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Effects of Magnetite Nanoparticles on Soybean Chlorophyll

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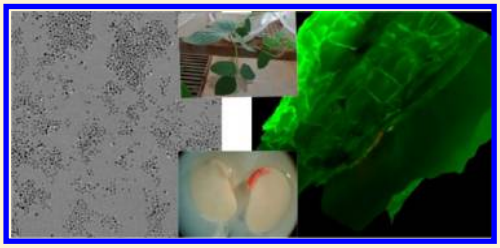
Supporting Information

ABSTRACT: Nanoparticles (NPs) have emerged as one of the most innovative and promising application in agriculture. Since plants are recognized as essential component of all ecosystems, the effects of NPs on plants may pave a new insight to the ecosystems. Here, uptake and translocation of superparamagnetic iron oxide NPs (SPIONs), with various surface charges, on soybean has been probed; in addition, the effects of SPIONs on variations of chlorophyll, in hydroponic condition, together with their ability for reduction of iron deficiency chlorosis were explored. We find that SPIONs, which were entered and translocated in the soybean, increased chlorophyll levels, with no trace of toxicity. Furthermore, it was found that physicochemical characteristics of the SPIONs had a crucial role on the enhancement of chlorophyll content in subapical leaves of soybean. The equivalent ratio of chlorophyll a to b, in all treatments with conventional growth medium iron chelate and SPIONs (as iron source), indicated no significant difference on the photosynthesis efficiency. Finally, it was observed that the effect of SPIONs on the soybean chlorophyll content may have influence on both biochemical and enzymatic efficiency in different stages of the photosynthesis reactions.



INTRODUCTION

Iron is an essential element for both plant and animal nutrition[1] that is required for respiration, photosynthesis, and many other cellular functions, such as DNA synthesis, nitrogen fixation, and hormone production.[2] Although iron is the fourth most abundant element of earth's crust and soil[3] iron deficiency occurs in more than 30% of Earth's arable land.[4] The solubility and availability of iron to plants is strongly depended on the chemical properties of the growth medium (e.g., pH and E_h)[5] and plant mechanism for iron acquisition from soil.[6] In soils with aerobic conditions at physiological pH (i.e., the physiological pH for mainly crop plants is varied between 4 and 8), the concentrations of Fe ions in soil solution are less than 10^{-15} M,[7] while to achieve optimal growth of soybean (*Glycine max*), the plant needs to maintain Fe in the concentration of $10^{-7.7}$ M.[8]

To date, different agronomic approaches have been applied for alleviation of Fe-deficiency by increasing the availability of Fe ions with protonation, chelation, and reduction.[9] In the past six decades, iron chelates, such as the ethylenediamine tetraacetic acid (EDTA) and the ethylenediamine-*N*-*N*′-bis(*o*-hydroxyphenylacetic) acid (EDDHA) have been recognized as the most widely supplements for improving Fe availability to plants.[10] Synthetic Fe-chelate fertilizers are expensive and have direct or indirect effects in the environment.[11] They may avoid the precipitation and enhance mobilization of heavy[12,13] and radioactive[14] metals. Moreover, ligands such as EDTA have been shown to exhibit toxic effects on cellular division, chlorophyll synthesis, and biomass production of photosynthetic organisms.[15] Therefore, new approaches should be developed for alleviation of Fe-deficiency in plants.[16]

Recently, there has been interest in the application of nanomaterials for agronomic purposes, such as smart delivery systems of fertilizers, herbicides, and pesticides, that are more efficiently.[17] Metal release from nanoparticles (NPs) may be a potential nutritional source for biological systems.[18] NP applications on plants need to be studied to determine the mechanism of uptake and translocation of NPs and their possible toxic effects on plants. Despite several studies on NPs effects on micro and macro organism, there are only few reports about the positive or negative or inconsequential effects on plant species.[19] In addition, there are conflicting reports about the ability of uptake and translocation of a variety of nanomaterials (CuO,[20] SiO_2,[21] C_{70},[22] TiO_2,[23,24] Au,[25] ZnO,[26] CeO_2 [27]) by different plant species (*Zea mays*,[20,27] *Triticum aestivum*,[25] *Vigna radiate*,[28] *Arabidopsis thaliana*,[24,29] *Nicotiana tabaccum*,[25] and *Oryza sativa* [22]). There are a few reports on the

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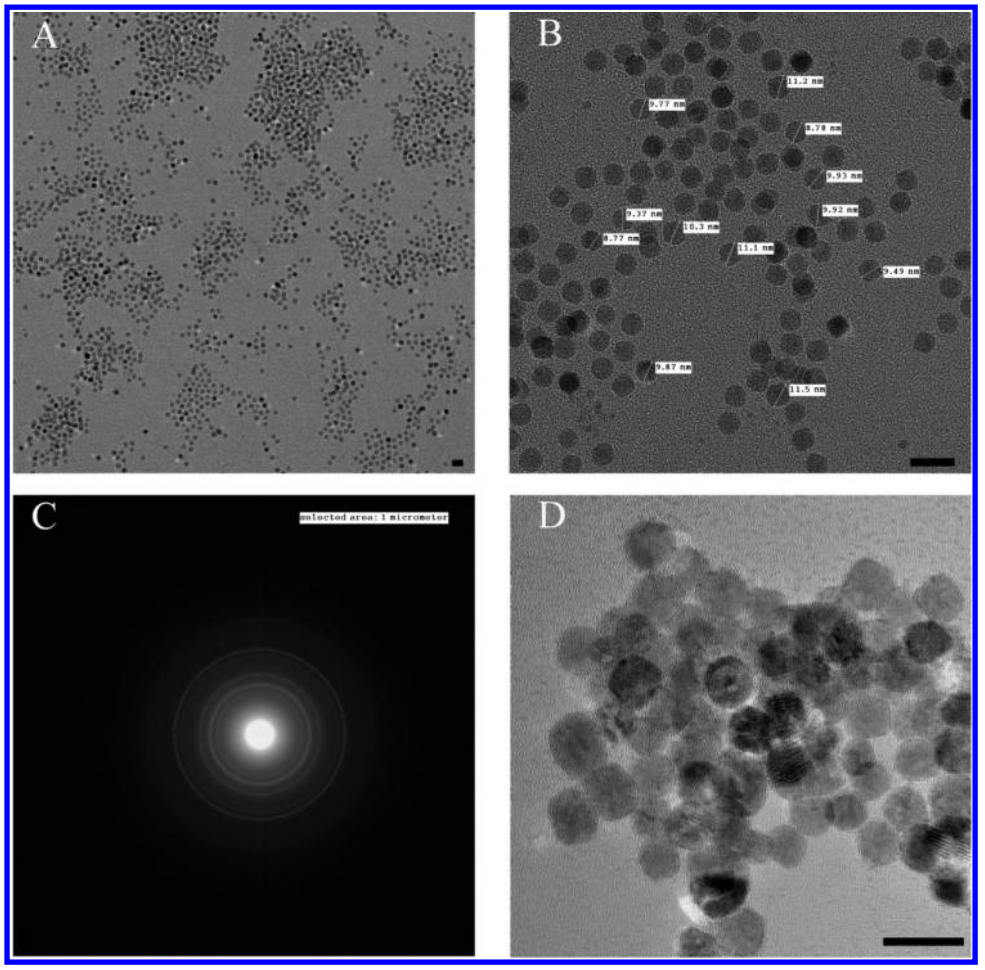

Figure 1. (A and B) TEM images of 9 nm SPIONs at various magnifications together with their (C) diffraction patterns. (D) TEM images of the coated SPIONs. The black size bars are 20 nm.

effects of SPIONs (their absorption, translocation, and accumulation in plants) on various plants (e.g., bean seedlings,[30] *Z. mays*,[30−32] pumpkin,[33,34] cucumber,[18,35] *A. thaliana*,[29] *T. aestivum*,[36] tomato,[36] sunflower,[36] pea,[36] and perennial ryegrasses[37]). Furthermore, the comprehensive effects of NPs (i.e., putative toxic effect, magnetic effect on life cycle, and uptake and translocation mechanisms) have not been probed in the these reports. Here, in this work, the putative toxic effects, magnetic effects on life cycle, uptake, and translocation together with the ability of SPIONs to improve chlorophyll content of soybean in liquid medium are studied and the results compared to Fe-EDTA.

MATERIALS AND METHODS

Full information on the synthesis and coating process of the SPIONs with very narrow size distribution is provided in the Supporting Information (SI).

Phytoxicity Assay. Soybean seeds (*G. max* L., "Oxley") were purchased from Gorghan, Iran Oilseed Research Center. Soybean seeds were sterilized in a 1% sodium hypochlorite (NaClO) solution for 5 min and rinsed three times with deionized water to ensure surface sterility. To determine phytotoxicity effects of various SPIONs, a factorial experiment was designed in randomized complete block (RCB). Four treatments of SPIONs suspensions (at various concentrations including 0.2, 0.4, 1, and 2 mg/mL) were prepared. It is noteworthy to mention that the SPION concentrations were chosen by U.S. EPA and OECD guidelines to evaluate the SPIONs phytotoxicity; however, the concentration of SPIONs in nutrient solution were selected based on iron concentration needed for plant growth. A piece of 11 μm filter paper was put into 100 mm Petri dish and 5 mL of SPIONs suspensions were added to the dishes. Then seeds were transferred to filter paper with around 1 cm between each seeds. The test suspension was applied in concentric circles over the seeds, completely covering the filter paper. Seeds germinated in deionized water were used as control and treatments were performed in triplicate. Covered Petri dishes were placed in a growth chamber at 25 °C with 75% relative humidity in the dark. After five days of seed planting that completed emergence stage,[38] root length was defined as the length from root tip to radicle (embryonic root) by vernier caliper and seed germination was defined by the tetrazolium test (TZ). It is noteworthy to mention that the emergence stage (VE) normally takes five to ten days depending on the temperature, moisture conditions, variety, and planting depth.[39] After five days of seed planting, lateral roots are growth from the radical and root hairs can be visible. During this time, whole needed nutrients would be absorbed from media.[39]

Plant Cultivation and Treatments. Sterilized soybean (*G. max* L., Oxley) seeds were germinated in 11 μm filter paper. After 5 days, healthy seedlings were transferred into perlite and irrigated with distilled water for a week. Then, seedlings (2-pair leaflet stage) were transferred into a continuously aerated

Table 1. Description of the Various Particles in DI Water and PBS

		DI water				PBS			
	functional group	D_H (nm)[a]	PDI[b]	$\langle D_H \rangle$ (nm)[c]	ζ-potential (mV)	D_H (nm)[a]	PDI[b]	$\langle D_H \rangle$ (nm)[c]	ζ-potential (mV)
SPIONs	COOH	18.3 ± 0.2	0.111	19.9 ± 0.2	−26.7 ± 0.5	19.8 ± 0.2	0.112	20.6 ± 0.2	−14.3 ± 0.8
	plain	19.7 ± 0.4	0.089	19.9 ± 0.3	−17.9 ± 1.1	20.4 ± 0.3	0.123	20.9.8 ± 0.2	−12.8 ± 1.6
	NH_2	18.8 ± 0.2	0.103	20.4 ± 0.2	+36.5 ± 1.8	20.7 ± 1.3	0.097	21.8 ± 0.2	+21.6 ± 1.2

[a]ζ-average hydrodynamic diameter extracted by cumulate analysis of the data. [b]Polydispersity index. [c]Average hydrodynamic diameter determined from Contin size distribution.

solution (i.e., 400 mL air min^{-1}) in 2 L plastic bucket. The nutrient solution was composed of (concentrations in $mg \cdot L^{-1}$): KH_2PO_4 68, KNO_3 505, $K_2HPO_4 \cdot 3H_2O$ 114, $Ca(NO_3)_2$ 102, $MgSO_4$ 240, H_3BO_3 2.86, $MnSO_4 \cdot H_2O$ 0.31, $ZnSO_4 \cdot 7H_2O$ 2.29, $CuSO_4 \cdot 5H_2O$ 0.71, $CoCl_2$ 0.22, and $NaMoO_4 \cdot 2H_2O$ 0.023. The solution pH was controlled between 5.8 and 6.2 (6.0 ± 0.2) using H_2SO_4 or NaOH solutions.[40] Water consumed by soybean was replenished and the solution was renewed every 7 days. All experiments were carried in a greenhouse under controlled conditions. The concentrations of SPIONs and Fe-EDTA in hydroponic solution were calculated by hydrobuddy version 1.5 software according to these factors: Fe requirement for soybean, solubility and compatibility of SPIONs and Fe-EDTA, and water quality. The SPIONs and Fe-EDTA (control) with concentrations (0.03, 0.045, and 0.06 mg mL^{-1}) were directly suspended in nutrient solutions and were dispersed using ultrasonicator (100 W, 40 khz) for 30 min. The studies were done to evaluate the ability of SPIONs to uptake, translocate and improve chlorophyll content of soybean in liquid medium and the results were compared to those obtained with Fe-EDTA, which is one of the most commonly used in agriculture to remediate Fe chlorosis. According to Meyer−Berthenrath's method, modified by Stirban in 1985,[32] the subapical leaves chlorophyll content extracted with acetone 90% was determined at 645 and 663 nm by a Shimadzu UV1700 UV/vis spectrometer with quartz cells.

Sample Preparation and Staining. The localization of the SPIONs was studied during the vegetative phase of the soybean hydroponic cultivated plant. Collection of samples was performed from beginning of the treatment until the inception of flowering with an interval of 7 days. In each sampling, a number of 2−3 well-developed stems and roots were randomly selected. Plant fresh materials were made by longitudinal sectioning of root and stem using a hand microtome. Prepared samples were immediately stained with the aqueous solution of Ouramin O (0.1% in 100 mL water).

Epifluorescence Microscopy. To study the tissue of the SPIONs treated plants, 3D microscopic techniques were employed. The stained samples were viewed through an Olympus BX51 (Olympus optical Co., Ltd. Tokyo, Japan) research fluorescence microscope equipped with the catadioptric objectives UMPlanFL-BDP and the BX-RFA (Olympus optical Co., Ltd. Tokyo, Japan) fluorescence illuminator. The best fluorescence excitation was observed when U-MWB3 (480−510 nm) and U-MWG3 (510 - 550 nm) mirror cube units were used. Consecutive image series from successive focal plates (with 5 μm increment per focal step) was acquired by means of an Evolution MP cooled CCD (Media Cybernetics, USA) according to a methodology introduced by Dadpour et al. ..[41] Digital images were taken by TIFF format using RGB mode (12 bits per channel) with a resolution of 2560 × 1920 pixels. Details on the image analysis is provided in the SI.

Statistical Data Analysis. All the experimental data were examined by using an analysis of variance (one-way ANOVA) and the results were presented as mean ± SD (standard deviation). The SPSS 15.0 package software (SPSS Inc., Chicago, U.S.A.) was used, followed by least significant difference (LSD) between treatments to test the significance as compared to control ($p < 0.05$) or between various treatments/physiological parameters. Each of the experimental values was compared to its corresponding control. The level of significance is indicated by an asterisk as compared to control.

RESULTS AND DISCUSSION

Characterization of SPIONs. To probe the effects of SPIONs on the soybean chlorophyll, highly uniform SPIONs with core size of 9 nm, and either plain, or dextran based negative or positive surface charges were synthesized (see Figure 1 and Table 1 for details). The SPIONs have very narrow size distribution as determined by transmission electron microscopy (TEM). Dynamic light scattering (DLS) and ζ potential measurements show that the electrokinetic potential and average sizes of the SPIONs in different solutions are highly dependent on the surface charges of the SPIONs. The average DLS sizes of the SPIONs with different coatings are 18.9 and 20.3 nm in DI water and phosphate buffered saline (PBS), respectively. The DLS results are in good agreement with TEM data. It is essential to plant research had monodisperse NPs, with same physicochemical properties for evaluation real interaction effects with plants. In this study, magnetite NPs was synthesized monodisperse and suitable magnetic properties.

Effect of SPIONs on Germination Index and Plant Elongation. There are no specific testing manual for nanomaterials phytotoxicity. The U.S. EPA and OECD are guidelines for testing of chemicals, frequently applied to nanotoxicity assay for plant recommended by these guidelines.[19] The plant species recommended have different germination time so exposure to nanomaterials at different stage of growing in the same time. In this study, it was used the TZ method because measurement of seed germination percent in this method is not time-dependent. The plants were treated with NPs and the effects on germination index and root elongation were probed. According to the results, various concentrations of SPIONs, with different charges, do not have significant effects on the germination index (see Table S1 in SI). Interestingly, positive and negative SPIONs show significant ($p < 0.01$) positive influence on root elongation, where as plain SPIONs have no significant effect; more specifically, plain SPIONs slightly inhibit (see Table 2). This may happened due to the lower protective effect of the polymeric shell in plain SPIONs, compared to the negative and positive SPIONs, resulting in release of more Fe^{2+} ions in the treatment. To evaluate this hypothesis, the measured Fe^{2+}concentrations in the growth medium containing SPIONs

Table 2. Seed Germination of Soybean Exposed to SPIONs

treatments	degrees of freedom	relative seed germination	relative root elongation	germination index
factor A (type of coating)	2	0.622[a]	3.467[b]	0.026[b]
factor B (concentration)	4	15.911[b]	17.389[b]	0.014[b]
A × B	8	0.428[a]	0.939[a]	0.003[a]
error	30	0.222	0.533	0.002
coefficient of variation (%)		0.504	1.672	1.938

[a]Non-significant. [b]Significant at 1% level.

with and without soybean cultured condition were measured by the Snell 2007 method at 510 nm. Results confirm that the concentration of Fe^{2+} in the growth medium is increased by the cultivating soybean. The NPs accumulate on the root and seed surface and occlude some water and ion channels. Nanomaterial phytotoxicity is related to its dimensions, chemical composition, surface properties, and ionization of the surface.[42,43] To remove the slight observed toxicity of SPIONs, lower concentrations (e.g., 0.06 mg/mL) are employed for the further stages.

Magnetization in Plants after Treatments with SPIONs. Selected VSM measurement results of various soybean tissues treated with 0.06 mg/mL of SPIONs, with various charges, are shown in Figure 3. It is important to mention that there is no trace of the magnetization signal in the control plant tissue. Because of the accumulation of SPIONs in the root, stem, crown, and leaf, regardless of surface charge of the SPIONs, in all sub apical leaf of treatment plants were detected magnetization signal (see Figures 3 (A series)). The weakest magnetic signal was detected from stem tissue in all treatment (see Figures 3 (B series)). The highest magnetic signal in aerial tissue is detected in the crown, where the root vascular systems change to stem and where we observed the maximum accumulation of NPs (Figure 3 (C series)). The strongest magnetizations (i.e., 138 memu/g) are observed in the roots for soybeans cultured in nutrient solution with plain SPIONs (Figures 3 (D series)); this may happen because the majority of SPIONs may be absorbed/trapped on the root tissue. It is now well recognized that the surface of NPs are covered by various macromolecules, upon their entrance into the fluids containing biomolecules.[44−46] Thus, the abundance of magnetic signal in the root tissue clearly indicates that the absorbance of root exudates macromolecules at the surface of NPs caused SPIONs accumulation on the root surface; this result was completely in agreement with the previous reports[47] (e.g., it was shown that the absorbance of plant exudates macromolecules (e.g., protein and polysaccharide) on the surface of gold NPs could varies their physicochemical properties and affect their uptakes and traslocations into rizhospher and xylem sap[25]). It is now well-recognized that NPs surfaces are affected by various chemical and biological elements in biological media.[48] The biological identity of NPs changes in biological milieu with absorption of biomolecules.[49] According to the magnetization results (see Table 3 for details), it is confirmed that positive and negative SPIONs can penetrate well from growth medium into various soybean tissues, rather than plain NPs; furthermore, positive and negative SPIONs can traverse to stem and leaf and less aggregated in aerial plant tissue, compared to the plain NPs. Zhu et al. [33] investigated uptake and translocation of Fe_3O_4 NPs (20 nm) by pumpkin (*Cucurbita mixta*) in hydroponic conditions by using a vibrating sample magnetometer. The study showed similar result that magnetic signals were detected in root, stem, and leave of plant grown in medium with magnetite NPs.[33] On the other hand, Wang et al.[37] did not observe any uptake of 25 nm Fe_3O_4 NPs by pumpkin plants. It is hypothesized that it is difficult for the larger size NPs to penetrate through the cell walls and transport across the plasma membranes.

Microscopic Evidence of Uptake, Transport, and Accumulation of Nanoparticles. Figure 4 shows how SPIONs penetrate into the root, traverse to the xylem, and translocate into the shoot. Figure 4A indicates that accumulation of SPIONs inside the root tissue is much broader than the aerial part of the plant. The largest amount of agglomerated SPIONs cannot be uptaken by the plant cells and a number of them is incorporated into the cell wall. Figure 4B confirms that SPIONs are diffused toward the interior of the stem parenchyma. Since SPIONs dimension (9 nm) have significant smaller size compared to the cell wall pore and the plasmodesmata width, the SPIONs traverse through the biomembranes and other plant pathways. Figure 4C displays SPIONs infiltrated into the mesotome and parenchyma cell from the leaf veins. SPIONs diffuse from xylem's sap to aerial tissue with apoplastic flow and symplastic transport. Transpiration and evaporation stream of water from stomata leaf are responsible for accumulated of SPIONs in the margin of leaves. However, the mechanisms underlying these processes are not

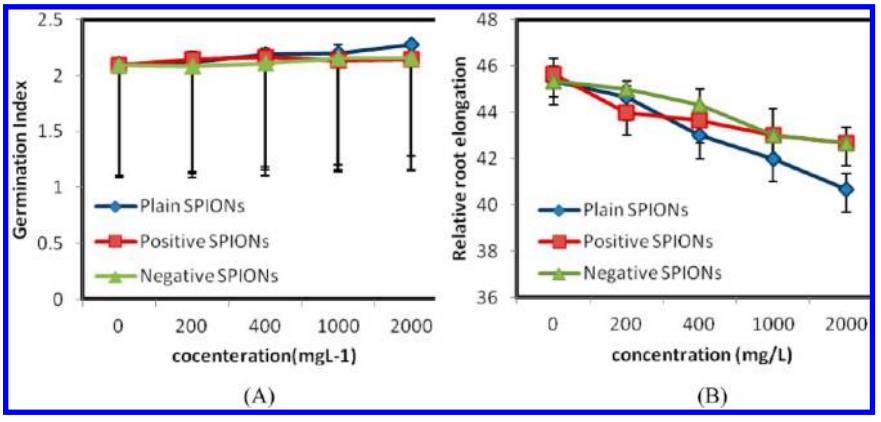

Figure 2. Effect of magnetite NPs on the soybean germination index in (A) and root elongation in (B) Bars represent means ± SD of four separate experiments.

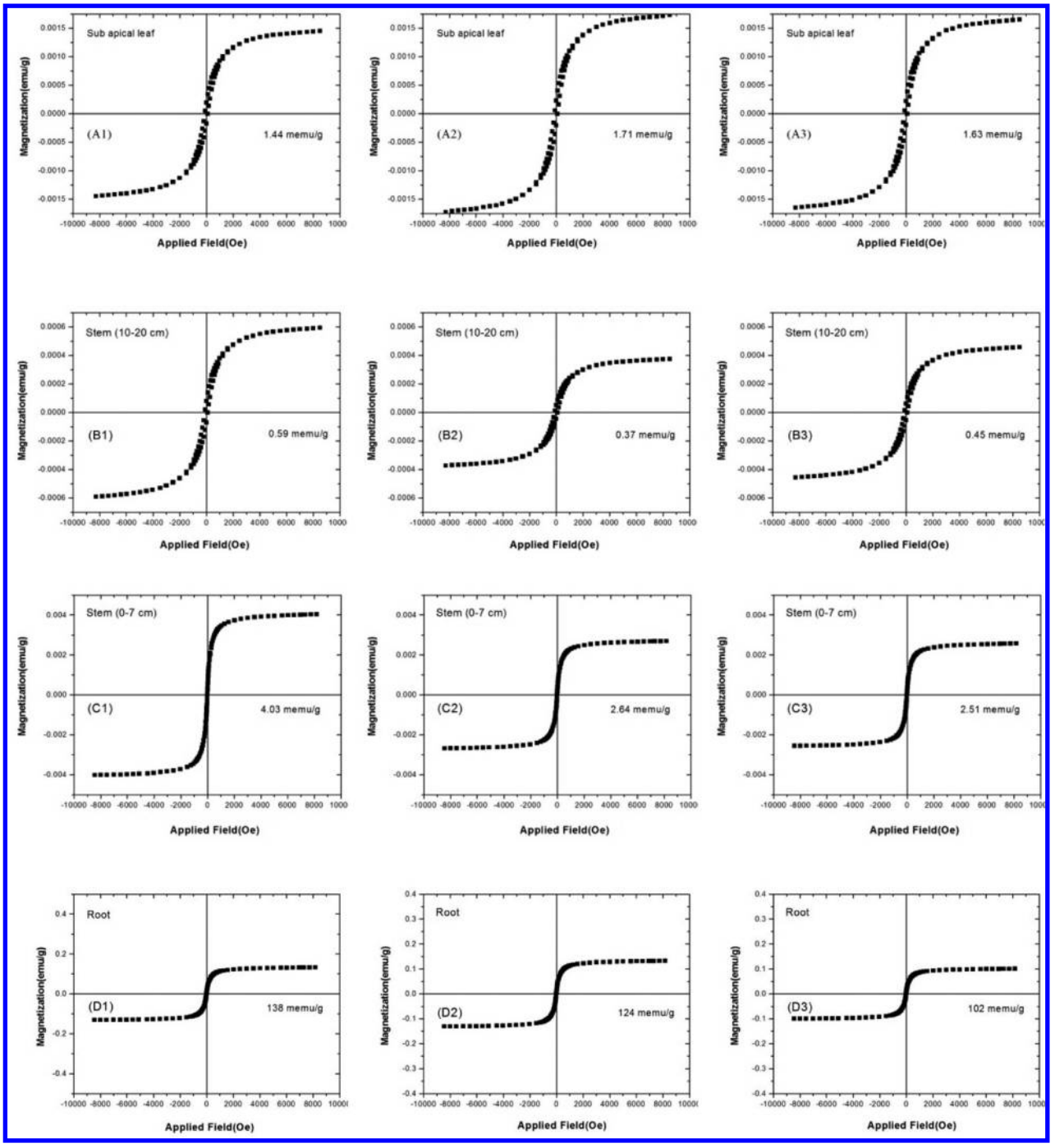

Figure 3. Selected VSM curves of soybean shoot and root samples. Symbols A_1, A_2, and A_3 show the VSM loops of subapical soybean leaf treated with 0.060 mg/mL SPIONs for 28 days. Symbols B, C, and D show the sample treated with same condition A but for stem (10−20 cm), crown (stem 0−7 cm), and root of soybean (X_1, X_2, and X_3 refers to plain, positive and negative SPIONs, respectively).

Table 3. Magnetize Signal Measured by VSM at Various Sampling Locations

plant tissue	plain SPIONs (memu/g)	positive SPIONs (memu/g)	negative SPIONs (memu/g)
subapical leaves	1.44	1.71	1.63
stem	0.59	0.45	0.37
crown	4.03	2.64	2.51
roots	138	124	102

understood. Nowack and Bucheli[50] speculated NPs may enter plant roots through osmotic pressure, capillary forces, through pores in cell walls and intercellular plasmodesmata, or via the highly regulated symplastic route. Plants have selective uptake and translocation of NPs. The NPs could also diffuse into the intercellular space, the apoplast, and then be adsorbed or incorporated into chelates.[51] The properties of NPs, such as composition, size, shape, and surface charge may affect the uptake and translocation inside plant.[47,52]

Chlorophyll Content. Enhancement of chlorophyll content in subapical leaves of soybean is depended on the

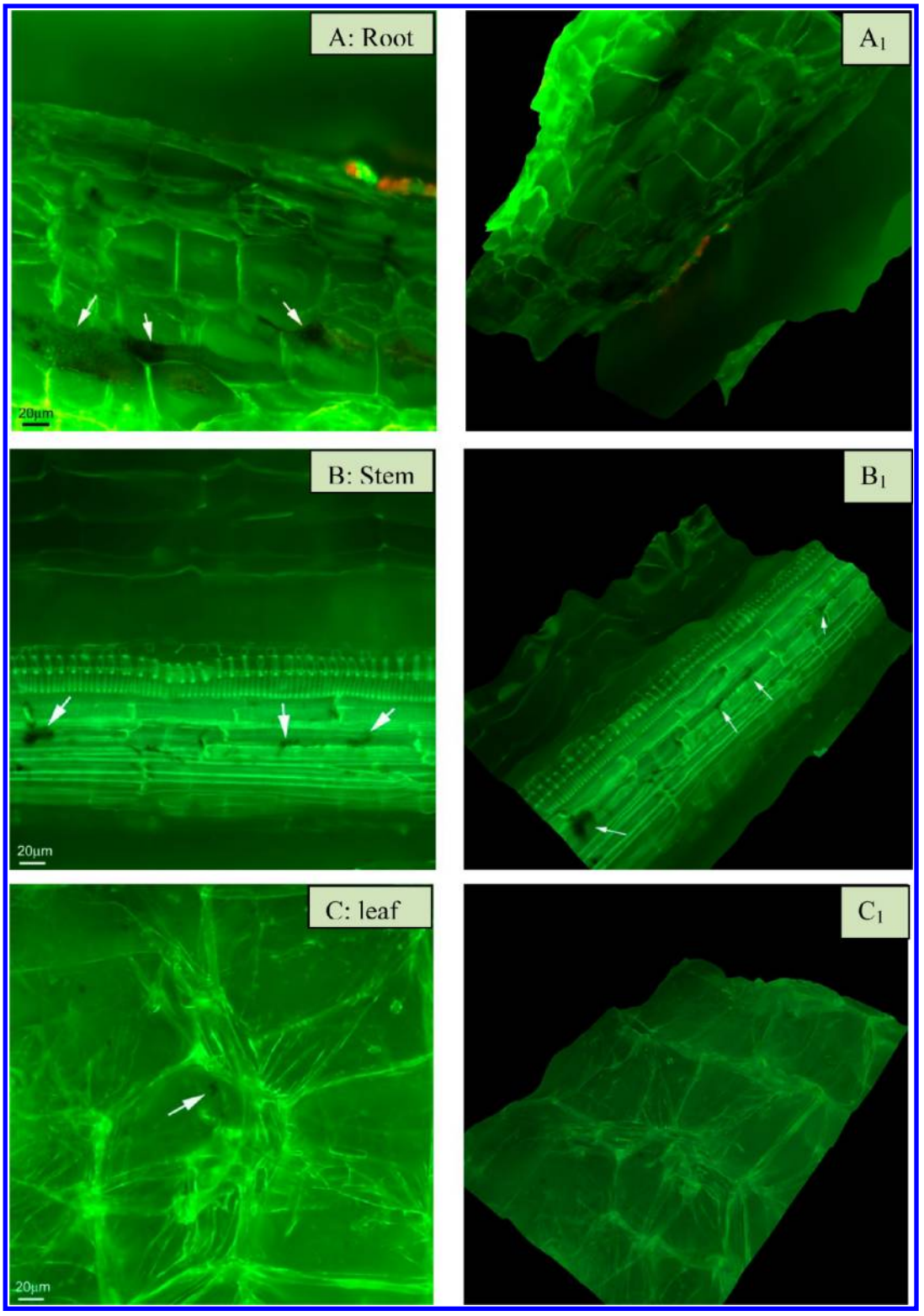

Figure 4. Sections from different samples of soybean. Detail of longitudinal section in root, stem, and leaf of soybean after 5 days of exposure to plain SPIONs in fluorescence microscopic images. Arrows (dark coloration) indicate accumulation of the plain SPIONs in vascular and parenchyma tissues in pictures A–B. Arrowheads indicate accumulation of SPIONs (because of its fluorescence quenching capability[53]) in cortical cells in picture C. A_1, B_1, and C_1 correspond to 3D format of A, B, and C.

concentration SPIONs in the growth medium and surface charge of NPs. The mean chlorophyll concentration in the soybean fresh weight exposed to SPIONs is significantly ($p < 0.01$) lower than those treated with Fe-EDTA at the same concentration (see Table 4). There is no significant difference in the ratio of chlorophyll a/b in all treatments. For these reasons we suggest that the biosynthesis of chlorophyll a is influenced differently in comparison to that of chlorophyll b. A suitable linear correlation between chlorophyll a and b, with correlation coefficient over 0.9, suggests that the biosynthesis of

main photosynthetic pigments is affected by SPIONs. In this experiment, the SPIONs are sole source of iron in the treatment. The soybean rhizosphere is acidified by protons for releasing of Fe ions from SPIONs and then the iron ions are used inside the plant. But iron ions concentrations are not adequate for soybean growth (10^{-9} M). The SPIONs could provide iron ions with redox reactions involved in the chloroplast. The biochemical reactions in chloroplast stroma, siderophore in the tylakoidal membranes,[54] and photocatalytic reaction are suggested as factors for iron availability from SPIONs. Other results of previous investigations show low ferrofluid concentrations increase chlorophyll level in bean plantlets.[30] The SPIONs effects on the soybean photosynthesis performance may have not only a biochemical influence but also a magnetic field of the particle influence on the enzymatic structures in the different stages of the photosynthesis reactions.[55] The effect of magnetic NPs coated with tetra methyl ammonium hydroxide (TMA−OH-8 nm) with super paramagnetic properties could influence the ion flows by changing ion channels properties.[32] Chlorophyll a and b concentrations at subapical leaves of soybean are diminished at more than 45 mg·L^{-1} of plain SPIONs in the growth medium (see Figure S1 of SI). The ratio of chlorophyll a to b in all treatments indicates that there is no significant difference on the photosynthesis efficiency between Fe-EDTA and SPIONs as sources of iron. We notice toxicity symptoms lead to brown spots covering the leaf surface in the plants for a culture medium with 60 mg·L^{-1} Fe-EDTA and plain SPIONs. Iron excess in this treatment could be generating oxidative stress in the leaf cells.

Table 4. Chlorophyll Content in Subapical Leafs of Soybean Exposed to SPIONs

treatments	degrees of freedom	chlorophyll *a*	chlorophyll *b*
factor A (concentration)	2	0.122[a]	0.046[a]
factor B (coating)	3	2.205[a]	0.865[a]
A × B	6	0.146[a]	0.057[a]
error	12	0.002	0.001
coefficient of variation (%)		1.877	2.452

[a]Significant at 1% level.

In summary, nanoparticles have emerged as one of the most innovative and promising field in agriculture. In this report, SPIONs with narrow size distribution and various chemical properties were prepared and their uptake and translocation in soybean, in hydroponic conditions, and the effect on chlorophyll production were probed. From the obtained results, the SPIONs were translocated into the soybean and increased chlorophyll levels. The SPIONs significantly enhanced the chlorophyll content in subapical leaves of soybean. To our knowledge, this is the first comprehensive report on probing SPIONs effects on soybean plant grown in solution with NPs.

■ ASSOCIATED CONTENT

Supporting Information

Nanoparticle synthesis, synthesis of various copolymers, coating process, SPION characterization, epifluorescence image analysis, figure showing the effect of magnetite, table comparing the mean effect of surface characterization, and additional references. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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