Synthetic Phytosiderophore, Proline-2'-Deoxymugineic Acid, is Efficiently Utilized by Dicots

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Abstract

Purpose: Phytosiderophores (PS) from grasses solubilize sparingly soluble iron (Fe), and the resultant PS-Fe is an Fe source, even for dicots. Recently, the synthetic PS proline-2'-deoxymugineic acid (PDMA) has been developed as a moderately biodegradable Fe fertilizer for grasses. We aimed to investigate whether PDMA-Fe is also a good Fe source for dicots.

Methods: The availability of PDMA-Fe to cucumber was evaluated in calcareous soil and hydroponic cultures under pH 7.0–9.0 by determining chlorophyll concentration, PSII activity, and Fe uptake. EDDHA-Fe, EDTA-Fe, and citrate-Fe were used as controls. The reducibility of Fe chelates by roots was measured to determine the mechanism underlying differences in availability. Expressions of Fe deficiency-inducible genes (*CsFRO1* and *CsIRT1*) were analyzed to estimate the Fe status in plants.

Results: Application of PDMA-Fe and EDDHA-Fe to calcareous soil reduced Fe-deficient chlorosis to a similar extent; however, shoot Fe concentration was higher in the PDMA-Fe treatment. In the hydroponic culture, PDMA-Fe had higher availability than the other chelates at every pH, which was confirmed by higher PSII activity and lower expression of Fe deficiency-inducible genes. The reducibility assay revealed that the reduction level of PDMA-Fe was greater than that of EDTA-Fe and citrate-Fe under alkaline pH.

Conclusion: PDMA-Fe is utilized by cucumber roots more efficiently than traditional synthetic chelates in both calcareous soil and hydroponic cultures. The higher availability of PDMA-Fe may be attributed to its higher reducibility. Our findings suggest that PDMA-Fe could be a good Fe fertilizer for dicots.

Introduction

Iron (Fe) is the third most abundant mineral in the Earth's crust; however, under aerobic conditions, the concentrations of Fe^{3+} and Fe^{2+} ions in soil solution are below 10^{-15} M at pH > 6, thus limiting plant growth at neutral or alkaline pH (Lindsay and Schwab 1982; Guerinot and Yi 1994). Therefore, plants have developed two mechanisms for Fe acquisition (called "Strategy I" and "Strategy II") to cope with this limitation (Marschner and Römheld 1986). Strategy II plants, which are mostly grasses, respond to Fe deficiency by secreting phytosiderophores (PS) into the rhizosphere which solubilize Fe(III) (Takagi 1976; Ma and Nomoto 1996; Ueno et al. 2007; Nozoye et al. 2011). Plants then take up PS-Fe complexes through yellow stripe 1-like (YSL) transporters without prior reduction (Curie et al. 2001; Murata et al. 2006; Inoue et al. 2009). In contrast, Strategy I plants, including non-grass monocots and dicots, release organic compounds including flavins and iron-mobilizing coumarins in response to Fe deficiency to mobilize rhizosphere Fe through reduction and/or chelation (Römheld and Marschner 1983; Jin et al. 2007; Sisó-Terraza et al. 2016; Tsai and Schmidt 2017). Fe chelates are then reduced by plasma membrane-bound Fe(III) reductase and subsequently taken up via Fe^{2+} transporters, such as iron-regulated transporter 1 (IRT1) (Eide et al. 1996). The acidification of the rhizosphere by H^+ release is also required to increase Fe solubility and to ensure Fe(III) reductase activity in this strategy (Römheld and Nikolic 2007). Based on the difference in mechanism, sparingly soluble Fe(III) is considered a substrate

for the Strategy II system, while Fe(III) chelates are major Fe sources for the Strategy I system (Römheld and Marschner 1986).

To correct Fe deficiency in crops that insufficiently induce adaptive responses to Fe starvation, synthetic Fe chelates such as Fe-ethylenediamine-*N,N,N',N'*-tetraacetic acid complex (EDTA-Fe) and Fe-ethylenediamine-*N,N'*-bis(2-hydroxy-phenyl acetic acid) complex (EDDHA-Fe) are supplied to the foliage or soil as Fe fertilizers (Lucena 2006; Römheld and Nikolic 2007). Among them, EDDHA-Fe is the most effective fertilizer for increasing soluble Fe in calcareous soil for uptake by the roots because of its higher stability constant at high pH (López-Rayó et al. 2009). However, the non-biodegradability of these chelates and their accumulation in the environment remains a concern (Nowack 2002; Hyvönen et al. 2003; Schenkeveld et al. 2012). Several biodegradable chelates have been studied as alternatives to traditional chelates (Pinto et al. 2014). Some of them were demonstrated to have a similar or higher phytoavailability than EDTA-Fe for various plant species when applied to hydroponic or soilless culture media (Villén et al. 2007; Nowack et al. 2008; Hasegawa et al. 2011, 2012). However, none of them had greater efficacy than EDDHA-Fe under both culture conditions (Albano and Merhaut 2012; López-Rayó et al. 2019). Application of microbial siderophores to roots has been proposed as an environmentally friendly alternative to provide Fe to plants (Vansuyt et al. 2007; Nagata et al. 2013; Ahmed and Holmström 2014). However, the redox potential of Fe(III) captured by microbial siderophores is generally too low to be reduced by the roots. Thus, microbial siderophore-Fe has a low availability to plants unless high reducibility is imparted (Ueno et al. 2019) or the ligand is substituted with PS (Ahmed and Holmström 2014).

Recently, a novel synthetic PS, proline-2'-deoxymugineic acid (PDMA), has been developed as a promising Fe fertilizer (Suzuki et al. 2021). The key structural difference between PDMA and natural 2'-deoxymugineic acid (DMA), that is, substitution of L-proline for L-azetidine, contributes to moderate biodegradability and low synthesis cost. Application of PDMA with or without Fe to calcareous soil promoted Fe uptake in rice plants more effectively than EDDHA-Fe and EDTA-Fe. The higher availability of PDMA-Fe was attributed to the fact that PDMA-Fe can be directly taken up by YSL transporters. Since it has been suggested that dicot plants can utilize PS when intercropped with grasses (Zuo et al. 2000; Ma et al. 2003; Cesco et al. 2006; Ueno and Ma 2009), PDMA-Fe could also be a promising Fe source for dicots. Therefore, the present study aimed to evaluate the availability of PDMA-Fe to the Strategy I system using dicots. We investigated the effects of PDMA-Fe application on calcareous soil and pH-dependent availability in hydroponic cultures, and revealed that PDMA-Fe could be a superior Fe fertilizer to the traditional synthetic chelates, even for Strategy I plants.

Materials And Methods

Preparation of Fe(III) chelates

Chemical synthesis of PDMA has been reported previously (Suzuki et al. 2021). To prepare the Fe(III) complex, 2.47 M FeCl₃ and 10 mM PDMA were mixed at the molar ratio of Fe:PDMA = 2:1. The pH of the

solution was adjusted to 7.0 with 0.5 M NaOH to precipitate Fe^{3+} as low-soluble Fe(III) oxide-hydroxide or Fe(III) oxide. The suspension was then incubated at 50 °C for 1 h with occasional mixing and then centrifuged at 15,000 rpm for 3 min. The supernatant containing PDMA-Fe was further filtered through a 0.22- μm syringe filter (Hawach Scientific, Xi'an, China) to exclude precipitated Fe. Fe(III) citrate (Cit-Fe) was prepared similarly using citric acid (Cas No. 5949-29-1, Wako, Tokyo, Japan) instead of PDMA. Both Fe chelates were stored at -30 °C until use to avoid biodegradation. EDDHA-Fe (Dissolvine Q-Fe-6, Akzo Nobel, Amsterdam, the Netherlands) and EDTA-Fe (Cas No. 15708-41-5, Dojindo Laboratories, Kumamoto, Japan) were also used in the experiments.

Plant material and culture condition

The availability of PDMA-Fe to Strategy I plants was tested using cucumber (*Cucumis sativus* L., cv Hokushin, Takii, Kyoto, Japan). For the soil culture, seeds were germinated in moistened vermiculite at 27 °C for 4 d. After germination, seedlings were transferred to pots (one plant per pot), which were 4.5–6 cm in diameter and 5 cm tall, filled with 100 g calcareous soil consisting of shelly fossils (pH 9.1, 15 g Fe kg^{-1} soil dry weight) purchased from Nihonkai Hiryo Co. Ltd., Japan. The soil was fertilized with N-P-K fertilizer (15-15-10; Chiyodakasei, SunAgro, Toyama, Japan) at 3 g kg^{-1} soil dry weight and watered daily with distilled water to saturation level (35 mL/100 g soil dry weight/pot). Seedlings were grown for 10 d until true leaves expanded. Seedlings with true leaves showing chlorosis were selected, and the soil was supplemented with or without PDMA-Fe, EDDHA-Fe, or Cit-Fe. The concentration of each Fe chelate in 35 mL soil solution was adjusted to 30 μM (0.586 mg Fe kg^{-1} soil dry weight). Plants were grown in a controlled growth chamber (14 h of light at 27 °C / 10 h of dark at 22 °C; light intensity 75–100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$; relative humidity 50–60%) for 4 d. SPAD values in expanded true leaves were analyzed daily using a chlorophyll meter (SPAD-502Plus, Konica Minolta, Tokyo, Japan). This experiment was also performed on pumpkin (*Cucurbita moschata* L., cv YūYūikki white type, Saitama Gensyu Ikuseikai, Kuki, Japan).

For the hydroponic culture, seeds were germinated on moistened filter paper in Petri dishes at 25–27 °C for 1–2 d in the dark. Germinated seedlings were transferred to a net floated on 0.5 mM CaCl_2 and incubated for 2–3 d. Then, seedlings were pre-cultured with a 1/5 Hoagland nutrient solution (pH 5.8) containing the following macroelements (in mM): KNO_3 (1), $\text{Ca}(\text{NO}_3)_2$ (1), MgSO_4 (0.4), and $(\text{NH}_4)_2\text{H}_2\text{PO}_4$ (0.2), and microelements (in μM): H_3BO_3 (3), MnCl_2 (0.5), ZnSO_4 (0.4), CuSO_4 (0.2), and $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ (0.2). Fe was not added to the nutrient solution to induce Fe-deficient chlorosis. To investigate the effect of PDMA-Fe on relieving Fe deficiency, seedlings grown for a week were exposed to a treatment solution containing macroelements and 0.5 μM PDMA-Fe, EDDHA-Fe, or Cit-Fe. The treatment solution was buffered with 1 mM piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES)-NaOH (pH 7.0), 3-[4-(2-hydroxyethyl)-1-piperazinyl]propanesulfonic acid (EPPS)-NaOH (pH 8.0), or *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES)-NaOH (pH 9.0). The solution was continuously aerated and replenished daily, and the pH was adjusted twice per day. The SPAD value in expanded true leaves was recorded daily during the 4 d of treatment. The availability of PDMA-Fe was also compared with that of a

synthetic chelate with high stability in the high pH range (7.5–12), *N,N'*-bis(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid (HBED)-Fe(III) (Adob, Poznań, Poland) (Ma et al. 1994); a natural microbial siderophore, deferroxamine B (DFOB)-Fe(III) (Sigma-Aldrich); and a synthetic microbial siderophore with high reducibility, tris[2-((*N*-acetyl-*N*-hydroxy)glycylamino)ethyl]amine (TAGE)-Fe(III) (Matsumoto et al. 2001; Ueno et al. 2019) at pH 8.0.

After the treatments, plants were divided into roots, true leaves, and the other aerial parts (seed leaves and stems), washed twice with distilled water, blotted, and subjected to element concentration measurements.

Determination of element concentrations

Harvested samples were dried at 70 °C, weighed, digested with 60% (v/v) HNO₃ at 140 °C, and then diluted with distilled water to appropriate concentrations. The concentrations of Fe, Zn, Mn, and Cu in the digested solution were determined using atomic absorption spectrometry (AA-6800, Shimadzu, Kyoto, Japan).

Quantitative RT-PCR

Expression of Fe deficiency-responsive genes, *ferric reduction oxidase 1* (*CsFRO1*) (Acc. No. AY590765.1, <https://www.ncbi.nlm.nih.gov/>) and *iron-regulated transporter 1* (*CsIRT1*) (Acc. No. XM_004145406.3), were compared among Fe chelate treatments to estimate differences in availability. Seedlings were pre-cultured in the absence of Fe for 4 d, then exposed to a nutrient solution (pH 9.0, 1 mM CHES-NaOH) containing 0.5 µM PDMA-Fe, EDDHA-Fe, or Cit-Fe for 72 h. The pH of the nutrient solution was adjusted three times per day. The nutrient solution was replenished 48 h after the Fe treatment. Total RNA was extracted from roots with the ISOSPIN Plant RNA kit (Nippon Gene, Tokyo, Japan), treated with DNase I (Toyobo, Osaka, Japan), and converted to cDNA using ReverTra Ace (Toyobo). Gene expression was measured using quantitative reverse transcription polymerase chain reaction (qRT-PCR) with the primers 5'-TGTGGGCAACAACCTATTCCTC-3' and 5'-AGGAGATGCCAACATGGAAG-3' for *CsFRO1*, and 5'-CTCATTGCGAGTGTTCATTGG-3' and 5'-GAATGATACCTGCTGCGAAAG-3' for *CsIRT1*. *Actin 7* (Acc. No. XM_011659465.2), used as an internal control, was analyzed using the primers 5'-TTGCAGACAGGATGAGCAAG-3' and 5'-ACCCTCCAATCCAAACACTG-3'. qRT-PCR was carried out using a KOD SYBR qPCR mix (Toyobo) on a Prism 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA).

Reducibility assay

The reducibility of Fe(III) chelates by plant roots was analyzed according to Romera et al. (1996), with some modifications. Roots of cucumber grown without Fe for 5 d were exposed to 10 mL of assay solution (0.2 mM CaSO₄, 5 mM PIPES at pH 7.0, EPPS at pH 8.0, or CHES at pH 9.0; 0.1 mM PDMA-Fe, EDTA-Fe, or Cit-Fe; and 0.2 mM bathophenanthroline disulfonic acid [BPDS] [Cas. No. 98645-86-4, Dojindo Laboratories]) for 1 h with occasional mixing at 25 °C in the dark. The solution was read at 535 nm using a spectrophotometer (V-630Bio, Jasco, Tokyo, Japan). After subtracting the A₅₃₅ of the solution without

the plant from that of the respective solution, the BPDS-Fe(II) concentration was calculated using the extinction coefficient of $22.14 \text{ mM}^{-1} \text{ cm}^{-1}$. The fresh weight of the roots was recorded.

Measurement of O₂-evolution rate

O₂ exchange was monitored using a ROS Field Master (RFM) with a closed leaf-type chamber (Bunkoukeiki Co., Ltd, Tokyo, Japan). An RFM is a device that can simultaneously take a P700 absorption measurement and determine the oxygen evolution rate. The device consists of a measurement light, far-red light, actinic red light, LED light source unit, closed chamber (including light detector, oxygen measurement sensor, and temperature/humidity/pressure sensor), signal processing unit, and touch panel display as the user interface. The device is powered by a 12V lithium-ion battery. The sample was irradiated with $16 \times 16 \text{ mm}$ light from the light guide path, and the transmitted light was received by a photodetector. In addition, the oxygen concentration in the closed chamber was measured using a galvanic oxygen sensor. The conversion of the sensor signal for oxygen measurement was calculated from the oxygen concentration in 1 mL of air and the amount of signal change, and the oxygen change was proportional to the measurement signal. In addition, the temperature, humidity, and atmospheric pressure inside the chamber were measured to compensate for the signal value of the oxygen sensor. The closed chamber has two doorways, one of which can be fitted with a tube to allow human exhalation to saturate the interior of the chamber to a saturated CO₂ state. As a result, the inside of the closed chamber can be brought into a saturated CO₂ state, and the maximum photosynthetic activity can be measured. A leaf disc (2.5 cm^2) excised from the true leaves of seedlings cultured with Fe chelate (PDMA-Fe, EDDHA-Fe, or Cit-Fe) at pH 9.0 for 6 d was placed in the chamber. Actinic red light (660 nm) was illuminated from the top of the chamber, and the photon flux density (PFD) was adjusted to $1000 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. As the chamber is a closed system, CO₂ in the chamber was consumed during photosynthesis; therefore, additional CO₂ was supplied with expiratory air (assumed to be CO₂ saturated air).

Statistical analysis

Data were analyzed using Tukey's tests with BellCurve for Excel (Social Survey Research Information, Tokyo, Japan). Significant differences ($P < 0.05$) are indicated by different letters.

Results

Effects of PDMA-Fe application to calcareous soil

To evaluate PDMA-Fe as an Fe source for Strategy I plants, we used cucumber and examined the effect of $30 \mu\text{M}$ PDMA-Fe application as a soil solution by comparing it with other Fe chelates. At 4 d after treatment, cucumber treated with Cit-Fe and without Fe treatment (control) showed Fe-deficient chlorosis, whereas plants treated with PDMA-Fe and EDDHA-Fe did not (Fig 1a). SPAD values in the Cit-Fe treatment and control groups were similar, ranging from 17 to 21 within 4 d (Fig. 1b). In contrast, the SPAD value linearly increased from 20 to 35 under the PDMA-Fe and EDDHA-Fe treatments (Fig. 1b).

For elemental analysis, shoots were divided into true leaves and other parts containing seed leaves and stems. Fe accumulated in the true leaves was mainly derived from the soil solution containing Fe chelates because Fe is rarely re-translocated from older leaves to younger leaves (Römheld and Nikolic 2007), while Fe accumulated in the other parts originated from both the seed and soil solutions. Thus, the true leaves were useful for estimating the rate of Fe uptake from Fe chelates in comparison with the whole shoot. The Fe concentration in the true leaves in the PDMA-Fe treatment was 34% higher than that in the EDDHA-Fe treatment, and more than twice as high as that in the Cit-Fe treatment and control (Fig. 2a). The Fe concentration in the other aerial parts was also significantly higher in the PDMA-Fe treatment than in the other treatments, but to a lesser degree (Fig. 2a). A greater effect of PDMA-Fe than EDDHA-Fe was also observed in pumpkin (Supplementary Fig. S1). In the true leaves, Zn concentration was more than 1.5-times higher than that in the other treatments (Fig. 2b), whereas the Mn concentration was almost half that in the control and the Cit-Fe treatment but did not differ from that in the EDDHA-Fe treatment (Fig. 2c). In seed leaves and stems, both Zn and Mn concentrations showed similar tendencies to those in the true leaves but to a lesser extent (Figs. 2b, c). The Cu concentration did not differ significantly among the treatments (Fig. 2d). The higher shoot Fe concentration under the PDMA-Fe application suggests that PDMA-Fe is more available than the other Fe chelates in alkaline soil.

Efficacy of PDMA-Fe to relieve Fe deficiency at various pH levels

To investigate the pH-dependent efficacy of PDMA-Fe to improve Fe chlorosis, we hydroponically applied Fe chelates under neutral–alkaline pH. Four days of exposure to PDMA-Fe improved Fe chlorosis more effectively than EDDHA-Fe at every pH (Fig. 3a). The SPAD value increased with time under the PDMA-Fe treatment and exhibited 1.4-, 2.1-, and 1.5-times higher rates at pH 7.0, 8.0, and 9.0, respectively, than that under the EDDHA-Fe treatment at 4 d ($P < 0.01$; Fig. 3b). In the Cit-Fe treatment, Fe-deficient chlorosis did not improve regardless of pH, and the SPAD value decreased with time and reached significantly lower levels than that in the EDDHA-Fe treatment at 4 d ($P < 0.01$; Figs. 3a, b). Recovery from Fe deficiency was also determined by analyzing the rate of O_2 -evolution as an index of PSII activity in true leaves of cucumber grown at pH 9.0. The rate of O_2 -evolution increased and reached a steady state at about 3 min in the PDMA-Fe and EDDHA-Fe treatments, while it did not change from a low rate in the Cit-Fe treatment (Fig. 4). The rate in the PDMA-Fe treatment was twice as high as that in the EDDHA-Fe treatment at the steady state ($P < 0.01$; Fig. 4).

The Fe concentration in true leaves was 1.6- and 5.5-times higher under the PDMA-Fe treatment than under the EDDHA-Fe and Cit-Fe treatments at pH 7.0, respectively (Fig. 5a). The concentration in the PDMA-Fe treatment decreased with elevated pH but was still more than twice as high as that in the other treatments (Fig. 5a). In seed leaves and stems, Fe concentration was also significantly higher under the PDMA-Fe treatment than under the other treatments at pH 7.0 and 8.0, but did not differ at pH 9.0 (Fig. 5b). In roots, the Fe concentration did not differ among the treatments at pH 7.0 or 8.0, but it was higher in the Cit-Fe treatment than in the PDMA-Fe treatment at pH 9.0 (Fig. 5c).

The comparative analysis of Fe chelates showed that the effect on chlorophyll concentration was in the following order: PDMA-Fe > EDDHA-Fe and TAGE-Fe > EDTA-Fe, HBED-Fe, Cit-Fe, DFOB-Fe, and -Fe (Supplementary Fig. S2a), whereas that on shoot Fe concentration was in the following order: PDMA-Fe > EDDHA-Fe and TAGE-Fe > EDTA-Fe, HBED-Fe and Cit-Fe > DFOB-Fe and -Fe (Supplementary Fig. S2b).

Effects of PDMA-Fe on expression of Fe deficiency-inducible genes

The higher availability of PDMA-Fe at various pH levels was further verified by analyzing the expression of Fe deficiency-inducible genes, *CsFRO1* and *CsIRT1*, which have been demonstrated to be involved in ferric reduction and ferrous uptake in cucumber roots, respectively (Waters et al. 2007). At 72 h after Fe chelate application to Fe-deficient plants, *CsFRO1* expression in the PDMA-Fe treatment was approximately one-fifth of that in the Cit-Fe treatment and two-fifths of that in the EDDHA-Fe treatment (Fig. 6a). *CsIRT1* expression under the PDMA-Fe treatment was similar to that under the EDDHA-Fe treatment, but 41% lower than that under the Cit-Fe treatment (Fig. 6b).

Reducibility of PDMA-Fe

To determine the mechanism underlying the higher availability of PDMA-Fe, we assayed the reducibility of Fe from Fe chelates by cucumber roots. Colorless EDTA-Fe was used instead of EDDHA-Fe because EDDHA-Fe has a strong absorption band in the near region as BPDS-Fe(II) forms in this assay. The rate of PDMA-Fe reduction was 34% higher than that of EDTA-Fe at pH 7.0 (Fig. 7). The difference in reducibility increased with pH; the rates were 3.4- and 5.6-times higher at pH 8.0 and 9.0, respectively, in PDMA-Fe compared to EDTA-Fe. The reducibility of Cit-Fe was significantly lower than that of PDMA-Fe and EDTA-Fe at pH 7.0 and 8.0 but was similar to that of EDTA-Fe at pH 9.0.

Discussion

Grass-borne PS and microbial siderophores can solubilize Fe in soil (Takagi et al. 1988; Ahmed and Holmström 2014), and the resultant Fe complex can be utilized by dicots as substrates of the reduction-based Fe acquisition system (Römheld and Marschner 1986). In the first study of synthetic PDMA (Suzuki et al. 2021), it was demonstrated that the PDMA-Fe complex can be directly taken up by grasses, leading to a higher availability than traditional chelates to the Strategy II system. Here, we provide evidence that PDMA-Fe could also be a good Fe source for Strategy I plants.

In the present study, PDMA-Fe provided more Fe than the other chelates in calcareous soil and hydroponic culture (Figs. 2, 5). Consistent with Fe accumulation, chlorophyll concentration and PSII activity were recovered greatly by PDMA-Fe application (Figs. 1, 3, 4) because Fe is involved in chloroplast development and the electron transport chain (Broadley et al. 2012). Application of PDMA-Fe lowered the expression of Fe deficiency-inducible genes more than the other Fe chelates (Fig. 6), suggesting that Fe status was more effectively improved. Although the constant stability of PDMA to Fe(III) ($\log K = 17.1$) was lower than that of EDTA ($\log K = 25.1$), EDDHA (*o,o*-EDDHA [$\log K = 35.1$], and *o,p*-EDDHA [$\log K = 28.7$]), the higher Fe uptake from PDMA-Fe may be attributed to higher reducibility (Fig. 7). In the case of

EDDHA-Fe, its high availability is due to the formation of highly reducible species, which are induced by the lowering of pH by root H^+ release (Gómez-Gallego et al. 2005; Escudero et al. 2012). Therefore, decreased Fe uptake with increasing pH (Fig. 5) may be due to decreased formation of such species as well as inhibited reductase activity. Although the chemical mechanism underlying the higher reducibility of PDMA-Fe requires clarification, the reduction of PDMA-Fe may be structurally less sensitive to high pH in comparison with that of traditional synthetic Fe chelates. Thus, PDMA-Fe is effective for crops relying on the reduction-based Fe uptake system.

Another possible explanation for the higher availability of PDMA-Fe is that a portion of this complex may be directly taken up by roots via YSL transporters. There is evidence that Strategy I plants can directly take up PS-Fe in intercropping systems. Xiong et al. (2013) reported that DMA secreted from intercropped maize was detected in peanut (*Arachis hypogaea* L.) roots in the same pot, and AhYSL1 expressed in the root epidermis showed transport activity for DMA-Fe in yeast. Recently, several reports have implied the secretion of PS from dicots, such as tomato (Astolfi et al. 2020) and grapevine (Marastoni et al. 2020). In addition, endogenous DMA has been reported to be present in the leaves and xylem of olive plants (Suzuki et al. 2016). These findings suggest that dicots also utilize exogenous and endogenous PS for the uptake and translocation of Fe, supporting our hypothesis. The direct uptake system is thought to be less sensitive to alkaline pH than the reduction-based uptake system, which requires acidification of the soil. Therefore, PDMA-Fe may be more useful for dicot species that highly depend on the direct uptake of PS-Fe.

The first study that compared the availability of PS-Fe and EDDHA-Fe in cucumber was reported by Römheld and Marschner (1986). In contrast to PDMA-Fe, the availability of PS-Fe was similar to or less than that of EDDHA-Fe. This discrepancy may be due to differences in the experimental design. In the previous study, PS secreted from Fe-deficient barley was supplied to the cucumber. Barley is known to secrete hydroxylated analogs of PS in addition to DMA (Ma et al. 1999). Although the capability of PDMA-Fe and DMA-Fe to be transported by YSL is similar (Suzuki et al. 2021), it is possible that the reducibility of PS-Fe is different between analogs, and the reducibility of PDMA-Fe could be equal to or higher than that of natural PS-Fe. For reduction-based uptake systems, highly reducible analogs can be a better Fe source. In fact, there is evidence that the synthetic microbial siderophore-Fe with high reducibility could provide more Fe than natural siderophore-Fe (Ueno et al. 2019).

In conclusion, PDMA-Fe is utilized by cucumber roots more efficiently than traditional synthetic chelates in both calcareous soil and hydroponic cultures. The pH-dependent tests showed that the higher availability of PDMA-Fe may be attributed to the higher reducibility at alkaline pH. Our findings suggest that PDMA-Fe can be a good Fe fertilizer in alkaline soil for Strategy I plants.

Declarations

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Code availability: Not applicable.

Authors contributions: D.U. and M.S. conceived and designed the study. D.U. and Y.I. performed the experiments. C.M., M.O., and T.S. contributed to analysis of photosynthesis. All authors analyzed data. D.U., C.M., and M.S. wrote the manuscript. All authors read and approved the manuscript.

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Figures

Fig. 1

Figure 1

Effects of PDMA-Fe(III) on chlorophyll concentrations in cucumber grown in calcareous soil Seedlings were treated with or without Fe chelates for 4 d. (a) Shoot image and (b) change in chlorophyll concentration (SPAD value) in expanded true leaves. Data are presented as the mean $\pm$ standard deviation (n = 6). Different letters indicate significant differences ($P < 0.05$) using Tukey's test

Fig. 2

Figure 2

Effects of PDMA-Fe(III) on microelement concentrations in cucumber grown in calcareous soil Seedlings were treated with or without Fe chelates for 4 d. Concentrations of Fe (a), Zn (b), Mn (c), and Cu (d) in true leaves and other aerial parts (seed leaves and stems) are shown. Data are presented as the mean $\pm$ standard deviation ($n = 6$). Different letters indicate significant differences ($P < 0.05$) using Tukey's test. n.s., not significant

Fig. 3

Figure 3

Effects of PDMA-Fe(III) on improving Fe chlorosis in hydroponic culture Seedlings were pre-cultured in the absence of Fe for a week and were treated with 0.5 μ M Fe chelates under neutral–alkaline pH (7.0, 8.0, and 9.0) for 4 d. (a) Representative images of shoots and (b) changes in chlorophyll concentration (SPAD value) in expanded true leaves. Data are presented as the mean $\pm$ standard deviation ($n = 4$)

Fig. 4

Figure 4

Effects of PDMA-Fe(III) on improving O₂-evolution in hydroponic culture Seedlings were treated with 0.5 μ M Fe chelates under pH 9.0 for 6 d. Time course analysis of O₂-evolution in the leaf disc excised from the true leaf is shown. Data are presented as the mean $\pm$ standard deviation (n = 4)

Fig. 5

Figure 5

Effect of PDMA-Fe(III) on Fe accumulation in hydroponic culture Seedlings pre-cultured in the absence of Fe for a week were treated with 0.5 μM Fe chelates under neutral–alkaline pH (7.0, 8.0, and 9.0) for 4 d. Concentration of Fe in true leaves (a), seed leaves and stems (b), and roots (c) are shown. Data are presented as the mean ± standard deviation (n = 4). Different letters indicate significant differences (P < 0.05) using Tukey's test. n.s., not significant

Fig. 6

Figure 6

Effects of PDMA-Fe(III) on Fe status Seedlings pre-cultured in the absence of Fe for 4 d were exposed to a nutrient solution (pH 9.0) containing 0.5 μ M Fe chelates for 72 h. Relative expression levels of ferric reduction oxidase 1 (CsFRO1) and iron-regulated transporter 1 (CsIRT1) in the roots grown under the Cit-Fe(III) treatment are shown. Actin 7 was used as the internal control. Data are presented as the mean $\pm$ standard deviation (n = 3). Different letters indicate significant differences (P < 0.05) using Tukey's test

Fig. 7

Figure 7

Reducibility of Fe(III) from Fe chelates by cucumber roots Roots of cucumbers grown hydroponically without Fe for 5 d were used. Reducibility was examined under neutral–alkaline pH (7.0, 8.0, and 9.0) using the bathophenanthroline disulfonic acid assay. Data are presented as the mean $\pm$ standard deviation ($n = 3$). Different letters indicate significant differences ($P < 0.05$) using Tukey's test

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