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Transgenic Soybean

Natural abundance

Enrichment

ICP-MS

84x47mm (300 x 300 DPI)
Evaluation of Fe uptake and translocation in transgenic and non-transgenic soybean plants using enriched stable $^{57}$Fe as tracer

Silvana R. Oliveira, * Amauri A. Menegário and Marco A. Z. Arruda

A tracer experiment is carried out with transgenic – T (variety M 7211 RR) and non-transgenic – NT (variety MSOY 8200) soybean plants to evaluate if genetic modification could influence the uptake and translocation of Fe. A chelate of EDTA with enriched stable $^{57}$Fe is applied to the plants cultivated in vermiculite plus substrate and the $^{57}$Fe acted as a tracer. The exposure of plants to enriched $^{57}$Fe caused the dilution of the natural previously existing Fe in the plant compartments and then a changed Fe isotopic ratio ($^{57}$Fe/$^{56}$Fe) is measured using a quadrupole-based inductively coupled plasma mass spectrometer equipped with a dynamic reaction cell (DRC). Mathematical calculations based on the isotope dilution methodology allow distinguishing the natural abundance Fe from the enriched Fe (incorporated during the experiment). The NT soybean plants acquire higher amounts of Fe from natural abundance (originally present in the soil) and from enriched Fe (coming from the $^{57}$Fe-EDTA during the experiment) than T soybean ones, demonstrating that the NT soybean plants probably absorb higher amounts of Fe, independently of the source. The percentage of newly incorporated Fe (coming from the treatment) was approximately 2.0 and 1.1% for NT and T soybean plants, respectively. A higher fraction (90.1%) of enriched Fe is translocale to upper parts, and a slightly low fraction (3.8%) is accumulated in the stems by NT plants than by T ones (85.1%; 5.1%). Moreover, in both plants, the Fe-EDTA facilitates the transport and translocation of Fe to the leaves. The genetic modification probably is responsible for differences observed between T and NT soybean plants.

Introduction

Global trade in soybeans and soybean products, a multi-billion-dollar business, has risen rapidly since the early 1990s and it is expected to keep growing throughout the next decade as a result of a continued increase in the global demand for food and feed consumption, production of biodiesel and other industrial uses. To meet this high demand for soybeans, a worldwide growth in the production has been observed, which can be significantly attributed to the widespread cultivation of transgenic plants.  

The Roundup Ready® soybean from Monsanto, genetically modified to tolerate the glyphosate, which is currently used as herbicide, is the most well-known and used transgenic soybean product owing to cost efficiencies for weed control and high yield. However, its cultivation and use has caused enormous scientific and public debates in the same way as occurs to other genetically modified plants. The most important concern has been whether the transgenic is as safe as the conventional with respect to human and animal food consumption, as well as in their environmental impact.

Some previous comparative studies involving transgenic and non-transgenic soybeans revealed the presence of changes in the proteome, enzymes and metallome of transgenic seeds and in the proteome and enzymes of transgenic leaves. These differences indicate that this genetic modification can provide not only tolerance to herbicide but also cause many changes in the whole metabolism of the transgenic plants.

Recently, a comparative study revealed that Fe is present at higher concentration in transgenic (variety MSOY 7575 RR) than in non-transgenic soybean seeds (variety MSOY 7501). As a result, the transgenic seeds seem to have the ability to take up and store higher amounts of Fe. However, considering other soybean plant parts (roots, stems and leaves) as well as other Roundup Ready® transgenic varieties, there is little or no information about the possible differences in the distributions of Fe among transgenic and non-transgenic soybeans. Additionally, a better comprehension of the specific aspects of the transgenic soybean plant metabolism related to absorption, transporting and storage of Fe would be of utmost importance for bio-fortification purposes since the Fe deficiency is a widespread agricultural problem, that hinders plant growth, lowers crop yields and reduces nutritional quality, and the plants are a primary food source for humans.

A potential tool for obtaining a better insight in the processes of absorption, transporting and storage of Fe is the use of tracer experiments employing stable isotopes. In the last few years the use of stable isotopes have gained importance.
for tracer experiments in biological and medical research. In these studies, stable isotopic tracers with an isotopic composition sufficiently different from the corresponding natural one are added to the studied system and changes in as selected isotope ratio is monitored. The absorption or bioavailability of an element can be determined with this approach as well as information about element redistribution over various compartments of an organism. Experiments based on enriched stable isotopes require accurate and precise determination of elemental isotope ratios, generally, by mass spectrometric techniques. Nowadays, Inductively Coupled Plasma Mass Spectrometry (ICP-MS) has been an excellent choice for enriched stable isotope tracer experiments because of the numerous recent developments in its instrumentation, such as high resolution, reaction/collision cell and multicollector systems. Tracer experiments aiming to study Fe metabolism, such as Fe uptake by plants, have been made employing ICP-MS in the last years. The Fe uptake of cucumber plants from chelates containing enriched stable Fe, Fe(III)-o,oEDDHA and Fe(III)-o,pEDDHA (which are compounds commonly applied to overcome Fe deficiency) have been studied in nutrient solution. The efficiency of both isomers (o,o and o,p) EDDHA to provide Fe to Strategy I plants (tomato and peach) in nutrient solution and in calcareous soil has also been investigated using chelates containing enriched stable Fe, which as far as we know, is the unique study performed directly in soil.

According to our knowledge, no tracer study for essential or toxic metals evaluating transgenic and non-transgenic soybeans has been exploited so far. Therefore, the aim of this work was to study and compare the uptake and translocation of Fe as well as its distribution among the plant compartments of transgenic and non-transgenic soybeans. For this task, soybean plants were cultivated in a mixture of vermiculite plus substrate and treated with a chelate of Fe-EDTA containing enriched stable Fe, where the Fe acted as a tracer. After appropriate sample preparation, Fe isotope ratio measurements and Fe quantification were carried out by ICP-MS and in order to discriminate between natural abundance Fe and the enriched-Fe, mathematical calculations based on the isotope dilution methodology were done.

Experimental

Reagents and materials

All solutions were prepared using polypropylene flasks with deionized water (≥18.2 MΩ cm) from a Milli-Q water purification system (Millipore). Nitric and hydrochloric acids from Merck were purified in a sub-boiling system (Berghof) before using. All reagents used were from Merck or Sigma Aldrich. Fe certified standard solution and Ca, Cr, Mg and Ni standard solutions (1000 mg L⁻¹) were purchased from Merck.

All working standard solutions were prepared daily from the stocks by simple dilution with Milli-Q water. The CRMs (1573a Tomato Leaves and 1515 Apple Leaves) from the National Institute of Standards and Technology (NIST) were used for method optimization and validation. Enriched Fe was obtained from Cambridge Isotope Laboratories (CIL) as iron oxide powder, dissolved in a minimum volume of sub-boiled hydrochloric acid and diluted with high purity water. The isotopic abundances of the Fe-enriched solution were determined by ICP-MS equipped with dynamic reaction cell (DRC) using a certified natural isotopic abundances Fe standard from Institute for Reference Materials and Measurements (IRM) - IRMM-634 to avoid error from mass discrimination. The concentration of the spike solution was determined by reverse isotope dilution analysis using the certified Fe solution (IRM-634) as reference. Ultra-clean procedures were used for sample preparation and analysis. Plastic bottles and glassware were cleaned by soaking in 10% (v/v) HNO₃ at least 24 h and rinsed abundantly in de-ionized water before use.

Instrumentation and ICP-MS measurements

A forced air oven (Quimis) was used to dry the finely ground plant compartments. Samples and certified reference materials were digested in a DGT 100 Plus microwave oven (Provecto Analitica) equipped with 50-ML PTFE vessels. An ICP-OES (iCAP 6300 Series, Thermo Scientific) equipped with an Elveflow optical design and a Charge Injection Device (CID) solid-state detector was used to provide Fe and Ca concentrations. A quadrupole-based ICP-MS equipped with a dynamic reaction cell (Elan DRC-e, PerkinElmer) was used for Fe total concentration determination and Fe isotope ratio measurements. An optimized hydrogen gas flow was introduced into the quadrupole reaction cell to eliminate some interferences in the Fe isotopes. The solutions were introduced into the ICP-MS using a Meinhard concentric nebulizer attached to a cyclonic spray chamber together with a peristaltic pump. All measurements were carried out in a clean room class 1000. The optimized instrumental operating conditions and measurement parameters for total Fe concentration and Fe isotopic ratios by ICP-MS are shown in Table 1.

In this work, Fe of natural isotope abundance is doped, in the plants, with the same element isotopically enriched in ⁵⁷Fe. As a result, two different isotope signatures were followed in the samples: the natural abundance Fe and the enriched Fe as ⁵⁷Fe. In order to distinguish between natural abundance and enriched Fe, the Fe isotope ratio measurements were processed on the basis of the classical approach according the mathematical procedure described before.

Soybean cultivation, tracer experiment and sample preparation

Transgenic (variety M 7211 RR) and non-transgenic (variety MSOY 8200) soybean seeds were kindly donated by Monsanto (Brazil). The transgenic soybean confers resistance to the herbicide glyphosate-N-(phosphonomethyl)glycine. For handling the Roundup Ready® type transgenic soybean seeds, the Institute of Chemistry has a Biosafety Quality Certificate (issued on July 24, 2007; number 240/2007). Transgenic and non-transgenic soybean seeds were germinated and grown in plastic pots (200 mL capacity) containing a 50% (m/m) mixture of substrate (Basaplant, Brazil) plus 50% (m/m) of vermiculite (Vermifloc Agro, Brazil) at pH 6.2, using the structure of the laboratory devoted to transgenic plants. The plants were allowed to grow for 21 days in a growth chamber under a controlled temperature (27 ± 0.1 °C) and photoperiod (12 h). Forty eight plants were cultivated, 24 treated with ⁵⁷Fe-EDTA chelate (12 T and 12 NT) and 24 non-treated – control plants (12 T and 12 NT). Only one biological replicate was done. For the preparation of ⁵⁷Fe-EDTA chelate solution, the EDTA was dissolved in NaOH (1:3 molar ratio) and ⁵⁷Fe (chloride form, 90.07% isotopic enrichment, CIL), previously dissolved in Suprapur hydrochloric acid, was slowly added at an equimolar amount of ligand. During the reaction, the pH was maintained between 6 and 8, and at the final adjusted to 7. After 24 h, the solution was filtered.
through a 0.45 µm PVDF membrane to eliminate possible iron hydroxides produced in the synthesis and diluted to volume with deionized water.

Table 1. Instrumental operating conditions and measurement parameters for total Fe concentration and Fe isotopic ratios by ICP-MS.

<table>
<thead>
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<th>Instrument settings</th>
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<td>Rf power (W)</td>
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<tr>
<td>Nebulizer gas flow - argon (L min⁻¹)</td>
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<td>Auxiliary gas flow - argon (L min⁻¹)</td>
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<td>Sampler and skimmer cones</td>
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<td>Solution uptake (mL min⁻¹)</td>
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<table>
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<th>Dynamic Reaction cell parameters</th>
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<td>H₂ gas flow (mL min⁻¹)</td>
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<tr>
<td>Rpq (V)</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Data acquisition parameters – total Fe concentration

- Reading mode: Peak Hopping
- Dwell time (ms): 50
- Sweeps/reading: 50
- Integration time (ms)*: 2500
- Detector dead time (ns): 60
- Lens voltage (V): 5
- Detector acquisition mode: Pulse Counting
- Replicates: 5
- Measurement time for n=5: 530 s
- Monitored Isotopes: ⁵⁶Fe, ⁵⁷Fe, ⁵⁸Fe and ⁵⁹Fe

*The integration time for each point is determined by multiplying the dwell time by the number of sweeps.

Exposition to light was avoided during preparation and storage to prevent photo decomposition. Daily, during 7 days (between the 7th and 14th day of cultivation), the plants were irrigated with this solution after its proper dilution and MES addition (to maintain the pH at 6). Each plant received in the end of treatment 632.92 nmol of ⁵⁷Fe-EDTA or 0.036 mg of Fe enriched in the ⁵⁷Fe isotope. After the 21 days of cultivation, the plant parts were separated in roots, stems and leaves and washed with deionized water. The plant compartments of each treatment were pooled, finely ground in a mortar with liquid nitrogen and dried at 40 ºC until a constant weight. About 100 mg of the dried, homogenized and powdered plant samples were digested with a mixture of 1.0 mL of H₂O₂, 1.75 mL of HNO₃ and 0.25 mL of HCl in closed TFEV vessels submitted to the following microwave program: 5 min @ 240 W; 5 min @ 420 W; 5 min @ 600 W and 15 min @ 800 W. After digestion all samples were dried on a hotplate, treated with a mixture of 30% (m/m) H₂O₂ and concentrated HNO₃ to oxidize the organic compounds and ferrous to ferric iron species, and dissolved in 1 mL of HCl 6 mol L⁻¹. The total Fe concentrations in T and NT soybean control plant parts were determined by ICP-MS after dilutions of the digested samples. In the case of T and NT soybean plant parts enriched in ⁵⁷Fe, the isotope dilution procedure was used to quantify total Fe.

In order to reduce Ca interferences for Fe isotopic measurements, Fe purification by anion-exchange chromatography was necessary and performed according an adapted procedure. Polypropylene columns of 10 mL (Poly-Prep Columns BioRad) were used for Fe separation, filled with 2 mL of AG1-X8 resin (100–200 mesh). The exchange capacity of 1 mL wet resin is 1.2 mmol FeCl₃, corresponding to approximately 90 mg of Fe₁⁶. After a cleaning procedure and conditioning of the resin, 500 µL of each sample, dissolved in HCl 6 mol L⁻¹, was loaded to the column. Matrix elements in the interstice of the column were washed out with HCl 6 mol L⁻¹ and afterwards Fe was eluted with HNO₃, 5 mol L⁻¹. Samples were dried down, treated with drops of concentrated HNO₃ to oxidize the residual organic compounds and re-dissolved in 1 mL of HNO₃ 0.2% (v/v). These fractions were diluted to around 250 µL L⁻¹ Fe and the isotopic ratio measurements were carried out by ICP-MS. Quantitative recovery of Fe and removal of Ca was checked with Fe and Ca concentration measurements in small aliquots of the samples and in the certified reference material of Apple Leaves (1515) before and after the separation step by ICP-OES.

Results and discussion

Overcoming spectral interferences under the Fe isotopes

Reliable isotopic analysis of Fe by ICP-MS is traditionally hindered by spectral interferences and the Ar- and Ca-based molecular ions are the most problematic ones. The main interferences that hamper accurate determination of Fe isotope ratios are ⁴⁰Ar, ⁴⁰Ar⁺N and ⁴⁰Ca⁺N for ⁵⁶Fe; ⁴⁰Ar⁺O and ⁴⁰Ca⁺O for ⁵⁷Fe; ⁴⁰Ar⁺OH and ⁴⁰Ca⁺OH for ⁵⁷Fe; ⁵⁴Ni, ⁴⁰Ar⁺O, ⁴⁰Ca⁺OH and ²³Na⁺Cl for ⁵⁸Fe. For resolving the isobaric overlap of the ⁵⁴Cr and ⁵⁵Ni signals with the corresponding Fe signals, the required mass resolution is so far beyond the capabilities of a quadrupole ICP-MS. Fortunately, the concentration of Cr in soybean tissues is usually low and did not create serious problems. In fact, the concentration of Cr in soybean seeds [˂ 0.15 µg g⁻¹] is around 490 times lower than Fe [ca. 73.5 µg g⁻¹], while ⁵⁴Cr is a minor abundant isotope. The Ni concentration in soybean tissues is also usually low, but may be a problem during Fe determination. For soybean seeds the Ni concentration [ca.0.34 µg g⁻¹] is around 175 times lower than Fe, but the ⁵⁸Ni is the isotope with the highest natural abundance, and the corresponding signal shows an overlap with the least abundant isotope of Fe. Therefore, this isobaric overlap was mathematically corrected for the contribution from ⁵⁸Ni.

The quadrupole ICP-MS was tested without using DRC available in the instrument. The maximum sensitivity to Fe isotopes and the minimum intensities to the interfering ions were attained with a RF power of 1240 W and a nebulizer gas flow rate as 0.91 L min⁻¹. However, under these conditions, the isotopic ratios ⁵⁶Fe/⁵⁷Fe, ⁵⁷Fe/⁵⁸Fe and ⁵⁸Fe/⁵⁹Fe were around 10 times higher than the natural ones due to the presence of Ar-based interferences. Furthermore, when a solution containing a Fe/Ca elemental ratio of ca. 1/13 (which simulates a soybean seed digested sample), the Fe isotopes intensities increased up to around 10%. Aiming to overcome these interferences under the Fe isotopes, DRC was used. The DRC is a powerful mean of overcoming spectral overlap. By appropriate selection of a reaction gas and a proper setting of the mass bandpass the signal intensity of unwanted ions can be reduced by orders of magnitude as a result of selective ion/molecule reactions.
The CH₄ and H₂ were tested as reaction gases and their performances were evaluated by monitoring the signal intensities and the signal to noise ratios at mass-to-charge ratios of 54, 56, 57 and 58, as well as the isotopic ratios ⁵⁴Fe/⁵⁶Fe, ⁵⁷Fe/⁵⁶Fe and ⁵⁸Fe/⁵⁶Fe employing 0.028 mol L⁻¹ HNO₃, 50 µg L⁻¹ Fe and 1250 µg L⁻¹ Ca, as a function of the RPq and reactor gas flow rate.

With RPq of 0.7 V and a reaction gas flow rate of 0.4 mL min⁻¹, the H₂ was more efficient to remove the Ar and Ca-based interferences under all Fe isotopes. As can be observed in the Table 2, the found isotopic ratios ⁵⁴Fe/⁵⁶Fe, ⁵⁷Fe/⁵⁶Fe and ⁵⁸Fe/⁵⁶Fe to the 50 µg L⁻¹ Fe standard solution were relatively close to the natural ones, remembering the measurement parameters of isotopic ratios had not been yet optimized. On the other hand, when the solution containing a Fe/Ca elemental ratio of approximately 1/13 (which simulates a soybean seed digested sample), and the digested certified reference material of tomato leaves (SRM 1573a) with a Fe/Ca elemental ratio of approximately 1/137 were analyzed, the found Fe isotopic ratios deviate considerably from the natural ones due to the presence of Ca which caused interferences and mass discrimination (Table 2). Besides, the presence of Ca also precluded the accurate mathematical correction for the contribution of ⁵⁸Ni, since the ⁶⁰Ni used in the correction was overlapped by ⁴⁴Ca⁰O and ⁴⁴Ca⁰O²⁻. The use of a Ca-matrix-matched blank to correct Ca interferences improved the results for the solution simulating the digested soybean seed and for the digested certified reference material of tomato leaves (1573a) as observed in the Table 2. However, as the soybean samples may present a high variability for Ca concentrations, the sample preparation would be exhaustive. Therefore, the Fe separation from the sample matrix was carried out by anion-exchange chromatography to ensure accurate Fe isotopic ratio measurements with ICP-MS.

The sorption of Fe(III) on anion exchange resin increases with HCl concentration, while Cr, Ni, Ca and several other elements were not quantitatively retained. Fe was bound on the column as FeCl₄⁻ (with a partition coefficient higher than 103 in favor of the resin) while matrix elements were eluted from the column with HCl 6 mol L⁻¹. Fe was then eluted from the column using 5 mol L⁻¹ HNO₃. The certified reference material of Apple Leaves (1515) with a Fe/Ca elemental ratio of approximately 1/184 was analyzed by ICP-OES to validate the method and the average yield of Fe was 100 ± 8 %. Additionally, all Ca was eluted with HCl 6 mol L⁻¹. Anion exchange resins are known to fractionate Fe isotopes, but, this fractionation could be only measured by MC-ICPMS. A quadrupole ICP-MS is not capable of measuring this subtle level of variation.

The Fe isotopic ratios ⁵⁴Fe/⁵⁶Fe, ⁵⁷Fe/⁵⁶Fe and ⁵⁸Fe/⁵⁶Fe measured (with optimized measurement parameter) for the certified reference material of Apple Leaves (1515) and soybean samples from the control group before the anion exchange separation were different from the natural ones, as expected. However, after the separation, the values found were in agreement at 95% confidence level (t-test) with the natural ones demonstrating that the Ca interference was eliminated.

Optimization of the measurement parameters for Fe isotopic ratios

Some isotopic ratio applications require a high measurement precision such as those ones which subtle natural variations in the isotopic composition of an element need to be revealed and quantified. For a tracer study, the necessary precision is modest, but achieving precision values approaching the counting statistics is desirable. The isotope ratio precision of a quadrupole-based ICP-MS is rather modest due to the ICP being a rather ‘noisy’ ion source and the presence of a single detector only, which allows monitoring only one single signal at any given time. This drawback is usually counteracted by choosing the scanning or peak hopping rate as high as possible (residence time as low as possible, but not too short that insufficient ions are counted) in order to compensate for signal drift and/or instability to the largest possible extent. In other words, the more simultaneous the measurement, the better the precision becomes.²⁴,²⁵

Aiming to achieve the best precisions possible for the Fe isotopic ratios measured by DRC-ICP-MS, the signal acquisition parameters were optimized. Initially, the residence time was varied from 2 to 50 ms maintaining the number of sweeps per reading at 500 (which resulted in integration times from 1000 to 25000 ms) for a 50 µg L⁻¹ Fe standard solution. The precisions (expressed as % RSD) obtained for 5 successive measurements of the isotopic ratios ⁵⁴Fe/⁵⁶Fe, ⁵⁷Fe/⁵⁶Fe and ⁵⁸Fe/⁵⁶Fe improved significantly at 25 and 50 ms of residence time, reaching 0.1, 0.6 and 0.8% at 25 ms. However, the little improvement offered by a residence time of 50 ms compared with 25 ms was not worth, taking into account the measurement time spent was approximately 2 times longer.

As the counts depend directly of the concentration, this parameter was evaluated in order to attain sufficient counts and consequently improving the precision. For the concentration range evaluated (25 to 500 µg L⁻¹ Fe) under the acquisition parameters established before (25 ms of residence time; 12500 ms of integration time and 500 sweeps per reading) it was observed a significant improvement in the precision as the concentration increased. However, for the 500 µg L⁻¹ Fe standard solution, the detector suffered shutdown and thus, the best results, obtained for 250 µg L⁻¹ Fe, were 0.05%, 0.5% and 0.6% to the isotopic ratios ⁵⁴Fe/⁵⁶Fe, ⁵⁷Fe/⁵⁶Fe and ⁵⁸Fe/⁵⁶Fe, respectively.

When the 250 µg L⁻¹ Fe standard solution was analyzed with the same integration time (12500 ms), but increased number of sweeps per reading or peak hopping rate (10000) as well as short residence time (12.5 ms) [which resulted in a more simultaneous measurement], the precisions were 0.045%, 0.21% and 0.37% to the isotopic ratios of ⁵⁴Fe/⁵⁶Fe, ⁵⁷Fe/⁵⁶Fe and ⁵⁸Fe/⁵⁶Fe, respectively. The optimization of the measurement parameters for Fe isotopic ratios combined with the use of a collision/reaction cell, pressurized with H₂, which probably promoted a temporal homogenization of the ions extracted from the ICP (mixing of ions sampled at slightly different time) provided better precisions. The obtained values were in agreement with the reported by the literature with a sector field instrument, operated at low mass resolution, (0.05% RSD).²⁴

When using an electron multiplier in pulse counting mode for measuring the signal intensity, a certain dead time of the detector and its associated electronics has to be taken into account to avoid that the measurement data for isotope ratios differing from unity show biases with respect to the corresponding true values. The detector dead time of 60 ns was determined experimentally according the method used by Russ.²⁵ This value was subsequently loaded into the instrument’s operation software and the signals appropriately corrected for dead time losses in order to obtain accurate isotope ratio results.
The differences in the efficiency of ion extraction, transmission and detection as a function of analyte mass can generate an isotope ratio measured by ICP-MS significantly biased with respect to the corresponding true value. This phenomenon is recognized as mass discrimination and it may amount to several percent per mass unit. Matrix elements also affect the mass discrimination, and in this case, they were previously eliminated by anion-exchange chromatography.

Adequate correction for mass discrimination for a quadrupole ICP-MS, characterized by its modest isotope ratio precision is not of great concern, and usually is done by running an external reference standard of certified isotopic composition, comparing the isotope ratio with the theoretical value, and then mathematically compensating for the difference. The mass discrimination for Fe was corrected with the sample-standard bracketing approach using the Fe solution of certified natural isotopic abundances, IRMM-634. The found accuracies of the bias-corrected isotope ratio measurements were 1.3% for $^{54}\text{Fe}/^{56}\text{Fe}$, 1.2% for $^{57}\text{Fe}/^{56}\text{Fe}$ and 3.2% for $^{58}\text{Fe}/^{56}\text{Fe}$. The optimized analytical method presented enough conditions to perform the tracer experiment on soybean plants.

### Tracer study

In this work, the Fe enriched stable isotope $^{57}\text{Fe}$ was applied as $^{57}\text{Fe}$-EDTA to increase the availability of Fe to soybean plants. The application of enriched $^{57}\text{Fe}$ as $^{57}\text{Fe}Cl_2$ ($^{57}\text{FeCl}_2$ was obtained after dissolution of $^{57}\text{Fe}_2\text{O}_3$ with HCl) to soybean plants, probably, would turn Fe unavailable to these plants since stable oxides could be formed in the mixture of substrate plus vermiculite. On the other hand, the application of Fe-EDTA chelate is well-known to be an effective practice to provide Fe to crops. The highest uptake of Fe was required due to the low mass of enriched stable isotope applied that had to be the lowest possible according the tracer experiment principle, in which, the amount of tracer should not disturb the natural conditions.

The 21-day-old T and NT soybean plants from the control and treated groups cultivated in the mixture of substrate plus vermiculite grew similarly and no symptoms of chlorosis were observed. Besides, the fresh and dry plant weights were measured for T and NT soybean plants from both groups (control and treated), and the differences were not statistically significant. The amount of Fe naturally available in this mixture of substrate plus vermiculite most likely was enough to accomplish the Fe requirements of the soybean plants.

The total concentrations of Fe in the T and NT soybean plant samples exposed to $^{57}\text{Fe}$-EDTA were determined by isotope dilution methodology. Samples and blanks were spiked with a known amount of the characterized $^{57}\text{Fe}$ and a collision-cell quadrupole ICP-MS was used to obtain the isotopic ratios. The analytical method was validated by analyzing a plant certified reference material (1515, Apple leaves). The found concentration was $81.5 \pm 2.3$ µg g$^{-1}$ (n=5, 95% confidence) which is in agreement with the certified value of $83 \pm 5$ µg g$^{-1}$. The total concentrations of Fe in the T and NT soybean samples from the control group were also determined using standard analytical curve. The concentrations of Fe obtained for the enriched T and NT soybean plant parts were, respectively: 523.8 ± 11.3 and 545.7 ± 9.0 µg g$^{-1}$ for the roots; 100.6 ± 6.6 and 92.4 ± 4.2 µg g$^{-1}$ for the stems; and 214.6 ± 2.2 and 230.7 ± 6.3 µg g$^{-1}$ for the leaves. According to t test, the total concentrations of Fe found for T soybean plant parts were significantly different from NT soybean ones. It is possible to observe that T stems presented a higher value than the NT ones, but, roots and leaves presented lower values. The same profiles were observed for the T and NT plants from the non-exposed group (control), but the Fe concentrations were lower, as expected.

Iron isotopic ratios and the abundances of Fe isotopes were obtained for T and NT soybean plant samples from the control and treated groups by ICP-MS. The Table 3 presents the abundances of Fe isotopes for T and NT soybean plant samples after performing the tracer study. As the abundances of Fe isotopes for T and NT soybean plant samples from the control group were very close to the natural ones only the IUPAC values are shown in the Table 3. This agreement proves the employed method was accurate and precise for the tracer experiment requirements. In the Table 3, it is possible to observe that the natural abundances of Fe isotopes (observed for T and NT soybean samples of the control group) slightly changed in the exposed plants, indicating these plants were enriched with $^{57}\text{Fe}$, or in other words, the $^{57}\text{Fe}$ was incorporated in them. Therefore, the amount of Fe used in the tracer experiment was suitable to generate a measurable level of enrichment in all samples. In practice, the difference determined for the isotopic ratio exceeded ten times the standard deviation of the basal isotopic ratio (in agreement with the IUPAC definition regarding the limit of quantification). The levels of enrichment for the NT soybean plant compartments were higher than those obtained for T ones as it can be seen by means of the $^{57}\text{Fe}$ abundances. The total Fe concentrations found for T and NT soybean plant compartments do not allow, by itself, to discriminate between the accumulated Fe coming from the mixture substrate plus vermiculite (natural abundance Fe) and the newly incorporated Fe coming from the $^{57}\text{Fe}$-EDTA during the experiment (enriched Fe) as well as to follow it within the plants. However, once the total concentrations have been computed, the concentrations of natural abundance Fe and enriched Fe can be calculated in the plant parts based on the
but, more studies are necessary to verify this hypothesis.

Figure 1. Relative distribution of natural abundance Fe between the different compartments of the T and NT soybean plants exposed to \textsuperscript{57}Fe

The total and natural abundance Fe concentrations found for T and NT soybean plants demonstrated that the NT ones were able to take up higher amounts of Fe. On the other hand, the data of relative distributions of natural abundance Fe established that the only remarkable difference between T and NT plants is the slightly high fraction of Fe accumulated in the T stems. Therefore, it is reasonable to suppose the genetic modification was able to affect the amount of Fe absorbed by one plant as well as the fraction of Fe translocate from stems to the leaves, but, it does not seem to disturb the means used by one soybean plant to take up and translocate Fe since the distribution profiles were similar between T and NT soybeans.

Table 3. Measured isotope abundances (at %) of Fe in T and NT soybean plant samples after the tracer study. For comparison, the IUPAC natural abundances for Fe are shown in the first column. Last row shows the \( ^{57}\text{Fe}/^{56}\text{Fe} \) ratio in each case. \( n=3 \)

<table>
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<tr>
<th>Isotope</th>
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<th>Non-Transgenic Soybean</th>
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<td></td>
<td>Roots</td>
<td>Stems</td>
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<td>58</td>
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</tr>
<tr>
<td>( ^{57}\text{Fe}/^{56}\text{Fe} )</td>
<td>0.02309 ± 0.00007</td>
<td>0.02762 ± 0.00012</td>
</tr>
</tbody>
</table>

Table 4. Average plant concentrations (µg g \(^{-1} \)) of total, natural abundance and \( ^{57}\text{Fe} \)-enriched Fe for T and NT soybeans after the tracer study. \( n=3 \)

<table>
<thead>
<tr>
<th>Soybean</th>
<th>Concentration of Fe (total)</th>
<th>Concentration of natural abundance Fe</th>
<th>Concentration of enriched Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transgenic</td>
<td>223.8 ± 2.8</td>
<td>221.3 ± 2.8</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>Non-transgenic</td>
<td>233.8 ± 3.7</td>
<td></td>
<td>4.6 ± 0.1</td>
</tr>
</tbody>
</table>

molar fractions measured previously and the atomic weights of natural abundance Fe and enriched \( ^{57}\text{Fe} \).

When the total dry weight of a plant is taken into account the average concentration of Fe for this plant can be calculated. For T and NT soybean plants the total Fe concentrations were 223.8 ± 2.8 and 233.8 ± 3.7 µg g \(^{-1} \), respectively (Table 4).

The concentrations of natural abundance Fe and enriched Fe obtained were: 221.3 ± 2.8 and 229.2 ± 3.9 and 46.0 ± 1 µg g \(^{-1} \) for NT soybean plants (Table 4). According to these results, the NT soybean plants acquired higher amounts of Fe from natural abundance (coming from different compounds present in the mixture substrate plus vermiculite) and from enriched Fe (coming from the \( ^{57}\text{Fe} \)-EDTA during the 7 days of experiment) than T soybean ones. The percentage of newly incorporated Fe in the NT soybean plants reached almost 2.0 % while only 1.1% of new Fe was found in the T soybean ones. When the total mass of Fe provided in the treatment is considered (36 µg of Fe for each plant), by means of a mass balance it was possible to determine that one NT soybean plant took up ca. 1.4% of all Fe provided, whereas a T soybean plant took up 0.8%. The capacity of NT soybean plants of taking up higher amounts of Fe seems not be dependent of the Fe source, since the sources of natural and enriched Fe were different, but, more studies are necessary to verify this hypothesis.

The relative distribution of natural abundance Fe between the different compartments of the T and NT plants for the treated group is shown in Figure 1, and they were similar. The fractions (in %) of natural abundance Fe increased according the following order: stems, with 13.3 ± 0.9 (T) and 11.8 ± 0.5 % (NT); roots, with 32.7 ± 0.7 (T) and 32.8 ± 0.5 % (NT); and leaves, with 54.0 ± 0.6 (T) and 55.4 ± 1.5 % (NT). When each plant part is compared individually, it is possible to verify that the relative distributions of natural abundance Fe in leaves and roots presented no significant differences between T and NT soybean, whereas, differences were found between T and NT soybean stems. Of all Fe absorbed by one plant, the same fraction (around 67%) was translocate to upper compartments (stems and leaves) of T and NT plants. However, even the same fraction of Fe have been translocate to upper compartments of T and NT plants, the T stems accumulated a slightly high fraction of Fe than NT ones.
The relative distribution of enriched $^{57}$Fe between the different plant compartments for the treated group is shown in Figure 2. As it was found previously for the natural abundance Fe, the distribution profiles of enriched Fe between the different parts of T and NT plants were very similar. The fractions (in %) of enriched Fe also increased according the order showed to natural abundance Fe: stems, with $5.1 \pm 0.4$ (T) and $3.8 \pm 0.2$ % (NT); roots, with $14.6 \pm 0.7$ (T) and $9.9 \pm 0.6$ % (NT); and leaves, with $80.3 \pm 2.6$ (T) and $86.3 \pm 3.9$ % (NT). When each plant compartment is compared individually, it is possible to verify that the relative distributions of enriched Fe in roots, stems and leaves showed significant differences between T and NT soybeans. Of all Fe absorbed by one plant, the fraction translocate to upper compartments (stems and leaves) was lower for T than NT soybean plant. Therefore, T soybean roots accumulated a higher fraction of Fe than NT ones, and, consequently, it translocate a lower fraction to superior parts. Moreover, of all Fe translocate to upper compartments, the T stems accumulated a slightly high fraction than NT ones, as it was observed to natural abundance Fe.

Figure 2. Relative distribution of enriched Fe between the different compartments of the T and NT Soybean plants exposed to $^{57}$Fe

It was observed that the fractions of enriched Fe present in the leaves of T and NT soybeans were considerably higher than those of natural abundance Fe, reaching more than 80%. Probably, the enriched Fe coming from the $^{57}$Fe-EDTA chelate used in the treatment was most easily translocate to leaves than the natural abundance Fe coming from different compounds present in the mixture substrate plus vermiculite. Other possibility is that all plants are growing on iron sufficiency – none of them is chlorotic and they all show the same fresh weight, even the supplemented ones. Therefore, the added iron is somehow “in excess”, and it could be being stored in leaves, as it was shown for transgenic soybean plants by Vasconcelos et al.\textsuperscript{26}

The enriched Fe concentrations found for T and NT soybean plants demonstrated that the NT ones were also able to uptake higher amounts of Fe. Concerning the relative distributions of enriched Fe between T and NT plant compartments, it is possible to identify that the NT plants translocate a higher fraction of Fe to upper parts than T ones and also accumulated a slightly low fraction of Fe in the stems.

The T and NT soybean plants presented differences related to the amount of Fe absorbed by one plant and with the fractions of Fe translocate to upper regions.

Conclusions

The main objective of this work was greatly attained, once the distribution and translocation of Fe were followed in T and NT soybean plants, by using $^{57}$Fe as tracer and a quadrupole-based ICP-MS as detector. From instrumental side, all the interferences were circumvented by carefully optimization of the ICP-MS conditions, using correction equations and using exchange resin for avoiding Ca interferences. From plant physiology side, those well optimized instrumental conditions allowed to obtain the abundances of the Fe isotopes close to the results established by IUPAC, and conclude that T and NT plants present similar profiles of Fe distribution between the plant compartments (stem Fe fraction $<$ root Fe fraction $<$ leaves Fe fraction), being NT plants able to accumulate higher amounts of Fe than T ones even when different sources of Fe were used. $^{57}$Fe-EDTA chelate or various compounds present in the mixture substrate plus vermiculite. Additionally, a higher fraction of Fe (coming from $^{57}$Fe-EDTA) was translocate to upper parts and a slightly low fraction of Fe was accumulated in the stems by NT plants than by T ones. Then, the genetic modification can be the cause of these differences, as already previously demonstrated by different works published by our research group. All these results are pushing our group to researches regarding transgenic plants.

Furthermore, the Fe coming from the $^{57}$Fe-EDTA chelate possibly, was most easily translocate to leaves than the Fe coming from different compounds (mixture substrate plus vermiculite) indicating the Fe-EDTA favoured transport and translocation of Fe to the leaves. As the plants are growing on Fe sufficiency there is also the chance this excess of Fe were stored in the leaves.

Finally, it is important to emphasize that this work did not have the intention to present arguments in favor or against transgenic plants or genetic modification, but, however, put some light for better understand these organisms.

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Notes and references

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