Final report (NKFI PD-111979)

Investigation of the main chemical factors influencing the effectiveness of iron uptake in plants – a Mössbauer spectroscopic study

Introduction

In the project, chemical factors influencing the iron uptake of plants were investigated with Mössbauer spectroscopy and plant physiological methods. The work can be divided into three main parts:

1. We have studied the iron speciation both in solution and in the plant tissues in the presence of organic compounds related to plants or synthetized by the plants. Among others, the effect of organic acids (citric and ascorbic acid), phenolics and additionally, synthetic chelates (*o-o'*-EDDHA, EDTA) and a carbonic amino acid (nicotianamine) was studied.

2. The effect of the oxidation state of the iron components (with the help of +2 and +3 Fe-complexes) were investigated. Treatments both through the roots and leaves were applied.

3. The effect of arsenic compounds (As(III)/As(V)) on the iron speciation in the nutrient solution and in roots of strategy I and II plants were investigated.

Main results achieved

1. Iron speciation in the presence of plant-related compounds – frozen solution Mössbauer measurements

$^{57}Fe^{3+}$-citrate (cit) complexes were prepared from $^{57}FeCl_3$ salt at different Fe:ligand ratios (1:1.1; 1:3; 1:10; 1:50, 1:100), and the speciation in solution was tested with Mössbauer spectroscopy in the pH range 4.5-7.5. In accordance with previous results obtained with other natural Fe^{3+} complexes, in the case of 1:1.1 Fe:ligand ratio, only oligomeric/polymeric Fe^{3+} species could be found, in distorted octahedral O_6 environment. At slightly basic pH, the changes of the Mössbauer parameters indicate a higher hydrolysis rate of Fe^{3+} than at pH 4.5,

however, no precipitate was formed at these circumstances. By applying more cit, the speciation of Fe^{3+} has changed significantly, namely, the high cit to Fe^{3+} ratio caused the formation of monomeric Fe^{3+}-cit complex while the concentration of oligomeric Fe^{3+}-species decreased.

The results also contribute to the Fe^{3+}-complex preference of the chloroplast Fe uptake system which is one of the most important factor in the utilization of iron in leaves. Since neither there are literature information on the *in vivo* chemical from of iron present in the cytoplasm (the concentration of iron is lower than the threshold of techniques such as Mössbauer spectrometry that are sensitive to the microenvironment of iron), nor there can be found any methods of cytoplasm isolation that guarantee the unchanged iron microenvironment, the indirect determination of this *in vivo* form is the only way to get valid information. For this, the above mentioned Fe^{3+}-complexes prepared in buffered solutions for Mössbauer measurements serve also as model systems for the iron uptake assay of chloroplasts. Beside the Fe^{3+}-cit complexes, Fe^{3+}-malate 1:1.1, Fe^{3+}-nicotianamine (NA) 1:1.2 (carbonic amino acid complex synthetized also by plant cells), and Fe^{3+}- *o-o'*-EDDHA (synthetic N-containing chelate) were measured. NA is also reported previously to form stable complexes also with ferrous iron, thus, Fe^{2+}-NA 1:1.2 complexes were also included in the investigations. The characteristics of iron-NA complexes were tested by Mössbauer spectroscopy both at 80 K and, in a frame of a cooperation, at liquid helium temperature in a presence of external magnetic field with Jiří Pechoušek, Libor Machala and Radek Zbořil (Department of Experimental Physics and Physical Chemistry, Palacký University, Czech Republic). In the case of high stability iron-complexes (NA and *o-o'*-EDDHA), the Mössbauer measurements showed mainly the presence of a monomeric, high spin Fe^{3+} in octahedral coordination. However, dimeric compound was also found in the case of Fe^{3+}-NA complex. The high cit content (Fe:ligand ration higher than 1:10) also resulted in the formation of more monomeric compound compared to the 1:1.1 sample. No significant redox transition of iron could be found. The uptake form ferric-carbonic acid complex sources at a ratio of low complexing compound to iron was significantly higher compared to that complexes and chelates where iron also binds to N ligands, but also compared to that complexes, where the ratio of the complexing citrate was high to iron. No direct correlation could be found between the speciation in water solution and the Fe-uptake rates, which suggests the preference of a well-defined Fe-complex structure (e.g. trimeric Fe_3-cit_3 complex) in the iron uptake system.

Data were presented on the 18th International Symposium on Iron Nutrition and Interactions in Plants, Madrid, Spain (2016), and a manuscript entitled "Müller B, Kovács K, Pham HD, Halász K, Kavak Y, Pechoušek J, Machala L Zbořil R, Fodor F, Klencsár Z, Solti Á: Iron uptake of chloroplasts prefers ferric-citrate over iron-nicotianamine complexes in *Brassica napus*" has been submitted recently to New Phytologist.

Fe^{2+}-ascorbic acid (asc) mixtures were prepared from both $^{57}FeCl_3$ and $^{57}FeSO_4$ salts. The main goal of the experiments was to find proper conditions to maintain iron in the +2 oxidation state in nutrient solution for studying the redox transformations of Fe^{2+} in iron sufficient and iron deficient roots. In our experiments, 1:3 Fe:asc mixtures were prepared at pH 4.5, 5.5, 6.5 and 7.5. In the case of pH 4.5 and 5.5, total reduction of Fe^{3+} could be found while in the case of pH 6.5 and 7.5, the solutions contained also significant amount of Fe^{3+} (10 and 90%, respectively). The Fe^{2+}-asc solutions prepared from $FeSO_4$ were stable against oxidation for at least 60 min at acidic pH (<5.5) while at pH~6.0-7.5, the partial oxidation of Fe^{2+} could not avoided. At pH 7.5, after 1 day aging at air, 15% Fe^{3+} could be found. According to these results, Fe^{2+}-asc complex for plant experiments was prepared from $FeSO_4$ and was used only in slightly acidic nutrient solutions. At calcareous soil conditions where the pH of the nutrient solution is >6.5, only Fe^{3+}-asc could be applied. The Mössbauer spectra of Fe^{3+}-asc indicate that the speciation of Fe^{3+} in presence of ascorbate is like those found in the Fe^{3+}-cit complex, namely, partial hydrolysis of Fe^{3+} occurs but without the formation of Fe^{3+}-hydroxide/oxide precipitates. According to the Mössbauer parameters one can suggest that not only ascorbic acid itself but also the oxidized products of it coordinate to Fe^{3+}.

For the studies of natural phenolic compounds (ph), water extract of green tea leaves was prepared since both the conditions of extraction and the chemical properties (composition, free radical scavenging activity, etc.) are well characterized. Extraction was performed in distilled water (2g/100 ml) without any organic solvents. The total phenolic content was measured with the Folin-Ciocalteu's method which revealed (after filtration) lower phenolic content (determined as gallic acid equivalent, ~31 mg/L) than reported in literature. Maximal complexing capacity (MCC) of Fe^{3+} with phenolics was measured applying the method of [Villén, M.; Cartagena, M.C.; Bravo, R.; García-Mina, J.M.; Martín de la Hinojosa, M.I.; Lucena, J.J. (2007). J. Agric. Food Chem. 55:5746-5753]. The titration curve obtained for Fe^{3+}-ph was similar as found for Fe^{3+}-amino acids by Villén et al. (2007) and thus, it can be suggested that ph-extract from tea leaves have lower complexing capacity compared to the

criteria for the inclusion of this Fe-product in European Regulations on Fertilizers. Mössbauer studies of $^{57}Fe^{3+}$-ph solutions (prepared from $^{57}FeCl_3$ and $^{57}Fe(NO_3)_3$) showed reduction of Fe^{3+} in the acidic pH-range (70% of total iron was reduced at pH 4.5) while at pH 7.5, only Fe^{3+} could be found. From our results, we concluded that at calcareous soil conditions, only Fe^{3+}-ph can be used while in the case of foliar treatments (at slightly acidic pHs), also the reduction of Fe^{3+} must be considered. It must be mentioned that in contrast to Fe^{3+}-cit and Fe^{3+}-asc complexes, in the Fe^{3+}-ph solutions the formation of Fe^{3+}-hydroxide/oxide precipitate (at pH 4.5-7.5) cannot be avoided. Like the phenolic compounds, other natural and biodegradable iron complexes with lignosulfonates, gluconates and humates were investigated. The results showed again the formation of ferrihydrite-like precipitates probably in a nanosized and/or in amorphous form. The efficacy of these compounds was tested later, too.

2. Iron speciation in Strategy I and Strategy II plants grown with the iron complexes characterized before. Distribution and utilization of iron from Fe^{2+}- and Fe^{3+}-complexes.

2.1. Mössbauer investigations of the roots

Iron sufficient cucumber, wheat and rice were grown with Fe^{3+}-cit and Fe^{2+}-asc in modified Hoagland solution. In both cases, in the roots of both strategy I and II plants, only Fe^{3+} compounds could be found indicating that iron is accumulated as Fe^{3+}-compounds. These species are suggested to be localized mainly in the apoplasm (see also the results of washing experiments described below) and are suggested to form nanosized iron-oxide/hydroxide aggregates.

Iron deficient cucumber, wheat and rice were grown without iron, then they were transferred into Fe^{3+}-cit and Fe^{2+}-asc containing solutions. The iron treatments were carried out applying different iron concentrations (10, 50, 100, 500 μM) for 30 min or 500 μM iron concentration for different time intervals (10, 20, 30, 60, 90, 180 min and 1 day). All samples were produced in duplicate: one of the plant roots was frozen after removing the nutrient solution from the root surface without chemical treatments, while the other one was washed with the reductive washing procedure (bipyridyl+natrium-dithionite).

In the case of Fe^{3+}-cit supply, the results showed the transient accumulation of Fe^{2+} in the apoplasm of strategy I plants, while no reduced iron occurred in strategy II plants. The main

chemical form of iron turned out to be Fe^{3+}-cit probably located in the xylem. In the iron deficient case, no Fe^{3+} accumulated in the apoplasm could be found. Data were published in: Kovács K, Pechoušek J, Machala L, Zbořil R, Klencsár Z, Solti Á, Tóth B, Müller B, Pham HD, Kristóf Z, Fodor F (2016) *Planta 244: 167–179*

In the case of Fe^{2+}-asc supply, the results indicated significant differences in the iron speciation in iron deficient plants. At the lowest Fe^{2+} concentration, only Fe^{3+} could be found that shows immediate oxidation of Fe^{2+} after uptake. The 50, 100 μM concentrations resulted in the appearance of Fe^{2+} (~5-10%) demonstrating that the uptake and oxidation rate is limited and thus the accumulation of Fe^{2+} is also possible, probably in the cell wall. However, to assure Fe^{2+} in excess, in the time range experiments, 500 μM iron concentration was applied (this resulted in ~30% Fe^{2+} excess). In cucumber, the relative Fe^{2+} content showed a sharp increase in 10-60 min while saturation after 180 min. This can be well understood in terms of the strategy I iron uptake. Namely, Fe^{2+} is transported through the membrane than oxidized inside the cell. The accumulation of iron in the cell is only in the +3-oxidation state while Fe^{2+} can be accumulated in the apoplasm bounded partially by the cell wall components. In contrast, wheat showed a much higher oxidation rate: even after 10 min Fe^{2+} supply, more than 80 % of the total iron was in the form of Fe^{3+}. Longer Fe^{2+} supply resulted in slightly decreasing Fe^{3+} (~80-95 %) content. The rapid oxidation indicates that accumulation of Fe^{2+} is not favoured even in the apoplasm and thus, a very effective oxidizing system must exist probably in the apoplasm. The experiments were completed with rice, Mössbauer spectra of the roots grown at iron deficient conditions and then supplied with 500 μM Fe^{2+}-asc for 10, 20, 30, 60, 180 min were measured. In the case of rice, the gradual increase of the relative Fe^{3+} content of the roots in the 10-60 min iron supply period was similar to that found in cucumber. However, the relative Fe^{3+} contents measured were higher: while 10 min Fe supply resulted in ~20% Fe^{3+} in the case of cucumber, in rice, it was found to reach even ~50%. After longer Fe^{2+}-asc supply, the relative Fe^{3+} content reached the same saturation level (~90%) as found in cucumber. The results indicate that rice, despite belonging to strategy II iron uptake mechanism, also exhibit about strategy I features. Namely, Fe^{2+} can be accumulated in the roots but this accumulation is not as favoured and thus, the reoxidation occurs faster than in the case of cucumber.

All the Mössbauer experiments (wheat, cucumber, rice) were completed with measurements of the ^{57}Fe concentration of the roots with the help of ICP-MS technique. Considering the

total ^{57}Fe concentrations and the Mössbauer spectral areas, the absolute Fe^{2+}/Fe^{3+} content of the roots could be calculated. To estimate the iron content of the simplast, the reductive washing procedure (bipyridyl+natrium-dithionite) was also applied before the ICP-MS measurements which is believed to remove the iorn from the apoplast. The ICP-MS results showed that in the case of cucumber and rice, the (absolute) Fe^{3+} content of the roots increased parallel with the simplastic iron content while in the case of wheat, the increase of the Fe^{3+} was much higher than that of the simplastic iron. This difference suggests that in Strategy I plants (cucumber, rice), the Fe^{2+}-oxidation occurs only after the Fe-uptake into the simplast (Fe^{3+} is formed mainly in the simplast) while in Strategy II plants, the oxidation takes place already in the apoplast (before the Fe-uptake). The oxidation process occurring in the apoplast may be driven by either H_2O_2 as a by-product of cell wall lignification, or by O_2 that is required for the respiration of the root cells.

To study the localization of the absorbed iron, the reductive washing with bipyridyl + sodium-dithionite was applied. The Mössbauer spectra of the washed roots showed no Fe^{2+}-compound which was found before that confirmed Fe^{2+} was localized in the apoplasm. However, in the same roots, a new Fe^{2+} component (~20-40% of the total iron) could be found which could be assigned to Fe^{2+}-bipyridyl complex. This unequivocally demonstrates that the reductive washing procedure cannot mobilize all iron and the relative high amount of Fe^{2+}-bipyridyl complex present in the cell wall must be considered by the calculation of the apoplasmic/symplasmic iron contents. The Fe^{3+} component detected after the washing suggests that Fe^{3+} is complexed by citrate in the cell after iron uptake. No significant differences could be found in iron component after the reductive washing of strategy I and II plants.

Data were presented as poster and oral (invited) talk at the International Conference on the Application of Mössbauer Effect in Hamburg and St Petersburg in 2015 and 2017.

2.2. Plant physiological measurements

The ability of Fe^{3+}-cit, Fe^{3+}-ph and Fe^{3+}-EDTA (control) was tested to overcome chlorosis under calcareous conditions. In the experiment, cucumber was tested at pH~7.5 because this plant was found to be very effective in iron accumulation also at calcareous conditions. Recovery from iron deficiency was followed by the iron content of the chloroplasts and by measuring the photosynthetic activity in cooperation with the Department of Plant Physiology

and Molecular Plant Biology. iron deficiency treatment significantly decreased the iron content of the chloroplasts that affected the photosynthetic pigment biosynthesis and the photosynthetic performance of the plants. One day application of iron compounds caused no significant alteration in the iron content of the chloroplasts thus remained significantly lower compared to the non-deficient control plants. Nevertheless, a tendentious but not significant increase was observed in the chlorophyll content of the leaves: together with the accumulation of chlorophylls, the chlorophyll a/b ratio increased as a first sign of the reorganisation of the photosynthetic apparatus. The largest increase was found in plants regenerated by Fe^{3+}-cit, and Fe^{3+}-ph complex was less effective. One day regeneration also increased the maximal and actual quantum efficiencies of the photosystem II centres as vitality markers. Again, Fe^{3+}-cit proved to be more effective than Fe^{3+}-ph complex. In a Hendrickson's-type excitation energy allocation analysis, the ratio of non-functioning photosystem II reaction centres in plants regenerated on Fe^{3+}-ph complex also remained between the value of iron deficient plant and that of regenerated on Fe^{3+}-cit.

The natural and biodegradable iron complexes (e.g. Fe^{3+}-lignosulphonate; Fe^{3+}-gluconate) along with synthetic chelates of high stability (e.g. Fe^{3+}- *o-o'*-EDDHA) used in the solution experiments were also involved into utilisation experiments. The reduction of synthetic Fe^{3+}-chelates was effective, whereas natural complexes proved to be inert in a ferric chelate reductase assay. In contrast, iron deficiency alleviation experiment indicated that over the widely-used Fe^{3+}- *o-o'*-EDDHA chelate, natural iron substances Fe^{3+}-lignosulphonate and Fe^{3+}-gluconate are also effective substrates of the iron uptake system. Data were published in: Martín-Fernández C, Solti Á, Czech V, Kovács K, Fodor F, Gárate A, Hernández-Apaolaza L, Lucena JJ (2017) *Plant Physiology and Biochemistry 118: 579-588.*

2.3. Mobility experiments

For studying the possible mobilization of iron accumulated in the apoplast, iron supplied cucumber plants were grown and then, iron supply was withdrawn until reaching an iron deficient state. After this period, ascorbic acid or phenolics extracted from tea leaves were added to the iron deficient nutrient solutions both at acidic (pH ~5) and alkaline (pH~7.5) conditions. The possible iron mobilization and reutilization of iron from the apoplast upon the addition of these agents was monitored by measuring the physiological status of the new leaves formed in the treatment period. The physiological status was investigated by

chlorophyll *a* fluorescence induction that indicates the activity of photosynthetic electron transport processes. The physiological status seemed to be somewhat better when the nutrient solution during the treatment was alkaline instead of slightly acidic. This rather surprising better physiological status included a higher maximal quantum efficiency of photosystem II (PSII) reaction centres, a lower PSII inactivation and a higher energy allocation (actual quantum efficiency) to PSII reaction centres. Further treatment with ascorbic acid and tea extracts caused a slight decrease in these physiological functions under all treatment, while no significant differences were found between the two types of treatments, independently on the pH of the original nutrient solution. For the rather surprising results, the experiments were repeated but no significant effect of the added ascorbic acid or phenolics could be demonstrated. Unfortunately, the difference between the alkaline and acidic conditions could not be clarified.

2.4. Ferric Chelate Reductase (FCR) activity measurements

FCR activity of iron deficient strategy I root (cucumber) were measured after treatment with both Fe^{2+}-asc and Fe^{3+}-ph. Since the Mössbauer measurements of the complex solutions showed that Fe^{2+}-asc can be easily oxidized on air above pH 6, we concluded to perform the iron supply experiments only at acidic pH (pH of the nutrient solution containing iron was set between 5-6). In both cases the initial FCR activity of the iron deficient roots (200 nmol Fe/ (g FW min)) was in good agreement with previous results. The Fe^{3+}-ph supply has decreased the FCR activity even after 30 min iron supply (like Fe^{3+}-cit complex) but the Fe^{2+}-asc complex had only long-term effects. In latter case, the FCR activity has significantly decreased only after 24 hour of iron treatment. However, after the long-term treatment, the lowest value of the FCR activity was very similar in both cases, it reached approx. 20-30 nmol Fe/ (g FW min).

According to the data, the Fe^{3+}-ph compound was more effective in decreasing the iron deficiency of the roots already in short time while Fe^{2+}-asc was only effective in long term supply. The relatively slower utilization of Fe^{2+} may be understood by the slow oxidation rate of Fe^{2+} as already shown in the Fe-uptake experiments described in section 1 (where the possible accumulation of Fe^{2+} in the apoplast was demonstrated). This suggests also that inside the plants, Fe^{3+} will be formed and utilized, independently of the oxidation state of the

iron supplied in the nutrient solution (supported also by the Mössbauer results presented in Kovács et al. 2016).

Mössbauer and FCR data together with the physiological results were presented at international conferences (International Symposium on Iron Nutrition and Interactions in Plants, Madrid (2016); European Symposium on Atomic Spectrometry, Eger (2016) and published in Solti Á, Kovács K, Müller B, Vázquez S, Hamar É, Pham HD, Tóth B, Abadía J, Fodor F (2016) *Planta 244: 1303-1313.*

2.5. Foliar treatments

First, iron deficient cucumbers were grown at calcareous conditions and foliar treatment was applied twice on the second (already iron deficient) leaf. The treatment solution was prepared according to previous experiments conducted at the Plant Physiological Department: ^{57}Fe concentration was set to 2 mM, the pH was 4.5-5.0 and Tween-40 was applied as surfactant. The solution was spread uniformly with the help of a paintbrush on leaf surface. However, despite several attempts, the hairs on the leaf of cucumber caused problems to equally dissipate the solution and thus, treated spots were formed. For this reason, in further experiments, a new Strategy I plant was chosen instead of cucumber: young white cabbage (*Brassica oleracea*) plants were grown and treated the same way as described before. The smooth leaves were suitable for the foliar treatments, so all the experiments were carried out with this plant.

Cabbage was grown at iron deficient conditions in the same nutrient solution as used before in the case of cucumber. The foliar treatment was done on 3-weeks old plants, on the 3rd leaves. 5 parallel plants were used for each compound. The treatment solution (250µl/leaf - applied on both sides) contained 2 mM (and in the further experiments 5 mM) $FeSO_4$ / Fe^{2+}-asc / Fe^{3+}-cit / Fe^{3+}-ph and 0,1% Tween-40 (surfactant). Treatments were carried out two times in three days. After the treatment, Mössbauer spectroscopy, photosynthetic activity, chlorophyll content and root FCR activity were measured. Unfortunately, there was a rather large difference between the plants used as parallels, thus, the whole experiment (with all the iron compounds) was repeated 4 times.

First, the treated cabbage leaves were measured with Mössbauer spectroscopy freshly (in frozen state) without any washing procedure than also after washing them twice with distilled water. Surprisingly, the original iron species present in the treatment solutions could not be detected (only a minor high spin Fe^{2+}-compound in the case of Fe^{2+}-asc, indicating also the presence of the original compound). In the Mössbauer spectra, in the case of Fe^{2+}-compounds, the oxidation of Fe^{2+} to Fe^{3+} could be observed. No magnetically ordered phase was found, suggesting no crystalline Fe-oxide/hydroxide formation on the leaf surface. However, one must note that amorphous or superparamagnetic Fe-oxide/hydroxide formation (represented mainly by a doublet subspectrum in the Mössbauer spectra at these conditions) cannot be entirely excluded. According to the evaluation of the Mössbauer spectra, the main part of the iron was taken up by the leaf and is suggested to be built in Fe-S proteins and/or hem-type compounds (e.g. cytochromes) with characteristic parameters of δ=0,4-0,5 mm/s; Δ=1,0 mm/s and δ=0,2-0,3 mm/s; Δ=0,3-0,5) mm/s. These iron compounds could be found in all the treatments independently from the oxidation state of iron in the treatment solution, however, in the case of the Fe^{3+}-ph compounds, the spectral evaluation was rather unreliable because of the poor statistics of the measurements.

Since Mössbauer results indicated the utilization of the iron compounds, the physiological status of the treated leaf was investigated also by chlorophyll content measurements (SPAD measurements) and chlorophyll *a* fluorescence induction. The results showed that even after the first treatment, all compounds could increase the chlorophyll content and the efficacy of the photosynthetic reaction centres. In the case of the $FeSO_4$ and Fe^{2+}-asc compounds, the positive effect was significant only after longer time (before the second foliar treatment) compared to the Fe^{3+}-cit complex. One must note, that the Fe^{3+}-ph compound was not so effective in overcoming the chlorosis because the measured physiological parameters were very close to the negative control plants (plants grown without any iron supply).

In parallel, FCR activity of the cabbage roots treated only through the leaves were also measured at the 2nd day after the 2nd treatment. The average value of the FCR activity was 40-60 nmol Fe/ (g FW min) which is significantly lower than the negative control (~100 nmol Fe/ (g FW min)). In this relatively short time range, none of the plants has reached the value of positive control (normal, iron sufficient cabbage plant, (~10 nmol Fe/ (g FW min)). The results indicate that there is a feed-back signal from the leaves to the roots due to the iron supply on the leaves and will be further investigated at the signal transduction level by the

Plant Physiological group. (FCR function and signal transductions were not studied in present project but are subjects in an ongoing research work, project No. NKFIH-PZ1110).

In summary, the foliar treatments were most effective in the case of Fe^{3+}-cit, while effective with Fe^{2+}-asc, and $FeSO_4$ (despite the oxidation of Fe^{2+}). The effectiveness resulted not only in the physiological status of the leaves but also in the reduced FCR activity of the roots. We could conclude that the oxidation state of the compounds had only minor effect on the success of the foliar treatments, while the presence of citrate increased the efficacy. Phenolics extracted from tee leaves are not well suitable for the applications through the leaves.

3. Effect of arsenic compounds

According to the experiments carried out with the roots, the two most effective iron compounds were chosen: Fe^{3+}-cit and Fe^{2+}-asc.

In the first experiments, the effect of arsenite and arsenate on the iron compounds were investigated in the nutrient solution prepared with Fe^{3+}-cit and Fe^{2+}-asc. The concentration of the As(III) and As(V) were kept the same as that of the iron (0,01 M). The Fe:ligand ratios were 1:1,1 and 1:3, respectively, as applied in all the measurements before. The frozen solution Mössbauer spectra showed no significant difference compared to the solutions prepared without As. In the case of arsenate-ions, at pH~5, slow oxidation of Fe^{2+}-asc was observed resulting in ~15% Fe^{3+} of the total iron in 30 min. This was later considered in evaluating the Mössbauer data of roots supplied with Fe^{2+}-asc complex. The possible reducing effect of arsenite could not be demonstrated so arsenite was suggested not to change the chemical state/microenvironment of Fe^{3+}-cit.

For the experiments with plants, cucumber (strategy I) and wheat (strategy II) were chosen. Plants were grown both at iron sufficient and deficient conditions for two weeks in hydroponics with aeration, thus, only the oxidized form of As was applied (As(V)). In the case of sufficient iron supply, the concentration of iron was 10 μM (with 10 μM As(V)-compounds) while in the case of iron deficiently grown plants, a short time, high concentration iron supply was applied (100 and 500 μM, with 100 and 500 μM As(V)). The iron uptake in iron deficient cucumber plants pre-treated with arsenate was also investigated: in the sensitive period of the growth (8-9th day), As(V) was added to the nutrient solution (without iron), and then, these plants were supplied with 500 μM iron for 30 min (without As(V)). In each experimental setup, both Fe^{3+}-cit and Fe^{2+}-asc were used. Mössbauer spectra

of the excised roots in frozen state were recorded freshly to avoid any changes in the iron distribution during measurements.

The evaluation of the Mössbauer spectra of iron sufficient plants showed entirely Fe^{3+} compounds, as already observed before without As(V) (section 2.1.). The large line width suggests again, that Fe^{3+} is in the form several similar chemical environments and no Fe^{2+} accumulation is favoured in the roots. In the case of the iron deficient cucumber plants, supplied with 500 μM iron, both +3 and +2 iron components were present while wheat roots did not contain any Fe^{2+}. This is in good agreement with previous findings of the group, and also suggests that the presence of As(V) does not change the Fe^{2+}/Fe^{3+} ratio in Strategy I plants, if iron is supplied in high concentration. However, one has to note, that in the case of lower (100 μM) iron concentration, despite of the reducing capability of the cucumber, no or only very little (~10%) Fe^{2+} could be found.

To get more information on the reducing capacity of the cucumber roots, FCR activity measurements were carried out with excised iron deficient roots in the presence of As(V) and without it. As(V) pre-treated plants were also measured. The FCR activity were 12(±3) nmol Fe/ (g FW min) in the presence of As(V) while 11(±2) Fe/ (g FW min) when no As(V) was applied. No significant difference was found in the case of As(V)-pre-treated plants, the FCR was 12(±3) nmol Fe/ (g FW min) again.

In summary, according to our measurements, no significant effect of the As(V) on the iron reduction in cucumber and on the iron distribution in wheat could be found. Moreover, no changes in the FCR activity were demonstrated at these conditions.

In further studies, according to recent literature data [Khan, N. et al, *Advances in Agronomy*, 2016, 138:1-96], hydrophyte plants could be investigated where iron plaque formation was shown at anaerobe conditions and the inclusion of As in the Fe-precipitate was also demonstrated. However, these experiments were not subject of recent project.

The results of the arsenic compounds will be presented as oral talk at the 4th Mediterranean Conference on the Applications of the Mössbauer Effect in 2018 May, 27th-31st, Zadar, Croatia.

4. Summary of the main results

In summary, we could conclude that the oxidation state had no significant effect on the iron uptake and distribution in roots and/or leaves. It seems, that in the efficacy of iron compounds, the complexing agent has a crucial role: the most effective Fe^{3+}-complex was found to be the Fe^{3+}-cit among the applied natural complexing agents. Fe^{2+} had no beneficial effect even in the case of strategy I plants which exhibit a reduction-based iron uptake. Moreover, in the case of strategy II plants, where no reduction occurs, the fast oxidation of Fe^{2+} to Fe^{3+} was demonstrated indicating that in these plants, the presence of divalent iron is not favoured. The applied phenolic compounds were not effective enough to overcome iron chlorosis.

Cumulative IF of the published articles: **9.250**

Publications related to the finishing project are:

full papers:

Martín-Fernández C, Solti Á, Czech V, **Kovács K**, Fodor F, Gárate A, Hernández-Apaolaza L, Lucena JJ (2017) Response of soybean plants to the application of synthetic and biodegradable Fe chelates and Fe complexes. *Plant Physiology and Biochemistry 118: 579-588.;* link to Real repository: http://real.mtak.hu/id/eprint/63027 **IF 2.724**

Solti Á, **Kovács K**, Müller B, Vázquez S, Hamar É, Pham HD, Tóth B, Abadía J, Fodor F (2016) Does a voltage-sensitive outer envelope transport mechanism contributes to the chloroplast iron uptake? *Planta 244: 1303-1313.;* link to Real repository: http://real.mtak.hu/id/eprint/40124 **IF 3.263**

Kovács K, Pechoušek J, Machala L, Zbořil R, Klencsár Z, Solti Á, Tóth B, Müller B, Pham HD, Kristóf Z, Fodor F (2016) Revisiting the iron pools in cucumber roots: identification and localization. *Planta 244: 167–179.*; link to Real repository: http://real.mtak.hu/id/eprint/40558 **IF 3.263**

conference abstract in periodical:

Müller B, **Kovács K**, Halász K, Kavak Y, Gyuris B, Fodor F, Sárvári É, Solti Á (2017): The chemical forms of Fe used as in vivo substrate in uptake process of chloroplasts. *Journal of Plant Physiology and Pathology 5:5 (Proceedings of 3rd Global Summit on Plant Science) doi: 10.4172/2329-955X-C1-012*

conference appearance & abstracts:

Kovács K, Fodor F, Solti Á (2015) Mössbauer spectroscopic investigation of plant roots supplied with ^{57}Fe(II)-ascorbate complex in: *Abstracts of the 33rd International Conference on the Application of Mössbauer Effect,2015, Hamburg, Germany, T-07-2*

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<u>*education*</u>

The frozen solution Mössbauer measurements with arsenic compounds were partly carried out by <u>Katalin Ágostai</u> in the frame of the summer experimental work 2017 (Chemistry BSc). These will be also included in her BSc thesis work started in 2018.

The foliar treatments were used in the undergraduate research work of <u>Vanda B Marosi</u> (Biology BSc) and presented as poster at national scientific conferences organized for students:

Marosi VB, Fodor F, Kovács K (2017) THE EFFECTS OF FOLIAR TREATMENT IN PLANT IRON DEFICIENCY, XI. Szent-Györgyi Albert Konferencia, 2017. április 7-8.