UNIVERSIDADE FEDERAL DE LAVRAS

MARINA JUSTI

AGRONOMIC VALUE OF METAL COMPLEXES WITH ORGANIC ACIDS AND NUTRITIONAL EFFECTS OF IRON SOURCES ON PLANTS

LAVRAS – MG

2021

MARINA JUSTI

AGRONOMIC VALUE OF METAL COMPLEXES WITH ORGANIC ACIDS AND NUTRITIONAL EFFECTS OF IRON SOURCES ON PLANTS

VALOR AGRONÔMICO DE COMPLEXOS DE METAIS E ÁCIDOS ORGÂNICOS E EFEITOS NUTRICIONAIS DE FONTES DE FERRO EM PLANTAS

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ciência do Solo, área de concentração em Fertilidade do Solo e Nutrição de Plantas, para obtenção do título de Doutor.

Prof. Dr. Carlos Alberto Silva
Orientador

LAVRAS – MG
2021

Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Justi, Marina.
Agronomic value of metal complexes with organic acids and nutritional effects of iron sources in plants / Marina Justi. - 2021.
100 p.

Orientador(a): Carlos Alberto Silva.

Tese (doutorado) - Universidade Federal de Lavras, 2021.
Bibliografia.

1. chelates. 2. metal complexation. 3. iron nutrition. I. Silva, Carlos Alberto. II. Título.

MARINA JUSTI

AGRONOMIC VALUE OF METAL COMPLEXES WITH ORGANIC ACIDS AND NUTRITIONAL EFFECTS OF IRON SOURCES ON PLANTS

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ciência do Solo, área de concentração em Fertilidade do Solo e Nutrição de Plantas, para obtenção do título de Doutor

Aprovada em 21 de dezembro de 2020.

Dr. Andrés Calderín García UFRRJ

Dr. Marcos Komogawa ESALQ/USP

Dr. Marcelo Eduardo Alves ESALQ/USP

Dr. Guilherme Lopes UFLA

Prof. Dr. Carlos Alberto Silva

Orientador

LAVRAS-MG

2021

Aos meus pais, Paulo e Maria Emília, pelo amor, apoio e ensinamentos sobre persistência, dedicação e felicidade; aos meus irmãos Joseane e Diego, eternos confidentes e parceiros de jornada

Dedico

AGRADECIMENTOS

Agradeço a Deus pela iluminação diante dos desafios, pelas oportunidades e conquistas alcançadas durante a vida.

Agradeço a minha família por todo apoio incondicional e companheirismo.

À Universidade Federal de Lavras (UFLA) e ao programa de pós-graduação do Departamento de Ciência do Solo, pela oportunidade concedida para realização do doutorado. À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pela concessão da bolsa de doutorado, e financiamento das ações de pesquisa (CAPES-PROEX-AUXPE 593-2018); ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, processo 307447-2019-7) e Fundação de Amparo À Pesquisa de Minas Gerais (FAPEMIG) pelo financiamento das ações de pesquisa.

Ao professor orientador Carlos Alberto Silva pela orientação, ensinamentos e compreensão mediante desafios.

Ao professor Matheus Puggina de Freitas e ao Josué Silla, por toda a colaboração, apoio, ensinamentos e paciência me ajudando com a química teórica e computacional. Ao DQI-UFLA por ceder a estrutura para os cálculos teóricos computacionais.

Ao professor Cleiton Antonio Nunes pela ajuda com os estudos de quimiometria.

À professora Dra. Maria Ligia de Souza Silva por permitir o uso da estrutura de hidroponia visando a condução dos experimentos com plantas em casa de vegetação do Setor de Nutrição Mineral de Plantas do DCS-UFLA.

Àqueles que foram minha segunda família em Lavras, companheiros de casa queridos que tornaram a minha vida alegre Jakeline, Pedro, Jéssica, Sibely, Matheus, Lívia, Monna Lysa e Mariane. Muito obrigada por todos os bons momentos e a amizade que ficarão para sempre na minha memória.

Ao Bruno, por ser parceiro, paciente e amoroso me ajudando sempre que possível e me apoiando incondicionalmente nessa jornada.

Aos meus amigos e colegas de trabalho do LEMOS, Sara, por ser minha companheira e confidente, por toda ajuda em experimentos e laboratório, Everton, por todo auxílio, experiência, amizade e conversas, Henrique e Pedro por toda ajuda, Rimena, Bruno e Murilo pela companhia e amizade. Todos vocês tornaram o trabalho muito mais leve.

Às minhas queridas Cynthia e Geila, por toda amizade e momentos maravilhosos, minha estada em Lavras não seria a mesma sem vocês.

Ao Osvaldo e ao Dr. Roberto que me acompanharam durante toda minha vida acadêmica, me orientando, me ajudando em todos os desafios e me ensinando a alcançar a saúde mental.

Aos funcionários do Departamento de Ciência do Solo, em especial, Dirce, Mariene, Lívia, Bethânia e Roberto, muito obrigada por tudo.

A todos aqueles que passaram pela minha vida neste período e contribuíram de alguma forma para minha vida acadêmica

Obrigada!

“A persistência é o caminho do êxito”

Charles Chaplin

ABSTRACT

Complexing agents are largely used for agricultural and environmental applications. For years, synthetic aminopolycarboxilic acids, such as EDTA, have been the most common complexing agents used for these applications. However, aminopolycarboxylic acids are expensive and hardly degraded in the environment. Thus, the investigation of natural molecules that can act as complexing agents is essential for sustainable applications. This study aimed at understanding the chemical properties of complexes formed between several metals and low-molecular-weight organic acids, and testing some of these complexes for agricultural applications. Complexes were synthesized at pH 7 using metals Fe, Mn, and Zn, as well as citric, tartaric, malic, and oxalic acids as complexing agents. The 1:1 and 1:2 stoichiometric ratios of reaction (SR; metal: ligand molar ratio) were tested. The chemical species formed were determined through VISUAL Minteq software. Molecular structure and stability were determined through theoretical calculations, and the complexes were submitted to the Fourier Transformed Infrared Spectra (FTIR analysis). The chemical characterization showed that complexed metal fraction, stability, and solubility depended on reaction stoichiometry, metal type, and complexing agent type. Oxalic acid was the smallest molecule used, and its complexes were predominantly of the 1:2 stoichiometry, presenting very low solubility and high stability. Citric and malic acid formed highly soluble complexes. Malic acid only formed 1:1 stoichiometry complexes with all metals and both stoichiometries tested. Citric and tartaric acid tended to form 1:2 complexes with Zn and 1:1 complexes with Mn and Fe. Considering the metals tested, the Fe complexes were more stable than Mn and Zn complexes. The chemometric partial least square technique was used to test the possibility to predict complex properties from spectra. Solubility and complexed-metal fraction were adequately predicted from FTIR spectra. The use of iron complexes as Fe sources in foliar application to maize and nutrient solution addition to soybean and maize was tested in greenhouse experiments. Foliar application of Fe-tartrate and Fe-citrate at 1:2 SR promoted similar plant growth and better Fe nutrition than the application of Fe-EDTA and FeSO4. When applied in the nutrient solution, iron-organic acid complexes kept less iron in the complexed form than EDTA. The effectiveness of complexes in promoting Fe accumulation and plant growth depended on plant species. Despite showing low complexation capacity, the Fe-tartrate at 1:2 SR promoted suitable Fe nutrition in maize plants. As for soybean, Fe-oxalate complexes promoted growth and Fe accumulation in plants similarly to that of the Fe-EDTA treatment.

Keywords: chelates. Visual Minteq. computational modeling. foliar fertilization. iron nutrition. metal complexation. molecular structure. stability.

RESUMO

Agentes complexantes são amplamente usados para aplicações agronômicas e ambientais. Durante anos os ácidos aminopolicarboxílicos sintéticos foram os agentes complexantes mais utilizados para tais aplicações. Entretanto, tais moléculas tem elevado custo e baixa degradabilidade no ambiente. Portanto, a investigação de moléculas naturais que possam agir como agentes complexantes é essencial no âmbito da aplicação sustentável. Este estudo objetivou investigar as propriedades químicas de complexos formados entre vários metais e ácidos orgânicos de baixa massa molecular, bem como testar alguns desses complexos para aplicação agronômica. Os complexos foram sintetizados utilizando os metais Fe, Mn, Zn e os ácidos cítrico, tartárico, málico e oxálico como agentes complexantes. Duas estequiometrias de reação, 1:1 e 1:2 (SR, metal: ligante em proporção molar), foram testadas. As espécies químicas formadas foram determinadas por meio do software VISUAL Minteq. A estrutura molecular e a estabilidade dos complexos foram determinadas por meio de cálculos teóricos, e os complexos foram submetidos análise de infravermelho com transformada de Fourier (FTIR). A caracterização química mostrou que a fração de metal complexado, a estabilidade e solubilidade dependeram da estequiometria de reação testada, do metal e do agente complexante utilizados. O ácido oxálico, o menor ligante utilizado, apresentou predominantemente complexos com estequiometria 1:2 com baixa solubilidade e alta estabilidade. O ácido cítrico e ácido málico formaram complexos altamente solúveis. O ácido málico apresentou a formação de complexes 1:1 com todos os metais e estequiometrias testadas. O ácido tartárico e o ácido cítrico tendem a formar complexos do tipo 1:2 com Zn e complexos do tipo 1:1 com Fe e Mn. Considerando os metais testados, os complexos de Fe foram mais estáveis que os complexos de Zn e Mn. A técnica quimiométrica de regressão de mínimos quadrados foi usada para verificar a possibilidade de predizer as características químicas dos complexos por meio do espectro de infravermelho. A solubilidade e fração de metal complexada foram adequadamente preditos pelo espectro de infravermelho. O uso de complexos de Fe como fonte do nutriente em aplicação foliar e em solução nutritiva foi testada em condições de casa de vegetação. A aplicação foliar de Fe-tartarato e Fe-citrato na estequiometria 1:2 promoveu crescimento vegetal similar ao Fe-EDTA e ao $FeSO_4$. Quando aplicados em solução nutritiva, os complexos de ácidos orgânicos com Fe mantiveram menos Fe na forma complexada do que o EDTA. A efetividade dos complexos em promover o acumulo de ferro e crescimento vegetal dependeu da espécie testada. Embora tendo menor capacidade complexante, o uso do Fe-tartarato na estequiometria 1:2 promoveu crescimento e acúmulo de Fe adequados nas plantas de milho. No caso da soja, o uso de Fe-oxalato promoveu crescimento e acúmulo de Fe similar ao uso de Fe-EDTA.

Keywords: quelatos. Visual Minteq. modelagem molecular. fertilização foliar. nutrição em Fe. complexação de metais. estrutura molecular. estabilidade

SUMÁRIO

SECTION 1

General Introduction

Complexing agents are molecules with one or more chemical groups able to donor electrons. Metals in general have a high affinity for accepting electrons. Complexing agents and metals reaction give rise to a molecule named coordination compound or complex (HOSMANE, 2017). If the complexing agent has two or more groups capable of donor electrons, it is also called chelating agent (HOSMANE, 2017). In the final molecular structure of the complex or chelate, the metal cation occupies the central position, and the complexing agent stays around it, a structural conformation that promotes high stability to the molecule formed (KOLODYNSKA, 2020).

Complexing agents are currently used in the health, industrial, environmental, and agricultural domains for several purposes (CLEMENS *et al.*, 1990; KALIA, 2005; RODRÍGUEZ-LUCENA *et al.*, 2010). In agriculture, complexing agents are used to keeping micronutrients in soluble forms and avoid undesirable reactions such as precipitation (e.g., the reaction of iron with phosphate) (ABADÍA *et al.*, 2011). Complexing agents are also used to increase the efficiency of plant uptake and translocation of nutrients (ÁLVAREZ-FERNÁNDEZ *et al.*, 2004; RODRÍGUEZ-LUCENA et al., 2010). Complexing agents are also used to increase desorption of metals from soil and sediments, increasing soluble metal forms, and improving their uptake by plants (GUO *et al.*, 2018). Additionally, complexing agents can be used to reduce metal bioavailability, fixing up the element in complexes of high stability and low solubility (SU *et al.*, 2015).

For several years, synthetic aminopolicarboxylic acids, such as EDTA and DTPA, were the most frequently chelating agents used in the agricultural and environmental fields (PINTO *et al.*, 2014). However, these chelating agents have a high cost and are hardly degraded in the environment (NOWACK and VANBRIESEN, 2005). EDTA and its metal complexes can remain for years in soils and sediments, altering the metal equilibria (NOWACK *et al.*, 2006). EDTA was found in significant amounts in subsurface waters and some metal-EDTA chelates like Cu-EDTA are hardly degraded by living microbial community in soils (NOWACK *et al.*, 2006), thus, representing a threat to soil fauna (DUO *et al*., 2019). Furthermore, the high cost of aminopolycarboxylic acids limits their application for valuable crops (ABADÍA *et al.*, 2011). Therefore, the use of biodegradable, environmentally friendly, and low-cost complexing agents is highly desirable.

Low molecular organic acids and humic substances have carboxylic and phenolic groups that can complex metals (PINTO *et al.*, 2014). Low molecular weight organic acids such as citric acid, malic, and oxalic acid were already successfully used as complexing agents in the remediation process of contaminated soils, increasing metals desorption and promoting high metal bioaccumulation by plants (EVANGELOU *et al.*, 2007). Furthermore, the use of citric acid as a complexing agent for iron in the foliar spray effectively promoted iron nutrition in plants, with a similar agronomic performance of Fe-EDTA (CHAKRABORTY *et al.*, 2014).

Several chemical aspects of the organometallic complexes can affect their applicability and effectiveness in supplying nutrients to plants (CARRASCO *et al.*, 2012; Fig. 1). Although some studies showed the efficacy of natural organic molecules as complexing agents, several chemical aspects of the complexes formed still lack to be clarified. The complexes can differ significantly concerning their solubility, complexation capacity, stability, and molecular structure (URRUTIA *et al.*, 2014; GARCIA-MINA, 2006). Molecular structure and spatial conformation of the complex molecule can affect their metabolism and uptake by the soil organisms (JOSHI-TOPE and FRANCIS, 1995), as well as their capacity to interact with mineral surfaces in soil and sediments (TANG *et al.*, 2017). Stability can affect the process of metal displacement from complexes by plants and microorganisms, and if exchange reactions with other cations will occur in the environment (GARCIA-MINA, 2006). The metal complexation capacity is crucial to define the complex agent effectiveness in keeping most of the metal in complexed and soluble forms in soil or nutrient solution (ZANIN *et al.*, 2019; GARCÍA-MINA *et al.*, 2004). Complexation capacity depends on the affinity of the metal for chemical groups of the ligand, and, therefore, can be affected by the metal ion that is supposed to be complexed, by the other ions present in the media, and pH and stoichiometry of reaction as well (GARCIA-MINA, 2006). Solubility of the complex should be high if the intended application is increase the bioavailability of the metal (GARCÍA-MINA *et al*. 2004), and can be low if the goal is to keep the metal in stable and low soluble forms, reducing its toxicity (SU *et al.*, 2015)

Although low molecular weight organic acids can be used for several environmental and agricultural purposes, some chemical properties of their complexes with metals are still not clear. It is important to investigate the stoichiometry of reaction to be used and the stability and molecular conformation of the complexed metal. The same ligand can result in complexes with different stabilities depending on the metal, pH, and stoichiometry used (CHU *et al.*, 2011). Considering the number of organic molecules that can be used in the complexes synthesis and the myriad of conditions that these complexes can be applied, it is necessary to elucidate how factors such as pH, stoichiometry, ligand, and metal affect stability, complexation capacity, and solubility of the complexes formed.

Regarding the agricultural use, the effectiveness of a molecule to act as complexing agent for a nutrient also depends on the genetic and physiological characteristics of the plant species (GARCÍA-MINA *et al.*, 2004). Differences in the root architecture, Kasparian strip thickness, and abundance of root transporters can also affect the metal complex absorption (LEŠTAN *et al.*, 2008; Fig. 1). On the leaf, the cuticle morphology and stomata abundance can affect the absorption (ABADÍA et al., 2011; Fig. 1). Plants can also exhibit different strategies to acquire nutrients with different oxidation states, such as iron. Monocotyledonous species release complexing substances (phytosiderophores) that allow them to uptake iron in oxidized forms (KOBAYASHI and NISHIZAWA, 2012). Dicotyledonous species release substances that promote reduction of iron before its acquisition by roots (KOBAYASHI and NISHIZAWA, 2012). Therefore, iron sources probably exhibit different effectiveness in nourishing monocots and dicots species. Additionally, the same micronutrient complex can have different effectiveness when applied *via* nutrient solution (root uptake) or *via* foliar spray (leaf penetration). Thus, a molecule used for nutrient solution application cannot be suitable for foliar application (RODRÍGUEZ-LUCENA *et al.*, 2010). Therefore, the growing of plant tests submitted to different application modes of complexed nutrients is essential to define their agronomic value according to the different fertilization management strategies.

Furthermore, organic molecules such as humic substances and low molecular weight organic acids play a role as biostimulants, affecting plant physiology and growth (ZANIN *et al.*, 2019). The effects of these molecules on the physiological process, such as respiration and photosynthesis, can vary considerably according to the plant species and used molecule concentration (CONSELVAN *et al.*, 2017). Organic acids are recognized by the ability to reduce abiotic stress effects in the plant physiology, replenishing of Krebs Cycle in nutrient starvation conditions, improvement of antioxidant system in plants, and by increasing metal uptake rate in plant cells (CARL-HUBER et al. 2016). Thus, the use of these molecules as complexing agents can promote effects on plants beyond plant nutrition.

In this sense, this study intended to assess several chemical attributes that affect the applicability of complexes formed between Fe, Zn, and Mn, and citric, malic, tartaric, and oxalic low molecular weight organic acids. Furthermore, we conducted experiments with soybean and maize in greenhouse conditions to determine the agronomic value of Fe-organic acid complexes applied to nutrient solution and via foliar spray in promoting adequate plant growth and iron nutrition.

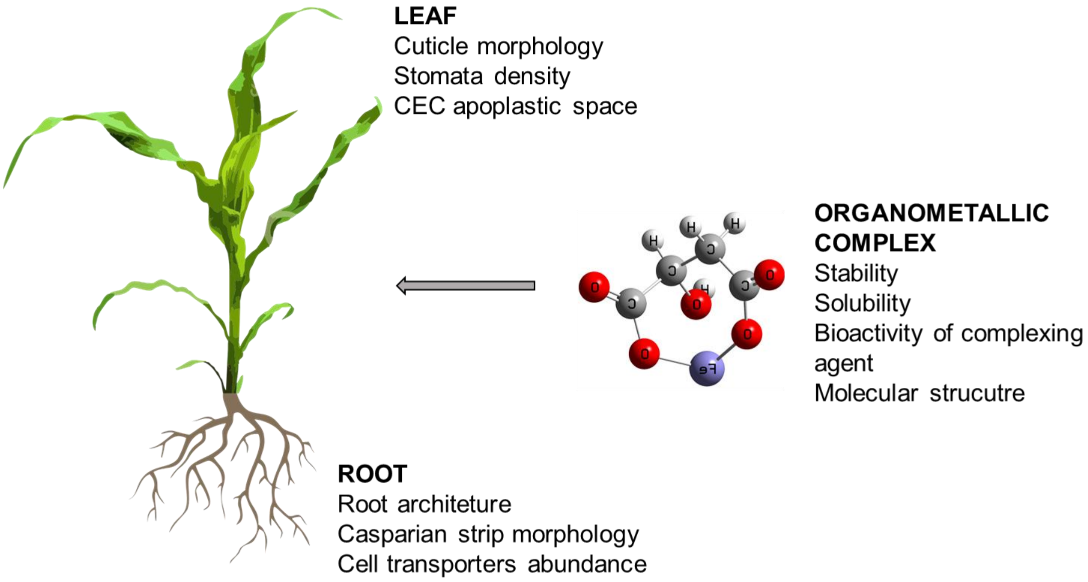

Figure 1 - Factors affecting the dynamic of complexed nutrients in plants.

Objectives

The aims of this study were:

i) to determine the thermodynamic stability, molecular structure, metal complexed fraction, and FTIR signature of the complexes formed between organic acids (citric, malic, tartaric, and oxalic acids) and metals (Mn, Fe, and Zn) at two different stoichiometries of reaction;

ii) to test the effectiveness of foliar-applied iron-organic acids complex in promoting adequate iron nutrition and plant growth, and to verify if the stability of the complex influences iron accumulation and redistribution in plants;

iii) to assess the effectiveness of iron-organic acid complexes applied to nutrient solution in promoting adequate iron nutrition and growth of maize and soybean plants.

This study was structured in three chapters, hereafter defined as articles, with the following titles: Article 1 – "Conformational structure and fast identification of chemical properties of metal-carboxylate complexes by FTIR and partial least square regression"; Article 2 – "Influence of Complex Stability on Iron Accumulation and Redistribution for Foliar-Applied Iron-Organic Acid Complexes in Maize"; and Article 3 – "Organic acids as complexing agents for iron and their effects on nutrition and growth of maize and soybean".

References

ABADÍA, J.; VÁZQUEZ, S.; RELLÁN-ÁLVAREZ, R.; EL-JENDOUBI, H. *et al.* Towards a knowledge-based correction of iron chlorosis. **Plant Physiology and Biochemistry**, 49, n. 5, p. 471-482, 2011.

ÁLVAREZ-FERNÁNDEZ, A.; GARCÍA-LAVIÑA, P.; FIDALGO, C.; ABADÍA, J. *et al.* Foliar fertilization to control iron chlorosis in pear (*Pyrus communis* L.) trees. **Plant and Soil**, 263, n. 1, p. 5-15, 2004.

CARRASCO, J.; KOVÁCS, K.; CZECH, V. r.; FODOR, F. *et al.* Influence of pH, iron source, and Fe/ligand ratio on iron speciation in lignosulfonate complexes studied using Mössbauer spectroscopy. Implications on their fertilizer properties. **Journal of agricultural and food chemistry**, 60, n. 13, p. 3331-3340, 2012.

CHU, C.; DARLING, K.; NETUSIL, R.; DOYLE, R. P. *et al.* Synthesis and structure of a lead(II)–citrate: $\{Na(H_2O)_3\}[Pb_5(C_6H_5O_7)_3(C_6H_6O_7)(H_2O)_3]\cdot 9.5H_2O$. **Inorganica Chimica Acta**, 378, n. 1, p. 186-193, 2011.

CLEMENS, D. F.; WHITEHURST, B. M.; WHITEHURST, G. B. Chelates in agriculture. **Fertilizer Research**, 25, n. 2, p. 127-131, 1990.

CONSELVAN, G. B.; PIZZEGHELLO, D.; FRANCIOSO, O.; DI FOGGIA, M. *et al.* Biostimulant activity of humic substances extracted from leonardites. **Plant and Soil**, 420, n. 1, p. 119-134, 2017.

DUO, L.; YIN, L.; ZHANG, C.; ZHAO, S. Ecotoxicological responses of the earthworm *Eisenia fetida* to EDTA addition under turfgrass growing conditions. **Chemosphere**, 220, p. 56-60, 2019.

EVANGELOU, M. W. H.; EBEL, M.; SCHAEFFER, A. Chelate assisted phytoextraction of heavy metals from soil. Effect, mechanism, toxicity, and fate of chelating agents. **Chemosphere**, 68, n. 6, p. 989-1003, 2007.

GARCIA-MINA, J. M. Stability, solubility and maximum metal binding capacity in metal–humic complexes involving humic substances extracted from peat and organic compost. **Organic Geochemistry**, 37, n. 12, p. 1960-1972, 2006.

GARCÍA-MINA, J. M.; ANTOLÍN, M. C.; SANCHEZ-DIAZ, M. Metal-humic complexes and plant micronutrient uptake: a study based on different plant species cultivated in diverse soil types. **Plant and Soil**, 258, n. 1, p. 57-68, 2004.

GUO, X.; ZHAO, G.; ZHANG, G.; HE, Q. *et al.* Effect of mixed chelators of EDTA, GLDA, and citric acid on bioavailability of residual heavy metals in soils and soil properties. **Chemosphere**, 209, p. 776-782, 2018.

HOSMANE, N. S. Chapter 5 - Ligands and d-Block Metal Complexes. *In*: HOSMANE, N. S. (Ed.). **Advanced Inorganic Chemistry**. Boston: Academic Press, 2017. p. 75-87.

JOSHI-TOPE, G.; FRANCIS, A. J. Mechanisms of biodegradation of metal-citrate complexes by Pseudomonas fluorescens. **Journal of Bacteriology**, 177, n. 8, p. 1989-1993, 1995.

KALIA, K.; FLORA, S. J. S. Strategies for Safe and Effective Therapeutic Measures for Chronic Arsenic and Lead Poisoning. **Journal of Occupational Health**, 47, n. 1, p. 1-21, 2005.

KOBAYASHI T.; NISHIZAWA N. K. 2012. Iron Uptake, Translocation, and Regulation in Higher Plants. **Annual Reviews in Plant Biology,** 63, n. 1, p.131–152.

LEŠTAN, D.; LUO, C.-l.; LI, X.-d. The use of chelating agents in the remediation of metal-contaminated soils: A review. **Environmental Pollution**, 153, n. 1, p. 3-13, 2008.

NOWACK, B.; SCHULIN, R.; ROBINSON, B. H. Critical Assessment of Chelant-Enhanced Metal Phytoextraction. **Environmental Science & Technology**, 40, n. 17, p. 5225-5232, 2006.

NOWACK, B.; VANBRIESEN, J. M. Chelating Agents in the Environment. *In*: **Biogeochemistry of Chelating Agents**: American Chemical Society, 2005. v. 910, cap. 1, p. 1-18. (ACS Symposium Series).

PINTO, I. S. S.; NETO, I. F. F.; SOARES, H. M. V. M. Biodegradable chelating agents for industrial, domestic, and agricultural applications—a review. **Environmental Science and Pollution Research**, 21, n. 20, p. 11893-11906, 2014.

RODRÍGUEZ-LUCENA, P.; HERNÁNDEZ-APAOLAZA, L.; LUCENA, J. J. Comparison of iron chelates and complexes supplied as foliar sprays and in nutrient solution to correct iron chlorosis of soybean. **Journal of Plant Nutrition and Soil Science**, 173, n. 1, p. 120-126, 2010.

SU, X.; ZHU, J.; FU, Q.; ZUO, J. *et al.* Immobilization of lead in anthropogenic contaminated soils using phosphates with/without oxalic acid. **Journal of Environmental Sciences**, 28, p. 64-73, 2015.

TANG, Q.; ZHOU, T.; GU, F.; WANG, Y. *et al.* Removal of Cd(II) and Pb(II) from soil through desorption using citric acid: Kinetic and equilibrium studies. **Journal of Central South University**, 24, n. 9, p. 1941-1952, 2017.

URRUTIA, O.; ERRO, J.; GUARDADO, I.; SAN FRANCISCO, S. *et al.* Physico-chemical characterization of humic-metal-phosphate complexes and their potential application to the manufacture of new types of phosphate-based fertilizers. **Journal of Plant Nutrition and Soil Science**, 177, n. 2, p. 128-136, 2014.

ZANIN, L.; TOMASI, N.; CESCO, S.; VARANINI, Z. *et al.* Humic Substances Contribute to Plant Iron Nutrition Acting as Chelators and Biostimulants. **Frontiers in Plant Science**, 10, n. 675, 2019.

SECTION 2 - ARTICLES

ARTICLE 1 - Experimental FTIR and theoretical investigation of chemical properties and molecular structure of metal-carboxylate complexes

Marina Justi*, Matheus P. Freitas, Josué M. Silla, Cleiton Antônio Nunes, Carlos Alberto Silva

Department of Soil Science, Federal University of Lavras, P.O. Box 3037, 37200-900, Lavras, MG, Brazil

Department of Chemistry, Federal University of Lavras, P.O. Box 3037, 37200-900, Lavras, MG, Brazil

E-mail:
Marina Justi* - marina.justi@gmail.com
* Corresponding author

This article was prepared in line with the guidelines of the ‘Journal of Molecular Structure’

Highlights

Molecular arrangement of metal-carboxylate complexes was unraveled

FTIR fingerprints of metal-carboxylate complexes are shown and discussed

Coordination modes of metals to carboxylate group can be estimated based on the infrared COO^- band shifts

Complexed fraction and solubility of metal complexes can be estimated from the combined use of FTIR spectra-partial least squares regression models

Abstract

Several chemical properties of complexes should be considered to base their suitable applications in agronomic and environmental domains. However, the determination of these characteristics is generally expensive and time consuming. Therefore, the prediction of properties of metal-complexes using a simple and expeditious technique, such as infrared spectroscopy, is highly desirable. This study aimed to investigate the molecular structure and chemical properties of metal complexes, such as stability, metal complexed fraction, and solubility. The complexes were submitted to experimental FTIR analysis and the spectra dataset were regressed against the metal-complexes properties aforementioned using partial least squares (PLS) to obtain a predictive model. Low molecular weight carboxylic acids (citric acid, malic acid, tartaric acid, oxalic acid) were used as complexing agents for three metals (Mn, Zn and Fe) in two stoichiometric ratios of reaction (1:1 and 1:2 metal: ligand molar ratio). Solubility and complexation ratio appeared to be dependent on the ligand and stoichiometry. Citrate and oxalate have formed the most stable complexes with the tested metals. Metal-oxalates tend to form 1:2 complexes at the pH and stoichiometries tested, whereas citrate, malate and tartrate tend to form 1:1 complexes. All the complexes exhibited monodentate coordination of carboxylic groups with metals. Overall, predictive models were built to estimate the solubility and the ratio of complexed metals. Furthermore, the coordination mode of ligands to metals could also be assigned from infrared band shifts of the carboxylate groups. This work revealed crucial chemical properties to base the use of metal-complexes, and show that the fast and non-destructive FTIR technique can be used for predict these properties.

Keywords: Partial least square regression, stability, monodentate coordination, stereochemistry

1. Introduction

Complexing agents are extensively applied for agricultural and environmental purposes [1–3]. In agriculture, they are commonly used to increase the bioavailability of nutrients, as well as to reduce the reactivity of these nutrients with soil colloids and other chemical species present in soil or nutrient solution [2,4]. In the environmental domain, complexing agents are commonly used for metal remediation processes, to desorb metals of environmental matrices, such as soil and sediments, and increase the metal bioavailability and efficiency of phytoextraction [1]. They can also be used for remediation processes that involve stabilization, and fixation of metals in stable and low soluble forms to prevent leaching of metals into the ground water[5]. In this case, the main goal is to make the metal less available for plants and microorganisms, reducing metal activity in soil solution [5,6].

Synthetic aminopolycarboxylic acids (such as EDTA and DTPA) have been used as the main chelating agents for agricultural and environmental applications for several years [1,7,8]. However, the low biodegradability of these molecules limits their use for large-scale field applications [8,9]. Therefore, the use of natural and biodegradable complexing agents, such as low-molecular-weight organic acids, is a more environmentally friendly option [1,10,11].

Organic acids (e.g., citric acid, malic acid, tartaric acid, and oxalic acid) are naturally found in soil and plant system and play several roles as complexing agents [12]. The release of these molecules by plants in the rhizosphere is a recognized mechanism to improve metal uptake and accumulation [13]. Although the organic acids exhibit a lower metal complexing ability than aminopolycarboxylic acids, they have been used successfully for environmental [14,15] and agricultural applications [16,17].

Several chemical conditions can control the formation of complexes in the environment, such as pH, metal type, and stoichiometry of reaction [18,19]. Furthermore, there are chemical properties of complexes that need to be understood to improve their performance in different applications and environments, such as their molecular conformation, stability, and solubility. It was found that the ability of soil bacteria to capture and metabolize metal complexes is associated with their stability and stereochemistry [20]. Several coordination modes in complexes involving the carboxylate group and metals are recognized, such as monodentate, bidentate and chelating, and these chemical bonds can affect the bioactivity of complexes [21]. Stability and solubility are also key factors influencing the availability of metal complexed for plant acquisition [4,22]. Stability is an index to infer the complexing agent and metal affinity to form the complex. It depends on the energy associated with bonds and molecular conformation [23]. Metal complexed fraction is related with stability; generally, the greater is the complexed fraction, the higher is the chemical stability of the complex [24]. The metal complexed ratio also indicates the ability of the complexing agent to keep the metal complexed in specified conditions of reaction (pH, stoichiometry, metal concentration) [25].

Several techniques are used for the determination of complex chemical properties. Thermodynamic stability determination is not a simple technique and requires a number of calculations with variables related to energetic parameters [23,26]. Thus, the use of theoretical calculations and computational modeling to assess the molecular structure and their thermodynamic parameters have been shown to be a very useful tool [27,28]. Determination of the complexation ratio usually involves chromatographic and spectrometric analyses or separation methods based on ultrafiltration, which are expensive and time consuming, limiting their application [18,29]. Therefore, the chemical speciation obtained by specific software has been largely used to determine the products of complexation reactions in solution [30–32].

Fourier Transform Infrared (FTIR) spectroscopy is a valuable tool to study the changes in functional groups signatures and in the molecular structure of metal complexes. This technique has been used to identify functional groups involved in the complexation reaction and conformational features of the formed complexes [33,34]. The band shift relative to specific vibrational modes can be used as an index of coordination modes and aspects of the molecular structure [35]. Multivariate calibration methods have been successfully applied to model spectroscopic data, with the aim of constructing predictive models for the determination of chemical properties of compounds [36]. Partial Least Squares (PLS) regression is one of the most popular multivariate regression techniques used to predict sample properties from FTIR spectra [37,38]. This technique is often used to predict the content of specific components from samples, such as fatty acids [39], flavonoids, and carotenoids [40] in food samples, as well as to estimate the content of soil organic matter and its properties in soils and sediments [41], and to predict aromaticity, C/N ratio, cation exchange capacity, and dissolved organic carbon content in plant substrates [42].

Considering that FTIR is a fast, non-destructive method and does not require sample pre-treatment, this analysis and the calibration techniques used to predict sample properties from spectra have attracted much interest within the green-chemistry scope and its multiple applications [36]. The possibility of determining properties that are conventionally highly cost and time-consuming from a simple, non-destructive, and inexpensive method, such as FTIR, is highly desirable [36,42]. Therefore, the main aim of this study was to find out key properties of metal-carboxylate complexes (molecular structure, stability, solubility, and metal complexed ratio), that affect their applicability and performance in environmental and agricultural uses, and to study the use of FTIR spectra dataset to predict these chemical properties. Accordingly, in the synthesis of complexes, three different metals (Fe, Mn, and Zn) were mixed with different low molecular weight organic acids as organic ligands (citric, malic, tartaric, and oxalic acids). The key chemical properties of complexes were determined with known methods and briefly discussed. Sequentially, the possibility to use FTIR spectra to verify aspects of molecular structure and to predict metal complexed fraction, solubility, and stability of complexes was investigated. Hypothetically, FTIR combined with PLS regression can be useful and a suitable approach to predict the chemical properties of metal organic acid complexes.

2. Material and Methods

2.1 Synthesis

The low molecular weight organic acids studied as complexing agents were: tricarboxylic citric acid ($pKa_1 = 3.13$, $pKa_2 = 4.76$, $pKa_3 = 6.40$), dicarboxylic *DL*-malic acid ($pKa_1 = 3.46$; $pKa_2 = 5.10$), dicarboxylic *L*-tartaric acid ($pKa_1 = 3.04$; $pKa_2 = 4.37$), and dicarboxylic oxalic acid ($pKa_1 = 1.23$; $pKa_2 = 4.19$). The 1:1 and 1:2 (metal: ligand molar ratio) stoichiometric ratios (SR) were chosen for organic acid reactions with metals because previous studies have shown that these are the most energetically favorable for transition metals and organic ligands, such as citric and oxalic acids [39,43]. Stock solutions of metal-sulfates ($MnSO_4.4H_2O$, $FeSO_4.7H_2O$, and $ZnSO_4.7H_2O$) and organic acids were prepared at 0.1 mol L^{-1}. The complexes were prepared by mixing 50 mL of the metal-sulfate stock solution with 50 mL (1:1 SR) or 100 mL (1:2 SR) of organic acid stock solutions. The pH was adjusted to 7.0 using a KOH 0.5 mol L^{-1} solution. The salts used in the formulation of complexes were analytical grade reagents. Regarding the complex solubility characterization, triplicate solutions at 0.01 mol L^{-1} concentration were prepared and shaken for 30 min. In sequence, the solutions were centrifuged to separate the precipitate, and the metal concentration was determined in the supernatant in an atomic absorption spectrometer (AAS).

2.2 Metal chemical speciation

To estimate the amount of metal complexed by the organic acids, as well as the main species of complexes formed in solution, the software of chemical speciation VISUAL Minteq version 3.1 was used. For the estimations, the metal (M) salts and complexing agents were added as initial compounds with the same concentration used in the laboratory synthesis (0.01

mol L^{-1}). The titration mode was used to reach the pH 7, and the titrant used was KOH 0.5 mol L^{-1}, mirroring the laboratory conditions. In the titration mode, several steps are previously defined, and the result of chemical speciation in each step can be selected to visualization. The temperature used was 25ºC and the CO_2 partial pressure and its dissolution in the solutions were not considered. The chemical species of the VISUAL Minteq outputs were classified as free-metal or complexed-metal. Free-metal represented the sum of ionic and non-complexed forms, such as M^{2+}, MOH^+ and MSO_4 (aq). The complexed metal represented the sum of all other forms of metals associated with the organic ligands (complexes), with negative, positive and neutral charges. Complexed-metals formed with one metal atom and one molecule of ligand were classified as 1:1 type, such as the $M(Citrate)^-$ and M(Malate) chemical species. Complexed-metals formed with one metal atom and 2 ligand molecules were classified as 1:2 type, such as the $[M(Oxalate)_2]^{-2}$ and $[M(Tartrate)_2]^{-2}$ chemical species.

2.3 Computational modeling

The geometry optimization of isolated organic acids and the complexes formed between metals and organic acids was quantum-chemically performed using the Gaussian 09W software. Both 1:1 and 1:2 (metal: ligand) stoichiometries were designed as input molecules for all metal complexes [28,43]. For complexes, the most likely geometry was considered with three carboxyl groups connected to metals for CA and with two carboxyl groups for MA, TA and OA at a 1:1 stoichiometry. Regarding the 1:2 stoichiometry, the most likely geometry was considered with three or two carboxyl groups of each organic acid molecules binding to metals as input geometries. All the input geometries were subsequently optimized at the density functional theory (DFT) ωWB97XD method, using the LanL2DZ basis set [28], which is a suitable and effective core potential for post-third row atoms, such as Fe, Mn and Zn. The calculations were performed considering both the gas phase and water as implicit solvents [28,39] (through the polarizable continuum model using the integral equation formalism)[44].

After the geometry optimization, the thermodynamic parameters were studied in order to compare the stability of the complexes tested. Accordingly, the standard Gibbs free energies (G^o) for the ligand, iron and complex were obtained and, then, used to calculate the standard Gibbs free energy of complexation (ΔG^o), according to the following Equation 1:

$$\Delta G^o = G^o \text{ complex} - (G^o \text{ organic acid} + G^o \text{ iron}) \qquad \text{(Eq. 1)}$$

2.4 FTIR Analysis

For the Fourier Transform Infrared analysis, the solutions of metal-complexes were frozen and lyophilized to obtain solid samples. The samples were homogenized and analyzed in a Cary 630 Agilent® equipment with a ZnSe crystal with an ATR configuration . The scans were done at 4 by 4 cm^{-1} resolution, with 64 scans, and from 650 to 4000 cm^{-1}. The software *Fityk* [44] was used for peak processing (peaks deconvolution).

2.5 Partial Least Squares Regression

Spectral data for 24 complex samples (Fe, Mn, and Zn complexes of citric, malic, oxalic, and tartaric acids, at both 1:1 and 1:2 stoichiometric ratios) were used for PLS regression analysis. The FTIR spectra (absorbance from 4000 to 650 cm^{-1}) of complexes were regressed against the respective chemical attributes (solubility, total metal complexed fraction, and stability) using partial least squares (PLS) regression, thus yielding PLS calibration models. The models obtained can be used to estimate chemical parameters of the complexes without to need using classical methods. The calibration performance was evaluated using the root mean square error of calibration ($RMSE_c$) and the squared correlation coefficient of calibration (R^2_{cal})[42,45]. $RMSE_c$ is a measure of how well the model fits the experimental results, and it was calculated through the equation 2 [45]:

$$\text{RMSEc} = \sqrt{\frac{\sum_{i=1}^{n}(\hat{y}i-yi)^2}{n}} \quad \text{(Eq. 2)}$$

where yi is the reference value of the dependent variable, $\hat{y}$i is the predicted value, y is the mean value, and n is the number of samples.

The leave-one-out cross validation method and a y-randomization test were used to validate the PLS models [46]. The root mean-square error of cross validation and the determination coefficient of cross validation were calculated according to the recommended literature [45]. The performance parameters root mean square of error of y-randomization and determination coefficient in the y-randomization were also calculated according to [45]. The models were tested through an external group of test samples, which were not included in the calibration process and used to verify the predictive capacity of the PLS models. From this step, the root mean square error of prediction ($RMSE_p$), and the coefficient of prediction (R^2pred) were determined and used as statistical parameters to evaluate the model performance on prediction [45]. The multivariate modeling and calculations were carried out using the Chemoface version 1.4 software [47].

3. Results and Discussion

3.1 Complex stability and molecular structure modeling

The computationally optimized molecular structures of the organometallic complexes are shown in Figure 1 (iron complexes), Figure 2 (manganese complexes), and Figure 3 (zinc complexes). Complexes of 1:2 type for all organic acids exhibited four bonds with metals, yielding a tetrahedral geometry (Figure 1, 2, 3). Although the citric acid has three carboxylic groups, in the 1:2 configuration only two groups of each molecule interacted with metals. The isomer with three carboxylic groups interacting with metal was tested, but it was less stable than the isomer with two carboxylic groups, for all metals. This probably occurred due to a high repulsion between the negatively charged oxygens, increasing the steric hindrance in the isomer with three carboxylates positioned in the center around the metal. Three bonds with the metal were formed in 1:1 citrate complexes, configuring a pyramidal arrangement. In 1:1 malate, tartrate and oxalate complexes, two bonds are formed with metals. Interactions of carboxylate groups with metal exhibited a monodentate configuration for all complexes studied (Figures 1, 2 and 3).

The Gibbs free energies also obtained through the theoretical calculations for the complexes formed between metal and organic acid as ligand are shown in Table 1. Complexes with Fe, in general, exhibited the lowest values of energy, suggesting greater stability of Fe complexes compared to Mn and Zn complexes. Zn complexes exhibited a lower stability compared with Mn and Fe. Stability of metal complexes generally increases with a decrease in the size of metal cations [23,50]. Zinc has an ionic radius (0.74 Å) larger than Mn and Fe (both of them around to 0.7Å), which partially explains the lower stability of zinc complexes. In general, the 1:2 complex type (with four bonds with metal) was more stable than 1:1 (with three or two bonds to metal) for all the ligands and metals considered.

The number of bonds formed around the metal ion could be ascribed to its coordination number [50]. The coordination number indicates the number of electron pairs that metal should receive to complete their orbitals in the ion valence layer [51]. This number is not unique because other factors, such as ligand size and charge, could affect their binding capacity [52]. Zn coordination number is usually 4, but it can also present a coordination number 6. Fe and Mn present octahedral coordination (coordination number 6) in most cases [52]. Therefore, differences in coordination number could explain the differences in stability. In the 1:2 stoichiometry ratio of metal-organic acids, more electron pairs are donated to metal ions, either achieving their coordination number (in the case of Zn) or approaching to it (in the case of Mn and Fe). The coordination number could be reduced in cases of high steric hindrance of ligands with large molecular size [43,52], such as in the 1:2 citrate complexes that exhibit only two carboxylic groups in each citrate ligand interacting with metals, instead of three groups. Furthermore, carboxylate groups rotate from its free to complexed forms [43], affecting the neat stability values.

Other variables, such as molar concentration, pH and reaction stoichiometry, can influence the formation and stability of the complexes [18], but these factors were not considered in the theoretical calculations done in this study. Thus, even being considered a more stable compound according to computational modeling, the formation of a complex could be conditioned by the solution parameters as well. For this reason, the chemical speciation, considering pH, molarity and stoichiometry, was also estimated using the Visual Minteq software.

Table 1. Sum of electronic and thermal free energies ($\Delta G°$, kcal mol^{-1}) of organometallic complexes in implicit water, according to the metal and stoichiometry.

Ligand	Stoichiometry	Fe	Mn	Zn
Citrate	1:1	-72.4	-58.0	-32.9
	1:2	-90.3	-74.0	-48.9
Tartrate	1:1	-48.7	-39.8	-12.0
	1:2	-88.6	-47.9	-35.8
Malate	1:1	-58.3	-23.8	-19.1
	1:2	-93.8	-34.9	-26.9
Oxalate	1:1	-56.8	-46.1	-18.6
	1:2	-91.9	-82.8	-56.5

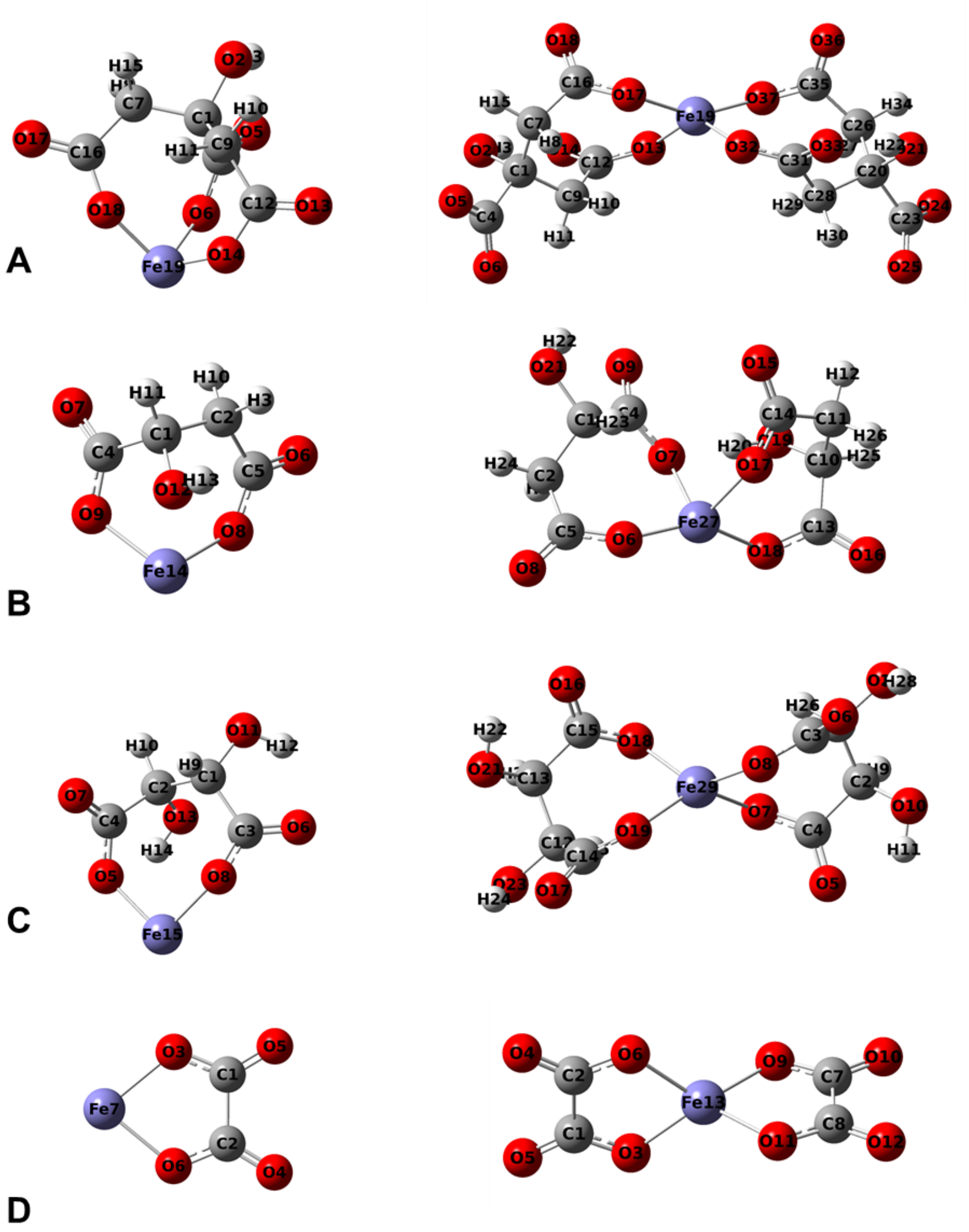

Figure 1. Molecular structure of iron complexes with citrate (A), malate (B), tartrate (C) and oxalate (D). The complexes at left are of 1:1 type, and 1:2 type at the right side.

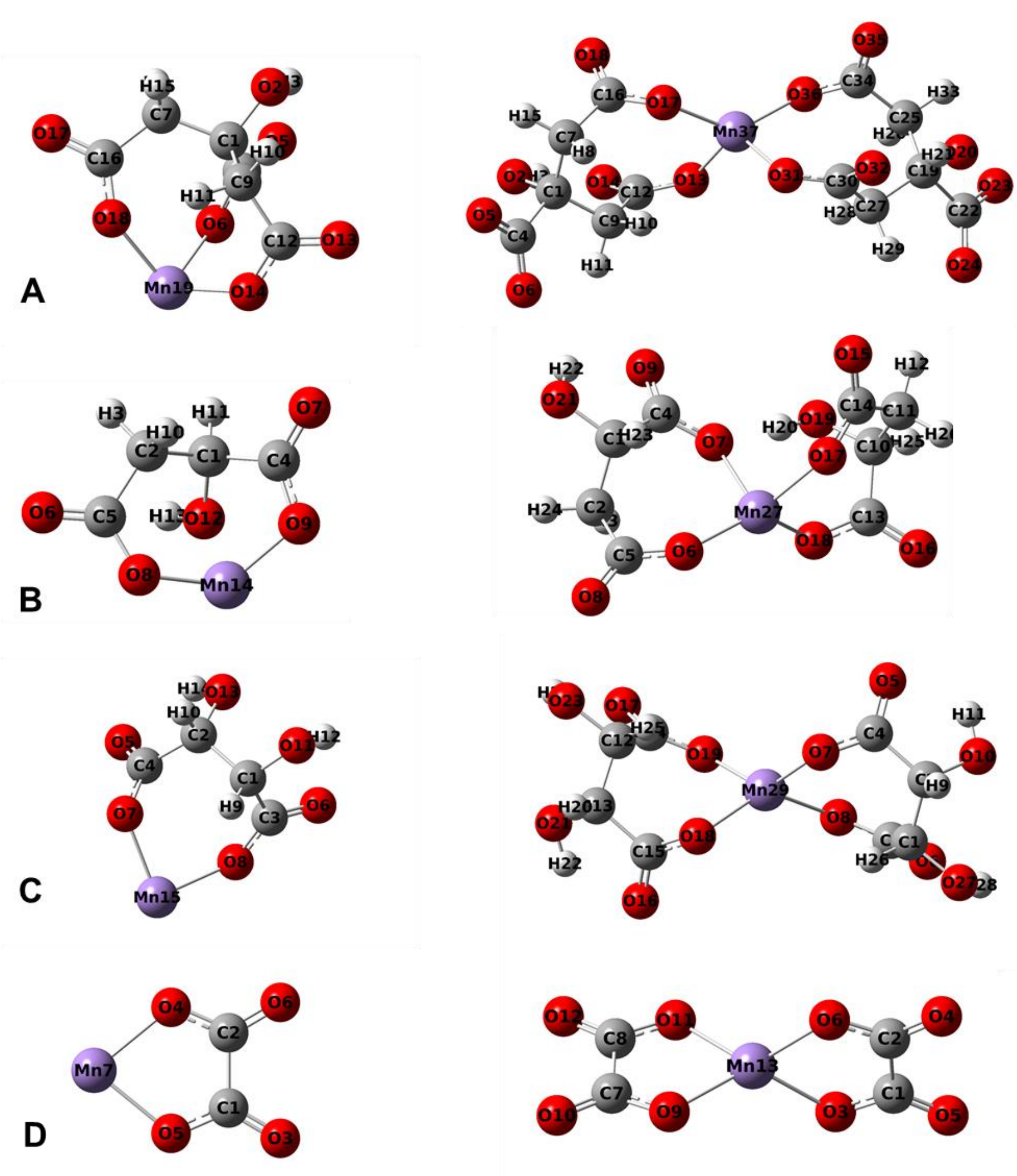

Figure 2. Molecular structure of manganese complexes with citrate (A), malate (B), tartrate (C) and oxalate (D). The complexes at left are of 1:1 type, and 1:2 type at the right side.

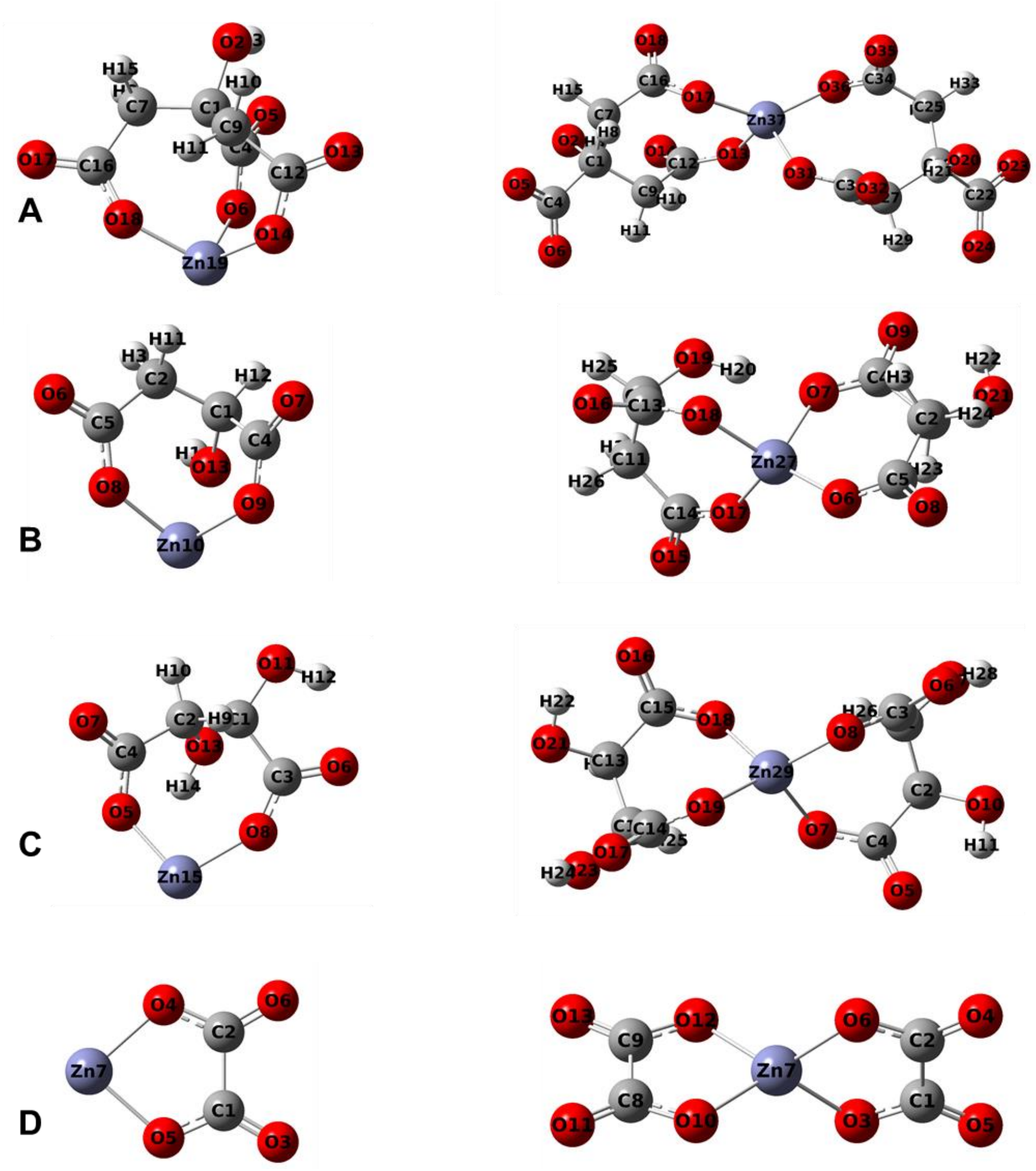

Figure 3. Molecular structure of zinc complexes with citrate (A), malate (B), tartrate (C) and oxalate (D). The complexes at left are of 1:1 type, and 1:2 type at the right side.

3.2 Chemical Speciation: Effect of Ligands, Stoichiometry and Metals

Organic acids, metals, and stoichiometry of reaction influenced the free and complexed metal ratios in solution, as well as the amount of each type of the complex formed (1:1 or 1:2) (Figure 4). For all three metals, citrate at 1:2 SR kept the highest ratio (>99%) of the metal in the complexed form, while TA at 1:1 SR has the lowest metal complexed ratio for Fe and Zn (58% and 56% for Fe and Zn, respectively). Regarding Mn complexes, the lowest complexed ratio was verified for MA at 1:1 SR (61%). Among the complexing agents, oxalate is more prone to form complexes of 1:2 type ($[Fe(Oxalate)_2{}^{-2}]$, $[Mn(Oxalate)_2{}^{-2}]$, and $[Zn(Oxalate)_2{}^{-2}]$).

Furthermore, regarding the metals, Zn is the most prone to form 1:2 type complexes. The 1:2 Zn complexes were formed with both tested SR for citrate, tartrate and oxalate, whereas Fe and Mn 1:2 complexes were formed only with oxalate. Metal malate complexes at 1:2 SR were not observed for any metal (Figure 4).

The chemical speciation is not in full agreement with the results found by the theoretical calculations. Although the 1:1 stoichiometry complex simulation, in general, presented lower stability than 1:2 stoichiometry complex according to the theoretical calculations, the former was predominant in solutions according to Minteq outputs. In line with above-mentioned, metal coordination number is not unique, and, in general, the metals can adopt more than one coordination number, depending on the ligand and reaction conditions [52]. Coordination number is highly dependent on metal size, ligand size and electron-donating ability [52]. According to chemical speciation (Figure 4), ligands with a larger size, such as citrate, formed 1:2 complexes with Zn, the element with a higher ionic radius (0.74 Å) than Mn and Fe (Figure 4). Metals with lower ionic radius than Zn, such as Mn^{2+} (0.70 Å) and Fe^{2+} (0.70 Å), did not present 1:2 complexes with citrate (Figure 1) in the tested conditions. Oxalate is more prone to form 1:2 complexes due to its low molecular weight/size, which minimizes the steric hindrance [39]. However, even with oxalate, the proportion of 1:2 for Mn and Fe is lower than with Zn, the element among the nutrients studied with the highest ionic radius. Although the 1:2 stoichiometry complexes were more stable according to the theoretical calculations, the occurrence of these complexes in solution should be associated to a lower pH (pH<4) and a higher proportion of ligand relative to metal [27]. In the phloem sap of Fe deficient plants, e.g. the Fe: citrate proportions found are around 1:75 – 1:250, and, in this case, the 1:2 and 1:3 Fe-citrate species are highly predominant over 1:1 species [53].

The stoichiometry also affects the amount of total complexed metals and the predominant type of complexes. Oxalate and Zn, the ligand and metal more prone to 1:2 complex type formation, exhibit almost the total of complexed ratio composed of 1:2 complexes at the 1:2 stoichiometry preparation (Figure 4). Conversely, in the 1:1 stoichiometric ratio solution, proportionally, less Zn-oxalate of 1:2 type is formed over 1:1 type, due to the insufficient amount of the organic ligand.

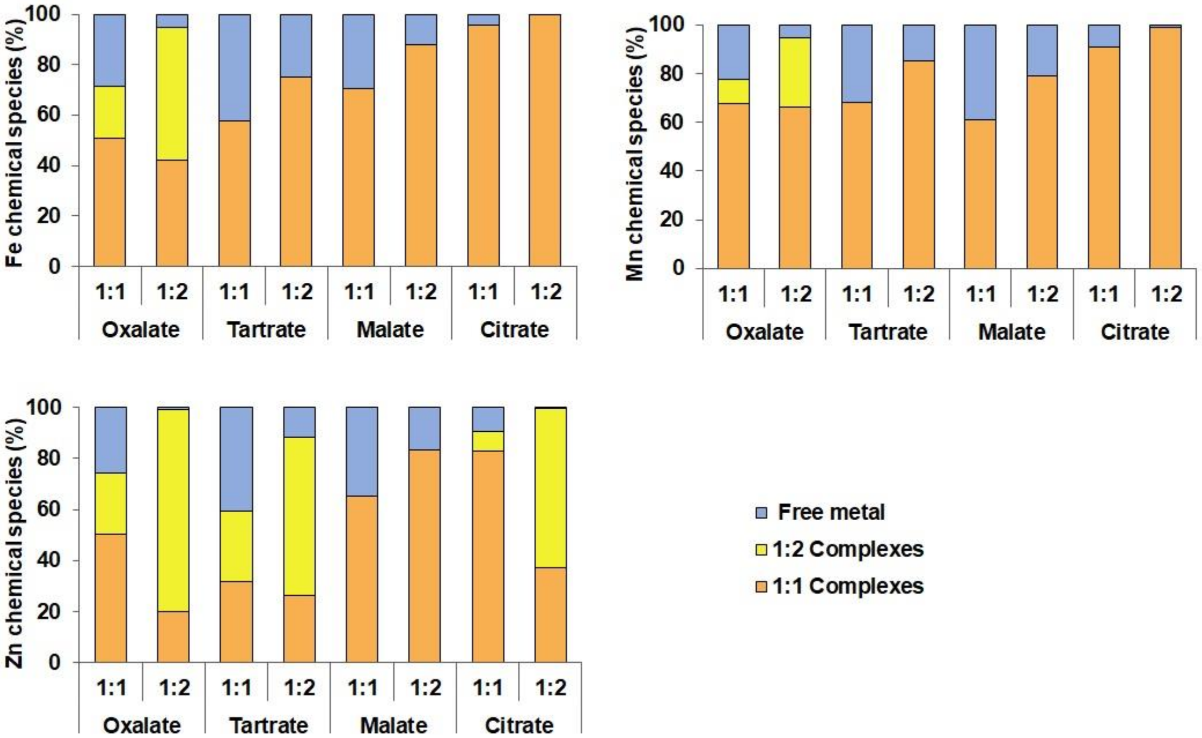

Figure 4. Metal complexed fraction and free metal fraction at pH 7 and 0.01 mol L^{-1} as related to metal (Fe, Mn, and Zn, Fig. 1A, 1B, and 1C, respectively), complexing agent (citrate, malate, tartrate, and oxalate), and the stoichiometric ratio of the reaction (1:1 and 1:2 metal: ligand molar ratio).

3.4 Solubility

Soluble and insoluble portions of the metals tested, as related to organic acids used as complexing agents and stoichiometry of reaction, are shown in Figure 5. Organic acids and stoichiometry influenced the soluble and insoluble metal ratios. In general, oxalate complexes exhibited the lowest soluble ratios for Fe (< 30%), Mn (<25%), and Zn (<30%) (Figure 5). Oxalate complexes generally have low solubility [54]. Metal-oxalate complexes form polymeric chains, resulting in crystalline precipitate materials [55]. Conversely, citrate and malate complexes with the three metals used in this study exhibited high solubility (Figure 5). Significant low ratios of insoluble material of citrate and malate (Figure 5) could be ascribed to the formation of metal oxides with the minimum metal ratio that remained not complexed (Figure 5). Tartrate generates salts with high or moderate solubility [56]. In this study, tartrate exhibited soluble metal ratio higher than oxalate, but lower than citrate and malate, for all the studied metals and stoichiometries. Therefore, the insoluble fraction of metals in tartrate solutions could be ascribed to the i) possible precipitation of tartrate salts [56] and/or ii) lower complexation capability of this ligand (Figure 5), resulting in more free metal ratios, which precipitates as hydroxide/oxide forms at pH 7.

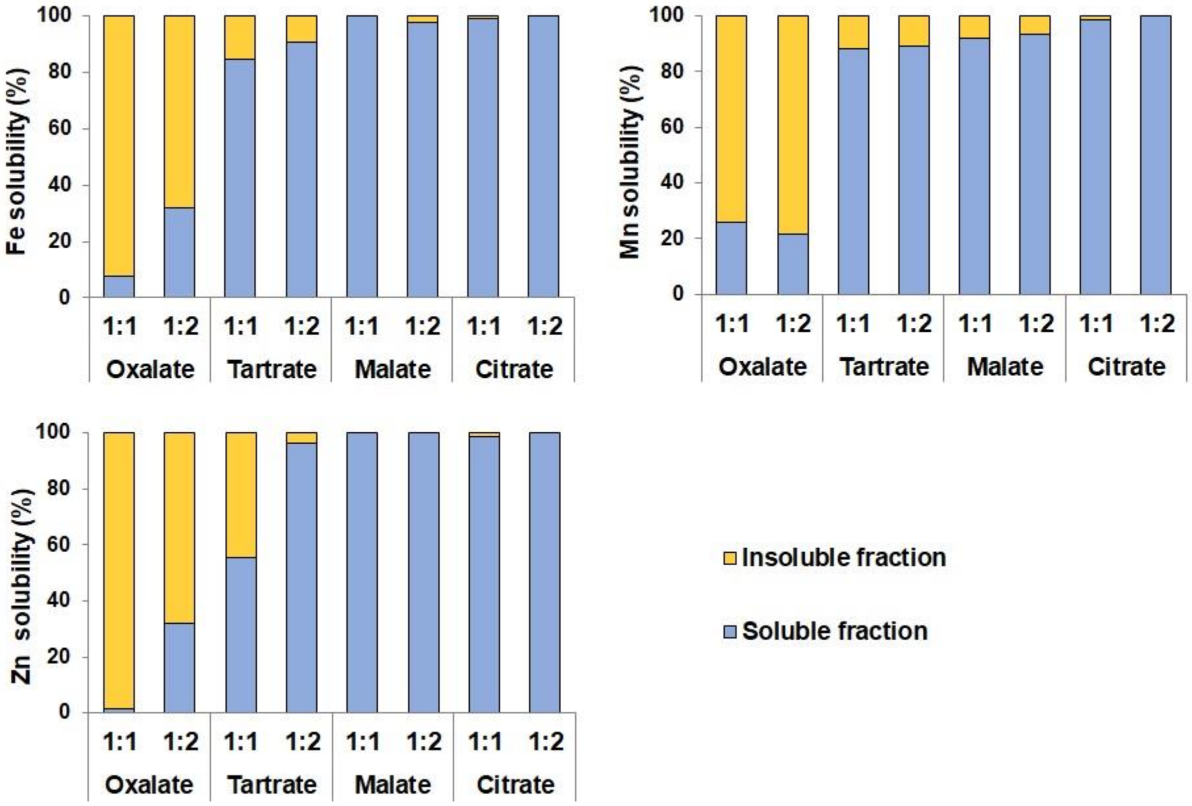

Figure 5. Soluble and insoluble ratios at pH 7 and 0.01 mol L^{-1} as related to metal (Fe, Mn, and Zn, Fig. 1A, 1B, and 1C, respectively), complexing agent (citrate, malate, tartrate, and oxalate), and stoichiometric ratio of the reaction (1:1 and 1:2 metal: ligand molar ratio).

3.3 Fourier Transform Infrared

The infrared spectra of citric, tartaric, malic, and oxalic acid and their complexes with Fe, Mn, and Zn are shown in Figure 6. A general assignment of characteristic vibrational modes found in the IR spectra according to theory and literature [33,34,43,57–60] are shown in Table 2.

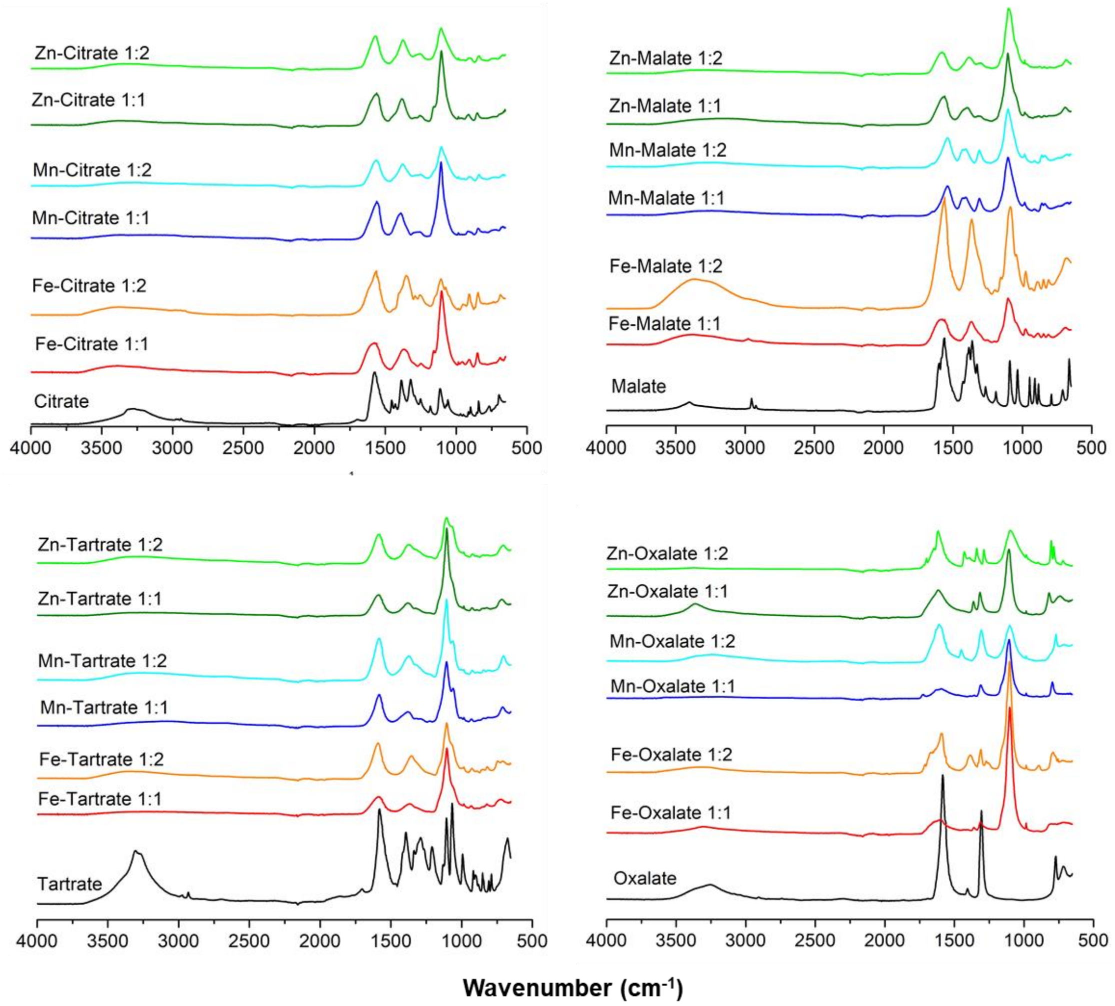

Figure 6. Infrared spectra of organic acids (citrate, malate, tartrate, and oxalate) and their respective metal-complexes at 1:1 and 1:2 stoichiometric ratios.

Table 2. General assignments of FTIR bands of metal-organic acid complexes according to theory and literature [33,34,43,57–60].

Vibration	**Wavenumber (cm^{-1})**
v (O−H)	3350-3250
v (C−H)	3100-2800
v $(COO^-)_{asym}$	1620-1540
v $(COO^-)_{sym}$	1440-1360
δ (O−H)	1440-1395
δ OH···O out of plane	1320-1280
v (C−O)	1080-1020
SO_2	1120-1050
v (C−C) + δ (O−C−O)	820-760

v: stretching*;* δ: rocking

3.3.1 Metal-carboxylate coordination according to COO- vibration stretching shifts

The interactions of metals with carboxylates occur through the carboxylic groups; thus, the most useful typical bands of metal-carboxylates are the COO^- stretching vibrations [33]. The frequencies and intensities of the carboxylate bands are sensitive to the structure and molecular environment of the carboxylate group, the nature of the complexed metal, and the structure of the complex formed [61]. The shift in the COO^- asymmetric stretching is associated to different coordination modes of the carboxylic groups, a stereochemical aspect that affects the metal-complex bioavailability. In the monodentate mode of coordination, a strong reduction of equivalence of the two oxygens of COO^- group occurs, and, in general, an increase in the COO^- asymmetric stretching frequency is observed [63,64]. A decrease in the COO^- asymmetric stretching wavenumber is particularly evident for chelating configuration of complexation [35]. Therefore, the formation of complexes with metals affects the distance between the asymmetric and symmetric COO^- bands. The separation of the bands (ΔCOO^-) is also used as an indicative of the type of metal-carboxylate coordination binding [61]. Thus, the detailed assignment of asymmetric and symmetric vibrations for each organic acid and their respective metal complexes are shown in Table 3.

In comparison with its corresponding free deprotonated ligand, a large splitting of COO^- stretching frequencies (generally higher than 200 cm^{-1}) is often an indication of monodentate coordination in a metal carboxylate [33,65]. Reduced splitting of COO^- stretching frequencies in comparison with ionic free deprotonate ligand are indicative of chelating or bridging configuration, but bridging splitting is larger than chelating [66,67].

According to chemical speciation outputs, at pH 7, the free carboxylic acids are completely deprotonated and, therefore, it can be assumed that the COO^- is present instead of COOH. In the citrate complexes, a larger splitting of carboxylic bands than free citrate ions were observed for Mn complexes at both stoichiometries, suggesting a monodentate coordination. This configuration is confirmed by the molecular structures obtained from computational modeling (Figure 2). Regarding Fe and Zn, at both stoichiometries, ΔCOO^- presents values approximately equal to citrate (Table 3). Although these ΔCOO^- values suggest a bidentate bridging interaction or ionic interaction with carboxylic groups, all these complexes exhibited monodentate interaction (Figure 1 and Figure 3). A low splitting of COO- stretching vibrations for monodentate complexes are also observed for Cd and Pb citrate complexes [27]. Furthermore, Deacon and Philips [66] also found acetate monodentate complexes of Zn with small ΔCOO^- values, similar to free ionic COO^-. The shift of COO^- asymmetric band in a ligand, and, in consequence, the ΔCOO^- values, is also dependent on the properties of metal ion interacting with carboxylate [68]. Furthermore, water molecules can interact with the free O from the carboxylate group bound to the metal, forming a pseudo-bridging mode, reducing the ΔCOO^- value [67].

For tartaric and malic acids, in general, all the metal complexes presented ΔCOO^- values higher than the corresponding free acid (Table 3), also indicating a monodentate interaction. The theoretical modelling mirrored this result, demonstrating that two carboxylate groups of tartrate and malate are bound to metals in a monodentate configuration (Figures 1, 2, and 3). In case of oxalate, all complexes presented ΔCOO^- values remarkably higher than free oxalate ions, indicating monodentate configuration, in line with the theoretical calculation outputs.

Therefore, a good association was found between the splitting of COO- vibrations and coordination modes of carboxylates in the structure of metal complexes with malate, tartrate, and oxalate. Only some exceptions to the rule were found for Zn and Fe complexes of citrate. Thus, an additional examination of the molecular structure performed in addition to FTIR spectroscopic analysis is recommendable to confirm the coordination modes.

Table 3. Carboxylate symmetric and asymmetric stretching frequencies for different metal carboxylic acid complexes.

Metal-Organic Acid	**Stoichiometry**	ν **(COO$^-$)$_{asym}$**	ν **(COO$^-$)$_{sym}$**	***Delta* (COO$^-$)**
Citrate	-	1559	1357	202
Fe	1:1	1580	1380	200
	1:2	1578	1377	201
Mn	1:1	1587	1364	223
	1:2	1586	1365	221
Zn	1:1	1573	1380	193
	1:2	1575	1375	200
Tartrate	-	1578	1394	184
Fe	1:1	1587	1358	236
	1:2	1592	1358	234
Mn	1:1	1584	1380	204
	1:2	1585	1374	211
Zn	1:1	1589	1379	210
	1:2	1586	1375	211
Malate	-	1582	1375	207
Fe	1:1	1611	1371	240
	1:2	1588	1365	223
Mn	1:1	1592	1383	209
	1:2	1604	1375	228
Zn	1:1	1590	1358	231
	1:2	1602	1354	248
Oxalate	-	1573	1306	267
Fe	1:1	1634	1311	323
	1:2	1629	1312	317
Mn	1:1	1628	1310	318
	1:2	1636	1307	329
Zn	1:1	1650	1315	335
	1:2	1637	1338	299

3.3.2 Partial least squares analysis and complex properties prediction from FTIR

PLS multivariate regression analysis was used to predict the relationship between the infrared spectral data and the solubility, stability, and total complexed metal fraction of the complexes. Although all these chemical attributes were tested, only solubility and stability were properly predicted using PLS. The statistical parameters obtained from the PLS regression for solubility and complexed fraction are shown in Table 4. Multivariate calibration with PLS was applied to a set of 24 complexes, which considered three metals and four organic acids at two stoichiometric ratios. The number of latent variables used for each model was stablished according to the minimal value of root mean square error of cross-validation ($RMSE_{cv}$) [45].

Table 4. Parameters of PLS regression for models of metal solubility and metal complexed ratio.

PLS Parameter	**Solubility (% metal in soluble form)**	**Complexed ratio (% metal in complexed form)**
LV	3	6
$RMSE_{c}$	13.39	3.33
R^2_{cal}	0.83	0.94
$RMESE_{y\text{-}rand}$	25.79	7.66
$R^2_{y\text{-}rand}$	0.38	0.64
$^{c}R^2_{p}$	0.61	0.51
$RMSE_{cv}$	22.07	8.98
R^2_{pred}	0.57	0.63

LV: latent variable; RMSE: root mean square error; R^2: determination coefficient; $RMSE_C$: RMSE of calibration; R^2_{cal}: R^2 of calibration; $RMSE_{y\text{-}rand}$: RMSE of y randomization; $R^2_{y\text{-}rand}$: R^2 of y-randomization; r^2p: r^2 of y-randomization prediction; $RMSE_{cv}$: RMSE of cross validation; $RMSE_{p}$: RMSE of prediction; R^2_{pred}: R^2 of prediction

For PLS regression-based models, values for the determination coefficients of calibration (R^2_{cal}) > 0.6 and prediction (R^2_{pred}) > 0.5 are acceptable [69]. Thus, the PLS models were highly predictive, both for solubility and ratio of complexation (Table 4). These models were also reliable and not prone to overfitting, since the $cR^2_{p} \geq 0.5$ for both solubility and complexation ratio models indicated R^2_{cal} are significantly reduced after randomization ($R^2_{y\text{-}rand}$) of the dependent variables block (y, the solubility and complexation ratio values) [45]. The predictive error of the models expressed as $RMSE_P$ is analytically acceptable [45] for the prediction of soluble ratio and for total complexed ratio, corresponding to a relative analytical of 13.39 and 3.33% (Table 4). In general, the PLS models had a greater performance to predict the metal complexed ratio than for complex solubility. The bands changes due to complexation is unequivocal [61,65]. In line with this study, several authors attested the relationship between complexation and the complex spectroscopic properties [33,43].

To illustrate the predictive ability of the developed models, examples of the regression lines of the cross-validated PLS-1 models for each parameter are shown in Figure 4.

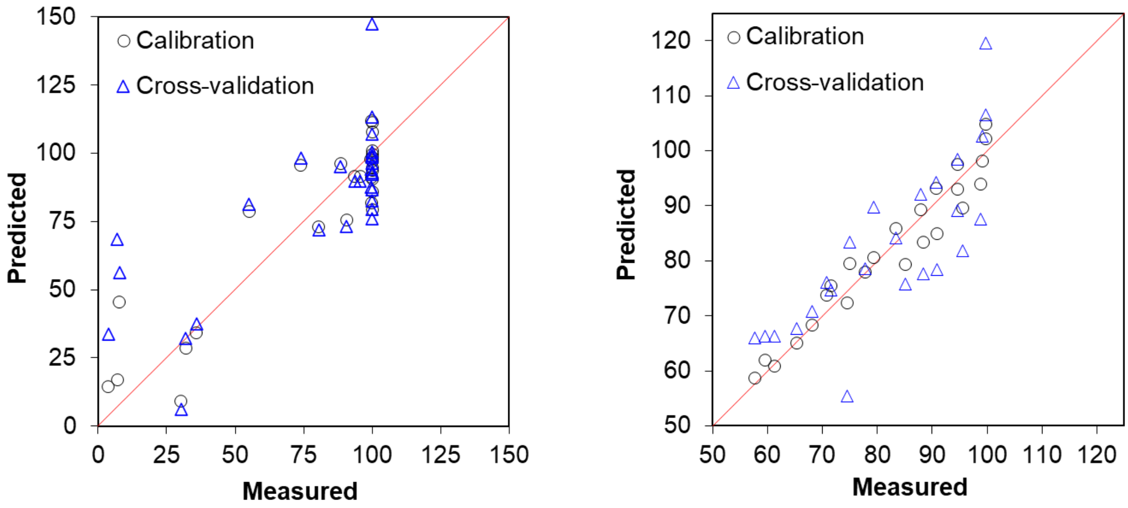

Figure 4. Predicted x Measured values for metal solubility (left) and metal complexed ratio (right).

The coordination mode of metal complexes can affect their recognition by cell membranes of soil bacteria and the uptake and metabolism of complexes by plants [25], and therefore the efficiency of bioremediation or plant nutrition. Complexation capacity, represented in this work as metal complexed ratio, and solubility are essential properties to base the choice for the most effective complexing agent for Fe, Mn, and Zn, depending on the end purpose. Agronomic applications require complexes with high solubility and high complexation capacity because the main objective is to keep the nutrient in soluble forms and avoid reactions with other chemical species to guarantee plentiful plant acquisition [4,24]. Regarding environmental applications, if the intention is to increase metal extraction by plants (phytoremediation), it is also suitable to use complexes with high solubility, stability and complexation capacity [22,70], such as citrate and malate. Otherwise, if the objective is to inactivate toxic metals, to avoid their negative effects on plants and microorganisms, metal complexes with high stability and low solubility [5,70], such as metal-oxalates, should be accounted for. Therefore, the prediction of these properties through PLS regression using FTIR data, a fast and non-destructive method, is a suitable technique to study the chemical properties of Mn, Zn, and Mn complexes and facilitate the decisions regarding their applicability.

4. Conclusions

Upon to the synthetic conditions of this study, Mn and Fe reacted with all organic acids and formed complexes with 1:1 stoichiometry, except for oxalate, which also presented complexes of 1:2 type. Regarding to Zn, 1:2 type complexes were also formed with tartrate and citrate at 1:2 stoichiometry of reaction. In the structure of all complexes, the carboxylate groups involved in the complexation interacts in a monodentate configuration with metals. The prediction of the structure and type of coordination interaction was provided by FTIR spectra combined with PLS regressions models. However, there are some few exceptions to general rules of splitting value association with coordination modes, which can affect the precision of conformational identification. The combination of FTIR spectroscopy with statistical multivariate techniques demonstrated a great potential of this approach in predicting complex properties such as solubility and stability.

Acknowledgements

Many thanks to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), by the scholarship provided to the first author and to the research funding (CAPES-PROEX-AUXPE 593-2018); to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, processo 307447-2019-7) for funding this research.

5. References

[1] M.W.H. Evangelou, M. Ebel, A. Schaeffer, Chelate assisted phytoextraction of heavy metals from soil. Effect, mechanism, toxicity, and fate of chelating agents, Chemosphere. 68 (2007) 989–1003. https://doi.org/10.1016/j.chemosphere.2007.01.062.

[2] S. López-Rayo, P. Nadal, J.J. Lucena, Novel chelating agents for iron, manganese, zinc, and copper mixed fertilisation in high pH soil-less cultures, J. Sci. Food Agric. 96 (2016) 1111–1120. https://doi.org/10.1002/jsfa.7183.

[3] C. Martín-Fernández, Á. Solti, V. Czech, K. Kovács, F. Fodor, A. Gárate, L. Hernández-Apaolaza, J.J. Lucena, Response of soybean plants to the application of synthetic and biodegradable Fe chelates and Fe complexes, Plant Physiol. Biochem. 118 (2017) 579–588. https://doi.org/10.1016/j.plaphy.2017.07.028.

[4] P. Rodríguez-Lucena, L. Hernández-Apaolaza, J.J. Lucena, Comparison of iron chelates and complexes supplied as foliar sprays and in nutrient solution to correct iron chlorosis of soybean, J. Plant Nutr. Soil Sci. 173 (2010) 120–126. https://doi.org/10.1002/jpln.200800256.

[5] Z. Derakhshan Nejad, M.C. Jung, K.H. Kim, Remediation of soils contaminated with heavy metals with an emphasis on immobilization technology, Environ. Geochem. Health. 40 (2018) 927–953. https://doi.org/10.1007/s10653-017-9964-z.

[6] A. Singh, S.M. Prasad, Remediation of heavy metal contaminated ecosystem: an overview on technology advancement, Int. J. Environ. Sci. Technol. 12 (2015) 353–366. https://doi.org/10.1007/s13762-014-0542-y.

[7] S. López-Rayo, I. Sanchis-Pérez, C.M.H. Ferreira, J.J. Lucena, [S,S]-EDDS/Fe: A new chelate for the environmentally sustainable correction of iron chlorosis in calcareous soil, Sci. Total Environ. 647 (2019) 1508–1517. https://doi.org/10.1016/j.scitotenv.2018.08.021.

[8] B. Nowack, Environmental chemistry of aminopolycarboxylate chelating agents, Environ. Sci. Technol. 36 (2002) 4009–4016. https://doi.org/10.1021/es025683s.

[9] D. Leštan, C. ling Luo, X. dong Li, The use of chelating agents in the remediation of metal-contaminated soils: A review, Environ. Pollut. 153 (2008) 3–13. https://doi.org/10.1016/j.envpol.2007.11.015.

[10] A. Ullmann, N. Brauner, S. Vazana, Z. Katz, R. Goikhman, B. Seemann, H. Marom, M. Gozin, New biodegradable organic-soluble chelating agents for simultaneous removal of heavy metals and organic pollutants from contaminated media, J. Hazard. Mater. 260 (2013) 676–688. https://doi.org/10.1016/j.jhazmat.2013.06.027.

[11] G. Renella, L. Landi, P. Nannipieri, Degradation of low molecular weight organic acids complexed with heavy metals in soil, Geoderma. 122 (2004) 311–315. https://doi.org/10.1016/j.geoderma.2004.01.018.

[12] R. Adeleke, C. Nwangburuka, B. Oboirien, Origins, roles and fate of organic acids in soils: A review, South African J. Bot. 108 (2017) 393–406. https://doi.org/10.1016/j.sajb.2016.09.002.

[13] D.L. Jones, Organic acids in the rhizosphere - A critical review, Plant Soil. 205 (1998)

25–44. https://doi.org/10.1023/A:1004356007312.

[14] C. Ash, V. Tejnecký, L. Borůvka, O. Drábek, Different low-molecular-mass organic acids specifically control leaching of arsenic and lead from contaminated soil, J. Contam. Hydrol. 187 (2016) 18–30. https://doi.org/10.1016/j.jconhyd.2016.01.009.

[15] J. Jiang, M. Yang, Y. Gao, J. Wang, D. Li, T. Li, Removal of toxic metals from vanadium-contaminated soils using a washing method: Reagent selection and parameter optimization, Chemosphere. 180 (2017) 295–301. https://doi.org/10.1016/j.chemosphere.2017.03.116.

[16] B. Chakraborty, P.N. Singh, S. Kumar, P.C. Srivastava, Uptake and Distribution of Iron from Different Iron Sources Applied as Foliar Sprays to Chlorotic Leaves of Low-Chill Peach Cultivars, Agric. Res. 3 (2014) 293–301. https://doi.org/10.1007/s40003-014-0128-4.

[17] S. Sharma, H. Malhotra, P. Borah, M.K. Meena, P. Bindraban, S. Chandra, V. Pande, R. Pandey, Foliar application of organic and inorganic iron formulation induces differential detoxification response to improve growth and biofortification in soybean, Plant Physiol. Reports. 24 (2019) 119–128. https://doi.org/10.1007/s40502-018-0412-6.

[18] J.M. Garcia-Mina, Stability, solubility and maximum metal binding capacity in metal-humic complexes involving humic substances extracted from peat and organic compost, Org. Geochem. 37 (2006) 1960–1972. https://doi.org/10.1016/j.orggeochem.2006.07.027.

[19] I. Guardado, O. Urrutia, J.M. García-Mina, Size distribution, complexing capacity, and stability of phosphate-metal-humic complexes, J. Agric. Food Chem. 55 (2007) 408–413. https://doi.org/10.1021/jf062894y.

[20] G. Joshi-Tope, A.J. Francis, Mechanisms of biodegradation of metal-citrate complexes by Pseudomonas fluorescens, J. Bacteriol. 177 (1995) 1989–1993. https://doi.org/10.1128/jb.177.8.1989-1993.1995.

[21] J.J. Lensbouer, A. Patel, J.P. Sirianni, R.P. Doyle, Functional characterization and metal ion specificity of the metal-citrate complex transporter from Streptomyces coelicolor, J. Bacteriol. 190 (2008) 5616–5623. https://doi.org/10.1128/JB.00456-08.

[22] J.M. García-Mina, M.C. Antolín, M. Sanchez-Diaz, Metal-humic complexes and plant micronutrient uptake: A study based on different plant species cultivated in diverse soil types, Plant Soil. 258 (2004) 57–68. https://doi.org/10.1023/B:PLSO.0000016509.56780.40.

[23] S. Muthaiah, A. Bhatia, M. Kannan, Stability of Metal Complexes, Stab. Coord. Compd. [Working Title]. (2020). https://doi.org/10.5772/intechopen.90894.

[24] W.L. Lindsay, Chemical reactions in soils that affect iron availability to plants. A quantative approach, Iron Nutr. Soils Plants. (1995) 7–14. https://doi.org/10.1007/978-94-011-0503-3_2.

[25] A.K. Pandey, S.D. Pandey, V. Misra, Stability constants of metal-humic acid complexes and its role in environmental detoxification, Ecotoxicol. Environ. Saf. 47 (2000) 195–200. https://doi.org/10.1006/eesa.2000.1947.

[26] F. Arnaud-Neu, R. Delgado, S. Chaves, Critical evaluation of stability constants and thermodynamic functions of metal complexes of crown ethers: (IUPAC technical report), Pure Appl. Chem. 75 (2003) 71–102. https://doi.org/10.1351/pac200375010071.

[27] A.C. Bertoli, R. Carvalho, M.P. Freitas, T.C. Ramalho, D.T. Mancini, M.C. Oliveira, A. De Varennes, A. Dias, Theoretical spectroscopic studies and identification of metal-citrate (Cd and Pb) complexes by ESI-MS in aqueous solution, Spectrochim. Acta - Part A Mol. Biomol. Spectrosc. 137 (2015) 271–280. https://doi.org/10.1016/j.saa.2014.08.053.

[28] A.C. Bertoli, R. Carvalho, M.P. Freitas, T.C. Ramalho, D.T. Mancini, M.C. Oliveira, A. De Varennes, A. Dias, Theoretical and experimental investigation of complex structures citrate of zinc (II), Inorganica Chim. Acta. 425 (2015) 164–168. https://doi.org/10.1016/j.ica.2014.10.025.

[29] M. Villén, J.J. Lucena, M.C. Cartagena, R. Bravo, J. García-Mina, M.I.M. De La Hinojosa, Comparison of two analytical methods for the evaluation of the complexed metal in fertilizers and the complexing capacity of complexing agents, J. Agric. Food Chem. 55 (2007) 5746–5753. https://doi.org/10.1021/jf070422t.

[30] L. Weng, E.J.M. Temminghoff, S. Lofts, E. Tipping, W.H. Van Riemsdijk, Complexation with dissolved organic matter and solubility control of heavy metals in a

sandy soil, Environ. Sci. Technol. 36 (2002) 4804–4810. https://doi.org/10.1021/es0200084.

[31] C. Catrouillet, M. Davranche, A. Dia, M. Bouhnik-Le Coz, R. Marsac, O. Pourret, G. Gruau, Geochemical modeling of Fe(II) binding to humic and fulvic acids, Chem. Geol. 372 (2014) 109–118. https://doi.org/10.1016/j.chemgeo.2014.02.019.

[32] S. Goldberg, Chemical modeling of boron adsorption by humic materials using the constant capacitance model, Soil Sci. 179 (2014) 561–567. https://doi.org/10.1097/SS.0000000000000098.

[33] S.K. Papageorgiou, E.P. Kouvelos, E.P. Favvas, A.A. Sapalidis, G.E. Romanos, F.K. Katsaros, Metal-carboxylate interactions in metal-alginate complexes studied with FTIR spectroscopy, Carbohydr. Res. 345 (2010) 469–473. https://doi.org/10.1016/j.carres.2009.12.010.

[34] M.C. D’Antonio, A. Wladimirsky, D. Palacios, L. Coggiola, A.C. González-Baró, E.J. Baran, R.C. Mercader, Spectroscopic investigations of iron(II) and iron(III) oxalates, J. Braz. Chem. Soc. 20 (2009) 445–450. https://doi.org/10.1590/S0103-50532009000300006.

[35] V. Zeleňák, Z. Vargová, K. Györyová, Correlation of infrared spectra of zinc(II) carboxylates with their structures, Spectrochim. Acta - Part A Mol. Biomol. Spectrosc. 66 (2007) 262–272. https://doi.org/10.1016/j.saa.2006.02.050.

[36] A. Gredilla, S. Fdez-Ortiz de Vallejuelo, N. Elejoste, A. de Diego, J.M. Madariaga, Non-destructive Spectroscopy combined with chemometrics as a tool for Green Chemical Analysis of environmental samples: A review, TrAC - Trends Anal. Chem. 76 (2016) 30–39. https://doi.org/10.1016/j.trac.2015.11.011.

[37] G.W. Small, Chemometrics and near-infrared spectroscopy: Avoiding the pitfalls, TrAC - Trends Anal. Chem. 25 (2006) 1057–1066. https://doi.org/10.1016/j.trac.2006.09.004.

[38] N. Zhao, Z.S. Wu, Q. Zhang, X.Y. Shi, Q. Ma, Y.J. Qiao, Optimization of Parameter Selection for Partial Least Squares Model Development, Sci. Rep. 5 (2015) 1–10. https://doi.org/10.1038/srep11647.

[39] T.A. Tolentino, A.C. Bertoli, M. Dos Santos Pires, R. Carvalho, C.R.G. Labory, J.S.

Nunes, A.R.R. Bastos, M.P. De Freitas, Applications in environmental bioinorganic: Nutritional and ultrastructural evaluation and calculus of thermodynamic and structural properties of metal-oxalate complexes, Spectrochim. Acta - Part A Mol. Biomol. Spectrosc. 150 (2015) 750–757. https://doi.org/10.1016/j.saa.2015.06.016.

[40] C. Gardana, A. Scialpi, C. Fachechi, P. Simonetti, Near-infrared spectroscopy and chemometrics for the routine detection of bilberry extract adulteration and quantitative determination of the anthocyanins, J. Spectrosc. 2018 (2018). https://doi.org/10.1155/2018/4751247.

[41] M.A. Jiménez-González, A.M. Álvarez, P. Carral, G. Almendros, Chemometric assessment of soil organic matter storage and quality from humic acid infrared spectra, Sci. Total Environ. 685 (2019) 1160–1168. https://doi.org/10.1016/j.scitotenv.2019.06.231.

[42] F.S. Higashikawa, C.A. Silva, C.A.Ô. Nunes, M.A. Sánchez-Monedero, Fourier transform infrared spectroscopy and partial least square regression for the prediction of substrate maturity indexes, Sci. Total Environ. 470–471 (2014) 536–542. https://doi.org/10.1016/j.scitotenv.2013.09.065.

[43] A.C. Bertoli, R. Carvalho, M.P. Freitas, T.C. Ramalho, D.T. Mancini, M.C. Oliveira, A. De Varennes, A. Dias, Structural determination of Cu and Fe-Citrate complexes: Theoretical investigation and analysis by ESI-MS, J. Inorg. Biochem. 144 (2015) 31–37. https://doi.org/10.1016/j.jinorgbio.2014.12.008.

[44] J. Tomasi, B. Mennucci, R. Cammi, Quantum mechanical continuum solvation models, Chem. Rev. 105 (2005) 2999–3093. https://doi.org/10.1021/cr9904009.

[45] R. Kiralj, M.M.C. Ferreira, Basic validation procedures for regression models in QSAR and QSPR studies: Theory and application, J. Braz. Chem. Soc. 20 (2009) 770–787. https://doi.org/10.1590/S0103-50532009000400021.

[46] C.A. Nunes, Vibrational spectroscopy and chemometrics to assess authenticity, adulteration and intrinsic quality parameters of edible oils and fats, Food Res. Int. 60 (2014) 255–261. https://doi.org/10.1016/j.foodres.2013.08.041.

[47] C.A. Nunes, M.P. Freitas, A.C.M. Pinheiro, S.C. Bastos, Chemoface: A novel free user-friendly interface for chemometrics, J. Braz. Chem. Soc. 23 (2012) 2003–2010. https://doi.org/10.1590/S0103-50532012005000073.

[48] O.C. Gagné, F.C. Hawthorne, Empirical Lewis acid strengths for 135 cations bonded to oxygen, Acta Crystallogr. Sect. B Struct. Sci. Cryst. Eng. Mater. 73 (2017) 956–961. https://doi.org/10.1107/S2052520617010988.

[49] R.G. Pearson, D.H. Busch, Hard and Soft Acids and Bases, 1963.

[50] N.S. Hosmane, Review of Bonding Theories for d -Block Metal Complexes, Adv. Inorg. Chem. (2017) 89–114. https://doi.org/10.1016/b978-0-12-801982-5.00006-0.

[51] N.S. Hosmane, Ligands and d -Block Metal Complexes, Adv. Inorg. Chem. (2017) 75–87. https://doi.org/10.1016/b978-0-12-801982-5.00005-9.

[52] M. Dudev, J. Wang, T. Dudev, C. Lim, Factors governing the metal coordination number in metal complexes from cambridge structural database analyses, J. Phys. Chem. B. 110 (2006) 1889–1895. https://doi.org/10.1021/jp054975n.

[53] A.F. Lopez-Millan, F. Morales, A. Abadia, J. Abadia, Effects of iron deficiency on the composition of the leaf apoplastic fluid and xylem sap in sugar beet. Implications for iron and carbon transport, Plant Physiol. 124 (2000) 873–884. https://doi.org/10.1104/pp.124.2.873.

[54] R.M. Ferreira, M. Motta, A. Batagin-Neto, C.F. De Oliveira Graeff, P.N. Lisboa-Filho, F.C. Lavarda, Theoretical investigation of geometric configurations and vibrational spectra in citric acid complexes, Mater. Res. 17 (2014) 550–556. https://doi.org/10.1590/S1516-14392014005000056.

[55] E.J. Baran, Review: Natural oxalates and their analogous synthetic complexes, J. Coord. Chem. 67 (2014) 3734–3768. https://doi.org/10.1080/00958972.2014.937340.

[56] Q. Gao, Y.B. Xie, D. Wang, Synthesis and crystal structure of the hydrated nickel(II) complex of tartrate, J. Chem. Crystallogr. 38 (2008) 587–590. https://doi.org/10.1007/s10870-008-9347-5.

[57] G. Socrates, Infrared and Raman characteristic group frequencies. Tables and charts, 2001. https://doi.org/10.1002/jrs.1238.

[58] C.C.R. Sutton, G. Da Silva, G. V. Franks, Modeling the IR spectra of aqueous metal carboxylate complexes: Correlation between bonding geometry and stretching mode wavenumber shifts, Chem. - A Eur. J. 21 (2015) 6801–6805. https://doi.org/10.1002/chem.201406516.

[59] M. Matzapetakis, N. Karligiano, A. Bino, M. Dakanali, C.P. Raptopoulou, V. Tangoulis, A. Terzis, J. Giapintzakis, A. Salifoglou, Manganese citrate chemistry: Syntheses, spectroscopic studies, and structural characterizations of novel mononuclear, water-soluble manganese citrate complexes, Inorg. Chem. 39 (2000) 4044–4051. https://doi.org/10.1021/ic9912631.

[60] B.H. Stuart, Infrared Spectroscopy: Fundamentals and Applications, 2005. https://doi.org/10.1002/0470011149.

[61] E.G. Palacios, G. Juárez-López, A.J. Monhemius, Infrared spectroscopy of metal carboxylates: II. Analysis of Fe(III), Ni and Zn carboxylate solutions, Hydrometallurgy. 72 (2004) 139–148. https://doi.org/10.1016/S0304-386X(03)00137-3.

[62] M. Clausén, L.O. Öhman, P. Persson, Spectroscopic studies of aqueous gallium(III) and aluminum(III) citrate complexes, J. Inorg. Biochem. 99 (2005) 716–726. https://doi.org/10.1016/j.jinorgbio.2004.12.007.

[63] M. Nara, M. Tanokura, Infrared spectroscopic study of the metal-coordination structures of calcium-binding proteins, Biochem. Biophys. Res. Commun. 369 (2008) 225–239. https://doi.org/10.1016/j.bbrc.2007.11.188.

[64] K.C. Lanigan, K. Pidsosny, Reflectance FTIR spectroscopic analysis of metal complexation to EDTA and EDDS, Vib. Spectrosc. 45 (2007) 2–9. https://doi.org/10.1016/j.vibspec.2007.03.003.

[65] P. Boguta, Z. Sokołowska, Interactions of Zn(II) Ions with Humic Acids Isolated from Various Type of Soils. Effect of pH, Zn Concentrations and Humic Acids Chemical Properties, PLoS One. 11 (2016) 1–20. https://doi.org/10.1371/journal.pone.0153626.

[66] G.B. Deacon, R.J. Phillips, Deacon G Phillips R, Coord. Chem. Rev. 33 (1980) 227–250.

[67] M. Nara, H. Morii, M. Tanokura, Coordination to divalent cations by calcium-binding proteins studied by FTIR spectroscopy, Biochim. Biophys. Acta - Biomembr. 1828 (2013) 2319–2327. https://doi.org/10.1016/j.bbamem.2012.11.025.

[68] S.C. Edington, A. Gonzalez, T.R. Middendorf, D.B. Halling, R.W. Aldrich, C.R. Baiz, Coordination to lanthanide ions distorts binding site conformation in calmodulin, Proc.

Natl. Acad. Sci. U. S. A. 115 (2018) E3126–E3134. https://doi.org/10.1073/pnas.1722042115.

[69] D.L.J. Alexander, A. Tropsha, D.A. Winkler, Beware of R2: Simple, Unambiguous Assessment of the Prediction Accuracy of QSAR and QSPR Models, J. Chem. Inf. Model. 55 (2015) 1316–1322. https://doi.org/10.1021/acs.jcim.5b00206.

[70] L. Liu, W. Li, W. Song, M. Guo, Remediation techniques for heavy metal-contaminated soils: Principles and applicability, Sci. Total Environ. 633 (2018) 206–219. https://doi.org/10.1016/j.scitoenv.2018.03.161.

ARTICLE 2 - Influence of complex stability on iron accumulation and redistribution for foliar applied iron-organic acid complexes

Marina Justi[†] [*]; Carlos Alberto Silva[†]; Everton Geraldo de Morais[†]; Josué Mariani Silla[§]; Matheus Puggina de Freitas[§]

[†] *Department of Soil Science, Federal University of Lavras/UFLA, Post office box 3037, 37200-000, Lavras, MG, Brazil*

[§]*Department of Chemistry, Federal University of Lavras, Post office box 3037, 37200-000, Lavras, MG, Brazil*

**Corresponding author*
Email address: marina.justi@gmail.com

This article was prepared in line with the guidelines of the journal 'Archives of Agronomy and Soil Science'

Abstract

The relation between chemical attributes of complexed Fe sources and the efficiency of foliar fertilization is still unclear. This study aimed to investigate the influence of Fe-complex stability on iron accumulation and redistribution in maize by foliar application. The iron complexing agents used were biodegradable citric acid (CA), malic acid (MA), tartaric acid (TA), and oxalic acid (OA). The prepared $FeSO_4$ based complexes in the foliar spray solutions had "Fe: organic acid" stoichiometric ratios of 1:1 and 1:2. Additionally, Fe-EDTA and $FeSO_4$ were also tested. The iron complexed fraction and the main complexed chemical species in the foliar spray solutions were determined using the Minteq software. Molecular modeling helped to obtain probable complex structures and stabilities. The parameters, such as maize dry matter, shoot N accumulation, SPAD index, Fe-shoot, and Fe-root accumulation were determined. The complexes with TA and CA prepared at the stoichiometric ratio 1:2 showed the most favorable results in plant growth, and iron nutrition. The stability and solubility of complexes affected iron accumulation and plant growth. The results indicate that iron redistribution from leaf to the root increased in tandem with complex stability. Additionally, shoot iron accumulation was more significant for complexes with high solubility and low stability.

Keywords: carboxylic acids, iron deficiency, iron complexes, Gibbs free energy.

1. Introduction

Iron is the most required micronutrient by plants, integrating proteins involved in photosynthesis processes (Broadley et al. 2011). Iron availability in soils is controlled by Fe-oxides, which have low solubility, mainly in high soil pH conditions (pH > 7) (Marschner & Rengel 2011), such as calcareous and weathered over-limed soils. Therefore, in alkalinized soils, the concentration of soluble iron is below the requirement for plentiful plant growth (Lindsay 1995). Iron uptake by plant roots is also restricted in conditions of low water availability conditions because diffusion is the primary process involved in iron transport from soil solution to the roots (Marschner & Rengel 2011). Leaf chlorosis, a common symptom of Fe-deficiency, is caused by the severe reduction of photosynthetic pigments, affecting crop development and yield (Abadía et al. 2002).

Foliar fertilization is a widely used method to overcome Fe chlorosis (Eichert & Fernández 2011; Malhotra et al. 2020) and increase cereal grain iron concentration in crops (He et al. 2013; Sharma et al. 2019a) It is common to use chelated or complexed forms of iron in foliar sprays because iron is easily oxidized and precipitated in aqueous solution. Additionally, the use of complexed forms of iron increased the mobility of iron from the leaves to non-fertilized plant tissue regions, along with efficient root redistribution (He et al. 2013). The most common chelators used are synthetic aminopolycarboxylic acids, such as EDTA (Eichert & Fernández 2011).

Iron chelated forms are highly stable, but in several foliar fertilization studies, their efficiency does not usually exceed that of inorganic iron forms (Shenker & Chen 2005; Rodríguez-Lucena et al. 2010). The high stability is a crucial factor in determining the efficiency of Fe-chelates applied to the soil and nutrient solution (Rodríguez-Lucena et al. 2010). However, the influence of this factor on iron accumulation and translocation for foliar-applied sources is still unclear. Despite the increased use of foliar sprays in crops, the current knowledge about foliar penetration and translocation of free and complexed nutrient forms is still limited (Fernandez & Eichert 2009; Fernández & Brown 2013). Synthetic Fe-chelates are expensive and mainly used for high-value crops. Furthermore, aminopolicarboxylic acids are hardly degraded in the environment, and could influence metal availability and mobility in soils (Nowack 2002). Therefore, the development of environmental-friendly fertilizers is an essential research theme on plant nutrition with iron (Abadía et al. 2011).

Soil solution and plant systems naturally contain organic acids, such as citrate, malate, and oxalate (Adeleke et al. 2017). Carboxylic and hydroxylic groups of organic acids can complex cationic elements, acting as natural complexing agents for micronutrients in the soil (Jones 1998). Citric and malic acids are complexing agents for iron transported in the xylem (Kobayashi & Nishizawa 2012; Malhota et al. 2019). Citric acid has also already be tested as Fe-complexing agent in foliar application, with appropriate agronomic value for iron supplying. However, the iron: ligand stoichiometric ratio in foliar fertilization studies with organic acids is not clearly defined (Álvarez-Fernández et al. 2004; Chakraborty et al. 2014).

The stoichiometric ratio between metals and ligands is an important variable that affects the stability and agronomic efficiency of the micronutrient complexed forms (Garcia-Mina 2006). Studies in the field of theoretical chemistry and electrospray mass spectrometry indicate that iron and organic acids, such as citrate and oxalate, are prone to form chemical species with 1:1 and 1:2 (metal: organic acid molar ratio) stoichiometric ratios, respectively (Bertoli et al. 2015a; Tolentino et al. 2015). Besides, Larbi et al. (2010) show that the apoplastic fluid of Fe-deficient plants contains Fe-citrate complexes of molar ratios 1:1 ($[\text{Fe-CitOH}]^{-}$) and 1: 2 ($[\text{FeCit}_2]^{-3}$). Polynucleate Fe-citrate complexes at molar ratio 1:1 (Fe_2Cit_2 and Fe_3Cit_3) were also found in xylem sap of Fe deficient and Fe sufficient leaves (Rellán-Álvarez et al. 2010).

The role played by natural complexes, a low-cost source, in delivering iron to plants is less studied than synthetic chelates. In the same way, the relation among complex stability, complexed fraction and iron foliar fertilization efficiency for both complexed and chelated sources is still unexplored (Abadía et al. 2011). Therefore, this study intended to investigate some chemical properties of Fe-complexes and verify if the complex stability and iron complexed fraction are associated with the efficiency of the foliar iron nutrition. Furthermore, this study explores if organic acids could be successfully used as iron complex agents in foliar fertilization, at two specific stoichiometric ratios (1:1 and 1:2 Fe: organic acid molar ratio). A chemical speciation software was employed to verify the Fe-complexed fraction and corresponding Fe-complex species for each tested ligand (EDTA, citric acid, malic acid, tartaric acid, and oxalic acid). Sequentially, theoretical calculations helped to estimate the chemical structure and the thermodynamic stability of the iron chemical species. Lastly, after the formulation of Fe-complexes in laboratory conditions, a greenhouse experiment was conducted to test the Fe-complexes capacity for iron supplying and redistribution in the foliar application, using maize as plant test. We hypothesized that the stability is a crucial factor determining the accumulation and redistribution of foliar-applied Fe-complexed sources.

2. Material and Methods

2.1 Foliar sprays solution preparation and analysis

The organic acids used as complexing agents for iron in the foliar sprays were: tricarboxylic citric acid (CA), dicarboxylic malic acid (MA), dicarboxylic tartaric acid (TA), and dicarboxylic oxalic acid (OA). The iron source used for Fe-complexes and Fe-EDTA preparation was $FeSO_4$ (Fe II). All the compounds used were of analytical grade. Stock solutions of all Fe-organic acids and Fe-EDTA in 0.01 mol L^{-1} concentration were prepared. Ligands were dissolved in water and KOH (0.05 mol L^{-1}) was used to adjust the solutions to pH 5.0-6.0. Subsequently, an amount of iron (as $FeSO_4$), calculated to be 2% above the molar amount of the ligand, was then slowly added while maintaining the pH between 5.0 and 6.0 (Rodríguez-Lucena et al. 2010; Martín-Fernández et al. 2017). The stoichiometric molar ratios (SR) used for organic acid reactions with iron were 1:1 and 1:2 (Fe: organic acid). Fe-EDTA was prepared in the recommended 1:1 SR (Fernández et al. 2006) Solutions were aerated and left to stand overnight to allow the excess iron to precipitate (Steiner & Van Winden 1970). All chelate/complex solutions preparation and storage were carried out in the dark to avoid potential photodecomposition of chelates (Steiner & Van Winden 1970).

In order to obtain a 0.005 mol L^{-1} Fe solutions for foliar spray (Rodríguez-Lucena et al. 2010), aliquots of Fe-organic acids or Fe-EDTA stock solutions were mixed with distilled water was added to complete 200 mL. After adjusting the solutions' pH to 5.0 (Rodríguez-Lucena *et al.,* 2010; Eichert & Fernández, 2011), the non-ionic Agral® surfactant (Syngenta) was added at the recommended rate of 0.5 mL L^{-1} to enhance the the foliar spray adherence. As a control, an uncomplexed source solution using $FeSO_4$ was prepared few minutes before the application, to avoid Fe^{2+} oxidation and precipitation. Additional solutions of 0.005 mol L^{-1} Fe were made in triplicate to measure the concentration of soluble iron. The solutions were mixed and centrifuged for precipitation of the insoluble material. The supernatant was collected and the iron concentration was determined using the atomic absorption technique. The measured soluble iron concentration was then divided by the total iron concentration (0.005 mol L^{-1}) to determine soluble iron fraction.

For Fourier Transform Infra-Red (FTIR) analysis, a separate batch of the iron complex foliar spray solutions was freeze-dried by lyophilization to obtain solid samples. The samples were analyzed by a Cary 630 Agilent® equipment containing a ZnSe crystal in an ATR (Attenuated Total Reflectance) configuration. The scans were recorded between 650 and 4000 cm^{-1} and at 4 cm^{-1} resolution.

2.2 Fe chemical species in the foliar spray solutions

The software Visual Minteq version 3.1 was used to estimate the proportion of iron complexed by the organic acids in the foliar spray solutions. The software determined the ionic strength after pH and temperature had been set at 5.0 and 25ºC, respectively. It was considered that only Fe(III) complexes were formed in the foliar spray solutions, due to the exposure to aeration and the fact that Fe(III)-organic acids complexes are more stable and preferably formed (Gomathl 2000; D'Antonio et al. 2009; Rodríguez-Lucena et al. 2010). The chemical species of the Minteq outputs were classified as Free-Fe or Complexed-Fe. Free-Fe represented the sum of ionic and non-complexed forms, such as Fe^{3+}, $FeOH^{2+}$, Fe^{2+}, and $FeSO_4$ (aq). Complexed-Fe represented the sum of all other forms of iron associated with the ligands (complexes), with negative, positive and neutral charges. Free-Fe and Complexed-Fe fractions were expressed as percentage of the total iron amount in the foliar spray solution.

2.3. Fe-complexes computational modeling

The complexed species identified in the Minteq were used to computational geometry optimization and thermodynamic calculations. The geometry optimization of reagents (organic acids and iron) and complexes formed between iron and organic acids was obtained using the Gaussian 09W software. Initially, the probable geometries were input for both the ligands (with the farthest carboxyl groups) and the complexes (with three groups for CA; or two carboxyl groups in SR 1:1 for MA and TA; or four carboxyl groups in SR 1:2 for OA). Subsequently, using the B3LYP method and the LanL2DZ basis set, the geometries were optimized by the Density Functional Theory (DFT) (Bertoli et al. 2015b). The B3LYP/LanL2DZ is a suitable effective core potential for post-third row atoms, such as the metal iron (Bertoli et al. 2015b). The calculations were performed considering both the gas phase and water as an implicit solvent (Bertoli et al. 2015b; Tolentino et al. 2015), through the polarizable continuum model using the integral equation formalism.

After the geometry optimization, the thermodynamic parameters aided the study of complex stability. Here, the standard Gibbs free energies (G^o) for the ligand, iron, and complex were obtained and then used to calculate the standard Gibbs free energy of complexation (ΔG^o), according to the following Equation 1(Forseman & Frisch 2015):

$$\Delta G^o = G^o \text{ complex} - (G^o \text{ organic acid} + G^o \text{ iron}) \qquad \text{(Eq. 1)}$$

2.4 Maize greenhouse experimental conditions

The effects of iron complexes in the foliar application on the maize plants were evaluated by a greenhouse experiment in the soil science department at Lavras Federal University, Lavras, Brazil. The treatments were arranged in a randomized block design with four replicates. Maize cv Pioneer 30F53 were germinated in a tray filled with vermiculite as a substrate. Ten days after germination, seedlings were transferred to the nutrient solution with 75% strength without iron supply for three days. After the transfer of seedlings to an iron-free full-strength solution, the Fe-complexes were applied to the maize leaves on day 7. Each plant's adaxial and abaxial leaf surfaces were sprayed with 4 ml of Fe-complexes solution (0.005 mol L^{-1} Fe). A second foliar application was made at the end of week 2 (day 14). Nutrient solution contamination was avoided by placing a plastic sheet and a polystyrene layer over the pot lid. The full-strength nutrient solution had the following nutrient concentration: 14, 196, 31, 235, 160, 49, 68, 0.8, 1, 0.2, 0.5 and 0.01 mg $L^{-1}$of N-NH_4^+, N-NO_3^-, P, K, Ca, Mg, S, B, Mn, Zn, Cu, and Mo, respectively (adapted from Hoagland et al. 1950). The salts used to formulate the nutrient solution were of analytical grade. To determine in the roots only the iron transported from the foliar spray in leaves, there was no iron addition to nutrient solution.

2.5 Maize growth and nutrition evaluations

SPAD readings were taken with a chlorophyll meter (Minolta SPAD-502) on day 21. The first wholly expanded leaf of the plant was assessed (Bin et al. 2016) and three specific leaf regions were chosen for readings: the basis, the middle, and the apex. The mean of these readings per plant was statistically analyzed. Maize plants were harvested ten days after the second iron foliar application (*i.e.* on day 24) and separated into shoot and root parts. Maize shoot parts were thoroughly rinsed with HCl (0.1%) and distilled water to remove any adhered Fe applied as a foliar spray. Dry matter (DM) of all these parts was determined after drying maize tissues at 60ºC for 72 hours. After the plant tissue digestion in a 4:1 HNO_3-$HClO_4$ solution (Silva, 2009), nitrogen and iron contents in the plant shoots, and iron content in the plant roots were determined by inductively coupled plasma optical emission spectrometry technique (Spectro, Blue®, Germany). Nitrogen and iron accumulation was calculated by multiplying the tissue nutrient concentration by its respective DM and expressed in mg (milligrams) or g (grams) per plant. Lastly, the iron accumulation in the root DM was divided by iron accumulated in the shoot DM to determine the "Fe root: shoot ratio".

2.6 Statistics and data analysis

The main effects of foliar treatments on plant attributes were identified by the Analysis of Variance (ANOVA) test. Differences among treatments were tested using the mean-separation Least Significant Difference (LSD) test at a p level ≤ 0.05. Simple and multiple regressions were done to correlate the iron accumulation and iron redistribution (iron root-to-shoot ratio) with iron complexes' solubility and stability. Iron sulfate was not included in these analyses, since the objective was to investigate the influence of the Fe-complexed sources' chemical parameters. The regressions analyses were further validated through the ANOVA and F tests. Moreover, multicollinearity analysis through the "faraway" package with the "VIF" function was conducted (Faraway, 2016). All other statistical analyses presented were done using the R software (R, the R project for statistical computing).

3. Results

3.1 Chemical speciation of Fe-organic acids complexes

The organic acids showed different capacities to keep Fe complexed in the foliar spray solution. The complexed and free Fe fraction, as well as the soluble Fe fraction in the foliar spray solutions, are shown in Table 1. The highest complexed Fe fraction was observed for the EDTA (100%) and CA at 1:2 SR (99.9%). Although the 1: 2 SR was used in the synthesis of Fe-complexes, only the oxalate presented formation of 1:2 complexes ($[\text{Fe-(Oxalate)}_2]^-$) (Table 1). Besides, oxalate also presents the formation of 1:1 and 1:3 complexes. However, the 1:2 SR complex majorly contributed to species of complexed fraction for both stoichiometries (37% and 57% of the complexed iron fraction for 1:1 and 1:2 SR, respectively). The other organic acids, even in the 1:2 molar ratio mixture, exhibited only the formation of 1:1 complex with a neutral (Fe-Citrate) or a single positive charge (Fe-Malate^+ and Fe-Tartrate^+) (Table 1). Therefore, CA, MA, and TA at 1:2 SR presented a higher proportion of free organic acid in the solution than at the 1:1 SR (Table 1). Regarding the solubility of iron in the foliar spray solutions, almost all complexes presented high iron soluble fraction ($\geq$ 95%), except Fe-OA, which showed 65.9% and 83.5% of iron in soluble form, at the 1:1 and 1:2 SR, respectively (Table 1).

The chemical species obtained with the Minteq software were used for the structure optimization and energy determination through molecular modeling calculations. For iron combined with OA, the species ($[\text{Fe-(Oxalate)}_2]^-$) was chosen for structure optimization. The corresponding Gibbs free energy of complexation (ΔG^o) is presented in Table 2, and the optimized structures are shown in Figure 1. All the Fe-complexes showed negative values of Gibbs free energy indicating a favorable formation of complex. Fe-EDTA presented the lowest value of ΔG^o, indicating the high stability of this complex, followed by $[\text{Fe-(Oxalate)}_2]^-$. After, the order of chemical species by stability was observed as follows: Fe-Citrate, $[\text{Fe-Malate}]^+$ and

[Fe-Tartrate]$^+$ complex (Table 2). Details about the standard orientation of the molecule geometries and their respective molecular volume are presented in the supplementary material.

Table 1. Free complexed agent fraction (Cag free; as a percentage of total complexing concentration), complexed, and water-soluble Fe (Fe complexed and Fe soluble; as a percentage of total Fe concentration), and the chemical species of complex in the foliar spray solutions. Complexed Fe, free complexing agents, and the chemical species of complex were determined using the Minteq software, and soluble Fe by atomic absorption technique.

Complexing	**Stoichiometry**	**CAg free**	**Fe soluble**	**Fe complexed**	**Chemical Specie**
	Fe: ligand molar ratio	% total complexing	% total Fe		
EDTA	1:1	0.0	100.0	100.0	[Fe-EDTA]$^-$
CA	1:1	0.5	99.8	99.5	Fe-Citrate
	1:2	49.7	100.0	99.9	Fe-Citrate
MA	1:1	22.0	96.0	77.6	[Fe-Malate]$^+$
	1:2	52.8	99.8	93.3	[Fe-Malate]$^+$
TA	1:1	32.5	95.0	66.3	[Fe-Tartrate]$^+$
	1:2	55.1	98.5	84.6	[Fe-Tartrate]$^+$
OA	1:1	0.1	82.4	50.6	[Fe-(Oxalate)$_3$]$^{-3}$, [Fe-(Oxalate)$_2$]$^-$, [Fe-Oxalate]$^+$
	1:2	0.2	64.9	88.2	[Fe-(Oxalate)$_3$]$^{-3}$, [Fe-(Oxalate)$_2$]$^-$, [Fe-Oxalate]$^+$
$FeSO_4$	-	0	95.5	0	$FeSO_4$; Fe^{2+}

Table 2. Gibbs free energy of the complexes, according to optimization at Density Functional Theory (DFT) and the B3LYP/LANL2DZ level, in aqueous phase.

Chemical Specie	**ΔGº (kcal mol^{-1})**
Fe-EDTA$^-$	-479.953
Fe-Citrate	-174.376
Fe-Tartrate$^+$	-139.903
Fe-Malate$^+$	-146.830
Fe-(Oxalate)$_2^-$	-209.098

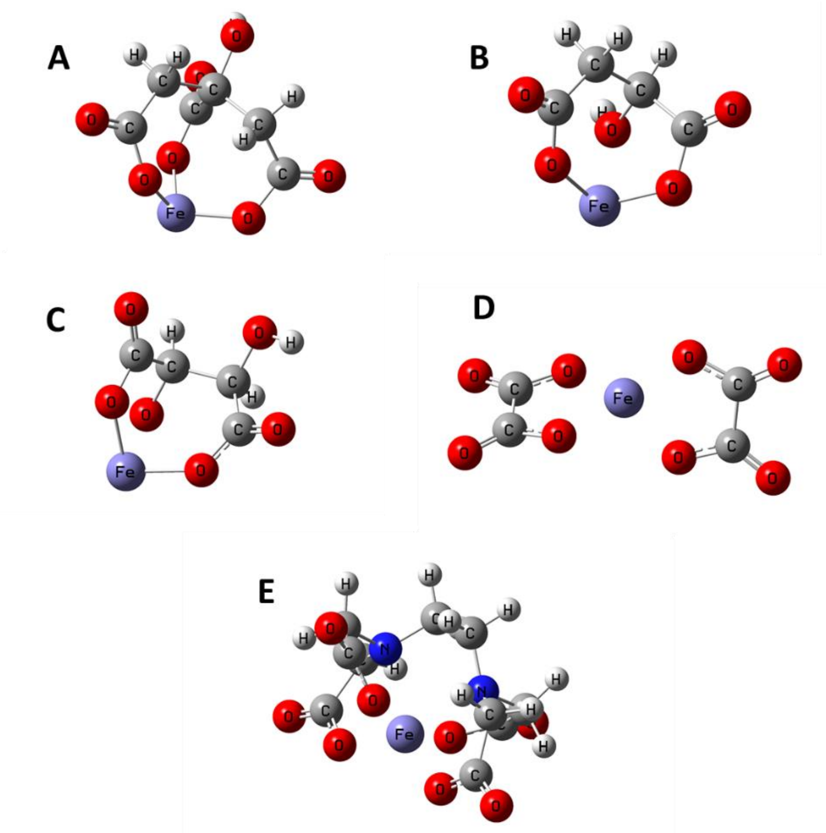

Figure 1. Optimized structures for the complexes tested. Iron citrate (A), Iron malate (B), Iron tartrate (C), Iron di-oxalate (D), and Iron EDTA (E).

The infrared spectra of the complexes are shown in Supplemental Material (Fig. S6) and the main characteristic bands to identify the organic acid complexes were the strong asymmetric stretching COO^- vibration at 1570-1540 cm^{-1}, and the medium-weak symmetric stretching COO^- vibration at 1440-1310 cm^{-1} [20]. The infrared spectra confirms the complex formation by the reduction of the intensity of these two bands associated to the carboxylic groups, as well as the wavenumber dislocation of the bands in the complex spectra in relation to the free organic acid spectra [37].

3.2 Growth and nutrition of maize plants

The Fe-sources added to foliar sprays significantly affected the maize shoot ($p = 0.001$) and root DM ($p = 0.039$), as shown in Figure 2A. Plants supplied with CA at 1:2 SR showed the highest shoot DM production (3.88 g; Figure 2A). Plants treated with EDTA, TA at 1:2 SR, and $FeSO_4$ showed a statistically similar shoot DM production (3.29 g, 3.22 g, and 2.94 g, respectively; Figure 2A). Plants supplied with OA at 1:2 SR and OA at 1:2 SR presented the lowest dry matter production (1.28 g, and 1.52 g respectively; Figure 2A). When applying Fe-CA and Fe-TA, the plants treated with complexes at 1:2 SR showed a higher DM accumulation than the 1:1 SR. In contrast, the use of Fe-MA at 1:1 SR showed a more significant DM accumulation than 1:2 SR (Figure 2A). Maize root growth responds to treatments similar to maize shoot DM. The use of CA at 1:2 SR and EDTA promoted the highest root biomass values (0.92 g and 0.91 g, respectively; Figure 2A). Plants treated with $FeSO_4$, Fe-CA at 1:1 SR, Fe-TA at 1:1 SR and 1:2 SR, and Fe-MA at 1:1 SR presented root DM statistically equal to those supplied with Fe-CA at 1:2 SR. Conversely, OA at 1: 2 SR showed the lowest root DM values (0.48 g plant^{-1}, Figure 2A).

Nitrogen (N) accumulated in maize shoot in response to the Fe-complexing agents used in foliar spray is shown in Figure 2B. Iron sources that promoted the highest N accumulation were Fe-CA at 1:2 SR, Fe-TA at 1:2 SR, Fe-EDTA and $FeSO_4$ (Figure 2B). Fe-OA at 1:2 SR showed the lowest N accumulated in the plant shoot (Figure 2B). Mirroring maize DM response to Fe-complexes, the 1:2 SR treatments showed more significant N accumulation for CA and TA than 1:1 SR treatments (Figure 2B).

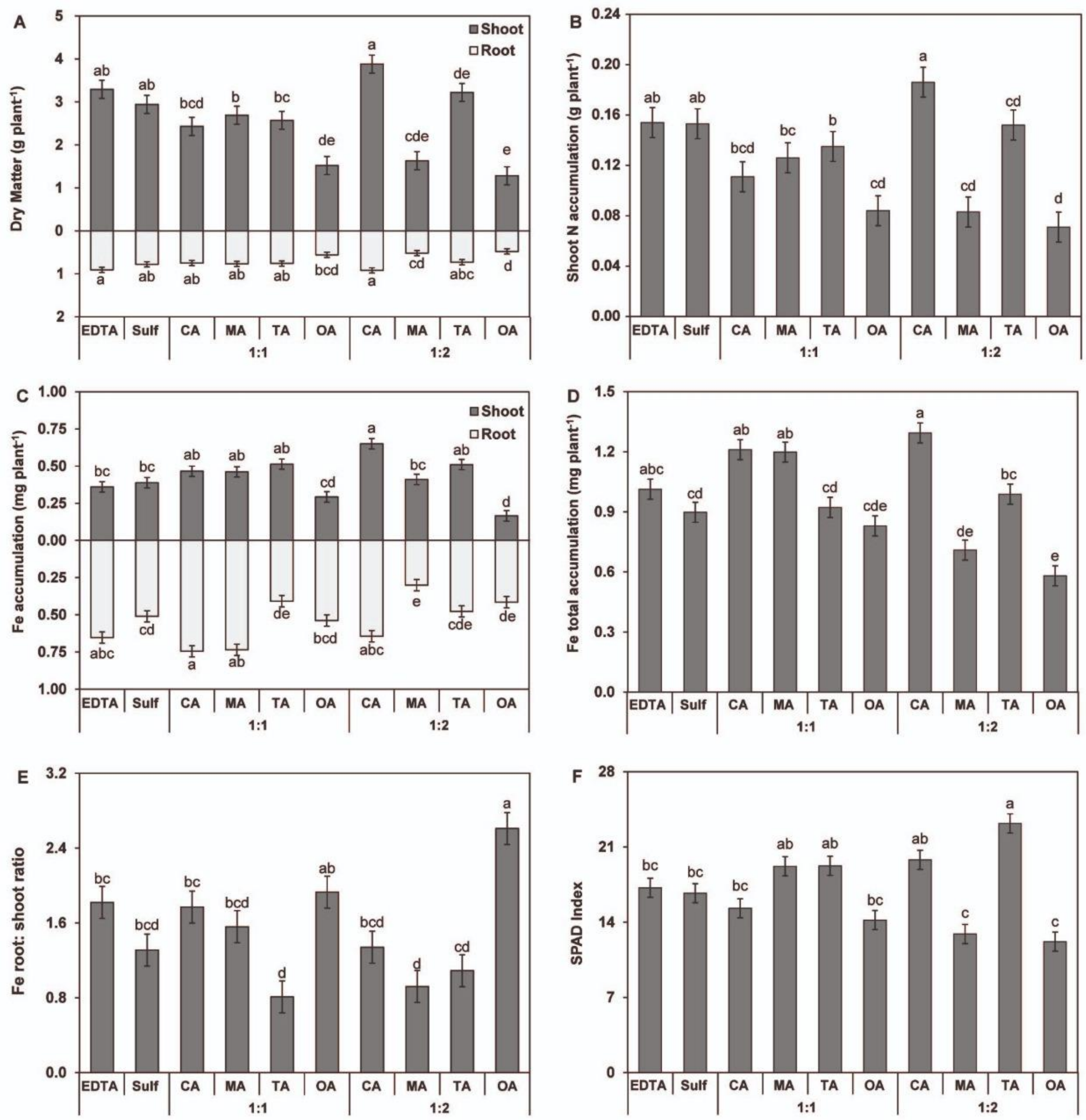

Figure 2. Dry matter of root and shoot (A), nitrogen accumulation in shoot (B), iron accumulated in shoot and root maize parts (C), iron total accumulated in plant (shoot + root) (D), Fe root: shoot ratio (E), and SPAD index (F) of maize plants as related to the stoichiometry ratio (1:1 or 1:2 molar ratio) and complexing agents for Fe in foliar sprays: EDTA, Sulf ($FeSO_4$), CA (citric acid), MA (malic acid), TA (tartaric acid), and OA (oxalic acid). Error bars represent the mean standard error. Letters refer to mean comparison in each compartment (root or shoot), and the same letters indicate no difference among treatments according to the LSD test at a 5% significance level.

The iron accumulated in shoot and root DM, total Fe accumulated in the plant (root + shoot), the Fe root: shoot ratio, and the SPAD index are also showed in Figure 2. The type of organic acid applied, and the SR affect the iron accumulated by the maize plants ($p=0.01$; Figure 2C and 2D). The Fe-CA at 1:2 SR had the greatest iron content in the maize shoot (0.65 mg plant^{-1}), which was statistically higher than those verified for $FeSO_4$ and EDTA. Fe-CA at 1:2

SR was followed by the TA at 1:2 (0.51 mg plant^{-1}) SR, TA at 1:1 SR (0.51 mg plant^{-1}), CA at 1:1 SR (0.47 mg plant^{-1}), and MA at 1:1 SR (0.46 mg plant^{-1}) (Figure 2C). In contrast, the most significant Fe accumulation was observed with the Fe-CA at 1:1 SR (0.74 mg plant^{-1}), followed by the Fe-MA at 1:1 SR (0.73 mg plant^{-1}), Fe-EDTA (0.65 mg plant^{-1}), and Fe-CA at 1:2 SR (0.64 mg plant^{-1}) (Figure 2C). Fe-CA and Fe-MA at 1:1 SR presented Fe-root accumulation similar to Fe-EDTA, but greater than $FeSO_4$ (Figure 2C). Fe-MA at 1:2 SR showed the lowest Fe-root accumulation (0.30 mg plant^{-1}; Figure 2A). Regarding the total Fe accumulated, in general, the De-complexed sources showed the highest values in maize plants. Plants supplied with Fe-CA at 1:2 SR presented the highest total iron content (1.29 mg plant^{-1}) followed by plants supplied with Fe-CA at 1:1 SR (1.21 mg plant^{-1}), and Fe-MA at 1:1 SR (1.20 mg plant^{-1}) (Figure 2D).

To verify the redistribution of iron from the shoot to the root regarding the different complexing agents, the Fe root: shoot ratio was calculated (Figure 2E). The use of Fe-OA at 1:2 SR showed the highest proportion (2.61). The other organic acids treatments showed proportions statistically similar to Fe-EDTA, except TA at 1:1 SR (0.81), and MA at 1:2 SR (0.91), that showed the lowest values, but statistically equal to $FeSO_4$ (Figure 2E).

Regarding the SPAD index (Figure 2F), maize plants treated with TA at 1:2 SR and CA at 1:2 SR showed the highest values. Conversely, treatments with OA at 1:2 SR and MA at 1:2 SR showed the lowest SPAD index. Plants treated with TA at 1:2 SR showed a SPAD index statistically higher than plants treated with $FeSO_4$ and Fe-EDTA (Figure 1E).

To investigate the influence of chemical parameters of complexes on maize iron nutrition, single and multiple correlations were performed. Iron complexed fraction did not correlate with any plant iron nutrition attribute. However, stability and corresponding solubility significantly influenced iron shoot accumulation and total iron accumulation (Figure 3A and 3B). There was a positive correlation between decreased complex stability and shoot iron accumulation for high solubility complexes (Figure 3A). Whereas, species with lower solubility (and higher stability) were associated with a decreased in shoot iron accumulation (Figure 3A). Lastly, total iron accumulated and increased solubility (and decreased stability) were positively correlated. When only the Fe-organic acids were considered (Fe-EDTA excluded), iron root to shoot ratio, an index for iron redistribution in the plant, was negatively correlated with the complex stability (Figure 3C).

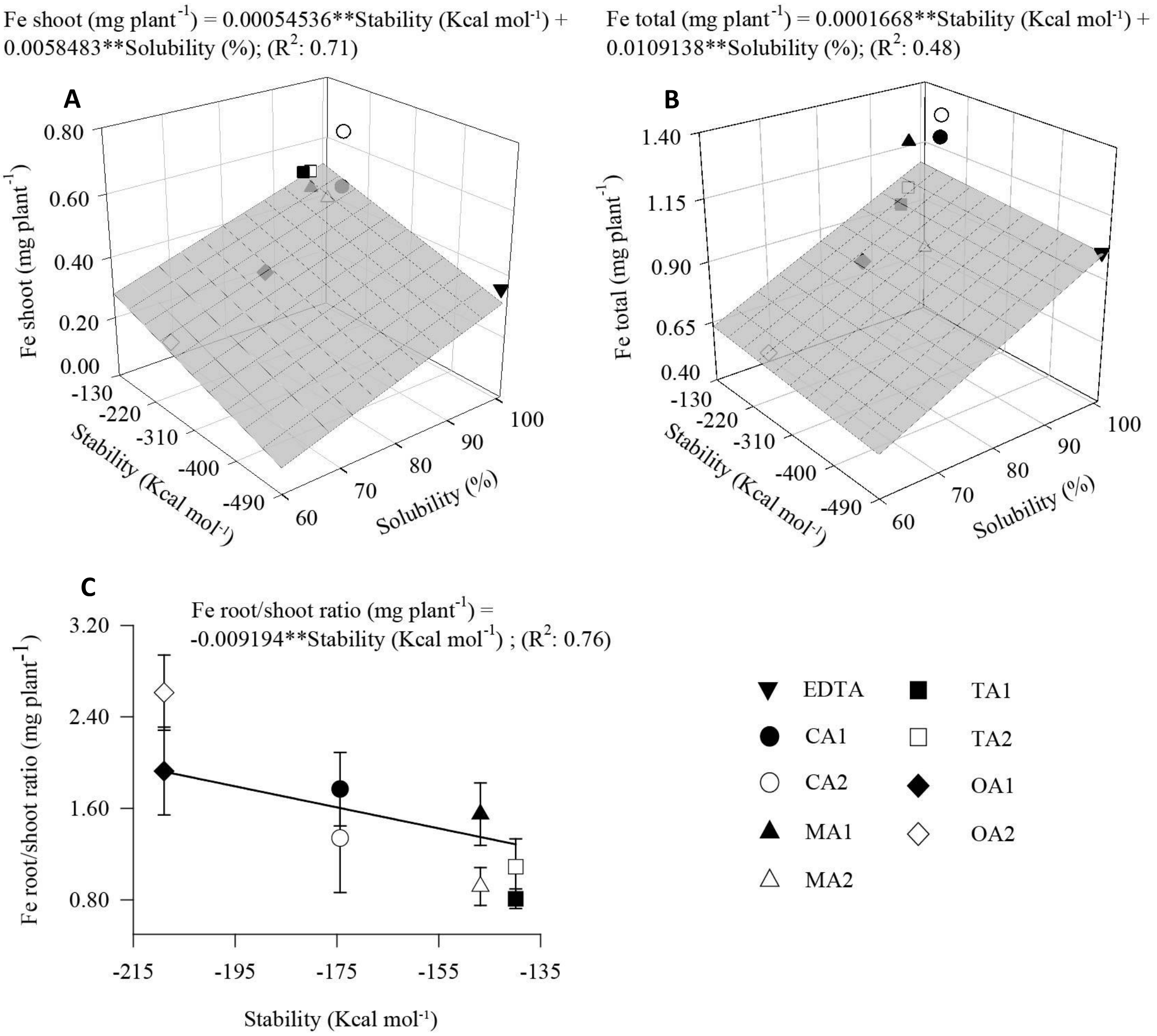

Figure 3. Multiple regressions correlating stability and solubility of iron complexes with shoot iron accumulation (A) and total iron accumulation (B) in maize plants. Simple regression correlated complex stability and Fe root to shoot ratio (C). The regression parameters were significative at $p<0.01$. Abbreviations: EDTA, CA (citric acid), MA (malic acid), TA (tartaric acid), and OA (oxalic acid). The numbers 1 and 2 indicate the stoichiometry (1 – 1:1 stoichiometric molar ratio and 2 – 1:2 stoichiometric molar ratio).

4. Discussion

4.1. Chemical properties of Fe-organic acid complexes

The organic acids used as complexing agents showed different abilities to keep iron complexed in foliar spray solution (Table 1). Since solution pH was similar, the changes in the Fe complexed fraction are mainly due to (a) the differences in dissociation constants of the organic acids (pKa), and consequently, the number of dissociated groups available for complexation, and (b) the stability of the complex formed between the organic acid and iron (Garcia-Mina

2006). The stability is affected by the type of functional group that is complexing iron, and the number of groups involved in the complexation (Hosmane, 2017).

For the stability parameters, EDTA was the most stable complex, followed by Fe-[(Oxalate)2]-. EDTA shows high stability when chelated with iron, mainly due to its six functional groups (four carboxylic groups and two amide groups; Figure 3) which can donate electrons to iron (Lindsay 1995).

In this study, CA and EDTA had highest capacity of chelation. Among the organic acids tested, CA at 1:2 SR showed the highest Fe complexed fraction and TA at 1:1 SR showed the lowest. The Fe-citrate complex had lower stability than EDTA, but higher than Fe-tartrate and Fe-malate (Table 1), as reported elsewhere (Gomathl 2000). Therefore, CA has a stronger ability to keep a high amount of iron complexed when compared to MA and TA.

Excluding oxalate, the stoichiometries tested do not affect the Fe-complexed species formed in the solutions (Table 1). Ferric ion has a coordination number of six; therefore, the donation of six electrons pairs should result in an octahedral geometry for the complexes. However, the ion coordination number is affected by the radius of the metal, and the ligand size and coordination ability of the ligand (Dudev et al. 2006). Since anionic ligands tend to repel, a reduction of the coordination number could occur (Bertoli et al. 2015b). Therefore, for CA, MA, and TA, only the 1:1 SR complex was observed (Table 1). Here, steric hindrance could disallow the formation of 1:2 SR complex. With EDTA, a single molecule can furnish six electron pairs to fulfill the coordination number of iron, which additionally contributes to the high stability of Fe-EDTA. OA was more prone to form 1:2 and 1:3 complexes because of its lower molecular size, and consequent spatial conformation that does not cause great repulsion forces between the two or three oxalate molecules around the iron (Bowes et al. 2003; Tolentino et al. 2015).

The solubility also showed variations among the organic acids used. Fe-OA showed the lowest solubility values. Oxalate salts have medium to low solubility (D’Antonio et al. 2009). Thus, these molecules are not suitable options to use as complexing agents since high solubility is one of the pre-requisites of a complex used as a micronutrient source (Garcia-Mina 2006). Moreover, the lower iron concentration in the foliar spray solution probably cause the inferior development of plants treated with these molecules (Figure 1).

4.2.Fe-complexes properties and maize growth and iron nutrition

Efficient iron foliar fertilization promotes adequate iron uptake by the leaves and iron translocation through the plant, resulting in improved crop growth and development (Abadía et al., 2011). Foliar-applied molecules penetrate the leaf through the cuticle or stomata (Abadía et al. 2011).Sequentially, the molecule should be taken up by the mesophyll cells, a process that involves a two-step mechanism related to a plasma membrane Fe (III) chelate-reductase and Fe (II) transporters (Kobayashi & Nishizawa 2012). Regarding Fe-complexed forms, before uptake, iron is released of the complex (Brüggemann et al., 1993). Therefore, several factors, such as Fe-complex stability, can affect the plant leaf uptake in each of the processes above.

The efficiency of Fe-complexes and synthetic Fe-chelates, such as Fe-EDTA, when applied to the soil, generally are related to these ligands' capacity in keeping iron in complexed form (Rodríguez-Lucena et al. 2010). However, studies have already shown that in foliar spray, the complexed fraction does not affect the efficiency of the Fe-source (Rodríguez-Lucena et al. 2010). In the present study, the complexed Fe fraction varied among the organic acids, but it does not influenced the iron accumulated in the shoot and root.

Conversely, complex solubility and stability affected the iron accumulation and redistribution in maize plants. Highly stable Fe-organic acid complexes increase the iron redistribution in plant (Figure 3C). Due to the Fe(III)s' potential toxicity of (prone to generate reactive oxygen species, ROS) and its low solubility in the free state, complexation is essential for efficient translocation in plant system (Kobayashi & Nishizawa 2012). Besides, the carboxylic and hydroxylic groups of the cuticle and other negative charges in the apoplastic space behaves as a cation exchange membrane (Fernandez & Eichert 2009). In the case of low stability complexes, iron could be displaced from the complex and bound to these charges. Furthermore, when the complex stability is low, exchange reactions with more potent ligands present in apoplast could occur (Doolette et al. 2018). High stability complexes prevent these phenomena, which could slow down the diffusion of nutrients across the leaf cell walls and apoplastic space and, consequently, reduced the iron redistribution on plant system (Fernández & Brown 2013).

Iron accumulation in maize shoots was higher when complexes with lower stability were used (Figure 3). In contrast, the high stability complex increases the redistribution and prevents iron adsorption to leaf apoplast and leaf cell walls (Fernandez & Eichert 2009). Therefore, when lower stability complexes were used greater amount of iron can remain in shoots due to exchange reactions. Furthermore, plant cells could displace and use iron from low stability compounds more easily than iron from high stability complexes.

Molecular weight and size of the Fe-source molecule are also influent factors on the efficiency of foliar-applied nutrients. In this study, the high stability complex (Fe-EDTA) also presented also the highest molecular weight and size (Fig. 1; Table S1). Maize treated with iron combined with CA, MA, and TA in general showed similar or greater iron content than plants sprayed with Fe-EDTA. The cuticle is size-selective because it has voids with limited size (Eichert & Fernández 2011). Thus, a Fe-source with a relatively large molecular size is supposed to slow down the diffusion through the leaf cuticle and cell walls (Shenker & Chen 2005; Chakraborty et al. 2014). The lower molecular weight and size of Fe-organic acid complexes compared to Fe-EDTA (Figure 1; Table S1) could also be an explanation for their improved efficiency of Fe acquisition (Chakraborty et al. 2014).

The application of Fe-organic acids and FeSO4 leads to a spotty regreening. Fe-EDTA promoted a more uniform regreening but also dark spots, characteristic of leaf damage. This damage is probably one reason for the lower SPAD index of plants treated with Fe-EDTA (Figure 2). Injury of plant tissues in treatments with Fe-synthetic chelates were used was reported elsewhere (Rodríguez-Lucena et al. 2010). Conversely, organic acid treatments at 0.005 mol L-1 showed no such symptoms (0.005 mol L-1)

Although both stoichiometries tested do not alter the type chemical species present in the foliar sprays, they produced different effects on maize growth. The stoichiometries used only differ in the amount of Fe-complexed and free organic acid present in the solutions (Table 1). In the case of MA, the use of both SR produced similar iron accumulation in shoot, but 1:1 SR showed higher iron accumulation in root and higher total iron accumulation. Regarding maize growth parameters, the use of 1:2 SR in foliar sprays resulted in lower plant growth, iron redistribution, and N accumulation. In this study, it was not possible to identify the physiological processes responsible for the lower maize growth and Fe nutrition when treated with Fe-MA at 1:2 SR. However, some explanations could be hypothesized. For example: the higher amount of free MA in the Fe-MA at 1:2 SR could have (a) triggered a dysregulation on iron homeostasis, leading to high ROS levels and metabolic dysregulation (Kobayashi & Nishizawa 2012; Sharma et al. 2019b), or (b) suppressive effects on growth of maize plants at concentrations used.

Conversely, maize treated with CA and TA at 1:2 SR showed more significant shoot DM production and numerically higher N content than maize treated with CA and TA at 1:1 SR. Thus, the possible benefits of these organic acids on plant growth or iron metabolism could be further explored. Other reports claim that the organic acids concentration and citrate: Fe ratio increase in the leaf apoplastic fluid of Fe deficient plants, (Larbi et al. 2010). The rise in citrate in Fe deficient plants may be a mechanism to replenish the Krebs cycle for increased citrate export from the mitochondria; thus, assuring its continuous operation in Fe-deficient conditions. (Abadía et al. 2002) Furthermore, foliar spray of citric acid reduced the oxidative damage and consequent lipid peroxidation in plants exposed to stressful conditions (An et al. 2014). The mechanism of stress alleviation is associated with an increase in antioxidant system activity or a decrease in the stress-induced ROS formation (An et al. 2014; Sharma et al. 2019b).

Plants treated with Fe-TA at 1:1 and 1:2 SR showed similar Fe-accumulation in shoot and root, but 1:2 SR produced higher shoot DM production, SPAD index, and N accumulation than 1:1 SR. There is little evidence in the literature of beneficial effects of using Fe-TA or even TA in a single application. However, in line with our results, the impact of TA on plant physiology and iron metabolism should be further investigated. The highest SPAD index observed in plants treated with Fe-TA indicates its high capacity for regreening. In plants, tartrate is evolved in the ascorbic acid route, and, thus, in the plant ROS scavenger system (DeBolt et al. 2006). Thus, similarly to CA, TA probably plays a role in decreasing of oxidative stress associated with iron deficiency. Therein, the use of organic acids as complexing agents can promote effects on plant iron homeostasis or plant physiological processes and, consequently, in plant growth and nutrition.

5. Conclusion

The tested stoichiometries 1:1 and 1:2 showed no influence on the type of chemical species observed in foliar spray solutions, altering only the Fe-complexed fraction. Complex stability and solubility significantly influenced iron accumulation and redistribution. High stability iron-organic acid complexes showed high iron redistribution. In contrast, iron accumulation in shoot was higher for Fe-complexes with low stability and high solubility. Fe-tartrate and Fe-citrate at 1:2 SR were as effective as $FeSO_4$ and Fe-EDTA in promoting maize growth. Additionally, Fe-citrate at 1:2 SR was more effective in increasing iron accumulation. Therefore, the studied complexes are suitable iron sources for application in foliar fertilization.

Abbreviations used

EDTA: ethylenediaminetetraacetic acid; CA: citric acid, MA: malic acid, TA: tartaric acid; OA: oxalic acid; SR: stoichiometric ratio; DFT: density functional theory; FTIR: Fourier Transform Infrared spectroscopy.

Acknowledgements

Authors acknowledge Prof. Maria Lígia Souza e Silva for providing the hydroponic facilities to carried out the experiments with plants in greenhouse conditions; Dr. Sara Dantas Rosa for helping to conduct the plant experiments; Coordenação de Aperfeiçoamento de Pessoal de

Nível Superior (CAPES) for the scholarship provided to the first author, and to fund this research (CAPES-AUXPE-PROEX 593-2018); and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, processo 307447-2019-7) by the financial support.

6. References

Abadía J, Álvarez-Fernández A, Morales F, Sanz M, Abadía A. 2002. Correction of iron chlorosis by foliar sprays. Acta Hortic. 594:115–121.

Abadía J, Vázquez S, Rellán-Álvarez R, El-Jendoubi H, Abadía A, Álvarez-Fernández A, López-Millán AF. 2011. Towards a knowledge-based correction of iron chlorosis. Plant Physiol Biochem. 49(5):471–482.

Adeleke R, Nwangburuka C, Oboirien B. 2017. Origins, roles and fate of organic acids in soils: A review. South African J Bot. 108:393–406.

Álvarez-Fernández A, García-Laviña P, Fidalgo C, Abadía J, Abadía A. 2004. Foliar fertilization to control iron chlorosis in pear (*Pyrus communi*s L.) trees. Plant Soil. 263(1–2):5–15.

An Y, Zhou P, Xiao Q, Shi D. 2014. Effects of foliar application of organic acids on alleviation of aluminum toxicity in alfalfa. J Plant Nutr Soil Sci. 177(3):421–430.

Bertoli AC, Carvalho R, Freitas MP, Ramalho TC, Mancini DT, Oliveira MC, De Varennes A, Dias A. 2015a. Structural determination of Cu and Fe-Citrate complexes: Theoretical investigation and analysis by ESI-MS. J Inorg Biochem. 144:31–37.

Bertoli AC, Carvalho R, Freitas MP, Ramalho TC, Mancini DT, Oliveira MC, De Varennes A, Dias A. 2015b. Theoretical and experimental investigation of complex structures citrate of zinc (II). Inorganica Chim Acta. 425:164–168.

Bin LM, Weng L, Bugter MHJ. 2016. Effectiveness of FeEDDHA, FeEDDHMA, and FeHBED in Preventing Iron-Deficiency Chlorosis in Soybean. J Agric Food Chem. 64(44):8273–8281.

Bowes KF, Ferguson G, Lough AJ, Glidewell C. 2003. Salts of maleic and fumaric acids with organic polyamines: Comparison of isomeric acids as building blocks in supramolecular chemistry. Acta Crystallogr Sect B Struct Sci. 59(1):100–117.

Broadley M, Brown P, Cakmak I, Rengel Z, Zhao F. 2011. Function of Nutrients: Micronutrients. In: Marschners's Mineral Nutrition of Higher Plants. 3 Ed. London: Academic Press p. 191-248.

Brüggemann W, Maas-Kantel K, Moog PR. 1993. Iron uptake by leaf mesophyll cells: The role of the plasma membrane-bound ferric-chelate reductase. Planta. 190(2):151–155.

Chakraborty B, Singh PN, Kumar S, Srivastava PC. 2014. Uptake and Distribution of Iron from Different Iron Sources Applied as Foliar Sprays to Chlorotic Leaves of Low-Chill Peach Cultivars. Agric Res. 3(4):293–301.

D'Antonio MC, Wladimirsky A, Palacios D, Coggiola L, González-Baró AC, Baran EJ, Mercader RC. 2009. Spectroscopic investigations of iron(II) and iron(III) oxalates. J Braz Chem Soc. 20(3):445–450.

DeBolt S, Cook DR, Ford CM. 2006. L-tartaric acid synthesis from vitamin C in higher plants. Proc Natl Acad Sci U S A. 103(14):5608–5613.

Doolette CL, Read TL, Li C, Scheckel KG, Donner E, Kopittke PM, Schjoerring JK, Lombi E. 2018. Foliar application of zinc sulphate and zinc EDTA to wheat leaves: Differences in mobility, distribution, and speciation. J Exp Bot. 69(18):4469–4481.

Dudev M, Wang J, Dudev T, Lim C. 2006. Factors governing the metal coordination number in metal complexes from cambridge structural database analyses. J Phys Chem B. 110(4):1889–1895.

Eichert T, Fernández V. 2011. Uptake and Release of Elements by Leaves and Other Aerial Plant Parts: Elsevier Ltd.

Faraway MJ. 2016. Package "faraway" Title Functions and Datasets for Books by Julian Faraway; [accessed 2020 Apr 15]. http://people.bath.ac.uk/jjf23/

Fernández V, Brown PH. 2013. From plant surface to plant metabolism: The uncertain fate of foliar-applied nutrients. Front Plant Sci. 4(JUL):1–5.

Fernandez V, Eichert T. 2009. Uptake of hydrophilic solutes through plant leaves: Current state of knowledge and perspectives of foliar fertilization. CRC Crit Rev Plant Sci. 28(1–2):36–68.

Fernández V, Del Río V, Abadía J, Abadía A. 2006. Foliar iron fertilization of peach (*Prunus persica* (L.) Batsch): Effects of iron compounds, surfactants and other adjuvants. Plant Soil. 289(1–2):239–252.

Forseman, James B., Frisch A. 2015. Exploring Chemistry with Elecronic Structure Methods: Gaussian, Inc. http://expchem3.com/about/

Garcia-Mina JM. 2006. Stability, solubility and maximum metal binding capacity in metal-humic complexes involving humic substances extracted from peat and organic compost. Org Geochem. 37(12):1960–1972.

Gomathl H. 2000. Chemistry and Electrochemistry of iron complexes. Bulletin Eletrochem 16:459–465.

He W, Shohag MJI, Wei Y, Feng Y, Yang X. 2013. Iron concentration, bioavailability, and nutritional quality of polished rice affected by different forms of foliar iron fertilizer. Food Chem. 141(4):4122–4126.

Hoagland D, Arnon D, Hoagland DR, Arnon DI, Hutchison CB. The water-culture method for growing plants without soil. California Agricultural Experiment Station. Circular 347

Jones DL. 1998. Organic acids in the rhizosphere - A critical review. Plant Soil. 205(1):25–44.

Kobayashi T, Nishizawa NK. 2012. Iron Uptake, Translocation, and Regulation in Higher Plants. Annu Rev Plant Biol. 63(1):131–152.

Larbi A, Morales F, Abadía A, Abadía J. 2010. Changes in iron and organic acid concentrations in xylem sap and apoplastic fluid of iron-deficient Beta vulgaris plants in response to iron resupply. J Plant Physiol. 167(4):255–260.

Lindsay WL. 1995. Chemical reactions in soils that affect iron availability to plants. A quantative approach. Iron Nutr Soils Plants.:7–14.

Malhotra H, Pandey R, Sharma S, Bindraban PS. 2020. Foliar fertilization: possible routes of iron transport from leaf surface to cell organelles. Arch Agron Soil Sci. 66(3):279–300.

Marschner P, Rengel Z. 2011. Nutrient Availability in Soils. In: Marchner's Mineral Nutrition of Higher Plants. 3 Ed. London: Academic Press p. 315-330.

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 Physiol Biochem. 118:579–588.

Nowack B. 2002. Environmental chemistry of aminopolycarboxylate chelating agents. Environ Sci Technol. 36(19):4009–4016.

Papageorgiou SK, Kouvelos EP, Favvas EP, Sapalidis AA, Romanos GE, Katsaros FK. 2010. Metal-carboxylate interactions in metal-alginate complexes studied with FTIR spectroscopy. Carbohydr Res. 345(4):469–473.

R: The R Project for Statistical Computing. [accessed 2020 Apr 15]. https://www.r-project.org/

Rellán-Álvarez R, Giner-Martínez-Sierra J, Orduna J, Orera I, Rodríguez-Castrilln JÁ, García-Alonso JI, Abadía J, Álvarez-Fernández A. 2010. Identification of a Tri-Iron(III), Tri-Citrate Complex in the Xylem Sap of Iron-Deficient Tomato Resupplied with Iron: New Insights into Plant Iron Long-Distance Transport. Plant Cell Physiol. 51(1):91–102.

Rodríguez-Lucena P, Hernández-Apaolaza L, Lucena JJ. 2010. Comparison of iron chelates and complexes supplied as foliar sprays and in nutrient solution to correct iron chlorosis of soybean. J Plant Nutr Soil Sci. 173(1):120–126.

Rodríguez-Lucena P, Ropero E, Hernández-Apaolaza L, Lucena JJ. 2010. Iron supply to soybean plants through the foliar application of IDHA/Fe^{3+}: Effect of plant nutritional status and adjuvants. J Sci Food Agric. 90(15):2633–2640.

Sharma S, Malhotra H, Borah P, Meena MK, Bindraban P, Chandra S, Pande V, Pandey R. 2019a. Foliar application of organic and inorganic iron formulation induces differential detoxification response to improve growth and biofortification in soybean. Plant Physiol Reports. 24(1):119–128.

Sharma S, Malhotra H, Borah P, Meena MK, Bindraban P, Chandra S, Pande V, Pandey R. 2019b. Foliar application of organic and inorganic iron formulation induces differential detoxification response to improve growth and biofortification in soybean. Plant Physiol Reports. 24(1):119–128.

Shenker M, Chen Y. 2005. Increasing Iron Availability to Crops: Fertilizers, Organo-Fertilizers, and Biological Approaches. Soil Sci Plant Nutr. 51(1):c17.

Steiner AA, Van Winden H. 1970. Recipe for Ferric Salts of Ethylenediaminetetraacetic Acid. Plant Physiol. 46(1): 862–863

Teixeira PC, Donagemma, GK, Fontana A, Tiexeira WG. 2017. Manual de Métodos de Analise de Solos [Manual of Soil Analysis Methods]. Brasilia (DF): Empresa Brasileira de Pesquisa Agropecuária. 573 p.

Tolentino TA, Bertoli AC, Dos Santos Pires M, Carvalho R, Labory CRG, Nunes JS, Bastos ARR, De Freitas MP. 2015. Applications in environmental bioinorganic: Nutritional and ultrastructural evaluation and calculus of thermodynamic and structural properties of metal-oxalate complexes. Spectrochim Acta - Part A Mol Biomol Spectrosc. 150:750–757.

Supporting Information

Influence of Complex Stability on Iron Accumulation and Redistribution for Foliar-Applied Iron-Organic Acid Complexes in Maize

Supporting Information description

Sum of electronic and thermal free energies, and molecule volume obtained from DFT calculations, according to Fe-complexes in Table S1; Optimized structure with standard orientation of atoms of iron citrate in Figure S1; Optimized structure with standard orientation of atoms of iron malate in Figure S2; Optimized structure with standard orientation of atoms of iron tartrate in Figure S3; Optimized structure with standard orientation of atoms of iron dioxalate in Figure S4; Optimized structure with standard orientation of atoms of iron EDTA in Figure S5; FTIR spectra of organic acids and their corresponding complexes with iron in Figure S6.

Supplemental Table

Table S1. Sum of electronic and thermal free energies, and molecular volume obtained from DFT calculations, for the Fe-complexes.

Fe-Complex	**Sum of electronic and thermal free energies (Hartree)**	**Molecular volume (cm^3 mol^{-1})**
Fe-EDTA-	-1222.937	189.521
Fe-Citrate	-881.341	130.124
$[Fe\text{-}Tartrate]^+$	-654.001	96.008
$[Fe\text{-}Malate]^+$	-729.177	87.210
$[Fe\text{-}(Oxalate)_2]^-$	-877.590	108.867

Supplemental Figures

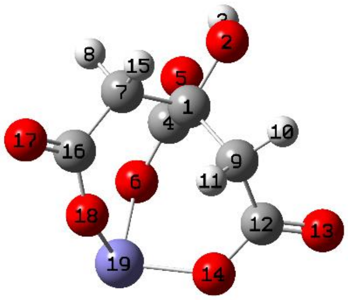

Figure S1. Optimized most stable structure of iron citrate

Standard Orientation:

Center Number	Atomic Number	Atomic Type	Coordinates (Angstroms)		
			X	Y	Z
1	6	0	-0.148207	1.387106	0.260480
2	8	0	-0.564456	2.753393	-0.464484
3	1	0	-0.924491	3.126353	0.368395
4	6	0	-0.49063	1.031774	1.227233
5	8	0	-0.972282	1,884351	1.974231
6	8	0	-0.169249	-0.214677	1.629401
7	6	0	1.397554	1.414680	-0.464402
8	1	0	1.838662	2.057170	0.305320
9	6	0	-0.877486	0.543405	-1.377852
10	1	0	-1.423986	1.282864	-1.962747
11	1	0	-0.131001	0.071043	-2.023218
12	6	0	-1.876317	-0.544566	-0.975800
13	8	0	-3.090019	-0.471904	-1.102650
14	8	0	-1.265939	-1.692827	-0.479563
15	1	0	1.564014	1.940837	-1.410580
16	6	0	2.261355	0.147464	-0.519523
17	8	0	3.466765	0.201013	-0.735366
18	8	0	1.632596	-1.061530	-0.323550
19	26	0	0.19926	-1.674615	0.575515

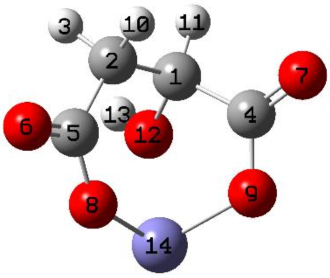

Figure S2. Optimized most stable structure of iron malate

Standard orientation:

Center Number	Atomic Number	Atomic Type	Coordinates (Angstroms) X	 Y	 Z
1	6	0	-0.424760	1.104510	0.920057
2	6	0	1.070887	1.365072	0.653799
3	1	0	1.593767	1.463395	1.611352
4	6	0	-1.289074	1.101757	-0.371952
5	6	0	1.859477	0.331705	-0.170422
6	8	0	2.944718	0.527488	-0.666305
7	8	0	-1.717100	2.100833	-0.905094
8	8	0	1.230360	-0.933443	-0.276901
9	8	0	-1.461570	-0.195281	-0.844751
10	1	0	1.174654	2.328055	0.143765
11	1	0	-0.817315	1.859907	1.602515
12	8	0	-0.621714	-0.247674	1.524183
13	1	0	-0.705883	-0.274568	2.492710
14	26	0	-0.443922	-1.492709	-0.103396

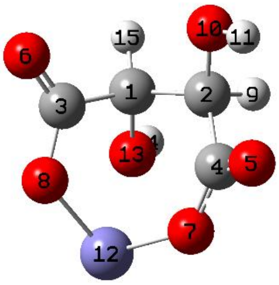

Figure S3. Optimized most stable structure of iron tartrate

Standard orientation:

Center	Atomic	Atomic	Coordinates (Angstroms)		
Number	Number	Type	X	Y	Z
1	6	0	0.450596	-0.959888	0.934171
2	6	0	1.357396	0.295926	0.849838
3	6	0	0.199905	-1.454727	-0.529150
4	6	0	0.855320	1.424303	-0.108921
5	8	0	1.684613	2.028798	-0.780605
6	8	0	1.227452	-1.987723	1.096308
7	8	0	-0.474907	1.618683	-0.120563
8	8	0	-0.930195	-1.219206	-1.061388
9	1	0	1.448516	0.714753	1.860180
10	8	0	2.599658	-0.236027	0.406408
11	1	0	3.062516	0.373170	-0.212991
12	26	0	-1.828683	0.365556	-0.111878
13	8	0	-0.865747	-0.668499	1.465095
14	1	0	-1.034235	-0.985692	2.369012
15	1	0	0.962674	-1.728591	1.515896

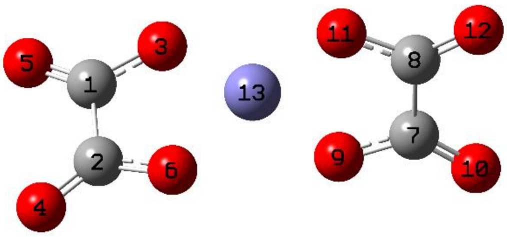

Figure S4. Optimized most stable structure of iron oxalate

Standard orientation:

Center Number	Atomic Number	Atomic Type	Coordinates (Angstroms) X	Y	Z
1	6	0	-2.661741	0.555237	0.563740
2	6	0	-2.661722	-0.555084	-0.563912
3	8	0	-1.397891	0.891768	0.905017
4	8	0	-3.689481	-1.025869	-1.042284
5	8	0	-3.689512	1.025786	1.042318
6	8	0	-1.397864	-0.891797	-0.904967
7	6	0	2.661740	-0.563942	0.555030
8	6	0	2.661723	0.563673	-0.555330
9	8	0	1.397891	-0.904931	0.891853
10	8	0	3.689512	-1.042343	1.025759
11	8	0	1.397864	0.904972	-0.891794
12	8	0	3.689481	1.042497	-1.025658
13	26	0	0.000000	0.000001	0.000034

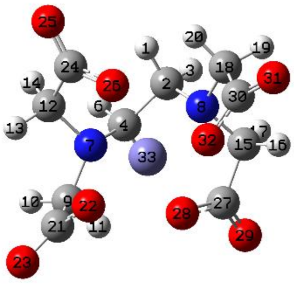

Figure S5. Optimized most stable structure of iron EDTA

Standard orientation:

Center Number	Atomic Number	Atomic Type	Coordinates (Angstroms) X	Y	Z
1	1	0	-0.592713	1.423908	2.172942
2	6	0	-0.698480	0.335559	2.146312
3	1	0	-1.231506	0.031218	3.063506
4	6	0	0.698664	-0.334305	2.146396
5	1	0	0.592823	-1.422638	2.173627
6	1	0	1.231858	-0.029484	3.063326
7	7	0	1.480594	0.027082	0.927695
8	7	0	-1.480502	-0.026612	0.927919
9	6	0	2.487414	-0.982971	0.475287
10	1	0	3.442225	-0.898019	1.011947
11	1	0	2.073516	-1.985406	0.619073
12	6	0	2.047137	1.406952	0.976533
13	1	0	2.934670	1.449446	0.335391
14	1	0	2.354232	1.686894	1.993874
15	6	0	-2.047083	-1.406417	0.977652
16	1	0	-2.935161	-1.449105	0.337270
17	1	0	-2.353414	-1.686049	1.995312
18	6	0	-2.487212	0.983228	0.474746
19	1	0	-3.442057	0.898678	1.011390
20	1	0	-2.073221	1.985724	0.617816
21	6	0	2.737993	-0.821808	-1.051142
22	8	0	1.708297	-0.286140	-1.712471
23	8	0	3.822332	-1.192962	-1.551386
24	6	0	1.055359	2.463243	0.432736
25	8	0	1.169669	3.663860	0.782662
26	8	0	0.148991	1.968130	-0.397165

27	6	0	-1.055840	-2.463045	0.433491
28	8	0	-0.149176	-1.968145	-0.396289
29	8	0	-1.170817	-3.663677	0.783020
30	6	0	-2.737768	0.821159	-1.051655
31	8	0	-3.822095	1.191954	-1.552168
32	8	0	-1.707960	0.285267	-1.712541
33	26	0	0.000118	-0.000166	-0.811393

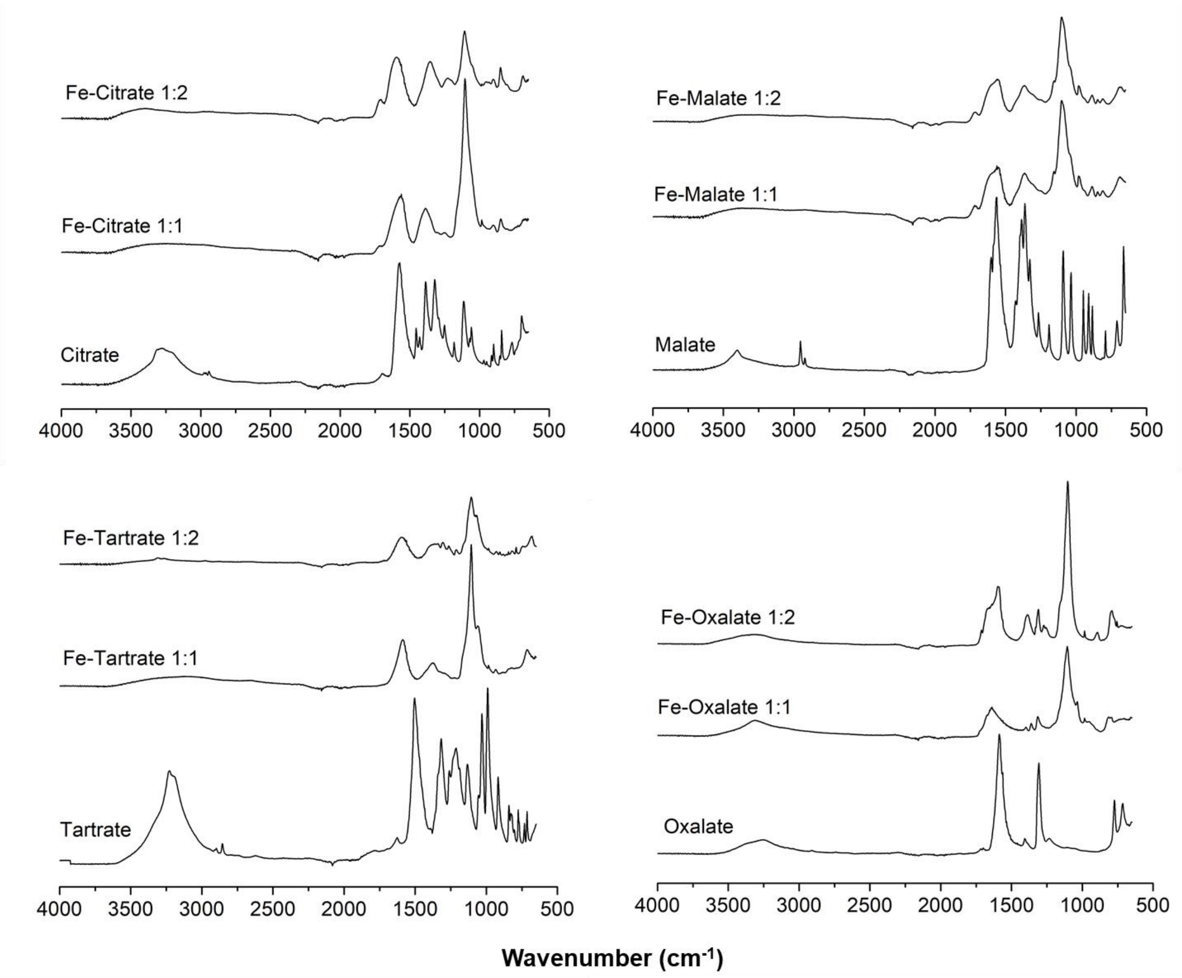

Figure S6. Infrared spectra of organic acids (citrate, malate, tartrate, and oxalate) and their respective Fe-complexes at 1:1 and 1:2 stoichiometric ratios.

ARTICLE 3 - Organic acids as complexing agents for iron and their effects on nutrition and growth of maize and soybean

Marina Justi[a*]; Carlos Alberto Silva[a]; Sara Dantas Rosa[b]

[a] Department of Soil Science, Federal University of Lavras, Av. Sylvio Meniccuci, Lavras, Minas Gerais, Brazil

[b] Faculty of Agronomy and Veterinary Medicine, University of Brasilia, Brasilia, Federal District, Brazil

*corresponding author: marina.justi@gmail.com

This article was prepared in line with the guidelines of the journal ‘Archives of Agronomy and Soil Science’

Abstract

Synthetic chelating agents have high cost and low biodegradation in the environment; thus, the use of natural complexing agents for iron increases the sustainability in crop production. The aim of this study was to verify the efficiency of natural organic acids added to the nutrient solution as complexing agents for iron. For that purpose, two experiments were carried out using soybean and maize. Citric acid (CA), malic acid (MA), tartaric acid (TA), and oxalic acid (OA) were used as complexing agents for iron. Fe-EDTA (ethylenediaminetetraacetic acid) and $FeSO_4$ were also tested. Soluble Fe and its chemical species in a nutrient solution were determined. Organic acids kept less complexed Fe in the nutrient solution than EDTA, but the complexed Fe fraction was not associated with the efficiency of the Fe organic acids complexes in supplying Fe to plants. Maize and soybean treated with Fe-MA and Fe-CA had reduced plant growth. For maize, the TA treatment at both SRs increased Fe accumulation and promoted Fe translocation similar to EDTA. For soybean, Fe-OA at both SRs improved biomass, Fe accumulation, and Fe translocation similar to EDTA. Therefore, the Fe-organic acid complexes were able to assure plentiful plant growth and nutrition, though Fe complexes sources effectiveness are crop-dependent.

Keywords: plant iron acquisition strategies, EDTA, Fe chlorosis, oxalic acid, tartaric acid

1. Introduction

Iron plays a role in several plant metabolic processes, such as physiological redox reactions and chlorophyll biosynthesis (Broadley et al. 2011). Although some plant families can take up and transport the Fe^{3+}, Fe^{2+} is the form used by plant cells in metabolic processes (Broadley et al. 2011). In aerated plant growth media and soils, the concentration of ionic Fe^{2+} in the solution is very low, due to its oxidation and conversion to Fe^{3+}, with subsequent formation of insoluble Fe hydroxides, oxyhydroxides, and oxides (Lemanceau et al. 2009). The solubility of Fe is also highly affected by pH, with insoluble (oxi) hydroxide forms, increasing as pH increases. Thus, complexed Fe forms maintaining Fe in the soil solution and in hydroponic media (Colombo et al. 2014). Additionally, due to the potential oxidative effects of Fe in plant physiological processes, Fe is stored and transported in the plant system in the complexed form (Kobayashi & Nishizawa 2012).

For agronomic applications, the most commonly used chelating agents for Fe are synthetic aminopolycarboxylic acids, such as EDTA (ethylenediaminetetraacetic acid) and EDDHA (ethylendiamine di(*o*-hydroxyphenylacetic) acid) (Abadía et al. 2011). These ligands have high stability when bound to Fe and are effective Fe chelating agents for soil application, fertigation, and hydroponics (Lindsay 1995; Rodríguez-Lucena et al. 2010). However, due to the high cost of aminopolycarboxilic acids, their use is more suitable only for the main cash crops (Fernández & Ebert 2005). Additionally, the low biodegradability and, therefore, high persistence of aminopolycarboxylic acids in the environment (Nowack 2002; Bloem et al. 2017) led to the study of natural, biodegradable products as complexing agents for Fe in order to replace aminopolycarboxylates (Rodríguez-Lucena, et al. 2010).

Organic acids are naturally found in plants and soils and have one or more carboxylic groups (e.g., tricarboxylic citric acid, dicarboxylic malic acid, tartaric, and oxalic acid) (Adeleke et al. 2017). In soil, organic acids are produced during organic matter decomposition and through microbial activity and root exudation (Adeleke et al. 2017). Organic acids increase the solubilization (Gerke 2015), transport (Pegoraro et al. 2005), and uptake of several nutrients and metals (Jones 1998). Therefore, they are appropriate for complexing micronutrients in soils and nutrient solutions (Abadía et al. 2011; Chen et al. 2017). However, organic acids usually have lower stability than aminopolycarboxylic acids when complexed with Fe (Rodríguez-Lucena, et al. 2010).

Nevertheless, organic acids play several roles in plant cell physiology; thus, their effectiveness can be determined by other factors in addition to the chemical stability. Organic acids are modulators of nutrient transport across membranes, acting as signaling messengers for metabolic routes, and they are involved in plant response to abiotic stress as well (Drincovich et al. 2016). The use of oxalic, malic, citric, and succinic acid in foliar sprays improved the growth of plants under stressful levels of toxic Al (An et al. 2014). For these reasons, organic acids need to be tested to evaluate their agronomic efficiency and effects on plant nutrition and growth.

Although some organic acids have been studied as complexing agents for micronutrients in foliar sprays and nutrient solutions (Rodríguez-Lucena et al. 2010), several factors remain to be clarified. The stoichiometry used to mix the micronutrient and complexing agent should be defined since this affects the solubility, stability, and Fe complexed fraction of the complexes formed (García-Mina et al. 2004). Thus, stoichiometry can affect the process of uptake and transport of the Fe-organic ligand in the plant. Complexes of the 1:1 and 1:2 type (metal: organic acid molar ratio) were formed when transition metals and citric and oxalic acids were combined in solution (Bertoli et al. 2015; Tolentino et al. 2015).

Based on the strategies for acquisition of iron, plants are classified as Strategy I and Strategy II. Strategy I plants (dicotyledonous and non-graminaceous monocotyledonous species) reduce the Fe^{3+} to Fe^{2+} through the ferric chelate reductase enzyme before uptake;strategy II plants (monocotyledonous species) release complexing compounds, such as phytosiderophores in the rhizosphere to complex the Fe^{3+}, increasing Fe availability to roots (Kobayashi & Nishizawa 2012). Due to these different mechanisms of Fe uptake, the efficiency of iron sources in nourishing plants of Strategy I and Strategy II plants may be different. The Strategy I plants are more prone to show iron deficiency symptoms under low Fe availability, and a set of studies is directed to the efficiency of Fe fertilizers for Strategy I plants (Rodríguez-Lucena et al. 2011; Bin et al. 2016). Although Strategy II plants are considered efficient in acquiring Fe, and have considerable ability to obtain Fe even in deficiency situations, low Fe nutritional status is also reported for maize and wheat (Zuchi et al. 2012; Li et al. 2014). Thus, Strategy II crops need to be fertilized to increase grain iron concentration and overcome Fe deficiency conditions, though far fewer studies are devoted to study the efficacy of iron sources for Strategy II plants than for Strategy I plants (Hopff et al. 2013).

Therefore, the aim of this study was to verify the efficiency of different organic acids (citric acid, malic acid, tartaric acid, and oxalic acid) as complexing agents for Fe in solution in nourishing contrasting plant species with contrasting strategies of iron acquisition (soybean - Strategy I, and maize, Strategy II). The Fe complexes were prepared at two stoichiometric ratios (1:1 and 1:2 molar ratio), and EDTA was evaluated as the standard chelating agent used to supply iron to plants grown in nutrient solution. Although the relations of chelation capacity and effectiveness of complexing agent in promoting plant nutrition are very well explored for synthetic chelating agents, this relation is still not defined for alternative complexing agents. Thus, in this study we also verified if the complexed iron and soluble iron in solution are determinants of the effectiveness of organic acids in promoting adequate plant growth and iron nutrient status. Contrarily to synthetic chelating agents, organic acids can present play a role in in plant physiological processes. Thus, it is necessary to elucidate the organic acids performance as Fe complexing agents through experiments with different plant species. The hypotheses of this study were i) organic acids are less effective than EDTA in complexing iron; ii) even though they are less effective than EDTA in complexing Fe, organic acids may promote suitable plant growth and Fe nutrition due to their physiological activity on the plant process; and iii) the effectiveness of Fe-organic acid complexes varies according to the iron acquisition strategy of the crop. Contrary to what occurs with synthetic chelating agents, we expected that the Fe complexation capacity of organic acids will not determine their effectiveness in promoting plant growth and iron acquisition. We also expected that the best Fe-organic acid source for maize will be different from that suitable for soybean.

2. Material and Methods

2.1. Preparation and characterization of Fe-complexes solution

The low molecular weight organic acids used as Fe complexing agents in the experiments were tricarboxylic citric acid (CA, $pKa_1 = 3.13$, $pKa_2 = 4.76$, $pKa_3 = 6.40$), dicarboxylic DL-malic acid (MA, $pKa_1 = 3.46$; $pKa_2 = 5.10$), dicarboxylic tartaric acid (TA, $pKa_1 = 3.04$; $pKa_2 = 4.37$), and dicarboxylic oxalic acid (OA, $pKa_1 = 1.23$; $pKa_2 = 4.19$). EDTA (chelated Fe source) and $FeSO_4$ (Fe free source) were used as controls. The 1:1 and 1:2 (metal: ligand molar ratio) stoichiometric ratio (SR) were chosen for organic acid reactions with Fe because previous studies showed that 1:1 and 1:2 complexes were the most stable for transition metals and organic ligands such as citric and oxalic acids (Bertoli et al. 2015; Tolentino et al. 2015). All Fe complexes were prepared as stock solutions with a concentration of 5 g L^{-1} Fe (or 0.089 mol L^{-1} Fe), as suggested by Hoagland et al. (1950). The complexes were prepared by adding an adequate amount of $FeSO_4$ to the solution with 100 mL of a 0.089 mol L^{-1} (1:1 molar ratio) or 100 mL of a 0.178 mol L^{-1} (1:2 molar ratio) citric/malic/tartaric/oxalic acid solution. The pH of solutions was adjusted to 5.5, and the solutions were left to stand overnight under aeration. The solutions were centrifuged to separate the precipitates, and the final concentration of soluble Fe in the supernatant was measured in an ICP-OES (inductively coupled plasma optical emission spectrometry) (Spectro, Blue, Germany). The salts used in the formulation of the Fe solutions were analytical grade reagents.

2.2 Greenhouse experiment and treatments

Two nutrient solution experiments were conducted to evaluate the effectiveness of Fe complexed sources on the maize cv Pioneer 30F53 (Experiment I) and the soybean cv CD350 (Experiment II) growth. A randomized block design was used for both experiments, with four replications in Experiment I (maize) and three replications in Experiment II (soybean). The treatments tested are as follows: Fe-citrate (Fe-CA), Fe-tartrate (Fe-TA), Fe malate (Fe-MA), and Fe-oxalate (Fe-OA), all of them prepared and applied at both the 1:1 and 1:2 SR. Fe-EDTA and FeSO4 were also tested, as a reference for a chelated Fe and salt Fe source, respectively. All Fe sources were tested in Experiment II, but the Fe-oxalate source was not used in Experiment I. Maize and soybean seedlings were germinated in a tray filled with vermiculite as a substrate. Ten days after sowing, seedlings were transferred to the nutrient solution at 75% strength with no addition of Fe. Following a three-day adaptation phase, the seedlings were placed in a full-strength nutrient solution. The full-strength solution had the following nutrient concentrations: 14, 196, 31, 235, 160, 49, 68, 5, 0.8, 1, 0.2, 0.5 and 0.01 mg L^{-1} of N-NH_4^+, N-NO_3^-, P, K, Ca, Mg, S, Fe, B, Mn, Zn, Cu, and Mo (Hoagland and Arnon, 1950). The salts used for formulation of the nutrient solution were analytical grade reagents. In both experiments, the nutrient solution was added to supply 5 mg L^{-1} Fe. Thus, the volume of Fe-complexing solution for each treatment used was calculated according to the soluble Fe concentration of the stock solutions. The treatments were added when the seedlings were placed in full-strength nutrient solution. After application of the treatments, an aliquot of the nutrient solution of each experimental unit was taken to determine the concentration of soluble Fe through ICP-OES (Spectro, Blue, Germany). The nutrient solution was replaced every seven days. At each nutrient solution replacement, a new application of all the Fe treatments were performed. Plants were cultivated for 24 days in both the experiments. Therefore, three nutrient solutions replacement and reapplications of Fe complexes treatments were performed during the whole experiment.

2.3 Chemical speciation in the solutions

To estimate the proportion of Fe complexed fraction by the organic acids in the stock solutions and the main chemical species of Fe formed when the treatments were applied to nutrient solution, the Visual Minteq® version 3.1 software was used. pH was fixed according to the levels set for stock (5.5) and nutrient solution (5.8-6.0), ionic strength was calculated by the software itself, and the temperature was set to 25ºC. The CO_2 partial pressure and its dissolution in the solutions were not considered.

2.4 Soil and plant analysis

SPAD (Soil Plant Analysis Development) readings (Minolta SPAD-502, Osaka, Japan) were performed on day 21 of Experiment I (maize) and II (soybean) through analysis of the last expanded leaf of maize and the last fully developed leaf of soybean. Maize and soybean plants were harvested, and separated into shoot and root parts. Shoot and root dry matter (DM) was determined after drying plant tissues at 60ºC for 72 hours. Micronutrient content in shoot and root were determined by atomic absorption spectrometry after plant tissue digestion in a HNO_3^- $HClO_4$ solution at a 4:1 ratio (Teixeira et al., 2017). The total N content was determined by digestion of plant tissues in an acid medium, followed by distillation of the digested plant material through the Kjeldahl method (Teixeira et al., 2017). N (g kg-1), Fe, Cu, Mn, and Zn (mg kg-1) and dry matter (g) of the respective plant tissues were used to determine the accumulation of nutrients in shoot and root (mg plant^{-1}).

2.5. Data analysis

The main effects of treatments on plant attributes were identified by the analysis of variance (ANOVA). Treatments mean differences were tested using the least significant difference (LSD) at $p \leq 0.05$. A principal component analysis (PCA) was performed to verify the patterns and clusters of dataset, and to identify the main associations among plant attributes and treatments. All the statistical analyses were performed using the R software version 4.0.

3. Results

3.1. Chemical speciation and solubility of iron according to the Fe sources

Complexing agents had different capacities for maintaining the iron complexed in the stock and nutrient solutions. The amounts of complexed Fe, free Fe, and free complexing agent represented as percentage of the total Fe/organic acid concentration in the stock solution are shown in Table 1. The highest complexed Fe fraction is for the EDTA (99.9%), CA at both the 1:1 and 1:2 SR (99.6 and 99.2%), and OA at the 1:2 SR (99.4%). Only OA had the 1:2 type complex ($Fe[Oxalate]_2^{-2}$), according to the Minteq outputs. All the other complexing agents had only the 1:1 type complex ($Fe[EDTA]^-$, FeCitrate, $Fe[Tartrate]^+$, $Fe[Malate]^+$), even in the 1:2 solutions. Therefore, a greater amount of the free complexing agent in the solution and a higher proportion of the Fe complexed fraction was observed in the 1:2 SR solutions than in the 1:1 SR solutions.

Table 1. Soluble and complexed iron in the stock solutions as related to the complexing agent and stoichiometric molar ratio used.

Complexing Agent	Stoichiometry	Complexed Fe	Soluble Fe
	Fe: ligand molar ratio	% of the total Fe concentration	
EDTA	1:1	99.9	100
Citrate	1:1	99.6	99.5
	1:2	99.2	100
Malic	1:1	77.7	99.8
	1:2	77.1	100
Tartaric	1:1	49.5	95.2
	1:2	50.6	87.5
Oxalic	1:1	55.9	14.5
	1:2	99.3	10.2
$FeSO_4$	-	0.0	95.2

The amount of complexed Fe and the proportions of other cations complexed with the organic acids in the nutrient solution are shown in Table 2. In general, only EDTA was able to maintain most of the Fe in complexed form (98.6% of the total Fe) in the nutrient solution. Among the organic acids, CA in the 1:2 and 1:1 SR treatments had the highest proportions of Fe complexed (70.2% and 48.1% of the total Fe, respectively; Table 2). OA at the 1:2 SR kept 30.1% of the total iron in the complexed form, and OA at the 1:1 SR kept 10.8% (Table 2). TA and MA at both SRs tested had less than 2% of the iron in the complexed form (Table 2). Thus, most of the Fe in the solution was free to react with other chemical species, such as HPO_4^{-2} and $H_2PO_4^-$, and also to form oxi(hydroxy)des. A high fraction of organic acids in the nutrient solution formed complexes with Ca and Mg (in general, >40% of the total complexing agent was complexed with Ca and Mg; Table 2). For TA and MA, more than 50% of the total amount added in the solution remains in free form (Table 2). Compared to Fe, the affinity of Mn, Cu, and Zn with complexing agents is low, considering that less than 1% of organic acids were bound to these micronutrients (Table 2).

The concentrations of iron in the nutrient solutions of the maize and soybean experiments are shown in Table 3. In accordance with the complexed Fe fraction (Table 2), the addition of Fe-EDTA in the nutrient solution results in the highest soluble Fe concentration, followed by Fe-CA at the 1:2 SR (Table 3). All the other Fe sources only promoted levels near 1.0 mg L^{-1} Fe in the nutrient solution (Table 2).

Table 2. Complexed Fe, soluble Fe, and complexing agent fractions in the nutrient solution, as related to the complexing agent and stoichiometric molar ratio (SR) used.

Complexing Agent	SR	Fe complexed	Free Complexing Agent	Complexing Agent binds to Fe	Complexing Agent binds to Mg and Ca	Complexing Agent binds to Mn, Cu, and Zn
	Fe: ligand molar ratio	% of total iron	% of total complexing agent concentration			
EDTA	1:1	99.6	0	99.6	0	0.4
Citrate	1:1	48.1	1.7	48.1	49.6	0.6
	1:2	70.2	2.1	35.0	62.3	0.5
Malate	1:1	0.7	58.0	0.3	40.3	0.4
	1:2	1.4	57.9	0.3	40.2	0.3
Tartrate	1:1	0.6	55.1	0.1	42.3	0.5
	1:2	1.2	54.8	0.1	42.1	0.5
Oxalate	1:1	10.3	10.8	25.0	58.2	1.0
	1:2	30.0	13.1	37.0	48.9	0.7

The concentrations of iron in the nutrient solution of maize and soybean experiment, are presented in Table 3. In line with complexed Fe fraction (Table 2), addition of Fe-EDTA in nutrient solution results in the highest soluble Fe concentration, followed by Fe-CA at 1:2 SR (Table 3). All the other Fe sources only promoted levels next to 1.0 mg L^{-1} Fe in the nutrient solution (Table 2).

Table 3. Soluble iron concentrations determined in the nutrient solution of soybean and maize experiments, as related to the complexing agent and stoichiometric molar ratio (SR) used.

Complexing Agent	SR	Maize	Soybean
	Fe: ligand molar ratio	mg L^{-1} Fe	
EDTA	1: 1	5.70	5.95
Citrate	1: 1	1.30	1.18
	1: 2	3.68	4.10
Malate	1: 1	0.65	0.59
	1: 2	1.01	1.10
Tartrate	1: 1	1.15	1.22
	1: 2	1.21	1.01
Oxalate	1: 1	-	0.95
	1: 2	-	1.19
$FeSO_4$	-	1.00	0.89

3.2. Experiment I: Maize growth and nutrition as affected by Fe sources

The effect of Fe sources added to the nutrient solution on maize shoot and root dry matter, Fe content in the shoots and roots, and the Fe root:shoot ratio values are shown in Figure 1. Plants treated with Fe-EDTA produced the greatest shoot dry matter (5.04 g plant^{-1}; Fig. 1A), though plants supplied with $FeSO_4$ and Fe-TA at the 1:2 SR produced shoot dry matter statistically similar to plants supplied with Fe-EDTA (4.78 and 4.43 g plant^{-1}, respectively; Fig. 1A). Furthermore, Fe-MA at the 1:1 SR (4.22 g plant^{-1}) and Fe-TA at the 1:1 SR (4.22 g plant^{-1}) led to dry matter similar to $FeSO_4$, but lower than Fe-EDTA. Excluding the treatment with no Fe added, plants exposed to Fe-MA at the 1:2 SR and Fe-CA at the 1:2 SR had the lowest shoot dry matter production (2.91 and 2.58 g plant^{-1}, respectively; Fig. 1A). For Fe-MA and Fe-CA, the 1:1 SR led to greater shoot dry matter production than the 1:2 SR (Fig. 1A). Regarding root dry matter, plants supplied with Fe-EDTA also produced the greatest dry matter (1.24 g plant^{-1}; Fig. 1B). Excluding the treatment with no Fe supplied, Fe-MA at the 1:1 SR and Fe-CA at the 1:2 SR led to the lowest root dry matter (0.77 and 0.67 g plant^{-1}; Fig. 1B).

Iron accumulation in shoots and roots, the Fe root: shoot ratio, and the SPAD index are also shown in Fig. 1. The greatest shoot Fe accumulation resulted from the TA at the 1:2 SR (0.90 mg plant^{-1}), followed by the TA at the 1:1 SR (0.73 mg plant^{-1}; Fig. 1C). Iron accumulation of plants supplied with Fe-TA at the 1:2 SR was almost two times the iron accumulation of plants supplied with Fe-EDTA (0.51 mg plant^{-1}). Plants supplied with Fe-EDTA, $FeSO_4$, and the other Fe-organic acid combinations had statistically similar shoot iron accumulations (Fig. 1C). Conversely, the highest accumulation of Fe in the roots was for plants supplied with $FeSO_4$ (5.33 mg plant^{-1}), followed by the TA at the 1:1 SR (4.66 mg plant^{-1}) and TA at the 1:2 SR (3.88 mg plant^{-1}) (Fig. 1D). Excluding the treatment with no Fe added, EDTA led to the lowest Fe content in the roots (2.49 mg plant^{-1}; Fig. 1D). To determine the effect of Fe sources on translocation of Fe from roots to shoots, the Fe root: shoot ratio was determined. The no added Fe and $FeSO_4$ treatments had the highest ratios (14.89 and 14.14, respectively), and EDTA (4.76) and TA at the 1:2 SR (4.78) had the lowest ratios (Fig. 1E). No differences were observed for the SPAD index among the treatments (Fig. 1F). The lowest SPAD index was found, as expected, for the plants with no Fe supplied (Fig. 1F).

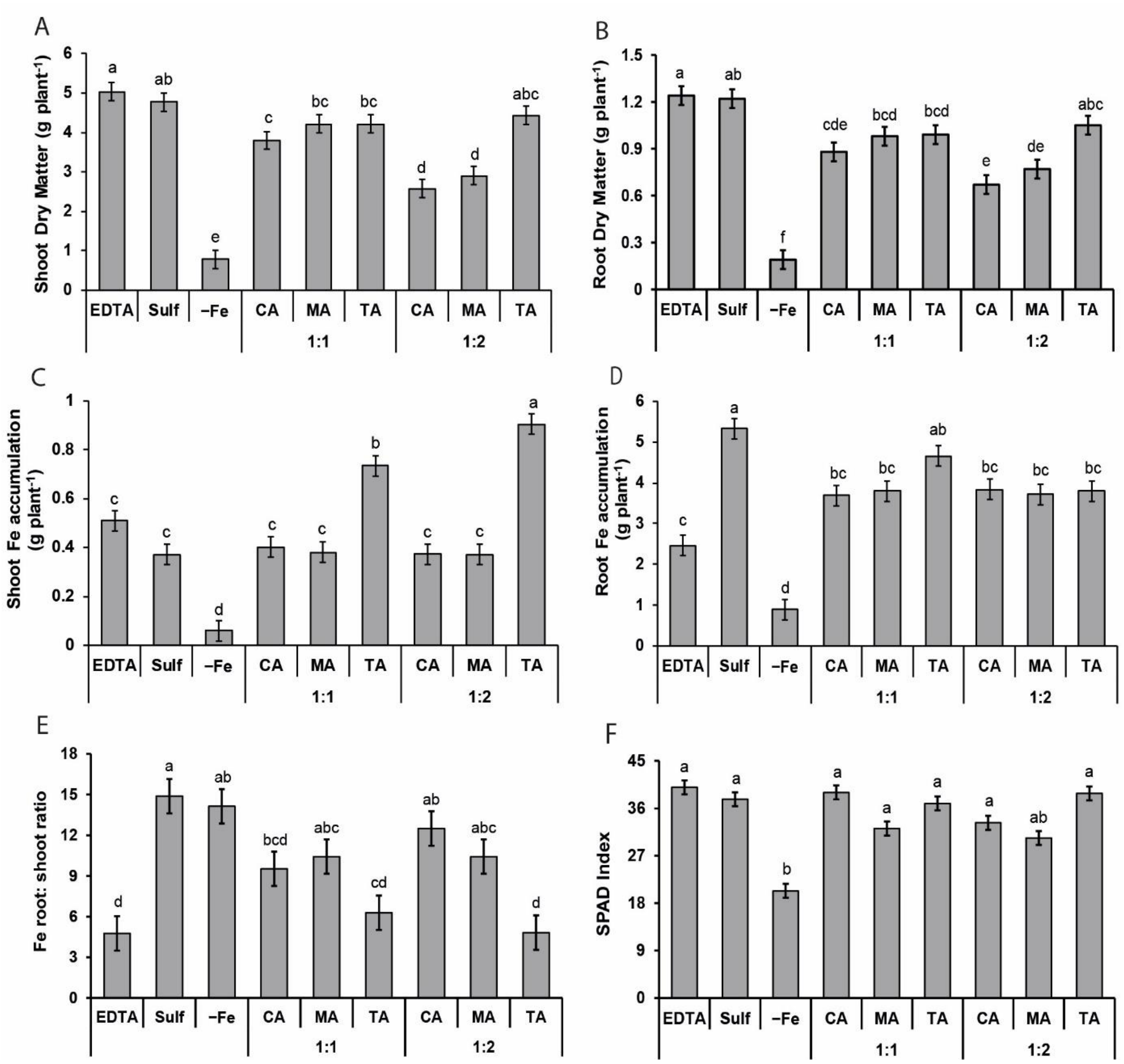

Figure 1. Shoot (A), and root (B) dry matter, shoot (C), and root (D) Fe accumulation, Fe accumulation root: shoot ratio (E), and SPAD index (F) of maize as related to stoichiometry ratio (1:1 or 1:2 molar ratio) and Fe complexing agents: EDTA, Sulf ($FeSO_4$), CA (citric acid), MA (malic acid), and TA (tartaric acid). Error bars represent the mean standard error. Letters refer to mean comparison, and the same letters indicate no difference according to the LSD test at $p<0.05$.

The accumulation of Mn, Zn, and Cu for maize, as well as the Fe/Mn, Fe/Zn, and Fe/Cu ratios, are presented in Table 4. Mn, Zn, and Cu accumulation in fact declined in the absence of iron in the solution. In addition, the greatest Fe accumulation brought about through use of TA at the 1:2 SR in the nutrient solution leads to decreased Mn accumulation (Table 4). The Fe/Mn ratio for TA at the 1:2 SR was the highest among the treatments. All Fe-organic acid sources led to Zn accumulation statistically similar to the EDTA, and only the negative control had a lower Zn accumulation (Table 4). Therefore, the variation in the Fe/Zn ratio is mainly due to variation in Fe accumulation. CA at the 1:2 SR and the no Fe added treatment led to the lowest Cu accumulation in maize plants (Table 4).

Table 4. Accumulation of Mn, Zn, and Cu by the maize plants as related to the Fe complexing agent and stoichiometric molar ratio used.

Fe source	Stoichiometry	Mn	Zn	Cu	Fe/Mn	Fe/Zn	Fe/Cu
	Fe: organic ligand molar ratio		mg plant^{-1}			-	
Fe-EDTA	1:1	0.50 ^{bc}	0.23 ^{abc}	0.038 ^{ab}	1.01 ^{bc}	2.24 ^{bc}	13.39 ^{bc}
$FeSO_4$	-	0.48 ^{bc}	0.21 ^{bc}	0.037 ^{ab}	0.79 ^{c}	1.80 ^{c}	10.02 ^{cd}
−Fe	-	0.15 ^{d}	0.15 ^{d}	0.010 ^{c}	0.41 ^{c}	0.42 ^{d}	6.07 ^{d}
Fe-Citrate	1:1	0.55 ^{ab}	0.26 ^{ab}	0.034 ^{ab}	0.73 ^{c}	1.52 ^{c}	11.98 ^{cd}
	1:2	0.62 ^{a}	0.27 ^{a}	0.026 ^{bc}	0.59 ^{c}	1.36 ^{cd}	14.19 ^{bc}
Fe-Malate	1:1	0.53 ^{abc}	0.23 ^{abc}	0.040 ^{ab}	0.75 ^{c}	1.66 ^{c}	9.41 ^{cd}
	1:2	0.51 ^{abc}	0.24 ^{abc}	0.047 ^{a}	0.71 ^{c}	1.53 ^{c}	10.18 ^{cd}
Fe-Tartrate	1:1	0.51 ^{abc}	0.26 ^{ab}	0.038 ^{ab}	1.53 ^{b}	2.95 ^{b}	19.41 ^{b}
	1:2	0.43 ^{c}	0.22 ^{bc}	0.032 ^{ab}	2.31 ^{a}	4.24 ^{a}	29,11 ^{a}

* Letters were used to compare means, and the same letter indicates no difference according to the LSD test at $p<0.05$.

3.3. Experiment II: Soybean growth and nutrition as affected by Fe sources

The effect of Fe sources added to the nutrient solution on soybean shoot and root dry matter is shown in Figure 3. Fe-OA at the 1:1 and 1:2 SR led to shoot dry matter production greater than all other organic acids, and similar to Fe-EDTA (Fig. 2A). TA at the 1:1 SR and CA at the 1:2 SR led to dry matter production (3.15 and 2.93 g plant^{-1}, respectively) lower than Fe-EDTA (4.03 g plant^{-1}; Fig. 2A). Fe-MA at both SRs, Fe-CA at the 1:1 SR, and Fe-TA at the 1:2 SR led to dry matter accumulation statistically similar to Fe-EDTA and $FeSO_4$. Shoot dry matter production of MA, TA, and CA at the 1:1 SR was similar to their respective treatments at the 1:2 SR. Root dry matter production mirrored the patterns observed for shoot dry matter (Fig. 2B).

Iron accumulation in shoots and roots, the Fe root: shoot ratio, and the SPAD index are also presented in Fig. 2. The Fe-OA led to the greatest Fe accumulation in the shoots at the 1:1 SR (0.508 mg plant^{-1}), which was statistically similar to Fe-EDTA (0.440 mg plant^{-1}) and Fe-OA at the 1:2 SR (0.403 mg plant^{-1}) (Fig. 2C). The other organic acids tested (CA, TA, and MA), resulted in shoot Fe accumulation statistically lower than Fe-EDTA (Fig. 2C). Conversely, considering root Fe accumulation, $FeSO_4$ (4.24 mg plant^{-1}) and Fe-OA at the 1:1

SR (4.15 mg plant^{-1}) led to the greatest accumulation (Fig. 2D). All Fe-organic acid treatments had root Fe accumulation statistically similar to Fe-EDTA (Fig. 2D).

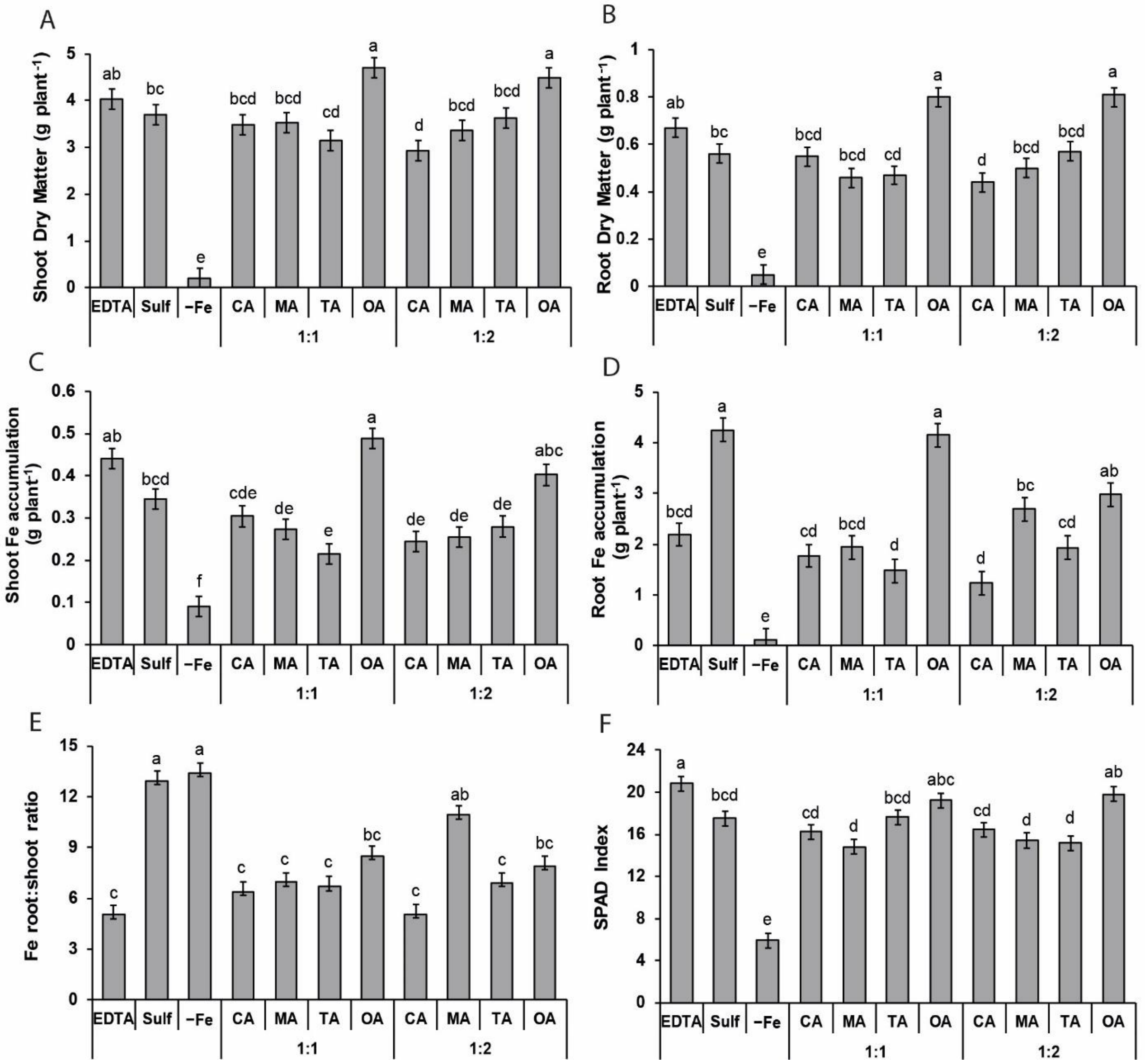

Figure 2. Shoot (A) and root (B) dry matter, shoot (C) and root (D) Fe accumulation, Fe accumulation root: shoot ratio (E) and SPAD index (F) of soybean as related to the the stoichiometry ratio (1:1 or 1:2 molar ratio) and Fe complexes agents in nutrient solution: EDTA, Sulf ($FeSO_4$), CA (citric acid), MA (malic acid), TA (tartaric acid) and OA (oxalic acid). Error bars represent the mean standard error. Letters refer to mean comparison, and the same letters indicate no difference according to the LSD test at $p<0.05$.

Similar to the results of Experiment I, the negative control and $FeSO_4$ had the highest Fe root: shoot ratio (14.66 and 12.95, respectively; Fig 2E). All the organic acids tested had ratios statistically similar to Fe-EDTA (5.03), except Fe-MA at the 1:2 SR (Fig. 2E). Regarding the Fe-MA treatments, the 1:2 SR led to a greater ratio than the 1:1 SR (Fig. 2E). For Fe-TA, Fe-CA, and Fe-OA, similar Fe root: shoot ratios were observed for both the 1:1 and 1:2 SR of each of these treatments (Fig. 2E). The SPAD index of the soybean plants is presented in Fig. 2F. Plants supplied with EDTA showed the highest SPAD index (20.8), and only OA at the 1:2 SR (19.8) and OA at the 1:1 SR (19.3) had statistically similar results (Fig. 2F). All the other organic acid treatments led to a plant SPAD index similar to FeSO$_4$ and lower than Fe-EDTA (Fig. 2F).

The accumulation of Mn, Zn, and Cu for soybean, as well as the Fe/Mn, Fe/Zn, and Fe/Cu ratios are shown in Table 5. Manganese accumulation is strongly affected by the complexing agents used ($p < 0.001$). TA at the 1:2 SR and CA at the 1:1 SR led to the greatest Mn accumulation in soybean plants. The treatment that led to the greatest Fe accumulation for soybean (Fe-OA at the 1:2 SR) had a lower Mn accumulation than the other Fe-organic acid treatments (Table 5). However, Mn accumulation in soybean treated with Fe-OA at the 1:2 SR was similar to Mn accumulation in the Fe-EDTA and FeSO$_4$ treatments. Copper and Zn accumulation also were affected by the Fe sources ($p < 0.0001$). Excluding the negative control, Fe-EDTA, Fe-TA at the 1:1 SR, and Fe-OA at the 1:2 SR led to the lowest Cu accumulation; and Fe-MA at the 1:2 SR, the greatest Cu accumulation (Table 5). Plants treated with Fe-EDTA and Fe-OA at the 1:2 SR had the greatest Fe/Cu ratio. Fe-MA at the 1:2 SR led to the greatest Zn accumulation; and the negative control, the lowest Zn accumulation. Conversely, the greatest Fe/Zn ratio was found for plants treated with Fe-OA at the 1:2 SR (Table 5).

Table 5. Accumulation of Mn, Zn and Cu by the soybean plants according to the Fe complexing agent and stoichiometric ratio used.

Fe source	**Stoichiometry**	**Mn**	**Zn**	**Cu**	**Fe/Mn**	**Fe/Zn**	**Fe/Cu**
	Fe: organic ligand molar ratio	mg plant^{-1}			-		
Fe-EDTA	1:1	0.35 ^{c}	0.17 ^{bcd}	0.020 ^{d}	1.24 ^{a}	2.54 ^{ab}	25.50 ^{a}
FeSO$_4$	-	0.36 ^{c}	0.16 ^{bcde}	0.020 ^{cd}	0.99 ^{b}	2.06 ^{bc}	16.45 ^{b}
−Fe	-	0.04 ^{d}	0.02 ^{f}	0.002 ^{e}	0.22 ^{d}	0.44 ^{e}	4.13 ^{d}
Fe-Citrate	1:1	0.59 ^{a}	0.19 ^{ab}	0.023 ^{bcd}	0.50 ^{c}	1.60 ^{cd}	12.82 ^{bc}
	1:2	0.44 ^{bc}	0.16 ^{cde}	0.021 ^{cd}	0.56 ^{c}	1.58 ^{cd}	12.62 ^{bc}
Fe-Malate	1:1	0.54 ^{ab}	0.21 ^{a}	0.023 ^{bcd}	0.50 ^{c}	1.32 ^{d}	11.86 ^{c}
	1:2	0.54 ^{ab}	0.19 ^{ab}	0.030 ^{a}	0.46 ^{c}	1.34 ^{d}	8.75 ^{c}
Fe-Tartrate	1:1	0.38 ^{c}	0.15 ^{de}	0.019 ^{d}	0.55 ^{c}	1.43 ^{d}	11.22 ^{c}
	1:2	0.64 ^{a}	0.18 ^{abc}	0.027 ^{abc}	0.44 ^{c}	1.54 ^{cd}	10.49 ^{c}
Fe-Oxalate	1:1	0.44 ^{bc}	0.19 ^{ab}	0.028 ^{ab}	1.10 ^{ab}	2.55 ^{ab}	17.11 ^{b}
	1:2	0.33 ^{c}	0.14 ^{de}	0.018 ^{d}	1.21 ^{ab}	2.89 ^{a}	21.88 ^{a}

* Letters were used to compare means, and the same letter indicates no difference according to the LSD test at $p<0.05$.

In order to visualize the relationships among the variables and summarize the results of the experiments, a PCA for Experiment I (maize) and Experiment II (soybean) was performed (Fig. 3). For the maize experiment (Fig. 3A), the PCA biplot explained 65.92% of the variance. DM and N accumulation are not related to the Fe shoot accumulation and Fe/micronutrient ratios. Fe shoot accumulation was associated with the free complexing agent percentage. Soluble and complexed Fe had little effect on the PC2 variation and even less on the PC1 variation. Soluble and complexed Fe were likewise not correlated with DM production and the SPAD index. The treatments were in three main clusters in the biplot: TA at both SRs, associated with the greatest Fe accumulation, Fe/micronutrient ratios, and free complexing agent fraction; EDTA and sulfate, associated with the DM, N accumulations, and SPAD index; and the other treatments (MA and CA at both SRs), associated with Mn and Zn accumulation and the highest Fe root to shoot ratios.

For the soybean experiment, the PCA biplot explained 76.06% of the variance (Fig. 3B). Contrary to the maize, shoot Fe accumulation was associated with DM production and the SPAD index. Soluble and complexed Fe fractions were not related to DM and Fe accumulation. The free complexed fraction and Mn, Zn, and Cu accumulation were negatively associated with the Fe/micronutrient ratios and Fe shoot accumulation. The treatments separated into two main groups: OA at both SRs and EDTA, associated with the SPAD index, DM, N, and shoot Fe accumulation; and the other organic acids treatments (MA, TA, and CA at both SRs), associated with Mn, Cu, and Zn accumulation and the free complexing agent fraction.

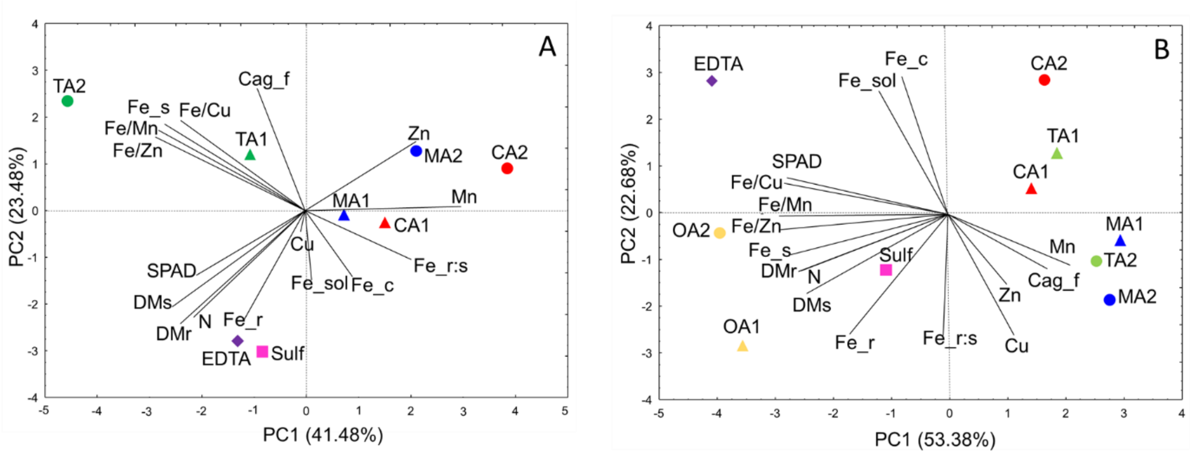

Figure 3. Principal component analysis with variables observed in the Experiment I (maize; Fig. 3A) and Experiment II (soybean; Fig. 3B): shoot dry matter (DMs); root dry matter (DMr); SPAD index (SPAD); Fe accumulated in shoot (Fe_s); Fe accumulated in root (Fe_r); root Fe to shoot Fe ratio (Fe_r:s); Mn accumulated in shoot (Mn); Cu accumulated in shoot (Cu); Zn accumulated in shoot (Zn); shoot Fe to shoot Cu ratio (Fe/Cu); shoot Fe to shoot Mn ratio (Fe/Mn); shoot Fe to shoot Zn ratio

(Fe/Zn); Fe complexed fraction (Fe_c); Fe soluble fraction (Fe_s); Complexing agent free fraction (Cag_f). EDTA, Sulf, CA, MA, TA and OA representing the complexing agents (EDTA, sulfate, citric acid, malic acid, tartaric acid, oxalic acid, respectively). The numbers 1 and 2 next to organic acids simbols refer to 1:1 and 1:2 stoichiometric ratios, respectively.

4. Discussion

4.1 Chemical properties of Fe as related to complexing agents

The organic acids had varied ability in maintaining iron in the complexed form (Table 1 and Table 2). Although high amounts of Fe in complexed form were found in the stock solutions of Fe-organic acids (in general >70%; Table 1), the Fe complexed fraction in the nutrient solution is low (in general < 10%, except for CA at the 1:1 and 1:2 SR, and OA at the 1:2 SR; Table 2). In the nutrient solution, there are several other cations competing with iron for the ligands. A considerable fraction of organic acids was complexed with Ca and Mg (Table 2). Thus, for the organic acids tested, the stability of the Ca and Mg complexes is higher than that of the Fe complexes (Ström et al. 2005). Furthermore, Ca and Mg are present at much higher concentrations than iron, displacing the reaction equilibria towards the formation of Ca and Mg complexes (Ström et al. 2005; Tan et al. 2011). Only EDTA was able to maintain most iron in complexed form (Table 2). EDTA is recognized for high stability of chelates formed with Fe(III), due to the six functional groups able to bind to iron (four carboxylic groups and two amide groups). The stability constants of Ca-EDTA and Mg-EDTA are lower than those of Fe-EDTA as well (Lindsay 1995).

The organic acids also had varied results regarding the solubility of their Fe complexes. Stock solutions with high nutrient concentrations are generally required for preparation of nutrient solutions on a large scale; thus, Fe soluble in concentrated solutions (5 g L^{-1}) was verified. OA showed formation of insoluble complexes, maintaining only 14.5% and 10.2% of iron in the soluble form for the 1:1 and 1:2 SR, respectively (Table 1). Oxalate salts with transition metals are characterized by their low solubility (Baran, 2014), which is a constraint for agronomic use of OA complexes. Complexes formed with tartrate are not completely soluble, but even with some precipitation, most Fe remained in the soluble form in the Fe-TA solutions (>85%; Table 1).

None of the organic acid solutions exhibited 1:2 complexes ($Fe[ligand]_2$), except for Fe-OA solutions. One possible explanation for the absence of 1:2 complexes is the steric hindrances between the ligands of higher molecular weight around the iron (Bertoli et al. 2015). OA had the lowest molecular weight among the organic acids, and this smaller size allows two molecules to bind with iron (Tolentino et al. 2015). Since the 1:2 complex does not form for CA, TA, and MA, in the 1:2 SR solutions, the higher content of complexing agents displaces the equilibria towards the formation of complexes, so a higher complexed Fe fraction is found at this SR than at the 1:1 SR (Table 2). Additionally, a higher amount of organic acid in free form (non-reacted) is found at the 1:2 SR than at the 1:1 SR (Table 2).

4.2 Agronomic efficiency of complexing agents

The organic acids exhibited different efficiency in Fe uptake and plant growth according to the species. Physiological, morphological, and biochemical differences among species are determinant factors affecting the efficacy of complexing or chelating agents. Even for the synthetic chelates such as EDTA and DTPA (diethylenetriaminepentaacetic acid), different results of metal absorption efficiency were reported when different species were tested, such as sunflower (dicot) and maize (monocot) (Shahid et al. 2013; Hasan et al. 2019). Differences regarding the concentration of transporters in the plasma membrane, translocation efficiency, transpiration rates, Casparian strip morphology, and degree of endodermis suberization may affect the efficiency of the complexed source on metal uptake by different species (Wang & Zhong 2011; Fu et al. 2015).

Regarding Fe nutrient status, maize and soybean have different mechanisms for acquiring Fe. Soybean, a dicot Strategy I plant species, reduces ferric ions and ferric complexes to the ferrous form at the root surface before uptake, through the action of the ferric chelate reductase enzyme and release of acidic compounds in the rhizosphere (Kobayashi & Nishizawa 2012). Maize, a monocot strategy II plant species, releases phytosiderophores that produce soluble complexes with the ferric ion, increasing the availability of iron in the rhizosphere. The ferric complex is taken up by the roots without Fe reduction (Kobayashi & Nishizawa 2012). Strategy I plants are considered less tolerant to Fe deficiency conditions than Strategy II plants (Marschner & Römheld 1994), which is in agreement with the results observed in this study. For maize, all the Fe-organic acid treatments and $FeSO_4$ lead to similar or higher iron accumulations than EDTA (Fig. 1), in contrast with the soybean experiment, in which all the treatments, except OA, led to lower Fe accumulation than EDTA. Maize roots can release phytosiderophores, which probably increased iron availability in the growth media (Marschner & Römheld 1994), even in treatments that have no Fe complexed or have a low complexed Fe fraction. Therefore, phytosiderophores release is a possibility that can explain the higher efficiency of maize in Fe accumulation.

The iron root:shoot ratio (Fig. 2E) is an index to estimate the amount of iron translocated from roots to shoots. Organic acid treatments, in general, bring about iron translocation from roots to shoots superior to an uncomplexed Fe source ($FeSO_4$) in soybean and maize experiments, showing the importance of complexing molecules for iron transport in a plant system (Kobayashi & Nishizawa 2012). Although organic acids (CA, MA, and TA) reduced soybean dry matter and Fe accumulation, the proportion of iron translocated to the shoots was not altered in comparison with EDTA (Fig. 2E). In maize, on the contrary, only TA and CA at the 1:1 SR led to Fe translocation statistically similar to EDTA (Fig. 1E).

Some complexing agents brought about Fe accumulation so high that it lowered the uptake of other elements, such as Mn, as found for Fe-TA in the 1:2 SR application in the maize experiment. This negative association between Fe and other micronutrients is shown by the PCA (Fig. 3A) In addition, increased Zn and Cu content was found in plants treated with organic acids that promote low iron accumulation, such as MA at the 1:1 and 1:2 SR in the soybean experiment. The supposition is that Fe competes with Zn, Mn, and Cu in the plant uptake phase (Broadley et al. 2011). Accordingly, excessive Fe uptake could reduce the acquisition of other micronutrients, such as Mn, due to competition during the uptake process. In contrast, reduced Fe availability and uptake could lead to increased Zn and Cu uptake, due to the small amount of Fe in solution to inhibit or compete with these micronutrients (Page & Feller 2015).

For both the maize and soybean experiments, the dry matter, Fe accumulation, and SPAD index were not closely related to the complexed Fe fraction and the soluble Fe fraction in the nutrient solution (Fig. 3). Since the organic acids are not able to maintain most of the iron in complexed form in the nutrient solution, a hypothesis to be confirmed in further studies is the organic acid effects might be attributable to their activity on plant physiology. Organic acids regulate several processes, such as oxidative stress scavenging, protein transcription and expression, photosynthesis, and plant growth (An et al. 2014; Drincovich et al. 2016).

OA brought about adequate Fe nutrient status for soybean, and TA for maize. In the maize experiment, PCA analysis showed an association of the free complexing fraction, Fe accumulation, and both TA treatments at the 1:1 and 1:2 SR (Fig. 3A). TA at the 1:2 SR increased iron accumulation (almost 2 times higher than the Fe-EDTA) in maize plants. OA at the 1:1 SR increased Fe content by 15% over Fe-EDTA. Oxalic and tartaric acids are both fates of ascorbic acid catabolism associated with antioxidant plant systems (Smirnoff 2018). Exogenous application of oxalic acid induced the activity of antioxidant enzymes (An et al. 2014) and maintained high levels of ATP and activity of energy metabolism enzymes in plant cells (Jin et al. 2014). Exogenous addition of tartaric acid in the nutrient solution alleviated Al stress and improved plant growth and the photosynthetic rate (Tian-Rodrigues et al., 2009). In addition, TA application was associated with high metal uptake in several plant species (Cui et al. 2007; Wang & Zhong 2011; Sharma et al. 2019), and these effects might be associated with an increase in influx rates of the metals in the presence of TA (Wang & Zhong 2011). The greater abundance of transport sites for these organic acid complexes in the cell membranes than for synthetic chelates such as EDTA may also explain the higher efficiency of the natural complexes in iron nutrient status (Wang & Zhong 2011). Therefore, a positive effect on plant biochemical processes related to iron nutrient status and growth may be a factor to consider in the agronomic efficiency of these organic acids.

In contrast, MA and CA use led to a decrease in plant growth for both maize and soybean, indicating a possible negative effect on the plant system (Fig. 1 and Fig. 2). For the soybean experiment, the effect of the free complexing agent fraction is negatively associated with plant growth and is related to the MA and CA treatments (Fig. 3B). A decrease in biomass at high concentrations of organic acids added to the soil was observed in some studies (Evangelou et al. 2005). An hypothesis to be confirmed in further studies is these results may be related to damage to the root endodermis and Casparian strip caused by the organic acids (Nunes et al. 2009). Plasma membranes are stabilized by Ca ions, which might be complexed and removed by the organic acids, leading to membrane damage and plant stress (Evangelou et al. 2007; Leštan et al. 2008).

In addition, MA and CA are associated with the tricarboxylic cycle of plant energy metabolism. Changes in cellular concentrations of citrate, and to a lesser extent, malate, impact nucleus-encoded transcript abundance and tricarboxylic cycle regulation (Drincovich et al. 2016). Thus, possible dysregulation of some plant physiological processes due to the concentration of the organic acids used (0.09 and 0.18 mmol L^{-1}) should not be ruled out. Nevertheless, it has also been reported that exogenous applications of CA and MA, as well as citrate, combined with iron led to suitable plant growth and increased photosynthetic and antioxidant activity (Chakraborty et al. 2014; Song et al. 2016; Chen et al. 2020). However, the concentrations used varied among these studies, and the toxicity limits for species varies greatly. In general, the effects of variable concentrations of organic acids on plant physiological processes are still lacking. Therefore, further research is necessary to elucidate the effects of organic acids on plant physiology and their relation to crop nutrient status and growth. Finally, phytosiderophores are ranked as potent complexing agents to improve iron acquisition by monocot crops (Kobayashi & Nishizawa 2012). For this reason, the release of phytosiderophores in response to the addition of organic acids alone and complexes with Fe should also be studied in future studies.

5. Conclusions

Organic acids were less efficient than EDTA in maintaining iron complexed in the nutrient solution, but maize and soybean growth and nutrient status were not associated with the Fe complexed fraction. Plant species was a key factor determining the effectiveness of the Fe complexes. The use of Fe combined with malic acid and citric acid at both SRs had negative effects on maize and soybean growth. Maize only had suitable growth and Fe nutrient status with the use of $FeSO_4$. However, the use of Fe combined with tartaric acid at the 1:2 SR was very effective in promoting Fe accumulation in maize plants. For soybean, OA at both 1:1 and 1:2 SRs in soybean assured plant growth, iron accumulation, and translocation equivalent to EDTA. Therefore, this study showed that, even with low complexation capacity, organic acids are low-cost and biodegradable sources that can be used to complex iron, ensuring plant growth and Fe nutrient status. However, further studies are required to investigate the effects of organic acid on plant physiological processes.

Acknowledgements

Authors acknowledge Prof. Maria Lígia Souza e Silva for provided the hydroponic facilities to carried out the plant experiments, FAPEMIG, CNPq (303899/2015-8 and 307447/2019-7 grants), and CAPES (PROEX/AUXPE 593/2018) for financial support and scholarship provided for the first author.

6. References

Abadía J, Vázquez S, Rellán-Álvarez R, El-Jendoubi H, Abadía A, Álvarez-Fernández A, López-Millán AF. 2011. Towards a knowledge-based correction of iron chlorosis. Plant Physiol Biochem. 49(5):471–482.

Adeleke R, Nwangburuka C, Oboirien B. 2017. Origins, roles and fate of organic acids in soils: A review. South African J Bot. 108:393–406.

An Y, Zhou P, Xiao Q, Shi D. 2014. Effects of foliar application of organic acids on alleviation of aluminum toxicity in alfalfa. J Plant Nutr Soil Sci. 177(3):421–430.

Baran EJ. 2014. Natural oxalates and their analogous synthetic complexes. J Coord Chem 67 (23–24): 3734–3768.Bertoli AC, Carvalho R, Freitas MP, Ramalho TC, Mancini DT, Oliveira MC, De Varennes A, Dias A. 2015. Structural determination of Cu and Fe-Citrate complexes: Theoretical investigation and analysis by ESI-MS. J Inorg Biochem. 144:31–37.

Bloem E, Haneklaus S, haensch R, Schung E. 2017. EDTA application on agricultural soils affects micorelement uptake of plants. Sci. Total Environ. 577: 166–173.

Broadley M, Brown P, Cakmak I, Rengel Z, Zhao F. 2012. Function of Nutrients: Micronutrients. In: Marschner's Mineral Nutrition of Higher Plants. 3 Ed. London: Academic Press p. 191-248.

Chakraborty B, Singh PN, Kumar S, Srivastava PC. 2014. Uptake and Distribution of Iron from Different Iron Sources Applied as Foliar Sprays to Chlorotic Leaves of Low-Chill Peach Cultivars. Agric Res. 3(4):293–301.

Chen H-C, Zhang S-L, Wu K-J, Li R, He X-R, He D-N, Huang C, Wei H. 2020. The effects of exogenous organic acids on the growth, photosynthesis and cellular ultrastructure of *Salix variegata* Franch. Under Cd stress. Ecotoxicol Environ Saf. 187:109790.

Chen YT, Wang Y, Yeh KC. 2017. Role of root exudates in metal acquisition and tolerance. Curr Opin Plant Biol. 39:66–72.

Cline GR, Powell PE, Szaniszlo PJ, Reid CPP. 1982. Comparison of the Abilities of Hydroxamic, Synthetic, and Other Natural Organic Acids to Chelate Iron and Other Ions in Nutrient Solution. Soil Sci Soc Am J. 46(6):1158–1164.

Colombo C, Palumbo G, He JH, Pinton R, Cesco S. 2014. Review on iron availability in soil: Interaction of Fe mineral, plants, and microbes. J Soils Sediments 14: 538–548.

Cui S, Zhou Q xing, Wei S he, Zhang W, Cao L, Ren L ping. 2007. Effects of exogenous chelators on phytoavailability and toxicity of Pb in *Zinnia elegan*s Jacq. J Hazard Mater. 146(1–2):341–346.

Drincovich MF, Voll LM, Maurino VG. 2016. Editorial: On the Diversity of Roles of Organic Acids. Front Plant Sci. 7:1592.

Evangelou MWH, Ebel M, Schaeffer A. 2005. Evaluation of the effect of small organic acids on phytoextraction of Cu and Pb from soil with tobacco *Nicotiana tabacum*. Chemosphere 63(6): 996–1004.

Evangelou MWH, Ebel M, Schaeffer A. 2007. Chelate assisted phytoextraction of heavy metals from soil. Effect, mechanism, toxicity, and fate of chelating agents. Chemosphere. 68(6):989–1003.

Fernández V, Ebert G. 2005. Foliar iron fertilization: A critical review. J Plant Nutr. 28(12):2113–2124.

Fu Y, Lei W, Shen Z, Luo C. 2015. Permeability of plant young root endodermis to Cu ions and Cu-citrate complexes in corn and soybean. Int J Phytoremediation 17(9): 822–834.

García-Mina JM, Antolín MC, Sanchez-Diaz M. 2004. Metal-humic complexes and plant micronutrient uptake: A study based on different plant species cultivated in diverse soil types. Plant Soil. 258(1–2):57–

68.

Gerke J. 2015. The acquisition of phosphate by higher plants: Effect of carboxylate release by the roots. A critical review. J Plant Nutr Soil Sci. 178(3):351–364.

Hasan M, Udin N, Ara-Sharmeen I, Alharby HF, Alzahrani Y, Hakeem KR, Zhang L. 2019. Assisting phytoremediation of heavy metals using chemical amendments. Plants 8(9): 295, https://doi.org/10.3390/plants8090295.

Hoagland D, Arnon D, Hoagland DR, Arnon DI, Hutchison CB. The water-culture method for growing plants without soil. California Agricultural Experiment Station. Circular 347.

Hopff D, Wienkoop S, Lüthje S. 2013. The plasma membrane proteome of maize roots grown under low and high iron conditions. J Proteomics 91:605–618.

Jin P, Zhu H, Wang L, Shan T, Zheng Y. 2014. Oxalic acid alleviates chilling injury in peach fruit by regulating energy metabolism and fatty acid contents. Food Chem. 161:87–93.

Kobayashi T, Nishizawa NK. 2012. Iron Uptake, Translocation, and Regulation in Higher Plants. Annu Rev Plant Biol. 63(1):131–152.

Lemanceausoils P, Bauer P, Kraemer S, Briat JF. 2009. Iron dynamics in the rhizosphere as a case study for analyzing interactions between soils, plants and microbes. Plant Soil. 321(1–2):513–535.

Leštan D, Luo C, Li X. 2008. The use of chelating agents in the remediation of metal-contaminated soils: A review. Environ Pollut. 153(1):3–13.

Li Y, Wang N, Zhao F, Song X, Yin Z, Huang R, Zhang C. 2014. Changes in the transcriptomic profiles of maize roots in response to iron-deficiency stress. Plant Mol Biol. 85: 349–363

Lindsay WL. 1995. Chemical reactions in soils that affect iron availability to plants. A quantative approach. In: Abadia J. (ed.) Iron Nutrition in Soils and Plants, Dordecht: Kluwer Academic Publishers, p.7–14.

López-Millán AF, Morales F, Gogorcena Y, Abadía A, Abadía J. 2009. Metabolic responses in iron deficient tomato plants. J Plant Physiol. 166(4):375–384.

Marschner H, Römheld V. 1994. Strategies of plants for acquisition of iron. Plant Soil. 165(2):261–274.

Nowack B. 2002. Environmental chemistry of aminopolycarboxylate chelating agents. Environ Sci Technol. 36(19):4009–4016.

Nunes FC, Miyazawa M, Pavan MA. 2009. Organic acid effect on calcium uptake by the wheat roots. Brazilian Arch Biol Technol. 52(1):11–15.

Page V, Feller U. 2015. Heavy metals in crop plants: Transport and redistribution processes on the whole plant level. Agronomy. 5(3):447–463.

Pegoraro RF, Silva IR, Novais RF, Mendonça ES, Alvarez V. VH, Nunes FN, Fonseca FM, Smyth TJ. 2005. Diffusive flux of cationic micronutrients in two Oxisols as affected by low-molecular-weight organic acids and cover-crop residue. J Plant Nutr Soil Sci. 168(3):334–340.

Rodríguez-Lucena P, Benedicto A, Lucena JJ, Rodríguez-Castrillón JA, Moldovan M, García Alonso JI, Hernández-Apaolaza L. 2011. Use of the stable isotope 57Fe to track the efficacy of the foliar application of lignosulfonate/Fe^{3+} complexes to correct Fe deficiencies in cucumber plants. J Sci Food Agric. 91(3):395–404.

Rodríguez-Lucena P, Hernández-Apaolaza L, Lucena JJ. 2010. Comparison of iron chelates and complexes supplied as foliar sprays and in nutrient solution to correct iron chlorosis of soybean. J Plant Nutr Soil Sci. 173(1):120–126.

Sharid M, Austruy A, Echevarria G, Arshad M, Sanaullah M, Aslam M, Nadeem M, Nasim W, Dumat C. 2013. EDTA-enhanced phytoremediation of heavy metals: A review. Soil Sediment Contam: An Int Journal. 23(4): 389–416.

Sharma M, Kumar V, Bhardwaj R, Thukral AK. 2019. Tartaric Acid Mediated Cr Hyperaccumulation and Biochemical alterations in seedlings of *Hordeum vulgare* L. J Plant Growth Regul. 39(1):1–14.

Shenker M, Chen Y. 2005. Increasing Iron Availability to Crops: Fertilizers, Organo-Fertilizers, and Biological Approaches. Soil Sci Plant Nutr. 51(1):1–17.

Smirnoff N. Ascorbic acid metabolism and functions: A comparison of plants and mammals. Free Radic Biol Med. 122: 116–129

Song J, Markewitz D, Liu Y, Liu X, Cui X. 2016. The Alleviation of Nutrient Deficiency Symptoms in Changbai Larch (*Larix olgensis*) Seedlings by the Application of Exogenous Organic Acids. Forests. 7(12):213.

Ström L, Owen AG, Godbold DL, Jones DL. 2005. Organic acid behaviour in a calcareous soil implications for rhizosphere nutrient cycling. Soil Biol Biochem. 37(11):2046–2054.

Tan K H. 2011. Principles of Soil Chemistry. Boca Raton (FL): CRC Press Taylor and Francis Group.

Tolentino TA, Bertoli AC, Dos Santos Pires M, Carvalho R, Labory CRG, Nunes JS, Bastos ARR, De Freitas MP. 2015. Applications in environmental bioinorganic: Nutritional and ultrastructural evaluation and calculus of thermodynamic and structural properties of metal-oxalate complexes. Spectrochim Acta - Part A Mol Biomol Spectrosc. 150:750–757.

Wang H, Zhong G. 2011. Effect of organic ligands on accumulation of copper in hyperaccumulator and nonaccumulator *Commelina communis*. Biol Trace Elem Res. 143(1):489–499.

Zuchi S, Cesco S, Astolfi S. 2012. High S supply improves Fe accumulation in durum wheat plants grown under Fe limitation. Environ Exp Bot 77: 25–32.

FINAL REMARKS

This study investigated the molecular structure and chemical properties of metal-organic acid complexes and the agronomic value of iron complexes in the plant nutrition domain. Metal, ligand, and stoichiometry of the reaction markedly affected the chemical properties of complexes. Therefore, it is crucial to investigate these properties before choosing a complexing agent for an agronomic or environmental purpose. FTIR proved to be a valuable tool to base the complexes properties investigation and prediction. The theoretical modeling unraveled aspects of the complexes molecular structure. Carboxylate band shifts in FTIR spectra were associated with coordination mode of metals with carboxylate group in the complex structure. Complex stability and solubility affected the effectiveness of complexes in promoting suitable plant nutrition and growth. In general, complexed Fe sources exhibit higher capacity of redistribution in plant than the free iron source. The application mode of complexes (foliar or in nutrient solution) determined the performance of complexes in supplying iron and promoting biomass production. The plant species also regulated the effectiveness of the complexes tested. Therefore, the newly developed complexes should be tested with different plant species and modes of application. Considering the use of complexing agents with bioactivity potential, such as low-molecular-weight organic acids, the possible action of complex on plant physiological processes should be investigated. These effects can potentialize the complex effectiveness as a nutrient source for plants, with the possibility to boost nutrient uptake and plant growth.