Thesis of PhD dissertation

Exploring Bioactive Compounds Involved in Iron Metabolism of Plants and Microorganisms – Analytical and Mössbauer Spectroscopy Studies

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Budapest, 2023

Introduction

Iron is an essential element for all living organisms as it plays a vital role in various processes such as cell construction, oxygen transport, energy production and DNA synthesis. Plants and microorganisms require iron in micromolar concentration for a proper development. For microorganisms the common way to acquire iron from the surrounding is the production of siderophores, which are small molecules that can complexate iron and make it more soluble and available for bacterial uptake. Higher plants typically take up minerals from the rhizosphere and translocate these elements towards the aerial tissues. Based on their strategy of iron uptake, plants can be divided into two groups: while Strategy I includes reduction of iron(III) as an obligatory step in making iron available to the plant, the plants following Strategy II release phytosiderophores into the rhizosphere to solubilize iron for uptaking.

If the uptake of iron is disturbed, it leads to iron deficiency - a condition where the plant or the bacterium lacks the necessary amount of iron required for its proper growth and development. Without sufficient amount of iron, microorganisms may experience growth reduction or even die. Since iron plays a crucial role in the synthesis of chlorophyll, the pigment that gives leaves their green color, and is essential for photosynthesis, the main symptoms of iron deficiency in plants are reduced growth and yellowing of the leaves. It is a common problem in alkaline and calcareous soils where iron can become less soluble and less available.

Iron deficiency in plants not only leads to a reduced quality and quantity of plant products, but as a consequence also promotes development of iron deficiency in human bodies, which is recognized as one of the most common nutritional disorder in the world. Therefore, one of the purposes of the research is to study the process of iron uptake in plants, to investigate the main chemical factors influencing its efficiency and to prevent problems caused by iron unavailability.

To develop new strategies for improving iron nutrition, it is important to understand the role and behaviour of bioactive iron compounds. Strategy I plants are long known to excrete various phenolic compounds and organic acids by their roots, the amount of which excretions sharply increases under Fe deficiency. However, systematic chemical studies of the complexation of iron by organic acids, its speciation in solutions at different physiological conditions, redox properties and photosensitivity are still not complete. One of the most important forms of complexed ferric iron in biosystems is iron(III) citrate which was also indicated to be present in the symplast and was shown to be the major complex in Fe transport in the xylem sap.

In the present work, the speciation of iron(III) citrate at biologically relevant conditions was investigated by means of Mössbauer and electron paramagnetic resonance spectroscopies applying the frozen solution technique, in order to clarify the nuclearity of the complexes depending on the pH and iron-to-ligand ratio. Additionally, the stability of the polynuclear iron citrate complexes was checked in the presence of organic solvents and compared to the stability of the μ-oxo dimeric form of iron ethylenediaminetetraacetate. As many plants and microorganisms are exposed to the light at their natural conditions, the photochemistry of iron citrate should be taken into account in describing its role in the iron metabolism. The photodegradation of iron(III) citrate solutions at different pH values and the processes occurred were compared with the transformation observed in the model plant.

To overcome iron deficiency, various strategies have been developed to increase the availability of iron for plants. The most common approach is to apply iron fertilizers, either as soil amendments or foliar sprays. These fertilizers can provide readily available source of iron for the plants, allowing them to reduce the symptoms of iron deficiency and improve growth and yield. In the present work, the preparation and the bioavailability of nanoparticles-based iron fertilizers was investigated and described. Several nanoparticle suspensions differing in the surfactant material were analysed from the point of view of their chemical stability and possible application in agriculture.

While describing the interaction of iron minerals with plants, the contribution of microorganisms cannot be neglected. The bacteria present in soil may either facilitate or disturb the iron uptake. Although most of microorganisms use iron as just one of the important micronutrients, as it was mentioned earlier, there are special categories of bacteria for whom the role of iron is much more crucial. Iron-oxidizing bacteria depend on ferrous iron as their primary energy source, utilizing iron as an electron donor with oxygen as the electron acceptor. After the oxidation, the newly formed iron(III) is almost immediately hydrolized and precipitates in the form of iron oxyhydroxide (ferrihydrite, goethite, etc.). As these organisms occur in both marine and fresh water environments, their metabolic activity contribute significantly to the geochemistry of the sediments. In the 1980s, a new physiological group of microorganisms was discovered, called dissimilatory iron-reducing microorganisms or iron reducers, which in contrast, obtain energy for life from the reduction reaction of various iron oxide compounds, using them as electron acceptors.

Redox reactions involving iron are one of the oldest forms of microorganism metabolism on Earth. The composition and physicochemical properties of the formed mineral phases strongly depend on the culture growth conditions and environment. As the mechanism of iron oxidation/reduction remains unresolved, the understanding of the exact procedure of mineral formation is a great multidisciplinary challenge for researchers. The study of minerals formed as a result of the growth of microorganisms is important for recreating the pattern of transformations of iron compounds and for searching for analogues of these processes in the modern iron biogeochemical cycle. Representative samples from one of the largest microbially mediated iron and manganese deposit (Urucum, Brazil) were comprehensively investigated and the results were interpreted with a special attention to the important signatures of microbial activity revealing the biogeochemistry of the biomat formation.

Experimental methods

The primary technique applied in this study was Mössbauer spectroscopy, which proves to be a powerful method for examining iron complexes, particularly when working with solution samples where obtaining crystallographic data on complex structures is not feasible. Establishing a correlation between the structure in the solid state and the structure in solution presents a significant challenge in general. Investigating the solution structure is intricate due to the presence of various species that often exhibit similar spectroscopic properties, making their differentiation challenging. However, Mössbauer spectroscopy offers an advantage in this regard. It exhibits high sensitivity to even subtle changes in the microenvironment and electronic structure of the Mössbauer active species, specifically the iron-containing species. Consequently, this technique enables high-resolution identification of distinct iron species. The application of Mössbauer spectroscopy for biological studies is of special interest, because this method was found to provide unique information about bioactive iron compounds, which play critical roles in a wide range of physiological processes. Iron-sulfur clusters involved in electron transfer and energy conversion in proteins have been studied recently in using Mössbauer spectroscopy, providing detailed information about the electronic and magnetic properties of the iron atoms in their structure. Mössbauer spectroscopy has proven to be a valuable tool for studying iron in various plant tissues, providing insights into the mechanism of uptake, transport, and storage of iron. By analysing the speciation of iron in roots, leaves, and seeds, valuable information can be obtained concerning the role of iron in plant metabolism, nutrient uptake, and response to environmental stress.

In this work, the results obtained through Mössbauer spectroscopy were complemented by additional investigations using electron paramagnetic resonance spectroscopy, X-ray powder diffraction, and transmission electron microscopy. These complementary techniques provided further insights into the composition and structure of the investigated samples. The physical background and the experimental setup for all techniques applied with a special attention to Mössbauer spectroscopy are described in the following chapter.

Summary and thesis points

The main aim of the work was revealing the new aspects of the chemistry of iron compounds and their role in the metabolism of plants and microorganisms.

As iron(III) citrate is known to be one of the most common complexed forms of ferric iron in many living organisms, including plants, I have prepared and investigated the properties of iron(III) citrate under various biologically relevant conditions. The speciation of iron(III) citrate was investigated by means of Mössbauer and electron paramagnetic resonance spectroscopies applying the frozen solution technique, in order to clarify the structure and the nuclearity of the complexes depending on the pH and iron-to-citrate ratio. Common application of both electron paramagnetic resonance and Mössbauer spectroscopies provided an accurate analysis of the spectra since the same theoretical concept of spin relaxation was used. The stability of the polynuclear complexes was checked in the presence of organic solvents and under exposition to visible light. The obtained results should be taken into account in plant biology/agronomy (e.g. foliar treatment of plants, modelling microbiotic systems). The main findings of the study are:

1. The nuclearity of iron(III) citrate complexes in aqueous solutions was revealed at different pH values and iron-to-citrate molar ratios. While at equimolar iron-to-citrate ratio, iron is found to be bond in a polynuclear complex, the excess of citrate leads to formation of monomeric complexes, whose concentration grows with the increase of pH.
2. The polynuclear nature of iron(III) citrate complex was not disturbed by addition of organic solvents, while dimers of iron(III)-EDTA were found to be broken by addition of dimethyl sulfoxide, forming monomeric complexes.
3. The photochemical properties of iron(III) citrate complexes were found to depend strongly on the pH: while at pH = 1.5 a complete photoreduction of iron(III) citrate into a stable mixture of iron(II) hexaaqua and citrate complexes occurred, at higher pH > 3 a subsequent reoxidation of the formed iron(II) citrate was detected, resulting in

the partial hydrolysis and polymerization of iron(III) into a photochemically stable compound.

In the second part of the work, I have investigated the properties of nanoparticles-based iron fertilizers. The transformation of the manufactured iron nanoparticles prepared with different surfactant materials was studied by means of Mössbauer spectroscopy, transmission electron microscopy, accompanied with energy dispersive spectroscopy. Nanomaterials at different stages of transformations were subject of plant physiological experiments aiming at comparing the behaviour and plant accessibility of manufactured suspensions of nanoscale ferrihydrite and iron(III) oxide particles. According to the results, following conclusions can be drawn:

4. Among the applied surfactants, polyethylene glycol polymer (mean molecular mass 1500 g/mol) was found to be the best to preserve the size and crystallinity of nanoferrihydrite.
5. Although ferrihydrite was believed to suppress iron deficiency symptoms in plants more effectively, it was shown that hematite obtained after transformation of ferrihydrite nanoparticles was more effective than the initial material, suggesting that not only the crystallinity but also the surfactant cover is crucial for nanoparticle utilization.

In the final part of the work a Neoproterozoic banded iron formations and ironstones occurring in the Urucum mining district (Brazil) and exhibiting signs of microbial origin were investigated by a combination of various techniques. The obtained results allowed to determine new features of the samples reflecting important signatures of microbial activity revealing the biogeochemistry of the biomat formation. The major conclusion of this study is summarized as followed:

6. The representative probes were found to contain iron and manganese nano-oxides and mixed carbonates supporting the proposed mechanism of its microbially mediated formation.

Publications

This thesis is based on the work published in the following papers:

1. **Gracheva, M.**, Homonnay, Z., Singh, A., et al. (2022). New aspects of the photodegradation of iron (III) citrate: spectroscopic studies and plant-related factors. *Photochemical & Photobiological Sciences*, *21,* 983–996, https://doi.org/10.1007/s43630-022-00188-1
2. **Gracheva, M.**, Klencsár, Z., Kis, V.K. et al. (2023), Iron nanoparticles for plant nutrition: Synthesis, transformation, and utilization by the roots of Cucumis sativus. *Journal of Materials Research, 38(4), 1035-1047*. https://doi.org/10.1557/s43578-022-00686-z
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Additional publications not included in this thesis:

1. Singh, A., **Gracheva, M.**, Kis, V.K., Keresztes, Á., Sági-Kazár, M., Müller, B., Pankaczi, F., Ahmad, W., Kovács, K., May, Z., Tolnai, G., Homonnay, Z., Fodor F., Klencsár, Z., Solti Á., (2023) Apoplast utilisation of nanohaematite initiates parallel suppression of RIBA1 and FRO1&3 in Cucumis sativus. *NanoImpact*, *29*, 100444. IF 6.038
2. Béres, K.A., Homonnay, Z., Barta Holló, B., **Gracheva, M.**, Petruševski, V.M., Farkas, A., Dürvanger, Z., Kótai, L., (2023). Synthesis, structure, and Mössbauer spectroscopic studies on the heat-induced solid-phase redox reactions of hexakis (urea-O) iron (III) peroxodisulfate. *Journal of Materials Research*, *38*(4), 1102-1118. IF 2.909
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Abstracts:

1. **Gracheva, M.**, Klencsár, Z., Homonnay, Z., Solti Á., Kovács, K., Iron Citrate Complexes at Biologically Relevant Conditions, Book of Abstracts XXXVII International Conference on the Applications of the Mössbauer Effect, p. 30
2. **Gracheva, M.**, Homonnay, Z., Klencsár, Z., Kovács, K., Iron(III) citrate complexes at physiological pH, Book of Abstracts of the Third International Conference on Radioanalytical and Nuclear Chemistry 2023, ISBN 978-963-454-902-4, p. 156.
3. **Gracheva, M.**, Kis Z., Sági-Kazár, M., Solti Á., Neutron imaging of plant rehydration, Central European Training School on Neutron Techniques 2023, Budapest Neutron Centre, ISBN: 978-963-7351-35-8, p. 13.
4. **Gracheva M.**, Homonnay Z., Solti Á., et al., (2022), Photodegradation of Iron(III) Citrate, the International Symposium on the Industrial Applications of the Mössbauer Effect, Book of Abstracts ISIAME 2022, p. 49.
5. **Gracheva M.**, Homonnay Z., Fodor F., et al., (2022), New Aspects of the Photodegradation of Iron(III) Citrate: Spectroscopic Studies and Plant Related Factors, Őszi Radiokémiai Napok 2022, Balatonszárszó, Hungary, Conference Program and Book of Abstracts, ISBN 978-615-6018-13-7, pp. 80.
6. Barnabás, C., Szilamér, K., **Gracheva M.**, et al., (2022), Leaf senescence initiation related alterations in the iron homeostasis of the chloroplasts. In Abstract Book of the 20th International Symposium on Iron Nutrition and Interactions in Plants. p. 83.
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8. **Gracheva, M.**, Homonnay, Z., Kovács, K., et al., (2021), Ferrihydrite formed from co-precipitated nanomagnetites in citric acid solution for plant nutrition. In International Conference on the Applications of the Mössbauer Effect - Book of Abstracts ICAME 2021. p. 237.
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10. **Gracheva, M.**, Homonnay, Z., Kovács, K., et al., (2021), Spectroscopic studies of iron(III) citrate complexes in aqueous solution. In International Conference on the Applications of the Mössbauer Effect - Book of Abstracts ICAME 2021. p. 232.

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12. **Gracheva, M.**, Homonnay, Z., Singh, A., et al. (2020), Mössbauer Study of Iron(III) Citrate Photodegradation in Solutions, 9th Interdisciplinary Doctoral Conference 2020 Book Of Abstracts, p. 195.

Oral presentations:

1. **Gracheva, M.**, Klencsár, Z., Homonnay, Z., Solti Á., Kovács, K., Iron Citrate Complexes at Biologically Relevant Conditions, XXXVII International Conference on the Applications of the Mössbauer Effect (ICAME 2023), Cartagena, Columbia
2. **Gracheva, M.**, Homonnay, Z., Klencsár, Z., Kovács, K., Iron(III) citrate complexes at physiological pH, The Third International Conference on Radioanalytical and Nuclear Chemistry (RANC-2023), Budapest, Hungary
3. **Gracheva, M.**, Kis Z., Sági-Kazár, M., Solti Á., Neutron imaging of plant rehydration, Central European Training School on Neutron Techniques 2023, Budapest Neutron Centre, Budapest, Hungary
4. **Gracheva M.**, Homonnay Z., Fodor F., et al., (2022), New Aspects of the Photodegradation of Iron(III) Citrate: Spectroscopic Studies and Plant Related Factors, Őszi Radiokémiai Napok 2022, 17-19 October, 2022, Balatonszárszó, Hungary
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6. **Gracheva, M.**, Homonnay, Z., Kovács, K., et al., (2021), Ferrihydrite/goethite/hematite nanoparticles: Transformation and utilization by the roots of *Cucumis sativus*, Őszi Radiokémiai Napok 2021, 18-20 October 2021, Balatonszárszó, Hungary
7. **Gracheva, M.**, Homonnay, Z., Singh, A., et al. (2020), Mössbauer Study of Iron(III) Citrate Photodegradation in Solutions, 9th Interdisciplinary Doctoral Conference 2020, Hungary

Posters:

1. **Gracheva M.**, Homonnay Z., Solti Á., et al., 2022. Photodegradation of Iron(III) Citrate, the International Symposium on the Industrial Applications of the Mössbauer Effect ISIAME 2022, Olomouc, Czech Republic

2. **Gracheva, M.**, Homonnay, Z., Kovács, K., et al., 2021. Spectroscopic studies of iron(III) citrate complexes in aqueous solution. International Conference on the Applications of the Mössbauer Effect ICAME 2021, Brasov, Romania
3. **Gracheva, M.**, Homonnay, Z., Kovács, K., et al., 2021, Ferrihydrite formed from co-precipitated nanomagnetites in citric acid solution for plant nutrition. International Conference on the Applications of the Mössbauer Effect ICAME 2021, Brasov, Romania
4. **Gracheva, M.**, 2021, Speciation of iron(III) citrate relevant to the nutrition of plants. Hercules School 2021, European Synchrotron Radiation Facility and Institut Laue-Langevin, Grenoble, France