OTKA NN 84307 – ERA Chemistry Project

Project closing report

As a global goal, the project tried to elucidate the constraints that limit the effectiveness of Fe-complexes and chelates as Fe fertilizers when applied through soils, nutrient solutions or foliar sprays. Since the efficacy of these compounds is related to the Fe speciation in the complex or chelate, Mössbauer spectroscopy proved to be an effective tool to achieve this objective. The results of the Fe speciation were completed with bioassay experiments that could reveal direct evidences on the biological efficacy of the natural Fe-complexes tested in the project.

For this aim, three specific objectives (9 Activities) were proposed and fulfilled. In the first year the accomplishment of project tasks were slow because the Spanish partner group had reduced financial support and they could not employ the planned personnel. The samples to be measured in Hungary arrived rather slowly from them during the whole project but the extension of the project duration with 6 months made it possible to accomplish all tasks. Both participants got acquainted with the others' methodologies during the visits to the laboratories. Three researchers from the Hungarian group visited the Spanish lab 4 times while 4 of researchers of the Spanish group visited the Hungarian lab a total of 4 times. The Hungarian participants have attended 1 Hungarian and 3 international conferences for the presentation of the results. The total number of publications accepted so far is 15 (6 in SCI journal, 3 in non-SCI journals and 6 international conference abstracts). Moreover there are still 3 manuscripts under evaluation submitted to SCI journals and we are currently preparing 3 manuscripts for submission to SCI journals. The results contributed to 1 PhD Theses (in Hungarian, with English summary) and to 1 Thesis of the Student Competition for Biologist Students (in Hungarian). The project results are summarized below:

OBJECTIVE 1: Study of Fe speciation and microenvironment in natural complexes.

Activity 1: Influence of the Fe:complex ratio in Fe speciation in the complex.
Activity 2: Evaluation of the influence of the pH on Fe speciation in the complex.

Gluconate (Sodium D-gluconate, GL), leonardite (water soluble alkaline extraction of leonardite coal, LN) and six different commercial lignosulfonates (LS) were used as complexing agents. The following lignosulfonates were tested: eucalyptus (hardwood) and spruce (softwood), both liquids; standard lignosulfonates, obtained from Sigma-Aldrich: lignosulfonic acid, sodium salt, lignosulfonic acid, sodium salt, sugared and lignosulfonic acid, calcium salt. Iron complexes were prepared with both $FeSO_4.7H_2O$ and $FeCl_3.6H_2O$. Three different Fe:complex ratios (1:1.1, 1:1.5 and 1:2) were studied at two different pH values (pH=4 and 7).
The Mössbauer analysis of the Fe-LS compounds demonstrated the complex formation between the Fe^{2+}/Fe^{3+} salts and the LS ligands at the studied Fe:LS ratios and pH values. In the Fe^{2+}-LS complexes, high oxidation rate of Fe^{2+} to Fe^{3+} was found that increased as the pH and the LS:Fe ratio were increased. This suggests a rather weak coordination of Fe^{2+} to LS ligands and indicates the sensitivity of the Fe^{2+}-LS compounds to oxidation. The observed oxidation has to be taken into account also as Fe^{2+}-LS complexes are applied in the field as Fe-fertilizers. In the case of Fe^{3+}-LS compounds, both Fe^{3+}-LS complex and amorphous Fe^{3+}-oxyde-hydroxide formation could be suggested. The frozen solution experiments demonstrated the presence of -OH/O-bridged $\mu(\text{-OH/O-})_xFe_yLS$ type complexes that hints at a significant difference compared to synthetic Fe^{3+}-chelates formed with EDTA or analogous ligands in the same concentration and pH range since these compounds were shown to build monomeric Fe^{3+}-species.
The Mössbauer spectra of the solid Fe-GL samples showed similar results as observed in the case of $Fe^{2+/3+}$-LS complexes. However, our results indicated that Fe^{2+}-GL complexes are more stable against oxidation than Fe^{2+}-LS complexes. The Mössbauer parameters of the Fe-GL complexes did not change

significantly as the pH or the Fe:ligand ratio were varied during preparation indicating well defined coordination sites for both Fe^{2+} and Fe^{3+} in the Fe-GL complexes.
According to the Mössbauer measurements of Fe-LN complexes, we can conclude that LN was not suitable to complex Fe^{2+} at neutral and alkaline pHs because the oxidation rate of the Fe^{2+} to Fe^{3+} was too high (the Mössbauer spectra showed only Fe^{3+} components). At lower pH, however, the relative amount of Fe^{2+} was 80% which indicates that Fe^{2+}-LN complexes can be prepared and used at slightly acidic pH. In the case of Fe^{3+}-LN complexes, the Mössbauer spectra showed an Fe^{3+} component in distorted octahedral O_6 coordination with relatively large line widths indicating slightly different Fe^{3+} microenvironments present in the samples. It is noteworthy, that in the case of Fe^{3+}-LN complexes no reducing effect of the ligand could be observed although many authors report about the possibility of reduction of Fe^{3+} ions by humic substances. In the case of Fe^{3+}-LN prepared at slightly acidic pH, no complex formation between Fe^{3+} and leonardite could be observed which indicates that Fe^{3+}-LN compounds are suggested to be prepared and used only at neutral or slightly alkaline pH.

From the results obtained in Objective 1 we can conclude that all the products tested have a lower maximum complexing capacity value for Fe^{2+} than for Fe^{3+} which suggests that these fertilizers could be prepared more effectively by adding an iron (III) inorganic salt to the complexing agent solution.
In respect to the agronomical relevance of the Fe^{2+}/Fe^{3+} speciation, in the case of leaf applications the strong Fe^{3+}-complexes could be preferred, while root uptake in hydroponics could be more related with the presence of weak bonding sites.

The results were presented at following international conferences (abstracts) and are summarized in the following SCI journals:

Kovács K; Lucena JJ; Hernández-Apaolaza L; Carrasco J; Czech V; Fodor F; Vértes A: Study of iron speciation in natural Fe(II) and Fe(III) complexes used as iron fertilizers, International Conference on the Application of Mössbauer Effect, Kobe, Japan, 2011 – abstract

Kovács K, Carrasco J, Czech V, Hernández-Apaolaza L, Lucena JJ, Fodor F, Vértes A†: Mössbauer study of iron speciation in natural Fe(II) and Fe(III) complexes used as iron fertilizers, 16th International Symposium on Iron Nutrition and Interactions in Plants, Amherst, MA, USA, 2012 – abstract

Carrasco J; Kovács K; Czech V; Fodor F; Lucena JJ; Vértes A; Hernández-Apaolaza L: Influence of pH, iron source and Fe/ligand ratio on the iron speciation in lignosulfonate complexes studied using Mössbauer spectroscopy. Implications on their fertilizer properties. *Journal of Agricultural and Food Chemistry*, 60:3331-3340, 2012 (IF =2.906)

Kovács K; Czech V; Fodor F; Lucena JJ; Santos-Rosell S; Hernandez-Apaolaza, L: Characterization of Fe-leonardite complexes, as novel natural iron fertilizers. *Journal of Agricultural and Food Chemistry*, accepted for publication (DOI: 10.1021/jf404455y) (IF=2.906)

OBJECTIVE 2: Study of the stability of Fe chelates and complexes under calcareous soil conditions or in interaction with calcareous soils.

Activity 3: Stability of Fe complexes and Fe chelates under calcareous soils conditions.

Fe^{2+}/Fe^{3+}-LS (three different lignosulfonates form hardwood, eucalyptus, and one form softwood, spruce) prepared with $FeSO_4.7H_2O$, $FeCl_3.6H_2O$ and were allowed to react in a 0.05 M Ca^{2+} solution at various pH (4-11) to study the stability of the complexes. After 3 days, the solutions were filtered and the Fe concentration in the filtrate was determined by AAS. The percentage of soluble Fe remaining in the Ca^{2+} containing solution showed an abrupt decrease between pH 8 and 9 in the case of LS eucalyptus, and between pH 9 and 10 in the case of LS spruce. At pH=8.5, the most effective complexing agent was the LS spruce (100% of total iron remained in solution) and one of the

chemically modified LS eucalyptus (50 % of total iron remained in solution) which presented higher percentage of sulfonic functional groups than the other LS eucalyptus ligands.
The same experiment was repeated with commercial Fe-complexes available at Spanish companies. A lignosulfonate based, an amino acid containing, a gluconate based and a humate product was used. Comparing the quantity of the iron remaining in the Ca^{2+} containing solution at different pH after 3 days, one can conclude that the most stable Fe-complex was the lignosulfonate based one showing similar behavior as Fe-LS eucalyptus complexes. The lowest Fe in the solution was found in the case of the above mentioned amino acid and humate complexes. These results are in good agreement with Fe speciation in the natural Fe-complexes studied in Activity 1 and 2.
Fe-chelates with *o,o*,-EDDHA, HBED, HJB, IDHA, EDTA were prepared with $Fe(NO_3)_3$ at pH=7. The solutions of the Fe-chelates were added to a buffered Ca^{2+} containing solution (pH=2-13) and shaken for three days. After filtration, the soluble iron was determined by AAS.
In the case of Fe^{3+}-*o,o*-EDDHA, -HJB, -HBED chelates, almost 100 % of the total iron remained in solution below pH=11, but at pH=13, all of the iron precipitated. Different result was found for the Fe^{3+}-IDHA and Fe^{3+}-EDTA, where the precipitation occurred between pH=8-9 and pH=9-10, respectively.
Mössbauer measurements were carried out at pH~5 and ~7 with $^{57}Fe^{3+}$-complexes/chelates prepared with 0,01 M $^{57}Fe(NO_3)_3$ solution. At both pH values, all the Fe^{3+}-complexes showed the presence of dimeric/oligomeric Fe^{3+}-componuds showing the partial hydrolysis of Fe^{3+} in these solutions. In contrast, all chelating agents (except of EDTA) formed monomeric Fe^{3+} species under the same conditions. Fe^{3+}-EDTA chelate was found to build monomeric Fe^{3+} species at acidic pH while dimerization occurred at pH higher than 6.

Activity 4: Stability of Fe complexes and Fe chelates when interacting with two calcareous and two other soil materials.

According to the results obtained in Activity 3, the chemically non-modified eucalyptus LS Fe^{3+}-complex was chosen for the stability experiments carried out with soil materials. For comparison, one the most stable chelating agent, the *o,o*,-EDDHA Fe^{3+}-chelate was applied.
The interactions of $^{57}Fe^{3+}$-LS eucalyptus and $^{57}Fe^{3+}$-*o,o*,-EDDHA with the different soil materials (calcium-montmorillonite, acid peat, calcium-carbonate and ferrihydrite) were repeated by the Spanish group. The ^{57}Fe concentration of the solutions was set to 0.1 M in the case of $^{57}Fe^{3+}$-LS Eucalypus and 0.02 M in the case of $^{57}Fe^{3+}$-*o,o*,-EDDHA. After the interaction of the soil component, the Fe concentration remaining in the solution was determined by AAS. In the case of *o,o*,-EDDHA, the retention of the ligand was also followed by HPLC measurements.
The iron in the soil after the interaction was found to be between 27-45% of the total iron depending both on the $^{57}Fe^{3+}$-compound and the soil material. No retention was observed in the case of calcium-carbonate treated with $^{57}Fe^{3+}$-*o,o*,-EDDHA.
According to the Mössbauer spectra of the soils after interaction measured at 80 K, different Fe-components could be identified. Both the $^{57}Fe^{3+}$-LS and $^{57}Fe^{3+}$-*o,o*,-EDDHA on acid peat resulted in the formation of a superparamagnetic Fe^{3+}-oxide, probably hematite, and a paramagnetic Fe^{3+}-component which can be associated to surface bound Fe^{3+}-OH-LS/ *o,o*,-EDDHA-type species. In the case of calcium-montmorillonite and calcium-carbonate, no magnetically ordered phase was observed suggesting the formation of a surface bound Fe^{3+}.
The interaction between ferrihydrite and $^{57}Fe^{3+}$-EDDHA was carried out with the help of synthetic ^{56}Fe-ferrihydrite and $^{57}Fe^{3+}$-*o,o*,-EDDHA in order to avoid the high contribution of the original ^{57}Fe present in the ferrihydrite soil material compared to the adsorbed ^{57}Fe. With this experimental setup we have succeeded to unequivocally show the decomposition of $^{57}Fe^{3+}$-*o,o*,-EDDHA on ferrihydrite forming a new, amorphous ^{57}Fe-ferrihydrite phase. The precipitation of Fe on the soil surface gives very important evidence on the decomposition of the Fe-complexes/chelates during the interaction and thus, it suggests that the mechanism of the retention supposed before needs to be reconsidered. (In the case of Fe^{3+}-*o,o*,-EDDHA, only surface bound Fe-species have been suggested so far in the literature.)

We can also suggest that the biological availability of iron from Fe-complexes and chelates in acid peat and ferrihydrite soils may strongly decrease because of the formation of a new Fe^{3+}-

oxide/hydroxide phase while in montmorillonite and carbonate, no such decomposition of the Fe^{3+}-compounds occurs (surface-bound Fe-compounds are thought to be mobilized easily by Strategy I plants). However, the question of the biological availability of these Fe-compounds for plants was not addressed in the present project but hints at a new significant problem in field applications that could to be studied in a further work.
The speciation experiments were complemented by the study of some possible redox transitions of $^{57}Fe^{3+}$-compounds at real soil conditions in the framework of an ongoing cooperation between the Hungarian research group and the Laboratory of Biochemistry of Plant-Bacterial Symbioses (Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences), focusing on the interactions of plant-associated microbes and the iron homeostasis of plants. For this reason, redox transitions of $^{57}Fe(NO_3)_3$ were studied in the presence of bacterial signaling molecules and of the rhizospheric bacterium, *Azospirillum sp*. In latter case, both transmission ^{57}Fe and emission ^{57}Co Mössbauer techniques were applied.

The results obtained are or will be summarized in following publications:

Kamnev AA; Dykman RL; Kovacs K; Pankratov AN; Tugarova AV; Homonnay Z; Kuzmann E: Redox interactions between structurally different alkylresorcinols and iron(III) in aqueous media: frozen-solution ^{57}Fe Mössbauer spectroscopic studies, redox kinetics and quantum chemical evaluation of the alkylresorcinol reactivities. *Structural Chemistry*, accepted for publication (DOI: 10.1007/s11224-013-0367-1) (IF=1.772)

Kamnev AA; Tugarova AV; Biró B; Kovács K; Homonnay Z; Kuzmann E; Vértes A: Cobalt(II) interaction with cells of the soil bacterium Azospirillum brasilense Sp7: A 57Co emission Mössbauer spectroscopic study, Hyperfine Interactions 206:91-94, 2012

Kamnev AA; Tugarova AV; Biró B; Kovács K; Homonnay Z; Kuzmann E; Vértes A: Aspartic acid interaction with cobalt(II) in dilute aqueous solution: A 57Co emission Mössbauer spectroscopic study, Hyperfine Interactions 206:101-104, 2012

Hernández-Apaolaza L, Kovács K; Machala L; Pechousek J; Czech V; Fodor F; Santos-Rosell S; Lucena JJ: A comprehensive study on the interaction of different soil compounds with synthetic and natural iron complexes applied as iron fertilizers. *European Journal of Soil Science*, under preparation

OBJECTIVE 3: Environment and speciation of Fe complexes and Fe chelates when used as Fe sources applied to roots or through foliar sprays.

Activity 5: Root $^{57}Fe^{3+}$ reduction and uptake by intact plants. Resupply experiments

Cucumber and soybean plants were grown in EDTA-buffered, aerated nutrient solution system. After a pre-culture period (10 days for cucumber and 4 days for soybean) the plants were transferred to a treatment solution (2 days without Fe for cucumber and 7 days still with Fe for soybean). Root ferric chelate reductase (FCR) activity was measured at pH 6 supplying the roots with Fe^{3+} complexes (Fe^{3+}-LS eucalyptus and spruce, Fe^{3+}-GL, Fe^{3+}-LN) and chelates (Fe^{3+}-*o,o*-EDDHA, -EDTA, -IDHA and -EDDS) by the BPDS method.
The root FCR activity for the chelates proved to be much higher for both plants than for the complexes. The FCR activity for Fe^{3+}-EDTA was similar after 10, 20 and 60 min of exposure. The average value of FCR activity was 68.9±8.1 nmol reduced Fe g^{-1} root fw min^{-1} for cucumber, the efficient plant and 23.6±2.2 for soybean, the inefficient plant. The FCR activity was similar for Fe^{3+}-IDHA, lower for Fe^{3+}- *o,o*-EDDHA but higher for Fe^{3+}-EDDS in both species. For these three chelates the enzyme activity was 1.5-2 times higher in cucumber than in soybean. Fe^{3+}-LSs, GL and LN proved to be inefficient substrates for the root FCR as the activity was practically zero.
The Fe uptake experiments were done with the same Fe^{3+}-compounds in the Spanish laboratory. The results show that the uptake of Fe is much lower than the Fe^{3+} reduction in the root. The uptake

(measured as Fe concentration of the xylem sap) was similar both in cucumber and soybean. In spite of the very low FCR activity, the Fe uptake from the Fe^{3+}-complexes was found to be slightly higher than from the Fe^{3+}-chelates.

Further FCR measurements together with Mössbauer analysis were done with iron deficient cucumber plants grown in hydroponics and supplied with $^{57}Fe^{3+}$-citrate at different pH values (4.5-7.5) since - according to the previous results of the Hungarian group - both FCR activity and Fe uptake are high with $^{57}Fe^{3+}$-citrate and thus these data can be further used as control data. The results demonstrated a sharp decrease in the FCR activity between pH 5.5 and 6.5. In parallel, based on the Mössbauer results, a change of the Fe^{2+} and Fe^{3+} components could be observed indicating the formation of a new –probably– hydrous iron oxide component at high pH values. The effect of calcareous conditions on the Fe-speciation was also studied in 1mM and 3 mM $KHCO_3$ containing nutrient solutions.

Activity 6: Leaf $^{57}Fe^{3+}$ reduction and uptake by leaf discs.

Cucumber and soybean plants were grown in EDTA-buffered, aerated nutrient solution system. The tests required somewhat larger plants with sufficiently developed leaf area. After a pre-culture period (10 days for cucumber and 15 days for soybean) the plants were transferred to a treatment solution for a longer time (without Fe 4 days for cucumber and 5 days for soybean). Leaf disk ferric chelate reductase (FCR) activity was measured at pH 6 supplying the disks (30 disks in one sample, 5 samples for each treatment) with different complexes (Fe^{3+}-LS eucalyptus and spruce, Fe^{3+}-GL, Fe^{3+}-LN) and chelates (Fe^{3+}-*o,o*-EDDHA, -EDTA, -IDHA and -EDDS) by the BPDS method. The procedure was based partly on previous experiments using vacuum infiltration to equilibrate the assay solution with the mesophyll intercellular spaces. The infiltration was made for 5 min twice at –65 kPa than (after taking a zero time blank sample) the vials were shaken for 60 min with 100 rpm. All procedures were made in darkness.
There was no significant difference between the leaf disk FCR activity of Fe deficient and Fe sufficient plants. It was found that the activity was much higher in soybean than in cucumber for all chelates and complexes. For Fe^{3+}-EDTA the activity was 8.44±3.95 and 9.97±2.00 nmol reduced Fe g^{-1} leaf fw h^{-1} for Fe-sufficient and Fe-deficient cucumber, while 13.39±3.30 and 26.41±2.10 for soybean, respectively. The highest reduction was found for Fe^{3+}-IDHA (between 80 and 146 nmol reduced Fe g^{-1} leaf fw h^{-1}). The activity for Fe^{3+}-EDDS and Fe^{3+}-*o,o*-EDDHA was similar to Fe^{3+}-EDTA. The FCR activity was very low for the studied complexes (0-7.5 nmol reduced Fe g^{-1} leaf fw h^{-1} for cucumber and 7.0-44.5 for soybean). In cucumber the highest value was measured for Fe^{3+}-LS spruce while in soybean for Fe^{3+}-LS spruce and -GL.

In order to measure the uptake of Fe from the different chelates and complexes plants were grown similarly to the ones for leaf disk FCR activity measurements. Three sets of disks were cut from the leaves: 25 disks to measure the initial Fe content of the leaves, 25 disks to assess the Fe adsorption during the infiltration-incubation with the Fe-chelates and Fe-complexes and 25 disks subjected to reductive washing ($Na_2S_2O_4$ + bypyridyle in $CaSO_4$) after the Fe uptake. For the uptake after the vacuum infiltration of the buffer solution Fe^{3+} complexes and chelates were individually added to the solution containing the disks and the vials were shaken with 100 rpm for 60 min in darkness. The Fe uptake for cucumber was different than for soybean. Iron deficient cucumber leaf disks took up similar amounts of Fe from Fe-EDTA as the Fe-sufficient ones. For the other chelates and complexes the disks from Fe-sufficient plants took up much more (5-20x as much) Fe than the Fe deficient ones. For soybean we could measure a net Fe release from leaf disks instead of uptake during the applied procedure. This refers to an accumulation of Fe in a highly mobile form in the leaf apoplast in soybean plant.

An attempt was made to measure the Fe speciation with Mössbauer spectroscopy in the cucumber and soybean leaf disks after the incubation with the same ^{57}Fe-complexes and chelates. Unfortunately, the quantity of the ^{57}Fe absorbed by the disks was not enough for measurements thus the reduction of Fe^{3+} could not be followed as it has succeeded in the case of the cucumber root samples. However, since the majority of Fe in leaves is located in the chloroplasts, Mössbauer spectra of intact chloroplasts

(supplied with $^{57}Fe^{3+}$ and isolated from sugar beet) could be recorded. The results indicated the incorporation of Fe into heme or Fe-S clusters (as Fe-sulphur proteins) and the formation of Fe^{3+}-carboxylate compounds, as discussed in the following two sections in detail.

Activity 7: Fe uptake when $^{57}Fe^{3+}$ complexes and chelates are applied to cucumber and soybean plants grown in hydroponics.

To overcome Fe chlorosis, the ability of eight Fe^{3+} complexes and chelates (Fe^{3+}-LS spruce, Fe^{3+}-LS eucalyptus, Fe^{3+}-GL, Fe^{3+}-LN, Fe^{3+}-*o,o*-EDDHA, Fe^{3+}-EDTA, Fe^{3+}-EDDS, Fe^{3+}-IDHA complexes) were studied for cucumber and soybean chlorotic plants in three replicates. Cucumber and soybean plants were grown in EDTA-buffered, aerated nutrient solution system. To obtain iron deficient plants showing strong iron chlorosis for the iron resupply bioassays, after a pre-culture period (one week), the plants were transferred to iron deficient, 0.1 g L^{-1} $CaCO_3$ containing (in order to model natural soil conditions) nutrient solution for 7 days. Iron sufficient control plants were grown on a nutrient solution containing 20 µM Fe^{3+}-EDTA during the whole time of treatment. In the iron resupply bioassay tests, Fe^{3+} chelates and complexes were applied in a 20 µM concentration according to the preliminary experiments as an effective concentration to obtain regeneration. In contrast to the project proposal, we found that after induced iron deficiency, the physiological recovery shows a much faster, three-days run than predicted previously thus samplings were performed one day after transferring plants to iron resupply media. The phytosynthetic activity of leaves developed under the iron deficiency treatment was recorded by chlorophyll (Chl) fluorometer. In case of cucumber plants, second leaves were used in the measurements. Among the natural complexes, , Fe^{3+}-LS eucalyptus and Fe^{3+}-LN were the most effective complexes in elevating the photosystem II maximal quantum efficiency (F_v/F_m=0.803±0.015 and 0.805±0.008, respectively), the major component of photosynthetic activity, thus it reached the values of iron sufficient control plants (0.803±0.010) in contrast to the iron deficient ones (0.0739±0.024). The Fe^{3+}-LS spruce treated plants showed significantly lower values (0.776±0.009). , Fe^{3+}-GL treatment caused an intermediate level of recovery (0.788±0.011). Among the synthetic chelates, Fe^{3+}-EDDS, Fe^{3+}-EDTA and Fe^{3+}-IDHA treatment caused a similar level of recovery to Fe^{3+}-LS eucalyptus and Fe^{3+}-LN, the differences were not significant whereas the recovery induced by Fe^{3+}-*o,o*-EDDHA was similar to Fe^{3+}-LS spruce (0.784±0.010). In the bioassay tests on soya, we used the prominent leaflet of the first trifoliate leaves. In one day, the recovery was significantly lower compared to cucumber. Whereas the values of iron sufficient and iron deficient controls did not differ significantly from cucumber, both the natural complexes and synthetic chelates caused a significantly lower elevation in values. In soya plants, Fe^{3+}-LS eucalyptus was the most effective natural complex to restore photosynthetic activity (0.785±0.013). Among the synthetic chelates, Fe^{3+}-EDTA and Fe^{3+}-IDHA were found to be the most effective. Nevertheless, the values remained below that of cucumber (0.768±0.014 and 0.766±0.014, respectively). Changes in the Chl content and ratio of Chl a and b forms (which refers to the composition changes in the photosynthetic thylakoid membrane) were also measured. In accordance to the activity measurements, the changes of Chl contents among treatments and plants showed a similar pattern. In one day recovery, Fe^{3+}-LS eucalyptus and Fe^{3+}-LN treated cucumber plants reached the Chl content of iron sufficient control plants (eucalyptus: 15.6±4.0, leonardite: 13.6±0.2, control: 13.8±1.1 µg Chl a+b cm^{-2}) whereas values of other complex or chelate treated plants reached an intermediate level between iron sufficient and iron deficient plants. We have measured that under iron deficiency recovery, the Chl a/b ratio shows an increasing-decreasing run where the increasing was induced by the accumulation of Chl a containing photosystem I particles, also the largest iron consuming complex in the chloroplasts, whereas the decrease occurred by the accumulation of other chlorophyll-protein complexes needed to obtain photosynthesis. Thus, increases in the Chl a/b ratios under Fe^{3+}-LS eucalyptus, Fe^{3+}-LN, and partially Fe^{3+}-GL treatment (ratios were 3.1±0.1, 3.2±0.2 and 3.1±0.3, respectively) were a clear sign of recovery processes. Soya plants were less able to recover, again. The Fe^{3+}-LS eucalyptus, Fe^{3+}-LN and Fe^{3+}-*o,o*-EDDHA treatments led to somewhat higher elevation of Chl content of leaves (14.5±3.9, 14.4±1.3 and 16.1±2.9, respectively, the iron sufficient control was 26.1±4.3) compared to other complexes and chelates, the differences were, however, weakly significant in one-way ANOVA. In soya leaves, Fe^{3+}-*o,o*-EDDHA treatment caused the highest elevation in Chl a/b ratio (3.23±0.1). Based on Chl fluorescence imaging, the recovery process started

close to the leaf veins and expanded over the whole leaf area which indicates that larger amount of iron started to be translocated to the leaves upon the start of recovery treatments. Among all the plants and treatment, no significant differences were found using native polyacrylamide gel electrophoresis PAGE, the major observation was that under recovery, the restoration of photosystem I particles was prior to the recovery of the other complexes. We found using BlueNative PAGE that in the Fe-deficient thylakoids, an increase in the ratio of PSI super-complexes and degrading PSII particles is more pronounced, together with higher zeaxanthin content.

The major point in the recovery was the iron accumulation process of the chloroplasts, as elevation in the iron content of chloroplast was necessary for both the increasing of the chlorophyll content, biosynthesis of photosystem I and restoration of photosynthetic activity. We have determined that the chloroplast iron uptake was dependent on the light periods of plant growth *in vivo*. To investigate the role of photosynthesis in the chloroplast iron uptake, isolated chloroplasts were tested. We found that darkness as well as photosynthesis inhibitors also inhibits chloroplast iron uptake *in vitro*. The K_M value for chloroplast iron uptake was determined to be 14.65±3.13 mM Fe^{3+}-complex. The reason for the light dependence was determined to be the NADPH demand of chloroplast ferric chelate oxidoreductase enzyme. According to the Mössbauer measurements of isolated chloroplasts, the iron taken up was found to incorporate into heme or Fe-S clusters. The chloroplast iron uptake was also affected by the ionic environment by the voltage-sensitive mechanism of the uptake of ferric-chelates across the chloroplast outer envelope membrane. Transition metal cations enhance the iron uptake of chloroplasts from a ferric-complex pool. The recovery treatments caused different elevations in the chloroplast iron content of cucumber and soya leaves. In case of cucumber, Fe^{3+}-LS eucalyptus, Fe^{3+}-LN, Fe^{3+}-EDHA and Fe^{3+}-EDDS were the most effective to elevate chloroplast iron content (they were 255±38, 197±50, 211±8 and 213±34 amol chloroplast^{-1}, respectively, after one light period of recovery), which were significantly higher compared to the iron deficient controls. Nevertheless they did not reach in one day the values of iron sufficient controls (269±15 amol chloroplast^{-1}).Chelates and complexes were much less effective in elevating chloroplast iron content of soya leaves, the difference between iron deficient controls and treated plants was only significant in case of Fe^{3+}-LN and Fe^{3+}-*o,o*-EDDHA treatments (where chloroplast iron contents were 20.3±3.8 and 17.2±6.6 amol chloroplast^{-1}, respectively). To compare cucumber and soya results it is important to mention that the average volume of soya chloroplasts is much smaller, in contrast to cucumber, according to our light microscopy observations.

Iron speciation in the root of cucumber and soybean chlorotic plants were studied with Fe^{3+}-LS spruce, Fe^{3+}-GL, $^{57}Fe^{3+}$-*o,o*-EDDHA and $^{57}Fe^{3+}$-EDTA complexes/chelates with two different sets of samples. In one experiment, iron deficient plants were grown at similar conditions as the FCR activity tests (described in Activity 5) but without BPDS and the $^{57}Fe^{3+}$- chelates and complexes were applied as a short-time treatment for 30 min at 500 μM concentrations. The Mössbauer spectra of these samples showed that in the case $^{57}Fe^{3+}$-*o,o*-EDDHA and Fe^{3+}-EDTA, approximately 20% of the total iron was reduced to Fe^{2+}. According to the Mössbauer parameters, no significant differences were found in the Fe-species formed in the root after the addition of different $^{57}Fe^{3+}$-chelates suggesting the decomposition of the $^{57}Fe^{3+}$-compounds during the reduction and uptake process. The accumulation of the Fe^{2+} compound (identified as $[Fe(H_2O)_6]^{2+}$ complex) indicates the accumulation of iron in the apoplast and thus it hints on a slower Fe uptake process compared to reduction. This is in good agreement with previous results obtained with Fe^{3+}-citrate complex. However, in the case of the $^{57}Fe^{3+}$-LS and GL complexes, no iron components (Fe^{2+} or Fe^{3+}) could be detected with Mössbauer spectroscopy because iron accumulation was very low in the root tissues. These results are in good agreement with the FCR activity and Fe uptake measurements since very low FCR activity and slow Fe uptake could be measured.

In order to study the iron species formed in the case of Fe^{3+}-LS spruce and Fe^{3+}-GL, the experimental conditions were modified. In the second experiment, plants were grown at similar conditions as in the iron resupply bioassay tests, described before. In this case, the $^{57}Fe^{3+}$- chelates and complexes were applied in 100 μM concentration for 7 days (long-term treatment). The roots and the regreened chlorotic leaves of the plants were measured after freeze-drying. In the case of $^{57}Fe^{3+}$-*o,o*-EDDHA and $^{57}Fe^{3+}$-EDTA, both samples showed the uptake and translocation of the iron in the cells but in the case of the studied natural complexes, iron could be found only in the roots. Since for Mössbauer spectroscopy a relatively high ^{57}Fe concentration is needed, the latter result does not mean that Fe^{3+}-

LS spruce and Fe^{3+}-GL were not able to supply iron through the root to the leaves (in the bioassay experiment, significant re-greening was observed, see before), it only indicates that the quantity of the total iron in leaves was much lower than after the application of $^{57}Fe^{3+}$-*o,o*-EDDHA or $^{57}Fe^{3+}$-EDTA. The Mössbauer parameters calculated show the presence only of +3 oxidation state of iron and suggests mainly the formation of Fe^{3+}-carboxylate complexes in roots and the formation of heme or Fe-S clusters (as Fe-sulphur proteins) in leaves. These results are in good agreement with the Mössbauer measurements of isolated sugar beet chloroplasts discussed before in the bioassay experiments and confirm the successful incorporation of iron in to the chloroplasts.

Altogether, among all the complexes and chelates, Fe^{3+}-LS eucalyptus, Fe^{3+}-LN and Fe^{3+}-*o,o*-EDDHA proved to be the most effective in recovering iron chlorosis symptoms. The results of the recovering efficiency of Fe^{3+}-GL were very controversial as values of Fe^{3+}-GL treated plants always have a very large standard deviation. In cucumber, the recovery results applying Fe^{3+}-LN was not significantly but tendentiously better compared to Fe^{3+}-LS eucalyptus. In soya, the ranking is rather controversial taking into consideration all the investigated parameters.

Activity 8: Fe uptake when $^{57}Fe^{3+}$ complexes and chelates are applied cucumber and soybean plants grown in a calcareous soil.

The ability of eight Fe^{3+} complexes and chelates (Fe^{3+}-LS spruce, Fe^{3+}-LS eucalyptus, Fe^{3+}-GL, Fe^{3+}-LN, Fe^{3+}-*o,o*-EDDHA, Fe^{3+}-EDTA, Fe^{3+}-EDDS, Fe^{3+}-IDHA complexes) in recovering developed iron chlorosis of plants was also tested in calcareous soil culture in cooperation with the Spanish partner. The plants were grown in the Spanish laboratory. Seedlings pre-cultivated in nutrient solution were transferred to pots filled with sandy clay irrigated daily with nutrient solution saturated with 0.1 g/L $CaCO_3$. The plants were grown in a growth chamber. Maximal quantum efficiency of photosystem II reaction centres (referring to the activity of photosynthesis; vitality factor) was tested by chlorophyll (Chl) fluorometer. Among chelates and complexes, Fe^{3+}-LS eucalyptus and Fe^{3+}-*o,o*-EDDHA were the most effective in restoring photosynthetic activity (F_v/F_m=0.796±0.041 and 0.790±0.035, respectively), the differences to the iron sufficient control were not significant, but were indeed significant compared to the iron deficient control (0.577±0.068). Fe^{3+}-GL and Fe^{3+}-IDHA also restored photosynthetic activity, but the values were significantly lower compared to iron sufficient controls. Chlorophyll contents of leaves were estimated by FMM chlorophyll fluorometer based on the ratio of red and far red fluorescent emission (reciprocal correlation). Chlorophyll content of leaves Fe^{3+}-LS eucalyptus treated plant was the highest (red to far-red ratio was the lowest among all treatments, 0.444±0.063, whereas the iron supplied control was 0.464±0.093). Chlorophyll content was significantly lower (fluorescence ratio significantly higher) in Fe^{3+}-*o,o*-EDDHA and Fe^{3+}-IDHA treated leaves. The chlorophyll content of leaves of Fe^{3+}-GL treated plants (red to far-red ratio was 0.662±0.076) did not differ to that of the iron deficient plants. According to BlueNative polyacrylamide gel electrophoresis, as it was mentioned in the Activity 7., again, the amount of photosystem I complexes showed the iron deficiency status of the plants whereas no difference was found between thylakoid composition of iron sufficient control and the Fe^{3+}-LS eucalyptus treated plants. Altogether, Fe^{3+}-LS eucalyptus was the most effective natural complex to recover calcareous soil-induced iron deficiency. These findings are in accordance with the nutrient solution results (see Activity 7.)

Activity 9: Fe uptake when $^{57}Fe^{3+}$ complexes and chelates are applied through foliar sprays to cucumber and soybean plants grown in hydroponics.

The ability of eight Fe^{3+} complexes and chelates (Fe^{3+}-LS spruce, Fe^{3+}-LS eucalyptus, Fe^{3+}-GL, Fe^{3+}-LN, Fe^{3+}-*o,o*-EDDHA, Fe^{3+}-EDTA, Fe^{3+}-EDDS, Fe^{3+}-IDHA complexes) to overcome Fe chlorosis were studied for cucumber and soybean chlorotic plants in three replicates. Cucumber and soybean plants were grown in EDTA-buffered, aerated nutrient solution system. To obtain iron deficient plants showing strong iron chlorosis for the iron resupply bioassays, after a pre-culture period (one week), the plants were transferred to iron deficient, 0.1 g L^{-1} $CaCO_3$ containing (in order to model natural soil conditions) nutrient solution for 7 day. Iron sufficient control plants were grown on a nutrient solution

containing 20 µM Fe^{3+}-EDTA during the whole time of treatment. In the iron resupply bioassay tests, Fe^{3+} chelates and complexes were applied in 5 µM concentration as foliar treatment. During the recovery period, two foliar spray treatments were performed, at the beginning of the recovery and on the 8th day of recovery. After experimental setup optimisation, second leaves were subjected to foliar treatment and third leaves were used in bioassays to also include iron remodelling in plant level. Physiological parameters were tested on the 7th and 14th day of recovery, but the efficiency of complexes and chelates was evaluated based on the 14th day of recovery.
In general, foliar treatments were more effective on cucumber compared to soya but foliar treatment caused significant recovery in both species. In cucumber, Fe^{3+}-LS eucalyptus, Fe^{3+}-LN and Fe^{3+}-IDHA treatment caused the highest photosystem II maximal quantum efficiency (F_v/F_m=0.764+0.007, 0.765±0.068 and 0.792±0.034, respectively. Iron sufficient control was: 0.808±0.005). In general, the use of natural complexes resulted stronger recovery compared to synthetic chelates, the one-way ANOVA test showed strong significant difference. In soya, all the chelates and complexes caused recovery in maximal quantum efficiency, but no significant difference was found among the treatments (all the treatments were around Fv/Fm=0.7 compared to iron deficient control: 0.520±0.036 and iron sufficient control: 0.810±0.007). Compared to photochemical efficiency, foliar treatment was less effective in restoring the total chlorophyll (Chl) content of leaves. Although all the complex and chelate treatments caused a significant increase in total Chl content of both cucumber and soya leaves, the difference between the treatments was not significant because of the high standard deviation within the treatment populations. In general, cucumber plants were more effective to increase total Chl content compared to soya plants. Based on Chl a/b ratio of the leaves, Fe^{3+}-LS eucalyptus and Fe^{3+}-LN were the most effective to restore the iron sufficient value in both of the cucumber and soya leaves. Nevertheless it has to be mentioned, that in foliar treatment, due the longer experimental period compared to root iron resupply (see Activity 7 and 8) the decrease of Chl a/b ratio (the full restoration of the thylakoid composition) showed the efficiency of the treatments, based on our findings. Compared to the root iron resupply experiments (Activity 7.), the Chl fluorescence imaging investigations did not show any significant pattern on the leaf area which indicates that iron translocation from the lower to the upper leaf storeys was a slow process which allowed to recover all the leaf area in the same run. Chl fluoresncence imaging investigation of the treated (second storey) leaves showed a measurable pattern, especially on Fe^{3+}-GL and Fe^{3+}-*o,o*-EDDHA treated leaves (both in soya and cucumber) which indicates that the adherence an uptake of the different complexes and chelates are different and this phenomenon may act also on the recovery processes. Similarly to chlorophyll pigment measurement, the BlueNative polyacrylamide gel electrophoresis measurement did not show any significant differences among the samples, either. Compared to photosynthetic pigment measurements, foliar treatments were much more effective in restoring chloroplast iron content. As foliar treatment, Fe^{3+}-LS eucalyptus and Fe^{3+}-*o,o*-EDDHA restored the iron content of cucumber chloroplast totally (they were 200.8±43.8 and 233.6±7.1 amol Fe chloroplast^{-1}, respectively, whereas the iron deficient and sufficient controls were 79.3±7.1 and 228.0±41.2 amol Fe chloroplast^{-1}, respectively). These two treatments also resulted in a significantly higher chloroplast iron content compared to other recovery treatments. In soya, no significant differences were found among the recovery treatments, all of which remained around 13 amol Fe chloroplast^{-1} (taking into consideration that the volume of soya chloroplasts is much smaller compared to cucumber).

For Mössbauer analysis of the iron speciation in chlorotic leaves of soya and cucumber, the plants were grown as in the iron resupply bioassay tests, described before. In the iron resupply, Fe^{3+}-LS spruce, Fe^{3+}-GL, $^{57}Fe^{3+}$-*o,o*-EDDHA and $^{57}Fe^{3+}$-EDTA complexes/chelates were applied in 5 µM concentration as foliar treatment. During the recovery period, two foliar spray treatments were performed, at the beginning of the recovery and on the 8th day of recovery. Plants were harvested on the 14th day of recovery; the second leaves (subjected to foliar treatment) and third leaves (grown in the recovery period) were measured. In the case of the treated (second) leaves, the ^{57}Fe containing treatment solution from the leaf surface was removed according to a washing procedure described in the literature: the leaves were washed with 0.1% HCl, 0.01% Tween-40 (surfactant) solutions and the twice with distilled water. Similarly as in the case of root applications (Activity 7), all samples were freeze-dried before Mössbauer measurements.

Although the bioassay tests showed significant recovery in the third leaves of both cucumber and soya, only the second (treated) leaves of both plants could be measured by Mössbauer spectroscopy. The total amount of ^{57}Fe transferred to the next (third) leaf was not enough for measurements indicating a very slow translocation of iron from the treated leaves in both (effective and ineffective) plants. The Mössbauer parameters calculated from the spectra suggest mainly the presence of the same components detected previously in the isolated chloroplasts (heme or Fe-S clusters and Fe^{3+}-carboxylates). However, the rather large line width found may indicate that partial hydrolysis of the Fe^{3+}-complex/chelate can occur and hydrous ferric oxides may also be formed.

Altogether, foliar spray treatments were effective on the efficient plants (cucumber) where Fe^{3+}-LS eucalyptus and Fe^{3+}-*o,o*-EDDHA were the most effective compounds. Foliar treatments were ineffective to restore the total iron homeostasis of the inefficient plants (soya). Mössbauer spectroscopy showed the accumulation of iron in both plants but only in the Fe-sprayed leaves, thus no translocation of Fe could be followed in further grown leaves.

The results were presented at following international conferences (abstracts) and are summarized in the following publications:

Fodor F, Kovács K, Solti A, Toth B, Lévai L, Sárvári É, Hernández-Apaolaza L, Lucena JJ, Vértes A†: Iron microenvironments in complexes, roots, leaves, chloroplasts – new aspects revealed by Mössbauer spectroscopy, 16th International Symposium on Iron Nutrition and Interactions in Plants, Amherst, MA, USA, 2012 – abstract

Solti Á; Kovács K; Sárvári É; Fodor F: Iron incorporation into chloroplasts: a Mössbauer spectroscopic study, 16th International Symposium on Iron Nutrition and Interactions in Plants, Amherst, MA, USA, 2012 – abstract

Kovács K; Fodor F; Czech V; Santos-Rosell S; Lucena JJ; Hernández-Apaolaza L; Mössbauer spectroscopic characterization of Fe-leonardite complexes, International Conference on the Application of Mössbauer Effect, Opatija, Croatia, 2013 – abstract

Solti Á; Gáspár L; Vági P; Záray G; Gémes-Juhász A; Sárvári É: Physiological traits of poplar relevant to cadmium phytoextraction, Summaries. XVII. National Meeting on Plant Breeding, Budapest, p. 119, 2011 - abstract

Solti Á; Gáspár L; Vági P; Záray G; Fodor F; Sárvári É: Cd, Fe and light sensitivity: interrelationships in Cd treated Populus, OMICS: Journal of Integrative Biology Special Issue 15: 811-818, 2011 (IF=1.944)

Fodor F; Kovács K; Czech V; Solti Á; Tóth B; Lévai L; Bóka K; Vértes A: Effects of short term iron citrate treatments at different pH values on roots of iron deficient cucumber: a Mössbauer analysis. *Journal of Plant Physiology*, 169: 1615-1622, 2012 (IF= 2.677)

Solti Á; Kovács K; Basa B; Vértes A; Sárvári É; Fodor F: Uptake and incorporation of iron in sugar beet chloroplasts, *Plant Physiology and Biochemistry* 52: 91-97, 2012 (IF=2.402)

Hajnal D (2013): Efficiency of natural ferric complexes in recovering plant iron deficiency [In Hungarian] Student Competition Thesis for Biologist Students, Eötvös Loránd University.

Solti Á (2012): Cd-Fe interference in iron homeostasis and in photosynthesis. [In Hungarian with English summary] PhD Thesis, Eötvös Loránd University, http://teo.elte.hu/minosites/ertekezes2012/solti_a.pdf

Fodor F., Cseh E., Solti Á., Czech V., Kovács K. Vaskelátok és vaskomplexek a vashiány orvoslásában: régi probléma, új ismeretek. in Hat évtized az agrár felsőoktatásért. Eds. Jávor A.,

Fürjné Rádi K., Veres Sz., Debreceni Egyetem Agrár- és Gazdálkodástudományok Centruma, ISBN 978-615-5183-97-3, pp49-55. (non-SCI journal; in Hungarian)

Solti Á, Müller B, Czech V, Sárvári É, Fodor F: Functional characterization of the chloroplast ferric chelate oxidoreductase enzyme. *New Phytologist*, submitted (28-11-2012; 10-10-2013 accepted with major revision) (IF=6.736)

Basa B, Lattanzio G, Solti Á, Tóth B, Abadía J, Fodor F, Sárvári É: Changes induced by cadmium stress and iron deficiency in the composition and organization of thylakoid complexes in sugar beet (*Beta vulgaris* L.). *Experimental and Environmental Botany*, submitted (07-11-2013)

Solti Á, Sárvári É, Kovács K, Tóth B, Vázquez S, Abadía J, Fodor F: The effects of anions and cations on chloroplast Fe uptake rates indicate the existence of a voltage-sensitive transport step in the outer envelope membrane. *Physiologia Plantarum*, submitted (25-07-2013)

Following publications are under preparation:

Kovács K; Czech V; Solti Á; Santos-Rosell S; Hernandez-Apaolaza, L; Lucena JJ; Fodor F: Comparative study of the efficiency of Fe-chelates and Fe-complexes to alleviate chlorosis I. Aplication in cucumber and soybean through the root system. (It will be sent to the *Journal of Plant Physiology*)

Solti Á; Kovács K; Czech V; Hajnal D; Santos-Rosell S; Hernandez-Apaolaza, L; Lucena JJ; Fodor F: Comparative study of the efficiency of Fe-chelates and Fe-complexes to alleviate chlorosis II. Foliar aplication in cucumber and soybean. (It will be sent to the *Journal of Plant Physiology*)

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