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Fe_3O_4 nanoparticles and *Rhizobium* inoculation enhance nodulation, nitrogen fixation and growth of common bean plants grown in soil

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ABSTRACT

The effects of Fe_3O_4 nanoparticles (NPs) and *Rhizobium* inoculation on nodulation, nitrogen fixation and plant growth of common bean (cv. Red Guama, *Phaseolus vulgaris*) plants were investigated in growth chambers. Plants were exposed to: Fe_3O_4 NPs (2000 mg/L) (T1), *Rhizobium* inoculation (T2) and Fe_3O_4 NPs + *Rhizobium* inoculation (T3); non-treated plants were considered as controls. Harvested 35-day-old treated plants showed improved symbiotic performance including increased nitrogenase activity (51.2–90.7%), nodule leghaemoglobin (44.8–80.9%) and iron content (83.4–84.2%), number of active nodules per plant (58.7–122%) and nodule dry weight (40.2–70.6%). This resulted in enhanced symbiotic nitrogen fixation,and increased shoot (26.5–50.2%) and root (24.1–48.2%) total nitrogen content in treated plants in comparison with the controls. The best result was obtained using treatment T3. Furthermore, Fe_3O_4 NPs were taken up by bean plants in treatments T1 and T3, and these accumulated in their organs, including in nodules. All treatments led to an increase in root (51.9–79.8%) and shoot (27.5–52.7%) lengths, in leaf area (10.9–16.8%) and in root (10.1–17.8%), stem (9.8–12.7%) and leaf dry weight (8–17.3%) compared to control plants. Thus applied treatments have the potential to improve common bean plant growth through enhancement of nodulation and nitrogen fixation during vegetative growth.This study also provides strong evidence that the presence Fe_3O_4 NPs in nodules improves the symbiotic performance between *Rhizobium* (*leguminosarum* CF1 strain) and the common bean plant, due to enhanced nodulation and nitrogen fixation.

1. Introduction

Nanoparticles are defined as materials with three external nanoscale dimensions in the range of 1–100 nm (Jeevanandam, 2018). They have attracted interest due to their unique properties (Gilroy et al., 2016; Liu and Di Valentin, 2019; Huang et al., 2020), including quantum confinement, a large surface area to volume ratio, high surface energy, and several other catalytic and magnetic properties (Handy et al., 2008; Vallabani et al., 2019). In particular, the effects of engineering nanoparticles (ENPs) on plants are of great interest because of their importance in ecological systems. Plants provide a potential pathway for ENP transportation into the environment and serve as a significant route for their bioaccumulation in the food chain.

There are several reports on the effects of Fe_3O_4 NPs on germination and plant growth. For example, there is an increase in chlorophyll in soybean seedlings treated with 9 nm Fe_3O_4 NPs applied in a

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concentration based on the quantity of iron needed for plant growth; no trace of toxicity has been observed despite the translocation of NPs into soybean stems (Ghafariyan et al., 2013). There are also significant positive effects of Fe_3O_4 NPs on plant growth characteristics of wheat and rocket (*Eruca sativa*)(Iannone et al., 2016; Plaksenkova et al., 2019). Fe_3O_4 NPs significantly increases plant root length (9%–32%), chlorophyll *a* fluorescence (1.94–2.8-fold) and miRNA expression (0.31–0.42-fold) compared to those of the control in yellow medick (*Medicagofalcata* L.) plants (Kokina et al., 2020). Addition of Fe_3O_4 NPs (2000 mg/L) in each heavy metal (Pb, Zn, Cd and Cu) solution (1 mM) significantly decreases the growth inhibition (193.91%, 37.56%, 97.72%, 31.89% in root and 65.75%, 25.06% 87.35%, 60.96% in shoot) and activates protective mechanisms to reduce oxidative stress induced by heavy metals in the wheat seedlings (Konate et al., 2017). A progressive and systematic increase in the magnetization signal occurs in leaves, stems, and root samples of common bean plants grown in soil irrigated with increasing concentrations of Fe_3O_4 NP suspensions. This indicates that they can be taken up by the roots, translocated to the aerial regions, and accumulated in different plant organs (Govea-Alcaide et al., 2016). Also, Fe_3O_4 NPs have remarkable positive effects on the chemical properties of the soil rhizosphere with increases in P (18–22%), K, (25–43%), Ca (35–43%), Mn (53–115%) and Fe (207–493%) and on accumulation of nutrients with increases in P (11–14%), K(21–32%), Ca (17–22%), Mn (10–17%) and Fe (192–277%) in common bean plants grown in this medium (De Souza et al., 2019). Thus their use opens up a wide range of possibilities in plant research and agronomy (Abd-Elsalam et al., 2019).

Very little information is available on the role of NPs in inducing nodulation and the biological fixation nitrogen in legumes, which depends on the effective formation of nodules by *Rhizobium*. Inoculation of legume seeds with the bacterium can assure its present in the root environment in adequate quantities to colonize the legume rhizosphere, thereby improving nitrogen fixation upon nodule formation (Schwember et al., 2019; Mahmud et al., 2020). However, the efficiency of *Rhizobium* inoculation depends on the host genotype, *Rhizobium* strain inefficiency, soil conditions and climatic factors (Thilakarathna and Raizada, 2017; Irisarri et al., 2019; Han et al., 2020; Yuan et al., 2020). Inoculation with Fe_3O_4 NPs-induced *Rhizobium* (MK358859 strain) enhances nodulation, leghaemoglobin content (110.4%), nitrogenase activity (3.7%), and growth of chickpea plants (22.3–32.7%) at salinities of 75 and 150 mM NaCl (Abd-Allaa et al., 2019), and multi-walled carbon nanotubes (3000 mg kg^{-1}) slightly increase nitrogen fixation (8%) in red clover plants (Moll et al., 2016). However, other studies have shown adverse effects of NPs on legume-rhizobia symbiosis. For instance, CeO_2 NPs diminish nitrogen fixation in soybeans (Coman et al., 2019), but there is no effect of TiO_2 and Fe_3O_4 NPs on nodule colonization (Burke et al., 2015). Delayed nitrogen fixation occurs in peas exposed to TiO_2 and ZnO NPs in hydroponic systems (Fan et al., 2014; Huang et al., 2014; Sarabia-Castillo and Fernández-Luqueño, 2016). Nodulation and nitrogenase activity in faba beans are delayed by Ag NPs (Abd-Alla et al., 2016), whereas arbuscular mycorrhizal colonization of white clover roots is increased by Ag and FeO NPs (Feng et al., 2013). The number of nodules is decreased by Ag and ZnO NPs in alfalfa plants (Moghaddam et al., 2017). Therefore, the effect of NPs on legume-rhizobia appears to be species-and NPs-dependent. Here, we assess whether *Rhizobium* inoculation, symbiotic performance, nodulation and nitrogen fixation in common bean plants grown in soil are affected by Fe_3O_4 NPs.

The common bean (*Phaseolus vulgaris* L.) is considered to be the most important grain legume (Jiang et al., 2020), but it is generally regarded as a rather inefficient fixer of nitrogen in comparison to other grain legumes (Argaw and Akuma, 2015; Wilker et al., 2019; Allito et al., 2020; Reinprecht et al., 2020).This is perhaps partly due to the absence of appropriate rhizobial strains, host cultivars, environmental variables (Chekanai et al., 2018; Reinprecht et al., 2020) and reduction in effective nodulation by competition from high populations of competitive but ineffective native *Rhizobia* spp. (Argaw and Akuma, 2015).Therefore, improvement of bean nitrogen fixation requires a multidisciplinary approach to increase the host capacity to fix nitrogen (Argaw and Akuma, 2015; diCenzo et al., 2018) and a selection of effective *Rhizobium* strains that can accomplish productive nodulation in the presence of native populations of bacteria present in most soils.

The aim of this study is to determine for the first time the effects of magnetite nanoparticles (Fe_3O_4 NPs) and *Rhizobium* (*leguminosarum* CF1 strain) inoculation treatments on nodulation, nitrogen fixation, iron content and vegetative plant growth of common bean plants in soil under growth chamber conditions, and evaluate whether their synergistic interaction can improve nodule activity, nitrogen fixation and the productivity of this important legume.

2. Materials and methods

2.1. Plant material

Genetically-uniform certified bean seeds (*Phaseolus vulgaris* L. cv. Red Guama) were provided by the Seed Laboratory of the Ministry of Agriculture in Granma Province, Cuba. Seeds without visible defects, insect damage or malformation were selected and stored in desiccators over 70% (v/v) glycerin. Seed moisture content was 10–12% on a fresh weight basis before the treatments, and final germination percentage was 90%.

2.2. Growth conditions and applied treatments

The seeds were sown in a soil medium placed in open black polyethylene bags of 29 cm high x 19 cm width with a capacity of about 483.6 cm^3, with four replications each, identified as N0, N1, N2 and N3. The number of replicates per treatment is stated in the Tables. Sterilized rhizosphere soil, placed in bags was a brown carbonate (USDA, 2003) with a loamy texture and pH 7.0. Its organic matter content was 3.2%, assimilable phosphorus 0.102 g kg^{-1} and potassium 0.815 g kg^{-1}. Cation exchange capacity (CEC) was 42.2 meq 100 g^{-1}; base exchange capacity 31.8 meq100 g^{-1}; Ca^{2+} 1.71 g kg^{-1}; K^+ 1.75 g kg^{-1}; Mg^{2+} 0.530 g kg^{-1} and the soil was low in total N (0.75 g kg^{-1}). According to soil analyses, plant nutrient content was adequate for the growth of common bean (Havlin et al., 2005). Plants were grown in a Conviron growth chamber under controlled conditions: 14 h light (4700 lux) and 10 h dark, at 25 ± 2 °C during the day and 20 ± 2 °C at night, and 60% relative humidity.

To apply the treatments, firstly 2000 mg of Fe_3O_4 NPs were suspended in 1 L water as reported in our previous paper (Govea-Alcaide et al., 2016). Then the treatments were arranged as follows: T1; 20 mL water and 20 mL water suspended Fe_3O_4 NPs at a concentration of 2000 mgL^{-1};T2; *Rhizobium* inoculation and with 40 mL of water; T3; 20 mL water and 20 mL water suspended Fe_3O_4 NPs and *Rhizobium* inoculation (Fe_3O_4 NPs (2000 mgL^{-1}) + *Rhizobium*), respectively. Bags N1 were irrigated daily in the morning with T1, bags N2 were irrigated with T2 and bags N3 were irrigated with T3. The seeds in the bag N0 were grown in soil free of Fe_3O_4 NPs (control) and irrigated every day with 40 mL of pure water.The amount of added water over the 35 days was 1400 mL per treatment for a total of 5600 mL. The daily dose of Fe was of 40 mg Fe_3O_4 NPs per treatment (T1 and T3) so that the total dose of Fe was of 1400 mg.The Fe_3O_4 NPs, with a log-normal distribution of median diameter $\overline{d}$ ~10 nm and standard distribution of $\sigma_d = 0.36$ nm, were prepared by the co-precipitation method (Rossi et al., 2007).

2.3. Nodulation

To study the effect of Fe_3O_4 NPs (T1), *Rhizobium* inoculation (T2) and Fe_3O_4 NPs + *Rhizobium* inoculation (T3) on symbiotic root nodule formation, one seed per bag (10 bags per treatment) was inoculated with

Rhizobium leguminosarum CF1 strain (1 mL of bacterial suspension and 108 bacteria mL^{-1} in mannitol (LM) yeast culture medium, (Vincent, 1970)) at sowing time and 7 days after sowing to induce root nodule formation. Nodules were collected at 28 days post-infection and the nodule number per plant was determined by counting all nodules on each of the plants and computing the average. Number of active nodules per plant was determined by cutting nodules on each of them and observing their internal color. Active nodules were identified as being pink to reddish. Samples were then oven-dried at 80 °C for 3 days to a constant weight, and nodule dry weight per plant determined.

2.4. Determination of leghaemoglobin in nodules

Nodules (500 mg) were homogenized in aliquots of Drabkin's reagent (10 ml) and leghaemoglobin was quantified spectrophotometrically at A540 as described by Wilson and Reisenauer (1963). Bovin haemoglobin was used as a standard, and values are expressed as mgg^{-1} nodule mass (fresh weight). Drabkin's solution was obtained by dissolving 52 mg potassium cyanide (KCN), 198 mg of potassium ferricyanide ($K_3Fe(CN)_6$) and 1 mg of sodium bicarbonate ($NaHCO_3$) in 1 L distilled water (Wilson and Reisenauer, 1963).

2.5. Nitrogenase activity

Nitrogenase activity was determined by the acetylene reduction assay (Hardy et al., 1968) in detached nodulated roots from 10 plants from each treatment as an indirect measurement of the biological nitrogen fixation. The root system of each plant was cleaned and placed inside a 1.1 dm^3 plastic jar. Immediately, 10% of the gaseous phase of each jar was replaced with pure C_2H_2 at room temperature for 2h. After incubation, 9 cm^3 of gas from each jar was transferred to non-additive vacutainer tubes (BD Vacutainer). The amount of ethylene was determined with a Varian Star 3400 CX gas chromatograph (CA, USA) equipped with a Chrompack Q type paraplot and a flame ionization detector. For each analysis, a 0.5 mm^3 aliquot was injected into the chromatograph using a Teflon sealed glass syringe (Hamilton, USA). Specific activity is expressed as μmol C_2H_4 reduced h^{-1} $plant^{-1}$. Afterward, nodules of each individual root were counted and nodule fresh and dry mass (at 70 °C for 48h) were measured. Total nitrogen content was determined, according to the Kjeldahl method, at the beginning and the end of the treatments. Nitrogen fixed was then calculated as the N content at harvest minus the N content of the plants at the onset of the treatment (Sassi et al., 2008).

2.6. Evaluation of the effect of treatments on the growth of common bean plants

Plants were allowed to grow for 35 days after sowing until there was established vegetative growth. Then they were removed from the bags and rinsed with deionized water to remove excess soil. Finally, shoot length, root length (from the root neck to the tip), leaf area per plant (ADC Bio Scientific Area Meter AM350, UK), root, stem and leaf dry weight (ventilated oven at 80 °C for 72 h until constant weight) were determined. Later, rhizosphere soil, root and shoot total nitrogen concentration of each sample was measured by the Kjeldahl method using concentrated H_2SO_4, K_2SO_4 and $CuSO_4$ to digest the sample (Bremner, 1996). The amount of fixed nitrogen was determined using a nitrogen difference technique by calculating the difference in uptake of nitrogen of the nitrogen-fixing-treated plants and control plants as the reference (Unkovich et al., 2008). The fundamental parameters to ensure the acceptability of the performance of the Kjeldahl method validation, accuracy (0.1%), precision (0.24 %N), linearity of the calibration function and working concentration range (determination coefficients, $r^2 \leq 0.98$), method detection limits (0.06 %N for soil samples and 0.03 % N for plant samples), limit of quantification (0.8 %N for soil samples and 0.5 %N for plant samples) were all within acceptable limits.

2.7. Iron determination

Samples of soil rhizosphere, nodule, root and shoots were dried at 70 °C for 72 h and ground to a fine powder using a grinder with agate bags. An analysis for Fe of each soil and dried plant sample was performed in an inductively coupled plasma optical emission spectrometer (ICP-OES) Spectro Arcos(Spectro) after calibrating the instrument using calibration blank and five working calibration standard solutions of iron to analyze. Reference solutions with a high degree of analytical purity were used to obtain the calibration curves.Deionized water (Milli-Q) was used to prepare all solutions. All samples were subjected to microwave-assisted digestion in a microwave oven (Speed Wave Four, Berghof Analytik) in a mixture of HNO_3 (2 mL), HF (2 mL) and H_2O_2(1 mL). The samples were digested at 200 °C for 15 min,170 °C for 10 min and then 160 °C for 5 min. The volume of the samples was then adjusted to 25 mL using deionized water before analysis.The underlying parameters to ensure the acceptability of the performance of the ICP-OES method validation, accuracy (0.1%), precision (1.7% relative standard deviation), linearity of the calibration function and working concentration range (determination coefficients, $r^2 \leq 0.99$), method detection limits (0.08% $mgkg^{-1}$ for soil samples and 0.01% $mgKg^{-1}$ for plant samples), limit of quantification (0.8% $mgkg^{-1}$ for soil samples and 0.5% $mgkg^{-1}$ for plant samples) and instrument stability were all within acceptable limits.

2.8. Magnetization curves

The magnetization curves, $M(H)$, for all the nodule samples were measured using a commercial superconducting quantum interference device (SQUID) magnetometer (MPMS, Quantum Design, San Diego, CA) in powder samples in the range -70 kOe $\leq H \leq +70$ kOe at 10 K and 300 K, after cooling the sample in zero field. SQUID sensitivity is of is $< 1 \times 10^{-8}$ emu ($\leq$2500 Oe) and $<1 \times 10^{-8}$ emu (>2500 Oe) and this characterization has been amply used for determining magnetic features of several biological systems, including plants organs (Govea-Alcaide et al., 2016).

2.9. Statistical analyses

Data on nodule number, number of active nodules, nodule dry weight, nitrogenase activity, leghaemoglobin content, total Fe content in soils, nodules, roots and shoots, rhizosphere soil, root and shoot total nitrogen concentration, amount of fixed nitrogen in plants and plant growth parameters were statistically analyzed by two-way ANOVA ($p < 0.05$) to determine the effects of applied treatments compared to the control. Means were compared using the Newman–Keuls test (Steel et al., 1997).The Kolmogorov–Smirnov procedure was used to test data normality; the Bartlett's test was used to test homogeneity of variances among treatments (Yandell, 1997). All of the statistical analyses were performed using the "Statistica for Windows'' software package, version 10 (StatSoft, Tulsa,OK).

3. Results

3.1. Nodulation, nodule activity and nitrogen fixation

The applied treatments induced a significant increase ($P < 0.05$) in nodule number per plant (50% for T1 and T2 and 100% for T3), number of active nodules per plant (58.7% for T1, 58.7% for T2 and 122% for T3) and nodule dry weight (40.2% for T1, 34.8% for T2 and 70.6% for T3) compared to in control plants (Table 1).The recorded values for T3 of mean nodule number (16), number of active nodules (14) and nodule dry weight (34.3 mg $plant^{-1}$) were markedly higher than in plants given treatments T1, T2 and the control.

The plants treated with T1, T2 and T3 improved significantly their biological nitrogen fixation as shown by nitrogenase activity (acetylene

Table 1
Effect of Fe_3O_4 NPs (T1), *Rhizobium* inoculation (T2) and Fe_3O_4 NPs + *Rhizobium* inoculation (T3) on nodulation of common bean plants grown in soil in growth chambers.

Parameters	T1	T2	T3	Control	ASE (±)	CV (%)
Nodule number per plant	12.0^b	12.0^b	16.0^a	8.0^c	0.8	8.5
Number of active nodules per plant	10.0^b	10.0^b	14.0^a	6.3^c	0.8	8.2
Total dry weight of nodules per plant (mg)	28.6^b	27.5^b	34.3^a	20.4^c	0.6	10.2

In rows, means followed by the same letter did not show significant differences ($P < 0.05$) according to the Newman–Keuls test. ASE: average standard error of mean; CV: coefficient of variation. An average of 30 plants per treatment was used.

reduction assay) compared to the controls ($P < 0.05$)(Table 2). T1 plants exhibited an increase of 51.2% in nitrogenase activity, T2 plants an increase of 58.1% while T3 plants increased by 90.7%. Hence, the plants treated with T3 showed the highest nitrogenase activity, significantly more than in T1, T2 and the control.

The leghaemoglobin contents of nodules were significantly increased by the treatments, with increases of 44.8% with T1, of 43.3% with T2 and of 80.9% with T3 compared to controls (Table 2). Among the treatments, T3 was the most positive for the bean plants.

Table 3 shows an appreciably greater shoot and root total nitrogen content (26.5% and 24.1% for T1, 25.2% and 18.7% for T2 and 50.2% and 48.2% for T3), and more shoot-nitrogen in treated plants (8.1 mg plant^{-1} for T1, 7.4 mg plant^{-1} for T2 and 15.1 mg plant^{-1} for T3) when compared to control plants. T3 generated the highest shoot and root nitrogen content and amount of shoot nitrogen (50.2%, 48.2% and 15.1 mg plant^{-1}), followed by T1 (26.5%, 24.1% and 8.1 mg plant^{-1}) and T2 (25.2%, 18.7% and 7.4 mg plant^{-1}), respectively, with marked differences between treatments. However, the nitrogen content of surrounding rhizosphere soil was unaffected by the treatments (Table 3).

3.2. Effects of applied treatments on vegetative growth of common bean plants

The plants treated with T1, T2 and T3 showed significantly higher shoot and root lengths compared to the controls ($P < 0.05$)(Table 4). T1 plants exhibited an increase of 27.5% in shoot length, T2 plants an increase of 23.6% while T3 plants an increase of 52.7%; similarly, T1 plants showed a 51.9% increase in root length, T2 plants a 45.2% increase while T3 plants a 79.8% increase. Hence, the plants treated with T3 showed the greatest shoot and root growth, which was significantly larger than after T1, T2, and the control.

Table 4 indicates that leaf area per plant was also enlarged ($P < 0.05$) by the treatments, with increases of 10.9% with T1, 9.6% with T2 and 16.8% with T3 compared to controls. T3 was the most beneficial.

Significantly higher root dry weights were found for all the treated plants, which had an increase of 10.1% with T1, 8.3% with T2 and 17.8% with T3 compared to controls. Correspondingly, stem dry weights were noticeably influenced by T1, T2 and T3, with increases of 9.8%, 9.5% and 12.7%, respectively (Table 4). Likewise, leaf dry weights were notably increased by 8% with T1, 7.3% with T2 and of 17.3 with T3 (Table 4). T3 resulted in the greatest root, stem and leaf dry weights.

Table 2
Effect of Fe_3O_4 NPs (T1), *Rhizobium* inoculation (T2) and Fe_3O_4 NPs + *Rhizobium* inoculation (T3) on nitrogenase activity and leghaemoglobin content of common bean plants grown in soil in growth chambers.

Parameters	T1	T2	T3	Control	CV (%)
Nitrogenase activity (μmol C_2H_4 h^{-1} plant^{-1})	6.5 ± 0.07^b	6.8 ± 0.04^b	8.2 ± 0.05^a	4.30 ± 0.03^c	6.7
Leghaemoglobin content (mg g^{-1} FW nodules)	3.04 ± 0.05^b	3.01 ± 0.01^b	3.80 ± 0.08^a	2.10 ± 0.04^c	5.2

In rows, means followed by the same letter did not show significant differences ($P < 0.05$) according to the Newman–Keuls test. CV: coefficient of variation. An average of 30 plants per treatment was used.

Table 3
Effect of Fe_3O_4 NPs (T1), *Rhizobium* inoculation (T2) and Fe_3O_4 NPs + *Rhizobium* inoculation (T3) on shoot, root and rhizosphere soil total nitrogen content and shoot –nitrogen of common bean plants grown in soil in growth chambers.

Parameters	T1	T2	T3	Control	ASE (±)	CV (%)
Shoot total N content (mg plant^{-1})	38.2^b	37.8^b	45.4^a	30.2^c	0.8	9.4
Shoot - N content (mg plant^{-1})	8.1^b	7.4b	15.1^a	–	0.4	10.8
Root total N content (mg plant^{-1})	11.3^b	10.8^b	13.5^a	9.1^c	0.6	10.2
Rhizosphere soil total N content (g kg^{-1})	0.77^a	0.76^a	0.79^a	0.75^a	0.08	2.8

In rows, means followed by the same letter did not show significant differences ($P < 0.05$) according to the Newman–Keuls test. ASE: average standard error of mean; CV: coefficient of variation. An average of 30 plants per treatment was used.

Table 4
Effect of Fe_3O_4 NPs (T1), *Rhizobium* inoculation (T2)and Fe_3O_4 NPs + *Rhizobium* inoculation (T3) on growth of common bean plants grown in soil in growth chambers.

Growth parameters	T1	T2	T3	Control	ASE (±)	CV (%)
Shoot length (cm)	23.2^b	22.5^b	27.8^a	18.2^c	0.4	8.3
Root length (cm)	15.8^b	15.1^b	18.7^a	10.4^b	0.6	9.7
Leaf area per plant (cm^2)	233.1^b	230.3^b	245.6^a	210.2^c	2.7	12.4
Root dry weight (mg)	112.3^b	110.5^b	120.2^a	102.0^c	0.8	10.5
Stem dry weight (mg)	224.6^b	223.8^b	230.3^a	204.2^c	1.0	12.1
Leaf dry weight (mg)	265.7^b	264.0^b	288.6^a	246.0^c	1.2	14.3

In rows, means followed by the same letter did not show significant differences ($P<0.05$) according to the Newman–Keuls test. ASE: average standard error of mean; CV: coefficient of variation. An average of 30 plants per treatment was used.

3.3. Iron contents

With the Fe_3O_4 NP and Fe_3O_4 NP and *Rhizobium* treatments, the treated soils showed a noticeable increase in the concentrations of total Fe compared with the control soils. The concentration increased equally

Table 5
Effect of Fe_3O_4 NPs (T1), *Rhizobium* inoculation (T2) and Fe_3O_4 NPs + *Rhizobium* inoculation (T3) on iron content of soil and different parts of common bean plants grown in soil in growth chambers.

Iron content (mg kg^{-1})	T1	T2	T3	Control	CV (%)
Soil	330.4 ± 6.5^a	105.2 ± 5.7^b	330.2 ± 5.3^a	104.6 ± 5.1^b	8.6
Nodules	450.0 ± 4.3^a	264.1 ± 5.4^b	452.2 ± 3.7^a	245.4 ± 4.8^c	8.2
Roots	382.6 ± 5.2^a	250.5 ± 4.6^b	381.4 ± 5.4^a	246.2 ± 4.1^b	8.5
Shoots	135.0 ± 4.6^a	94.3 ± 4.2^b	134.5 ± 4.7^a	90.8 ± 3.7^b	7.8

In rows, means followed by the same letter did not show significant differences ($P<0.05$) according to the Newman–Keuls test. CV: coefficient of variation. An average of 30 plants per treatment was used.

by 215.8% in T1 and by 215.6% in T3 with respect to the control (Table 5).

The nodules showed a considerable increase in concentrations of total Fe content of 84.2% in T1, 7.6% in T2 and 83.4% in T3 with respect to the control plants (Table 5). The highest concentration of total Fe was found in T3 (462.0 ± 3.7 mg kg^{-1}), which does not differ significantly from T1 (450.0 ± 4.3 mg kg^{-1}). Also, the roots registered a significant increase of 58.6% in T1 and 58.1% in T3 in total Fe content with respect to the control plants (Table 5). Differences in total Fe concentration occurred in shoots between the Fe_3O_4 NP and Fe_3O_4 NP and *Rhizobium* treatments, it was improved similarly by 48.6% and 48.1% in T1 and T3 with respect to the untreated plants, respectively (Table 5). Nodules had higher Fe concentrations than roots and shoots.

3.4. Magnetization measurements

The effect of Fe_3O_4 NPs on the bean plants was also assessed by magnetization measurements. As the focus of this study is on nodulation, only the magnetic effects of Fe_3O_4 NPs on nodules are presented here. However, it is important to mention that a detailed study of the magnetic response of isolated NPs used in the present experiment was reported in detail in a previous work (Govea-Alcaide et al., 2016).

The Fe_3O_4 NPs exhibited a superparamagnetic (SPM) behavior, with a blocking temperature (T_B) of ~130 K, which was analyzed previously (Govea-Alcaide et al., 2016). Below T_B, the magnetic moment (μ) of each nanoparticle are in the blocked state and the $M(H)$ curves showed a large hysteresis compared to their bulk counterpart, although the magnetic properties of nanoparticles depend on several chemical and morphological characteristics (Leslie-Pelecky and Rieke, 1996). However, above T_B, the magnetic moments are in the SPM state, generating a reversible magnetization curve with no coercive field or remanent magnetization, even though the magnetic material comprising the NPs is well below its Curie temperature. In the SPM state, $M(H)$ curves follow the Langevin function ($\mathscr{L}(x) = \coth x - 1/x$), weighted by a log-normal distribution function, $f(\mu)$, given by

$$M(H) = \frac{\int_0^\infty M_s \mathscr{L}\left(\frac{\mu H}{k_B T}\right) \mu f(\mu) d\mu}{\int_0^\infty \mu f(\mu) d\mu}, \quad (1)$$

where

$$f(\mu) = \frac{1}{\sqrt{2\pi}\,\mu\sigma_\mu} \exp\left(-\frac{\ln^2(\mu/\mu_0)}{2\sigma_\mu^2}\right), \quad (2)$$

M_S is the saturation magnetization, μ_0 the NP median magnetic moment, σ_μ the width of magnetic moment distribution, and k_B the Boltzmann constant (Fonseca et al., 2002).Firstly, values of μ_0 and σ_μ were obtained by numerical fitting the experimental *M(H)* curves with SPM behavior to the Eqs (1) and (2). From fitting results, the size distribution of NPs can be estimated by using the relations $\overline{d} = (6\mu_0/(\pi M_s))\exp(\sigma_d^2/2)$ and $\sigma_d = \sigma_\mu/3$, where $\overline{d}$ is de mean diameter and σ_d the standard deviation (Govea-Alcaide et al., 2016).

The magnetization curves measured at T = 300 K for the nodules of samples under treatments T1 and T3 are shown in Fig. 1. The obtained results display the typical SPM behavior of systems comprised by magnetic nanoparticles (Fonseca et al., 2002). For both samples, the $M(H)$ curves at 10 K exhibited hysteresis with a coercive field of ~160 Oe and remanent magnetization of 40 memu/g for T1 and 28 memu/g for T3, while the $M(H)$ curves at 300 K are reversible with negligible coercive field and remanent magnetization. However, the magnitude of magnetization for nodules from control samples and under treatment T2 is very low when compared to the ones treated with Fe_3O_4 NPs (T1 and T3) (Fig. 1). Also, a small hysteresis is observed for the $M(H)$ curves taken at 10 K and 300 K for both samples, indicating that the magnetic behavior of nodules T2 and control is very different from the SPM behavior shown

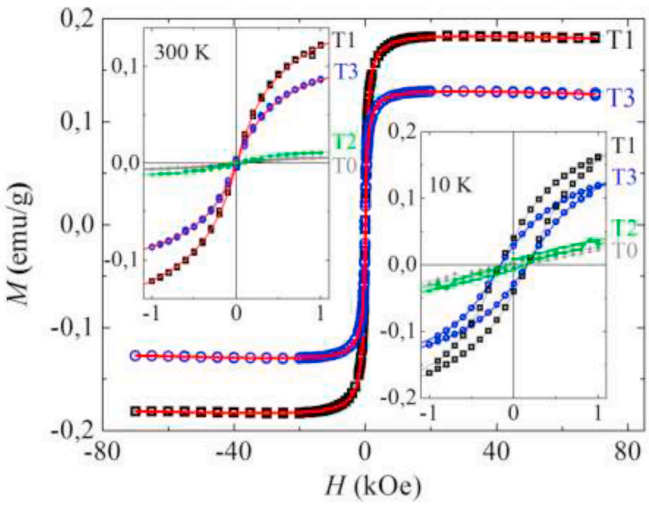

Fig. 1. Magnetization curves, $M(H)$, as a function of applied magnetic field measured at 300 K for nodules given treatments T1, T2 and T3 and the control (T0). Solid red lines are fittings using the Langevin function given by Eq. (1). The upper and lower insets show the $M(H)$ curves taken at 300 K and 10 K, respectively, for the nodules under treatments T1, T2, T3 and control (T0). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

in nodules T1 and T3.

In these samples, from the fittings of $M(H)$ curves at 300 K to Eqs. (1) and (2), it is estimated that there are Fe_3O_4 NPs of $\overline{d}$ = 6.7 and 6.8 nm accumulated in the nodules from samples of T1 and T3, respectively. The same value of σ_d = 0.35 nm was obtained for both samples. These findings are very close to those extracted fitting the data for Fe_3O_4 NPs used previously (Govea-Alcaide et al., 2016). Additionally, a concentration of 9.5 and 6.6 × 10^{14} Fe_3O_4 NPs/g in the nodules T1 and T3 was determined by computing the ratio $M_S/\overline{\mu}$, respectively (Govea-Alcaide et al., 2016). This indicates that Fe_3O_4 NPs are accumulated in the nodules T1 and T3. It is important to highlight that the SPM behavior is the universal.

4. Discussion

The marked increase in nitrogenase activity, leghaemoglobin and iron contents in nodules, nodule number per plant, number of active nodules per plant, and nodule dry weight in response to applied treatments indicates an improved symbiotic performance between *Rhizobium* and common bean plants. Accordingly, the increase in nodule number implies an increased area of bacteroids for fixing nitrogen and hence producing ammonium (Ghalamboran, 2011).The number of nodules present on roots is directly related to the amount of nitrogen fixed (Unkovich et al., 2008). About 85% of the nodules in T1-T3 plants were pink to dark red, denoting the presence of active leghaemoglobin and therefore of active nodules (Lindström and Mousavi, 2019) in the treated plants. The observed increase in nodule dry weight is generally associated with increased fixation in legumes (Fatima et al., 2007). A higher symbiotic performance and nodulation due to treatment T1 (Fe_3O_4 NPs) may have resulted from the stimulating effect of these NPs on nodulation, while in treatment T2 (*Rhizobium* inoculation) it may be attributed to the efficiency of the introduced *Rhizobium* strain. Consequently, the induced effect by T3 (Fe_3O_4 NPs + *Rhizobium* inoculation) could be ascribed to the combined effect of both. These results are in agreement with Abd-Allaa et al. (2019) who found the interaction Fe_3O_4 NP-induced *Rhizobium* (MK358859strain) significantly improves nodulation, leghaemoglobin content, nitrogenase activity, and growth of chickpea grown in enhanced salinity (75 and 150 mM). Burke et al. (2015) revealed that positively charged Fe_3O_4 NPs enhance nodulation

in soybean, compared to the negatively charged Fe_3O_4 NPs. However, Fan et al. (2014) found a negative impact of TiO_2 NPs on Rhizobium-legume symbiosis (*leguminosarum* bv. Viciae) in garden peas that resulted in morphological changes to the bacterial cells and a delay in root nodule development and nitrogen fixation.

Sajid et al. (2011) reported that *Rhizobium* inoculation significantly enhances nodule number. Similar effects of seed inoculation on nodule dry weight were also reported by Yoseph and Shanko (2017) and Bhuiyan et al. (2008) in soybeans; inoculation significantly increased nodule dry weight under field conditions. However, Fan et al. (2014) found a negative impact of TiO_2 NPs on *Rhizobium*-legume symbiosis (*leguminosarum* bv. Viciae) in garden peas that resulted in morphological changes to the bacterial cells and a delay in root nodule development and nitrogen fixation.

The increase of shoot and root total nitrogen content in treated plants may be attributed to the accumulation of significant amounts of symbiotic nitrogen compared to the control plants. This improvement in nitrogen nutrition results from an enhanced nitrogen supply via nitrogen fixation promoted by introduced Fe_3O_4 NPs and *Rhizobium*. In the same way, the present study indicates that higher shoot and root total nitrogen accumulation and better nodulation was obtained in T3, implying that improved nodulation is important to enhance the total nitrogen content in plant tissues. These results agree with the findings of Ghalamboran (2011) who found an increase in nodule dry weight and the quantity of fixed nitrogen in soybean plants grown in soil due to effect combined of Fe_3O_4 NPs and *Bradyrhizobia japonicum* bacteria strains. Medina-Perez et al. (2018) reported a significant rise in total nitrogen in roots and shoots of common bean plants irrigated with suspensions of Fe_3O_4 NPs grown in soil. Mehropouyan (2011) reported an increase in number and dry weight of nodules in common bean plants inoculated with *Rhizobium leguminosarum* strains.

Common bean plants have a shallow root system, extract very little water below a soil depth of 50 cm and require frequent irrigation or rainfall throughout the growing season. Consequently, the substantial increase in root length of treated plants with Fe_3O_4 NPs, *Rhizobium* inoculation and Fe_3O_4 NPs + *Rhizobium* inoculation may result in increased water uptake from the soil, better stand establishment in the field and enhanced plant performance. Better root growth in young plants has been suggested to result in better root systems throughout the lifetime of a plant (Leskovar and Stoffella, 1995; Lynch, 1995).Our result is consistent with that reported by Iannone et al. (2016) who found a marked increase in root length in wheat plants treated with Fe_3O_4 NPs (5 mgL^{-1}). Duran et al. (2018) reported an increase in radicle elongation of bean plants exposed to Fe_3O_4 NPs, as did Plaksenkova et al. (2019) who found a five-fold increase in root length in similarly treated rocket plants.

Increased shoot length of exposed plants to the treatments may also be favorable for the longer-term growth of the plants. The increase in shoot length following Fe_3O_4NPs and *Rhizobium* inoculation could be due to increased vegetative growth and nitrogen fixation. T3 resulted in the highest shoot length, as reported by Plaksenkova et al. (2019) for rocket plants. Iannone et al. (2016) showed an increase in aerial-part length of wheat plants treated with Fe_3O_4 NPs (5 mgL^{-1}). Other authors have also reported similar results from studies conducted on chickpeas and faba beans, El-Wakeil and El-Sabai (2007) indicating *Rhizobium* inoculation significantly increased plant growth.

T1-T3 had positive effects on the root, stem and leaf dry weights; therefore, supporting the hypothesis that these treatments increase production of dry matter in common bean plants, particularly in the T3 due to positive synergistic effect of Fe_3O_4 NPs and *Rhizobium* inoculation.The increases in dry matter accumulation could be attributed to increases in nodulation and nitrogen fixation and enhance acquisition of iron by nanoparticles.These results are in line with those of Pariona et al. (2017) who found an increase in dry biomass of oak plants irrigated with Fe_3O_4 NPs grown in soil. Kather (2015) revealed the ability of Fe_3O_4 NPs to increase dry weights of peppermint plants (*Menthapiperita* L).

Increased leaf area in treated plants likely resulted in a greater interception of light in treated plants, resulting in more assimilates for vegetative growth. This is suggested by AbouEl-Nasr et al. (2015) who found an increase in leaf area in pear saplings due to foliar spray with Fe_3O_4 NPs.

The increase of Fe contents in soil, nodules, roots and shoots by magnetite NPs (Fe_3O_4 NP and Fe_3O_4 NP and *Rhizobium* treatments) indicates that they induce the ability of plants to extract Fe from soils. This may lead to an enhancement of both iron mobilization in the rhizosphere and the uptake rate of iron, or may NPs supply enough Fe to nodules, roots and shoots to increase content of this ion. Fe plays a critical role in nodule initiation in legume crops, determining nodule number and nodule mass (Brear et al., 2013; Stambulska and Bayliak, 2019; Schwember et al., 2019), and there is a positive correlation between Fe concentration in the nodule and rate of nitrogen fixation (Mus et al., 2016) due to its positive effect on the activity of leghaemoglobin and nitrogenase (González-Guerrero et al., 2014; Roy et al., 2020).

The magnetization curves for the nodules of samples under treatments T1 and T3 shown in Fig. 1 indicate the characteristics of superparamagnetic systems. These confirm the uptake of Fe_3O_4 NPs by the plants with treatments T1 and T3 and the subsequent accumulation of these NPs in the nodules. From the fittings of $M(H)$ curves at 300 K to Eq. (1), it is estimated that there are Fe_3O_4 NPs of $\overline{d} =$ 6.7 and 6.8 nm accumulated in the nodules from samples of T1 and T3, respectively. The same value of $\sigma_d = 0.35$ was obtained for both samples.These findings agree with the data for Fe_3O_4 NPs used previously (Govea-Alcaide et al., 2016).

The measurement of magnetic signals arising from any organ of the plants may not be related specifically to stoichiometric Fe_3O_4 NPs. The high instability of tiny magnetite particles in air and/or water may cause a partial, superficial oxidation of the NPs leading to the occurrence of material with a core-shell morphology comprised of magnetite (core)-maghemite c-Fe_3O_4 (shell) (Frison et al., 2013). If this were the case, in plants grown under aerobic soil conditions, one would expect: (1) a superficial oxidation of the Fe_3O_4 NPs due to a reaction with air and/or water inthe soil and in other parts of the plants; (2) changes inthe magnetic volume of both magnetic specimens, or more appropriately in the ratio of the volume fraction maghemite/magnetite. In fact, such a ratio increases when the Fe_3O_4 mean size decreases below 20 nm (Salazar et al., 2011). Also, the NPs are functionalized in certain way when they are in soil. It is unlikely that NPs break down or degrade into ions when they are in water, soil or plants (Govea-Alcaide et al., 2016) because a magnetic signal would not appear in this environment. In any event, our estimated values of the mean diameter $\overline{d}$ of the pristine Fe_3O_4 NPs and the ones accumulated in soils and in different parts of the plants (roots, stems, and leaves) were found to vary little, being in a very narrow range between 6.0 and 7.7 nm. All these findings indicate that the width ofthe maghemite shell does not change appreciably during the uptake, translocation, and accumulation of the Fe_3O_4 NPs by the plant organs.

It is important to note that the presence of Fe in nodules of treatments T1 and T3 can be related directly to the use of Fe_3O_4 NPs in treatments T1 and T3. The superpamagnetic behavior observed in *M(H)* curves measured at 300 K provide evidence of this. Moreover, the estimation of size distribution of NPs of $\overline{d} =$ 6.7 and 6.8 with $\sigma_d = 0.35$ nm is similar to that reported for the isolated particles of $\overline{d} = (10 \pm 0.36)$ nm, and confirms the accumulation of these NPs in the nodules of these treatments.

The novelty of this study consists of providing strong evidence that the Fe_3O_4 NPs improve the symbiotic performance between *Rhizobium leguminosarum* (CF1 strain) and the common bean plant, in the form of nodulation and nitrogen fixation. This is important because the common bean is considered to be an inefficient fixer of nitrogen (Argaw and Akuma, 2015; Wilker et al., 2019; Allito et al., 2020; Reinprecht et al., 2020); the presence of Fe_3O_4 NPs in the nodules induce greater

nodulation and nitrogen fixation specifically when they act synergistically with the *Rhizobium* resulting in an increase in plant growth and dry matter production and higher concentration of Fe, Mn, P, Ca, K in the root, stem and leaf (De Souza et al., 2019). It still remains to be determined how nanoparticles combine with *Rhizobium* inoculation modify symbiotic performance, nodulation, nitrogen fixation and plant growth in legume plants.

The use of Fe_3O_4 NPs reveals a new strategy to increase the nodule population and nitrogen fixation that can be explored as a potential fertilizer for sustainable agriculture practices of legumes to reduce nitrogen fertilizer. These results point to a new niche in legume research, which is a non-genetic approach of improving legume crop; this is reinforced by the findings of Srivastava et al. (2014) and Jangir et al. (2019) in chickpea plants grown from pre-treated seeds with FeS_2 (iron pyrite) NPs and iron pyrite material.

5. Conclusions

Fe_3O_4 NPs (2000 mg/L), *Rhizobium* inoculation and Fe_3O_4 NPs + *Rhizobium* inoculation treatments notably enhance symbiotic performance and nodulation of common bean plants. These treatments result in increased nodule number per plant, number of active nodules per plant and nodule dry weight, with an improvement in symbiotic nitrogen fixation through increased nitrogenase activity, leghaemoglobin and Fe contents, shoot and root nitrogen content and amount of root fixed-nitrogen. The enhanced nodulation and nitrogen fixation in treated plants leads to a significant increase in growth and dry matter production as shown by root and shoot lengths, leaf area per plant, and root, stem and leaf dry weights of treated plants. Treatment T3 results in a synergistic effect between Fe_3O_4 nanoparticles and *Rhizobium* inoculation. The magnetic properties of the nodules indicate that Fe_3O_4 NPs are accumulated therein in common bean plants treated with nanoparticles. These results provide strong evidence that the presence of Fe_3O_4 NPs in nodules improves the symbiotic performance between *Rhizobium* (*leguminosarum* CF1 strain) and common bean plant, nodulation and nitrogen fixation. It is a non-genetic approach that brings out a long-term change in common bean growth.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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