Fe-humicin as a new source of iron in plants

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Abstract

Fe plays an essential role in the photosynthetic processes of plants. Iron deficient plants show limitations in growth, biomass production and ultimately in yield. The synthesis of chlorophylls is also negatively affected and carotenoid pigments take precedence, thus the leaves turn yellow or *chlorotic*. In practice, iron deficiency is rarely caused by the general lack of the element itself. Rather, it may form biologically inaccessible, stable precipitates due to unfavourable chemical conditions in the soil, thus its uptake may only proceed through high affinity pathways. Alkaline pH, strong buffering effect and an oxidative environment normally present under such conditions all hinder the mobilization of iron compounds and the acidification of the rhizosphere by the plant. In the modern-day agriculture, Fe is mostly supplemented via highly stable chelates, however prolonged treatments have proven to be environmentally harmful. For this reason, safe and reliable sources of iron and other microelements need to be developed.

In our experiments, we explored the possibilities of using Fe-humicin, a medicinal iron supplement as an Fe source in hydroponic cultures. Using Mössbauer sprectroscopy, we determined that the ascorbic acid added to the product does indeed contribute to keeping the Fe ions in a reduced state, which may in turn improve their bioavailability. The plant experiments were conducted on cucumber (*Cucumis sativus* L. cv. Joker), in hydroponic culture, on ¼ strength Hoagland nutrient solution. The product has been shown to be a potent source of iron in a wide range of concentrations on slightly acidic pH, similarly to our controls (Fe-citrate and Fe-EDTA). In the plants treated with Fe-humicin, biomass production, chlorophyll and carotenoid concentrations, as well as the maximum quantum efficiency of photosystem II have all been determined to be superior to iron deficient controls, and comparable to controls supplied with iron. However, on alkaline pH, Fe-EDDHA, a potent iron source along a wide pH-range outperformed the product, while humicin-treated plants performed better than their Fe deficient peers. Further experiments are being conducted to determine the impact of Fe-humicin made with and without ascorbic acid on high affinity Fe uptake pathways by measuring Fe-chelate reductase activity.

Abbreviations

EDTA – Ethylenediaminetetraacetic acid

EDDHA – Ethylenediamine di-2-hydroxyphenyl acetate

SPAD – Soil Plant Analysis Development

PRI – Photochemical reflectance index

BPDS - Bathophenanthroline disulfonic acid

MES - 2-(*N*-morpholino)ethanesulfonic acid

ICP-OES – Inductively coupled plasma-optical emission spectrometer

Chl – chlorophyll

IDHA – N-(1,2-dicarboxyethyl)-D,L-aspartic acid

FRO2 – ferric reduction oxidase 2

ITR1 – iron-regulated transporter 1

AHA2 – autoinhibited plasmamembrane H^+ ATPase 2

Introduction

Iron is an important, essential microelement for plants. It is a constituent to many enzymes, important for chlorophyll synthesis and 80-90% of Fe is found in chloroplasts (TERRY AND LOW, 1982). Fe plays a key role in photosynthesis and its deficiency is linked to a decreased production of photosynthetic pigments and a lower efficiency of the process. Iron deficient plants are suffering from *chlorosis* and exhibit a set of physiological symptoms. Yield loss resultant from commonplace iron deficiency can exceed 25% in case of some cereals and legumes (BRIAT ET AL., 2015) Based solely on iron uptake, two strategies can be differentiated. Strategy I plants, such as cucumber (*Cucumis sativus* L.) reduce ferric iron (Fe(III)) to its ferrous (Fe(II)) form at the root plasma membrane, before transporting it inside the cells. Strategy I high-affinity iron uptake is tied together by the interactome described by MARTÍN-BARRANCO ET AL. (2020). The team recorded ferric reduction oxidase 2 (FRO2) to operate closely with iron-regulated transporter 1 (IRT1), a transmembrane transporter of iron and other metals and autoinhibited plasmamembrane H^+ ATPase 2 (AHA2), a proton pump, perhaps involved in the acidification of the rhizosphere. This association described first by RÖMHELD AND MARSCHNER (1986), allows for tight, local regulation of Strategy I high-affinity iron uptake. Strategy II plants (from the clade *Poaceae*), on the other hand, release their own chelator molecules into the rhizosphere, which bind the iron and then are taken up by specific transport proteins (CONNORTON ET AL., 2017). The valence of the iron atoms may also change within the plant on demand, as it is transported in the xylem mostly in ferric form. (NIKOLIC AND PAVLOVIC, 2018) Chloroplasts of sugarbeet are shown to prefer ferric iron over ferrous iron for uptake. (SOLTI ET AL., 2012)

Under specific circumstances such as highly alkaline environment, the mobility and availability of Fe significantly decreases and the plants cannot take up sufficient amount. This leads to Fe deficiency which drastically reduces the rate of plant growth and crop or fruit yield. For this reason agricultural techniques are continuously developed to maintain an adequate supply of Fe to the plants. This includes the application of Fe-chelates which remain mobile in the soil (MCGRATH ET AL., 2002). However, the majority of the Fe-chelates are not biodegradable and thus may build up in the soil, necessitating the development of new, environmentally friendly alternatives.

Other research also indicates that the pre-existing iron content of the soil may also be utilized better, such as with the addition of accessible Si compounds, that seem to improve the efficiency of Fe metabolism, stockpiling and mobilization (PAVLOVIC ET AL., 2013), while other substances may influence

Humic substances are naturally plentiful in agricultural soils and also in greater depths. Humic and fulvic acids originate during the decomposition of biomass, which is also a safe and effective method of industrial scale production. A method similar to hydrocharring can be used on biomass to achieve thorough humification under a fraction of the time traditional composting takes. This process is rather simple and tame compared to the complicated leonardite path used mostly for smaller scale production. These substances have many agricultural uses already, and thus most of their beneficial effects are well-understood. (YANG AND ANTONIETTI, 2020)

Fe-humicin, a commercially available product, may have advantageous properties in this respect, as its constituents, humic acids and ascorbic acid have been confirmed to form large complexes and precipitates with the iron ions that may work similar to other known, stable compounds in a variety of circumstances. The aim of our work was to assess the potential of Fe-humicin as Fe source and reveal the effect of added ascorbic acid on Fe uptake and metabolism across a range of environments.

Materials and methods

Fe-humicin products

Fe-humicin (commercially available product of Pannon-Trade Kft.) containing 30 g humic/fulvic acids, 3 g Fe (as FeSO4) and 45 g ascorbic acid was used in the experiments. For the Fe-chelate reductase tests the product was prepared with the composition of 30 g humic/fulvic acids and 30 g ascorbic acid and of 30 g humic/fulvic acids, 3 g Fe. The products were kept refrigerated until use.

Plant material

Our model of choice was cucumber (*Cucumis sativus* L. cv. Joker), raised from seed in hydroponic culture. The culture medium was ¼ strength Hoagland solution; for the exact composition see Table 1. The seeds were first germinated for one day in the dark at 30°C on filter paper moistened with deionized water in Petri dishes. The germinated seeds were planted on Styrofoam floaters inside 400 ml pots, lined with plastic bags against iron contamination as the experiment made it necessary. The pots were kept on constant temperature of 23°C and 60% relative humidity and under a strict day/night 14/10 hours cycle of 200 μE PPFD in a climate controlled growth chamber. For two days the plants were kept in a 0.5 mM $CaSO_4$ solution, covered by a tray to induce growth in diffuse light. Then, when their roots and shoots both reached proper size, nutrient solution was applied. The nutrient solution was changed three times a week, at these times the pots were washed as well and replaced as needed. Any treatments were also applied upon the imminent change of the nutrient solution. The plants were usually grown until the 2nd leaf reached significant size, or the root was deemed developed enough, this usually took no more than 18-20 days, or longer for the 3rd leaf. Iron was supplied as Fe-humicin, Fe-Ethylenediaminetetraacetic acid (EDTA) or Fe-citrate in 0 or 10 μM concentration.

Table 1. Components and respective concentrations in the culture medium.

Component	Final concentration (M)
KNO_3	$1{,}25 \times 10^{-3}$
$Ca(NO_3)_2$	$1{,}25 \times 10^{-3}$
$MgSO \times 7H_2O$	5×10^{-4}

KH_2PO_4	$2{,}5 \times 10^{-4}$
H_3BO_3	$1{,}156 \times 10^{-5}$
$MnCl_2 \times 4\ H_2O$	$4{,}6 \times 10^{-6}$
$ZnSO_4 \times 7\ H_2O$	$1{,}9 \times 10^{-7}$
$Na_2MoO_4 \times 2\ H_2O$	$1{,}2 \times 10^{-7}$
$CuSO_4 \times 4\ H_2O$	8×10^{-8}

Measurements

To obtain exact data regarding the iron concentration in the received Fe-humicin, the product was digested in ccH2O2 for 1 h, then ccHNO3 for about 15 min at 60°C and 45 min at 120°C. The solution was thoroughly homogenized, and eventually filtered through MN 640 W filter paper (Macherey-Nagel, Düren, Germany). Measurements on the resultant fluid were performed with an Inductively coupled plasma-optical emission spectrometer (ICP-OES, Spectro Genesis, SPECTRO, Freital, Germany) device to determine its exact elemental composition. The Fe concentration of every treatment was calculated based on these results.

The oxidization states of the iron within the product was investigated with Mössbauer spectroscopy to better understand its properties as an iron source. These measurements were conducted and evaluated at the ELTE Institute of Chemistry, in the Laboratory for Nuclear Chemistry. Mössbauer spectroscopy is based on the on the recoil free resonance absorption of gamma photons by certain nuclei such as ^{57}Fe, a relatively common isotope of iron. The hyperfine interactions between the nuclei and their environment can be measured, from which the oxidation state and even the particular molecular environment of the investigated atom may be discerned.

Several properties of the plants were measured during the treatments. The chlorophyll contents of select leaves were measured with a handheld device (Soil Plant Analysis Development (SPAD) 502+, Konica Minolta, Japan) , based on the tissue's absorption of a monochromatic red light. The device is sensitive to leaf thickness and leaf veins in particular. The resulting SPAD index is a good approximation to actual chlorophyll content. This measurement was regularly repeated on several days of treatments, in order to follow the regeneration of the iron deficient plants.

The photochemical reflectance index (PRI) was also measured on living plants, with a handheld device (Plant Pen PRI, PSI Instruments, Drásov, Czech Republic) as well. This involved measuring the reflectance of the leaves at 530 nm and 590 nm wavelengths. The resulting index is indicative of the presence and concentration of carotenoid pigments in the plant tissue. More positive values are characteristic of a healthy plant and more negative ones indicate iron deficiency.

PRI is calculated according to the following formula:

$$PRI=(\rho530-\rho590)/(\rho530+\rho590),$$

where ρ530 and ρ590 indicates reflectance at 530 nm and 590 nm, respectively.

The maximum quantum efficiency of photosystem II. was measured after no less than 20 minutes of dark adaptation, performed either inside a cabinet, or with special clips locked onto the leaves. This measurement utilizes the Kautsky effect to provide information regarding the state of the electron transport chain, and as such is moderately sensitive to iron deficiency. Both a desktop and a handheld device was available for this purpose (Fluorpen, PSI Instruments, Drásov, Czech Republic; PAM 101,102,103, Waltz, Effeltrich, Germany).

At harvest, the plants were split up to their major organs (roots, shoots with the cotyledon, and leaves separately). These were then weighed on an analytical scale to record fresh weight and after being dried in a thermostat on 70°C for no less than three days for dry weight. Both the final, post-harvest pH and the starting pH of the culture medium was measured with a digital pH meter, while under moderate stirring.

Ferric chelate reductase activity was measured in young iron deficient plants suppled with different Fe substrates as pre-treatment. For this purpose, the plants were grown without any iron for 17 days until their first leaf mostly developed. They were then subjected to the treatments and incubated for one or two hours to assess the utilization of the Fe delivered by the applied sources, then the roots were cut off and smaller, mostly uniform sections were selected. These were incubated in the dark for 15 minutes, using a solution based on ½ strength Hoagland nutrient solution, containing only the macro salts, 500μM Fe(III)-EDTA, the iron binding dye 400μM Bathophenanthroline disulfonic acid (BPDS), and 5 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) (pH=6) buffer. Following incubation, the solutions were

sampled and an hour later the absorbance was measured in an UV-VIS spectrophotometer (Shimadzu 2101 PC, Japan) at 800 and 535 nm wavelengths. The fresh mass of the root pieces was also measured. From this data, the activity of Fe-chelate reductase could be calculated based on the specific extinction coefficient of Fe(II)-$BPDS_3^{4-}$ (22.14 mM^{-1} cm^{-1}).

Statistical analysis

Statistical treatment was conducted in Excel and R. All data were recoded and all figures were created in the former, while statistical tests (chiefly one-way ANOVA and Tukey *post hoc* test) were performed in the latter. Statistically significant values ($P<0.05$) are indicated with different letters in the figures.

Results

Fe-humicin

Mössbauer spectroscopic measurements were performed on several different Fe-humicin samples, to determine the effect of added ascorbic acid. In the sample without ascorbic acid, based on the isomer shift of 0.52219 and quadrupole splitting of 0.35352 roughly 30% of Fe was in +3 oxidation state (Fig. 1), while in the commercial Fe-humicin sample, the isomer shift was 1.36194 with a quadrupole splitting of 3.52480, all Fe was present in a reduced +2 state (Fig. 2). In both cases a portion of Fe ions formed complexes with humate ions.

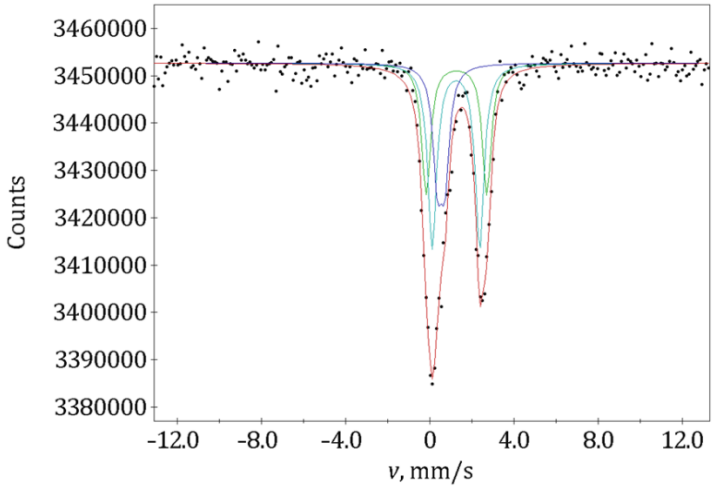

Figure 1. Representative Mössbauer spectrum of the undiluted Fe- humicin product without ascorbic acid.

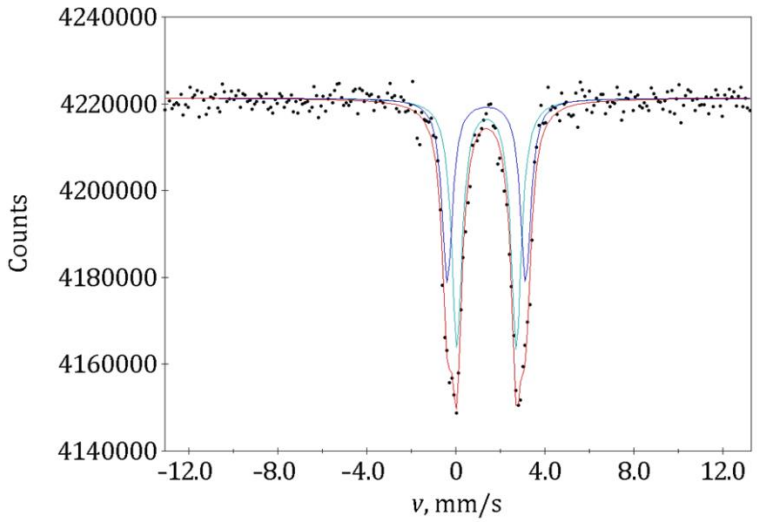

Figure 2. Representative Mössbauer spectrum of the undiluted Fe-humicin product containing ascorbic acid.

Experiment 1

The first experiment aimed to establish and confirm that Fe-humicin is indeed an effective iron source. For this purpose, the plants were supplied with different concentrations of the Fe-humicin product and grown in nutrient solution for 28 days. During and after the experiment, relative Chl content, fresh and dry mass of the plant parts and the maximum quantum efficiency of photosystem II were measured. Different dilutions of Fe-humicin (5, 10, 50, 100, 500 and 1000 µM for final iron concentration) and one control (ctrl) with 10 µM of Fe-EDTA were applied in the experiment.

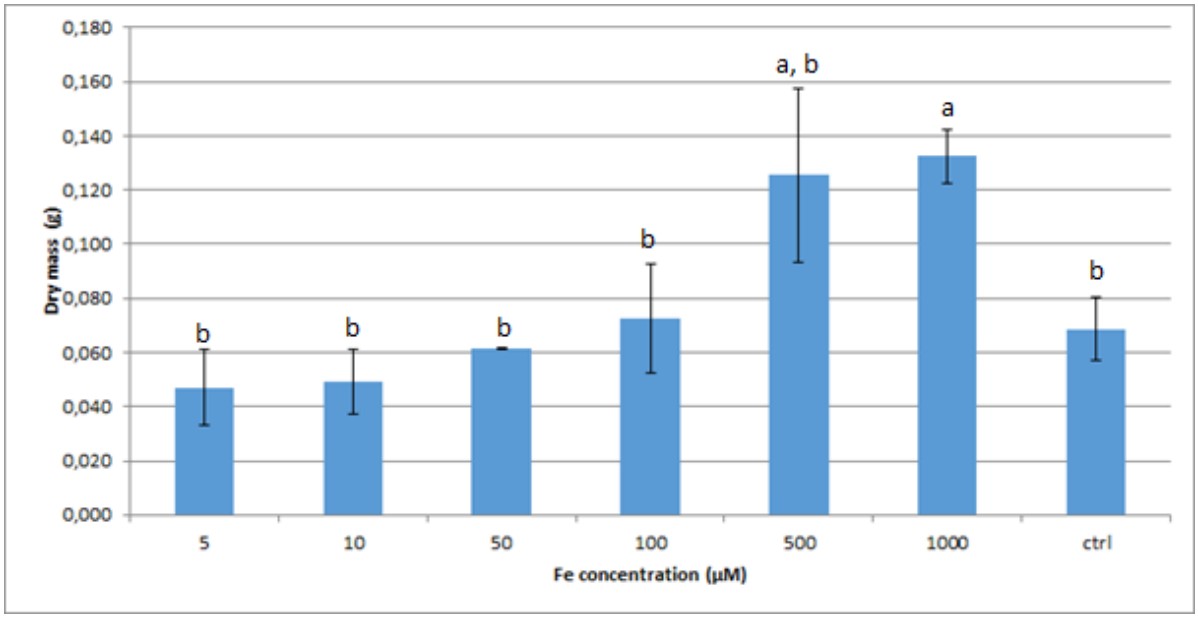

Figure 3.: Dry mass of the roots of cucumber corresponding to the different concentrations of Fe-humicin treatments. Control (Ctrl) plants were supplied with was 10 µM Fe-EDTA. The data are presented as means of 6 plants, error bars represent SD. Different letters indicate that the means are statistically different at the 0.05 confidence level.

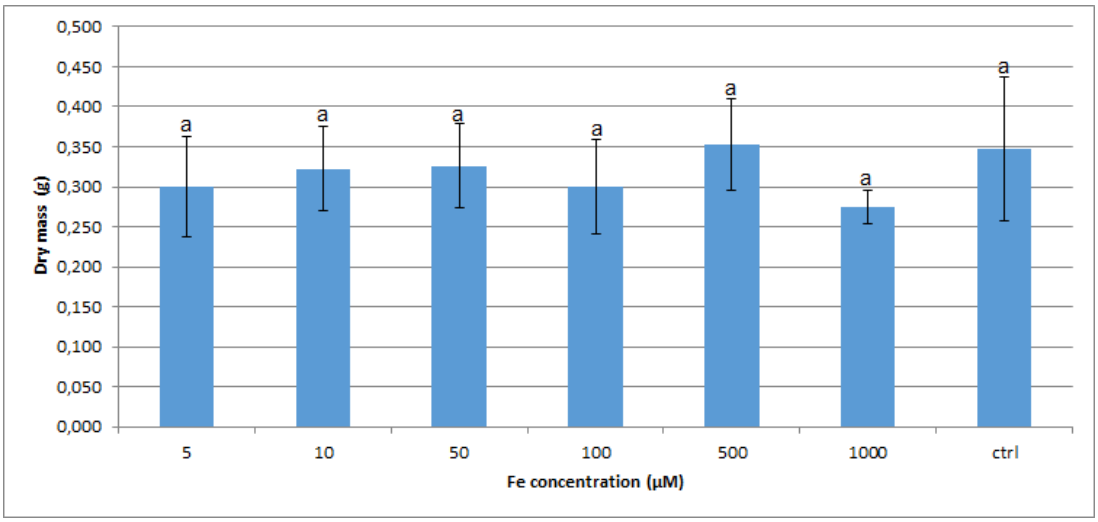

Figure 4.: Dry mass of the shoots+ 2nd leaves of cucumber corresponding to the different concentrations of Fe-humicin treatments. Control (Ctrl) plants were supplied with was 10 µM Fe-EDTA. The data are presented as means of 6 plants, error bars represent SD. The same letters indicate no statistically significant difference on the 0.05 confidence level.

Statistical analysis revealed no significant difference between the means of second leaf's mass corresponding to the treatments on the 0.05 confidence level, (Fig. 4). The dry masses of roots treated with 500 and 1000 µM Fe-humicin were significantly larger than the rest.

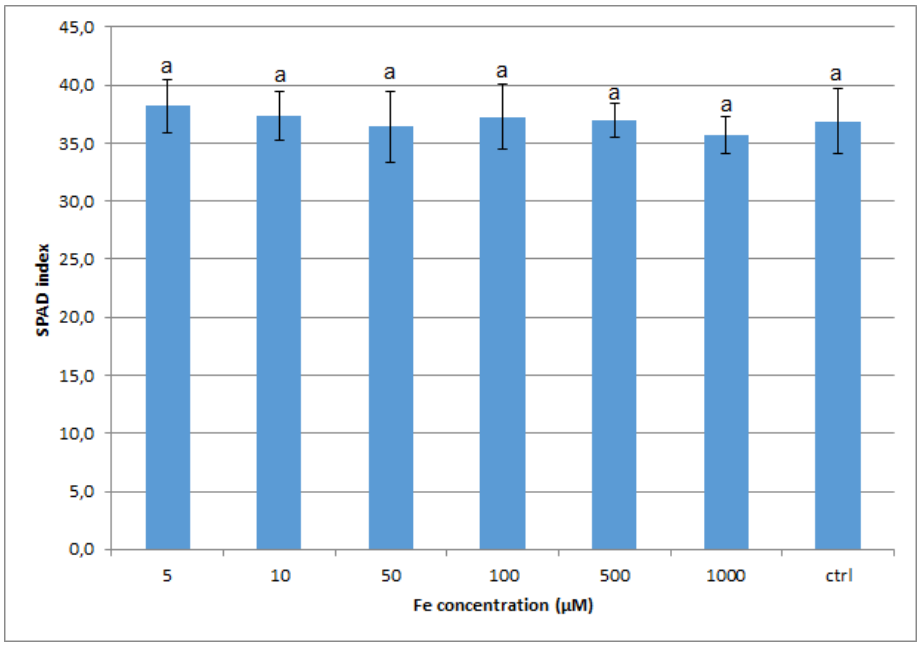

Figure 5.: Chl content (SPAD index) of the 2nd leaves of cucumber plants corresponding to the different concentrations of Fe-humicin treatments. Control (Ctrl) plants were supplied with was 10 µM Fe-EDTA. The data are presented as means of 6 plants, error bars represent SD. Different letters indicate that the means are statistically different at the 0.05 confidence level.

Table 1.: Maximum quantum efficiency of photosystem II measured on the 2nd leaves of cucumber plants corresponding to the different concentrations of Fe-humicin treatments. Control (Ctrl) plants were supplied with was 10 μM Fe-EDTA. The data are presented as means of 6 plants and SD. Statistical analysis revealed no significant difference between the treatments.

Fe concentration (μM)	**Fv/Fm**	**SD**
5	0.807	*0.008*
10	0.812	*0.008*
50	0.816	*0.002*
100	0.809	*0.009*
500	0.808	*0.015*
1000	0.82	*0.005*
ctrl	0.81	*0.011*

The relative Chl concentrations did not differ significantly among the treatments (Fig.5), and neither did the maximum quantum efficiency of photosystem II (Table 1).

Experiment 2

The second experiment aimed to determine the efficiency of Fe-humicin as an iron supplement under adverse conditions. For this purpose, the plants received a small amount (1 μM) of Fe-EDTA for three days at the start of the experiment. The nutrient solution did not contain Fe afterwards, and the plants developed iron deficiency by the time of the treatment. This resulted in somewhat larger leaves that enabled more accurate measurements. The plants were divided into 4 groups with 5 parallels each and were supplied with 10 μM Fe-citrate, Fe-humicin and Fe-Ethylenediamine di-2-hydroxyphenyl acetate (EDDHA) for 2 days before harvest. One group was left without Fe as control. The culture media was amended with $CaCO_3$) during the treatment time to maintain alkaline conditions. Throughout and after the experiment, relative Chl content, PRI, the fresh mass of plant organs and the maximum quantum efficiency of photosystem II were measured, along with the final pH of the culture media.

Table 2.: The final pH of the culture media post-harvest in Experiment 2.

Treatment	**pH**	**SD**

Fe-citrate	7.8	*0.01*
Fe-EDDHA	7.72	*0.08*
Fe-humicine	7.76	*0.13*
ctrl	7.75	*0.01*

The pH values of the culture media did not differ significantly and are severely alkaline, the iron supplementation had no apparent effect on the acidification, meaning that the buffer was sufficient (Table 2).

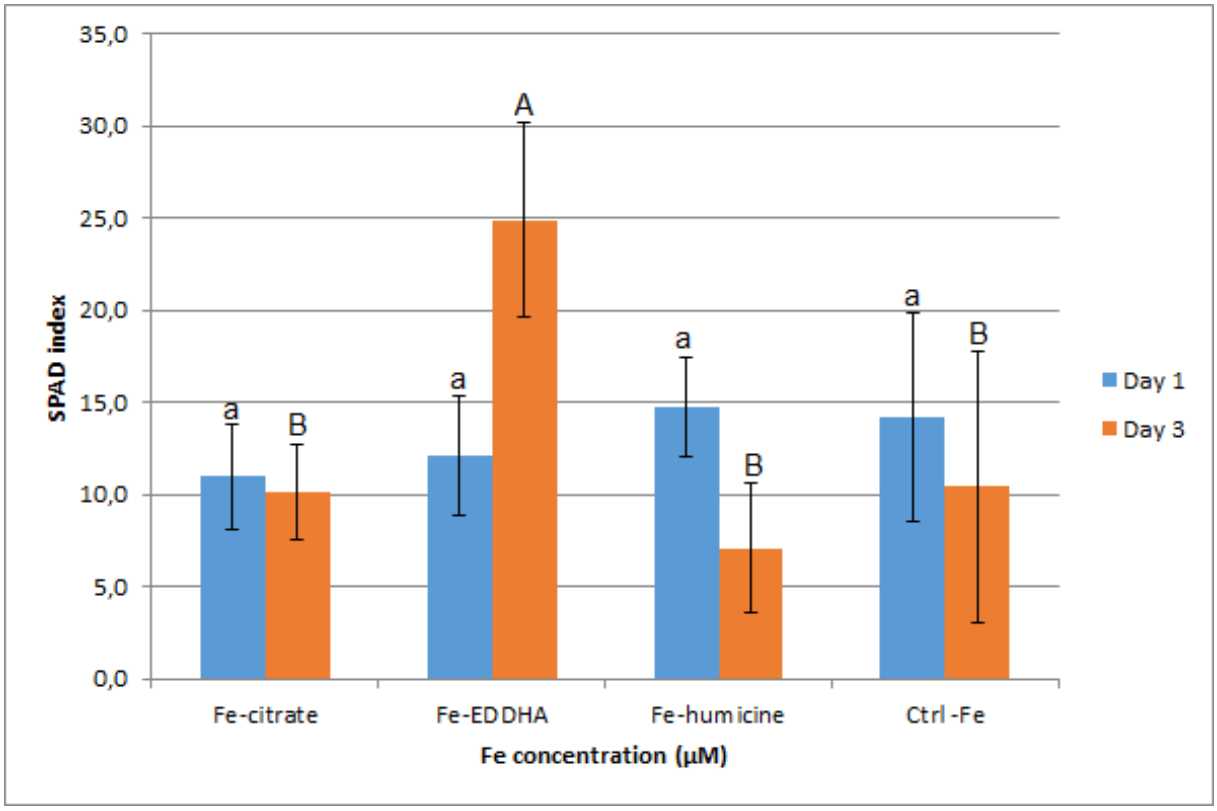

Figure 6.: Chl content (SPAD indexes) corresponding to each treatment, measured on the 2nd leaves.The treatments with different Fe sources were applied in 10 µM concentration, while ctrl was left iron deficient. The different letters designate statistically significant differences at the 0.05 confidence level. The lowercase letters compare values from the day of the treatment, the capital letter compare the values after 3 days of regeneration.

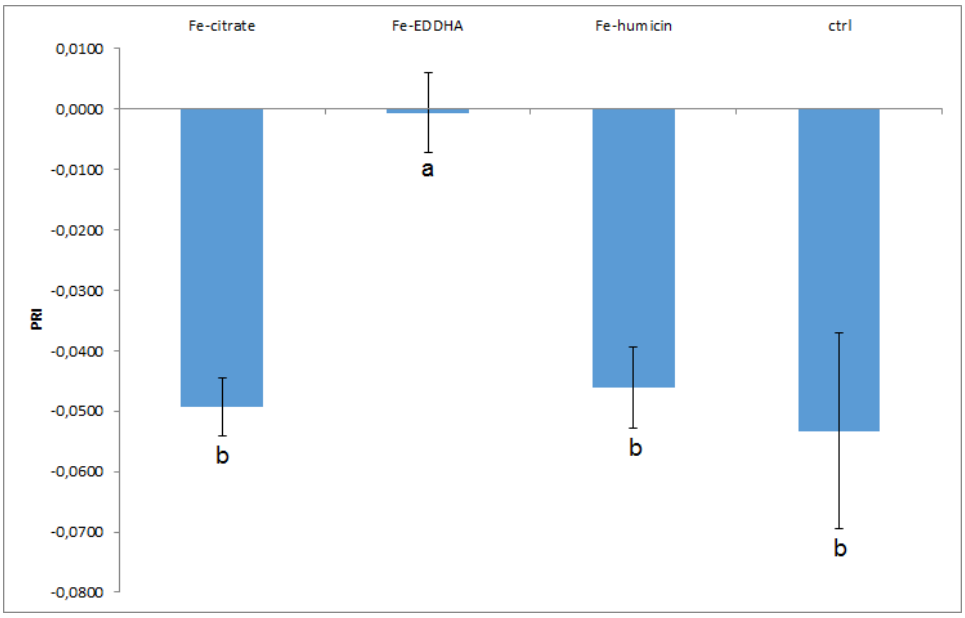

Figure 7.: PRI values corresponding to different treatments, measured on the second leaf. The treatments with different Fe sources were applied in 10 µM concentration, while ctrl was left iron deficient. The different letters designate statistically significant differences on the 0.05 confidence level.

Measurements regarding the Chl content revealed that Fe-humicin was unable to supply Fe to the plants similarly to Fe-citrate as the SPAD index in the 2nd leaves of the plants remained at the level of iron deficient plants (Fig. 6). On the contrary, Fe-EDDHA highly outperformed all the other treatments in Chl synthesis. The other treatments were statistically not different from each other. The photochemical reflectance index showed similar picture with Fe-EDDHA performing best and all the other treatments representing similarly low photosynthetic efficiency (Fig. 7). The same was found for the maximal quantum efficiency of photosystem II, showing that Fe-humicin was ineffective under alkaline conditions (Table 3).

Table 3.: The maximal quantum efficiency of photosystem II measured on the 2nd leaves. The treatments with different Fe sources were applied in 10 µM concentration, while ctrl was left iron deficient. The different letters designate statistically significant differences on the 0.05 confidence level.

Treatment	**Fv/Fm**	**SD**
Fe-citrate	0,785[a, b]	*0,046*
Fe-EDDHA	0,833[a]	*0,003*
Fe-humicine	0,760[b]	*0,025*
ctrl	0,717[b]	*0,054*

Experiment 3

We aimed to determine the effectiveness of Fe-humicin in comparison with other iron sources under acidic conditions. The plants received a small amount (1 µM) of Fe-EDTA for three days at the start of the experiment, but then the nutrient solution did not contain Fe and the plants developed iron deficiency by the time of harvest. The plants were then regenerated with different iron sources for 4 days before harvest. During and after the experiment, relative Chl content, Fv/Fm, PRI and pH were measured. The iron deficient plants further acidified the originally slightly acidic nutrient solution to a significant degree compared to the Fe treatments, especially Fe-EDTA and Fe-citrate. Fe-humicin decreased this effect to a lesser degree, albeit this difference is not significant on the 0.05 confidence level. (Table 4.)

Table 4.: pH of the culture media upon the start and at the end of the treatment period in Expriment 3. Neither the means of the final pH values nor the pH change differed significantly between the treatments, as indicated by the same letters.

Treatment	**Starting pH**	**Final pH (mean)**	**Final pH (SD)**
Fe-citrate	5.3	4.35[a]	*0.11*
Fe-EDTA	5.26	4.34[a]	*0.15*
Fe-humicin	5.27	4.12[a]	*0.09*
Ctrl -Fe	5.29	4.02[a]	*0.22*

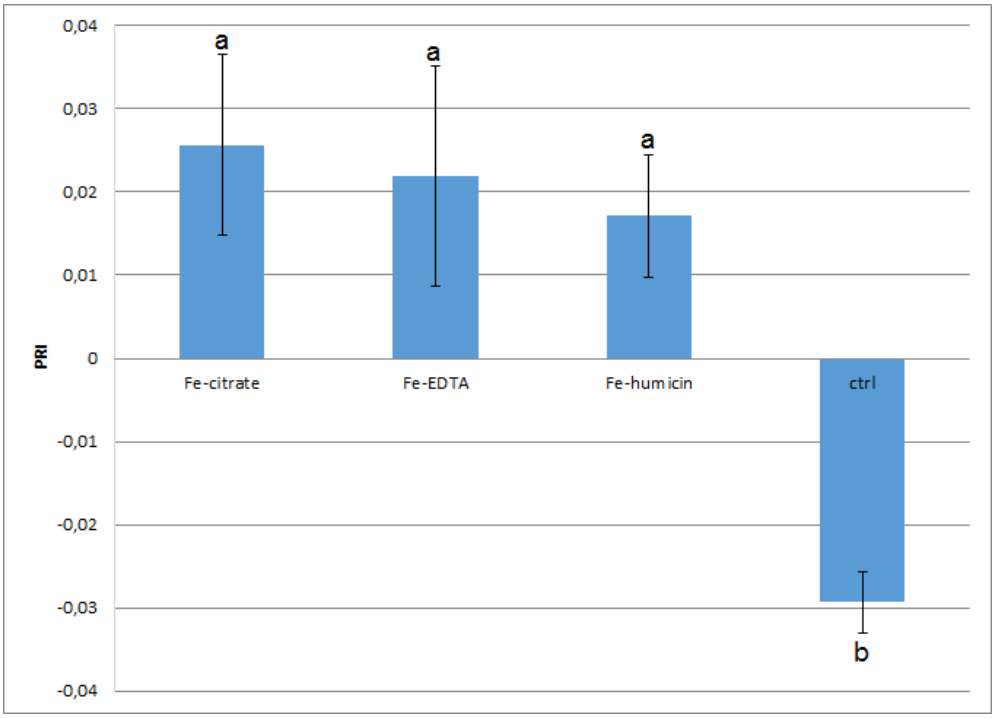

Figure 8.: PRI values corresponding to different treatments, measured on the third leaf. The treatments with different Fe sources were applied in 10 µM concentration, while ctrl was left iron deficient. The different letters designate statistically significant differences on the 0.05 confidence level.

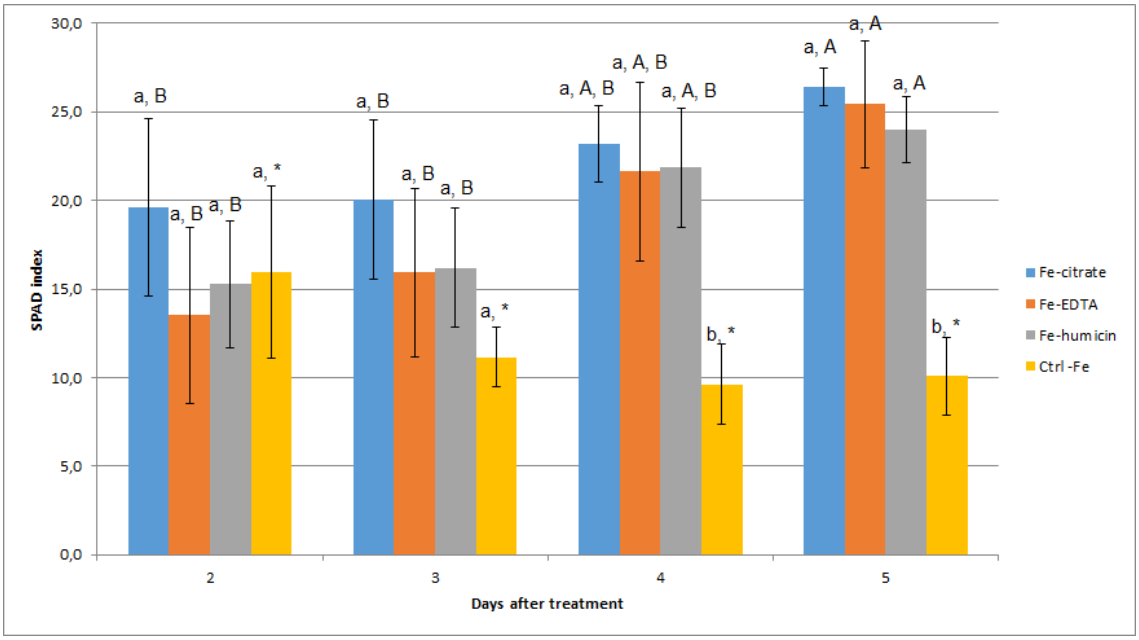

Figure 9.: SPAD indexes corresponding to each treatment, measured on the third leaves, organized according to the specific treatments, all of which deliverediron in 10 µM concentration, while ctrl was left iron deficient. The different lowercase and capital letters designate statistically significant differences on the 0.05 confidence level within days and within treatments, respectively.

The photochemical reflectance index as well as the Chl content (SPAD index) in the second leaves were greatly improved by all the treatments over time as compared to the iron deficient control, however the Fe positive treatments did not show significant differences compared to each other (Fig. 8 and 9).

Experiment 4

In the fourth experiment we aimed to assess the effective concentration of Fe-humicin as an iron source in regenerating plants from iron deficiency. For this purpose, fully iron deficient plants were grown for two weeks, then supplied with 3 concentrations of Fe-humicin for 5 days. During and after the experiment, fresh and dry mass, relative Chl content, Fv/Fm, PRI and the final pH of the culture media were measured.

Table 5.: The final pH of the culture media post-harvest in Experiment 4. The different letters indicate statistically significant differences on the 0.05 confidence level.

Fe concentration (µM)	pH	SD
50	4,67[a]	*0,19*
10	4,42[a]	*0,14*
5	3,99[b]	*0,03*
Ctrl -Fe	3,92[b]	*0,28*

The greater Fe-humicin concentration resulted in a lower degree of acidification, while sustained iron deficiency corresponds to the lowest pH value.

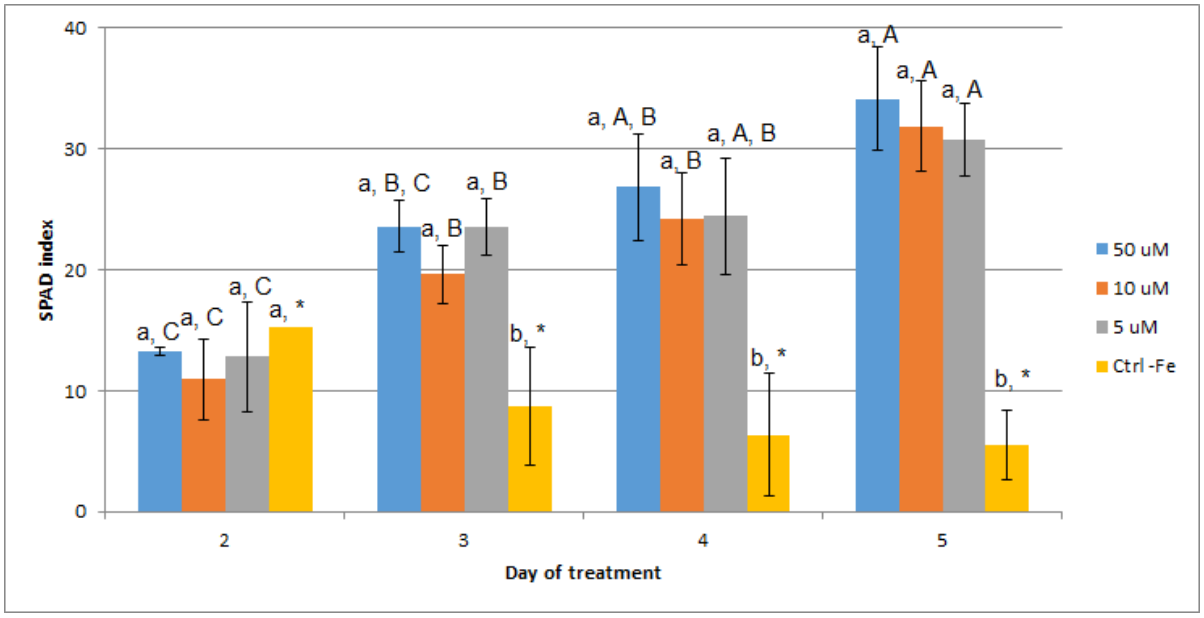

Figure 10.: SPAD indexes corresponding to each treatment, measured on the second leaves. The x axis represents the days spent under treatments, which were Fe-humicin in 50 µM, 10 µM and 5µM concentration, while Ctrl -Fe was left iron deficient. The different lowercase and capital letters designate statistically significant differences on the 0.05 confidence level within days and within treatments, respectively. Ctrl –Fe did not show any meaningful change over the course of the experiment.

Table 6.: The maximum quantum efficiency of photosystem II, as measured on the first and second leaves. In all cases, ctrl is statistically different on the 0.05 confidence level, the other treatments are identical.

	Second leaf		**First leaf**	
Treatment	**Mean**	**SD**	**Mean**	**SD**
50 µM	0.832	*0.009*	0.82	*0.022*
10 µM	0.834	*0.006*	0.822	*0.008*
5 µM	0.838	*0.006*	0.82	*0.004*
Ctrl -Fe	0.733	*0.058*	0.625	*0.027*

The treatments with different concentrations of Fe-humicine showed no significant difference in Chl content and the maximum quantum efficiency of photosystem II, but they are sharply separated from the values measured on the iron deficient plants. According to Figure 10., the plants treated with Fe-humicin rapidly regenerated from iron deficiency, meanwhile the Chl content in the leaves of the control plants remained virtually unchanged. (Fig. 10, Table. 6)

Experiment 5

The fifth experiment aimed to determine the efficiency of Fe-humicin to provide Fe to the ferric chelate reductase enzyme. For this purpose the manufacturer provided Fe-humicin made with or without ascorbic acid and also humate with ascorbic acid but without Fe. For the enzyme assay, plants were grown all the way under totally iron deficient conditions and then they were supplied with the products for two hours. We have applied Fe deficient control and another one supplied with Fe-EDTA. The assay was performed after the two-hour pre-treatment time to assess the extent of downregulation of enzyme activity upon Fe utilization.

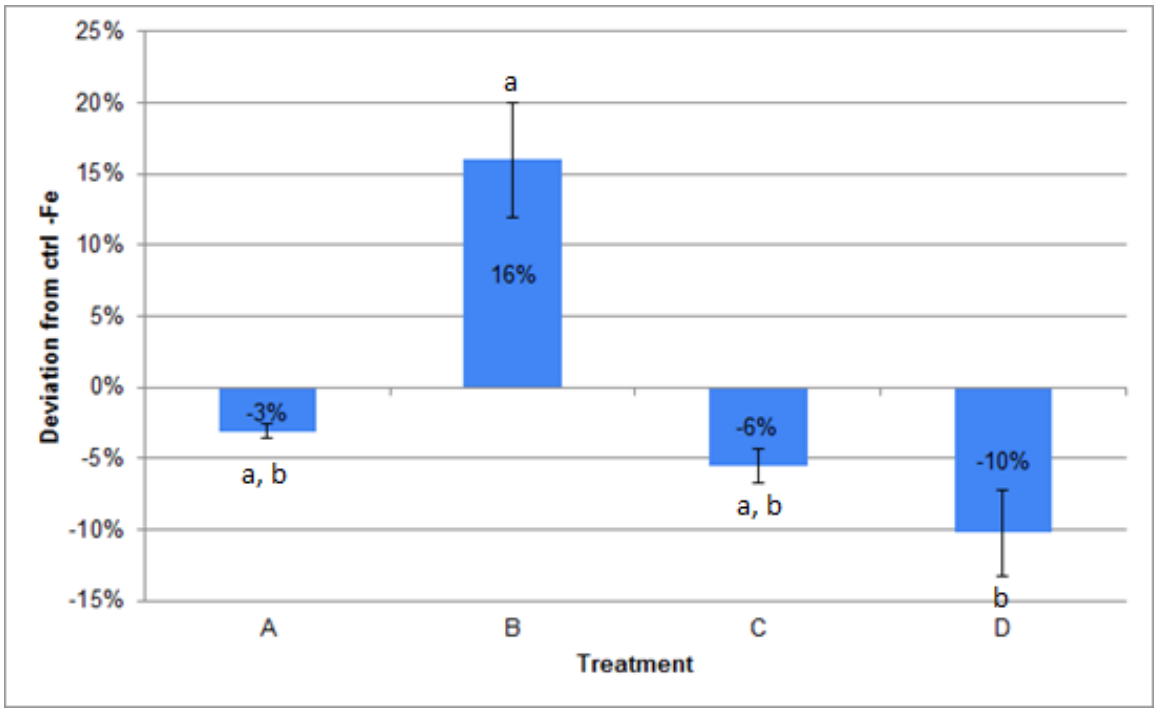

Figure 11.: Fe reduction in root samples as measured in experiment six, expressed as percentage of deviation from the iron deficient control. "A" – Fe-EDTA, "B" – humate and ascorbic acid, "C" – Fe-humicin without ascorbic acid, "D" – Fe-humicin with ascorbic acid. The different letters indicate that the treatments are significantly different on the 0.05 confidence level.

Table 7.: Ferric chelate reduction (FCR) in root samples as measured in experiment six, expressed in nmole Fe reduced / gram of root fresh mass in one minute.

Treatment	**FCR**	**SD**
ctrl -Fe	130,0[a, b]	28,1
ctrl Fe-EDTA	125,9[a, b]	19,9
Humate + ascorbic acid	150,7[a]	38,1
Fe-humicin – ascorbic acid	122,8[a, b]	26,0
Fe-humicin	116,7[b]	33,9

Humate with ascorbic acid induced a great increase in the rate of Fe(III) chelate reduction relative to both controls, while the addition of Fe-humicin contributed to the downregulation as expected.

Figure 12. and 13.: Chlorotic leaves and swollen root tips, two notable symptoms of young, iron deficient cucumber plants (my own pictures).

Discussion

Humic and fulvic acids are brown, thick substances with a characteristic smell. They are present naturally in soils and decaying plant matter, and due to their useful traits are also important resources in modern agriculture. These relatively large molecules, often polymeric organic acids are known to improve the organic carbon content of soils, and thus their structure, porosity and humidity as well. Humic and fulvic acids can be applied to soils in simple ways, such as via decaying plant matter fermented to specifically aid the creation of these compounds, or artificial products could be used, the manufacturing of which is generally well understood. (YANG et al., 2019) (SUSIC, 2016) (CANELLAS et el., 2015)

According to DE HITA ET AL. (2020), foliar and root application of sedimentary humic acid alters the hormonal balance of the treated plants. The affected hormones among others are cytokinins, auxins and jasmonic acid, while other measurements seem to indicate that these changes and their positive effects are connected to an eustress state induced by the treatment. The article also argues that foliar application may differ greatly from soil treatment in its effect on the roots, and the hormonal system as well. VAN TOL DE CASTRO ET AL. (2021) also state that the slight stress effect prompted by humic

substances contribute to increased development of the root system and an enhanced rate of photosynthesis. Moreover, they argue that humic substances obtained from different sources may induce effects of different strengths. In their experiments, the compounds were extracted from vermicompost, river sediment and potting soil, and these induced changes in chlorophyll, carotenoid, nitrogen and sap sugar balance in different ways.

Di Iorio et al. (2019) created several different Fe(II)-humic acid co-precipitates from different iron compounds and their own humic acid, and used them on cucumber plants. Their work proves that humic acids do not only contribute to iron solubilisation and uptake, but also aid mobilization within the plant, so that Fe may get to the leaves, where it is most needed. Moreover, the original source of added iron may play a role in determining solubility.

Humic substances do have other agricultural applications as well. The introduction of humic substances to the soil system has been found to positively affect the diversity of bacterial and fungal communities within the rhizosphere. These changes were accompanied by a lower occurrence of potentially pathogenic species also. (Jiang et al., 2022) The addition of humic substances appears to inhibit nematode pests throughout most of their life cycles, both by itself and due to its interaction with root metabolism, as well as probably enhancing biocontrol from beneficial soil-dwelling organisms. (Nagachandrabose and Baidoo, 2021) Different forms of iron within the soil also interact with all the other nutrients available to the plants. Wu et al. (2020), working with biochar systems, found that chars treated with Fe(II) were significantly better at absorbing, retaining and providing P.

As compared to other commercial products, Fe-humicin contains a reducing agent, ascorbic acid. Without this additive, the Fe-humicin product contained about 10% ferric component but the rest was preserved from the original constituent $FeSO_4$ as ferrous Fe. The ascorbic acid added to Fe-humicin was proven to be changing the ratios of different oxidation states and all Fe remained in ferrous form. In all cases, ferrous ions were present as multiple different components, either in complex with humate side chains' moieties or in hexaaqua coordination. Since no Fe(III) ions were recorded in the commercial product, it can be concluded that ascorbic acid is effective in maintaining Fe ions in a reduced state. (*Fig. 1 and 2*) Di Iorio et al. (2019) used different concentrations of FeSO4, reacted with their humic acid solution (extracted from

leonardite) at neutral and alkaline pH. The resulting co-precipitates have been determined to contain the iron in crystalline magnetite or less orderly gethite and ferrihydrite forms.

The Fe metabolism and general photosynthetic capability of plants is known to be influenced by organic compounds. AHHAMED ET AL. (2020) found that both Fe stressed and deficient cucumber plants benefited from foliar melatonin treatment in all metrics measured, including biomass production, gas exchange rate, photosynthetic pigment concentration and leaf iron content. Foliar or soil application of gamma-aminobutyric acid (GABA) may also alleviate the effects of iron deficiency through influencing iron accumulation as well as photosynthetic pigment and auxin production. (GUO ET AL., 2020)

Fe-humicin generally performed similarly to Fe-EDTA, a widely applied Fe fertilizer at equal and lower iron concentration. Higher doses of Fe-humicin caused a statistically significant increase in root mass, while not having a meaningful effect on the mass and appearance of aboveground parts. (*Fig. 3 and 4*) The relative Chl contents of the second leaves reveal that a steady supply of even 5 μM Fe-humicin was sufficient to achieve healthy levels of Chl production in the young plants. (*Fig. 5*) The uniformity of the maximal quantum efficiency of photosystem II also indicates a healthy construction of the photosynthetic apparatus, compared to iron deficient and abundantly supplied plants (*Table 2.*). These findings are in accordance with the results of DI IORIO ET AL. (2019).

Fe supplementation beyond the amount minimally necessary for the establishment of healthy photosynthetic pigment levels and a functional electron transport chain has been described to cause no further iron accumulation inside the leaf chloroplasts, but rather in other tissues and organelles in rape (*Brassica napus* L. cv DK Exquisite). The results from Experiment 1 therefore coincide with the findings of SÁGI-KAZÁR et al. (2021)

In alkaline and buffered conditions, the iron content of Fe-citrate and Fe-EDTA, the conventional Fe supplying agents, becomes unavailable to the plant, as it is unable to mobilize them through acidification (*Table 3.*) Despite this, the plants' Fe-reducing system remained functional, thus the iron content of Fe-EDDHA, being stable in a wide pH range (KLEM-MARCINIAK et al., 2021) was still available, as seen on the photosynthetic

pigment production and the functionality of the electron transport chain (*Fig. 6 and 7, Table 4*). Fe-humicin behaved similarly to Fe-citrate and Fe-EDTA, therefore it is not stable and accessible across a wide pH range in nutrient solutions. MARTÍN-FERNÁNDEZ et al. (2012) produced similar results with soybean (*Glycine max* cv. Klaxon) when comparing the availability of Fe-EDDHA to Fe- N-(1,2-dicarboxyethyl)-D,L-aspartic acid (IDHA) in a calcareous environment.

In the slightly acidic range of the unbuffered culture medium, the efficacy of Fe-humicin was measured in comparison with that of Fe-citrate and Fe-EDTA in regenerating iron deficient plants. When applied in equal concentrations, there seems to be no significant difference between the three iron compounds in the speed and final grade of regeneration, as shown by the photosynthetic pigment production (*Fig. 8 and 9*). Therefore it can be concluded that under unstressed conditions, the iron content of Fe-humicin is available unimpeded, much like that of the other two compounds. How much of this effect might be due to the presence of ascorbic acid was the aim of another experiment.

During a 5-day treatment period, different concentrations of Fe-humicin downregulated the acidification response of Fe deficient plants in a dose-dependent manner (*Table 6.*). The concentration of Chl in the second leaf and the maximal quantum efficiency of photosystem II, measured on both leaves on the fifth day, also indicate that recovery was complete in all treatments, except the iron deficient control (*Fig. 10., Table 7.*). Thus, increasing the Fe-humicin concentration in the nutrient solution may accelerate the regeneration but the measurements made on photosynthetic pigments do not conclusively support this. Nevertheless, even in a short while (5 days), the lowest applied concentration (5μM) has proved to be sufficient in restoring the plants to healthy condition.

Fe-humicin may provide ferrous ions to the root cells without enzymatic reduction. Based on this assumption it might be more efficient as compared to stable ferric chelates that require reduction prior to the uptake. It was found that humate containing ascorbic acid but not added iron increased the ferric chelate reductase activity of root cells after a two hour exposure before the treatment. In contrast, Fe-humicin (with ascorbic acid) somewhat decreased the ferric chelate reduction while Fe-EDTA did not modify it compared to untreated, iron deficient plants. This seems to

confirm our assumption, but, unfortunately, the dataset from the experiment did not support statistically confirmed conclusions, therefore further repetitions of this experiment are necessary to reach a conclusion (*Fig. 11.*, *Table 8.*).

Conclusions

Young, Fe deficient cucumber plants show specific symptoms e.g. short lateral roots with swollen tips, leaf chlorosis and intense acidification of the culture medium. The application of Fe-humicin as Fe source results in a gradual downregulation of acidification, greening of the leaves due to newly commenced Chl synthesis and the recovery of photosystem II functions. Fe-humicin proved to be a viable Fe supply in slightly acidic environment while it is ineffective under alkaline conditions perhaps because the mobilization of Fe ions for uptake is not possible. The efficiency of the Fe-humate complex is most probably based on the oxidation state of Fe and the ascorbic acid content of the product, but the specific reactions must be further studied to reach reliable, applicable conclusions.

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