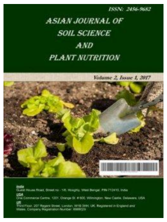

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Enhancement of Seed Zinc and Iron Density in Cowpea (*Vigna unguiculata*): A Physiological Approach

Namita Yadav[1*] and Yogesh Kumar Sharma[1]

[1]Department of Botany, University of Lucknow, Lucknow (U.P.), India.

Authors' contributions

This work was carried out in collaboration between both authors. Author NY designed the experiment, performed the statistical analysis and wrote the protocol and first draft of the manuscript. Author YKS helped in the designing and analysis of the whole experimental work.

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ABSTRACT

The present study was conducted to enhance the zinc (Zn) and iron (Fe) concentration in cowpea (*V. unguiculata* L.) var. Rituraj using physiological approach. Cowpea was grown in soil-pot culture subjected to soil application at presowing and foliar application of Zn, Fe and their combination at different time period of plant growth during the growing season February to April 2016 at Lucknow University campus. In soil application, the rate of $ZnSO_4.7H_2O$ and $FeSO_4.7H_2O$ application was 10 mg kg-1 soil each and foliar application was done at 0.5% of Zn and Fe at 35, 45 and 55 days of plant growth after sowing. The concentration of photosynthetic pigments, specific activities of catalase, peroxidase and carbonic anhydrase in leaves and tissue Zn and Fe concentrations in single treatment 17.41% & 68.90% and in combined treatment 26.92% & 8.83% respectively in seeds were significantly increased as compared to control. Zinc translocation from flag leaves to seed and its accumulation was more significant in the combined treatment of Zn and Fe foliar application at 35 days after sowing.

**Corresponding author: E-mail: namita0788@gmail.com;*

Keywords: Zinc; iron; translocation; accumulation; foliar application.

1. INTRODUCTION

Zinc (Zn) and iron (Fe) deficiencies are major micronutrient disorder and an important soil constraint for crop yield in certain soils [1]. There is great need to focus on the availability of Zn and Fe in soil because main reason for Zn and Fe deficiencies are low availability of this micronutrient to plant roots rather than low percentage of that nutrient in soil. Sometimes plant roots cannot absorb required amount of essential nutrients to plants. In that condition foliar application is more effective and supportive technique for supplying nutrients to plants more easily, when soil conditions are not suitable to plant growth [2,3].

Zinc is an important micronutrient that affects flowering, floral development, fertilization and seed maturation in crop. Insufficient supply of zinc affects more the seed yield than vegetative growth because zinc has a specific role in pollen fertilization. Iron is another micronutrient that is directly involved in the photosynthetic activity [4], chlorophyll synthesis, respiration [5] and nitrogen metabolism. In enzyme system iron serves as a cofactor that catalyzes biochemical reaction and occurs with a heam group in some enzymes like catalase, peroxidase and cytochrome oxidase [6].

In the present study effort has been made to focus on improving cowpea yield, biomass and tissue Zn and Fe concentration in edible plant parts (seed) by applying foliar application of Zn, Fe and their combination at different time points of plant growth in addition to soil Zn and Fe application.

2. MATERIALS AND METHODS

Cowpea *(V. unguiculata L.) var. Rituraj plants were grown in pots containing 10 kg soil in a glass house at an ambient temperature (25-30°C) during the growing season February to April 2016 at Lucknow University campus and subjected to treatment of iron and zinc singly and their combination* in addition to *soil application* at pre-sowing *and foliar application at different* time period *of plant growth*. Experimental soil was loamy- sand with pH 7.30, electrical conductivity 0.303 dsm−1 and Diethylenetriaminepentaacetate (DTPA)–extractable iron (Fe), and Zinc (Zn) was 7.93 and 1.16 mg kg−1 soil respectively before mixing. In soil *Zn and Fe* were applied at the rate of *10 mg kg-1* soil each. *Foliar application was applied at the rate of 0.5%* $ZnSO_4$ and 0.5% $FeSO_4$ singly and their combination at 35, 45 and 55 days of plant growth after sowing. Pots were divided into four lots: control, Zn, Fe and Zn+Fe treatment and arranged in a complete randomized block design (CRD). In each treatment, there were three replicates. After emergence, seedlings in each pot were uniformly thinned to maintain two plants per pot.

The concentration of photosynthetic pigment (chl a, chl b and carotenoids) and activities of certain enzymes catalase (CAT), peroxidase (POX), carbonic anhydrase (CA) were studied in young leaves at day 35, 45 and 55 after sowing. Total chlorophyll, chlorophyll a, chlorophyll b and carotenoid were measured according to Lichtenthaler [7]. Hundred miligrams leave were crushed in 10 ml 80% chilled acetone. Extract were centrifuged at 2000xg for 10 minutes. Absorbance of supernatant was observed in spectrophotometer at wavelength 662 nm, 645 nm and 470 nm. Chlorophyll contents were expressed in terms of mg chlorophyll present g^{-1} fresh weight of tissue.

Catalase (CAT) is a H_2O_2 splitting enzyme. The activity of CAT (EC 1.11.1.6) was assayed as described by Euler and Josephan [8] in a reaction mixture (10 ml) containing 500 µM H_2O_2 and 1.0 mmoL KH_2PO_4 buffer at pH 7.0 stabilized at 25°C. The reaction was allowed to proceed for 5 min and stopped by adding 5.0 ml 2N H_2SO_4. The reaction mixture was titrating with 0.1N potassium permangate ($KMnO_4$). CAT activity is expressed as µmol H_2O_2 decomposed in the given conditions of the assay.

Peroxidase (POX) (EC 1.11.1.7) activity was assayed by method of Luck [9]. The reaction mixture (10 ml) contained 5.0 ml of 0.1M of KH_2PO_4 buffer (pH 6.5), 1.0 mL of 0.5% p-phenylenediamine, 1.0 mL of 0.01% (v/v) H_2O_2, and 1.0 mL tissue extract and reaction proceed for 5.0 min and was stopped by adding 2.0 ml 5N H_2SO_4. The colour intensity of the supernatant after centrifugation at 10,000xg for 5.0 minutes was measured at 485 nm.

Carbonic anhydrase (CA) is a zinc-containing carbonate hydro-lyase enzyme. CA (EC 4.2.1.1) used 2 ml of 0.02 M vernol buffer (pH 8.15), 0.10% bromothymol blue, 0.2 ml enzyme extract and 2 ml distilled water saturated with CO_2. The change in colour blue to greenish yellow measured to record the time in second and unit of CA activity expressed enzyme unit 100 mg mg^{-1} protein [10].

Plants were harvested at 90d and studied number of pod $plant^{-1}$, number of seed pod^{-1}, seed weight pod^{-1} and weight of 100 seeds. Plants were sampled and separated into various parts for determination of dry matter, Zn and Fe tissue concentration. Harvested plants were washed properly under running tap water, and finally rinsed with distilled water to avoid surface contamination. Plants of all treatments were separated into their parts, which were chopped and placed in paper bags to be dried in an electric oven at 70 ◦C for 48 h. Concentrations of iron and zinc in cowpea were measured in different plants parts by atomic absorption spectrophotometer (Thermo Jerrell Ash Video 12E: AA⁄AE Chicago, IL) in clear digests after a nitric acid–perchloric acid (10:1) digestion [11]. After harvesting the crop soil samples were collected from surface horizon (0-15 cm) of the earthen pots, oven dried, passed through 100 mesh sieve for analysis of soil Zn and Fe concentration [12].

The values presented in tables and figures are the mean of three observations (±SE; n = 3). The data were analyzed by one way ANOVA using software program Sigma Stats 3.5. It was followed by comparison of mean value using Holm Sidak method at $P \leq 0.05$.

3. RESULTS

Number of pod $plant^{-1}$, number of seed pod^{-1}, seed weight pod^{-1} and weight of 100 seeds were increased in all treatments as compared to control (Table 1). Single treated Fe was more significant as compared to combined treatment, its obtained maximum number of pod $plant^{-1}$ (12.66), number of seed pod^{-1} (13.00), seed weight pod^{-1} (1.95 g) and weight of 100 seeds (12.98 g) at 35d. Single and combined treatment of Zn was observed 17.41% and 26.92% at 45d and 35d respectively, which was significantly higher as compared to control and other Zn treatments (Table 1).

Biomass of plant increased significantly and variably in single and combined treatments of iron and zinc. The increased biomass was more pronounced in combined treatment (Zn+Fe) at 35 d (Table 1). Zn and Fe tissue concentration in seed were significantly increased in all treatments as compared to control. Combined Zn treated plants might be obtained maximum Zn translocation and accumulate in seed. The data in Table 2 indicated significant effect on translocation and accumulation of Zn and Fe concentrations in seed in both treatments as compared to without any treatment at different time periods.

Chlorophyll concentration in leaves was increased with foliar application of Fe single and combined Zn and Fe treatments as compared to single treated zinc. The effect of foliar application of Fe and Zn on total chlorophyll, chlorophyll a, chlorophyll b and carotenoid was shown in Fig. 1 (a-d). With increase in period of foliar application, chlorophyll content was decreased and carotenoid was slightly increased.

In both single and combined treatments of Fe, the specific activity of catalase (CAT) and peroxidase (POX) showed a significant result at 45 d. Foliar application after 45 d marked decrease in activity of CAT and POX was found in single and combined treated Fe plants (figure 2a-b). The specific activitiy of carbonic anhydrase increased with supply of Zn single and combined treatments. Maximum enzyme activity of CA was obtained at 45d in single Zn treatment. The specific activity of that enzyme was marked significant effect in single and combined treatments as compared to control (Fig. 2c).

In soil analysis after mixing DTPA extractable Zn and Fe were varied from to 7.31 to 6.23 mg kg^{-1} and 12.07 to 11.56 mg kg^{-1} respectively and after harvesting the crop it varied from 6.04 to 5.22 mg kg^{-1} Zn and 10.74 to 9.67 mg kg^{-1} Fe in single and combined treated soil respectively. The results indicate that 16.22% to 17.38% applied Zn and 11.02% to 16.35% applied Fe was available to plants.

4. DISCUSSION

In the present experiment the plants grown without any application of nutrients later showed reduction in the number of pod due to suppression of branching; reduction in a number of seeds per plant and marked delay in flowering. Flower buds and open flowers were shed prematurely which resulted into reduced pod formation (Table 1). Seed yield might be due to increase in shoot branching, early flowering and decrease in premature fall of flower buds and flowers. Metal accumulation in the edible plant part (seed) is correlated with the metal concentration in the substrate. In the foliar application leaves are the primary sites to which metal entered through stomata and showed that foliar application of Zn in leaves directly related to Zn concentration in both treatments single and combined (Table 2). In combined treatment of Zn and Fe enhanced the mobility of Zn in their interaction. The leaves showed least accumulation of Zn in combined treatment of Zn+Fe and Fe in single treatment which might reflect that most of metal is translocated to edible plant part (seeds).Gravel et al. reported least content of heavy metals accumulation in stem and most of metal translocated to leaves in some medicinal plants [13]. In combined foliar application of Fe and Zn concentration there was decrease in Fe content in the various parts of cowpea might be due to interaction between Zn and Fe for the same physiological binding site and interference the availability of Fe, leading to impairment of Fe metabolism at same concentration [14]. In combined treatment, Fe assesses competitive effect, showed less translocation and accumulation from leaf to seed and stem as compared to single Fe treated plants. The data indicated that foliar application of single treatment of Zn at 45 d and combined treatment Zn+Fe at 35 d markedly increased the Zn seed density in cowpea respectively. These

Table 1. Effect of single and combined Zn and Fe foliar application at different time period on seed yield and dry weight of cowpea plants

Days of foliar spray	Treatments	No. of pod plant $^{-1}$	No. of seed pod $^{-1}$	Seed weight pod $^{-1}$ (g)	Weight of 100 seeds (g)	Dry weight (g plant $^{-1}$)**
	Control	7.33±0.666	9±0.577	1.32±0.032	11.75±0.017	16.87±0.054
35 d	Zn	10.66±0.666*	11.66±0.333*	1.44±0.024	11.92±0.005	24.48±0.043*
	Fe	12.66±1.33*	13.00±0.577*	1.95±0.017	12.98±0.004	34.81±0.012*
	Zn+Fe	11.33±0.666*	11.33±0.333*	1.64±0.033	11.97±0.006	28.09±0.014*
45 d						
	Zn	11.66±0.666*	12.00±0.577*	1.53±0.015	12.52±0.014	28.67±0.034*
	Fe	12.00±1.15*	12.66±0.666*	1.80±0.011	12.20±0.020	28.29±0.023*
	Zn+Fe	10.66±1.33*	10.00±0.577*	1.58±0.005	11.96±0.006	25.04±0.021*
55 d						
	Zn	9.33±0.666*	11.00±0.333*	1.51±0.013	11.98±0.041	23.10±0.010*
	Fe	10.00±1.15*	11.33±0.333*	1.41±0.021	11.82±0.003	26.86±0.018*
	Zn+Fe	9.66±0.333*	10.00±0.577*	1.37±0.008	11.85±0.012	23.03±0.026*

**Significant difference from the control at P≤0.05*
*** including pod*

Table 2. Effect of single and combined Zn and Fe foliar application at different time period on tissue Zinc and iron concentrations in different plant parts of cowpea

Days of foliar spray	Treatments	Root	Stem	Leaf	Pod
		Zinc concentration(µg g^{-1} dry matter)			
	Control	8.91±0.620	25.15±0.606	18.44±0.692	29.86±0.092
35 d	Zn	31.81±0.219*	31.6±0.103*	31.74±0.976*	33.80±0.346*
	Zn+Fe	32.07±0.875*	32.20±0.499*	29.92±0.798*	37.63±0.941*
45 d	Zn	29.11±0.793*	32.44±0.049*	32.02±0.470*	35.06±0.199*
	Zn+Fe	30.61±0.155*	31.17±0.586*	30.41±0.280*	35.41±0.536*
55 d	Zn	19.14±0.333*	30.05±0.890*	33.47±0.733*	31.96±0.369*
	Zn+Fe	28.50±0.800*	30.99±0.505*	31.39±0.176*	34.32±0.730*
		Iron concentration (µg g^{-1} dry matter)			
	Control	105.75±0.016	52.52±0.660	85.56±1.11	58.08±0.396
35 d	Fe	116.95±1.48*	85.01±0.626*	106.20±0.680*	98.10±0.736*
	Zn+Fe	109.45±0.396*	69.69±0.733*	100.75±0.666*	60.20±0.386*
45 d	Fe	117.85±0.548*	93.41±0.303*	109.50±0.00*	95.07±0.726*
	Zn+Fe	121.15±0.375*	78.95±0.623*	95.30±1.40*	61.45±0.416*
55 d	Fe	108.75±0.750*	78.57±0.763*	104.00±0.750*	78.57±0.946*
	Zn+Fe	111.55±0.779*	83.67±0.333*	92.90±0.660*	63.21±0.196*

**Significant difference from the control at P≤0.05*

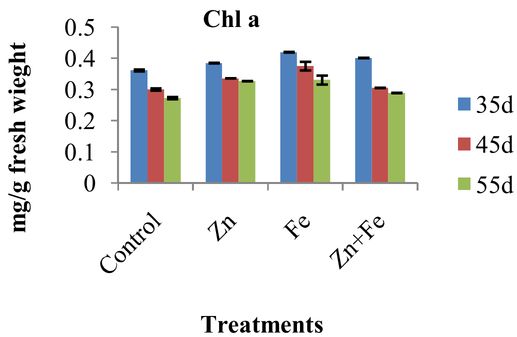
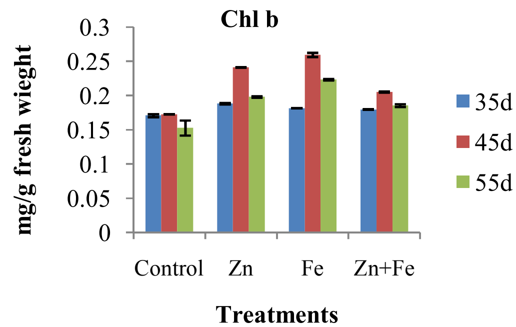


Fig. 1(a)



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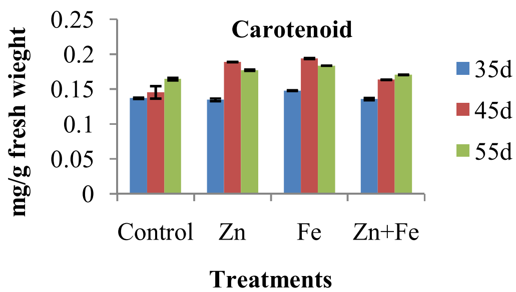

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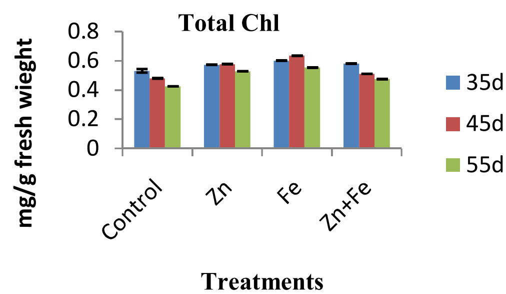

Fig. 1(d)

Fig. 1(a-d). Effects of foliar application of Zn, Fe and Zn+Fe on chlorophyll a, chlorophyll b, carotenoid and total chlorophyll content in cowpea leaf at different time period of plant growth. Vertical line in each represents ± SE

results are in support with the results of Phattarakul, et al. [15]. After harvesting crop from experimental soil, zinc becomes slightly available to the plant might be due to high capacity of fixation [16,17] and iron due to its oxidizing nature ($Fe^{2+} = Fe^{3+} + e^{-}$) [18, 19,20,21].

The chlorophyll contents were increased in leaves of cowpea treated with Fe in single and combined with Zn (Fig. 1 a-d). The increase in chlorophyll concentration ultimately increased photosynthesis and production of the photosynthetic product, might be a probable reason for the enhanced plant biomass. The

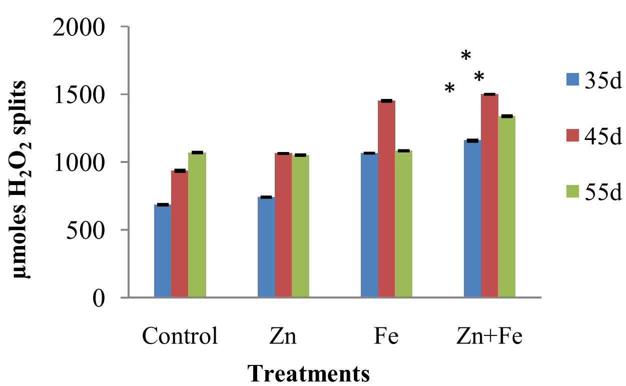

Fig. 2(a)

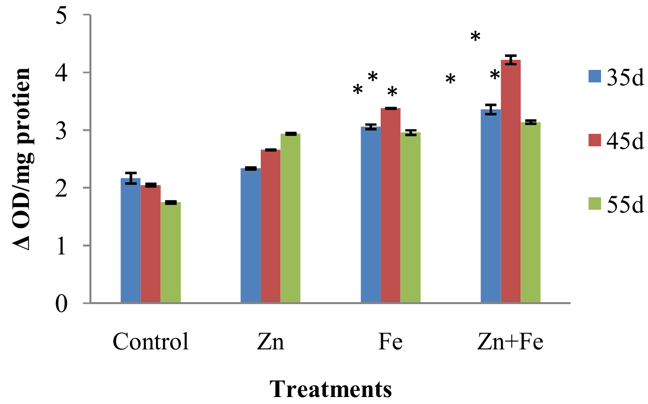

Fig. 2(b)

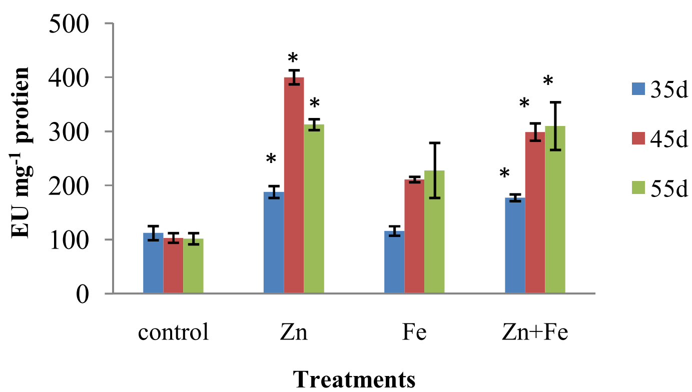

Fig. 2(c)

Fig. 2(a-c). Effect of foliar application of Zn, Fe and Zn+Fe on catalase, peroxidase and carbonic anhydrase activity in cowpea leaf at the different time period of plant growth. Vertical line in each represents ± SE. Asterisk (*) denotes significant difference from the control at P≤0.05

chlorophyll contents in leaves are directly linked to Fe concentration applied to the plants [22]. Catalase and peroxidase are free radical scavenging enzymes; their activity might depend upon the availability of oxidizing substrate (H_2O_2). Foliar treatment of Fe at 45 d was more significant and increased catalase activity, level of H_2O_2 decreased in leaves which resulted in enhancement of plant growth and yield (Fig. 2a). Peroxidase decomposes H_2O_2 by oxidation of phenolic compounds which might be accumulated under Fe stress condition at 55 d and Fe supply is not useful for plant growth (Fig. 2a). The increased specific activity of peroxidase might be attributed to the concentration of free radicals and re-synthesis of membrane bound enzyme [23]. At 45 d activity of carbonic anhydrase enzyme was more and significantly increased in Zn treated plants as compared to control and other treated plants (Fig. 2c). Carbonic anhydrase is hydro-lyase enzyme that catalyzes the reversible hydration of carbon dioxide, increasing absorbtion of carbon dioxide per unit leaf area, thereby increasing photosynthesis and biomass production [24]. At 45d Zn foliar application significantly increased yield and tissue Zn density in cowpea seed.

5. CONCLUSION

From the observed results in cowpea, it finds that combined treatment of Zn with Fe significantly increased biomass, seed yield, enzyme activities (catalase and peroxidase) and tissue Zn concentration in cowpea seed at 35 d. But carbonic anhydrase activity was more significant in the single treatment of Zn. Fe in single treatment was more significant as compared to the combined treatment of Zn with Fe. The observed data of foliar application of Zn and Fe at 35d might be more profitable for seed Zn and Fe density in cowpea.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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