Guelke-Stelling, M., von Blanckenburg, F. (2012): Fe isotope fractionation caused by translocation of iron during growth of bean and oat as models of strategy I and II plants. - Plant and Soil, 352, 1-2, 217-231

DOI: 10.1007/s11104-011-0990-9

Fe isotope fractionation caused by translocation of iron during growth of bean and oat as models of strategy I and II plants

Plant Soil, *doi: 10.1007/s11104-011-0990-9*

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Keywords: Fe isotopes, higher plants, isotope fractionation, Fe translocation in plants

Abstract

Background: The determination of the plant-induced Fe-isotopic fractionation is a promising tool to better quantify their role in the geochemical Fe cycle and possibly to identify the physiological mechanisms of Fe uptake and translocation in plants. Here we explore the isotope fractionation caused by translocation of Fe during growth of bean and oat as representatives of strategy I and II plants.
Methods: Plants were grown on a nutrient solution supplemented with Fe(III)-EDTA and harvested at three different ages. We used the technique of multi-collector ICP-MS to resolve the small differences in the stable iron isotope compositions of plants.
Results: Total bean plants, regardless of their age, were found to be enriched in the light iron isotopes by -1.2‰ relative to the growth solution throughout. During growth plants internally redistributed isotopes where young leaves increasingly accumulated the lighter isotopes whereas older leaves and the total roots were simultaneously depleted in light iron isotopes. Oat plants were also enriched in the light iron isotopes but during growth the initial isotope ratio maintained in all organs at all growth stages.
Conclusions: We conclude that isotope fractionation in bean as a representative of strategy I plants is a result of translocation or re-translocation processes. Furthermore we assume that both uptake and translocation of Fe in oat maintains the irons' ferric state, or that Fe is always bound to high-mass ligands, so that isotope fractionation is virtually absent in these plants. However, in contrast to our previous study in which strategy II plants were grown on soil substrate, oat plants grown on Fe(III)-EDTA contain iron that enriches ^{54}Fe by 0.5 permil over ^{56}Fe. A possible explanation for the enrichment is the prevalence of a constitutive reductive uptake mechanism of iron in the nutrient solution used which is non-deficient in iron.

Introduction

New tools are nowadays increasingly used in the effort to understand mechanisms for iron uptake and transport in plants and in humans. The use of stable iron isotopes has now been recognized (e.g. Walczyk and von Blanckenburg 2002; Walczyk and von Blanckenburg 2005; Guelke and von Blanckenburg 2007; Álvarez-Fernández 2006; Stuerup et al. 2008, von Blanckenburg et al. 2009). In studies of iron uptake stable iron isotopes can be used in two different ways: (1) isotope fractionation studies, utilizing minute natural shifts in the isotopic abundances of iron isotopes as driven by binding form and reaction kinetics; and (2) and tracing studies, using compounds enriched in a specific non-radioactive isotope. Both approaches permit to track the natural cycles of iron and to study metabolic processes, e.g. in humans or plants.
Measuring the stable iron isotope fractionation during iron uptake in plants has so much scientific potential since iron metabolism in plants involves many changes in its binding forms and most likely also changes in the redox state. Iron (mostly Fe(III)) from the soil or nutrient solution diffuses into the

apoplast, which is the extracytosolic compartment of plant cells, of plant roots. Under non-limiting iron supplies, all plant roots reduce Fe(III)chelates and transport Fe(II) through the plasma membrane by a constitutive plasma membrane-bound ferric reductase (Bienfait et al. 1985; Briat et al. 1995). However, in soils iron is present in sparingly soluble Fe(III) compounds which are not directly available for root uptake. Therefore higher plants were forced to develop different strategies to make iron in soil available for their needs.

Strategy I plants, which comprise the dicots and non-grass monocots, excrete protons via a plasmalemma H^+-ATPase to acidify the rhizosphere, thus making Fe(III) more soluble. The inducible ferric chelate reductase activity of FRO2 reduces Fe(III) to Fe(II) (Robinson et al. 1999). Fe(II) is subsequently transported into the plant by IRT1, which is the major iron transporter of the plant root (Vert et al. 2002). This strategy is induced under Fe-deficiency. In addition it has recently been demonstrated that besides Fe(II) after reduction complete Fe-chelates can be taken up by plants, but amounts should not exceed 1 % of total Fe (Orera et al. 2010).

Strategy II plants, which are represented by graminaceous plant species, acquire iron by exuding phytosiderophores (PS) and the Fe(III)-PS complexes are then transported into plant roots via a specific membrane transport system.

The mechanisms of iron transport in plants, once taken up by the roots, are less clear. It has recently been suggested that younger leaves receive their iron primarily from the phloem whereas older leaves receive iron from the xylem (Tsukamoto et al. 2009). In the xylem iron is transported as Fe(III)-citrate (Tiffin 1966), in the phloem as Fe(III)-ITP (Iron Transporter Protein) or Fe-nicotianamine (NA) (Kruger et al. 2002; Curie et al. 2009). The species of Fe-NA transported in the phloem has still to be identified. NA is a precursor of phytosiderophores. It is present in all plants and has the ability to bind various metals including Fe^{2+} and Fe^{3+} (von Wirén et al. 1999) but the kinetic stability is higher for the Fe(II)-NA complex than for the Fe(III)-NA complex. Seeds receive iron from the roots and from senescent leaves (Morrissey and Guerinot 2009). At daylight iron moves to the seeds most likely via the phloem, because the flow of the xylem is driven by transpiration and seeds hardly transpire (Grusak 1994). At night, iron is also transported to the seeds through the xylem due to root pressure. The level of remobilization from shoot to seed varies by species (Marr et al. 1995; Garnett and Graham 2005). In the seed, iron is thought to be stored mainly in the vacuoles of the embryo and endosperm as Fe(III), but Fe storage in the vacuole is species-dependent and some legumes store most Fe as ferritin (Morrissey and Guerinot 2009, Ravet et al. 2009).

Iron has four naturally occurring stable isotopes with the following approximate composition: ^{54}Fe (5.8 %), ^{56}Fe (91.8 %), ^{57}Fe (2.1 %) and ^{58}Fe (0.3 %) (Rosman and Taylor 1998). The relative abundances are virtually constant in nature but tiny differences due to small chemical or physical differences between the iron isotopes can now be identified using advanced mass-spectrometric techniques (Weyer and Schwieters 2003; Schoenberg and von Blanckenburg 2005). The partitioning of isotopes between two substances or two compartments of the same substance with different isotope ratios is called isotope fractionation. The main phenomena producing isotope fractionations are isotope exchange reactions (equilibrium isotope fractionation) and kinetic processes, the latter depending primarily on differences in reaction rates between isotopically substituted molecules of different mass. Isotope fractionation is preserved in the form of differences between compartments provided that transfer of the element is not complete. Iron isotope fractionation is expressed in the delta notation, which provides the permil deviation of the isotopic ratio (e.g. $^{56}Fe/^{54}Fe$ or $^{57}Fe/^{54}Fe$) of the sample relative to that of the IRMM-014 standard (Taylor et al. 1992): $\delta^{56}Fe/\ [‰] = [(^{56/54}Fe_{sample}/^{56/54}Fe_{standard}) - 1] \cdot 10^3$.

Iron isotope fractionation factors α(expressed here as permil difference between the $\delta^{56}Fe$ of a target compartment and $\delta^{56}Fe$ of a source compartment) have been calibrated for a variety of chemical reactions. Crosby et al. (2007) have shown a fractionation of −3 ‰ from a solid Fe(III) substrate into $Fe(II)_{aq}$ using dissimilatory iron-reducing bacteria. This value is similar to the abiotic fractionation factor (Welch et al. 2003; Anbar et al. 2005). In soil $\delta^{56}Fe$ of the $Fe(II)_{aq}$ taken up can still be lowered by re-adsorption of $Fe(II)_{aq}$ as adsorption processes are known to preferentially sequester the heavier isotope at soil particle surfaces (Icopini et al. 2004). These predictions have been confirmed for reduction of ferric iron in marine sediments (Severmann et al. 2006; Staubwasser et al. 2006). Additionally it has been shown that iron isotope fractionation might occur during ligand exchange

reactions. Dideriksen et al. (2008) described the equilibrium isotope fractionation between complexes of the siderophore Fe(III)-desferrioxamine B (FeDFOB) and inorganic aqueous Fe(III) complexes with 0.5 ‰, which is similar to what Wiederhold et al. (2006) found for the ligand-controlled dissolution of goethite with oxalate to be about 0.3 ‰.

In the first study of the stable iron isotope signature of higher plants the δ^{56}Fe values in vegetables and crops grown on two distinct soil substrates were determined (Guelke and von Blanckenburg 2007). It was found that strategy I and strategy II plants differ in their stable iron isotope composition. Iron in strategy I plants was found to yield significantly lower δ^{56}Fe values than the plant-available iron pool in the soil substrates, whereas iron in strategy II plants yielded slightly heavier δ^{56}Fe values than the soil substrates. The first observation was explained with the preferential reduction of the lighter iron isotopes during uptake (e.g. Welch et al. 2003; Staubwasser et al. 2006) while the latter could be due to the preferential chelation of heavier iron isotopes during complexation to phytosiderophores (Brantley et al. 2004; Dideriksen et al. 2008). In addition it was found that δ^{56}Fe values in strategy I plants decreased from soils to stems, from stems to leaves and from leaves to seeds with seeds having the lowest δ^{56}Fe value of −1.6 ‰. In contrast, all measured parts of strategy II plants displayed similar δ^{56}Fe values. This finding led to the assumption that these plant types differ in the numbers of oxidation and reduction cycles during translocation as these processes are known to induce significant iron isotope fractionation. Very recently Kiczka et al. (2010) found a δ^{56}Fe of −1.0 to −1.7 ‰ in three Alpine plant species, two of them being strategy I plants and one a strategy II plant, grown under natural growth conditions. Mass balance calculations revealed that fractionation towards lighter Fe isotopic composition occurred before uptake, probably during mineral dissolution, and during selective uptake of iron at the plasma membrane. Iron isotopes were further fractionated during remobilization from old into new plant tissue, which changed the isotopic composition of leaves and flowers over the season.

However, these previous studies have raised several questions. First, the transport mechanisms responsible for the fractionation processes need to be identified. Second, the question arises whether the fractionation during uptake depends on the iron speciation in the growth medium. For example, the observed trends might be characteristic of plants grown on soil substrates, and might differ in plants grown in nutrient solutions.

To address these open questions bean (*Phaseolus vulgaris L.*) as a model for strategy I plants and oat (*Avena sativa L.*) as a model for strategy II plants were planted on purified quartz sand, watered with a nutrient solution of known iron isotopic signature, and different plant organs were harvested at several growth stages. The iron concentration and the iron isotopic signature were determined and compared to that of the nutrient solution.

Materials and Methods

Plant growth with nutrient solution

Seeds of *Avena sativa L.* (oat) and *Phaseolus vulgaris L.* (bean) were immersed into deionised water on a tissue for two days and then planted onto approximately 5 L quartz sand at a density of 6 plants in 5 L pots which were open at the bottom to avoid reducing conditions through flooding. Plants were watered as needed with deionisied water. Every two days approximately 200 mL of a nutrient solution with the following composition was added: 1000 µM $Ca(NO_3)_2$, 375 µM K_2SO_4, 325 µM $MgSO_4$, 100 µM KH_2PO_4, 8 µM H_3BO_3, 0.2 µM $CuSO_4$, 0.2µM $ZnSO_4$, 0.2 µM $MnSO_4$, 10 µM NaCl, 0.05 µM $MoNa_2O_4$ and 20 µM Sodium-Fe(III)-EDTA, all dissolved in deionised water. The Fe(III)-EDTA solution was not part of the nutrient solution containing the other elements, but was added separately before every watering. The speciation of the nutrient solution was modelled with the PHREEQC software using the minteq database (Packhurst and Appelo 1999). The pH without Fe(III)-EDTA in the nutrient solution amounts to 5.5 and with the addition of Fe(III)-EDTA it stays approximately the same. About 99.3 % of the EDTA is present as Fe(III)-EDTA$^-$ and displacement of Fe with other elements is unlikely. This nutrient solution was used for both plant species. Plants grew in a daylight climate chamber with a temperature of 16 – 18 °C. At different points in time, one pot of plants was harvested; plants in the other pots continued to grow. Bean plants were harvested 17 days, 30 days, 47 days and 74 days after germination. Oat plants were harvested 14, 28 and 50 days after germination. Complete plants were rinsed with ultrapure water and separated into roots, stem, the different leaves

and seeds/buds. Roots were rinsed to remove adherent nutrient solution. Since apoplastic iron was not removed (Bienfait et al. 1985) measured roots concentration and isotope data integrate over both, intracellular iron and apoplastic iron. The pedicel from the leaves was removed prior to cleaning with ultrapure H_2O. The plant parts were dried in an oven for at least 3 days at 80 °C and their dry weight was determined afterwards. Finally they were ground to mince and homogenize them. The same procedure was applied for original seeds.

Sample decomposition and iron separation

All reagents used during sample preparation were *suprapure* grade and prepared with ultrapure water. Hydrochloric and nitric acids were *pro analysi* grade and were further purified by sub-boiling distillation. All preparation work was carried out in a clean lab class 1000 in laminar-flow hoods, class 10. Approximately 200 mg of each dried plant sample was digested via microwave digestion in 7 mL concentrated HNO_3 and 1 mL concentrated H_2O_2 at 200 °C for more than half an hour. The Fe(III)-EDTA solution given to the bean and oat plants was also digested in order to determine its isotopic signature. The EDTA complex breaks down completely at temperatures of about 200 °C after 2 hours (Martell et al. 1975). 10 mL of the Fe(III)-EDTA solution (3 repetitions) were dried down, the residues were dissolved in 7 mL concentrated HNO_3 and 1 mL concentrated H_2O_2 followed my microwave digestion at 220 °C for two hours. After this procedure the Fe(III)-EDTA solutions were clear, indicating that all EDTA was destroyed. As an additional test for the initial composition the nutrient solution (including Fe(III)-EDTA) was digested in the same way.

As it is also possible that plants mobilize small amounts of iron from the quartz sand which does contain traces of adsorbed iron, the iron concentration in a quartz leach which best represents the mobilization of iron by plants was determined. In three replicates 2 g of the quartz sand were weighed into 50 mL centrifuge tubes and 40 mL 0.5 M HCl were added. The samples were placed into an over-head shaker at room temperature. After 24 hours of shaking the tubes were centrifuged (15 min, 5000 rpm, 4472 x g) and the supernates decanted and filtered through 0.2 µm PTFE membrane filters wetted with ultrapure water. This procedure is thought to extract all poorly-crystalline iron (oxyhydr)oxides and iron bound to organic compounds (Wiederhold et al. 2007; Guelke et al. 2010) which are most likely to be available for plant nutrition (e.g. Borggaard 1992; Bertrand and Hinsinger 2000). The total iron concentration and stable iron isotope composition of the quartz sand was also determined. For this purpose the quartz sand was digested via microwave agitation with a 1:2 HF/HNO_3 mixture at 200 °C for about an hour. Fluoride complexes that form in silicate samples were destroyed by treating the evaporated sample with concentrated aqua regia and heating to 170 °C for several hours.

After digestion or extraction all samples were dried down on a hotplate and full oxidation of iron to its trivalent state was achieved by adding a drop of concentrated HNO_3 to the samples, heating them to 150 °C and careful drying them down. For isotope measurements with MC-ICP-MS it is essential to present an iron solution totally free of its natural matrix to avoid interferences and introduction of an instrumental mass bias (Schoenberg and von Blanckenburg 2005). For that reason all samples were dissolved in 1 mL of 6 M HCl for iron purification by anion-exchange chromatography following the procedure described by Schoenberg and von Blanckenburg (2005). As iron concentrations in plant materials are quite low (50-100 µg/g) microcolumns were used for iron separation, filled with ca. 300 µL DOWEX AG© 1x8 (100-200 mesh) resin. For the Fe(III)-EDTA and nutrient solution samples 7.5 mL Spectrum® 104704 polypropylene columns filled with 1 mL of the resin were used. The exchange capacity of 1 mL wet resin is 1.2 mmol $FeCl_4^-$ corresponding to approximately 90 mg iron (Schoenberg and von Blanckenburg 2005). After a cleaning procedure and conditioning of the resin, samples, dissolved in 6 M HCl, were loaded to these columns. Matrix elements were washed out with 6 M HCl and afterwards iron was eluted with 5 M HNO_3. Samples were dried down and redissolved in a drop of 15 M HNO_3. After taking samples almost to dryness, they were dissolved in 1 mL of 0.3 M HNO_3. An additional precipitation step was applied for the plant samples that ensures complete precipitation of all Fe(III) as Fe(III)OOH while e.g. Zn, which cannot be separated from Fe by anion-exchange-chromatography, stays in solution (Schoenberg and von Blanckenburg 2005). The samples were precipitated at pH 10 with ammonia. After one hour the samples were centrifuged, the supernate

solutions were discarded and the precipitates washed twice with ultrapure H_2O before they were redissolved in 0.3 M HNO_3.
Quantitative recovery and removal of matrix elements during iron separation and precipitation was controlled by iron concentration measurements with small aliquots of the samples before and after each step by optical emission spectroscopy with inductively coupled plasma (ICP-OES: *Varian Vista PRO CCD Simultaneous*). This check is important because non-quantitative recovery could result in artificial isotope fractionation (Anbar et al. 2000; Roe et al. 2003). Additionally the iron concentrations of all samples were obtained and total procedural iron blanks were measured with mostly less than 60 ng. This was less than 1 % of the processed iron (with a minimum measureable Fe content of 6 µg) and was considered to be insignificant.

Iron isotope measurements

The iron isotope compositions of the Fe(III)-EDTA-solution, and for comparison also that of the nutrient solution, the quartz sand, and the different plant tissues were determined with the use of a multiple-collector inductively coupled plasma mass spectrometer (MC-ICP-MS; Neptune, *ThermoFinnigan*) by means of a high-mass resolution mode. Molecular interferences were resolved routinely by increasing mass resolution on this instrument (Weyer and Schwieters 2003). The mass discrimination was corrected with the sample-standard bracketing approach (Schoenberg and von Blanckenburg 2005) using the iron isotopic reference material IRMM-014 (Institute of Reference Material and Measurement, Geel, Belgium).
Sample and standard solutions were introduced into the mass spectrometer in 0.3 M HNO_3 at concentrations of 5-7 ppm Fe. All values are reported as δ^{56}Fe and δ^{57}Fe relative to the IRMM-014 standard of which the isotopic composition is close to that of rocks at the Earth's surface (Johnson et al. 2004; Dauphas and Rouxel 2006; Schoenberg and von Blanckenburg 2006).
δ^{56}Fe and δ^{57}Fe of all samples were plotted against each other and were found to follow a mass-dependent fractionation law which demonstrates the absence of molecular or elemental interferences. Within each analytical session the internal laboratory standard JM (Johnson & Matthey, Fe Puratronic wire) was measured to test the accuracy of the measurements. During the course of this study the measured Fe isotope composition of the JM standard was δ^{56}Fe = 0.421±0.050 ‰ and δ^{57}Fe = 0.625±0.090 ‰ (2σ, n = 62), which is in agreement with previous measurements (δ^{56}Fe = 0.423±0.046 ‰ and δ^{57}Fe = 0.624±0.073 ‰) given by Schoenberg and von Blanckenburg (2005). The reproducibility of replicate measurements and chemical replicates according to Schoenberg and von Blanckenburg (2005) of the samples processed in this and our previous studies (Guelke and von Blanckenburg 2007; Guelke et al. 2010) were determined as well. It was found to be 0.07 ‰ (2σ; n= 29) for the δ^{56}Fe of chemical replicates and 0.11 ‰ for replicate measurements (2σ; n=108). These values are less reproducible than those obtained by Schoenberg and von Blanckenburg (2005).
A mass balance approach is used to determine the δ^{56}Fe of bulk plants and above-ground organs (without the roots) which is calculated according to the following formula, where Fe_n is the fraction of the iron amount of plant tissue n (dry weight multiplied with Fe concentration) and $\delta^{56}Fe_n$ the isotopic composition of plant tissue n:

$$\delta^{56}Fe_{total} = \sum_n \left(\delta^{56}Fe_n \times [Fe]_n \right) \quad (1)$$

Errors of the calculated total δ^{56}Fe are the propagated errors of the δ^{56}Fe of the individual plant tissues.

Results

The iron isotopic composition of the Fe(III)-EDTA solution, the nutrient solution and the quartz sand is shown in Table 1. The iron concentration for the quartz sand HCl-extract was about 60 ng/g. As this comprised only about 5 % of the iron contained in the Fe(III)-EDTA it is considered to be negligible. The iron concentration leached from quartz was too low for iron isotope measurements. However, even if the δ^{56}Fe of HCl-extracted Fe and Fe(III)-EDTA differed significantly, this would result in a bias of less than 0.1 ‰, which is within the 2 standard deviation of the analysis (assuming a δ^{56}Fe of the HCl-extracted Fe of −2 ‰, which is an upper bound). Total quartz sand had a Fe concentration of 2.5 µg/g. About 2.5 % of this Fe was available for the plants in form of mobile Fe (extracted with

HCl). The residual Fe contained in the quartz sand is considered to be negligible as plants are not able to extract iron of crystalline oxides or silicates (Bertrand and Hinsinger 2000).
Iron in Fe(III)-EDTA had a δ^{56}Fe of 0.56 ± 0.11 ‰. For comparison, the isotopic composition of a small aliquot of the nutrient solution (after Fe(III)-EDTA was added) was also determined. It is in agreement with the value found for Fe(III)-EDTA. In the following discussion the isotopic difference between plant parts and the Fe(III)-EDTA of the nutrient solution will be expressed as: $\Delta^{56}Fe_{plant\text{-}Fe(III)\text{-}EDTA}$ (note that $\Delta^{56}Fe_{B\text{-}A}$ commonly denotes a measured permil difference in normalized isotope ratios between a target compartment B and a source compartment A, not a fractionation factor: $\Delta^{56}Fe_{B\text{-}A} = (\delta^{56}Fe)_B - (\delta^{56}Fe)_A$) (Figures 1 and 2). The precision on the δ^{56}Fe values was better than 0.11 ‰ (2SD) (Table 2). The error of the nutrients' δ^{56}Fe value was not propagated into $\Delta^{56}Fe_{plant\text{-}Fe(III)\text{-}EDTA}$ since this error was the same during the entire growth experiment, assuming uniform iron composition in the growth solution.

Bean

All parts of bean plants except for the roots exhibited iron concentrations within the range expected for green plant tissues (Marschner 1995). Original and new grown seeds showed the lowest iron concentrations of approx. 50 ppm (Table 2). Roots had the highest Fe concentrations of mostly more than 200 ppm and differed between the harvests which might be explained by the presence of apoplastic iron that was not removed prior to sample digestion. Therefore the δ^{56}Fe of roots includes apoplastic iron, precipitated in the "free space" after reduction. This pool was potentially available for plants; precipitated iron could be re-reduced and taken up. As roots were washed with ultrapure water after harvesting most Fe(III)-EDTA will be washed out and it is assumed that the apoplast comprises mostly iron which was reduced once and then precipitated again.
The concentration of iron in the cotyledon and first leaf decreased during growth. The iron concentration of the stem decreased from the first (38 µg/g) to the second (22 µg/g) harvest but then increased again to 44 and 81 µg/g. Envelopes of seeds had lower iron concentrations (ca. 20 µg/g) than seeds (ca. 45 µg/g). Together they showed similar values as the original seeds (53 µg/g).
All measured tissues of the bean plants were found to be enriched in the lighter iron isotopes compared to the Fe(III)-EDTA solution by up to −2.5 ‰. Iron in the different plant tissues became increasingly lighter from older to younger plant parts, i.e. from roots to cotyledon, to stem, to leaves and to seeds. At every harvest point this trend was visible. It is also obvious that during first growth the earlier leaves accumulated iron with high δ^{56}Fe while the young leaves of the later growth stages obtained iron with lower δ^{56}Fe than that obtained by the earlier leaves during their growth. Iron in roots and the cotyledon shifted to slightly higher compositions during growth. Iron in the stem and the first leaf evolved towards heavier compositions from the third harvest point on. The second and third leaf shifted to heavier isotopes during growth as well, whereas the seeds developed towards a lighter iron isotope composition when they grew further (Figure 1).
With mass balance (equation *1*) above-ground organs' and bulk plant isotope compositions were calculated for each point of harvest (Table 2). Complete bean plants were found to be lighter by about 1.2 ‰ than Fe(III)-EDTA at all three growth stages. In contrast, the composition of the above-ground plant changed from −1.2 ‰ at stage 1 to −1.75 ‰ at stage 4 when compared to the growth solution.

Oat

Similar to the bean plants, oat roots showed very high iron concentrations (400 to 1000 µg/g, Table 3) which can be explained by the lack of apoplastic iron removal prior to digestion.
The iron concentration in the cotyledon decreased from 77 µg/g at the first point of harvest to 62 µg/g at the second point of harvest. In the stem it decreased from 42 to 15 µg/g whereas in the first leaf and fourth leaf the iron concentration increased during growth. In the second and third leaf the iron concentration was slightly diminished.
All δ^{56}Fe values of the oat plants covered a small range of (0 to 0.2 ‰). All measured tissue samples of the oat plants were enriched in the lighter iron isotopes compared to the nutrient solution ($\Delta^{56}Fe_{plant\text{-}Fe(III)EDTA}$ = −0.34 to −0.62 ‰). In contrast to the bean plant, this finding holds regardless of whether roots were included in the mass balance or whether only the above-ground organs are taken into account (Figure 2).

At all three harvests the roots, stems and the cotyledons had iron isotopic compositions that are indistinguishable within the 2 standard deviations. From the second to the third point of harvest the first leaf evolved towards a lighter iron isotope composition by 0.3 ‰, at the same time the iron concentration doubled. The iron isotopic composition of the second and third leaf and the seed remained constant during growth whereas leaf 4 evolved towards slightly lighter compositions.
With mass balance (equation *1*) the total iron isotopic composition of the oat plants for each point of harvest was calculated (Table 3). It was found that oat plants were uniformly lighter by about 0.5 ‰ than Fe(III)-EDTA at all three growth stages.

Discussion

Iron isotope fractionation in bean as a model of strategy I plants

Fractionation during uptake of iron by the bean plant

Mass balance (equation *1*) shows that iron of the complete strategy I plant bean was about 1.2 ±0.11 ‰ lighter than the Fe(III)-EDTA and 0.2 ±0.11 ‰ lighter than iron of the original seeds at every point of harvest. Therefore (i) uptake of iron by the bean plants from a Fe(III)-EDTA solution led to an enrichment of light iron isotopes, (ii) the fractionation factor for iron uptake by bean plants grown in nutrient solution was constant during all growth stages and (iii) this enrichment of light iron isotopes is compatible with a reduction step before uptake.
A similar enrichment of light iron isotopes has been found in strategy I plants grown on two distinct types of soil substrate (Guelke and von Blanckenburg 2007), where iron in some plant parts was up to 1.6 ‰ lighter than the plant-available iron in the respective soil substrate. This similarity indicates that the sense of isotope fractionation of strategy I plants do not depend on the type of the growth medium or iron availability. Further evidence for reductive uptake of light iron isotopes is provided by the roots of the bean plants, which were all depleted in heavy iron isotopes when compared to the growth solution. However, in contrast to the bulk plants, the composition of the roots also changed during growth. Roots of bean plants were enriched in the light iron isotopes by 1 ‰ compared to the Fe(III)-EDTA at the first point of harvest, by 0.9 ‰ at the second point of harvest and 0.8 ‰ at the third and fourth point of harvest. With 200-400 µg/g the roots furthermore yielded much higher iron concentrations than the plant tissues with 60-80 µg/g. Therefore, it is likely that these elevated iron concentrations are due to iron contained in solids ($FePO_4$ or $Fe(OH)_3$) that were precipitated in the apoplast. The change of the root $\delta^{56}Fe$ cannot be attributed to a change in the $\delta^{56}Fe$ of the nutrient solution as the nutrient solution was renewed every 2 days. Thus, iron isotope fractionation by bacterial growth within the nutrient solution is unlikely. Reduction of iron by the reductase in the plasma membrane of root cells most likely leads to the observed enrichment in ^{54}Fe over ^{56}Fe in the above-ground plant organs and also within the roots tissue and the roots apoplast (Table 2). The latter, however, contains a partially re-oxidized solid reservoir. Following from this finding it can be hypothesized that roots were successively depleted in lighter iron isotopes, as light iron isotopes from storage molecules in the roots or apoplast were transported preferentially into younger plant parts, probably involving another reduction step of the apoplast iron. Nevertheless, it is also possible that not all Fe(III)-EDTA was washed out but small amounts remained in the apoplast of the roots which biased the $\delta^{56}Fe$ value of the roots. In addition it has recently been demonstrated that besides Fe(II) after reduction complete Fe-chelates can be taken up by plants (Orera et al. 2010). However, the uptake of Fe(III)-EDTA should not result in detectable mass-dependent isotope fractionation as this complex is too big to cause detectable iron isotope fractionation. Hence Fe(III)-EDTA uptake is not a mechanism that can explain the ^{56}Fe-depleted signature of the bean plant.

Fractionation during translocation of iron by the bean plant

With that isotope fractionation model during uptake in mind the distribution of iron isotopes between the different organs of bean plants can be discussed. It was previously observed that from the oldest to the youngest leaf iron in leaves of soil-grown plants became increasingly enriched in the lighter isotopes (Guelke and von Blanckenburg 2007). The same pattern emerged in bean plants in the present study although plants were grown on Fe(III)-EDTA. Hence in bean as a representative of strategy I plants the isotope fractionation patterns during both uptake and translocation do not differ depending on whether natural soil or an artificial solution is used as growth medium.

Iron isotope fractionation during uptake alone cannot be responsible for the observed patterns in the bean plants. Such an open-system fractionation during uptake was discussed in Guelke and von Blanckenburg (2007). If an infinite iron pool was to supply the roots, and the iron isotope fractionation during uptake was also constant, the δ^{56}Fe values in all parts of strategy I plants would be identical. Such uniform compositions have never been observed in strategy I plants. Rather, during growth, iron in older leaves of bean evolved towards heavier and iron of new young leaves towards even lighter compositions. Newly grown seeds showed the lightest iron isotope composition with a $\Delta^{56}Fe_{plant\text{-}Fe(III)\text{-}EDTA}$ of up to −2.5 ‰ at the fourth harvest point. Therefore fractionation during translocation of iron is adding to the iron isotopic shift induced into strategy I plants during uptake. This scenario, called "fractionation during uptake and translocation as an open system" in Guelke and von Blanckenburg (2007) can be seen as a series fractionation steps supplying light iron from older into younger plant parts. A series of consecutive iron reduction steps lead to increasingly fractionated isotope ratios during growth of the plant.

Since mass balance indicates, however, that translocation steps alone can explain the enrichment of light isotopes during growth, but not the amounts required for growth, fresh iron from uptake is continuously mixed into the plant, too. This additional iron can be supplied by the apoplast (which evolves towards heavier residual iron in the process), or by fresh iron taken up by reduction from the growth solution. This effect can be demonstrated with a simple example. The amount of iron lost from the cotyledon and leaves 1-3 during growth was ca. 700 µg if not all iron was remobilized from the dying cotyledon and first leaf (Table 2). Iron contained in the fruits (fruit 1 and 2 including fruit shells) amounts to ca. 1900 µg, therefore about ca. 40 % of the fruits' iron has been retranslocated from the early plant parts. The residual 60 % originated from iron uptake via the roots. The combination of these two processes leads to a decrease of the δ^{56}Fe value during growth of the plants. Regardless of the actual details of the process, fractionation during translocation is the mechanism that best fits the decrease in δ^{56}Fe from older to younger organs of strategy I plants.

Which mechanisms during translocation may potentially be responsible for the fractionation of iron isotopes? Changes of the binding form and redox state of iron are expected to result in isotope fractionation whenever they are not complete. An enrichment of the lighter iron isotopes can occur during non-congruent reduction of iron in the root apoplast. In this process the heavier iron isotopes are concentrated in the oxidized and precipitated apoplastic iron pool - which is observed. Fractionation of iron isotopes can also occur during release of iron into xylem vessels where it is transported as Fe(III) (Tiffin 1966) or uptake into leaf tissues where iron has to be reduced for transport across the plasma membrane (Briat et al. 2007). Non-complete oxidative phytoferritin fixation during storage would result in light residual Fe(II)-NA. Also ligand exchange and change of the redox state during loading of iron to the phloem, transport inside it or unloading from the phloem can cause iron isotope fractionation (von Blanckenburg et al., 2009). As it has been shown that iron is transported as Fe^{2+} into the cell's vacuole and is stored probably as Fe(III)-complexes (Kim et al. 2006), it can be assumed that the mobilization of iron involves a reduction step and can therefore result in isotope fractionation favoring a relative accumulation of lighter iron isotopes in the soluble iron pool. Especially when the plant enters the generative growth phase, root activity usually decreases, so elements become retranslocated to sink tissues like the seeds or fruits (Curie et al. 2009). In summary it is concluded that seeds contain iron that mainly originates from light iron upon reduction in the root apoplast and from light remobilized iron from older leaves.

Iron isotope fractionation in oat as a model of strategy II plants

Fractionation during uptake of iron in oat as a model of strategy II plants

Mass balance (equation *1*) showed that iron of total oat plants was around 0.54 ‰ lighter than the Fe(III)-EDTA solution (Table 2) at all growth stages. This finding is in contrast to that of the first study on iron isotope fractionation in higher plants (Guelke and von Blanckenburg 2007; Guelke et al. 2010). In these studies iron in strategy II plants appeared to be similar or even slightly heavier than to that of the iron assumed to be plant-available in the soil substrate. The new results is consistent with Fe isotope data of an alpine strategy II plant (above ground biomass) determined by Kiczka et al. (2010) with a δ^{56}Fe of approx. −1 ‰ compared to the cortex which consists mainly of apoplastic iron. The main difference between these studies was the type of growth medium and therefore the iron

availability. In Guelke and von Blanckenburg (2007) plants were grown on two soil substrates, a sandy Cambisol and a loamy Stagni-Haplic Luvisol. Iron solubility is as low as 10^{-10} M in these kinds of soils (Briat and Lobreaux 1997). In this regard it is important to note that our speciation analyses resulted in 99.3 % of the EDTA being present as Fe(III)-EDTA$^-$ and displacement of Fe with other elements is unlikely (section "Materials and Methods"). Hence, plants in the growth solution were supplied with sufficient iron and were not required to induce their plant-specific iron mobilization strategies. All plant roots reduce Fe(III)chelates and transport Fe(II) through the plasma membrane by a constitutive plasma membrane-bound ferric reductase (Bienfait et al. 1985; Briat et al. 1995). Therefore it is likely that oat plants grown on a Fe(III)-EDTA nutrient solution reduce iron and take up the resulting Fe^{2+} as under non-limiting iron supplies.
However, the oat plant did not enrich ^{54}Fe as strongly as the bean plant. Therefore, a competing uptake mechanism, involving a different isotope fractionation factor, must have been in operation. In growth solution oat plants exude phytosiderophores too but they do this to a lesser extent than oat plants grown on soil, because upregulating of the strategy-specific processes only occurs when plants suffer from Fe-deficiency (Grusak and DellaPenna 1999). Additionally it is also possible that the Fe(III)-EDTA complex has entered the plant directly as has been demonstrated by Orera et al. (2010). Hence the reductive pathway competes with both phytosiderophore complexation and direct Fe(III)-EDTA uptake, and the respective isotope fractionation factors are weighted by the process in the resulting binary mixture. As both the Fe(III)-PS and the Fe(III)-EDTA complex as a whole are too big for mass-dependent isotope fractionation (relative mass differences are too small), the Fe(III)-PS membrane transport process should not result in any further fractionation. The Fe(III)-PS pathway might still involve isotope fractionation before uptake when ligands and chelates are exchanged. The direction of the reaction is determined by the stability constant of the respective complexes. Siderophores form multi-dentate and very stable complexes with dissolved Fe(III), with stability constants up to ~10^{50} (Hider 1984) but phytosiderophores are considerable less stable (stability constant 10^{18}, von Wirén et al. 2000). Fe(III)-EDTA has a stability constant of ~10^{25}. In an aqueous solution where both chelates are present at the same concentration, the more stable chelator will bind the metal. Therefore, when phytosiderophores are exuded into the nutrient solution, an equilibrium isotope fractionation allows for the partitioning of iron isotopes, where preferentially the light iron isotopes are bound to the phytosiderophores as the heavier iron isotopes are favoured by the complex with the strongest bonds (Urey 1947; Schauble 2004). This Fe(III)-PS complex can be subsequently taken up by YS1-type membrane transporters that mediate root uptake by the cotransport of metal-phytosiderophores with protons (Curie et al. 2001).
Additionally it is likely that precipitated $FePO_4$ or $Fe(OH)_3$ in the apoplast is remobilized by exuded phytosiderophores. Here the PS bind Fe(III) more strongly than $FePO_4$ or $Fe(OH)_3$ as Fe(III)-PS has a higher stability constant and thus the heavy iron isotopes will be enriched in the Fe(III)-PS complex. Thus iron in the oat plant is a mixture of light Fe(II) released during reduction of Fe(III)-EDTA or light Fe(III)-PS and this enrichment of light iron isotopes is damped by the amount of Fe remobilized from the apoplast. This Kiczka et al. (2010) study no artificial chelate was present but iron in the strategy II plant *Agrostis* was lighter than in the soil too. For that reason it is likely that the constitutive reductase reduced iron and which therefore lead to an enrichment of the light iron isotopes in the *Agrostis* and also in our oat plants.

Fractionation during translocation of iron in the oat plant
In contrast to the bean plants, all parts of the oat plants obtained similar δ^{56}Fe values at all growth stages. Furthermore, the iron isotope ratios of roots were identical to those of the above-ground tissues and remained constant during growth. We therefore conclude that iron isotope fractionation remains constant during growth and even if iron is remobilized from the roots this does not lead to fractionation. This finding points to differences in the way iron is translocated within bean and oat as representatives of strategy I and II plants.
As described in section 2 no consensus exists on the fate of the imported Fe(III)-PS complex in strategy II plants. Current thinking is that strategy I plants more frequently change the redox state of Fe during translocation, while in strategy II plants, iron remains to a larger extent in its ferric form,

also during ligand exchange. These differences potentially result in the substantial differences in the distribution of stable iron isotopes between these two plant types as found in this study.

Conclusions and potential applications

Previous studies have made use of radiolabelled iron (^{55}Fe or ^{59}Fe) to trace iron uptake and distribution in plants and to image its distribution within the plant (Brown et al. 1965). These studies have focused on the uptake and breakdown of synthetic or natural chelates, as well as on the shoot translocation rates of the iron supplied by those compounds (e.g. Reid and Crowley 1984; Roemheld and Marschner 1986; Crowley et al. 1992; Johnson et al. 2002; Cesco et al. 2004). Our new study demonstrates that stable isotopes too provide a novel tool to trace biogeochemical pathways of iron which possibly complement studies employing radiotracers. Stable iron isotopes can be used in two different ways: fractionation and tracer studies (e.g. Rodríguez-Castrillón et al. 2008), the latter employing an enriched stable iron isotope label. Both approaches permit to follow the natural cycles of iron and to study metabolic processes. In the present study fractionation study, the first of its kind, we show that a strategy I and a strategy II plant grown with artificial chelates differ in the way they induce redox processes during their iron translocation mechanisms. These are superimposed onto the distinct Fe acquisition systems these plants employ. In addition it is shown that uptake mechanisms of the strategy II plant oat depend on the Fe availability in its growth substrate. These findings suggest that fractionation studies with stable iron isotopes will become a complementary tool in the study of Fe uptake and translocation in plants as suggested recently by Álvarez-Fernández (2006) or Baxter (2009).

Acknowledgements

We are grateful to E. Neitzel-Rode and H. Stuetzel (Institute of Biological Production Systems, Leibniz Universitaet Hannover) for plant growth experiments and two anonymous reviewers are thanked for their constructive comments. This research was supported by the GermanNational Science Foundation DFG grant BL562-6.

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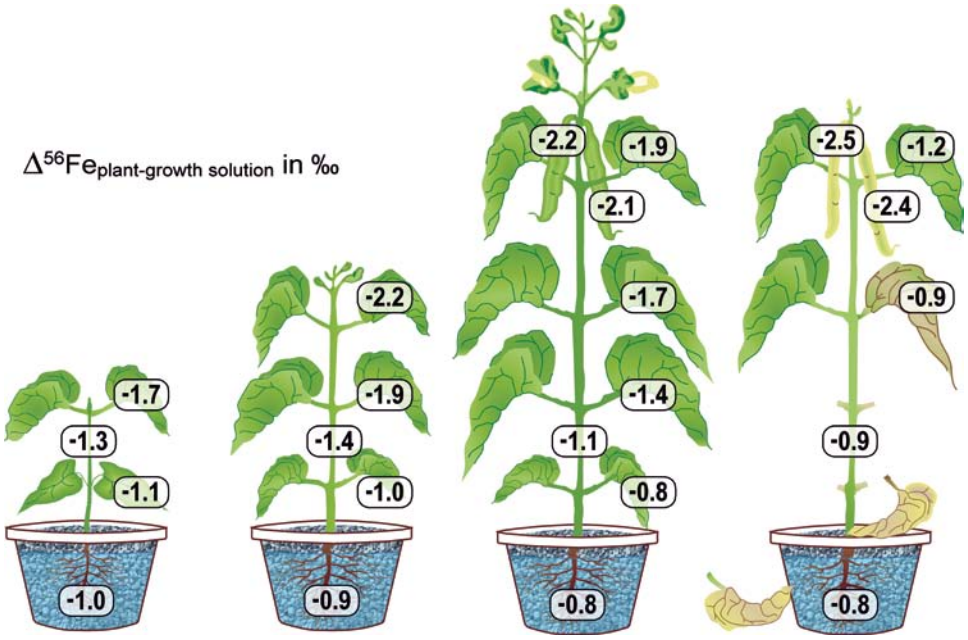

Figure 1 $\Delta^{56}Fe_{plant\text{-}Fe(III)\text{-}EDTA}$ in ‰ of bean plant tissues during growth. Note that compositions shown for roots are those total roots (tissue and apoplast) and are not those of the growth solution which is 0 ‰ at all growth stages.

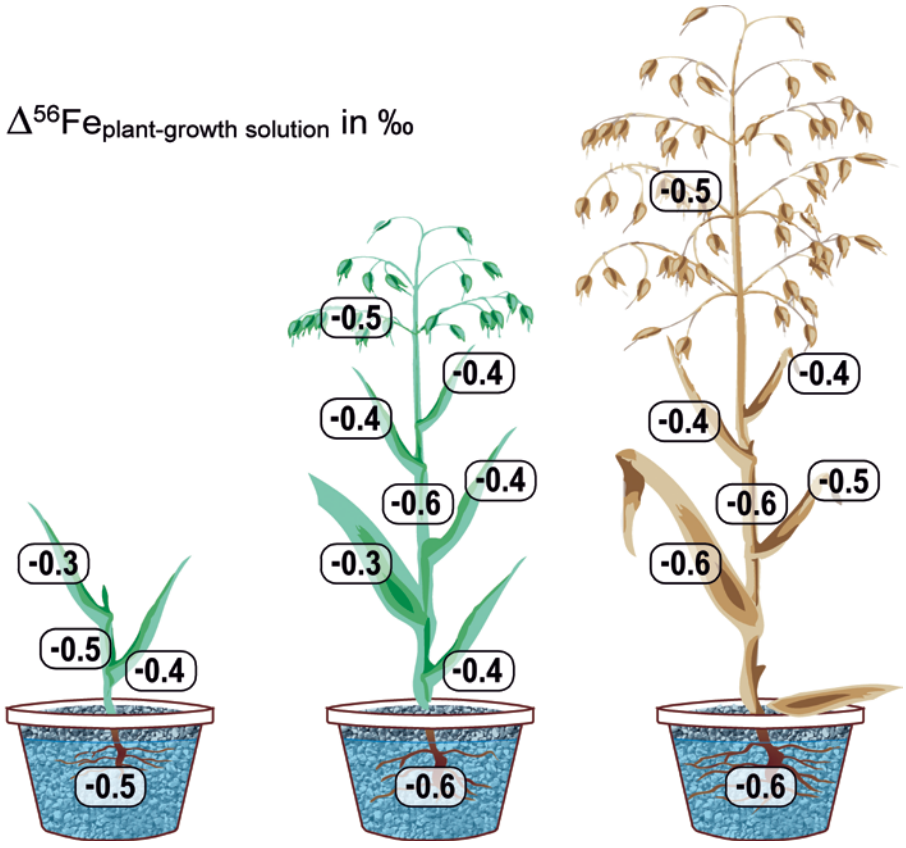

Figure 2 $\Delta^{56}Fe_{plant\text{-}Fe(III)\text{-}EDTA}$ in ‰ of oat plant tissues during growth. Note that compositions shown for roots are those total roots (tissue and apoplast) and are not those of the growth solution which is 0 ‰ at all growth stages.

Table 1 Iron concentration and $\delta^{56}Fe$ of the Fe(III)-EDTA solution, nutrient solution and quartz sand

	Fe concentration [µg/g]	$\delta^{56}Fe$ [‰]	2SD [‰][1]
Fe(III)-EDTA	1.10	0.56	0.11
nutrient solution	1.10	0.55	0.11
quartz sand HCl extract	0.06	----------	
quartz sand	2.50	0.25	0.11

[1] given as the 2 standard deviation reproducibility of replicate measurements

Table 2 Iron concentrations and stable Fe isotope compositions of plant tissues and total bean plants

		Bean					
number of harvest	plant part	dry mass [g] per pot	Fe concentration [µg/g]	error[1]	δ^{56}Fe [‰]	2SD [‰][2]	$\Delta^{56}Fe_{plant-FeEDTA}$ [‰]
	original seeds		53	± 2	-0.53	0.11	
1st harvest	roots	0.4	205	± 7	-0.45	0.11	-1.00
	cotyledon	6.0	128	± 4	-0.57	0.11	-1.12
	stem	2.2	38	± 1	-0.76	0.11	-1.31
	leaf 1	5.7	133	± 4	-1.19	0.11	-1.74
	above-ground organs[4]				*-0.72*	*0.19*[3]	*-1.27*
	Total plant[4]				***-0.70***	***0.22***[3]	***-1.25***
2nd harvest	roots	1.5	199	± 7	-0.35	0.11	-0.90
	cotyledon	5.0	70	± 3	-0.44	0.11	-0.99
	stem	5.5	22	± 1	-0.87	0.11	-1.42
	leaf 1	1.6	57	± 2	-1.32	0.11	-1.87
	leaf 2	7.9	64	± 2	-1.63	0.11	-2.18
	bud	0.4	62	± 2	-1.69	0.11	-2.24
	above-ground organs[4]				*-0.96*	*0.24*[3]	*-1.51*
	Total plant[4]				***-0.70***	***0.27***[3]	***-1.25***
3rd harvest	roots	3.0	311	± 11	-0.27	0.11	-0.82
	cotyledon	1.2	99	± 3	-0.21	0.11	-0.76
	stem	5.0	44	± 2	-0.54	0.11	-1.09
	leaf 1	3.1	63	± 2	-0.86	0.11	-1.41
	leaf 2	0.5	56	± 2	-1.14	0.11	-1.69
	leaf 3	11.6	78	± 2	-1.38	0.11	-1.93
	seed 1	0.9	52	± 2	-1.59	0.11	-2.14
	seed 2	3.5	48	± 2	-1.65	0.11	-2.20
	above-ground organs[4]				*-0.85*	*0.29*[3]	*-1.40*
	Total plant[4]				***-0.62***	***0.31***[3]	***-1.17***
4th harvest	roots	6.0	433	± 15	-0.25	0.11	-0.80
	stem	1.5	81	± 3	-0.31	0.11	-0.86
	leaf 2	1.3	59	± 2	-0.38	0.11	-0.93
	leaf 3	8.0	68	± 2	-0.69	0.11	-1.24
	seed 1	3.1	49	± 2	-1.83	0.11	-2.38
	seed 2	19.6	43	± 2	-1.90	0.11	-2.45
	envelope of seed 1	1.8	24	± 1	-1.15	0.11	-1.70
	envelope of seed 2	6.7	17	± 1	-1.05	0.11	-1.60
	above-ground organs[4]				*-1.2*	*0.29*[3]	*-1.75*
	Total plant[4]				***-0.65***	***0.31***[3]	***-1.20***

[1] Errors are combined from weighing, dilution, instrumental count statistics and calibration error. Numbers refer to the last digits given for the concentration values

[2] given as the 2 standard deviation reproducibility of replicate measurements

[3] propagated from the 2 standard deviation reproducibilities of replicate measurements from all plant parts

[4] calculated with wt% fractions of the different plant tissues (equation *1*)

Table 3 Iron concentrations and stable iron isotope compositions of plant tissues and total oat plants

			Oat				
number of harvest	plant part	dry mass [g] per pot	Fe concentration [µg/g]	error[1]	δ^{56}Fe [‰]	2SD [‰][2]	$\Delta^{56}Fe_{plant-FeEDTA}$ [‰]
	original seeds		33	± 1	0.22	0.11	
1 st harvest	roots	0.4	1053	± 37	0.04	0.11	-0.51
	cotyledon	1.2	77	± 3	0.18	0.11	-0.37
	stem	0.6	42	± 2	0.04	0.11	-0.51
	leaf 1	2.3	48	± 2	0.21	0.11	-0.34
	above-ground organs[4]				*0.09*	*0.19*[3]	*-0.46*
	Total plant[4]				***0.06***	***0.22***[3]	***-0.49***
2 nd harvest	roots	1.0	405	± 14	-0.05	0.11	-0.60
	cotyledon	0.1	62	± 2	0.14	0.11	-0.41
	stem	5.3	24	± 1	-0.06	0.11	-0.61
	leaf 1	0.7	55	± 2	0.27	0.11	-0.28
	leaf 2	0.5	67	± 2	0.19	0.11	-0.36
	leaf 3	0.5	63	± 2	0.18	0.11	-0.37
	leaf 4	0.2	55	± 2	0.19	0.11	-0.36
	seed	2.7	27	± 1	0.05	0.11	-0.50
	above-ground organs[4]				*0.03*	*0.29*[3]	*-0.52*
	Total plant[4]				***-0.01***	***0.31***[3]	***-0.56***
3 rd harvest	roots	2.0	469	± 16	-0.01	0.11	-0.56
	stem	3.6	15	± 1	-0.05	0.11	-0.60
	leaf 1	0.1	120	± 4	-0.07	0.11	-0.62
	leaf 2	0.5	52	± 2	0.09	0.11	-0.46
	leaf 3	0.1	53	± 2	0.14	0.11	-0.41
	leaf 4	0.1	62	± 2	0.03	0.11	-0.52
	seed	3.3	11	± 0	0.07	0.11	-0.48
	above-ground organs[4]				*-0.04*	*0.27*[3]	*-0.59*
	Total plant[4]				***-0.02***	***0.29***[3]	***-0.57***

[1] Errors are combined from weighing, dilution, instrumental count statistics and calibration error. Numbers refer to the last digits given for the concentration values

[2] given as the 2 standard deviation reproducibility of replicate measurements

[3] propagated from the 2 standard deviation reproducibilities of replicate measurements from all plant parts

[4] calculated with wt% fractions of the different plant tissues (equation *1*)