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Nano- and Pheroid technologies for development of foliar iron fertilizers and iron biofortification of soybean grown in South Africa

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Abstract

Background: Foliar iron (Fe) fertilization of crops may increase Fe concentrations in edible portions of plants and improve yield in soils with low available Fe. However, the role of foliar Fe fertilization in increasing seed Fe has not been studied in soybeans (*Glycine max*). In this study, the Pheroid® nutrient delivery technology was combined with $FeSO_4$ or nanostructured $FePO_4$ to develop potential new Fe foliar fertilizers. Eight different treatments including different combinations of $FeSO_4$ and Pheroids were foliarly applied on field-grown soybeans in Northern Cape province in South Africa to investigate their influence on seed nutrient composition and yield.

Results: Confocal and optical microscopy images indicate that $FeSO_4$ or $FePO_4$ was not entrapped in the Pheroids but formed separate precipitates. The average seed Fe of the non-treated plants was 56 ± 3 mg kg^{-1}, and none of the treatments (including the positive controls, $FeSO_4$ and $FeSO_4$ with citrate) significantly increased seed Fe over the control. There was also no significant change in yield or seed Zn, P, protein, or phytic acid. Thus, Pheroids as well as $FeSO_4$ are not suitable as delivery system for Fe to soybean seeds due to Pheroid incompatibility with $FeSO_4$ and poor dispersibility of $FePO_4$.

Conclusions: Because none of the Fe treatments (including positive controls) affected seed Fe concentrations, foliar Fe application may not be effective to increase seed Fe in crops such as soybean that already have high native Fe.

Keywords: Biofortification, Iron, Foliar application, Soybean, Pheroids, Nanotechnology, Fertilizer

Background

Anemia affects close to 2.2 billion people worldwide, with approximately half of the cases being caused by iron (Fe) deficiency (ID) [1]. The high prevalence of ID has major negative impacts on health and economy, and Fe deficiency anemia increases risk for maternal death, low birth weight, and infant mortality [2]. Biofortification is a potentially sustainable and cost-effective approach to reduce micronutrient deficiencies. While selective breeding can provide long-term improvements, agronomic biofortification (i.e., fertilizer application to the soil and/or foliage) can provide a short-term solution [3]. Foliar application has been successful for selenium [4] and particularly for zinc (Zn) to increase grain levels in wheat and rice [5–7]. Since water-soluble ferrous compounds (like ferrous sulfate ($FeSO_4$)) applied to the soil are rapidly converted into unavailable ferric forms [8], it has been suggested that foliar Fe application may be the only way to increase Fe levels in edible plant parts [8], e.g., in rice, wheat, maize, and pea [9–13]. In a few studies, however, no

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clear effect of foliar Fe treatment on grain Fe for barley and oats [14], and wheat [15] was observed.

Legumes, especially soybeans, represent an important part of the diet in many populations [16]. Soybeans are a good source of high-quality protein and non-heme Fe [16, 17]. Due to the wide genetic variety, Fe content of soybean seeds [18] can range from 40 to 90 mg kg^{-1} dry weight (DW) and may, thus, present a good target for agronomic biofortification and selective breeding [19]. Previous studies on foliar or soil treatments focused on correcting Fe deficiency chlorosis in soybean [20]. Yet, soil application of a chelated Fe compound also increased seed Fe concentrations in chlorotic soybeans [21].

The Pheroid® technology (hereafter called Pheroid or Pheroids) is a patented delivery system comprised of essential fatty acids and other ingredients emulsified in water [22]. Proven for active ingredient delivery in the pharmaceutical industry [23], Pheroids also seem to enhance root and foliar uptake and translocation of minerals in plants [24]. Pheroids may, therefore, be a promising vehicle or tool to deliver Fe to the edible plant parts of staple crops.

This study had two main aims: first, the potential of newly developed Pheroid-based nano-Fe foliar fertilizers was studied and compared to positive and negative control treatments. To the best of our knowledge, nanostructured FePO$_4$ has not yet been tested as a source of Fe or P to plants. Second, this study aimed to evaluate whether soybean could be a potentially promising crop for biofortification with Fe through foliar application. Most studies have been carried out on cereal crops [9, 11, 12], while agronomic biofortification of dicotyledonous crops with Fe is relatively unexplored [13].

Two Fe compounds were selected for foliar application onto soybean, namely FeSO$_4$ and nanostructured ferric phosphate (FePO$_4$). The Fe salt FeSO$_4$ is commonly used as an inexpensive foliar fertilizer and has been shown to increase grain Fe in rice [9, 11], wheat [12], and pea [13]. First, Pheroid treatments containing FeSO$_4$ and FePO$_4$ were formulated and characterized. Subsequently, Pheroid-emulsified and bare FeSO$_4$ and FePO$_4$ were applied as foliar sprays to field-grown soybean with the aim to increase Fe concentration in seeds. In addition, seed Zn, P, protein, and phytic acid (PA) were measured, and seed aluminum (Al) was used as indicator for a possible Fe contamination from soil dust or particles during harvest and transport [25].

Methods
Treatment preparation
Eight different treatments were applied (Table 1): distilled water as control treatment (T1), Pheroids (T2), FeSO$_4$ (T3), Pheroids with FeSO$_4$ (T4), FePO$_4$ (T5),

Pheroids with FePO$_4$ (T6), FeSO$_4$ with pH adjusted (T7), and FeSO$_4$ with citrate (T8). The Pheroid-containing treatments (T2, T4, and T6) were prepared as a concentrate in the laboratory [24] and diluted to contain 1.2 wt% Pheroids and 0.4 g Fe L^{-1} (7 mM). This Fe concentration is comparable to previous foliar Fe spray studies [26]. The non-Pheroid treatments were all prepared directly before spray application. Treatment T3 was prepared by dissolving FeSO$_4$ (dried, micronized powder, FCC 6, Dr. Paul Lohmann, Emmerthal, Germany) in distilled water to obtain a final concentration of 0.4 g Fe L^{-1} for foliar spray. In the first two spray preparations, some Fe precipitated due to the high pH (around 8) of the local deionized water source. Therefore, two treatment groups T7 and T8 were included for the second and third spray applications as positive (dissolved Fe) control groups. These two Fe treatments were acidified to prevent Fe precipitation. In the case of T7, the required amount of FeSO$_4$ was dissolved in distilled water with pH = 5.5 (adjusted using 0.1 M HCl). For T8, FeSO$_4$ was dissolved in a citric acid solution (Fe:citrate = 1:1 molar ratio) with pH adjusted to 5.5 using NaOH. The resulting pH of T7 and T8 was 4.5 and 4.2, respectively, which is in good agreement with previous studies [27, 28].

For the preparation of T5, FePO$_4$ (specific surface area 28 m^2 g^{-1}, fine powder, FCC 6, Dr. Paul Lohmann) was dispersed in the required amount of distilled water to get 0.4 g Fe L^{-1}, and the sample was shaken vigorously before spray application. No sedimentation was observed between sample preparation and after spray application.

Treatment characterization
The size distribution of the Pheroid vesicles (T2, T4, and T6) was measured in duplicate by light scattering on a Malvern Mastersizer 2000 fitted with a Hydro 2000MU sample dispersion unit (Malvern Instruments, UK). The laser obscuration was kept constant at 12% by diluting the sample in distilled water (stirred at 2000 rpm). A refractive index of 1.481 was used for the Pheroids. Analysis of T5 was done at a lower obscuration of 1–2%, using refractive index 1.7 for FePO$_4$. Zeta potentials of the different treatments were measured on a Malvern Zetasizer Nano ZSP (Malvern Instruments). Samples were diluted in distilled water (1500× dilution for T2, T4, and T6; 100× dilution for T5). Measurements were done twice in duplicate and the average zeta potential was recorded. The infrared spectra of Pheroid vesicles were measured using a Bruker ALPHA FT-IR spectrometer (Bruker Optics, USA). Spectra were collected from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1} and corrected by subtracting the absorbance spectrum of water.

Optical microscopy analysis of the Pheroid treatments was done on an inverted microscope (Nikon Eclipse

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TE2000-E, Nikon Instruments, USA) equipped with a DS-Ri1 Nikon digital camera with real-time imaging. Confocal laser scanning microscopy images were taken with an inverted Nikon D-Eclipse C1 microscope with a 30-mm pinhole and a 60×1.40 ApoPlanar oil immersion objective. This microscope was equipped with a diode laser (emission at 409 nm), an Argon Ion laser (emission 488 or 515 nm) and a Helium–Neon polarized laser (emission at 543 nm). Pheroids were labeled with Nile red (1 g L^{-1}) which fluoresced at 650 nm and longer wavelengths. Approximately, 5 µL of sample was placed on a microscope slide, covered with a glass coverslip, and placed with the coverslip face towards the objective.

Trial location
The field trial was carried out in the Northern Cape Province in South Africa (GPS coordinates 28°09′29.1″ S; 024°32′13.7″ E). Soil and fresh leaf analyses were carried out by SGS (Cape Town, South Africa). The soil had a high clay content (40%) with a pH of 6.9 (KCl), with 17.4 mg kg^{-1} available N (1.9 mg kg^{-1} as NH_4^+ and 15.5 mg kg^{-1} as NO_3^-), and 15.4 mg kg^{-1} available P (Bray 1). The available macronutrient concentrations measured by ammonium acetate method were (in mg kg^{-1}): K 446; Na 244; Ca 5116; Mg 1269. The available micronutrient concentrations measured by 0.1 M HCl method were (in mg kg^{-1}): Fe 2.42; Zn 0.41; Mn 1.69; Cu 0.23. Micronutrient concentrations are comparable to the study of Pahlavan-Rad and Pessarakli [12] but lower compared to others [10, 11].

Soybeans (*Glycine max*, cultivar DM4670RR, *Rhizobium* inoculate FDP) were planted with a density of 380,000 plants per hectare, row spacing 300 mm. The plants received 200 kg ha^{-1} monoammonium phosphate. During the study, there was no visual indication of Fe deficiency chlorosis in plants. Leaf samples taken during BBCH growth stage 69 to determine mineral nutritional status of the plants showed that the plants were adequately supplied with mineral nutrients (Additional file 1: Table S1) [29, 30].

Experimental outline
In the field study, eight different treatments were applied, as described in Treatment preparation. The plots (each 10 m^2) for the first 6 treatments were set up in a randomized complete block design (5 by 6) with 5 replicates each, while the plots for treatment 7 and 8 were set up in a chessboard-like pattern (3 by 4) with 6 replicates each. Treatments were applied at the following BBCH growth stages [29] with 2 weeks in between: first spray at stage 69 (end of flowering), second spray at stage 77 (~70% of pods have final length), and third spray at stage 79 (during seed filling). This application sequence was selected based on the recommendations for Zn on wheat [5]. Treatments 1–6 were applied at all three spray occasions, while T7 and T8 were only applied at second and third sprays. Spray application was done in the early mornings (after sunrise) at 500 L ha^{-1} (equivalent to 1.3 mL/plant) using a pressure-regulated knapsack sprayer equipped with a double (spray 1) or single even fan nozzle (spray 2 and 3), each nozzle having spray width 0.5 m. These spray conditions correspond to approximately 20 mg Fe/spray/m^2, similar to previous studies on soybean [31], pea [13] and tomato [32]. Weather conditions were recorded before and after foliar spray; on average, ambient temperature was 21–33 °C, relative humidity was 45–66%, and wind speed was $< 2.5 \text{ m s}^{-1}$. There was no rain for at least 36 h after each application.

Plants were manually harvested at full maturity. In each plot, 10 plants were randomly selected and cut off directly above the ground, avoiding the outer rows and at least 0.5 m from each plot edge to avoid side effects. Harvested plants were dried in a drying oven, and dry pods were manually separated from the stems.

Table 1 Composition, pH, and zeta potential of the various applied foliar fertilizer treatments

	Composition	Fe (g L^{-1})	pH (—)	Zeta potential (mV)	Spray application
T1	Control (dH$_2$O)	0	7.5 ± 0.4		1, 2, 3
T2	Pheroids	0	7.4 ± 0.6	− 25 ± 3	1, 2, 3
T3	FeSO$_4$	0.4	5.3 ± 0.7		1, 2, 3
T4	Pheroids + FeSO$_4$	0.4	3.9 ± 1.2	− 14 ± 2	1, 2, 3
T5	FePO$_4$	0.4	6.7 ± 0.8	− 14 ± 3	1, 2, 3
T6	Pheroids + FePO$_4$	0.4	6.4 ± 0.9	− 24 ± 4	1, 2, 3
T7	FeSO$_4$, pH adjusted	0.4	4.5 ± 0.5		2, 3
T8	FeSO$_4$ + citrate	0.4	4.2 ± 0.1		2, 3

The BBCH growth stages of the spray applications are: 1, stage 69; 2, stage 77; and 3, stage 79

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Analysis of seeds

Number of seeds per pod (averaged over 100 pods) and 100-seed weight (averaged over 400 seeds) were determined manually, and seed samples were stored in zip lock bags at room temperature. Before chemical analysis, the seeds were rinsed under flowing nanopure water and dried in a drying oven for 48 h at 50 °C. The dry seeds were ground in a titanium mill (ZM1, Retsch, Germany) with titanium sieve (mesh size 0.25 mm), and ground seed samples were stored in zip lock bags at room temperature until analysis.

The moisture content of the milled samples was measured using a halogen moisture analyzer (HE53, Mettler Toledo, Switzerland). For each measurement, 2 g of sample was dried at 115 °C for 12 min. Averaged over all samples, the moisture content was 50 ± 2 g kg^{-1}. All chemical analyses are reported as normalized per dry weight (DW) of each individual sample.

For mineral analysis, 250 mg of ground sample was digested in triplicate using 65% HNO_3 (sub-boiled, in-house production) in a pressurized microwave digestion system (turboWAVE, MLS, Germany). In each run, a single blank and duplicate reference sample material (rice flour, SRM 1568b, National Institute of Standards & Technology, USA) were used as internal controls. The Fe and Al concentrations were analyzed by graphite furnace atomic absorption spectroscopy (GF-AAS, AA240Z with GTA-120 Graphite Tube Atomizer, Agilent Technologies, USA), and Zn concentrations by flame AAS (FAAS, AA240FS, Agilent Technologies). For each mineral, external calibration was performed using standard Tritisol solutions (Merck, USA).

Seed phytic acid (PA) contents were measured in triplicate by a modified method described by Makower [33]. In each run, a single blank and duplicate in-house reference sample material (hard red wheat bran) were used as controls. Using 500-mg seed powder, the PA was extracted with 12 wt% trichloroacetic acid ($\geq 99\%$, Sigma-Aldrich, USA) and subsequently precipitated with cerium $(Ce(SO_4)_2 \cdot 4 \ H_2O, \geq 98\%$, Merck). After mineralization for 240 min at 338 °C on a Digest Automat K-438 (Büchi, Switzerland) in concentrated sulfuric acid (95–97%, Sigma-Aldrich), inorganic phosphate was determined according to van Veldhoven and Mannaerts [34] by a microplate spectrophotometer (PowerWave HT, BioTek, USA) and converted into PA concentrations.

Total phosphorus (P) content was measured in triplicate by digesting 250-mg DW ground sample for 90 min at 420 °C on a Digest Automat K-438, using 20-mL concentrated sulfuric acid with a Kjeldahl tablet (Merck). One reference sample material (rice flour, SRM 1568b) and one blank sample were included in each run. The inorganic phosphate concentration was determined as described for the PA analysis.

The total nitrogen (N) content was measured in triplicate according to the Kjeldahl method. To each 250-mg ground sample, 20-mL concentrated sulfuric acid with a Kjeldahl tablet was added, and the sample was digested at 420 °C for 90 min on a Digest Automat K-438. One blank sample was included in each run. The N concentrations were determined by titration on a KjelFlex K-360 (Büchi) connected to a titrator (877 Titrino plus, Metrohm, Switzerland), and 0.1 M HCl (made from Fixanal ampoule, Fluka) titration volumes were converted into seed N content after subtracting the blank. A conversion factor of 6.25 was used to convert N into protein content.

Statistical analysis

Statistical analyses were done using IBM SPSS Statistics (version 23, IBM) and Microsoft Excel (version 2010, Microsoft). Data were subjected to one-way ANOVA, and differences were considered statistically significant for $p \leq 0.05$.

Results

Treatment characterization

Treatment pH and zeta potential

The Pheroid vesicles consist primarily of unsaturated essential fatty acids dispersed in water [35]. Without any Fe, Pheroids (T2) had a neutral pH and zeta potential of -25 mV (Table 1) [35]. The pH of Pheroids with $FeSO_4$ in T4 was significantly lower compared to T2; the presence of dissolved Fe^{2+} most likely catalyzed the fatty acid oxidation that resulted in the formation of acidic oxidation products including carboxylic acids [36] that further reduced the pH to 3.9 in T4. Pheroids are susceptible to oxidation due to their small size, and thus high surface–volume ratio [37]. Any change in Pheroid chemical composition, however, could not be confirmed by infrared spectroscopy analysis (Additional file 1: Figure S1). The pH of $FePO_4$ in water (T5) was 6.7, with zeta potential of -14 mV [38]. Treatment T6 had neutral pH and its zeta potential was the same as that of plain Pheroid (T2) thus $FePO_4$ did not appear to change Pheroid properties. Treatments T7 and T8 had pH of 4.5 and 4.2, respectively, to keep the $FeSO_4$ dissolved and avoid Fe precipitation.

Treatment particle size and morphology

The frequency (Fig. 1a) and cumulative (Fig. 1b) particle size distributions of the different Pheroid-based treatments (T2, T4, and T6) did not show major differences. The bimodally shaped size distributions are typical for Pheroids [39], here with main mode at 2 μm and a smaller mode around 0.2 μm. Characteristic sizes

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of the size distributions (d_{10}, d_{50}, and d_{90}; inset table in Fig. 1b) are comparable to that of Pheroids analyzed by flow cytometry [23]. The inclusion of $FeSO_4$ (T4) or $FePO_4$ (T6) caused a slight shift towards larger sizes, as is evident from the increase in d_{50} and d_{90}. In T5, the $FePO_4$ agglomerates were larger than the Pheroids, with their main peak at 30 μm, and a smaller, secondary peak around 5 μm.

Optical and confocal microscopy techniques were applied to verify if any Fe entrapment in the Pheroid vesicles had taken place [35]. The Pheroids are spherical and uniform in shape (Fig. 2a), and confocal microscopy clearly shows red-fluorescent Pheroids in contrast to background (Fig. 2d). Addition of $FeSO_4$ to the Pheroids (in T4) did not change Pheroid size or shape (Fig. 2b). Few separate orange–red aggregates/agglomerates with size up to 20 μm are visible (green in the confocal image, Fig. 2e). In both confocal and optical microscopy images, no clear Fe entrapment is visible, and agglomerates are separated from the Pheroids. Most likely, dissolved Fe^{2+} reacted with unsaturated fatty acids in the Pheroids, causing the Fe^{2+} to be oxidized to poorly soluble ferric hydroxides that precipitated. In the presence of Pheroids, the $FePO_4$ agglomerates in T6 (Fig. 2c) were considerably smaller than those in T5 (Fig. 1); possibly the Pheroids inhibited $FePO_4$ re-agglomeration. As for $FeSO_4$, no visual entrapment could be verified (as indicated by the arrows in Fig. 2f).
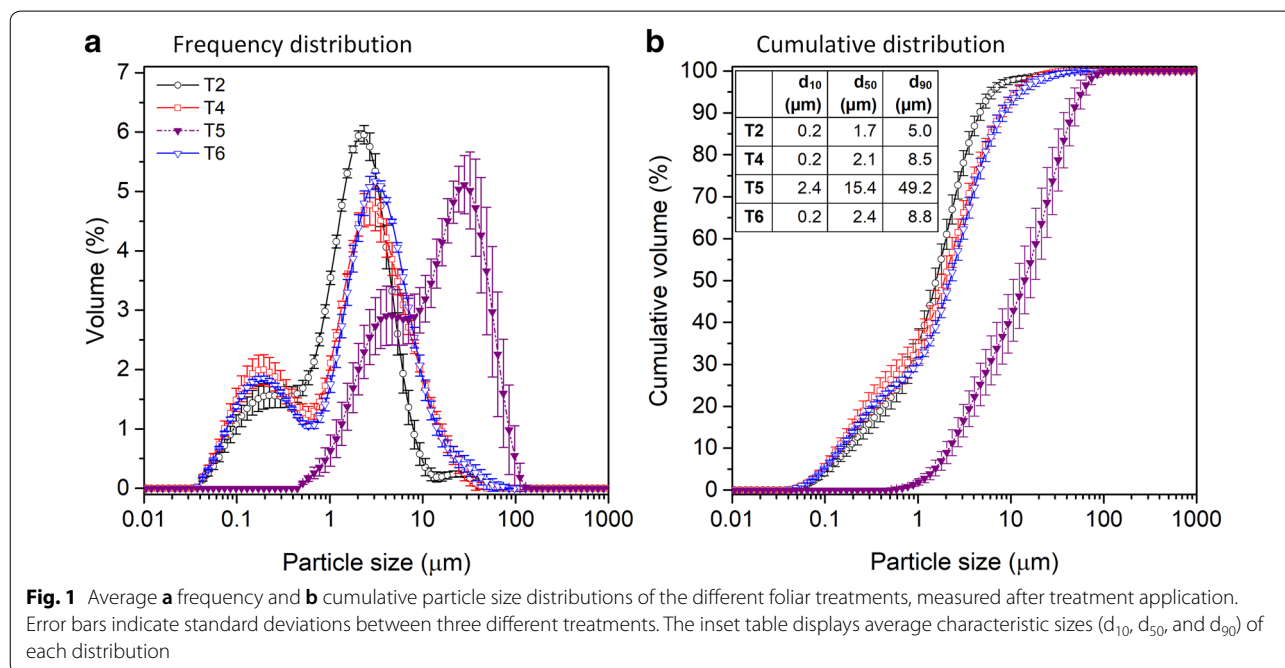
Yield parameters and soybean composition

Yield analysis

The average number of seeds per pod over all treatments was 2.39 ± 0.10, and average 100-seed weight was 18.2 ± 0.6 g, and there was no statistically significant difference between treatments (Table 2). Leaf analysis during flowering stage indicated that in the current study, there was no Fe deficiency stress in the untreated soybeans (Additional file 1: Table S1). In other studies, there was also no increase in yield after foliar Fe spray [12, 14], probably due to the lack of any visible Fe deficiency chlorosis.

Seed analyses

The average seed Fe concentration over all treatments was 57 ± 4 mg kg^{-1} and foliar application did not increase seed Fe concentrations (Table 2). The average seed Al content was < 4 mg kg^{-1}, confirming that there was no Fe contamination from soil dusts or soil particles [25], as typical Al levels in soybean seeds without soil contamination [40] are 4–12 mg kg^{-1}. Average seed Zn content was 42 ± 2 mg kg^{-1} and not significantly different among the groups. Total P and PA concentrations were also not influenced by the treatments (average concentration of 6.0 ± 0.2 g kg^{-1} and 10.1 ± 1.2 g kg^{-1}, respectively). It was estimated that approximately 54% of seed P is present in the form of PA. Similarly, no significant difference in protein concentration was detected (average concentrations 385 ± 11 g kg^{-1}, Table 2). The nutrient concentrations found in the current study are in the range typical

Fig. 1 Average **a** frequency and **b** cumulative particle size distributions of the different foliar treatments, measured after treatment application. Error bars indicate standard deviations between three different treatments. The inset table displays average characteristic sizes (d_{10}, d_{50}, and d_{90}) of each distribution

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for soybeans [18, 21, 40, 41]. Using a higher statistical significance level ($p \leq 0.10$) made no difference for any of the outcomes.

Discussion

The main objectives of this study were to combine the Pheroid technology with $FeSO_4$ or nanostructured $FePO_4$ to develop a novel foliar Fe fertilizer, and to test it for enhancement of seed Fe concentrations in soybeans compared with commonly used foliar fertilizers like $FeSO_4$ and $FeSO_4$ with citrate. The results obtained showed that none of the formulations caused any change in soybean seed Fe, Zn, P, PA, and protein concentrations. The cause for this could either come from the formulation of the treatment, or from the chosen crop.

The particle size distribution and surface charge (zeta potential) of the treatment may have an influence on foliar penetration [42, 43]. Plant cuticles have an isoelectric point around 3.0 [44] leading to negatively charged cuticle at pH higher than 3. Since negatively charged Fe

compounds enhance foliar penetration [43, 45], our compounds should theoretically have penetrated the leaves (Table 1). Foliar uptake of nanoparticles via stomatal penetration was seen for metal oxides (< 50 nm) in watermelon plants [42] and carboxylate-coated polystyrene nanoparticles (45 nm) in broad bean [46]. Larger particles (1.1 μm) were not taken up [46], since particles with size over 100 nm may block stomata [42]. In our study, the $FePO_4$ agglomerates (in T5) were predominantly larger than 1 μm (Fig. 1) making leaf penetration unlikely [42, 46]. There was no clear evidence for Fe entrapment in the Pheroid vesicles for T4 and T6 (Fig. 2). Ideally, Fe packaging in the Pheroids would have improved foliar penetration and translocation to the seeds. The lack of Fe loading may have resulted in the Pheroids being foliarly absorbed without Fe. In T4, the Fe^{2+} did likely react with the Pheroids causing precipitation of ferric hydroxides outside the Pheroids, which may have partially contributed to the poor soybean response. In one study, ferric chloride showed low penetration compared to chelated
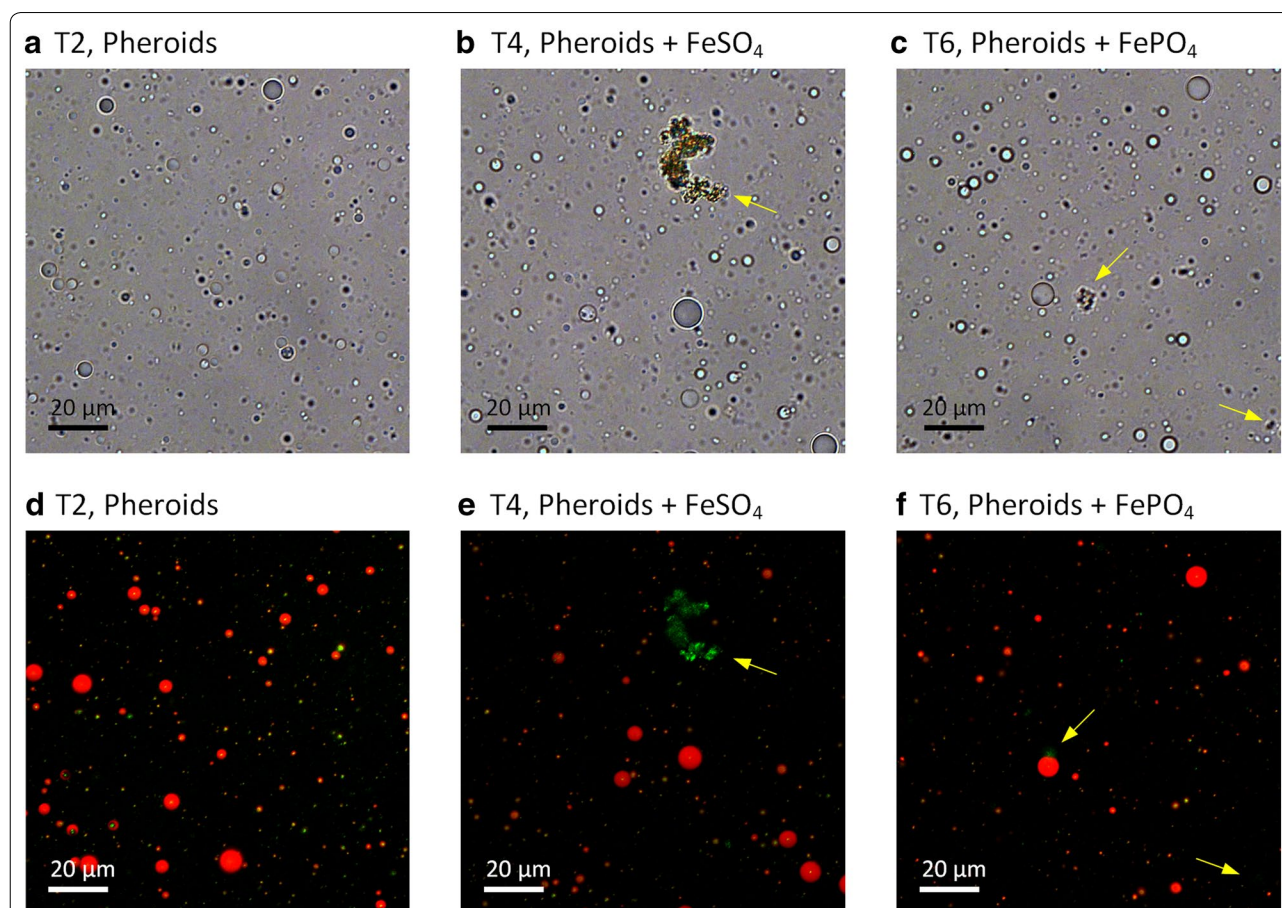

Fig. 2 **a–c** Optical and **d–f** confocal microscopy images of **a**, **d** T2, **b**, **e** T4, and **c**, **f** T6. Scale bars correspond to 20 μm. Confocal images are overlay images of red and green excitation. Pheroids fluoresce red by association with Nile red. Green spots in **d** are most probably an artifact from background noise. Arrows highlight particulate agglomerates, namely ferric precipitates for T4 and $FePO_4$ for T6

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Table 2 Number of seeds per pod and weight of 100 seeds, and Fe, Zn, P, and protein concentration of mature soybean seeds after application of various foliar treatments T1–T8

	Seeds/pod (—)	100-seed weight (g)	Fe (mg kg^{-1})	Zn (mg kg^{-1})	P (g kg^{-1})	Protein (g kg^{-1})
T1	2.4 ± 0.1(4)	18.6 ± 0.5(3)	56.0 ± 3.0(5)	42.9 ± 2.3(5)	5.8 ± 0.2(4)	388.1 ± 13.6(4)
T2	2.4 ± 0.1(5)	18.1 ± 0.3(1)	60.1 ± 5.4(9)	42.9 ± 1.3(3)	6.1 ± 0.2(3)	383.8 ± 6.6(2)
T3	2.4 ± 0.1(3)	18.4 ± 0.6(4)	56.3 ± 3.0(5)	41.4 ± 1.0(3)	5.9 ± 0.3(4)	388.4 ± 12.4(3)
T4	2.3 ± 0.1(4)	17.8 ± 0.6(4)	57.2 ± 2.0(4)	41.6 ± 1.4(3)	5.8 ± 0.2(3)	385.6 ± 9.8(3)
T5	2.4 ± 0.1(3)	18.2 ± 0.3(2)	58.1 ± 5.3(9)	42.3 ± 1.7(4)	6.0 ± 0.2(4)	386.1 ± 12.9(3)
T6	2.4 ± 0.1(2)	17.8 ± 0.7(4)	58.0 ± 4.1(7)	42.3 ± 1.8(4)	6.1 ± 0.2(4)	378.0 ± 15.0(4)
T7	2.4 ± 0.1(2)	18.4 ± 0.4(2)	56.0 ± 3.3(6)	43.3 ± 1.4(3)	6.1 ± 0.2(3)	387.3 ± 10.1(3)
T8	2.4 ± 0.1(6)	18.3 ± 0.7(4)	55.8 ± 3.0(5)	42.6 ± 1.9(4)	6.1 ± 0.3(5)	382.8 ± 8.0(2)
	NS	NS	NS	NS	NS	NS

Values are given as mean ± SD, and the coefficient of variation (CV) is given in brackets. The last row shows the statistical significance from one-way ANOVA. NS: no statistically significant difference ($p > 0.05$). For each treatment, $n \geq 5$

Fe compounds caused by precipitation of Fe hydroxides [47]. Also, ferric iron precipitation from inorganic Fe compounds sprayed onto leaves may occur on leaves of plants grown on alkaline soils and, thus, inhibit reduction towards mobile Fe^{2+} [48, 49]. Such reactivity and subsequent precipitation can be limited by protecting Fe^{2+} using chelating agents like EDTA [36], which, however, would increase the cost and decrease attractiveness of such formulations. Combining $FeSO_4$ with citrate (T8), however, also did not increase seed Fe.

The foliar penetration half-life of ferric compounds in poplar leaves was 20–30 h at 100% humidity, with longer times at lower humidity [47]. In common bean, approximately 8% of foliarly applied $FeCl_3$ was absorbed after 48 h [50]. Therefore, the low foliar Fe absorption rate combined with a possible event of rain (even after more than 48 h) may have resulted in the Fe compounds being washed off before foliar absorption. The $FePO_4$ agglomerates in T6 were not reactive but were nevertheless not entrapped in the Pheroids, most likely because the hydrophilic nanoparticles were not compatible with the mainly hydrophobic Pheroid phase [51]. Also, since both Pheroids and $FePO_4$ phase exhibited a negative surface charge (Table 1) they would electrostatically repel each other. Indeed, both hydrophobicity and surface charge are important for Pheroid entrapment: positively charged polymeric nanoparticles could be successfully entrapped in Pheroids, while negatively charged ones were likely not [35].

It is recommended to use a surfactant to improve contact between the treatment and the leaves [14, 43, 52]. In the present study, we opted against a surfactant because the Pheroids were designed to take over this role. The $FeSO_4$ and $FeSO_4$ with citrate were selected as positive control treatments because they have previously shown to be effective foliar fertilizers to increase grain Fe [9–11, 13], even without surfactant [12]. The absence of any response to the positive controls indicates that the crop itself (rather than the formulation) is responsible for the lack of change in seed Fe.

Nitrogen plays a major role in plant mineral accumulation, in both mineral transport and storage. During seed filling, micronutrients such as Fe and Zn are transported to the developing seed via the phloem [53]. If Fe was indeed foliarly absorbed, as would be expected for the positive control treatments (T7 and T8), the remobilization of Fe from leaves to seeds may have been the limiting factor [27]. Seed Fe and Zn concentrations (Table 2) are similar to or lower than leaf Fe and Zn concentrations (Additional file 1: Table S1), respectively, indicating low mineral remobilization efficiency [54]. In pea, it was not the availability of Fe in source leaves, but more likely the synthesis of such chelating agents that limited Fe phloem loading and transportation to seeds [55]. Indeed, improving nutritional N status of plants by soil and/or foliar applications, either with or without foliar Fe fertilizers, enhanced grain Fe concentration in wheat [15]. Under Fe deficiency, a fraction of Fe may be remobilized from senescing leaves [56]. Despite the low available soil Fe in the current study, the soybeans were not Fe deficient (Additional file 1: Table S1), providing no additional incentive for the plants to remobilize Fe from leaf.

Generally, soybean has at least twice as much seed protein as wheat [57, 58]. Because soybean seeds are already rich in protein and Fe, it may be difficult to increase such levels even further, and additional Fe from foliar spray may, thus, have no effect on seed Fe. Analogously, foliar spray of Zn fertilizer resulted in higher increase in grain

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Zn of wheat compared to common beans [7]. The smaller increase was ascribed to the high native Zn and protein concentrations in common bean. Moreover, soybean seed mineral concentrations are mostly determined by their genotype and are difficult to overcome using agronomic approaches [59]. Low seed Fe varieties are not likely to increase their seed Fe levels [60] and in general Fe concentrations do not vary more than 10% for a given cultivar [18].

Summarizing, several potential flaws in the formulations have been pointed out, and we conclude that Pheroids may not be suitable for Fe delivery due to their reaction with Fe^{2+}, the difficulty to break up and encapsulate $FePO_4$ agglomerates, and hydrophilic/hydrophobic interactions. The positive controls, however, did not cause any seed response either. It appears that the major obstacle to increase seed Fe concentration is the selection of the crop. Thus, optimization of the formulation may elicit only a limited response because genetics may largely control seed composition limits of soybeans [18, 60].

The HarvestPlus program (http://www.harvestplus.org/) is currently developing more nutritious crops (mainly through plant breeding) with higher amounts of primarily three nutrients: vitamin A, Fe or Zn [61]. The current study demonstrates that Pheroids may not be compatible with Fe. Zinc, however, may be more compatible with Pheroid vesicles due to its redox inactivity and higher stability in solution. While Pheroids may enhance plant uptake of Zn [24], it is not known whether the Zn reaches edible plant parts. In addition, entrapment of vitamin A or provitamin A (carotenoids) in Pheroids could be promising for agronomic biofortification. Given the success of Pheroids in entrapment of pharmaceutical compounds [23] and the fatty acid nature of Pheroids, organic molecules may be easier to entrap than minerals. Their translocation to edible plant parts, however, must still be evaluated.

Conclusions

To our knowledge, this is the first evaluation of nanostructured $FePO_4$ and the Pheroid delivery system for foliar application, and the first attempt to increase Fe content in soybeans by foliar Fe spray application. Novel Pheroids containing $FeSO_4$ and nanosized $FePO_4$, as well as bare $FeSO_4$ and nanosized $FePO_4$, that were foliarly applied on soybeans did not change yield or seed concentrations of Fe, Zn, P, protein, and phytic acid. The lack of soybean response can be partly ascribed to the formulation (reactivity of $FeSO_4$ and no Pheroid entrapment), and the Pheroid technology may not be suitable for foliar Fe fertilization. However, since the positive controls also did not elicit a response,

it appears that soybeans are ill-suited for agronomic biofortification due to their inherently high Fe and protein concentrations and tight genetic constraints. Thus, not only formulation optimization, but also careful crop selection is crucial for successful agronomic biofortification.

Additional file

Additional file 1. Fresh leaf analysis results and infrared spectra of the treatments T1–T8.

Authors' contributions
JTNK designed the study, produced and analyzed the foliar treatments, collected the data, analyzed and interpreted the data, and wrote the manuscript. FMH designed the study, analyzed and interpreted the data, and co-wrote the manuscript. JO designed and carried out the foliar application experiments. RB designed and carried out the foliar application experiments. MBZ designed the study and revised the manuscript. IC designed the study, analyzed and interpreted the data, and co-wrote the manuscript. AFG designed the study and the foliar treatments and revised the manuscript. All authors read and approved the final manuscript.

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