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REGULAR ARTICLE

A synthetic phytosiderophore analog, 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 a calcareous substrate and hydroponic cultures at pH 7.0–9.0 by determining chlorophyll level, 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 were analyzed to estimate the Fe status in plants.

Results The application of PDMA-Fe and EDDHA-Fe to a calcareous substrate reduced Fe-deficient chlorosis to a similar extent; however, the shoot Fe concentration was higher in the PDMA-Fe treatment. In the hydroponic culture, the availability of PDMA-Fe was higher than that of the other chelates at all pH levels, and this was confirmed by higher PSII activity and lower expression of Fe deficiency-inducible genes. The reducibility assay revealed that

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the reduction level of PDMA-Fe was greater than that of EDTA-Fe and citrate-Fe under alkaline pH.
Conclusions PDMA-Fe is utilized by cucumber roots more efficiently than traditional synthetic chelates in both calcareous substrate 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.

Keywords Mugineic acid · Iron deficiency · Chlorosis · Chelate · Reduction · EDDHA

Introduction

Iron (Fe) is the fourth most abundant element 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). Iron chelates are then reduced by a 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 mechanisms, sparingly soluble Fe(III) is considered a substrate for the Strategy II system, whereas Fe(III) chelates are the 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 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 because of its higher stability constant at high pH (López-Rayo 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 high phytoavailability compared with 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-Rayo 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) is the substitution of L-proline for L-azetidine, which contributes to moderate biodegradability and decrease 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. 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). Furthermore, recent studies have implied the secretion of PS from dicots, such as tomato (Astolfi et al. 2020) and grapevine (Marastoni et al. 2020). Therefore, in the present study, we aimed to evaluate the availability of PDMA-Fe to the Strategy I system using dicots. We investigated the effects of PDMA-Fe application on a calcareous substrate and the availability depending on pH in hydroponic cultures, and revealed that PDMA-Fe could be an alternative to the traditional synthetic chelates, even for Strategy I plants.

Materials and methods

Preparation of Fe(III) chelates

The chemical synthesis of PDMA has been described previously (Suzuki et al. 2021). To prepare the Fe(III) complex, 2.47 M $FeCl_3$ and 10 mM PDMA were mixed at a molar ratio of 2:1. The pH of the solution was adjusted to 7.0 with 0.5 M NaOH to precipitate the excess Fe^{3+} as Fe(III) oxide-hydroxides or Fe(III) oxides. 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 passed through a 0.22-μm syringe filter (Hawach Scientific, Xi'an, China) to further 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., 'Hokushin'; Takii, Kyoto, Japan). For the calcareous substrate culture, the seeds were germinated in moistened vermiculite at 27 °C for 4 d. After germination, the seedlings were transferred to pots (one plant per pot), 4.5–6.0 cm in diameter and 5 cm tall, filled with 100 g calcareous substrate consisting of shelly fossils [pH(H_2O) 9.1, 10–15 g Fe kg^{-1} substrate dry weight] purchased from Nihonkai Hiryo Co., Ltd., Takaoka, Japan. The calcareous substrate was mined from a site analyzed previously (Morikawa et al. 2004). The substrate was fertilized with N-P-K fertilizer (15-15-10; Chiyodakasei, SunAgro, Toyama, Japan) at 3 g kg^{-1} substrate dry weight and watered daily with distilled water to the saturation level (35 mL/100 g substrate dry weight/pot). Seedlings were grown for 10 d until true leaves expanded. The seedlings with true leaves showing chlorosis were selected, and the substrate was supplemented with or without PDMA-Fe, EDDHA-Fe, or Cit-Fe. The concentration of each Fe chelate in 35 mL substrate solution was adjusted to 30 μM (0.586 mg Fe kg^{-1} substrate 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 (metal halide lamp) 75–100 μmol photons m^{-2} 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). The experiment was also performed on pumpkin (*Cucurbita moschata* L. '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. Subsequent culture was carried out under the same controlled condition described above. Seedlings were transferred to 1.25 L pots (four plants per pot) and pre-cultured with 1/5 Hoagland nutrient solution (pH 5.8) containing the following macroelements (in mM): KNO_3 (1), $Ca(NO_3)_2$ (1), $MgSO_4$ (0.4), and $(NH_4)H_2PO_4$ (0.2), and microelements (in μM): H_3BO_3 (3), $MnCl_2$ (0.5), $ZnSO_4$ (0.4), $CuSO_4$ (0.2), and $(NH_4)_6Mo_7O_{24}$ (0.2). Fe was not added to the nutrient solution to induce Fe-deficiency 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, deferoxamine 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 (cotyledons and stems), washed twice with distilled water, blotted, and used for elemental concentration measurements.

Determination of elemental concentrations

Harvested samples were dried at 70 °C, weighed, digested with 60% (v/v) HNO_3 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, and 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. The total RNA was extracted from the roots using the ISOSPIN Plant RNA kit (Nippon Gene, Tokyo, Japan) at 4 h after lighting, 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′-TGTGGGCAACAACTATTCCTC-3′ and 5′-AGGAGATGCCAACATGGAAG-3′ for *CsFRO1*, and 5′-CTCATTGCGAGTGTCATTGG-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′-ACC CTCCAATCCAAACACTG-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 (approx. 0.15 g fresh weight) of intact cucumber grown without Fe for 5 d were exposed to 10 mL of assay solution (0.2 mM $CaSO_4$, 5 mM PIPES at pH 7.0, EPPS at pH 8.0, or CHES at pH 9.0), supplemented with an Fe source (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 absorbance of 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 an extinction coefficient of 22.14 mM^{-1} cm^{-1}. The fresh weight of the roots was also recorded to calculate the reduction rates. In this assay, both plasma membrane bound reductase and reducing-compound exudations mediated the reduction.

Measurement of O_2-evolution rate

O_2 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 measure P700 absorption 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 12 V lithium-ion battery. The sample was irradiated with a 16 mm × 16 mm light spot 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 CO_2 state. As a result, the inside of the closed chamber can be brought into a saturated CO_2 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 μmol photons m^{-2} s^{-1}. As the chamber is a closed system, CO_2 in the chamber was consumed during photosynthesis; therefore, additional CO_2 was supplied with expiratory air (assumed to be CO_2 saturated air).

Statistical analysis

Data were analyzed using Tukey's tests with Bell-Curve for Excel (Social Survey Research Information, Tokyo, Japan). Significant differences ($P < 0.01$ or $P < 0.05$) are indicated by different letters.

Results

Effects of PDMA-Fe application to a calcareous substrate

To evaluate PDMA-Fe as an Fe source for Strategy I plants, we used cucumber and examined the effect of 30 μM PDMA-Fe application to the substrate using other Fe chelates as controls. At 4 d after the treatment, plants treated with Cit-Fe and with no Fe (control) showed Fe-deficiency chlorosis, whereas plants treated with PDMA-Fe and EDDHA-Fe did not (Fig. 1a). The SPAD values in the Cit-Fe and control plants were similar, ranging from 17 to 21 during the treatment (Fig. 1b). In contrast, the SPAD value

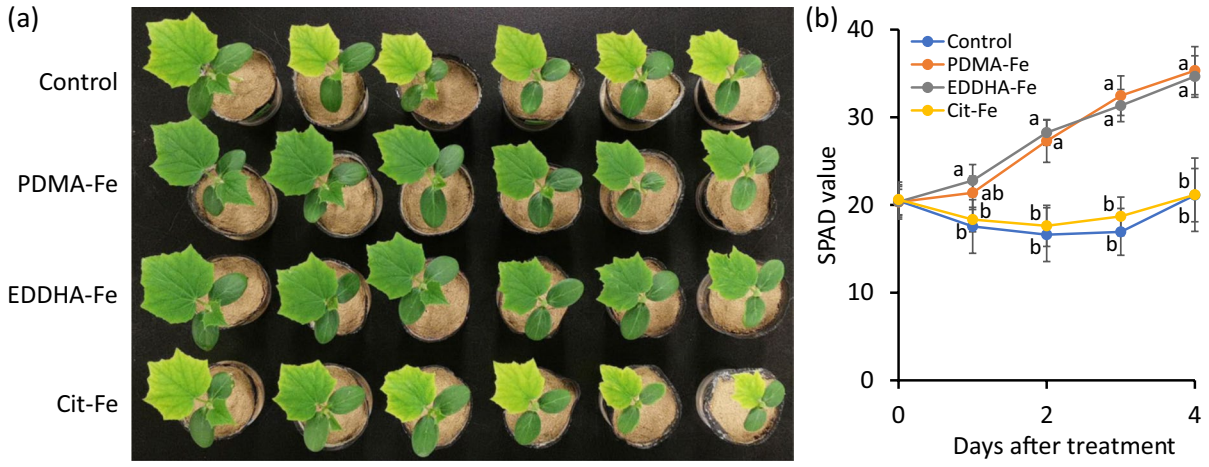

Fig. 1 Effects of PDMA-Fe(III) on leaf chlorophyll in cucumber grown in a calcareous substrate. Iron-deficient seedlings were treated with or without Fe chelates. (**a**) Shoot image on day 4 and (**b**) time course of chlorophyll level (SPAD value) in expanded true leaves. Data are presented as mean ± standard deviation ($n = 6$). Different letters indicate significant differences ($P < 0.01$) using Tukey's test

increased progressively from 20 to 35 in the PDMA-Fe and EDDHA-Fe treatments (Fig. 1b).

For elemental analysis, the shoots were divided into true leaves and other parts, including cotyledons and stems. Iron accumulated in the true leaves is likely to be derived from the soil solution containing Fe chelates, because Fe is rarely re-translocated from older leaves to younger leaves (Römheld and Nikolic 2007), whereas Fe accumulated in the other parts originate from both seed and substrate solutions. Thus, the true leaves can provide an estimate of 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 (differences significant at $P < 0.05$), but to a less degree (Fig. 2a). A greater effect of PDMA-Fe than EDDHA-Fe was also observed in pumpkin (Supplementary Fig. S1). In the true leaves, the Zn concentration was more than 1.5-fold higher than that in the other treatments (Fig. 2b), whereas the Mn concentration was almost half of that in the control and Cit-Fe treatment, it but did not differ from that in the EDDHA-Fe treatment (Fig. 2c). In cotyledons and stems, both Zn and Mn concentrations showed similar tendencies to those in the true leaves but to a less extent (Fig. 2b, c). The Cu concentration did not differ significantly among the treatments (Fig. 2d). The higher shoot Fe concentration under PDMA-Fe application supports that PDMA-Fe is more available than the other Fe chelates in alkaline substrates.

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

To investigate the pH-dependent efficacy of PDMA-Fe to improve Fe chlorosis, we applied Fe chelates in hydroponics under neutral–alkaline pH. After 4 d

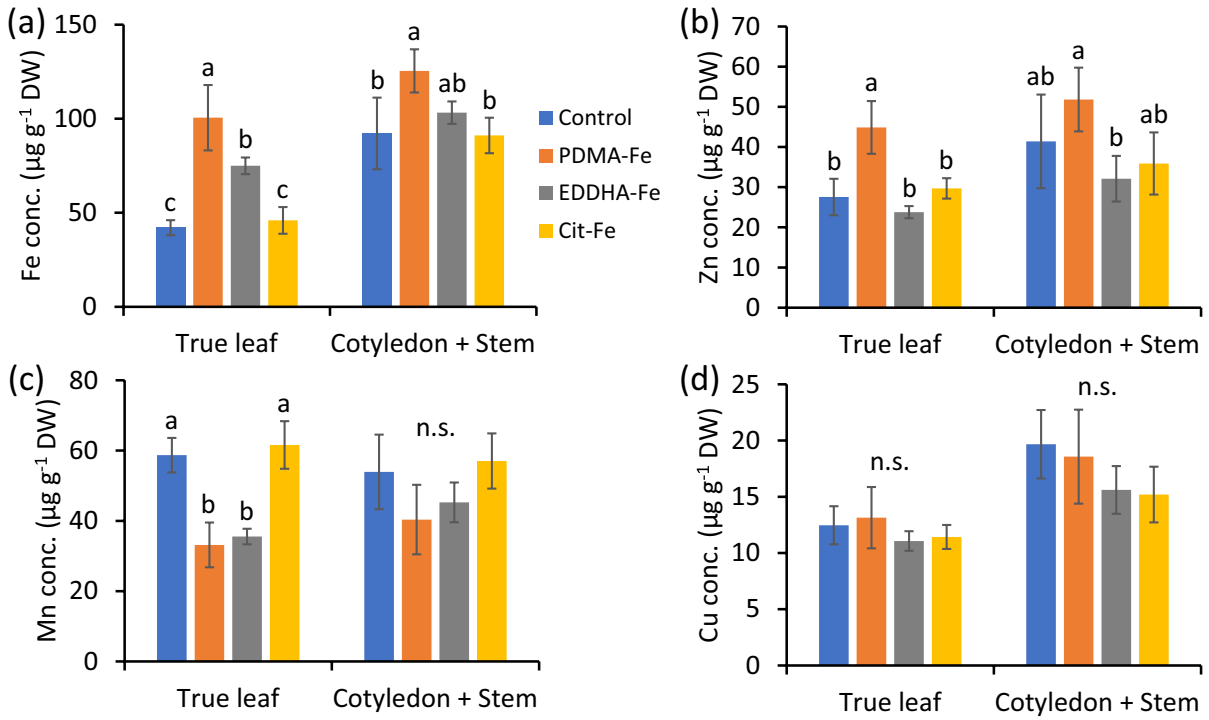

Fig. 2 Effects of PDMA-Fe(III) on microelement concentrations in cucumber grown in a calcareous substrate. Seedlings were treated with or without Fe chelates for 4 d. Concentrations of Fe (**a**), Zn (**b**), Mn (**c**), and Cu (**d**) in true leaves and other aerial parts (cotyledons and stems). Data are presented as mean ± standard deviation ($n = 6$). Different letters indicate significant differences ($P < 0.01$) using Tukey's test. n.s., not significant

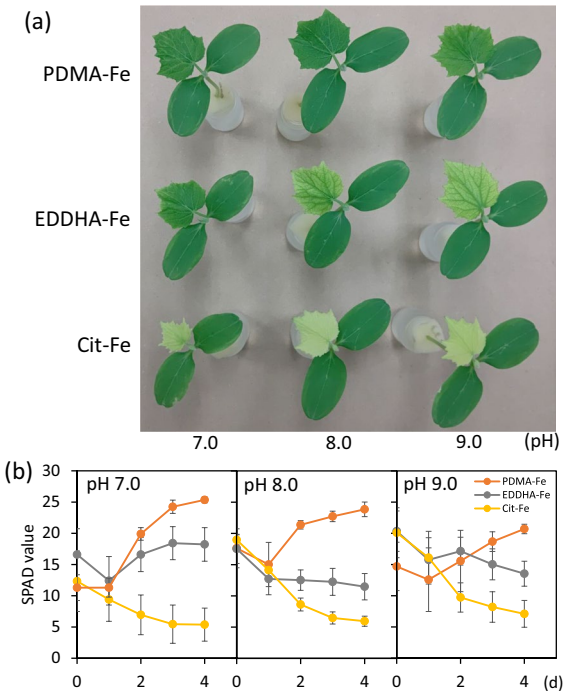

Fig. 3 Effects of PDMA-Fe(III) on improving Fe chlorosis in hydroponic culture. Seedlings were pre-cultured in the absence of Fe for a week and were treated with 0.5 μM Fe chelates under neutral–alkaline pH (7.0, 8.0, and 9.0) for 4 d. (**a**) Representative images of shoots on day 4 and (**b**) time course chlorophyll level (SPAD value) in expanded true leaves. Data are presented as mean ± standard deviation ($n = 4$)

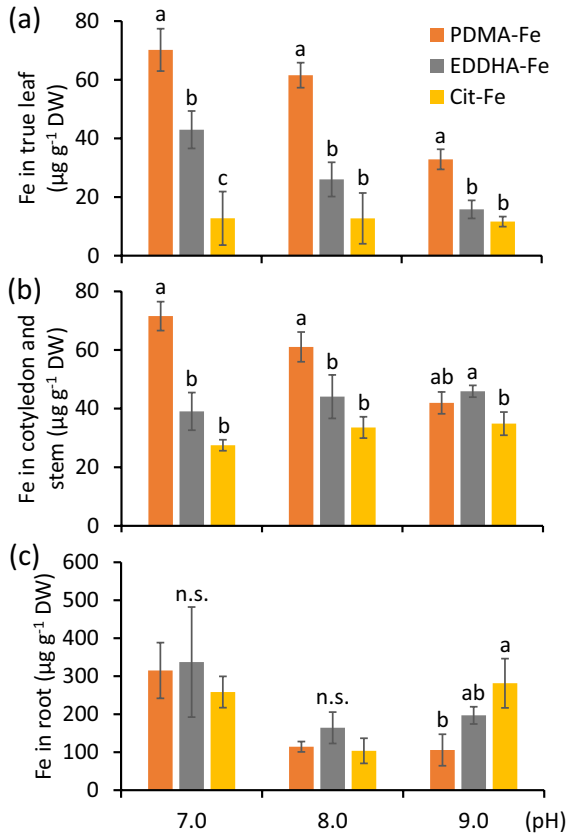

Fig. 4 Effect of PDMA-Fe(III) on Fe accumulation in hydroponic culture. Seedlings pre-cultured in the absence of Fe for a week were treated with 0.5 μM Fe chelates under neutral–alkaline pH (7.0, 8.0, and 9.0) for 4 d. Concentration of Fe in the true leaves (**a**), cotyledons and stems (**b**), and roots (**c**). Data are presented as mean ± standard deviation ($n = 4$). Different letters indicate significant differences ($P < 0.01$) using Tukey's test. n.s., not significant

of exposure, PDMA-Fe relieved Fe chlorosis more effectively than EDDHA-Fe at all pH levels (Fig. 3a). The SPAD value increased with time in the PDMA-Fe treatment and exhibited 1.4-, 2.1-, and 1.5-fold higher rates at pH 7.0, 8.0, and 9.0, respectively, than that under the EDDHA-Fe treatment on day 4 ($P < 0.01$; 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 on day 4 ($P < 0.01$; Fig. 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, whereas a steady negative rate indicating respiration was observed in the Cit-Fe treatment (Supplementary Fig. S2). The rate in the PDMA-Fe treatment was twice as high as that in the EDDHA-Fe treatment under the steady state ($P < 0.01$; Supplementary Fig. S2).

The Fe concentration in the true leaves was 1.6- and 5.5-fold higher under the PDMA-Fe treatment than under the EDDHA-Fe and Cit-Fe treatments at pH 7.0, respectively (Fig. 4a). The concentration in the PDMA-Fe treatment decreased with elevated pH, but it was still more than twice as high as that in the other treatments (Fig. 4a). In the cotyledons and stems, the Fe concentration was also significantly higher under the PDMA-Fe treatment than under the other treatments at pH 7.0 and 8.0, but it did not differ at pH 9.0 (Fig. 4b). In the 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. 4c).

The comparative analysis of Fe chelates showed that the effect on the chlorophyll level decreased in the following order: PDMA-Fe > EDDHA-Fe and TAGE-Fe > EDTA-Fe, HBED-Fe, Cit-Fe, DFOB-Fe, and −Fe (Supplementary Fig. S3a), 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 (differences significant at $P < 0.05$; Supplementary Fig. S3b).

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 two 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 two-fifths of that in the EDDHA-Fe treatment (differences significant at $P < 0.05$) and one-fifth of that in the Cit-Fe treatment (Fig. 5a). *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. 5b).

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. 6). 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

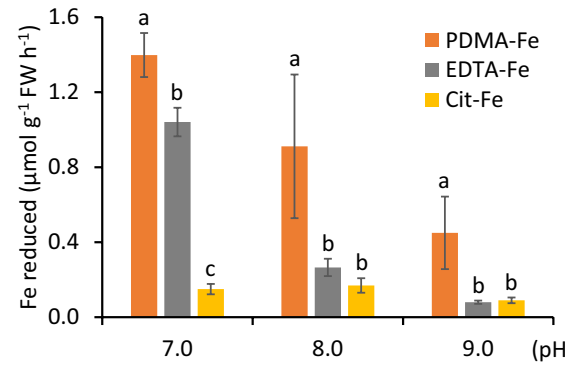

Fig. 6 Reducibility of Fe(III) from Fe chelates by cucumber roots. Roots of cucumber plants grown hydroponically without Fe for 5 d were used. Reducibility was examined under neutral–alkaline pH (7.0, 8.0, and 9.0) using the bathophenanthroline disulfonic acid assay. Data are presented as mean ± standard deviation ($n = 3$). Different letters indicate significant differences ($P < 0.05$) using Tukey's test

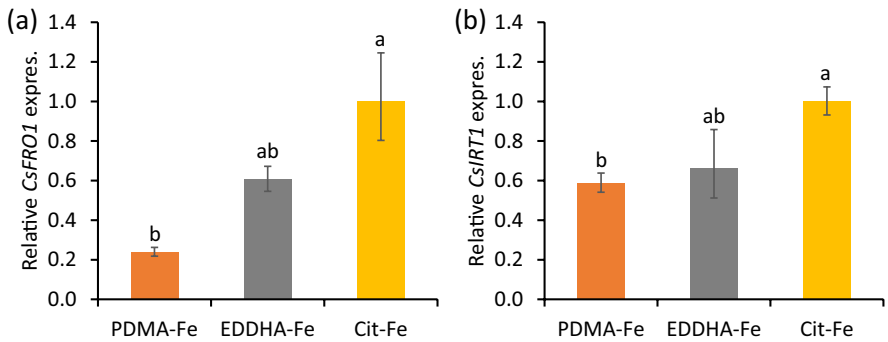

Fig. 5 Effects of PDMA-Fe(III) on Fe status. Seedlings precultured in the absence of Fe for 4 d were exposed to a nutrient solution (pH 9.0) containing 0.5 µM Fe chelates for 72 h. Relative expression levels of ferric reduction oxidase 1 (*CsFRO1*) (**a**) and iron-regulated transporter 1 (*CsIRT1*) (**b**) in the roots grown under the Cit-Fe(III) treatment. *Actin 7* was used as the internal control. Data are presented as mean ± standard deviation ($n = 3$). Different letters indicate significant differences ($P < 0.01$) using Tukey's test

PDMA-Fe than in 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 the 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 substrate and hydroponic culture (Figs. 2 and 4). Consistent with Fe accumulation, the chlorophyll level and PSII activity were recovered substantially by PDMA-Fe application (Figs. 1 and 3, Supplementary Fig. S2) because Fe is needed for many chloroplast components (Broadley et al. 2012). The application of PDMA-Fe lowered the expression of Fe deficiency-inducible genes more than the other Fe chelates (Fig. 5), suggesting that the 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. 6). 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). Therefore, decreased Fe uptake with increasing pH (Fig. 4) may be due to decreased formation of such species as well as to the inhibition of reductase activity (Römheld and Marschner 1986). Although the chemical mechanism underlying the higher reducibility of PDMA-Fe requires clarification, the reduction of PDMA-Fe could be structurally less sensitive to high pH in comparison with that of traditional synthetic Fe chelates. Thus, PDMA-Fe seems to be effective for crops relying on the reduction-based Fe uptake system.

Another possible explanation for the high 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 by 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, studies have implied the secretion of PS from tomato and grapevine, and the presence of endogenous DMA in the leaves and xylem of olive plants (Suzuki et al. 2016; Astolfi et al. 2020; Marastoni et al. 2020). Although the dependence of this on the Fe nutritional status has not been proven, these findings suggest that dicots also utilize exogenous and endogenous PS for the uptake and translocation of Fe. 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 possible direct uptake of PS-Fe by dicots should be investigated in the future.

The first study that compared the availability of PS-Fe and EDDHA-Fe in cucumber was 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 (Takagi 1993; 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 can be utilized by cucumber roots more efficiently than traditional synthetic chelates in both calcareous substrate and

hydroponic cultures. The pH-dependency tests showed that the higher availability of PDMA-Fe may be attributed to a higher reducibility at alkaline pH. Our findings suggest that PDMA-Fe can be a good Fe fertilizer in alkaline soil for Strategy I plants.

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Declarations

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