Superoxide dismutase (SOD)

Superoxide dismutase (SOD) activity was determined by measuring the inhibition in photoreduction of Nitroblue Tetrazolium (NBT) by SOD enzyme [17]. The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.6), 0.1 mM EDTA, 50 mM sodium carbonate, 12 mM L-methionine, 50 μ M NBT, 10 μ M riboflavin and 100 μ L of crude extract in a final volume of 3.0 mL. A control reaction was performed without crude extract. The SOD reaction was carried out by exposing the reaction

mixture to white light for 15 min at room temperature. After 15 min incubation, absorbance was recorded at 560 nm using a spectrophotometer. One unit (U) of SOD activity was defined as the amount of enzyme causing 50% inhibition of photochemical reduction of NBT.

2.3 Chlorophyll Measurements

Leaf chlorophyll content was measured by SPAD leaf chlorophyll meter [18]. Twelve independent SPAD measurements were made per treatment, using several different plants.

2.4 Data Analysis

Statistical analyses of experimental data were carried out by using SPSS software. Analysis of variance was carried out to test the significance of treatment effect and least significant difference (LSD) was calculated by standard method [19]. Data were expressed as mean \pm SD. Differences were considered statistically significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

Change in the activity of antioxidant enzymes is a defense mechanism of plants under stress induced by environmental conditions. The results revealed that the application of Fe and phytohormones significantly increased enzymes activity like catalase, peroxidase and superoxide dismutase.

3.1 Catalase Activity

In Fe-deficient soil (no application of iron in the soil at the time of experiment) a lower activity of catalase was recorded in both the cultivars, 'Suwan' and 'Shaktiman-3'. The value was 172.3 μ mole $min^{-1} g^{-1}$ fresh protein in cultivar 'Suwan' and 129.0 μ mole $min^{-1} g^{-1}$ fresh protein in Shaktiman-3 cultivar in control (Table 1). A significant increase in activity of catalase was observed with application of GA_3 plus cytokinin at 10 ppm in both the cultivars. In cultivar 'Suwan', the value was 196.0 μ mole $min^{-1} g^{-1}$ fresh protein and while it was 153.7 μ mole $min^{-1} g^{-1}$ fresh protein in Shaktiman-3. Similar trend of catalase activities in control and phytohormone treatments were also observed when Fe was applied as foliar spray to the aforesaid maize cultivars. The values were higher than the Fe-deficient (no iron application) conditions. While

Table 1. Effect of iron application and phytohormones on catalase activity (μ mole $\text{min}^{-1} \text{g}^{-1}$ protein) in contrasting cultivars of maize

Treatment	No iron			Iron application			Overall mean
	'Suwan'	'Shaktiman-3'	Mean	'Suwan'	'Shaktiman-3'	Mean	
Control	172.3	129.0	150.7	191.7	156.3	174.0	162.3
GA ₃ at 10 ppm	188.3	149.7	169.0	212.0	181.3	196.7	182.8
GA ₃ at 20 ppm	187.3	146.3	166.8	209.7	176.0	192.9	179.8
Cytokinin at 10 ppm	183.3	139.3	161.3	206.0	167.7	186.9	174.1
Cytokinin at 20 ppm	182.3	134.3	158.3	203.7	166.7	185.2	171.8
GA ₃ + Cytokinin at 10 ppm	196.0	153.7	174.9	217.7	182.0	199.9	187.4
GA ₃ + Cytokinin at 20 ppm	180.3	134.3	157.3	200.3	163.0	181.7	169.5
	Cv.	Fe	PH	Cv.× Fe	Cv.× PH	Fe × PH	Cv.× Fe × PH
LSD (P=0.05)	2.17	1.73	2.92	5.19	NS	2.11	NS

LSD = Least significant difference, Cv. = Cultivar, Fe = Iron, PH = Phytohormone, NS=Non significant

Table 2. Effect of iron application and phytohormones on peroxidase activity (Units mg^{-1} protein) in contrasting cultivars of maize

Treatment	No iron			Iron application			Overall Mean
	'Suwan'	'Shaktiman-3'	Mean	'Suwan'	'Shaktiman-3'	Mean	
Control	61.7	53.3	57.5	64.7	57.0	60.9	59.2
GA ₃ at 10 ppm	81.7	68.7	75.2	87.0	79.0	83.0	79.1
GA ₃ at 20 ppm	79.2	67.3	73.3	86.7	76.7	81.7	77.5
Cytokinin at 10 ppm	68.5	60.3	64.4	75.0	69.0	72.0	68.2
Cytokinin at 20 ppm	68.0	59.3	63.7	74.3	66.0	70.2	66.9
GA ₃ + Cytokinin at 10 ppm	90.0	75.0	82.5	103.0	88.3	95.7	89.1
GA ₃ + Cytokinin at 20 ppm	65.0	58.0	61.5	71.0	60.3	65.7	63.6
	Cv.	Fe	PH	Cv. × Fe	Cv. × PH	Fe × PH	Cv. × Fe × PH
LSD (P=0.05)	1.42	1.06	2.65	3.63	NS	3.76	NS

LSD = Least significant difference, Cv. = Cultivar, Fe = Iron, PH = Phytohormone, NS=Non significant

the interaction effects between iron application and cultivars were statistically significant, the interaction with phytohormones was non-significant. Interaction effects between iron application and phytohormones showed a synergistic effect with respect to catalase activity.

From perusal of the data it is clear that catalase activity of efficient cultivars were significantly higher than inefficient cultivar. Iron deficiency could induce alteration in reactive oxygen species and H_2O_2 content in leaves. Catalase was greater under Fe-sufficient treatment, suggesting higher amounts of physiological iron in leaf tissue of both cultivars. These results suggested that, antioxidant compounds are the key compounds to protect cell from oxidative injury. According to Salama et al. [20] antioxidant enzymes have potential role in protecting plant from the deleterious effect of iron deficiency in different flax cultivars. They also reported that significantly increased activity of this enzyme in Fe- deficient treatment compared to Fe-sufficient treatments.

3.2 Peroxidase Activity

Peroxidase activity declined under iron stress condition in the cultivars, 'Suwan' and Shaktiman-3', and the value ranged between 53.3 to 103.0 unit mg^{-1} fresh proteins. Peroxidase activity was highest in the treatment (GA_3 +cytokinin at 10 ppm). A significant increase in activity of peroxidase was observed with application of GA_3 and cytokinin and a negative dose dependent response was apparent. The value of peroxidase ranged between 61.7 to 90.0 unit mg^{-1} proteins in cultivar 'Suwan' and 53.3 to 75.0 unit mg^{-1} proteins in cultivar 'Shaktiman-3' where iron was not applied (Table 2). Under iron application, values were the highest in treatment GA_3 +cytokinin at 10 ppm in both the cultivar 'Suwan' (103.0 unit mg^{-1} protein) but in 'Shaktiman-3' (88.3 unit mg^{-1} protein). According to Kavita and Kumar [21] the highest value of peroxidase activity was observed in GA_3 + cytokinin at 10 ppm with foliar application of iron in rice. Guo et al. [11] also reported enhancement of uptake of iron in rice plants after GA_3 application.

3.3 Superoxide Dismutase Activity

The superoxide dismutase (SOD) activity showed significant increase in maize cultivars (Suwan and Shaktiman-3) with phytohormone

and iron application compare to control (Table 3). The highest value of SOD was noted in the GA_3 + cytokinin at 10 ppm with iron application condition. The highest mean value of SOD for the two cultivars were 12.7 unit mg^{-1} protein with iron application and 10.2 unit mg^{-1} protein in the iron deficient condition in treatment GA_3 + cytokinin at 10 ppm. SOD constitutes the first line of defence against ROS. SODs are a group of metalloenzymes that constitute the primary line of antioxidative defense by catalyzing the dismutation reaction of superoxide anion radical (O_2^-) to oxygen (O_2) and hydrogen peroxide (H_2O_2). In plants, three types of SODs (Cu/ZnSODs, FeSODs and MnSODs) have been identified, differing in the metal cofactor present within the active site. Cu/ZnSOD isoforms are found in the cytosol, chloroplasts, mitochondria, peroxisomes, and extracellular space. Significant modulations in SOD activity have been observed in a variety of plants exposed to a broad range of environmental stresses, such as drought, high or low temperature, ultraviolet-B irradiation, darkness, high salinity, nitrogen deficiency, supplementation with carbohydrates, herbicide treatment, heavy metal exposure, magnetic field influence, and pathogen infection. According to Sun et al. [22], the increment of SOD activity may account for the increased accumulation of superoxide radicals (O_2^-) in iron-deficient leaves. The results suggest that the greater SOD response of maize might be an important factor in the ability to alleviate oxidative burst and thus may be the basis for greater ability to survive in stress condition in resistant cultivars.

3.4 Chlorophyll Content

The leaf chlorophyll content was significantly lower in Fe-deficient condition in the cultivars, 'Suwan' (21.0) and 'Shaktiman-3' (14.9) and the SPAD value increased with application of phytohormones ranging between 18.1 to 28.2 (Table 4). Application of GA_3 + cytokinin at 10 ppm resulted in significantly increased chlorophyll content in both the cultivars of maize. Under Fe-sufficient condition (foliar spray of iron), the lowest SPAD value was observed in control (without phytohormone application) and it increased with application of both the phytohormones. The highest SPAD value under Fe application condition was observed by application of GA_3 + cytokinin at 10 ppm (27.1 - 32.7). According to Zaharieva et al. [23], iron deficiency primarily affects structure and function of the chloroplasts thus, under Fe

Table 3. Effect of iron application and phytohormones on superoxide dismutase (unit mg⁻¹ protein) in contrasting cultivars of maize

Treatment	No iron			Iron application			Overall Mean
	'Suwan'	'Shaktiman-3'	Mean	'Suwan'	'Shaktiman-3'	Mean	
Control	10.2	8.8	9.5	11.0	12.3	11.7	10.6
GA ₃ at 10 ppm	10.7	9.2	10.0	11.8	12.9	12.3	11.1
GA ₃ at 20 ppm	10.7	9.1	9.9	11.8	12.8	12.3	11.1
Cytokinin at 10 ppm	10.4	9.0	9.7	11.4	12.5	12.0	10.8
Cytokinin at 20 ppm	10.4	8.9	9.6	11.4	12.4	11.9	10.8
GA ₃ + Cytokinin at 10 ppm	11.2	9.3	10.2	12.2	13.3	12.7	11.5
GA ₃ + Cytokinin at 20 ppm	10.3	8.9	9.6	11.3	12.5	11.9	10.7
	Cv.	Fe	PH	Cv. × Fe	Cv. × PH	Fe × PH	Cv. × Fe × PH
LSD (P=0.05)	0.39	0.13	0.51	0.68	NS	0.32	NS

LSD = Least significant difference, Cv. = Cultivar, Fe = Iron, PH = Phytohormone, NS=Non significant

Table 4. Effect of iron application and phytohormones on chlorophyll content (SPAD value) in contrasting cultivars of maize

Treatment	No iron			Iron application			Overall Mean
	'Suwan'	'Shaktiman-3'	Mean	'Suwan'	'Shaktiman-3'	Mean	
Control	21.0	14.9	18.0	24.2	18.1	21.2	19.6
GA ₃ at 10 ppm	27.1	20.1	23.6	29.6	24.9	27.3	25.4
GA ₃ at 20 ppm	24.3	18.1	21.2	27.0	19.0	23.0	22.1
Cytokinin at 10 ppm	26.4	19.9	23.2	28.9	24.7	26.8	25.0
Cytokinin at 20 ppm	24.3	18.0	21.2	27.7	18.9	23.3	22.2
GA ₃ + Cytokinin at 10 ppm	28.2	22.2	25.2	32.7	27.1	29.9	27.6
GA ₃ + Cytokinin at 20 ppm	22.9	16.2	19.6	25.9	21.7	23.8	21.7
	Cv.	Fe	PH	Cv. × Fe	Cv. × PH	Fe × PH	Cv. × Fe × PH
LSD (P=0.05)	0.25	0.19	0.38	0.27	NS	0.54	NS

LSD = Least significant difference, Cv. = Cultivar, Fe = Iron, PH = Phytohormone, NS=Non significant

Table 5. Effect of iron application and phytohormones on grain yield (q ha⁻¹) in contrasting cultivars of maize

Treatment	No iron			Iron application			Overall Mean
	'Suwan'	'Shaktiman-3'	Mean	'Suwan'	'Shaktiman-3'	Mean	
Control	50.3	54.1	52.2	53.2	61.1	57.2	54.7
GA ₃ at 10 ppm	56.8	64.3	60.6	61.9	70.8	66.4	63.5
GA ₃ at 20 ppm	55.2	61.3	58.3	59.3	68.2	63.8	61.0
Cytokinin at 10 ppm	52.8	60.4	56.6	56.1	65.1	60.6	58.6
Cytokinin at 20 ppm	52.8	61.4	57.1	56.5	64.3	60.4	58.8
GA ₃ + Cytokinin at 10 ppm	58.6	67.0	62.8	64.6	72.2	68.4	65.6
GA ₃ + Cytokinin at 20 ppm	52.1	59.2	55.7	55.0	63.3	59.2	57.4
LSD (P=0.05)	Cv.	Fe	PH	Cv. × Fe	Cv. × PH	Fe × PH	Cv. × Fe × PH
	1.87	1.21	2.20	3.5	NS	1.43	NS

LSD = Least significant difference, Cv. = Cultivar, Fe = Iron, PH = Phytohormone, NS=Non significant

deficiency the reduction of leaf Fe content accompanied by a marked reduction of chlorophyll level.

The results obtained showed that foliar application of phytohormones (GA₃, cytokinin) had improved the state of Fe-deficient plants. The effect of these hormone applications might be explained by changes in Fe transport towards the leaves, by increased efficiency of Fe and by effects on plant metabolism [24]. Under Fe deficiency, the reduction of leaf Fe content accompanied by a marked reduction of chlorophyll level was also reported by Gogorcena et al. [25]. The simultaneous increase of the chlorophyll content along with activities of both heme enzymes (catalase and peroxidase) might also be regarded as sign for enhanced Fe supply in leaves. Cytokinins are known to stimulate the structural and biochemical differentiation of chloroplasts [26]. Drazkiewicz [27] reported that Fe deficiency and cytokinin act in opposite ways on chlorophyllase activity which also corroborates with our experimental results.

3.5 Grain Yield

Grain yield of maize increased with the foliar application of phytohormones and iron in Fe-deficient soil (Table 5). Application of Fe and GA₃ + cytokinin at 10 ppm significantly increased grain yield from 50.3 to 53.2 q ha⁻¹ in cv. 'Suwan' and 54.1 to 61.3 q ha⁻¹ in cv. 'Shaktiman-3'. Interaction effect between cultivar and Fe-application as well as Fe and phytohormone were significant and positive. The result showed that significantly higher yield was observed in Fe with phytohormone application (GA₃ + cytokinin at 10 ppm) was 68.4 q ha⁻¹ (mean value of two cultivars). Thus it is clear that significantly higher yield was obtained in Fe-deficient soil with phytohormone application even when no Fe was applied, indicating that phytohormones may compensate for the need of exogenous application of Fe by way of enhanced translocation of this micronutrient. A strong synergetic effect of GA₃ application on Fe was also reported by Guo et al. [11]. It seems that application of phytohormones increased translocation of Fe from soil resulting in significantly higher grain yield of rice. Liu et al. [28] reported that exogenous hormones regulate tiller bud growth through changing the contents of endogenous ABA, IAA, and zeatin + zeatin riboside contents in rice plants.

4. CONCLUSION

Foliar application of GA₃ and cytokinin significantly enhanced the activity of antioxidant enzymes, and increased chlorophyll content in leaves. They apparently increased the translocation of Fe from soil resulting in significantly higher grain yield of maize. It was also evident that application of Fe and growth regulators acted synergistically. Hence, application of GA₃ + cytokinin at 10 ppm may be done to alleviate Fe stress and to improve grain yield of maize.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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