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EFFECT OF ZINC, IRON AND MANGANESE FERTILIZATION ON CONCENTRATIONS OF THESE METALS IN THE STEM AND LEAVES OF SOYBEAN AND ON THE CHLOROPHYLL CONTENT IN LEAVES DURING THE REPRODUCTIVE DEVELOPMENT STAGES

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Abstract

In order to investigate the effect of zinc, iron and manganese fertilization on Zn, Fe and Mn concentrations in the stem and leaves and on the chlorophyll content in soybean leaves at different reproductive growth stages, two experiments were conducted in a research field of the Islamic Azad University of Kermanshah, Iran, during the 2010 and 2011 growing seasons. The experimental design consisted of a factorial experiment based on the randomized complete block method with three replicates. The treatments included three levels of Zn (0, 20 and 40 kg ha⁻¹), Fe (0, 25 and 50 kg ha⁻¹), and Mn (0, 20 and 40 kg ha⁻¹), which were all applied to the soil. At 30, 60, 90 and 120 days after sowing (DAS), the SPAD readings were done on five leaves from each experimental plot for all replicates. The results indicated that the zinc application had significantly affected Zn, Fe and Mn concentrations in soybean stems at all sampling times except for the soybean stem Mn concentration at the maturity stage. In contrast, the concentration of Mn at seed filling was unaffected by iron fertilization. Also, the maximum Fe concentration in soybean stems resulting from zinc application was achieved in Zn₀ treatment. The highest Zn and Mn concentrations in leaves were recorded when iron was applied in smaller amounts. Furthermore, zinc, iron and manganese applications had significant effects on the leaf chlorophyll concentration during all of the growth stages of soybean plants. In addition, increased iron and manganese fertilization raised the soybean leaf chlorophyll concentrations in all the samples. The maximum chlorophyll concentration in soybean leaves at 60 DAS was recorded in Zn₂₀Fe₅₀, Zn₂₀Mn₄₀ and Fe₅₀Mn₄₀ treatments (34.7, 34.0 and 35.2, respectively).

Keywords: antagonistic effect, Atomic Absorption Spectrometry, flowering stage, leaf chlorophyll, SPAD value.

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INTRODUCTION

Availability of micronutrients is one of the most important factors in their absorption by plant roots and transfer to shoots. According to ALLOWAY (2004), there are significant relationships between the availability of micronutrients in soil and photosynthesis as well as assimilation in plants. Furthermore, some previous studies have shown that deficiency of iron and manganese in a human organism causes anaemia, skin diseases and, in children, a weaker response to environmental factors (SAN 2006). The uptake of micronutrients from the soil and their transfer to the edible parts of plants plays a vital role in human health (TSUI et al. 2006).

The problem of micronutrient deficiency in human populations, with the underlying high social costs, cannot be solved by using enriched food or drugs (HU-LIN et al. 2007). Contrarily, the production of crops with a high content of micro- and macronutrients in edible parts can be a successful solution (WELCH, GRAHAM 2004), particularly because plant crops make up a large part of our diets (HU-LIN et al. 2007). Many researchers have emphasized the fact that application of micronutrients can result in their higher concentrations in plant tissues (CUI, WANG 2005, SHARMA, AGRAWAL 2006, HANSCH, MENDEL 2009). Concentrations of micronutrients are different in individual parts of the plant, but their highest content is frequently reported to be in the roots (BONNET et al. 2000, KHUDSAR et al. 2008) or shoots (SHARMA, AGRAWAL 2006). Also, the content of micronutrients in plants fluctuates at different time and/or different of plant growth stages (GUPTA et al. 2008). Micronutrient deficiency limits plant growth (GHOLAMALIZADEH et al. 1995, FAGERIA 2000) and chlorophyll concentration (HEITHOLT et al. 2003, WIERSMA 2005). In addition, the leaf chlorophyll content in soybean has been found to decrease in response to Zn, Mn and Fe deficits (ADAMS et al. 2000).

The biosynthetic pathway of chlorophyll depends on zinc, iron and manganese; similarly, electron transport reactions are influenced by these metals (SPILLER et al. 1982, PUSHNIK, MILLER 1984). Chlorophyll content can be determined with a SPAD (Soil and Plant Analyzer Development) meter, which is a nondestructive tool for measuring chlorophyll. YAMAMATO et al. (2002) reported that using an SPAD 502 chlorophyll meter for the estimation of chlorophyll content in leaves was more useful than the extraction method. The usefulness of an SPAD 502 chlorophyll meter as a nondestructive analytical instrument for determination of chloroplast pigments was mentioned in some earlier studies (PARVIZI et al. 2004, NEVES et al. 2005, BONNEVILLE, FYLES 2006, GIRMA et al. 2006). Application of zinc and manganese fertilizers leads to higher chlorophyll values read with an SPAD 502 meter (HEITHOLT et al. 2002). Hence, an SPAD 502 chlorophyll meter was chosen as a diagnostic tool in the current experiment to determine the chlorophyll content in leaves at different growth stages of soybean plants.

In brief, the main objective of this study has been to monitor zinc, iron and manganese concentrations in the leaves and stems of soybean plants

at different reproductive growth stages, after zinc, iron and manganese fertilization. Additionally, the relationships between leaf chlorophyll levels in soybean and the use of zinc, iron and manganese fertilizers were assessed.

MATERIAL AND METHODS

Two field experiments were carried out on a research field of the Islamic Azad University of Kermanshah Province, Iran (34023' N, 4708' E; 1351 m elevation) in 2010 and 2011. The experimental design was a 3×3×3 factorial experiment based on the randomized complete block method with three replicates. Every year, cv. Williams soybean [*Glycine max* (maturity group III), supplied by the oilseed company of the Kermanshah Agricultural Administration, Iran], was selected as the experimental material. Soil samples were collected from an experimental area at 0-30 cm depth for soil analyses. The texture of the soil corresponded to silty clay.

The soil had the following properties: pH 7.6, total organic matter 2.3%, electrical conductivity (ECe) 0.61 ds m⁻¹, total nitrogen 0.18%, available phosphorus 9.9 mg kg⁻¹, available potassium 563 mg kg⁻¹, zinc 0.71, iron 6.2 and manganese 4.3 mg kg⁻¹ soil for year of 2010 and pH 7.4, total organic matter 2.1%, electrical conductivity (EC) 0.52 ms m⁻¹, total nitrogen 0.14%, available phosphorus 10.1 mg kg⁻¹, available potassium 389 mg kg⁻¹, zinc 0.83, iron 3.6 and manganese 4.0 mg kg⁻¹ soil for year of 2011. Before planting soybean seeds, fertilizers were applied as follows: 200 kg P₂O₅ ha⁻¹ and 50 kg N ha⁻¹ mixed with soil, which was then ploughed once and harrowed twice. Before planting, seeds were presoaked in 10% sugar solution and then inoculated with *Bradyrhizobium japonicum* (2 g kg⁻¹ of seeds). The experiment included 27 treatments placed on 81 plots. The plots consisted of six rows, 5 m in length and spaced at 60 cm. The distance between plants in a row was 5 cm and the plant density was 333,000 plants/ha. The plant density was achieved by overplanting and thinning at V3 stage. The amounts of fertilizers to be applied, i.e. zinc (0, 20 and 40 kg ha⁻¹ from ZnSO₄ source), iron (0, 25 and 50 kg ha⁻¹ from FeSO₄ source) and manganese (0, 20 and 40 kg ha⁻¹ from MnSO₄ source), were calculated based on the surface area of each plot; next, appropriate doses of fertilizers were mixed with soft soil at a ratio of 1: 5 and placed in furrows made manually next to the stacks. The plots were irrigated when necessary to avoid water deficits. At intervals of 30, 60, 90 and 120 days after sowing, the SPAD readings (Minolta SPAD-502 chlorophyll meter with wide display range: -9.9 to 199.9 SPAD units) were performed on five leaves (from the uppermost leaf of the youngest fully developed trifoliolate leaf) from each experimental plot for all replicates. Five plants were randomly selected from each plot at consecutive reproductive growth stages of soybean (flowering stage, pod setting stage, seed filling period stage, and maturity stage), distinguished by FEHR, CAVINESS (1977). To measure leaf

concentrations of micronutrients, the topmost trifoliolate leaves of the plants were cut off the stem. Samples (leaves and stems, separately) were washed with distilled water, dried in an oven at 70°C for 48 hours, weighed, and incinerated at 550°C. Dry ash samples were solved in concentrated HNO₃ and HClO₄. The Zn, Fe and Mn content was determined by Atomic Absorption Spectrometry (AAS) according to KACAR (1984).

All the data obtained from the measurements were evaluated statistically with MSTATC software. Combined variance analysis was performed after the Bartlett test for checking uniformity of data variance ($P = 0.05$). LSD (Least Significant Difference Test) was used to compare the means. Excel software was used to plot diagrams.

RESULTS AND DISCUSSION

Combined analysis of variance performed to test the impact of zinc, iron and manganese fertilization on their concentrations in soybean stems at different reproductive growth stages yielded the results, shown in Table 1, which indicated that – except for the Mn concentration in the stem at the maturity stage – zinc application significantly affected the Zn, Fe and Mn concentrations in soybean stems at all the sampling times.

At the flowering stage, the effect of zinc fertilization was significant at 1% with respect to Zn concentration and at 5% regarding Fe and Mn concentrations. Moreover, the Zn and Fe concentrations in soybean stems at the pod setting, seed filling and maturity stages and the Mn concentration at the seed filling stage were affected by zinc application at the level of significance equal to 1%. Iron fertilization had a significant effect on the Zn and Fe concentrations in soybean stems at all sampling times ($P < 0.01$) and on the Mn concentration at the maturity stage at 5%. The concentration of Mn at seed filling was unaffected by iron fertilization.

Except for the Zn concentration at flowering, manganese application had significantly affected the Zn, Fe and Mn concentrations in soybean stems at all the reproductive growth stages. ALLOWAY (2004) reported that manganese nutrition could enhance the transfer of zinc to other plant organs. Zn, Fe and Mn concentrations at the seed filling period stage, Fe concentration at the flowering, pod setting and maturity stages and Mn concentration at the pod setting stage were significantly affected by interaction effects of zinc with iron (Zn×Fe). Furthermore, the interaction of zinc with manganese (Zn×Mn) had a significant effect on Zn and Fe concentrations in the soybean stem at pod setting and at seed filling, whereas its influence on the other evaluated traits was not statistically significant. On the other hand, the interaction of iron with manganese (Fe×Mn) affected the Fe concentration at the flowering, pod setting and seed filling stages but had no significant effect on concentrations of the other micronutrients.

The results of combined analysis of variance applied to test the impact of

Table 1
 Combined analysis of variance for the impact of zinc, iron and manganese fertilization on their concentrations in soybean stem
 at different reproductive growth stages of soybean

Source of variation	df	MS											
		flowering stage			pod set stage			seed filling period stage			maturity stage		
		Zn	Fe	Mn	Zn	Fe	Mn	Zn	Fe	Mn	Zn	Fe	Mn
Year (Y)	1	60.8	613.7	693.9	9.5	1024.5	2.0	6.1	1680.1	75.6	1.2	11.5	0.1
Zn	2	110.7**	698.4*	16.5*	214.4**	1400.8**	11.9*	33.1**	457.3**	29.4**	19.3**	165.5**	0.9 ^{ns}
Y×Zn	2	0.3 ^{ns}	156.3 ^{ns}	19.6*	46.7**	634.0**	26.1**	4.5**	95.2**	14.5**	3.5**	52.2 ^{ns}	15.3**
Fe	2	12.4**	30242.4**	284.3**	16.5**	18373.3**	219.3**	12.5**	6109.2**	5.6 ^{ns}	3.4**	5483.2**	9.7*
Y×Fe	2	1.3 ^{ns}	2521.3**	162.5**	3.7**	3542.1**	142.7**	1.9**	1654.7**	11.6*	0.1 ^{ns}	3851.9**	12.9*
Zn×Fe	4	0.7 ^{ns}	433.3*	2.5 ^{ns}	3.9**	249.5*	25.3**	2.3**	213.5**	12.9**	0.5 ^{ns}	73.3**	2.6 ^{ns}
Y×Zn×Fe	4	0.9 ^{ns}	387.5*	5.3 ^{ns}	0.9 ^{ns}	820.3**	13.5*	0.7**	125.7**	9.9*	0.1 ^{ns}	38.8 ^{ns}	3.5 ^{ns}
Mn	2	2.3 ^{ns}	6618.7**	437.3**	10.7**	4564.4**	354.2**	1.8**	287.1**	230.4**	1.6*	148.9**	295.1**
Y×Mn	2	1.8 ^{ns}	3231.4**	31.3**	1.8*	1782.7**	38.2**	0.3 ^{ns}	59.1**	22.9**	0.1 ^{ns}	134.6**	35.6**
Zn×Mn	4	0.6 ^{ns}	20.3 ^{ns}	8.9 ^{ns}	1.6*	280.3*	0.9 ^{ns}	0.7**	21.2*	3.4 ^{ns}	0.4 ^{ns}	20.7 ^{ns}	0.1 ^{ns}
Y×Zn×Mn	4	0.2 ^{ns}	80.7 ^{ns}	6.2 ^{ns}	0.7 ^{ns}	582.7**	4.3 ^{ns}	0.2 ^{ns}	16.5 ^{ns}	8.2*	0.1 ^{ns}	32.6 ^{ns}	0.5 ^{ns}
Fe×Mn	4	1.6 ^{ns}	1080.4**	6.2 ^{ns}	0.4 ^{ns}	989.5**	5.2 ^{ns}	0.2 ^{ns}	61.2**	1.4 ^{ns}	0.5 ^{ns}	31.3 ^{ns}	1.8 ^{ns}
Y×Fe×Mn	4	3.7 ^{ns}	627.3**	3.0 ^{ns}	2.9*	1018.4**	8.6 ^{ns}	0.3 ^{ns}	69.9**	3.3 ^{ns}	0.1 ^{ns}	26.2 ^{ns}	2.5 ^{ns}
Zn×Fe×Mn	8	0.9 ^{ns}	166.3 ^{ns}	8.4 ^{ns}	0.4 ^{ns}	345.1**	6.3 ^{ns}	0.2 ^{ns}	18.7*	9.5*	0.3 ^{ns}	13.2 ^{ns}	3.8 ^{ns}
Y×Zn×Fe×Mn	8	0.9 ^{ns}	151.9 ^{ns}	6.1 ^{ns}	0.2 ^{ns}	429.4**	8.2 ^{ns}	0.4*	19.7**	6.3*	0.1 ^{ns}	16.5 ^{ns}	3.1 ^{ns}
Error	104	1.6	149.1	4.9	0.4	95.4	2.8	0.1	7.1	2.6	0.3	17.8	3.0
Coefficient of variation (%)	-	10.0	8.2	9.9	7.6	7.3	7.8	9.6	6.8	10.2	9.8	11.5	12.4

ns, * and **: non-significant, significant at 5% and 1% levels of probability, respectively

zinc, iron and manganese fertilization on concentrations of these elements in soybean leaves at different reproductive growth stages are contained in Table 2. Zinc fertilization had significant effects on Zn, Fe and Mn concentrations in soybean leaves at pod setting and seed filling as well as Zn and Fe at the flowering and maturity stages. The Mn concentration in leaves at the flowering and maturity stages was unaffected by the zinc application. On the other hand, iron applied to soil had significant effects on the Zn, Fe and Mn concentrations in soybean leaves at all the growth stages. Except for the Zn concentration at the pod setting stage and the Mn concentration at the maturity stage, manganese application had a significant effect on the Zn, Fe and Mn concentrations in soybean leaves (Table 2). The Zn and Fe concentrations in soybean leaves at flowering and maturity as well as Zn, Fe and Mn concentrations at pod setting and seed filling were affected by interaction effects of zinc with iron (Zn×Fe). In addition, the interaction of zinc with manganese (Zn×Mn) had a significant effect on the Zn concentration in leaves at flowering and pod setting, and on the Mn concentration at pod setting and seed filling. The interaction of iron with manganese (Fe×Mn) significantly affected the Fe concentration in leaves at flowering and pod setting ($P < 0.05$), and Mn at pod setting and seed filling ($P < 0.01$).

The impact of different zinc, iron and manganese levels applied to soil on their concentrations in soybean stems at reproductive growth stages of soybean is shown in Table 3. The highest Zn concentration at flowering, pod setting, seed filling period and maturity was observed in Zn₄₀ treatment (14.1, 12.2, 4.9, and 4.5 mg kg⁻¹ dry weight of soybean stem, respectively). Also, at all the sampling times, the maximum Fe concentration in the soybean stem resulting from zinc application was achieved in Zn₂₀ treatment. Zinc application up to 20 kg ha⁻¹ had no significant effect on the Mn concentration at flowering and pod setting, while an excess amount of zinc decreased the Mn concentration in the soybean stem at those times. Application of iron increased the stem Fe concentration by 175.9, 157.2, 53.9, and 45.8 mg kg⁻¹ dry weights from the flowering to maturity stages, respectively. HODGSON et al. (1992) found that iron use increased the Fe concentration in soybean leaves by 42% compared to the control treatment. In our experiment, there were antagonistic effects between iron versus zinc and manganese, so that the Zn and Mn concentrations were the highest in treatments with smaller amounts of iron (Fe₀ treatment). GHASEMI-FASAEI et al. (2003) demonstrated that an application of iron decreased the mean Mn concentration by 91%. These results are in agreement with data from other experiments (MANDAL et al. 2000, ALAM et al. 2001, IZAGUIRRE-MAYORAL, SINCLAIR 2009).

With manganese applied to soil in a range from 0 to 40 kg ha⁻¹, the Mn concentration in the soybean stem increased at all of the sampling times. GUPTA et al. (2008) believed that the response of soybean to manganese fertilization was positive. At different reproductive growth stages, the maximum Zn concentration in soybean stems was achieved in Mn₂₀ treatment (Table 3). Similar results were observed in soybean leaves. This is consistent with the findings

Table 2
 Combined analysis of variance for the impact of zinc, iron and manganese fertilization on their concentrations in soybean leaves at different reproductive growth stages

Source of variation	df	MS											
		flowering stage			pod set stage			seed filling period stage			maturity stage		
		Zn	Fe	Mn	Zn	Fe	Mn	Zn	Fe	Mn	Zn	Fe	Mn
Year (Y)	1	1.7	2075.1	1955.2	167.2	1892.5	271.4	0.2	8416.8	529.9	78.1	1191.3	131.8
Zn	2	1252.6*	208.4	28.8 ^{ns}	591.6**	3147.2*	977.5**	1402.5*	16065.9*	391.5**	1438.5**	2041.5**	13.3 ^{ns}
Y×Zn	2	191.8**	121.8 ^{ns}	251.8**	75.7**	4834.4**	1054.5**	47.7**	6162.5**	444.9**	61.5**	10010.2**	144.5 ^{ns}
Fe	2	73.8**	96159.7**	1314.8*	116.8**	49062.5**	2004.5**	37.9**	15300.9*	1079.8**	35.3*	36308.5**	345.0**
Y×Fe	2	2.1 ^{ns}	1125.6**	28.5 ^{ns}	7.3 ^{ns}	7090.2*	311.1**	3.5 ^{ns}	3511.4**	815.4**	53.0**	1369.4**	176.2 ^{ns}
Zn×Fe	4	36.1**	3243.1*	79.0 ^{ns}	10.3*	589.2*	283.7**	30.1**	739.6*	488.5**	30.0*	365.8**	103.6 ^{ns}
Y×Zn×Fe	4	16.8*	934.1 ^{ns}	18.9 ^{ns}	30.1**	490.9*	132.9**	19.0**	392.5 ^{ns}	291.9**	22.8*	815.6**	3.9 ^{ns}
Mn	2	175.6**	16523.8**	4543.8**	6.8 ^{ns}	13320.1**	8289.9*	32.1**	4894.2*	5329.7**	5.9 ^{ns}	2840.3**	8766.8**
Y×Mn	2	95.9**	7830.9**	996.6*	1.5 ^{ns}	297.9 ^{ns}	14.5 ^{ns}	10.6*	453.8 ^{ns}	143.6**	13.0 ^{ns}	156.8 ^{ns}	440.7**
Zn×Mn	4	26.8**	891.8 ^{ns}	48.4 ^{ns}	19.0**	75.7 ^{ns}	82.5**	7.6 ^{ns}	84.2 ^{ns}	67.8**	10.1 ^{ns}	229.9 ^{ns}	68.3 ^{ns}
Y×Zn×Mn	4	45.2**	327.4 ^{ns}	27.3 ^{ns}	4.7 ^{ns}	489.8*	104.5**	9.9*	36.6 ^{ns}	18.1 ^{ns}	11.9 ^{ns}	55.2 ^{ns}	37.3 ^{ns}
Fe×Mn	4	13.5 ^{ns}	1965.8*	51.9 ^{ns}	3.5 ^{ns}	622.1*	162.9**	9.2*	238.9 ^{ns}	97.1**	3.5 ^{ns}	200.9 ^{ns}	42.6 ^{ns}
Y×Fe×Mn	4	37.9**	625.2 ^{ns}	38.6 ^{ns}	13.2*	705.5**	84.2**	22.7**	13.8 ^{ns}	33.9 ^{ns}	4.3 ^{ns}	100.1 ^{ns}	32.3 ^{ns}
Zn×Fe×Mn	8	9.0 ^{ns}	1927.8**	33.9 ^{ns}	6.6 ^{ns}	549.8**	175.7**	6.7 ^{ns}	143.2 ^{ns}	132.6**	11.9 ^{ns}	203.9 ^{ns}	47.9 ^{ns}
Y×Zn×Fe×Mn	8	8.7 ^{ns}	261.9 ^{ns}	50.4 ^{ns}	8.9*	117.6 ^{ns}	102.4**	14.4**	330.4 ^{ns}	171.2**	15.0 ^{ns}	170.0 ^{ns}	42.6 ^{ns}
Error	104	5.6	615.7	34.0	3.8	186.6	23.0	3.3	264.0	14.2	8.2	204.8	60.1
Coefficient of variation (%)	-	8.5	7.9	8.3	8.1	5.6	8.7	7.9	10.3	7.8	10.7	12.1	11.2

ns, * and **: non-significant, significant at 5% and 1% levels of probability, respectively

Table 3

The impact of zinc, iron and manganese fertilization on their concentrations in soybean stem at different reproductive growth stages

Treat- ment	Means														
	concentration in stem (mg kg ⁻¹ dry weight)														
	flowering stage				pod set stage				seed filling period stage				maturity stage		
Zn	Fe	Mn		Zn	Fe	Mn		Zn	Fe	Mn		Zn	Fe	Mn	
Zinc															
Zn ₀	11.3±1.7	135.4±27.0	20.7±4.7	7.8±0.8	123.6±25.7	21.5±3.6	3.4±0.5	28.4±7.2	16.5±2.8	3.1±0.5	36.2±11.5	14.0±2.8			
Zn ₂₀	12.9±1.2	151.2±27.8	20.7±4.4	9.4±0.9	140.6±23.9	21.5±3.6	4.6±0.7	32.3±7.5	16.0±2.7	3.6±0.8	39.6±12.0	13.8±2.6			
Zn ₄₀	14.1±1.3	145.6±26.9	18.6±4.6	12.2±1.6	121.2±21.7	19.2±3.9	4.9±0.9	30.7±7.9	14.2±2.7	4.5±0.6	32.5±11.9	13.8±2.3			
Iron															
Fe ₀	11.4±1.9	130.7±13.9	21.2±3.8	7.9±2.2	121.9±13.8	21.9±3.3	3.6±0.9	28.8±3.9	15.9±2.9	3.2±0.8	33.7±3.6	14.1±2.8			
Fe ₂₅	9.7±1.8	175.9±15.7	18.6±4.4	7.1±1.9	157.2±17.2	20.7±3.4	3.2±0.9	53.9±10.8	15.5±2.7	3.0±0.8	45.8±9.8	14.1±2.7			
Fe ₅₀	7.2±1.7	153.2±24.9	16.8±4.3	6.7±1.6	135.9±23.4	17.6±3.4	2.9±0.6	43.7±7.6	15.1±2.7	2.7±0.7	52.3±11.0	13.4±2.5			
Manganese															
Mn ₀	11.5±1.9	142.6±29.6	19.9±3.2	7.7±2.2	127.6±27.4	21.3±3.1	3.4±1.0	29.8±8.4	15.9±2.4	3.0±0.8	38.2±12.6	14.0±1.8			
Mn ₂₀	13.0±1.8	143.2±23.9	22.2±4.1	8.9±2.1	108.7±13.4	23.8±2.9	3.9±0.9	26.8±8.1	17.6±2.0	3.3±0.8	37.3±12.5	15.3±1.6			
Mn ₄₀	12.7±1.8	125.0±22.4	25.7±4.4	8.2±1.8	95.5±14.9	26.9±3.1	3.2±0.8	20.5±7.5	18.3±2.4	3.1±0.7	28.4±9.8	17.6±2.2			

Values are means of three replicates and standard deviations

obtained by ALLOWAY (2004). Zinc application up to 40 kg ha⁻¹ increased the Zn concentration in leaves. In contrast, the Fe concentration decreased with zinc fertilization (Table 4). The effects of zinc application on Mn concentrations were diverse in different reproductive growth stages. The highest Mn concentration in the flowering stage was observed in the Zn₄₀ treatment, while the maximum Mn concentration at pod setting and seed filling was found in Zn₂₀ treatment. Iron and manganese added to soil in doses up to 25 and 20 kg ha⁻¹ increased the Zn concentration, but the leaf zinc concentration declined when higher amounts of iron and manganese had been incorporated into soil. A similar result was obtained by RONAGHI, GHASEMI-FASAEI (2008).

The antagonistic effects between iron and zinc or manganese that were observed in soybean stems also occurred in leaves. CHEN et al. (2004) reported that iron deficiency could reduce the zinc absorption and concentration in plants. The highest Zn and Mn concentrations in leaves were recorded when iron was applied in smaller amounts. ROMBOLA et al. (2005) stated that iron deficiency in plants leads to increased concentrations of copper and zinc in plant tissues. Manganese fertilization increased the Mn concentration in leaves at the flowering to maturity stages. In addition, the maximum concentrations of Zn and Fe in leaves were observed in Mn₂₀ treatment. Indeed, manganese added to soil in a dose up to 20 kg ha⁻¹ had favorable effects on zinc and iron transfer from the stem to leaves. The results of the variance analysis of leaf chlorophyll concentrations (SPAD value) in different growth stages (30, 60, 90 and 120 days after sowing) of soybean plants are shown in Table 5. These data indicated that zinc, iron and manganese fertilizers had significant effects on the leaf chlorophyll content (SPAD value) in all the growth stages (30, 60, 90 and 120 days after sowing) of soybean plants at 1% level of significance ($P < 0.01$). In addition, the interaction of zinc and iron (Zn×Fe) influenced the leaf chlorophyll concentration at 30 and 60 days after sowing ($P < 0.01$), while producing no significant effect on the same trait at 90 and 120 DAS (Table 5). Furthermore, the leaf chlorophyll concentration in soybean plants at 30 and 90 DAS was affected by zinc and manganese (Zn×Mn) and 30, 90 ($P < 0.01$) and 120 ($P < 0.05$) DAS by iron and manganese (Fe×Mn) interactions. In contrast, the leaf chlorophyll concentration (SPAD values) at 60 and 120 DAS was not affected significantly by zinc and manganese (Zn×Mn) interaction. Also, triple interaction effects (Zn×Fe×Mn) had significant influence on leaf chlorophyll at 30 and 90 DAS at 1% significance level and at 120 DAS at 5% (Table 5). In an experiment of ZAYED et al. (2011), the simultaneous application of zinc, iron and manganese on rice had a stronger impact on leaf chlorophyll (SPAD value) than the separate use of these elements.

In this experiment, there was no significant difference in the leaf chlorophyll concentration at all the growth stages of soybean plants between 2010 and 2011. The readings of leaf chlorophyll concentrations achieved with an SPAD-502 chlorophyll meter showed that the effect of iron and manganese on the above trait at all the growth stages of soybean plants was similar. With

Table 4
The impact of zinc, iron and manganese fertilization on their concentrations in soybean leaves at different reproductive growth stages

Treat- ment	Means													
	flowering stage						concentration in leaves (mg kg ⁻¹ dry weight)							
	pod set stage		seed filling period stage		maturity stage		pod set stage		seed filling period stage		maturity stage			
Zn	Fe	Mn	Zn	Fe	Mn	Zn	Fe	Mn	Zn	Fe	Mn	Zn	Fe	Mn
Zinc														
Zn ₀	28.8±3.5	276.5±48.9	60.5±12.3	22.6±2.9	226.5±35.8	48.4±11.4	21.1±2.4	159.3±27.0	37.4±7.6	20.8±2.4	124.6±29.3	43.1±12.4		
Zn ₂₀	30.3±4.8	249.4±50.7	66.3±11.0	26.7±2.4	209.8±33.7	57.2±13.4	23.6±1.9	158.7±21.7	41.6±10.1	24.7±3.4	117.4±33.2	42.5±14.2		
Zn ₄₀	33.4±3.5	237.5±47.8	69.8±11.5	29.3±3.7	195.0±32.4	49.6±13.6	29.3±3.7	131.7±23.6	38.2±10.3	30.3±4.2	106.8±32.2	42.1±14.3		
Iron														
Fe ₀	28.6±5.9	269.4±26.1	61.2±11.5	22.9±2.0	218.3±27.8	51.2±13.5	21.8±5.1	156.2±20.9	38.6±11.2	21.4±5.9	121.0±18.9	45.0±14.5		
Fe ₂₅	28.2±5.3	351.7±32.6	60.3±10.5	22.7±3.8	276.3±29.8	44.6±14.2	22.7±5.6	188.7±25.8	38.0±7.6	21.4±5.7	147.9±19.5	42.7±13.7		
Fe ₅₀	25.6±5.3	326.5±44.2	52.7±10.7	18.7±3.6	252.1±31.2	35.9±9.7	20.4±4.1	162.8±28.2	30.8±7.8	19.6±4.4	165.4±31.1	38.9±12.2		
Manganese														
Mn ₀	26.3±5.8	265.2±56.6	60.5±6.9	22.7±3.8	221.3±35.2	47.3±11.7	21.2±4.5	155.4±32.5	37.5±8.5	20.8±4.9	120.3±32.8	43.7±9.4		
Mn ₂₀	27.8±4.9	282.5±43.3	70.9±7.0	24.3±3.9	225.4±29.4	56.9±9.6	21.4±5.4	168.7±26.6	49.8±9.5	22.5±5.6	126.1±31.7	49.6±7.9		
Mn ₄₀	25.5±5.6	224.9±40.4	78.7±11.9	23.2±4.5	173.9±24.0	68.2±9.6	19.8±4.2	142.6±26.4	57.0±10.3	20.6±5.6	107.2±29.8	63.8±8.8		

Values are means of three replicates and standard deviations

Table 5

Combined analysis of variance for the impact of zinc, iron and manganese fertilization on leaf chlorophyll concentration (SPAD value) in different growth stages (30, 60, 90 and 120 days after sowing) of soybean plant at 2010 and 2011 years

Source of variation	df	MS			
		chlorophyll concentration (SPAD value)			
		30 days after sowing	60 days after sowing	90 days after sowing	120 days after sowing
Year (Y)	1	9.53 ^{ns}	4.11 ^{ns}	0.02 ^{ns}	1.14 ^{ns}
Zn	2	323.76 ^{**}	50.07 ^{**}	83.23 ^{**}	24.23 ^{**}
Y×Zn	2	0.07 ^{ns}	0.40 ^{ns}	3.61 ^{ns}	0.23 ^{ns}
Fe	2	482.94 ^{**}	245.62 ^{**}	298.85 ^{**}	288.83 ^{**}
Y×Fe	2	0.28 ^{ns}	0.62 ^{ns}	12.36 ^{ns}	4.65 ^{ns}
Zn×Fe	4	12.81 ^{**}	48.60 ^{**}	6.03 ^{ns}	0.67 ^{ns}
Y×Zn×Fe	4	0.12 ^{ns}	1.57 ^{ns}	16.09 [*]	1.06 ^{ns}
Mn	2	227.16 ^{**}	281.98 ^{**}	153.68 ^{**}	202.04 ^{**}
Y×Mn	2	0.35 ^{ns}	0.16 ^{ns}	19.11 [*]	2.66 ^{ns}
Zn×Mn	4	12.40 ^{**}	0.99 ^{ns}	24.15 ^{**}	2.37 ^{ns}
Y×Zn×Mn	4	0.19 ^{ns}	0.52 ^{ns}	24.84 ^{**}	1.02 ^{ns}
Fe×Mn	4	8.57 ^{**}	7.13 ^{ns}	36.09 ^{**}	8.77 [*]
Y×Fe×Mn	4	0.11 ^{ns}	1.37 ^{ns}	3.31 ^{ns}	1.63 ^{ns}
Zn×Fe×Mn	8	10.58 ^{**}	4.23 ^{ns}	21.21 ^{**}	6.30 [*]
Y×Zn×Fe×Mn	8	0.19 ^{ns}	0.59 ^{ns}	8.58 ^{ns}	1.09 ^{ns}
Error	104	2.25	4.00	5.20	2.88
Coefficient of variation (%)	-	6.87	6.54	6.26	6.70

ns, * and **: non-significant, significant at 5% and 1% levels of probability, respectively

increased iron and manganese doses, soybean leaf chlorophyll concentrations increased in all the samples. However, with respect to zinc fertilization, it was observed that an application of 20 kg Zn ha⁻¹ increased leaf chlorophyll concentrations at 30 and 60 days after sowing (DAS), while the highest chlorophyll concentration in soybean leaves was observed at 90 and 120 DAS by 40 kg Zn kg⁻¹ soil application (Figure 1). These results are in agreement with NAIEMA (2008), OBAID, and AL-HADETHI (2013).

In this study, the maximum content of chlorophyll in the leaves of soybean plants in all the treatments was observed 90 days after sowing. AI-QING et al. (2011) reported that leaf chlorophyll concentration in wheat cultivars increased 1.2 to 2.6 times more with Fe and 1.2 to 2.7 times more with Zn fertilizations compared to the control treatments (Fe₀ and/or Zn₀). A similar result on *Oryza sativa* was obtained by ZAYED et al. (2011). Interaction effects

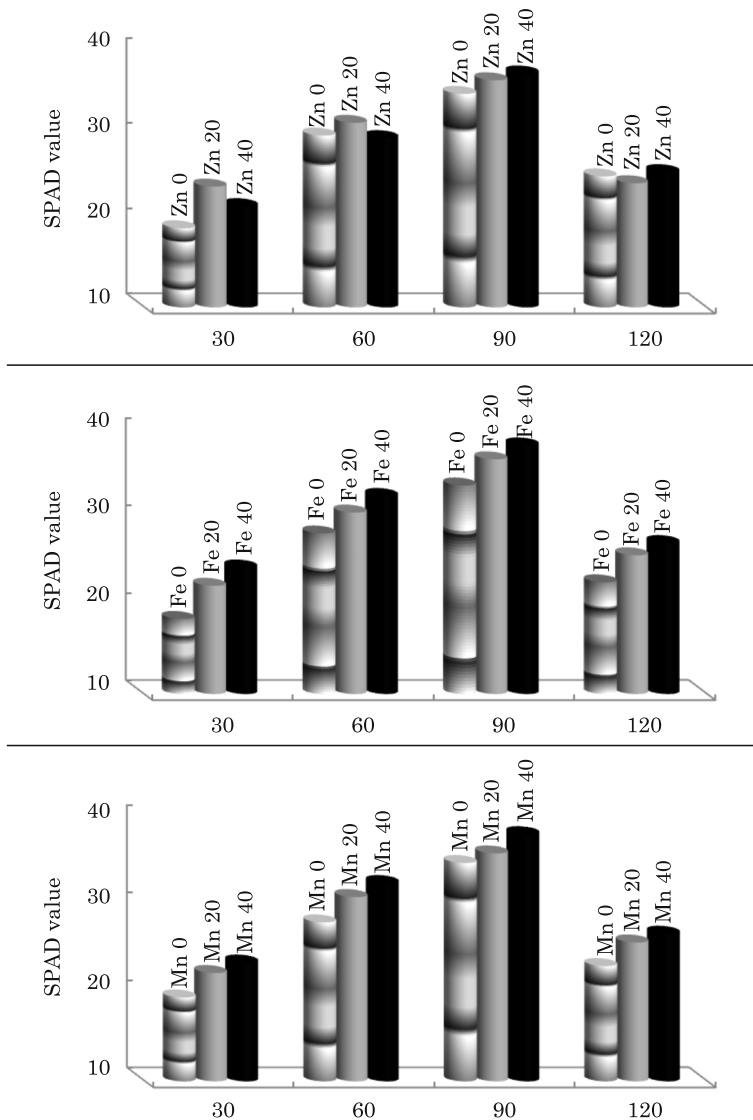


Fig. 1. The effect of different levels of zinc, iron and manganese (kg ha^{-1}) (individually) on leaf chlorophyll concentration (SPAD value) in soybean plant at 30, 60, 90 and 120 days after sowing

of zinc, iron and manganese ($\text{Zn} \times \text{Fe}$, $\text{Zn} \times \text{Mn}$ and $\text{Fe} \times \text{Mn}$) on leaf chlorophyll concentrations (SPAD value) at 30 DAS are illustrated in Figure 2. Based on the results, it is demonstrated that an application of 20 kg Zn ha^{-1} with 50 kg Fe ha^{-1} and 20 kg Zn ha^{-1} with 40 kg Mn ha^{-1} simultaneously had the most significant effect on the leaf chlorophyll concentration in soybean plants. Also, the treatment of 50 kg Fe ha^{-1} with 20 kg Mn ha^{-1} was found to have stimulated the highest leaf chlorophyll concentration (Figure 2). These

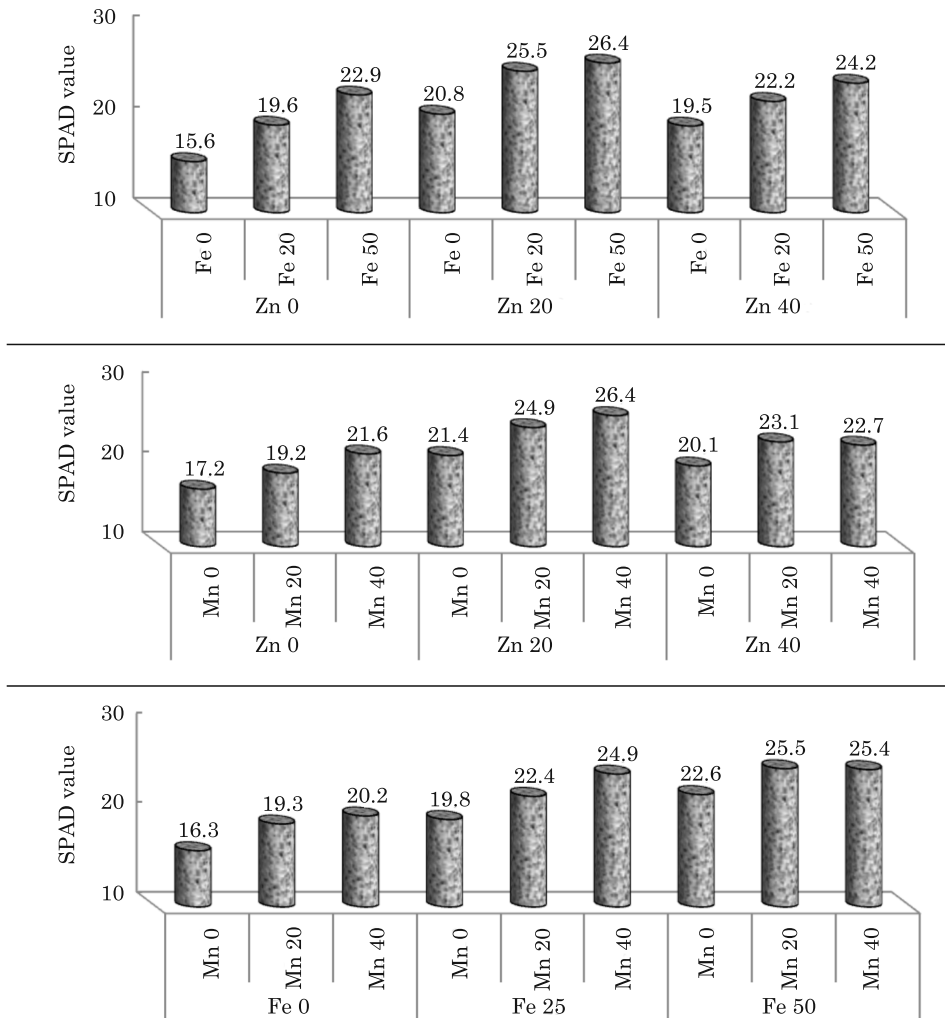


Fig. 2. Interaction effects of zinc, iron and manganese (kg ha^{-1}) on leaf chlorophyll concentration (SPAD value) at 30 days after sowing

results are in line with the report by KAYA et al. (1999). Similar results were observed in SPAD values measured at 60 DAS (Figure 3).

The maximum chlorophyll concentration in soybean leaves was recorded in $\text{Zn}_{20}\text{Fe}_{50}$, $\text{Zn}_{20}\text{Mn}_{40}$, and $\text{Fe}_{50}\text{Mn}_{40}$ treatments (34.7, 34.0 and 35.2, respectively). On the other hand, $\text{Zn}_{40}\text{Fe}_{50}$ and $\text{Fe}_{50}\text{Mn}_{40}$ treatments, when compared to the other variants, increased the leaf chlorophyll concentration in soybean leaves at 90 and 120 DAS (Figures 4 and 5). It is important to note that there was no difference in the chlorophyll concentration in soybean leaves when 0 and 40 kg zinc ha^{-1} was applied simultaneously with 40 kg of manga-

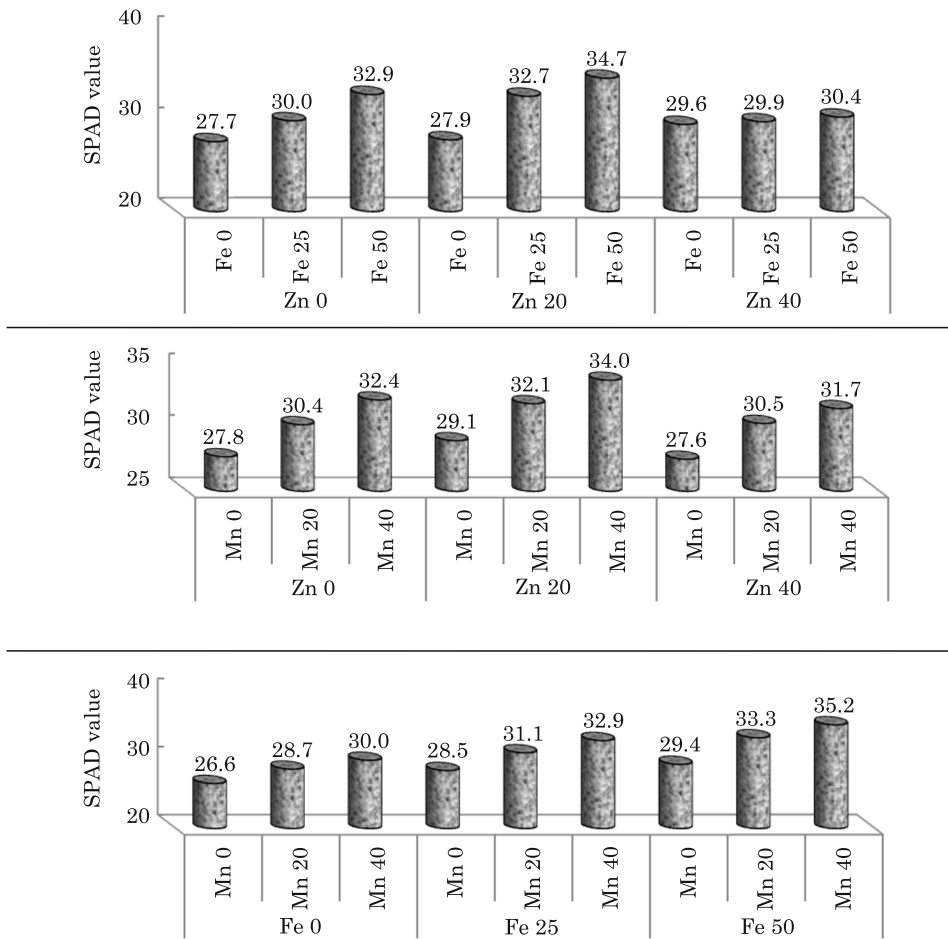


Fig. 3. Interaction effects of zinc, iron and manganese (kg ha^{-1}) on leaf chlorophyll concentration (SPAD value) at 60 days after sowing

nese (Figure 5). DOS SANTOS et al. (2013) emphasized that the application of manganese had a stronger impact on the SPAD values than zinc fertilization. At 90 days after sowing, the highest value of SPAD was recorded after the application of 20 kg Zn ha^{-1} and 40 kg Mn ha^{-1} (Figure 4).

CONCLUSION

In this experiment, the highest Zn and Mn concentrations in leaves were recorded when iron was applied in smaller amounts. Therefore, there were antagonistic effects (negative relationships) between these elements in soybean organs. During the R1 to R8 growth stages, $[\text{Zn}]_{\text{leaf}}/[\text{Zn}]_{\text{stem}}$, $[\text{Fe}]_{\text{leaf}}/$

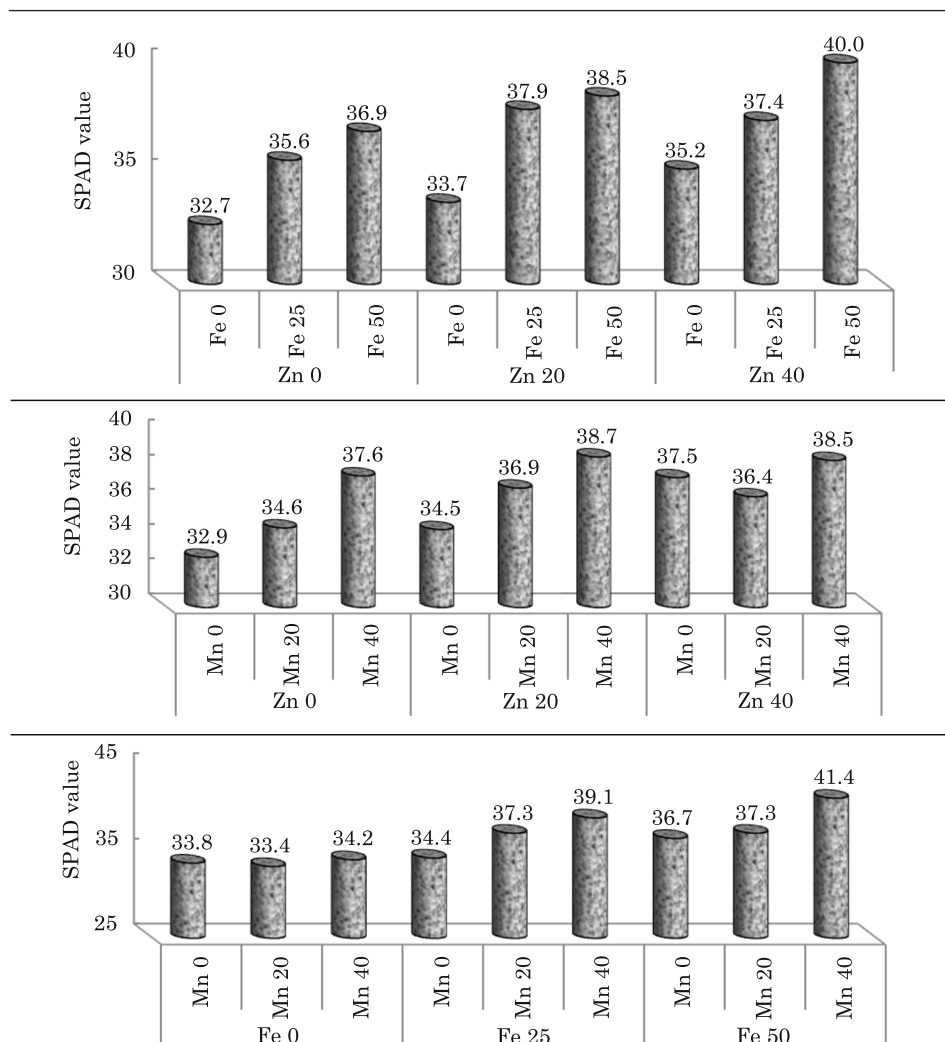


Fig. 4. Interaction effects of zinc, iron and manganese (kg ha^{-1}) on leaf chlorophyll concentration (SPAD value) at 90 days after sowing

[Fe]stem and [Mn]leaf/[Mn]stem ratios were altered by about 2 to 7.5, 2 to 4 and 3 to 3.5 times. Thus, in soybean reaching maturity, the role of leaves as a reservoir for the accumulation of zinc and iron is becoming more important. Alternatively, the effect could be due to higher transfer of zinc and iron and/or poorer transport of manganese from the stem to leaves. Zn, Fe and Mn concentrations in stems and leaves increase owing to fertilization with micronutrients.

In this experiment, Zn, Fe and Mn concentrations are reduced in stems and leaves when soybean is ripening because the final destination of micronutrients is the grains. The effects of iron and manganese on the chlorophyll

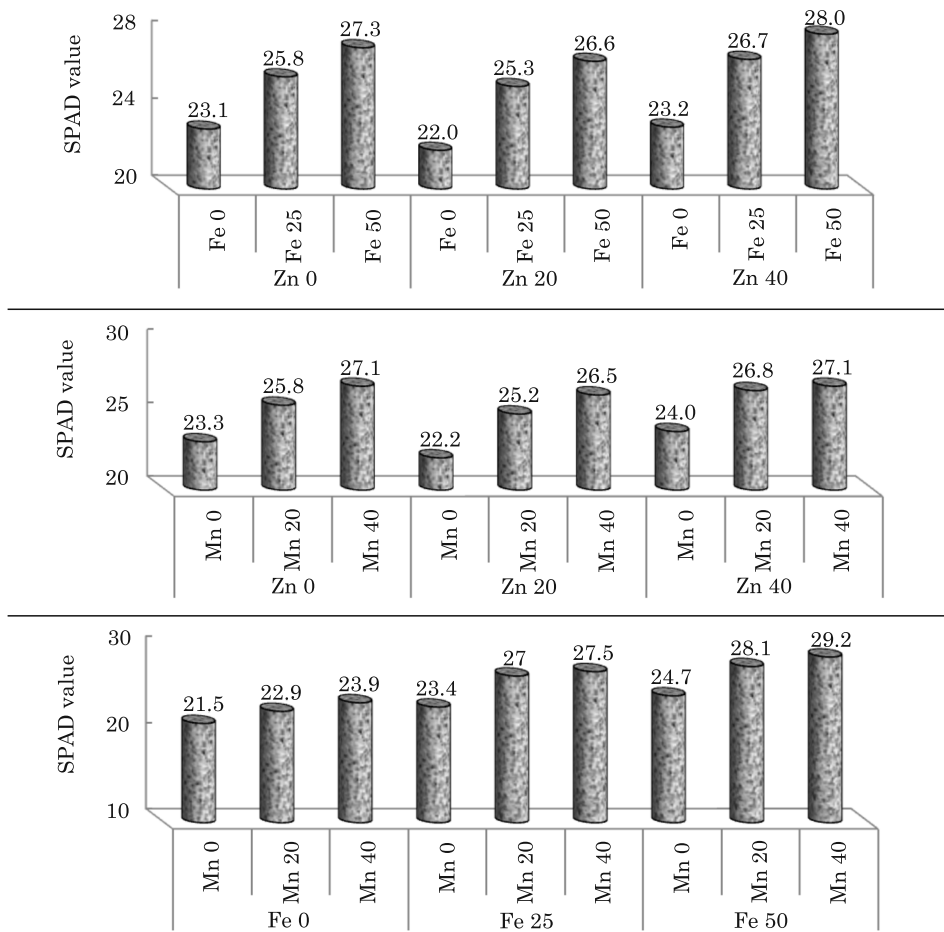


Fig. 5. Interaction effects of zinc, iron and manganese (kg ha⁻¹) on leaf chlorophyll concentration (SPAD value) at 120 days after sowing

content at all the growth stages of soybean plants are similar. With increased iron and manganese doses used as fertilizers, soybean leaf chlorophyll concentrations increased in all the samples.

With respect to zinc fertilization, an application of 20 kg Zn ha⁻¹ increased leaf chlorophyll concentrations at 30 and 60 days after sowing, while the highest chlorophyll concentration in soybean leaves was observed at 90 and 120 days after sowing following the 40 kg Zn kg⁻¹ soil application.

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