Research Article

Increased Plant Growth with Hematite Nanoparticle Fertilizer Drop and Determining Nanoparticle Uptake in Plants Using Multimodal Approach

Armel Boutchuen,¹ Dell Zimmerman,¹ Nirupam Aich,² Arvid Mohammad Masud,² Abdollah Arabshahi,³ and Soubantika Palchoudhury¹

¹Department of Civil and Chemical Engineering, University of Tennessee at Chattanooga, Chattanooga, Tennessee 37403, USA

²Department of Civil, Structural, and Environmental Engineering, University of Buffalo, Buffalo, New York 14260, USA

³SimCenter, University of Tennessee at Chattanooga, Chattanooga, Tennessee 37403, USA

Correspondence should be addressed to Soubantika Palchoudhury; soubantika-palchoudhury@utc.edu

Received 22 February 2019; Accepted 16 May 2019; Published 9 June 2019

Academic Editor: Paulo Cesar Morais

Copyright © 2019 Armel Boutchuen et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

There is an emerging scientific interest in the use of nanoparticle fertilizers for enhanced agricultural and bioenergy crop production to meet the growing food and energy demands of the world. The objective of designing the nanoparticle fertilizers is to effectively deliver the required nutrients for the plants without adding large quantities of fertilizer to the environment. However, most reports on nanoparticle fertilizers so far, involved the addition of nanoparticles to the hydroponic system or the soil. In this study, we report a new modified seed presoak strategy using a drop of Fe-enriching hematite nanoparticle dispersion to enhance plant growth and production in four different legume species, i.e., chickpea, green gram, black bean, and red bean. The hematite nanoparticle fertilizer drop promoted a 230-830% increase in plant growth with green gram showing the highest increase, based on our prolonged and statistically reliable growth studies. In general, we observed an increase in the survival span of plants, a twofold increase in fruit production per plant, nearly two times faster fruit production, and healthy second-generation plants with the nanoparticle treatment; however, there were slight species-specific variations. We used a novel multimodal material characterization approach combining three techniques, hyperspectral imaging, Fourier transform infrared spectroscopy (FTIR), and inductively coupled plasma optical emission spectroscopy (ICP-OES), to evaluate the internalization and transport of the nanoparticle fertilizer within the plants. Our results indicated that the hematite nanoparticles were transported through the roots and stems and were localized in the leaves after 10 days of growth in pots of soil. Therefore, the modified seed presoaking method using a drop of hematite nanoparticle will be highly attractive in enhancing plant growth and health, while minimizing environmental impacts.

1. Introduction

Iron (Fe) is a key element for several cellular reactions in plants such as respiration and the formation of chlorophyll required for photosynthesis. Plants have adopted a mechanism to acquire this essential nutrient from the soil using the apoplastic pathway through the roots, but limited Fe is available in some soil types or in soils with excessive agricultural use [1, 2]. Fe deficiency is known to cause chlorosis in plants [3]. Therefore, Fe-enriching fertilizers are required to

ensure optimum Fe delivery to the plants. Recently, there has been a thrust to develop innovative fertilizer formulations like nanoparticle (NP) fertilizers because conventional fertilizers are required in large quantities owing to their slower absorption by the plants [4–6]. The NP fertilizers can facilitate tunable delivery of the required nutrients to the plants. Therefore, NP fertilizers are seen as highly promising candidates for enhanced production of agricultural and bioenergy crops to meet the growing food and energy demands of the world population [5, 7–9].

Recently, Yuan et al. demonstrated a concentration-specific role of Fe NPs in promoting growth in *Capsicum annuum* plants [10]. The Fe NPs increased growth in these plants through reorganization of the leaf, increasing chloroplast per grana stacking, and regulating the vascular tissues within the leaf and stem. Raju et al. reported the role of Fe NPs in increasing the radical length and biomass of green gram sprouts during germination [11]. In the study by Srivastava et al., iron pyrite NPs induced a marked increase in the growth of spinach sprouts [12]. Iron pyrite NPs also facilitated a denser network of roots and a significantly (~2.5 times) higher yield in both chili and marigold plants [13]. These NPs served as a suitable equivalent of nitrogen, phosphorus, and potash fertilizer for the production of rice, a staple food crop [14]. Li et al. demonstrated the stimulated root growth of peanut plants under the influence of Fe NPs [15]. In addition to Fe NPs, iron oxide NPs have also been adopted as Fe-enriching fertilizers to replenish Fe content in plants as they are inherently nontoxic. For example, Jeyasubramanian et al. showed that hematite NPs boosted the growth rate of spinach plants in a slightly acidic hydroponic system via conversion to Fe^{2+} ions [16]. Ren et al. reported increased physiological activity of green gram plants with iron oxide ($\gamma\text{-Fe}_2\text{O}_3$, maghemite) NPs. The NPs translocated through the plant roots to the stem and leaves in this case [17]. In another study, Ghafariyan et al. demonstrated that iron oxide NPs were absorbed and translocated within the soybean plants under hydroponic conditions. The iron oxide NPs boosted chlorophyll production in these plants without showing any toxic impact [18]. Zhu et al. also reported the absence of any toxic impact of iron oxide NPs in pumpkin plants during a prolonged period of exposure [19]. These studies demonstrate the immense potential of iron oxide NPs as Fe-enriching fertilizers and chlorosis treatment agents for agricultural and bioenergy crops. However, most of these methods involved the direct addition of the NP fertilizer to the soil or the hydroponic system, which is less attractive in terms of environmental sustainability. Studies have shown stress response and reduction of amino acids in plants with excess addition of NP fertilizers [20]. There is a need for a new environmental-friendly strategy that increases plant growth with a minimum quantity of the fertilizer while also minimizing the addition of fertilizer to the environment. Recently, we reported a seed presoak strategy where soaking the embryonic seeds of legumes in liquid dispersions of iron oxide NPs showed enhanced root growth by 88–366%, but more than 4 mL of NPs were required for each seed [21]. Minimizing the quantity of NP dispersions required would make the seed presoak strategy more effective for practical applications.

We also need to understand the pathway of internalization, interaction, and translocation of the NP fertilizer within the plant both for risk assessment and for synthesizing high-efficiency NP fertilizers [1, 22–24]. Most plant-NP interaction studies to date have focused on the physiological aspects as it is challenging to find a material characterization method capable of detecting the low concentrations of NPs uptaken within the complex biological matrix of plants [25]. Imaging techniques like optical and electron microscopy have been

traditionally used to detect NPs within the plant cells [15, 21, 26, 27]. However, optical microscopy cannot resolve objects less than 250 nm apart due to the diffraction limit of visible light even though it is rapid and requires negligible sample preparation. Electron microscopy can easily resolve nanoscale objects, but the required sample preparation stages such as staining, ultrathin sectioning, sputter-coating, and labeling increase artifacts in the images. Recently, darkfield hyperspectral imaging has emerged as a highly promising visualization tool to both detect and map the localization of NPs within complex microenvironments [28]. The charge-dependent uptake and mobility of Au NPs by the roots of *Arabidopsis thaliana* could be demonstrated using this technique [29]. When combined with an elemental analysis technique like X-ray tomography or mass spectroscopy, hyperspectral imaging provided further reliable insights into plant-NP interactions [29, 30]. Another method requiring minimum sample preparation is FTIR, which has also proven useful in understanding the plant-NP interactions via chemical composition analysis in several reports.

In the present study, we investigated the effectiveness of an Fe-enriching hematite ($\alpha\text{-Fe}_2\text{O}_3$) NP fertilizer to boost plant growth and production using four different species of legumes as model plants (i.e., chickpea, green gram, black beans, and red beans). The plants were grown in same-sized pots filled with the same soil type to keep all growth conditions the same other than the NP treatment. A new “modified seed presoak” strategy was investigated to minimize the quantity of NP fertilizer required and to prevent the addition of NPs directly to the soil. Two different concentrations of the hematite NP fertilizer were used to investigate the Fe concentration-dependent growth trend in the plants. Another objective of this study was to develop a multimodal material characterization strategy combining hyperspectral imaging, FTIR, and ICP-OES for gaining insights into the internalization, transport, and localization of the NP fertilizer within the plants. This improved seed presoaking method with one drop of the NP fertilizer will be highly beneficial in promoting enhanced agricultural production in nutrient-deficient environments in a cost-effective and sustainable fashion. The multimodal material characterization strategy will be significant both in understanding the mechanism of plant-NP interactions and risk assessment of the new NP fertilizers.

2. Materials and Methods

2.1. Materials. All reagents were used as purchased. The reagents for NP synthesis included iron (III) acetylacetonate ($\text{Fe}(\text{acac})_3$, 99%, Alfa Aesar), polyvinylpyrrolidone (PVP, Mw 10 kDa, TCI, Thermo Fisher Scientific), polyethyleneimine (PEI, Mw 60 kDa, 50% aq, Alfa Aesar), triethylene glycol ($\text{C}_6\text{H}_{14}\text{O}_4$, TREG, 99%, Acros Organics), and deionized water (DI, Thermo Fisher Scientific). Potting soil, pots, and seeds of chickpea (*Cicer arietinum*), green gram or mung bean (*Vigna radiata*), and black and red beans (*Phaseolus vulgaris*) were purchased from local grocery stores in Chattanooga, Tennessee, USA, for plant growth experiments.

FIGURE 1: Images of chickpea seeds treated with a drop of (a) DI water, (b) a drop of low concentration NP fertilizer suspension (0.022 gL^{-1} Fe), and (c) a drop of high concentration NP fertilizer suspension (1.1 gL^{-1} Fe), immediately before planting on day 7.

2.2. Hematite NP Synthesis and Characterization. Hematite ($\alpha\text{-Fe}_2\text{O}_3$) NPs were synthesized using a highly reproducible modified polyol synthesis method developed and reported in our earlier studies [21, 31]. In a typical synthesis conducted on a Schlenk line, the $\text{Fe}(\text{acac})_3$ iron precursor (2 mmol) was added to a PVP/PEI (PVP, 0.7 g and PEI, 0.3 g) ligand mixture in the solvent, TREG. The reactant mixture was heated at 290°C for 1 h on a heating mantle with magnetic stirring (Thermo Fisher Scientific) under an inert atmosphere to form the hematite NP product. The NPs were cleaned with DI water three times via centrifugation at 14000 rpm (Thermo Fisher Scientific). Transmission electron microscopy and X-ray diffraction characterization of these hematite NPs were reported in our earlier studies [21].

2.3. Hematite NP Growth Dispersion Synthesis and Characterization. Finally, the precipitant hematite NPs were dispersed in DI water via sonication (Branson 1800, room temperature) for 15 min to obtain the target concentrations for use in subsequent plant growth studies. Two NP growth suspensions for plants were prepared with two different hematite NP concentrations, i.e., low NP concentration (0.022 gL^{-1} Fe), and high NP concentration (1.1 gL^{-1} Fe), while DI water without the addition of hematite NPs was used as a control.

The hydrodynamic diameter and zeta potential of the hematite NPs in the growth suspensions were analyzed on a Litesizer 500 Particle Analyzer (Anton Paar), prior to use as fertilizer drop for the legumes. Mean hydrodynamic diameter was reported based on an average of five consecutive runs. Zeta potential measurements were conducted at 25°C using omega cuvettes and reported as an average of five analyses (Figures S1a-b, SI).

2.4. Plant Growth Experiments Using Hematite NP Drop. Four different varieties of legumes, i.e., chickpea or *Cicer arietinum*, green gram or *Vigna radiata*, black beans and red beans or *Phaseolus vulgaris*, of varying seed sizes were used as test plants for our plant growth studies to investigate the general effectiveness of the NP fertilizer. A “modified seed presoak” method was developed to enhance the growth of

legumes using a drop of $\alpha\text{-Fe}_2\text{O}_3$ NP growth suspension. First, the seeds were cleaned with 75% ethanol and DI water and dried with filter paper for use in the plant growth experiments. In this modified presoak method, each legume seed was placed on a wet paper towel inside a sterilized petri dish (Thermo Fisher Scientific), prior to the addition of one drop of $\alpha\text{-Fe}_2\text{O}_3$ NP growth suspension. A set of three petri dishes with the same seed type was prepared for adding three different growth suspensions, i.e., one drop of DI water reference, one drop of a low concentration of hematite NPs (0.022 gL^{-1} Fe), and one drop of a high concentration of hematite NPs (1.1 gL^{-1} Fe), to investigate the applicability of the NP fertilizer in enhancing plant growth. The petri dishes were then loosely closed with lids to retain airflow for the growing seeds. Roots were observed from these seeds within 2-4 days, following which the shoots sprouted. Figure 1 and Figure S2 (SI) show the three sets of seeds for chickpea, green gram, black bean, and red bean immediately before planting. A summary of the experimental parameters used for our plant growth study is presented in Table 1. The three seeds of the same legume species treated with a drop of DI water, a drop of a low concentration of NPs, and a drop of a high concentration of NPs were planted in the same pot as soon as shoots were seen at around day 7. The pot was placed indoor near a window to ensure a controlled environment and access to sunlight. The growth of each plant was subsequently monitored each day by measuring the length of the shoot using a Vernier caliper for a total period of 60-100 days, depending on the species. The experiment was repeated six times for each legume species to ensure statistical reliability. Error bars for the average plant height were reported based on a 95% Student's *t*-distribution.

The NP-treated plants from all legume species except the chickpea produced fruit pods. Seeds were collected from the mature pods and cleaned with 75% ethanol and DI water. These seeds from NP-treated green gram, black bean, and red bean plants were planted in pots of soil and placed near a window, similar to the first generation plants. The second-generation seeds were planted on the same day after collection from the seed pods, without any NP treatment. The growth of the second-generation plants were then

TABLE 1: Summary of key experimental conditions used to investigate legume growth with hematite NP fertilizer.

Type of legume seeds Germination condition: seeds placed in moist paper towel inside a nearly covered petri dish	Type of suspension used for seed presoaking	No. of seeds tested	Potting conditions	Total time of plant growth monitoring (days)
Green gram Seed size: 4.76 ± 0.2 mm	Reference suspension: DI water (0 gL^{-1} Fe), 1 drop	6	Soil Seeds potted after 7 days of germination Plant placed indoor in sunlight beside the window	60
	Low-concentration suspension: hematite NP fertilizer (0.022 gL^{-1} Fe), 1 drop	6	Soil Seeds potted after 7 days of germination Plant placed indoor in sunlight beside the window	60
	High-concentration suspension: hematite NP fertilizer (1.1 gL^{-1} Fe), 1 drop	6	Soil Seeds potted after 7 days of germination Plant placed indoor in sunlight beside the window	60
Black bean Seed size: 8.50 ± 0.7 mm	Reference suspension: DI water (0 gL^{-1} Fe), 1 drop	6	Soil Seeds potted after 7 days of germination Plant placed indoor in sunlight beside the window	60
	Low-concentration suspension: hematite NP fertilizer (0.022 gL^{-1} Fe), 1 drop	6	Soil Seeds potted after 7 days of germination Plant placed indoor in sunlight beside the window	60
	High-concentration suspension: hematite NP fertilizer (1.1 gL^{-1} Fe), 1 drop	6	Soil Seeds potted after 7 days of germination Plant placed indoor in sunlight beside the window	60
Chickpea Seed size: 9.21 ± 0.7 mm	Reference suspension: DI water (0 gL^{-1} Fe), 1 drop	6	Soil Seeds potted after 7 days of germination Plant placed indoor in sunlight beside the window	100
	Low-concentration suspension: hematite NP fertilizer (0.022 gL^{-1} Fe), 1 drop	6	Soil Seeds potted after 7 days of germination Plant placed indoor in sunlight beside the window	100
	High-concentration suspension: hematite NP fertilizer (1.1 gL^{-1} Fe), 1 drop	6	Soil Seeds potted after 7 days of germination Plant placed indoor in sunlight beside the window	100
Red bean Seed size: 16.82 ± 0.7 mm	Reference suspension: DI water (0 gL^{-1} Fe), 1 drop	6	Soil Seeds potted after 7 days of germination Plant placed indoor in sunlight beside the window	60
	Low-concentration suspension: hematite NP fertilizer (0.022 gL^{-1} Fe), 1 drop	6	Soil Seeds potted after 7 days of germination Plant placed indoor in sunlight beside the window	60
	High-concentration suspension: hematite NP fertilizer (1.1 gL^{-1} Fe), 1 drop	6	Soil Seeds potted after 7 days of germination Plant placed indoor in sunlight beside the window	60

monitored to assess any adverse effect of the NP fertilizer on the next generation of plants.

2.5. Evaluating Plant Uptake of Hematite NPs

2.5.1. Fourier Transform Infrared Spectroscopy. The surface functional groups of the shoot and leaf samples from legumes grown by seed presoaking with hematite NP drops (Figure S1c, SI) were analyzed using a Bruker Alpha Fourier transform infrared (FTIR) spectrometer equipped with attenuated total reflectance (ATR) capability to better understand the role of NP fertilizers in promoting plant growth. Samples for FTIR measurements were prepared by cutting a 2 mm piece of the shoot and leaf from the potted plants after 10 days of growth. FTIR measurements conducted over a range of $400\text{--}4000\text{ cm}^{-1}$ were reported as an average of three consecutive measurements for reliability.

2.5.2. Hyperspectral Imaging. The leaf samples from the legume plants as collected were further characterized via CytoViva hyperspectral imaging to investigate the accumulation of the NP fertilizer within the leaf. For hyperspectral imaging, the leaf samples were placed on a standard 1 mm glass slide and covered with a glass cover slip for viewing (Figure S3, SI). The sample images were further analyzed by generating a spectral library from the images and filtering the library against respective images of hematite NP and control leaf samples from plants grown without NP treatment to eliminate false positive signals. The remaining spectral library was then mapped to determine the location of NPs within the leaf samples.

2.5.3. ICP-OES Measurements. Acid digestion of the plant leaves for ICP-OES analysis was carried out following an established protocol [32]. Leaves were collected from all the four species of potted plants after 10 days of growth, except for the green gram plant without NP treatment because the plant did not survive that long. The leaves were air dried at 70°C in an oven for 24 hours. Acid digestion was carried out with 100 mg of dried leaves for all the species. 5 mL of HNO_3 was added to 100 mg of each dried sample (from a specific plant type without or with NP treatment) in a glass vial, and the mixture was left unaltered for 24 hours to allow the reaction to take place. The acidified sample was then heated at 120°C on a hot plate for 1 hour. Four additions of 2 mL H_2O_2 were made after every 15 minutes within a 1-hour period. The digested sample turned colorless at the end of 1 hour and confirmed the completion of the digestion process. The sample was then completely air dried in an oven at 80°C for 48 hours. The dried sample was cooled and dissolved in 3 mL of 10% HCl (v/v) for 2 hours. This solution was diluted five times with DI water and was used as the stock solution for the ICP-OES measurement.

An iCAP 6000 ICP-OES (Thermo Fisher Scientific) was used to determine the iron content in the plant leaves. A high-purity Argon (Ar) gas was employed as a plasma, auxiliary (0.5 L/min), and nebulizing gas. Before operating with the sample, the ICP-OES was purged with Ar gas for 1 hour. The power of the radio frequency (R.F.) was kept at 1150 W. The sample pump rate was fixed at 50 rpm with a stabiliza-

tion time of 5 seconds. For iron content determination, the measurements were observed at the most sensitive emission wavelength of 259.9 nm. A standard calibration curve (1–5 ppm) for the known iron concentration was prepared ($R^2 = 0.999$). The end solution from the digestion step was used in ICP-OES to measure the unknown iron concentration by comparing its spectra with that from the calibration curve.

3. Results and Discussion

Iron is an essential element for the generation of chlorophyll in plants. Iron is added in the form of soil fertilizers or chelated compounds in soil-less cultivation to facilitate iron uptake for enhanced production of agricultural and bioenergy crops. Agricultural research facilities and commercial units are investigating innovative formulations of Fe fertilizers such as the Fe micronutrient containing iron and phosphates [33–35]. The two major targets in designing these fertilizers are to significantly increase the plant growth or production and to minimize the addition of excess fertilizers to the soil for environmental sustainability. In this study, we designed a “modified seed presoak” strategy to apply the minimum possible quantity of Fe fertilizer for enhanced plant growth. In this method, the seed was placed on a wet paper towel inside a nearly covered petri dish for germination and one drop of the fertilizer suspension was added to the seed once. The germinated seeds were potted in soil after 7 days. Using this modified seed presoak method, we investigated the role of our new hematite NP fertilizer on the shoot growth of four different species of legumes (e.g., chickpea or *Cicer arietinum*, green gram or *Vigna radiata*, and black beans and red beans or *Phaseolus vulgaris*). Each species of legume seed was treated with a drop of hematite NP fertilizer suspension of three different concentrations: reference suspension (DI water, $0\text{ gL}^{-1}\text{ Fe}$), low concentration ($0.022\text{ gL}^{-1}\text{ Fe}$), and high concentration ($1.1\text{ gL}^{-1}\text{ Fe}$). Figure 2 shows a schematic representation of the improved seed presoak strategy used in this study to promote enhanced plant growth and production.

The shoot length of each potted legume plant was measured every day for a maximum period of 100 days to determine the efficacy and concentration-dependent effect of the synthesized hematite NP fertilizer on plant growth enhancement. The growth experiments were repeated six times with a new set of seeds for each legume species to predict statistically reliable growth trends. Figure 3 shows representative images of the different legume plants treated with different NP suspensions at the end of the growth study and also the corresponding time-dependent plant growth plots.

As seen from the images, all three chickpea plants showed healthy growth, but the growth height of plants treated with a drop of high-concentration hematite NP fertilizer was 230% higher than the control DI water plant. The chickpea plants treated with a drop of low-concentration hematite NP fertilizer also grew 206% higher than the control DI water plant. Growth heights were reported based on the final height of the plant achieved before the plant death. In addition, the NP fertilizer-treated chickpea plants showed a higher

FIGURE 2: Schematic of the modified seed presoak method with hematite NP fertilizer.

FIGURE 3: Effect of the hematite NP fertilizer delivered via a modified seed presoak method on the growth of different legumes. (a) Images of plants taken on day 30 of growth after transferring the seeds to potted soil and (b) plots showing comparative time-dependent growth of the plants treated with control DI water, a low concentration of NPs, and a high concentration of NPs. Error bars on the plots are reported based on 95% normalized distribution.

survival time than the control DI water plants by 15 days. The DI water chickpea plant held an increasing growth trend for 72% of its total life span. In comparison, the hematite NP-treated plants showed an increasing growth for 82–85% of their life span, suggesting a healthier growth and vitality as compared to the DI water plant. We observed a difference in morphology of the leaves for chickpea plants treated with DI water, low-concentration NP fertilizer, and high-concentration NP fertilizer, which may suggest a possible dominance of a more stable genetic structure for the NP-

treated plants (Figure S4, SI) [10, 36]. However, the leaf structures are currently under further detailed investigation and further proof through genetic and molecular level analyses is required, which is not within the scope of this work. The Fe-enriching hematite NP fertilizer significantly boosted the growth of green gram plants as the seeds treated with low and high concentrations of the fertilizer grew 830% and 700% more than the control DI water plant. The NP fertilizer-treated green gram plants were stronger with a 5-fold higher survival span compared to the control

FIGURE 4: Hyperspectral imaging of black bean leaf samples. (a) Image of control DI water-treated leaf. (b) Image of leaf from a plant treated with a high concentration of hematite NPs ($1.1 \text{ gL}^{-1} \text{ Fe}$); the red dots indicate the localization of NPs. (c) Comparative spectral plots of leaves from control and NP-treated plants.

plant. These green gram plants produced pods after 18 days, two times faster than other literature reports on regular plants [37]. Images of the healthy second-generation plants from the NP fertilizer-treated green gram seeds are included in the SI (Figure S5). In the case of black beans, the plants grown with a drop of high-concentration hematite NP fertilizer showed the highest growth. The Fe-enriching hematite NP fertilizer significantly boosted the growth rate of black bean plants, even though the beans are richer in Fe content compared to the other legume species tested in this study [38]. Black bean plants treated with high- and low-concentration NP fertilizers grew 588% and 453% higher than the control plants, respectively. Growth percentages were reported based on the last day of measurement for the black bean plants. They also produced more seed pods than the control plants, and healthy second-generation plants were observed for the NP-treated black bean plants (Figure S5, SI) [39]. However, the survival span of the black bean plants was not affected by the NP fertilizer. This could be due to the high iron content in the beans [38]. The Fe-enriching NP fertilizer treatment increased the growth of red bean plants by 425% and 350% for high and low fertilizer concentrations, respectively, as compared to the control plant. Fruit production per plant increased twofold with NP treatment for the red bean plants, and healthy second-generation plants were observed (Figure S5, SI). In brief, the key insights from our growth studies was that the modified seed presoak method with the hematite NP fertilizer increased the growth of legumes by 230–830%, increased survival time of most legume species, enhanced fruit production per plant except for chickpeas, facilitated faster fruit production, and produced healthy second-generation plants. The highest impact in growth with the NPs was observed for the green gram plants.

These results indicated the strong potential of hematite NPs to serve as a leading Fe-enriching fertilizer for enhanced agricultural production. However, understanding

the method of uptake and interaction of these NPs with the plants is important both for practical applicability and safety. It is difficult to determine NP interactions in complex biological media like plants via a single independent material characterization technique due to the low concentrations of NPs encountered in the plant tissues, interference from plant tissues, and the similarity of the NPs to naturally occurring NPs or metal ions. Electron microscopy is traditionally used to visualize NPs within the plants, but the sample preparation required for this method increases the probability of artifacts in the images. Therefore, we chose hyperspectral imaging (CytoViva), a minimally invasive and enhanced darkfield imaging technique requiring negligible sample preparation to investigate the uptake of hematite NPs in the legumes [29, 40]. The enhanced darkfield illumination technology in this method minimized light loss and enabled scatter from the sample to be detected without source illumination interference. Figures 4(a) and 4(b) show representative hyperspectral images of control and NP-treated black bean leaves used for visually mapping the localization of the hematite NP fertilizer. The spectral signature of the NP fertilizer was collected as a reference for comparison. A spectral library was created from the image of the NP-treated leaf and filtered against the control image to generate the comparative map (Figure 4(c)). The peak around 475 nm and the shoulder at 600–650 nm indicated the presence of hematite NPs in the leaves of legumes treated with the NP fertilizer. The localization of hematite NPs within the leaf is marked in red in Figure 4(b). The images and spectra from chickpea leaves and the raw hematite NP fertilizer are presented in the SI for further confirmation of our conclusion (Figures S6 and S7). It should be noted that though hyperspectral imaging has been reported earlier in detecting NPs within animal tissues, the use of this technique for plant samples is relatively new [29].

We used FTIR with ATR as a second noninvasive characterization technique requiring minimum sample preparation

FIGURE 5: FTIR plots of stem and leaf samples from red bean plants grown from seeds treated with DI water, a drop of a low concentration of NPs, and a drop of a high concentration of NPs. Samples were taken after 10 days of growth in soil.

to further understand the uptake and translocation of the NP fertilizer within the legumes [16, 21]. The chemical composition of the shoot and leaves of the legumes grown from seeds presoaked in DI water, low-concentration NP fertilizer, and high-concentration NP fertilizer were investigated using an FTIR spectrometer. Figure 5 shows the representative FTIR plots from red bean leaves after 10 days of plant growth. The FTIR spectrum of the hematite NP fertilizer is presented in the SI (Figure S1c). Typically, the shoot and leaf exhibit characteristic peaks between 3500 and 3000 cm^{-1} representative of O-H and N-H groups, 3000 and 2800 cm^{-1} due to CH_3 and CH_2 stretching, 1800 and 1200 cm^{-1} attributed to C=O stretch, 1738 cm^{-1} attributed to membrane lipids and cell wall, 1656 cm^{-1} due to amide I, 1563 cm^{-1} from amide II, 1513 cm^{-1} attributed to lignin, 1235 and 1153 cm^{-1} due to carbonyl stretch in esters and amide III, and 1100 and 1000 cm^{-1} in the fingerprint region owing to cellulose [21, 41]. All these regions were visible in the FTIR spectra of all our shoot and leaf samples. The shoots of red bean plants treated with the high-concentration hematite NP fertilizer showed additional bands at 2345 and 2365 cm^{-1} , similar to the C-N triple-bond peaks in the FTIR spectrum of hematite NPs shown in Figure S1c (SI). This data suggested the internalization of the NP fertilizer by the plants treated with high concentrations of the fertilizer. The peaks at 2345 and 2365 cm^{-1} were absent in the shoot samples of both the plants treated with a low NP concentration and those treated with DI water, indicating the absence of NPs in the stem of these plants after 10 days growth. However, the leaves of plants treated with both low and high concentrations of NPs showed these two additional peaks. This suggested the translocation of the NP fertilizer from the roots through the stem to the leaves. The NP fertilizer localized in the leaves within 10 days of growth in plants treated with a low concentration of NPs. After 10 days of growth, the hematite NPs were being

transported through the shoots with some accumulation within the leaves for the plants treated with a high concentration of NPs. FTIR plots for the other legume samples were included in the SI (Figure S8). Therefore, the coupled hyperspectral imaging and FTIR characterization method provided significant insights into the transport of the NP fertilizer through the legume plants. The difference in the rate of transport of the NPs within the plants could account for the enhanced growth rate in the NP-treated plants, based on our FTIR results.

The ICP-OES measurements confirmed the presence of iron in the plant leaves; however, it indicated the absence of any dose-dependent relationship between the iron content in plant leaves and hematite NP dosing (Figure 6). The results did not suggest any significant difference in the iron content among the three different dose conditions (control, low NP concentration, and high NP concentration) for each species of legumes. Previous studies involving iron NPs in the soil matrix after seed germination have reported a dose-dependent relationship between the iron NP dosage and iron content within different parts of the plant [10, 16, 42]. However, when applied as only seed treatment, Srivastava et al. found no significant difference in iron content in spinach plant leaves with a different iron pyrite (FeS_2) NP dosing [12, 43]. Our study also confirms the absence of a significant dose-dependent iron content relationship when applying NPs in a “modified seed presoak” strategy. Hematite NPs facilitating enzymatic activity during germination, through surface chemistry rather than uptake, can be attributed as one of the reasons. These surface-mediated processes can contribute to the overall growth of the plants. Additionally, plants dosed with higher hematite NP loading exhibited higher and faster growth resulting in more chlorophyll production utilizing more iron content. This can also offset the added iron input for plants with NP dosing compared to the controls.

FIGURE 6: Different iron content in four different legumes with different hematite NP loading.

4. Conclusion

In summary, we reported a new seed presoak method using just a drop of hematite NP fertilizer to significantly increase plant growth in legumes by 230–830%, depending on the species. Though the growth trend varied between different species of legumes, in general, the seeds treated with a high concentration of the NPs ($1.1 \text{ gL}^{-1} \text{ Fe}$) showed the fastest growth compared to those soaked in a low concentration of the NPs ($0.022 \text{ gL}^{-1} \text{ Fe}$) and control DI water. The NP-treated plants exhibited twofold faster initial fruit production, two times more fruits per plant, and a longer life span compared to the control plants, in general. They produced healthy second-generation plants.

We also reported a minimally invasive material characterization strategy coupling hyperspectral imaging and FTIR to determine the internalization and transport of the NP fertilizer within the plants, while minimizing artifacts from sample preparation. The hematite NPs were transported through the shoot and localized in the leaves of the legumes. The ICP-OES measurements showed the presence of iron in the plant leaves; however, no correlation was found between the iron content in the leaves and input hematite NP fertilizer concentrations. This indicates that seed presoaking with hematite NPs actually led to more effective iron utilization which resulted in improved growth and faster fruit productions in the tested plants.

A primary advantage of our strategy is that the NP fertilizer was added in minimum quantity to a moist paper towel as a presoak solution for the seed. No NPs or additional ingredients were added to the soil. Therefore, our Fe-enriching NP fertilizer could drastically increase the

production rate and life span of plants with minimum impact on the environment. The hematite NP fertilizer and the reported strategy will be highly beneficial in enhancing the production of bioenergy crops.

Data Availability

The .xls data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

The authors acknowledge that the research reported in this publication was supported by the University of Tennessee Chattanooga's internal grants (2018 Center of Excellence for Applied Computational Science competition and 2018 Student SEARCH grant). The authors also acknowledge Mr. Kaan Alp for help with plant growth experiments.

Supplementary Materials

The supplementary materials include the following: hydro-dynamic size plot; zeta potential plot and FTIR characterization of hematite NPs; images showing difference in chickpea leaves for NP-treated plants; images of second-generation green gram, black bean, and red bean plants; photo demonstrating hyperspectral imaging of leaf samples without any special sample preparation; hyperspectral images and map for hematite NPs; hyperspectral images and mapping for chickpea leaf samples; and FTIR characterization of shoot and leaves of legumes. (*Supplementary Materials*)

References

- [1] H. Roschztardt, G. Conéjéro, F. Divol et al., "New insights into Fe localization in plant tissues," *Frontiers in Plant Science*, vol. 4, p. 350, 2013.
- [2] J. Morrissey and M. L. Guerinot, "Iron uptake and transport in plants: the good, the bad, and the ionome," *Chemical Reviews*, vol. 109, no. 10, pp. 4553–4567, 2009.
- [3] C. A. Price and E. F. Carell, "Control by iron of chlorophyll formation and growth in *Euglena gracilis*," *Plant Physiology*, vol. 39, no. 5, pp. 862–868, 1964.
- [4] R. Raliya, V. Saharan, C. Dimkpa, and P. Biswas, "Nanofertilizer for precision and sustainable agriculture: current state and future perspectives," *Journal of Agricultural and Food Chemistry*, vol. 66, no. 26, pp. 6487–6503, 2018.
- [5] N. Kottegoda, C. Sandaruwan, G. Priyadarshana et al., "Urea-hydroxyapatite nanohybrids for slow release of nitrogen," *ACS Nano*, vol. 11, no. 2, pp. 1214–1221, 2017.
- [6] P. K. Rai, V. Kumar, S. Lee et al., "Nanoparticle-plant interaction: implications in energy, environment, and agriculture," *Environment International*, vol. 119, pp. 1–19, 2018.
- [7] M. Rui, C. Ma, Y. Hao et al., "Iron oxide nanoparticles as a potential iron fertilizer for peanut (*Arachis hypogaea*)," *Frontiers in Plant Science*, vol. 7, p. 815, 2016.

- [8] A. Karny, A. Zinger, A. Kajal, J. Shainsky-Roitman, and A. Schroeder, "Therapeutic nanoparticles penetrate leaves and deliver nutrients to agricultural crops," *Scientific Reports*, vol. 8, no. 1, article 7589, 2018.
- [9] D.-Y. Kim, A. Kadam, S. Shinde, R. G. Saratale, J. Patra, and G. Ghodake, "Recent developments in nanotechnology transforming the agricultural sector: a transition replete with opportunities," *Journal of the Science of Food and Agriculture*, vol. 98, no. 3, pp. 849–864, 2018.
- [10] J. Yuan, Y. Chen, H. Li et al., "New insights into the cellular responses to iron nanoparticles in *Capsicum annuum*," *Scientific Reports*, vol. 8, no. 1, article 3228, 2018.
- [11] D. Raju, S. R. Beedu, and U. J. Mehta, "Biogenic green synthesis of monodispersed gum kondagogu (*Cochlospermum gossypium*) iron nanocomposite material and its application in germination and growth of mung bean (*Vigna radiata*) as a plant model," *IET Nanobiotechnology*, vol. 10, no. 3, pp. 141–146, 2016.
- [12] G. Srivastava, C. K. Das, A. Das et al., "Seed treatment with iron pyrite (FeS_2) nanoparticles increases the production of spinach," *RSC Advances*, vol. 4, no. 102, pp. 58495–58504, 2014.
- [13] H. Jangir, C. K. Das, J. Kumar et al., "Nano pyrite (FeS_2) root priming enhances chilli and marigold production in nutrients-deficient soil: a nano strategy for fertiliser tuning," *Applied Nanoscience*, vol. 9, no. 3, pp. 327–340, 2019.
- [14] C. K. Das, H. Jangir, J. Kumar et al., "Nano-pyrite seed dressing: a sustainable design for NPK equivalent rice production," *Nanotechnology for Environmental Engineering*, vol. 3, no. 1, p. 14, 2018.
- [15] X. Li, Y. Yang, B. Gao, and M. Zhang, "Stimulation of peanut seedling development and growth by zero-valent iron nanoparticles at low concentrations," *PLoS One*, vol. 10, no. 4, article e0122884, 2015.
- [16] K. Jeyasubramanian, U. U. Gopalakrishnan Thoppey, G. S. Hikku, N. Selvakumar, A. Subramania, and K. Krishnamoorthy, "Enhancement in growth rate and productivity of spinach grown in hydroponics with iron oxide nanoparticles," *RSC Advances*, vol. 6, no. 19, pp. 15451–15459, 2016.
- [17] H. X. Ren, L. Liu, C. Liu et al., "Physiological investigation of magnetic iron oxide nanoparticles towards Chinese mung bean," *Journal of Biomedical Nanotechnology*, vol. 7, no. 5, pp. 677–684, 2011.
- [18] M. H. Ghafariyan, M. J. Malakouti, M. R. Dadpour, P. Stroeve, and M. Mahmoudi, "Effects of magnetite nanoparticles on soybean chlorophyll," *Environmental Science & Technology*, vol. 47, no. 18, pp. 10645–10652, 2013.
- [19] H. Zhu, J. Han, J. Q. Xiao, and Y. Jin, "Uptake, translocation, and accumulation of manufactured iron oxide nanoparticles by pumpkin plants," *Journal of Environmental Monitoring*, vol. 10, no. 6, pp. 713–717, 2008.
- [20] M. Rui, C. Ma, J. C. White et al., "Metal oxide nanoparticles alter peanut (*Arachis hypogaea* L.) physiological response and reduce nutritional quality: a life cycle study," *Environmental Science: Nano*, vol. 5, no. 9, pp. 2088–2102, 2018.
- [21] S. Palchoudhury, K. L. Jungjohann, L. Weerasena et al., "Enhanced legume root growth with pre-soaking in $\alpha\text{-Fe}_2\text{O}_3$ nanoparticle fertilizer," *RSC Advances*, vol. 8, no. 43, pp. 24075–24083, 2018.
- [22] L. Grillet, S. Mari, and W. Schmidt, "Iron in seeds—loading pathways and subcellular localization," *Frontiers in Plant Science*, vol. 4, p. 535, 2014.
- [23] J. P. Stegemeier, B. P. Colman, F. Schwab, M. R. Wiesner, and G. V. Lowry, "Uptake and distribution of silver in the aquatic plant *Landoltia punctata* (duckweed) exposed to silver and silver sulfide nanoparticles," *Environmental Science & Technology*, vol. 51, no. 9, pp. 4936–4943, 2017.
- [24] X. Gui, Y. Deng, Y. Rui et al., "Response difference of transgenic and conventional rice (*Oryza sativa*) to nanoparticles ($\gamma\text{Fe}_2\text{O}_3$)," *Environmental Science and Pollution Research*, vol. 22, no. 22, pp. 17716–17723, 2015.
- [25] J. Lv, P. Christie, and S. Zhang, "Uptake, translocation, and transformation of metal-based nanoparticles in plants: recent advances and methodological challenges," *Environmental Science: Nano*, vol. 6, no. 1, pp. 41–59, 2019.
- [26] A. Milewska-Hendel, M. Zubko, J. Karcz, D. Stroz, and E. Kurczynska, "Fate of neutral-charged gold nanoparticles in the roots of the *Hordeum vulgare* L. cultivar Karat," *Scientific Reports*, vol. 7, no. 1, article 3014, 2017.
- [27] L. van Nhan, C. Ma, Y. Rui et al., "The effects of Fe_2O_3 nanoparticles on physiology and insecticide activity in non-transgenic and Bt-transgenic cotton," *Frontiers in Plant Science*, vol. 6, article 1263, 2016.
- [28] M. Mortimer, A. Gogos, N. Bartolomé, A. Kahru, T. D. Bucheli, and V. I. Slaveykova, "Potential of hyperspectral imaging microscopy for semi-quantitative analysis of nanoparticle uptake by protozoa," *Environmental Science & Technology*, vol. 48, no. 15, pp. 8760–8767, 2014.
- [29] A. Avellan, F. Schwab, A. Masion et al., "Nanoparticle uptake in plants: gold nanomaterial localized in roots of *Arabidopsis thaliana* by X-ray computed nanotomography and hyperspectral imaging," *Environmental Science & Technology*, vol. 51, no. 15, pp. 8682–8691, 2017.
- [30] R. Sekine, K. L. Moore, M. Matzke et al., "Complementary imaging of silver nanoparticle interactions with green algae: dark-field microscopy, electron microscopy, and nanoscale secondary ion mass spectrometry," *ACS Nano*, vol. 11, no. 11, pp. 10894–10902, 2017.
- [31] S. Palchoudhury and J. R. Lead, "A facile and cost-effective method for separation of oil-water mixtures using polymer-coated iron oxide nanoparticles," *Environmental Science & Technology*, vol. 48, no. 24, article 14558, 14563 pages, 2014.
- [32] A. Pequerul, C. Perez, P. Madero, J. Val, and E. Monge, "A rapid wet digestion method for plant analysis," in *Optimization of Plant Nutrition*, Springer, 1993.
- [33] F. Yunta, M. di Foggia, V. Bellido-Díaz et al., "Blood meal-based compound. Good choice as iron fertilizer for organic farming," *Journal of Agricultural and Food Chemistry*, vol. 61, no. 17, pp. 3995–4003, 2013.
- [34] M. D. Frost, J. C. Cole, and J. M. Dole, "Fertilizer source affects iron, manganese, and zinc leaching, nutrient distribution, and geranium growth," *Journal of Plant Nutrition*, vol. 26, no. 2, pp. 315–329, 2003.
- [35] G. A. Achari and M. Kowshik, "Recent developments on nanotechnology in agriculture: plant mineral nutrition, health, and interactions with soil microflora," *Journal of Agricultural and Food Chemistry*, vol. 66, no. 33, pp. 8647–8661, 2018.
- [36] R. P. S. Pundir, M. H. Mengesha, and K. N. Reddy, "Leaf types and their genetics in chickpea (*Cicer arietinum* L.)," *Euphytica*, vol. 45, no. 3, pp. 197–200, 1990.
- [37] J. Iqbal, M. Ahsan, M. Saleem, and A. Ali, "Appraisal of gene action for indeterminate growth in mungbean (*Vigna radiata* (L.) wilczek)," *Frontiers in Plant Science*, vol. 6, p. 665, 2015.

- [38] N. Petry, E. Boy, J. Wirth, and R. Hurrell, "Review: the potential of the common bean (*Phaseolus vulgaris*) as a vehicle for iron biofortification," *Nutrients*, vol. 7, no. 2, pp. 1144–1173, 2015.
- [39] C. Miles, K. Atterberry, and B. Brouwer, "Performance of Northwest Washington heirloom dry bean varieties in organic production," *Agronomy*, vol. 5, no. 4, pp. 491–505, 2015.
- [40] A. Moghimi, C. Yang, M. E. Miller, S. F. Kianian, and P. M. Marchetto, "A novel approach to assess salt stress tolerance in wheat using hyperspectral imaging," *Frontiers in Plant Science*, vol. 9, article 1182, 2018.
- [41] A. Ertani, O. Francioso, E. Ferrari, M. Schiavon, and S. Nardi, "Spectroscopic-chemical fingerprint and biostimulant activity of a protein-based product in solid form," *Molecules*, vol. 23, no. 5, p. 1031, 2018.
- [42] M. Mohammadi, N. Majnoun Hoseini, M. R. Chaichi, H. Alipour, M. Dashtaki, and S. Safikhani, "Influence of nano-iron oxide and zinc sulfate on physiological characteristics of peppermint," *Communications in Soil Science and Plant Analysis*, vol. 49, no. 18, pp. 2315–2326, 2018.
- [43] G. Srivastava, A. Das, T. S. Kusurkar et al., "Iron pyrite, a potential photovoltaic material, increases plant biomass upon seed pretreatment," *Materials Express*, vol. 4, no. 1, pp. 23–31, 2014.