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EDTA-Coated Fe_3O_4 Nanoparticles: a Novel Biocompatible Fertilizer for Improving Agronomic Traits of Sunflower (Helianthus Annuus)

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Abstract

In this study, a set of experiments were conducted to investigate the influence of EDTA-grafted iron oxide nanoparticles exposure on agronomic traits of sunflower (Helianthus annuus) plants. The experiment was implemented by applying Nano-Fe_3O_4-EDTA and Fe-EDTA fertilizers applied through spray or soil amendment. A variety of parameters including Aerial organ biomass, Number of leaves, Plant height, Chlorophyll content as well as elemental quantities of the plants were investigated. The results demonstrated considerably dominant effect of Nano-Fe_3O_4-EDTA fertilizer on many of the studied factors. The dramatically increased Fe content of plants (137% relative to the control) by using nano-Fe-EDTA, makes this novel fertilizer a promising candidate to obviate iron deficiency problem in plants. Moreover, it was clearly observed that more pronounced positive effects is obtained through soil amendment than by foliar application of fertilizers and only in some cases such as aerial organ biomasses and Fe content, the foliar treatment has turned out to be more effective.

1. Introduction

As one of the most essential micronutrients in the scope of agricultural science, iron is a crucial components for plant development and plays a vital role in photosynthetic process. Iron is known as an element prone to participating in redox reaction as well as generating reactive oxygen species [1]. Enzyme activation, assistance in respiration, RNA synthesis, siderophores biosynthesis, and enhancement of photosystem performance are some of the renowned stimulatory activities of the ferrous and ferric ions. [2-5]

By the advent of nanotechnology in life science, many of the agricultural problems started to be solved, progressively. Nanotechnology, as a potential practical field in the agriculture, pave new pathways to devise engineered nanomaterials with a variety of applications in plant growth due to their high reactivity, extremely small feature size, high surface: volume ratio and special electronic, magnetic, and optical properties [6-8]. Recently, there have been an increasing interest in using nanomaterials for stimulation of agronomic traits of many plants [8]. One of the latest progresses in the application of nanoparticles in plant protection and nutrition is their use as "nanofertilizers". For instance, iron containing nanoparticles have been widely used as a nano-fertilizer for nutrition of different plants. Many reports confirm the positive effect of iron containing nanoparticles on growth parameters. For example, Cotae and Creanga studied the influences of ferrofluid treatment and static magnetic field exposure on the light harvesting complex (LHC II) and they observed that low ferrofluid concentrations show increased influences rather than high concentrations [9]. Dhoke et al. investigated the effect of nano-FeO and nano-ZnCuFe-oxide particles on the growth of mung (Vigna radiata) seedling [5]. They observed positive effect of the nanoparticle treatment on the seedlings growth with the best performance of nano-ZnCuFe-Oxide. Effect of nano-iron oxide on soybean yield and quality was determined by Sheykhbaglou et al. and its positive influence on leaf + pod dry weight and pod dry weight of the plant was observed [10]

In spite of many devoted efforts toward application of iron containing nanomaterials, their uptake and translocation in the plants still remains as a challenging issue. In recent years, some reports have revealed certain commonplace routs for translocation of nanoparticles in plant [11-13]. One main point deduced from these reports is that an appropriate hydrophilic protecting sheet is crucial in order to make the nanoparticles more prone for uptake. Moreover, it is evident that high rate of agglomeration of iron oxide nanoparticles that leads to precipitation due to gravitational forces could be decreased by surface-coating materials [14]

Coated iron oxide nanoparticles are one of the novel nanomaterials which hold great promise regarding their nutritive role in improving agronomic traits of various plants [10]. There have been several reports on production of iron oxide nanomaterials modified with various chelating agents, for instance, Xiu-mei et al. demonstrated that Nano-Fe_2O_3 coordinated with humic acid improves the mobility of iron in peanuts [15]. Rãcuciu et al. investigated the effect of water-based ferrofluid, stabilized with citric acid on the growth of maize (Zea mays) plants in their early ontogenetic stages and observed stimulatory effects of ferrofluid and magnetic exposure upon the studied plant species [16]. By applying γ-Fe_2O_3 nanoparticles to soybean (Glycine max (L.) Merr.), Dorostkar and Isoda reported a significant positive effect on root elongation as well as photosynthesis parameters [17].

In this paper, a novel nano-iron fertilizer capped with EDTA chelate is synthesized and applied to sunflower (Helianthus annuus) by two various treatment methods including foliar and soil addition during their growth period.

2. Experimental procedure

2.1. Synthesis of nano-Fe_3O_4-EDTA and Fe-EDTA chelate fertilizers

$FeCl_2$ (Merck) and $FeCl_3$ (Merck) were weighed in the required molar proportion, 1:2. Then Fe^{3+} was dissolved in 200 mL of deionized water and Fe^{2+} was added to the solution. After five minutes, 10 mL of NH_3 (25%) and 0.615 mg of Ethylenediaminetetra acetic acid (EDTA) (Merck) as precipitation agent and as capping agent were added to the solution with stirring, respectively. The reaction was allowed to proceed for 1 h at 50 °C with constant stirring. Finally, the black precipitate of nano-Fe_3O_4-EDTA (hereafter, it is denoted as nano-Fe-EDTA) was washed with distilled water and dried at 80 °C for 3h.

For preparation of Fe-EDTA chelate fertilizer, stoichiometric amount of EDTA and $FeCl_3$ (1:1) were dissolved in 100 mL amount of water and the solution was allowed to equilibrate overnight. To obtain the required concentration of fertilizer, the concentrated solution was diluted.

2.2. Preparation of nano-Fe-EDTA suspension

The as-prepared nano-Fe-EDTA was suspended in distilled water and dispersed by ultrasonic vibration bath for 30 min (Bandelin, Sonorex Digitec). For foliar application, 10 mg of nano fertilizer was suspended in 1L water. Distilled water was used as a negative control.

2.3. Soil-grown plants exposed to nano-Fe-EDTA and Fe–EDTA

This experiment was conducted in pot (25×20 cm) filled with non-saline sandy loam soil in a greenhouse at university of Zabol in Iran (latitude of 30° 54 'N and longitude of 61° 41' E and altitude of 481 m above sea level) during September-October 2013. The chemical properties of the soil is as follows: potassium, 52 ppm; phosphorous, 2.2 ppm; pH, 7.8; EC, 7.98 Ds/m. The experiment was laid-out a completely randomized factorial design with three replicates. The plants were grown under greenhouse conditions with a 12 h photoperiod of natural daylight, maximum and minimum temperatures of 26 and 18°C, respectively and relative humidity of 70% on average. Five seeds were sown at uniform depth (2 cm) and after completion of emergence, thinning was done and two plants were maintained in each pot. The experiment was implemented by applying Nano-Fe-EDTA and Fe-EDTA fertilizers applied through spray treatment (1 mg/L and 500 µM, respectively) and soil amendment (1g/Kg for both samples) beside the concomitant controls. Treating the plants by spraying and soil consumption was done four times at 4-6 leaf stage and within 15 days after the first treatment. Irrigation operations was done once every 3 days and cultivation continued for 2 months. The content of chlorophyll a and chlorophyll b and carotenoid pigments were measured according to Arnon method. [18]. Vanadate/molybdate method (yellow method) was employed for measuring potassium and phosphorus elements. To measure Fe and Zn elements, atomic absorption GF AAS model device was used. Data analysis was carried out with SAS Institute Inc 6.12. All data were first analysed by ANOVA means were compared based on Duncan's multiple range test at 1% probability level.

3. Results and discussion

3.1. Characterization of nano-Fe-EDTA

The as-prepared nano-Fe-EDTA product was characterized by FT-IR to confirm the EDTA grafting on its surface. Fig. 1a and 1b show the FT-IR spectra of the free EDTA and nano-Fe-EDTA, respectively. According to the literature, FT-IR spectrum of magnetite shows two distinct bands at 570 cm^{-1} (ν_1) and 390 cm^{-1} (ν_2), while maghemite, a defective form of magnetite, has absorption bands at 590 and 430 cm^{-1}. [19, 20] In Fig. 1b, two absorption bands, one at 568 cm^{-1} and another one below 400 cm^{-1} are assigned to the vibration of the Fe-O bands of magnetite network. Since during the synthesis of nano-Fe-EDTA the concomitant addition of NH_3 and EDTA is performed, the carboxyl groups of EDTA are definitely deprotonated. According to Dobson et al., the deprotonated carboxylate (COO^-) show strong asymmetric (ν_{as}) and comparatively strong symmetric (ν_s) stretching at 1650-1510 and 1400-1280 cm^{-1}, respectively [21]. In Fig. 1b, bands at 1630 and 1400 cm^{-1} are assigned to to ν_{as} (COO-) and ν_s (COO-), respectively. The slight peak shifts compared with those of Fig. 1a are frequently due to carboxylate-metal chelation. [22, 23]. In Fig. 1b, two peaks between 2853 and 2924 cm^{-1} are attributable to stretching vibration of the $-CH_2$ groups of chelated EDTA on Fe_3O_4. Hence, EDTA grafting on the surface of Fe_3O_4 nanoparticles is approved by FT-IR spectroscopy.

3.2. Investigating plant growth parameters

The results for various plant growth parameters are investigated and presented in Fig. 2. Two different methods including foliar spraying and direct addition to the soil were employed to add Fe-EDTA chelate and nano-Fe-EDTA to the plants. The obtained results for both methods are analysed and the average values and standard deviations are shown. Variance analysis results are shown in Table 1. From the values presented in this table, it is clear that employing iron chelate fertilizers in both treating methods i.e. foliar spraying and soil adding have significant difference at 1% probability level on aerial organ dry and fresh biomass, number of leaves per plant, and plant height.

3. 2.1. Aerial organ dry biomass

Plant biomass is a key element in the study of functional plant biology which may anticipate the location on an axis of resource capture, usage and availability [24, 25]. The obtained results for mean aerial organ dry biomass (stem and leaves) of two various methods show that the mean values are significantly different at the 1% probability level (Fig. 2a).

On the whole, applying nano-Fe-EDTA by foliar treatment has resulted in improved aerial organ dry biomass of the plants in comparison with the control. Moreover, it is clearly observed that soil amendment of either of nano-Fe-EDTA or Fe-EDTA has no positive effect on this parameter. The highest value for aerial organ dry weight (4.36 g) belonging to the plants treated with sprayed nano-Fe-EDTA, is 50.36% higher than that of the control.

Table 1. Analysis of variance testing the effects of treating method on growth parameters of sunflower plants

Source of change	Degrees of freedom	Aerial organ dry biomass	Aerial organ fresh biomass	Number of leaves per plant	Plant height
Foliar treatment	3	2.36**	48.06**	7.66**	35.14**
error	8	0.169	2.009	0.074	0.320
Soil amendment	3	1.24**	37.39**	11.33**	84.66**
error	8	0.0986	0.1833	4.0000	0.4074
CV%		4.12	0.50	1.30	1.66

** one percent significance level using Duncan's multiple range test.

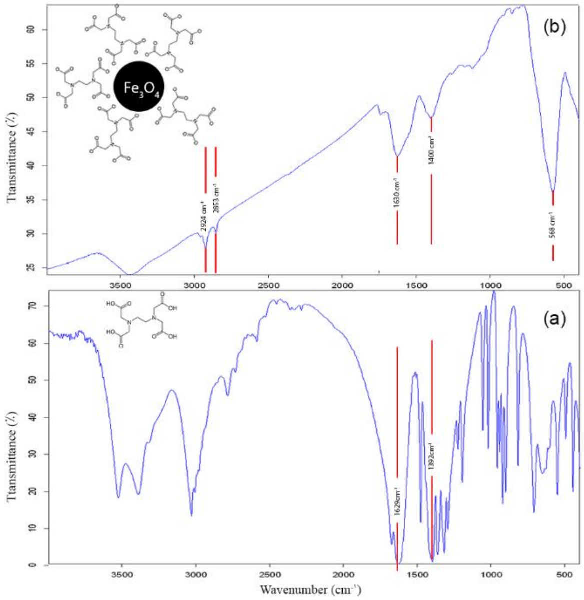


Fig. 1. FT-IR spectrum of (a) free EDTA ligand (b) nano-Fe-EDTA powder

Furthermore, spraying the plants with the conventional Fe-EDTA chelates had negative effect on aerial organ dry weight with respect to the control.

Increasing the dry weight in this study is consistent with previous report by Sheykhbaglou et al. for nano-iron oxide effect on soybean yield and quality [10]. It was reported that employing iron nanoparticles brings about rising pod and dry leaf weight as well as total yield.

3.2.2. Aerial organ fresh biomass

Fig. 2b shows a diagrammatic presentation for mean values of aerial organ fresh biomass. Reiteratively, it is observed that the mean values are different at the 1% probability level. From the diagram it can be observed that while both foliar and soil addition of nano-Fe-EDTA give rise to higher aerial organ fresh weight of the plants, subjecting the plants to conventional Fe-EDTA by either of the methods result in an abatement. Moreover, it is observed that the highest mean value (23.46 g) belongs to the plant treated with sprayed nano-Fe-EDTA which is 3.9% higher than that of the control.

Soil amendment of nano-Fe-EDTA has similar effect on the fresh biomass of the samples and the average mean value was reported to be 23.35g which is 3.4% higher than that of the control. Therefore, the results for novel nano-Fe-EDTA and the conventional Fe-EDTA fertilizers reveals that employing coated nanoparticles by either of the applied methods is striking in increasing aerial organ weight of the sunflower plants

3.2.3. Number of leaves per plant

From Fig. 2c it is observed that except for foliar application of Fe-EDTA, other fertilizers were effective in increasing the number of leaves per plant. In foliar treatment, the highest average number of leaves belongs to the plants treated with Fe-EDTA with mean value of 17 which is increased by 21.42% compared to the control.

Table 2. Analysis of variance testing the effects of treating method on physiological parameters of sunflower plants

Source of change	Degrees of freedom	Chlorophyll a	Chlorophyll b	Total Chlorophyll	Carotenoids
Foliar treatment	3	0.093**	0.021**	0.029**	0.040**
error	8	0.00244	0.00016	0.00339	0.00021
Soil amendment	3	0.219**	0.032**	0.187**	0.036**
error	8	0.03379	0.00008	0.00603	0.00011
CV%		8.99	1.78	2.73	1.81

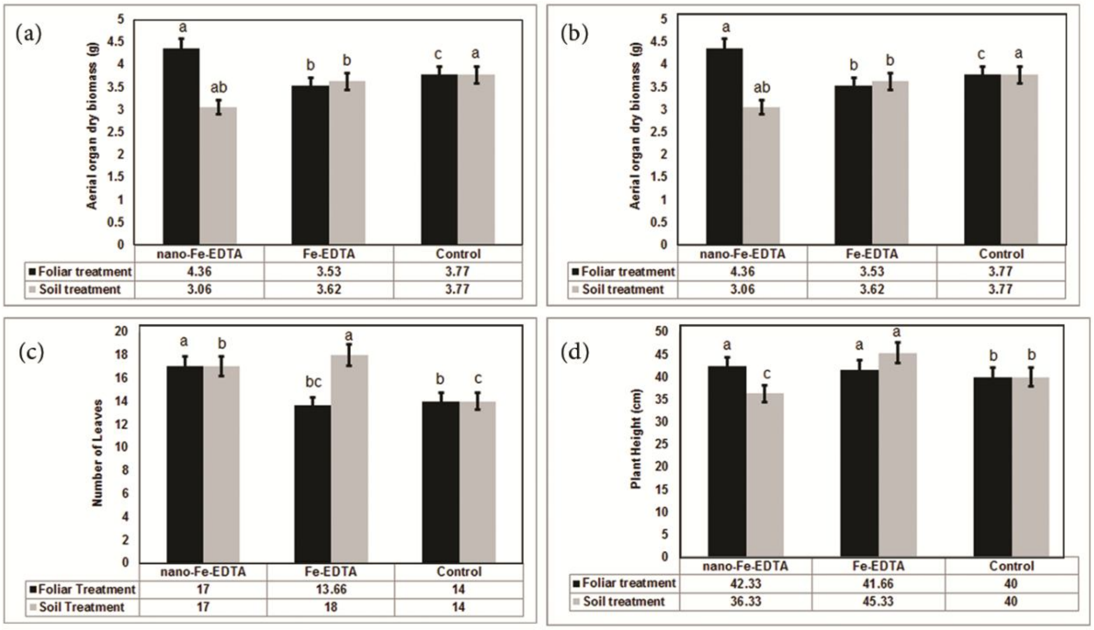


Fig. 2. Growth parameters for various treated plants. Values in each set of data followed by different letters are not significantly different at 1% probability level using Duncan's multiple range test plants.

On the other hand, the direct soil addition of fertilizers gave rise to the maximum mean value of leaves (18) for the plants interacted with Fe-EDTA chelates. In this method, applying nano-Fe-EDTA and Fe-EDTA chelate brought about increasing number of leaves by 21.42 and 28.57% compared with the control.

3.2.4. Plant height

The mean measured values for plant heights are shown in Fig. 2d. From the presented values, it is observed that employing both fertilizers have been effective on plant height so that the maximum mean value (45.33 cm) is reported for those treated with Fe-EDTA fertilizers through soil direct addition which is 13.32% higher than that of the control. Furthermore, although in foliar treatment either nano-Fe-EDTA or Fe-EDTA did not show significant difference at 1% probability level, they both resulted in an increase in plant height (5.8%) relative to the control.

From the results mentioned above it is deduced that the novel nano-Fe-EDTA fertilizer significantly affects sunflower growth. Previously, several attempts have been made to study the effect of iron or iron oxide nanoparticles on growing a variety of plants and the results are quite contradictory.

For instance, Trujillo-Reyes et al. reported that nano-Fe/Fe_3O_4 at specific concentrations did not affect lettuce growth and chlorophyll content [26]. However, Mazlomi Mamyandi et al. studied the effect of nano-iron spraying on sugar beet (Beta vulgaris L.) in the terms of plant growth stage and nano-iron concentration and they found out that interacting sugar beet in the primary stages of plant growth results in significant difference in length of peduncle with respect to the control [27]

In another study by Rãcuciu et al., the findings showed that small concentrations of TMA-OH coated magnetic nanoparticles added to cultural medium of popcorn plants have a stimulating effect on the growth of the plantlets, while the excess amount of the same solution have an inhibitory effect [28].

3.3. Physiological parameters of the plants

Table 2 presents the results for variance analysis of plants for their chlorophyll and carotene content. The findings indicate that the difference in chlorophyll and carotene contents of nano-Fe-EDTA- and Fe-EDTA-treated plants and the control is significant at 1% probability level. The details are discussed as follows.

3.3.1. Chlorophyll content

Fig. 3a-3c shows the results for photosynthesis pigments of the plants containing standard deviation bars before and after treating with the fertilizers by two methods. All fertilizer-treated plants have led to higher chlorophyll levels compared with the water control.

While the total chlorophyll content has been increased by applying both fertilizers, nano-Fe-EDTA has been the one leading to better results.

The analysis showed that the maximum values for both total chlorophyll content (2.69 mg/g) and hlorophyll a (2.34 mg/g) belongs to the treated plants with nano-Fe-EDTA through soil absorption which are 31.21 and 49.04% higher than that of the control, respectively.

This reveals a statistical tremendous difference between the nano-Fe-EDTA treated and non-treated plants especially in the case of chlorophyll a as the main photosynthesis pigment.

The mentioned immense distinction in chlorophyll contents after root absorption of nano-

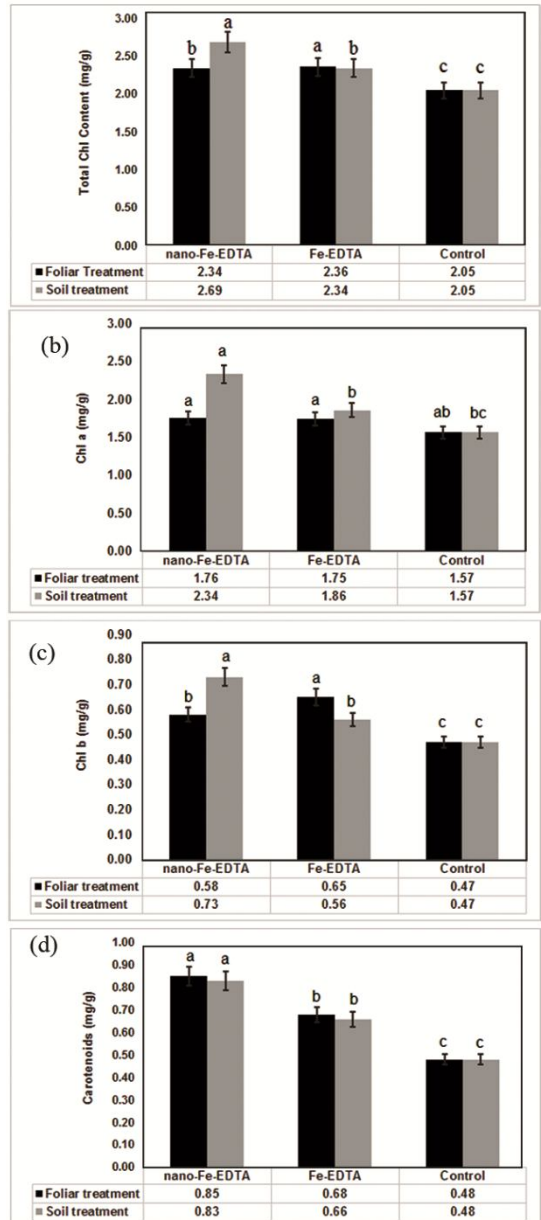


Fig. 3. Effects fertilizers by two method on physiological parameters: total chlorophyll (a), chlorophyll a (b) chlorophyll b (c), and carotenoids (d). Values in each set of data followed by different letters are not significantly different at 1% probability level using Duncan's multiple range test.

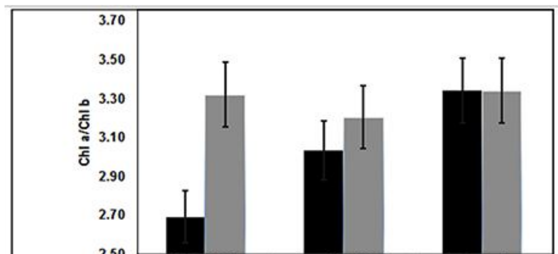
Fe-EDTA could be considered an evidence for transportation of coated nanoparticles through vascular tissues toward the aerial parts. This behaviour of engineered and biocompatible nanoparticles has been suggested by some recent reports, as well [29, 30]. A recent study has been done on adsorption of magnetic carbon-coated nanoparticles by a variety of plants including sunflower. In this study Cifuentes et al. reported that in sunflower, nanoparticles uptake occurs through the roots and there is an accumulation after 24 hours of treatment in both vascular and aerial parts [31].

Comparing two treating methods in current study indicates that soil addition of fertilizers turned out to be more effective than foliar treatment. These findings are consistent with some of the previous studies showing that root application is faster and more reliable than leaf treatments [11, 13, 12]. In spraying method, even though the chlorophyll level was enhanced by applying both fertilizers relative to the control, this enhancement was negligible and both fertilizers gave rise to statistically same values. These results propose that nano-Fe-nanoparticles have not been well absorbed through surface of the leaves which further supports the findings reported by Corredor et al. concerning the presence of nanoparticles chiefly in cells from the epidermis of the petiole not far from the application point [13].

3.3.2. Carotenoid content and LHC II

Carotenoid, the most important secondary pigments of the light harvesting complexes (LHC) I and II systems, is to maintain the photosynthesis process by transferring the absorbed energy from light to the molecules of chlorophyll a [16]. Fig. 3d shows the carotenoid level of the samples. It is observed that for both treatment methods, the mean obtained values show statistically significant difference. The highest value for carotenoid level is for nano-Fe-EDTA fertilizer (0.85 and 0.83 mg/g for foliar and soil amendment, respectively) which is exceedingly intensified in comparison with the control.

LHC II, a leading index for indicating the photosynthesis process efficiency and its enzymatic aggregates located in the chloroplasts membranes, is assessed on the basis of the chlorophylls ratio (chlorophyll a/chlorophyll b) [32]. This crucial parameter is diagrammatically shown in Fig. 4 and it is clearly observed that an inhibitory effect has been resulted by application of both fertilizers.



	Fe-EDTA	nano-Fe-EDTA	Control
Foliar treatment	2.69	3.03	3.34
Soil treatment	3.32	3.21	3.34

Fig. 4. Chlorophylls ratio level in treated plants.

This is consistent with previous results of Rãcuciu and Creang in which the chlorophyll ratio was decreased with increasing the concentration of water-based ferrofluid [16]. This admits the impressionability of the LHC II towards even slight changes in its physiological environment. The obtained values for chlorophyll ratio after fertilizer treatment indicates that soil application of both nutrients is dominant. In the case of Fe-EDTA foliar-treated plants, the inhibitory influence is tremendous so that 19.46% decrease is observed with respect to the control.

3.4. Elemental determination

Variance analysis of elemental contents in the plants are shown in Table 3. It can be observed that employing both fertilizers by either of the treating methods have significant difference at 1% probability level on elemental content of plants.

The obtained results for average Fe content of plants show that the mean values are significantly different at the 1% probability level (Fig. 5a). Despite some reports showing no difference in the concentration of Fe after treatment with iron nanoparticles, in this study it is evident that utilizing nano-Fe-EDTA has dramatically increased Fe content of the plant. Trujillo-Reyes et al. reported that applying nano-Fe/Fe_3O_4 to lettuce results in retaining Fe in the roots as insoluble compounds [26]. On the other hand, in many other investigations application of nano-iron oxides has been identified as an outstanding contributing element for enhancing Fe content in plants [33, 5]. Herein, the elemental content of various plants are presented in Fig. 5 and it is obvious that Fe concentration is drastically higher in nano-Fe-EDTA treated plants than those of the others in both foliar and soil treatments (98.4 and 82.67 ppm, respectively). However, it could not be discarded that this parameter is more intensified in the case of foliar application.

Although current analysis confirms lower concentration of Fe for soil treated plants, in above discussions it was observed that this treatment method turned out to be more effective. A possible explanation to this is that according to the previous findings, there are certain penetration points on the surface of the leaves including the stomata and the subestomatic chambers which are the routine pathways used by pathogens. Therefore, occasional penetration of nanoparticles with hydrophobic external surface is totally plausible through these pores [13, 34]. Nevertheless, as it was mentioned before, in foliar application of nanoparticles they are mainly stuck in cells from the epidermis of the petiole not far from the application point and therefore, they do not greatly contribute to plant growth process or photosynthesis reaction.

=In Fig. 5a, it can be observed that Fe-EDTA chelate has completely different effects so that it is effective in foliar method and inhibitory in soil application. This antithetical impact in two application methods could be attributed to a common phenomenon in absorption of metal-chelant complex by plant roots. Fe-EDTA is being dissociated before root uptake and after its translocation upward, the potential leaching of metals into the surrounding environment in the process of chelant-enhanced phytoextraction takes place and hence, the decreased amount of Fe concentration in soil treatment could be ascribed to this phenomenon.

The mean values for other nutrient elements including Zinc, Phosphorous, and Potassium are presented in Fig. 5b, 5c and 5d, respectively. It can be seen that all the results are significantly different in each column, except for potassium mean values of nano-Fe-EDTA and the control in foliar method which are statistically similar. The mean values for Zinc element is enhanced in soil amendment. The maximum Zinc content (46.42 ppm) which is around 112% higher than that of the control, belongs to the plants treated with Fe-EDTA through their root.

In the case of Potassium element, the maximum mean value is obtained for nano-Fe-EDTA soil treated plant (9.46 ppm) which is 97% higher than the value for the control. This result is consistent with recent report by Vattani et al. in which they reported that application of Nano-chelated iron in spinach results in accumulation of iron and potassium in the plant organs [35].

Finally, the results for Phosphorous content shows that the maximum value is for soil treated nano-Fe-EDTA which is 60% higher than that of the control. Contrary to effectiveness of this fertilizer in soil, it has only a slight positive impact on Phosphorous content of the foliar treated plants. This reveals that nano-Fe-EDTA could be considered as an effective agent for increasing Phosphorous in plants.

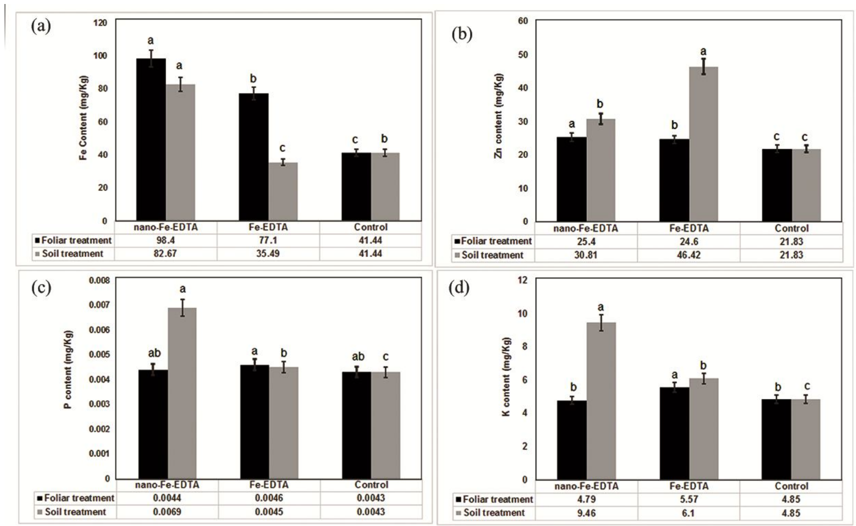


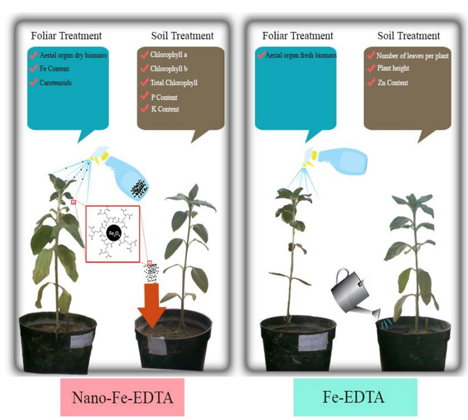
Fig. 5. Effects fertilizers by two method on elements uptake: iron (a), zinc (b) phosphorous (c), and potassium (d). Values in each set of data followed by different letters are not significantly different at 1% probability level using Duncan's multiple range test.

On the basis of the discussions presented above, the maximum values for each parameter is listed in Table 4 and schematically illustrated in Scheme 1. It can be observed that in most of cases, nano-Fe-EDTA applied in soil treatment has led to the
content should not be discarded.

4. Conclusion

In conclusion, in this study it was shown that grafting EDTA on the surface of Fe_3O_4 nanoparticles resulted in fabrication of a biocompatible fertilizer for plant development. Comparing soil and foliar application of the novel nanocompatible fertilizer indicated that the former method has substantial effect on a number of growth and physiological parameters of sunflower plants. It is noteworthy that by applying the novel

maximum value of the investigated trait. However, in some cases such as aerial organ biomasses and Fe content, its foliar treatment has turned out to be more effective. Moreover, the effect of Fe-EDTA on number of leaves per plant, plant height, and Zn fertilizer, the average value for Fe content has enhanced up to 137% relative to the control. From the obtained results, it can be deduced that providing an organic shell around nano-Fe has made it more compatible for entering and translocation in the plant.



Scheme 1. The listed highest mean values for

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