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To cite this article: Motofumi Suzuki, Yutaro Suzuki, Kensuke Hosoda, Kosuke Namba & Takanori Kobayashi (2024) The phytosiderophore analogue proline-2'-deoxymugineic acid is more efficient than conventional chelators for improving iron nutrition in maize, *Soil Science and Plant Nutrition*, 70:5-6, 435-446, DOI: [10.1080/00380768.2024.2385401](https://doi.org/10.1080/00380768.2024.2385401)

To link to this article: <https://doi.org/10.1080/00380768.2024.2385401>



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RESEARCH ARTICLE



The phytosiderophore analogue proline-2'-deoxymugineic acid is more efficient than conventional chelators for improving iron nutrition in maize

Motofumi Suzuki^{a*}, Yutaro Suzuki^{b*}, Kensuke Hosoda^a, Kosuke Namba^c and Takanori Kobayashi^b

^aAichi Steel Corporation, Tokai-shi, Aichi, Japan; ^bResearch Institute for Bioresources and Biotechnology, Ishikawa Prefectural University, Nonoichi, Ishikawa, Japan; ^cDepartment of Pharmaceutical Sciences, Tokushima University, Tokushima, Japan

ABSTRACT

Iron (Fe) is an essential nutrient but has poor bioavailability because of its low solubility under conditions of high pH, such as calcareous soils. Previously, we synthesized an analogue of the natural Fe(III) chelator 2'-deoxymugineic acid secreted by graminaceous plants for efficient Fe uptake, designated as proline-2'-deoxymugineic acid (PDMA). Soil application of Fe(III)-PDMA ameliorated symptoms of Fe deficiency in rice. In the present study, we explored the potential of PDMA as Fe nutrition supplement in maize, a major graminaceous crop, and rice for comparison. In calcareous soil pots, Fe deficiency chlorosis of maize was efficiently recovered by a single application of Fe(III)-PDMA. In contrast, maize plants treated with conventional Fe(III)-chelator complexes, such as Fe(III)-ethylenediaminetetraacetic acid (EDTA) or Fe(III)-N,N'-di(2-hydroxybenzyl)ethylenediamine-N,N'-diacetic acid monohydrochloride (HBED), showed little or no recovery. Similarly, rice Fe chlorosis in calcareous soil pots was efficiently recovered by a single application of Fe(III)-PDMA, but not by other conventional Fe(III)-chelator complexes. Application of Fe(III)-PDMA was also effective in ameliorating Fe deficiency chlorosis in hydroponically grown maize seedlings; other Fe(III)-chelator complexes showed minimal or no efficacy. Addition of low concentrations of metal-free PDMA recovered Fe chlorosis of maize or rice in the presence of Fe(III)-EDTA, suggesting possible ligand exchange from EDTA to PDMA for subsequent Fe(III)-PDMA uptake by ZmYS1 and OsYSL15 transporters. Similar effects were observed with Fe(III)-HBED, which has a higher stability constant, but to a lesser extent than Fe(III)-EDTA, suggesting that ligand exchange from HBED to PDMA might be less prone to occur than for chelators with moderate stability constants, such as EDTA. Ferric-chelate reductase assays of maize roots showed substantial reduction of Fe(III)-PDMA, but this reduction activity was not increased under Fe-deficient condition. These results suggested that PDMA is an efficient reagent for improvement of Fe nutrition in graminaceous crops, including maize, because of suitability of Fe(III)-PDMA as a substrate for chelation-based Fe uptake systems.

ARTICLE HISTORY

Received 11 January 2024
Accepted 12 July 2024

KEYWORDS

Chelation; iron fertilizer; maize; mugineic acid family phytosiderophores; proline-2'-deoxymugineic acid

1. Introduction

Iron (Fe) is an essential nutrient for nearly all living organisms. Although abundant in the Earth's crust, Fe has poor bioavailability because of its low solubility under aerobic conditions at neutral to alkaline pH. In calcareous soils, which cover approximately 30% of the global cultivated area, Fe solubility is extremely low and does not meet plant demand (Marschner 1995; Wallace and Lunt 1960). Consequently, plants growing on such soils often experience Fe deficiency, which typically manifests as interveinal yellowing of new leaves (Fe chlorosis), leading to impaired chlorophyll biosynthesis and reductions in agricultural productivity and quality. Fe uptake by plants is also a major source of Fe nutrition for animals and humans. Fe deficiency anemia is a global problem, affecting an estimated two billion people worldwide (Gardner et al. 2023), particularly in Asian and African countries (Aung et al. 2013; Connorton and Balk 2019).

Plants have developed two distinct mechanisms to acquire sparingly soluble Fe from soil. Nongraminaceous plants utilize

a reduction strategy (Strategy I), in which ferrous ions (Fe^{2+}) are taken up after the reduction of Fe(III)-chelator complexes by Fe deficiency-inducible ferric-chelate reductase (FCR) at the root surface (Marschner, Römhelt, and Kissel 1986; Römhelt and Marschner 1986). This activity is aided by the secretion of protons and organic substances, such as coumarins and flavins, which possess Fe(III)-chelating or reducing activity (Fourcroy et al. 2014; Paffrath et al. 2023; Rodríguez-Celma et al. 2013; Schmid et al. 2014; Sisó-Terraza et al. 2016). In contrast, graminaceous plants use a chelation strategy (Strategy II) mediated by the biosynthesis and secretion of efficient Fe(III) chelators designated as mugineic acid family phytosiderophores (MAs) (Takagi 1976). MAs secreted from the roots of graminaceous plants chelate Fe(III) in the rhizosphere; the resulting Fe(III)-MA complexes are absorbed into roots by Yellow Stripe 1 (YS1)/YS1-Like (YSL) transporters (Curie et al. 2001, 2009). Key molecules involved in these Fe-uptake strategies are increased under conditions of low Fe availability through the induction of corresponding genes at the transcriptional level (Kobayashi

CONTACT Takanori Kobayashi abkoba@ishikawa-pu.ac.jp Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University, 1-308 Suematsu, Nonoichi, Ishikawa 921-8836, Japan

*These authors contributed equally to this work.

Supplemental data for this article can be accessed online at <https://doi.org/10.1080/00380768.2024.2385401>.

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2019; Kobayashi and Nishizawa 2012). Recent molecular and physiological studies have detected partially overlapping strategies in some plant species. For example, rice, a graminaceous plant, utilizes Strategy II, using the 2'-deoxymugineic acid (DMA) among MAs; it also takes up Fe^{2+} using transporters such as OsIRT1. However, rice has very low FCR activity on the root surface, and this activity is not induced under conditions of Fe deficiency (Ishimaru et al. 2006). Rice also secretes phenolics to solubilize apoplastic Fe (Bashir et al. 2011; Ishimaru et al. 2011). On the other hand, peanut, a nongraminaceous legume plant, primarily utilizes Strategy I, but it also utilizes Fe(III)-DMA when it is present in the rhizosphere through DMA secretion from intercropped maize, possibly via direct uptake of Fe(III)-DMA by the AhYSL1 transporter (Xiong et al. 2013). Additionally, DMA is detected in the xylem sap of some nongraminaceous plants, such as soybean, tomato, and olive (Ariga et al. 2014; Suzuki et al. 2016).

Despite these specific mechanisms for Fe acquisition in the rhizosphere, some plant species are susceptible to low Fe availability in calcareous and alkaline soils. Strategy I systems are particularly susceptible under these conditions, mainly because of FCR inactivation by bicarbonates and high pH, as well as soil buffering activity against acidification (Marschner 1995; Marschner, Römheld, and Kissel 1986). Strategy II systems are more tolerant to bicarbonates and high pH, but they are limited by MA production capacity and secretion. Among graminaceous species, rice, maize, and sorghum are highly susceptible to low Fe availability because of their low MA secretion capacity (Marschner 1995; Mori et al. 1991; Römheld and Marschner 1986).

The improvement of Fe availability by fertilizers is a straightforward approach to enhance Fe nutrition in plants. However, this approach is often hindered by Fe precipitation, particularly under conditions of high pH. Metal chelation is an effective strategy for preventing metal precipitation and unwanted metal reactions. Indeed, synthetic Fe chelators are commonly applied in calcareous fields (Lucena 2006). Synthetic Fe chelators that are effective for nongraminaceous plants include Fe(III)-ethylenediaminetetraacetic acid (EDTA), Fe(III)-diethylenetriaminepentaacetic acid (DTPA), and especially the stable Fe(III)-*o,o*-ethylenediamine di(*o*-hydroxyphenylacetic) acid (EDDHA). These Fe(III)-chelator complexes are presumably reduced by FCR at the root surface for Strategy I Fe uptake. However, the application of these artificial chelators has limitations, such as environmental threat conferred by low biodegradability-related persistence (Pinto, Neto, and Soares 2014; Schenkeveld et al. 2012). Furthermore, chelating agents with high stability constants are reportedly ineffective for graminaceous plants (Lucena, Gárate, and Carpena 1988).

As an alternative to synthetic chelators, we previously applied chemically synthesized DMA to rice plants grown in calcareous soil pots (Suzuki et al. 2021). Compared with conventional synthetic chelators, the application of Fe(III)-DMA is more effective for recovery from Fe chlorosis, but its efficacy is transient because of high biodegradability (Takagi, Kamei, and Yu 1988). To overcome the high cost and poor stability of synthetic DMA for agricultural use, we developed a more stable and much less expensive analogue, proline-2'-deoxymugineic acid (PDMA) (Suzuki et al. 2021), in which a DMA analogue is

synthesized from L-proline instead of 2-azetidine carboxylic acid for DMA synthesis, as previously described (Namba et al. 2007). The application of Fe(III)-PDMA in calcareous soil pots ameliorates Fe chlorosis in rice plants to a greater degree than Fe(III)-DMA for long periods. Additionally, Fe(III)-PDMA at 3 μM showed efficacy similar to the representative conventional chelating agent Fe(III)-EDDHA at 30 μM in rice, indicating approximately 10-fold greater efficacy for PDMA (Suzuki et al. 2021). The application of unchelated PDMA had similar effects because it efficiently chelates and solubilizes Fe(III), which is abundant in calcareous soils. The efficacy of PDMA as Fe nutrition supplement was also substantiated by its promotion of rice growth in a calcareous soil field (Suzuki et al. 2021). Along with recovery from Fe chlorosis, the application of Fe(III)-PDMA or unchelated PDMA in calcareous soil pots resulted in increased Fe concentrations in rice leaves. In contrast, the application of zinc (Zn)(II)-PDMA increased leaf Zn concentrations, indicating the efficacy of PDMA as Zn nutrition supplement when chelated with Zn (Suzuki et al. 2021). Molecular experiments have shown that Fe(III)-PDMA is transported through rice OsYSL15, maize ZmYS1, and barley HvYS1 transporters (Suzuki et al. 2021), similar to their natural substrate Fe(III)-DMA (Curie et al. 2001; Inoue et al. 2009; Lee et al. 2009; Murata et al. 2006). These transporters are expressed in root surfaces for Strategy II-based Fe uptake in Fe deficiency-inducible manner (Curie et al. 2001; Inoue et al. 2009; Lee et al. 2009; Murata et al. 2006). The cryoelectron microscopic structure of HvYS1 with Fe(III)-PDMA is remarkably similar to the structure with Fe(III)-DMA (Yamagata et al. 2022). After application of Fe(III)-PDMA in hydroponic solution to rice, PDMA was detected in xylem sap, indicating direct uptake of the chelated form (Suzuki et al. 2021).

Recent studies showed that application of PDMA is also highly effective in dicot plants, including cucumber, pumpkin, and peanuts, for recovery from Fe chlorosis in calcareous soil (Ueno et al. 2021; Wang et al. 2023). Fe(III)-PDMA is readily reduced on the surface of cucumber roots (Ueno et al. 2021), suggesting effective uptake via Strategy I. Regardless of the extremely high potential efficacy of Fe(III)-PDMA as Fe fertilizer in many plant species, this efficacy has not been investigated for major graminaceous crops other than rice, including crops with high susceptibility to Fe deficiency (e.g., maize and sorghum).

In the present study, we aimed to demonstrate and delineate the potential of PDMA for improving Fe nutrition in maize, as a representative of major crops, and rice for comparison. Our results showed that PDMA is highly effective for improving Fe nutrition in maize in calcareous soil pots and hydroponics, whereas conventional synthetic chelators are less effective. Then, we explored underlying molecular mechanism in which PDMA improves maize and rice Fe nutrition more effectively than conventional synthetic chelators with higher stability constants for Fe(III). We showed that metal-free PDMA can substitute Fe from other synthetic chelators with higher stability constants for Fe(III), and ameliorates maize and rice Fe chlorosis, suggesting ligand exchange and subsequent Fe(III)-PDMA uptake. Our results indicate that PDMA is an effective fertilizing substance improving Fe nutrition for various graminaceous crops including maize.

2. Materials and methods

2.1. Preparation of Fe(III)-chelator complexes

Chemical structures of synthetic chelators used in the present study are shown in Figure S1. PDMA was chemically synthesized as previously described (Suzuki et al. 2021). For maize calcareous soil pot Experiments 1, 2, and maize FCR assay Experiment 1 (Figure 1), PDMA and citric acid monohydrate (WAKO Chemicals, Tokyo, Japan) were dissolved in Milli-Q water to prepare 0.20 mM solutions that were mixed with an equimolar amount of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, and stirred for 1 h at room temperature to chelate Fe(III) [resulting in pH 2.9 for Fe(III)-PDMA solution]. For maize hydroponic Experiment 1 (Figure 1), PDMA and citric acid monohydrate as above were dissolved in Milli-Q water to prepare 0.3 mM solutions that were mixed with an equimolar amount of $\text{Fe}_2(\text{SO}_4)_3 \cdot x\text{H}_2\text{O}$ (Sigma-Aldrich, Inc., Missouri, U.S.A.; calculated as 22% Fe), and stirred for 1 h at room temperature to chelate Fe(III) (resulting in pH 2.6). Fe(III)-*N,N'*-di(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid monohydrochloride [Fe(III)-HBED, 7% Fe; ADOB Ltd., Poznan, Poland], Fe(III)-EDTA (Dojindo Laboratories, Kumamoto, Japan), and Fe(III)-EDDHA [6% Fe (3% *o*, *o*-EDDHA); CresCal, Aglukon Spezialduenger GmbH & Co. KG, Dusseldorf, Germany] were dissolved in Milli-Q water to prepare 0.2 mM solutions [resulting in pH 7.1 and 8.0 for Fe(III)-EDTA and Fe(III)-EDDHA solutions, respectively].

2.2. Maize growth test in calcareous soil pots

The experimental frameworks of maize growth tests are summarized in Figure 1.

In Experiment 1 for calcareous soil tests (Figures 2 and S3), maize (*Zea mays* L. cv. Launcher) seeds were germinated on paper towels wetted with distilled water for 6 days in the dark at 25°C. Seedlings were individually transplanted into 600-mL pots (Yamato Plastic Co. Ltd., Nara, Japan) filled with 800 g of calcareous soil (pH 9; sandy calcareous soil containing fossil shell collected in Takaoka city, Toyama, Japan; Neo-Best, Nihonkai Hiryou, Takaoka, Japan; Morikawa et al. 2004). The soil was mixed with 2 g of controlled-release (70 days) nitrogen – phosphorus – potassium (NPK) fertilizer (EcoLong 313–70; JCAM Agri, Tokyo, Japan) with the following composition (in g/kg): N, 130; P_2O_5 , 110; K_2O , 130), and saturated with distilled water. The bottom of the pot was covered with paper to permit water drainage. The maize pots were cultured in a growth chamber under a 14-h light/10-h dark, 28°C/23°C cycle. The pots were supplied with tap water to saturation (270 mL/800 g soil) three times per week (every 2–3 days). At 16 days after transplantation, water solution of Fe(III)-chelator complex was applied as 10, 30, or 90 μM Fe(III)-PDMA or 90 μM Fe(III)-EDTA (final concentration; 200 mL in 800 g calcareous soil), instead of watering on the corresponding day. Relative chlorophyll levels

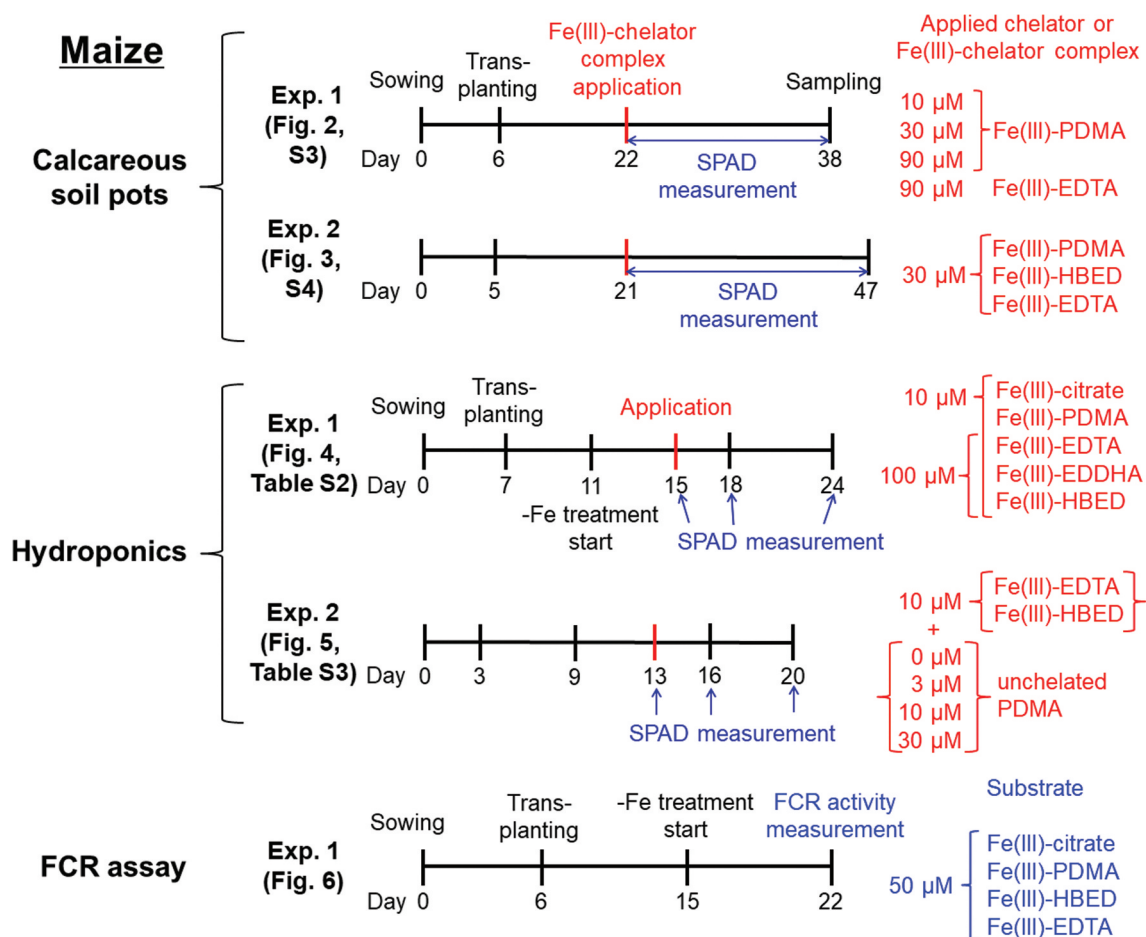


Figure 1. Experimental frameworks of maize cultures. Numbers of experiments (Exp.), figures (Fig.) and tables correspond to description in the main text. PDMA: proline-2'-deoxymugineic acid; EDTA: ethylenediaminetetraacetic acid; EDDHA: *o,o*-ethylenediamine di(*o*-hydroxyphenylacetic) acid; HBED: *N,N'*-di(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid monohydrochloride.

in the newest leaves were measured as soil plant analysis development (SPAD) values using a SPAD-502 chlorophyll meter (Konica Minolta, Tokyo, Japan) three times per week (every 2–3 days). Plants were harvested at 16 days after application of Fe(III)-chelator complex. Leaf blades of the newest and second newest leaves were collected as ‘upper leaves,’ leaf blades of other leaves were collected as ‘lower leaves.’ The remaining aboveground parts were collected as ‘leaf sheaths.’

In Experiment 2 (Figures 3 and S4), plants were cultured in pots as in Experiment 1, with the following modifications. Seedlings were transplanted into calcareous soil pots at 5 days after sowing. At 16 days after transplantation, water solution of Fe(III)-PDMA, Fe(III)-EDTA, or Fe(III)-HBED was applied as 200 mL of 30 μ M (final concentration in soil). Plants were harvested at 26 days after application of Fe(III)-chelator complex.

2.3. Metal concentration analysis

Nitric acid digestion and metal concentration analysis were conducted as previously described (Kobayashi et al. 2013), with the following modifications. Plant segments were cut into pieces (3–4 cm), then dried for 2–3 days at 70°C; portions weighing 100–200 mg were wet-ashed with 2.0 mL of 13.4 M HNO_3 and 2.0 mL of 8.8 M H_2O_2 for 20 min at 230°C. Fe, Zn, manganese (Mn), and copper (Cu) concentrations were measured by inductively coupled plasma optical emission spectrometry (5800 ICP – OES; Agilent Technologies, Santa Clara, CA, U.S.A.).

2.4. Rice growth test in calcareous soil pots

The experimental frameworks of rice growth tests are summarized in Figure S2.

In Experiment 1 for calcareous soil tests (Figure S5), Rice (*Oryza sativa* L. cv. Nipponbare) seeds were grown and analyzed as previously described (Suzuki et al. 2021), with the following modifications. After 6 days of soaking to induce germination, seedlings were transferred onto a Saran net floating on nutrient solution and grown for 7 days in a growth chamber under a 14-h light/10-h dark, 28°C/23°C cycle. Next, three seedlings grown similarly were transplanted into 600-mL pots filled with 800 g of calcareous soil described in 2.2., containing controlled-release (70 days) NPK-micronutrient type fertilizer (EcoLongTotal 313–70; JCAM Agri) with the following composition (in g/kg): N, 130; P_2O_5 , 110; K_2O , 130; MgO, 20; Mn, 1.0; B, 0.6; Fe, 2 (as Fe-EDTA); Cu, 0.5; Zn, 0.15; and Mo, 0.20). After 9 days, additional micronutrients excluding Fe were added to the soil solution as 4.6 mM H_3BO_3 , 0.9 mM MnCl_2 , 0.08 mM ZnSO_4 , 0.03 mM CuSO_4 , and 0.01 mM Na_2MoO_4 (final concentration of additional micronutrients in soil) to promote Fe deficiency; the temperature conditions were adjusted to a 14-h light/10-h dark, 30°C/25°C cycle. After 5 days, chelator complex solutions containing $\text{Fe}_2(\text{SO}_4)_3$ with PDMA, Fe(III)-HBED, Fe(III)-EDTA, and Fe(III)-EDDHA were applied to soil as 30 μ M (final concentration; 270 mL in 800 g calcareous soil).

2.5. Maize growth test in hydroponics

In Experiment 1 for hydroponics (Figure 4, Table S2), maize (cv. Launcher) seeds were germinated on paper towels wetted with distilled water for the first 2 days in the dark and the next 5 days in a growth chamber under a 14-h light/10-h dark, 28°C/23°C cycle. Seedlings were transplanted into 8 L of nutrient solution [4.0 mM $\text{Ca}(\text{NO}_3)_2$, 1.3 mM $\text{NH}_4\text{H}_2\text{PO}_4$, 2.0 mM MgSO_4 , 8.0 mM KNO_3 , 46 μ M H_3BO_3 , 9 μ M MnCl_2 , 0.8 μ M ZnSO_4 , 0.3 μ M CuSO_4 , 0.1 μ M Na_2MoO_4 , 100 μ M Fe(III)-EDTA] for 4 days, then transplanted into nutrient solution without Fe for 4 days. Subsequently, three plants were transplanted into 550 mL of nutrient solution with 10 μ M Fe(III)-citrate, 10 μ M Fe(III)-PDMA, 10 μ M Fe(III)-EDTA, 10 μ M Fe(III)-EDDHA, 10 μ M Fe(III)-HBED, 100 μ M Fe(III)-EDTA, 100 μ M Fe(III)-EDDHA, or 100 μ M Fe(III)-HBED. Each nutrient solution was adjusted to pH 7.0 with NaOH solution. Relative chlorophyll levels in the newest leaves were measured as SPAD values at 0, 3, and 9 days after application of Fe(III)-chelator complex.

In Experiment 2 (Figure 5, Table S3), maize (cv. Royaldent TX1235) seeds were germinated on paper towels wetted with distilled water for the first 2 days in the dark and then 1 day in a growth chamber under a 14-h light/10-h dark, 28°C/23°C cycle. Seedlings were transplanted into nutrient solution as described above, cultured for 6 days, and then transplanted into nutrient solution without Fe for 4 days. Subsequently, three plants were transplanted into nutrient solution including 10 μ M Fe(III)-EDTA or 10 μ M Fe(III)-HBED with 0, 3, 10, or 30 μ M unchelated PDMA. Each nutrient solution was adjusted to pH 7.0 with NaOH solution. Relative chlorophyll levels in the newest leaves were measured as SPAD values at 0, 3, and 7 days after application of Fe(III)-chelator complex.

2.6. Rice growth test in hydroponics

In Experiment 1 for hydroponics (Table S4), rice (cv. Nipponbare) seeds were soaked in distilled water to induce germination for 7 days in a growth chamber under a 14-h light/10-h dark, 28°C/23°C cycle. Next, seedlings were transferred onto a Saran net floating on 5.0 L of nutrient solution [4.0 mM $\text{Ca}(\text{NO}_3)_2$, 1.3 mM $\text{NH}_4\text{H}_2\text{PO}_4$, 2.0 mM MgSO_4 , 8.0 mM KNO_3 , 46 μ M H_3BO_3 , 9 μ M MnCl_2 , 0.8 μ M ZnSO_4 , 0.3 μ M CuSO_4 , 0.1 μ M Na_2MoO_4 , 100 μ M Fe(III)-EDTA], then transplanted into nutrient solution without Fe for 7 days. Subsequently, three seedlings grown similarly were transplanted into 550 mL of nutrient solution including 10 μ M Fe(III)-EDTA or 10 μ M Fe(III)-HBED with 0, 3, 10, or 30 μ M unchelated PDMA, or 10 μ M Fe(III)-PDMA alone for comparison. Each nutrient solution was adjusted to pH 7.0 with NaOH solution. Relative chlorophyll levels in the newest leaves were measured as SPAD values at 0, 3, and 8 days after application of Fe(III)-chelator complex.

2.7. Ferric-chelate reductase activity assay

In Experiment 1, maize plants were germinated as described above for calcareous soil tests for 6 days, then transferred into hydroponic solution with continuous aeration in a growth chamber under a 14-h/10-h light/dark, 28°C/23°C cycle. The composition of the maize hydroponic solution was as follows:

0.70 mM K_2SO_4 , 0.10 mM KCl, 0.10 mM KH_2PO_4 , 2.0 mM $Ca(NO_3)_2$, 0.50 mM $MgSO_4$, 10 μM H_3BO_3 , 0.50 μM $MnSO_4$, 0.50 μM $ZnSO_4$, 0.20 μM $CuSO_4$, 0.010 μM $(NH_4)_6Mo_7O_{24}$, and 100 μM Fe(III)-EDTA, at pH 5.5 (Kobayashi et al. 2019). The concentrations in the solution were reduced by half during the first 7 days. Solutions were renewed three times per week (every 2–3 days). At 9 days after transplantation, Fe deficiency treatment was initiated for half of the plants by omitting Fe(III)-EDTA from the hydroponic solution. At 7 days after the onset of Fe deficiency or control Fe sufficiency treatment, whole roots were cut, weighed, and used for FCR assays as follows. Roots were rinsed with distilled water and submerged in 37.5–40 mL of assay solution comprising 0.50 mM $CaSO_4$, 50 mM tris-(hydroxymethyl)aminomethane (pH 7.5), 0.30 mM bathophenanthroline disulfonic acid disodium salt (Dojindo Laboratories), and 50 μM Fe(III)-chelator complexes [Fe(III)-PDMA, Fe(III)-citrate, Fe(III)-EDTA, or Fe(III)-HBED, prepared as described above]. After incubation for 90 min in the dark at 25°C with occasional mixing, the absorbance of the solution at 535 nm was measured using a spectrophotometer (95044; Eppendorf, Stevenage, UK). The amount of Fe^{2+} produced was calculated from a standard curve prepared using standard solutions of $FeSO_4 \cdot 7H_2O$ mixed with the assay solution containing each Fe(III)-chelator complex.

2.8. Statistical analysis

Data were evaluated using one-way analysis of variance followed by parametric Tukey's honestly significant

difference (HSD) test using SPSS (IBM JAPAN, Ltd, Tokyo, Japan). In all analyses, $p < 0.05$ was considered indicative of statistical significance.

3. Results

3.1. Application of Fe(III)-PDMA improved Fe chlorosis in maize and rice plants grown in calcareous soil pots with greater efficacy than other synthetic chelator complexes

We examined the efficacy of Fe(III)-chelated PDMA for recovery of Fe chlorosis in maize plants, compared with Fe(III)-chelated EDTA as a conventional synthetic chelating agent. Maize seedlings were cultured in calcareous soil pots for 16 days when Fe deficiency-induced chlorosis was evident in new leaves. Next, a solution of 10, 30, or 90 μM Fe(III)-PDMA, or 90 μM Fe(III)-EDTA, was applied once to the soil; SPAD values of the newest leaves were subsequently measured over time (maize calcareous soil pot Experiment 1; Figure 2). Alteration of soil solution pH by the application of 30 μM Fe(III)-PDMA or Fe(III)-EDTA was less than 0.1, confirming that the buffering capacity of the calcareous soil tested was high enough relative to applied chemicals. The application of Fe(III)-PDMA improved Fe chlorosis at 5 days after application, when SPAD values of the newest leaves were higher in plants treated with 10, 30, or 90 μM Fe(III)-PDMA, but not with 90 μM Fe(III)-EDTA, relative to untreated plants (Figure 2(a)). Similar trends of SPAD values and leaf greenness were sustained until day 12 except for plants with 10 μM Fe(III)-PDMA (Figure 2(a, b)). The application of 90 μM Fe(III)-PDMA resulted in substantially increased SPAD

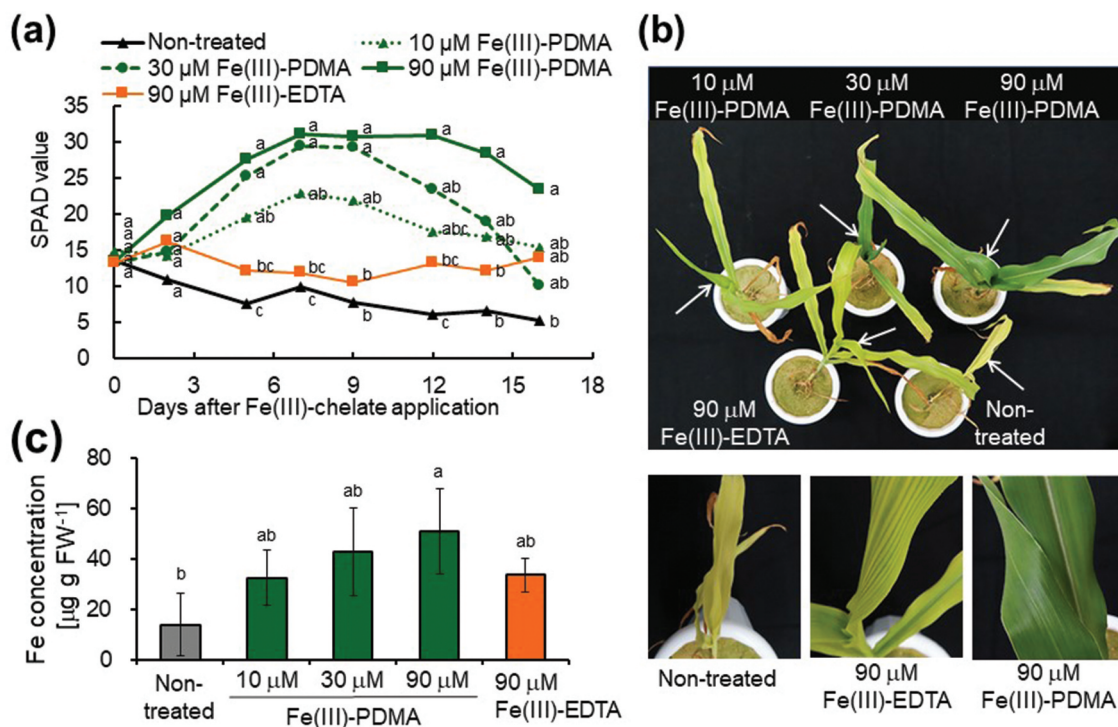


Figure 2. Efficacies of ferric iron-chelated proline-2'-deoxymugineic acid [Fe(III)-PDMA] and ethylenediaminetetraacetic acid [Fe(III)-EDTA] in maize calcareous soil pot Experiment 1. (a) Soil plant analysis development (SPAD) values of the newest leaves. Mean values are shown [$n = 3$ for 30 μM Fe(III)-PDMA; $n = 4$ for other blocks]. (b) Representative plants at 9 days after Fe(III)-chelator complex application. Upper: whole plants. Arrows indicate new leaves. Lower: magnified view of new leaves. (c) Fe concentrations in upper leaves after the experimental period. Values are shown as the mean \pm standard deviation (SD) [$n = 3$ for 30 μM Fe(III)-PDMA; $n = 4$ for other blocks]. In (a) and (c), groups with the same letters indicate no significant differences ($p < 0.05$; Tukey's HSD test).

value, reaching approximately 30 (Figure 2(a)), consistent with green color of new leaves (Figure 2(b)). Significant differences in SPAD values among the newest leaves compared with untreated controls were sustained for longer periods in proportion to the concentration of applied Fe(III)-PDMA; specifically, until day 16 (the end of the cultivation), day 12, and day 7 after the application of 90 μM Fe(III)-PDMA, 30 μM Fe(III)-PDMA, and 10 μM Fe(III)-PDMA, respectively (Figure 2(a)). The application of Fe(III)-EDTA resulted in minimal SPAD value improvement throughout the experiment, even at a concentration of 90 μM (Figure 2(a)).

After the cultivation period, shoot height, the fresh weight of each plant part, and metal concentrations in new leaves were examined (Figures 2(c) and S3). Shoot height was not affected by any treatment, except for a slight decrease after application of 30 μM Fe(III)-PDMA (Figure S3(a)). Treatment with 90 μM Fe(III)-EDTA increased the fresh weight of lower leaves, whereas 90 μM Fe(III)-PDMA increased the fresh weight of roots (Figure S3(b)). Fe concentrations in upper leaves tended to increase in proportion to the concentration of Fe(III)-PDMA applied, with a significant increase after treatment with 90 μM Fe(III)-PDMA compared with the untreated control (Figure 2(c)). The application of 90 μM Fe(III)-EDTA also tended to increase Fe concentrations in upper leaves (Figure 2(c)). Zn, Mn, and Cu concentrations were not significantly altered by these treatments (Figure S3(c)).

Next, we examined the efficacies of Fe(III)-PDMA and Fe(III)-EDTA application in comparison with Fe(III)-chelated HBED, another conventional synthetic chelator that displays a higher stability constant for Fe(III) (Table S1; Chaney 1988; Smith and Martell 1987). Maize plants were cultured on calcareous soil pots, to which a 30 μM solution of Fe(III)-PDMA, Fe(III)-HBED, or Fe(III)-EDTA was applied once (maize calcareous soil pot Experiment 2; Figure 3). The application of Fe(III)-PDMA improved Fe chlorosis within 5 days, and the effect was sustained for at least 9 days (days 5–14 after application); the application of Fe(III)-EDTA only slightly improved Fe chlorosis at ~14 days after application (Figure 3(a)). The application of Fe(III)-HBED resulted in no recovery from leaf chlorosis (Figure 3(a,b)). After the cultivation period,

shoot height, the fresh weight of each plant part, and metal concentrations in new leaves were similar among the treatments (Figure S4). Taken together, these results showed that Fe(III)-PDMA was highly effective in a dose-dependent manner for improving Fe chlorosis in maize plants experiencing low Fe availability in calcareous soils, whereas the synthetic chelators Fe(III)-HBED and Fe(III)-EDTA had negligible effects at similar or higher concentrations.

We then confirmed the efficacy of Fe(III)-chelated PDMA in rice compared with Fe(III)-chelated HBED, which has a remarkably high stability constant for Fe(III) (Table S1) (rice calcareous soil pot Experiment 1; Figure S5). As a comparison, we also used Fe(III)-EDTA and Fe(III)-EDDHA, which were shown to have negligible and only weak effects, respectively, in the previous study (Suzuki et al. 2021). The application of Fe(III)-PDMA recovered leaf chlorosis in rice plants within 3 days; this effect was sustained until the end of the experiment (Figure S5). In contrast, the application of Fe(III)-HBED had no effect, similar to Fe(III)-EDTA and Fe(III)-EDDHA, compared with non-treated controls (Figure S5). Therefore, Fe(III)-PDMA showed greater efficacy for recovery of Fe chlorosis, compared with other synthetic chelator complexes, in both maize and rice.

3.2. Application of PDMA in hydroponic solutions is effective for improving Fe chlorosis in maize and rice plants at lower concentrations than other synthetic chelators

We examined the dose-dependent efficacy of PDMA for recovery of maize plants from Fe chlorosis in hydroponics, compared with other synthetic chelates. Maize seedlings were hydroponically precultured with 100 μM Fe(III)-EDTA, and Fe deficiency was imposed by withdrawal of the Fe source. Maize seedlings displayed severe Fe deficiency after 4 days of this treatment, such that the newest leaves showed SPAD values of ~10 (Table S2). Then, nutrient solution was supplied with 10 μM Fe(III)-chelated citrate (a natural chelator) or Fe(III)-PDMA, or either 10 or 100 μM Fe(III)-EDTA, Fe(III)-EDDHA, or Fe(III)-HBED (maize hydroponic Experiment 1; Figure 4). After 3 days of this treatment, the

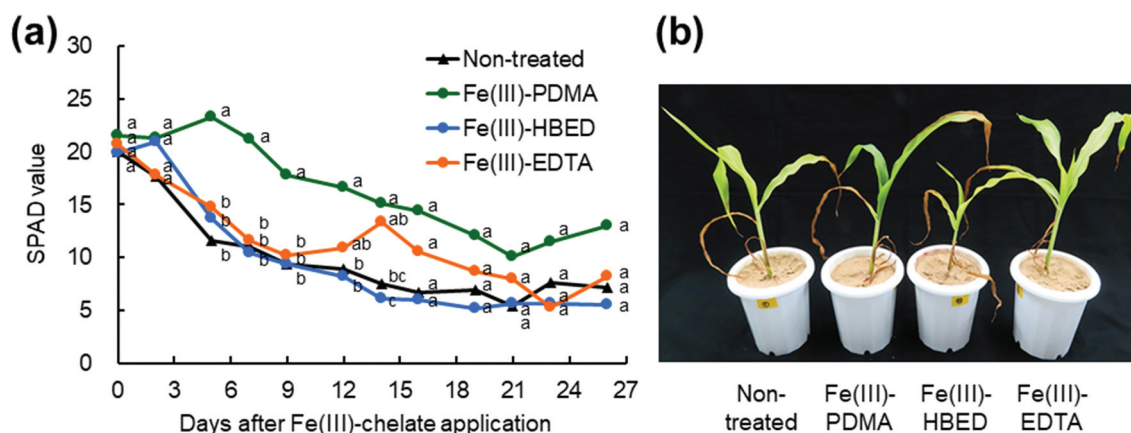


Figure 3. Efficacy of 30 μM Fe(III)-PDMA in maize compared with 30 μM Fe(III)-chelated *N,N'*-di(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid monohydrochloride (HBED) or EDTA in calcareous soil pot Experiment 2. (a) SPAD values of the newest leaves. Mean values are shown ($n = 4$ for non-treated block; $n = 3$ for other blocks). Groups with the same letters indicate no significant differences ($p < 0.05$; Tukey's HSD test). (b) Representative plants at 7 days after Fe(III)-chelator complex application.

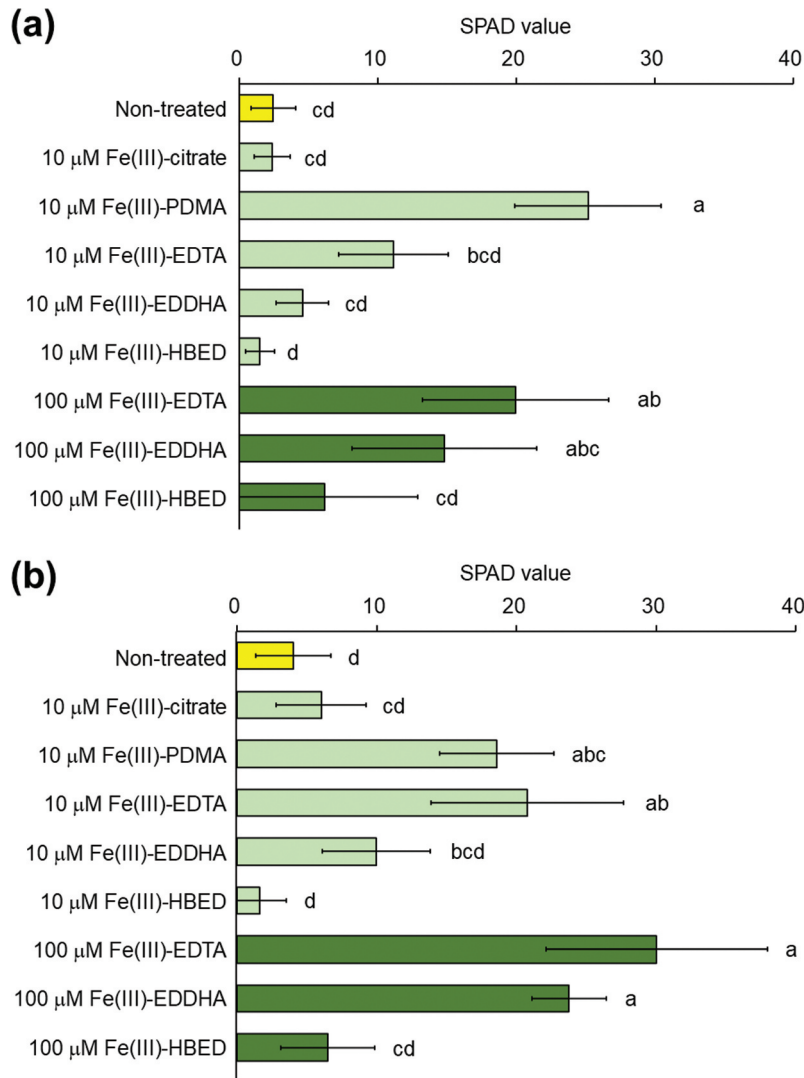


Figure 4. Efficacies of Fe(III)-PDMA and other Fe(III)-chelate complexes at different concentrations in maize hydroponic Experiment 1. SPAD values of the newest leaves were measured on days 3 (a) or 9 (b) after Fe(III)-chelator complex application. Values are shown as the mean \pm SD ($n = 3$). Groups with the same letters indicate no significant differences ($p < 0.05$; Tukey's HSD test).

degree of Fe deficiency symptoms was strongly dependent on the chelating agent. Chlorosis in the newest leaves continued to progress in non-treated control plants, such that SPAD values reached ~ 2.5 (Figure 4(a)). Fe(III)-citrate showed no efficacy. In contrast, application of 10 μ M Fe(III)-PDMA was highly effective in improving Fe chlorosis; SPAD values reached ~ 25 within 3 days (Figure 4(a)). Other synthetic chelators at 10 μ M had no significant efficacy, although Fe(III)-EDTA tended to show some recovery effects. Application of these synthetic chelators tended to be more effective at 100 μ M than at 10 μ M, with significant recovery by Fe(III)-EDTA, although the effects of Fe(III)-EDDHA and Fe(III)-HBED remained insignificant even at 100 μ M compared with non-treated controls (Figure 4(a)). At day 9 of treatment, Fe chlorosis was significantly recovered with 10 μ M Fe(III)-PDMA, 10 or 100 μ M Fe(III)-EDTA, and 100 μ M Fe(III)-EDDHA, but not with 10 μ M Fe(III)-citrate, 10 μ M Fe(III)-EDDHA, or 10 or 100 μ M Fe(III)-HBED (Figure 4(b)). These results showed that Fe(III)-PDMA is considerably more effective in improving Fe chlorosis than other synthetic chelator complexes at the relatively low concentration of 10 μ M.

Next, we examined whether metal-free PDMA can induce recovery of Fe chlorosis in maize in the presence of Fe(III)-chelated synthetic chelators with higher stability constants (i.e., EDTA and HBED; Table S1). For this purpose, we established another hydroponic experiment in which Fe-free hydroponic solution was simultaneously supplied with 10 μ M Fe(III)-EDTA or Fe(III)-HBED and 0, 3, 10, or 30 μ M of unchelated PDMA (maize hydroponic Experiment 2; Figure 5). The SPAD values of the newest leaves at the onset of chelator treatment were ~ 13 – 15 (Table S3). After 3 days, SPAD values further decreased to ~ 2 – 3 in non-treated control seedlings, as well as seedlings treated with 10 μ M Fe(III)-EDTA or Fe(III)-HBED but without application of unchelated PDMA (Figure 5(a)). In contrast, addition of 3 μ M free PDMA with 10 μ M Fe(III)-EDTA led to strong recovery of Fe chlorosis in maize, while addition of 10 or 30 μ M free PDMA with 10 μ M Fe(III)-EDTA resulted in full recovery of Fe chlorosis with SPAD values reaching ~ 30 (Figure 5(a)). Efficacy of free PDMA was weaker with 10 μ M Fe(III)-HBED solution, although addition of 10 or 30 μ M free PDMA induced significant recovery of maize Fe chlorosis in a dose-dependent

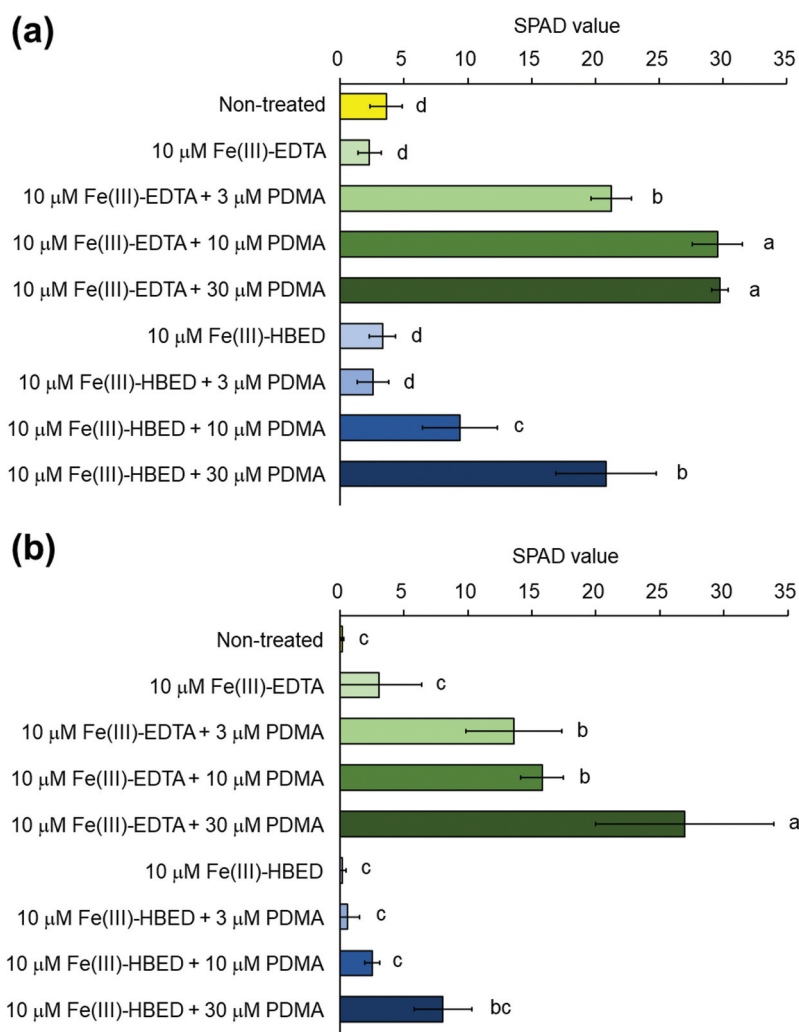


Figure 5. Efficacy of unchelated PDMA in combination with Fe(III)-EDTA or Fe(III)-HBED in maize hydroponic Experiment 2. SPAD values of the newest leaves were measured on days 3 (a) or 7 (b) after free PDMA and Fe(III)-chelator complex application. Values are shown as the mean \pm SD ($n = 3$). Groups with the same letters indicate no significant differences ($p < 0.05$; Tukey's HSD test).

manner (Figure 5(a)). The efficacy of free PDMA was sustained after day 7 from treatment onset with 10 μ M Fe(III)-EDTA solution, but not with 10 μ M Fe(III)-HBED solution (Figure 5(b)). These results indicated that the addition of metal-free PDMA efficiently recovered maize Fe chlorosis in the presence of Fe(III)-EDTA or Fe(III)-HBED, suggesting possible ligand substitution reaction of Fe(III) from EDTA to PDMA, and less effectively from HBED to PDMA, followed by Fe(III)-PDMA uptake in maize. We also conducted similar hydroponic experiment in rice (rice hydroponic Experiment 1; Table S4). Similarly to maize, metal-free PDMA had efficacy for recovery of Fe chlorosis also in rice plants in the presence of Fe(III)-EDTA or Fe(III)-HBED (Table S4). Taken together, these results suggested that PDMA can effectively substitute Fe(III) from other chelators and supply Fe to plants using Strategy II Fe uptake systems.

3.3. Fe(III)-PDMA is reducible on maize roots but without induction under Fe deficiency

We then examined whether Fe(III)-PDMA and other Fe(III)-chelator complexes could be reduced to Fe^{2+} on maize roots for

possible Strategy I-based Fe uptake. Maize plants were hydroponically grown under conditions of Fe sufficiency or deficiency; root FCR activity was assayed using Fe(III)-PDMA, Fe(III)-citrate, Fe(III)-HBED, and Fe(III)-EDTA as substrates (maize FCR assay Experiment 1; Figure 6). Maize roots exhibited substantial FCR activity with Fe(III)-PDMA; it was slightly lesser than the activity with Fe(III)-citrate and much greater than the activity with Fe(III)-HBED and Fe(III)-EDTA (Figure 6). These FCR activities were not induced by Fe deficiency, but they tended to be decreased in the presence of Fe(III)-PDMA and Fe(III)-citrate. These results indicated that Fe(III)-PDMA is reducible by maize roots for potential uptake by Fe^{2+} transporters, in addition to direct uptake of Fe(III)-PDMA through the ZmYS1 transporter.

4. Discussion

In the present study, we first explored the efficacy of Fe(III)-PDMA in maize grown in calcareous soil pots. The application of Fe(III)-PDMA was highly effective for recovering Fe chlorosis compared to conventional synthetic Fe chelator complexes (Figures 2 and 3). Significant effects of 90 μ M Fe(III)-PDMA

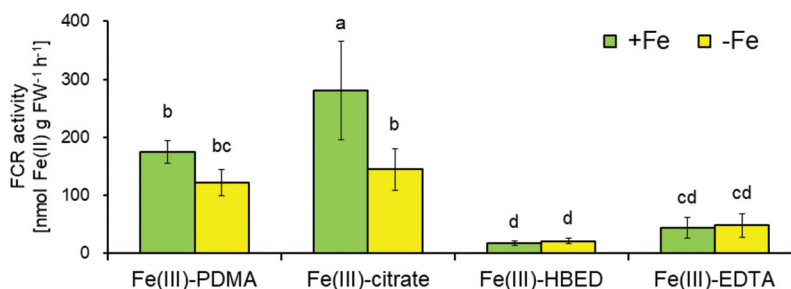


Figure 6. Ferric-chelate reductase (FCR) activities of maize roots with 50 μM Fe(III)-PDMA and other Fe(III)-chelator complexes as substrates in FCR assay Experiment 1. Maize plants were hydroponically grown under Fe-sufficient (+Fe) or Fe-deficient (–Fe) conditions for 7 days after preculture. Values are shown as the mean \pm SD [$n = 4$ for Fe(III)-PDMA –Fe and Fe(III)-citrate –Fe; $n = 3$ for other blocks]. Groups with the same letters indicate no significant differences ($p < 0.05$; Tukey's HSD test).

were sustained until 16 days after only once application at day 0 (Figure 2). Such sustainability of the PDMA effect has also been observed in rice soil pot culture (Suzuki et al. 2021), in contrast to the much lower sustainability of the effect of the natural chelator DMA, which is rapidly degraded by soil microorganisms (Takagi, Kamei, and Yu 1988). In the present study, 10 or 30 μM Fe(III)-PDMA showed less sustained effects than 90 μM Fe(III)-PDMA (Figure 2), suggesting possible occurrence of biodegradation or adsorption to soil. The application of Fe(III)-chelated HBED, another synthetic chelate, had no effect on maize and rice Fe chlorosis (Figures 3 and S5). In a previous study, we showed that Fe(III)-PDMA is transported through OsYSL15, ZmYS1, and HvYS1 (Suzuki et al. 2021), which are representative Fe(III)-DMA transporters in rice, maize, and barley, respectively. We also revealed the presence of PDMA in the xylem sap of rice plants supplied with Fe(III)-PDMA (Suzuki et al. 2021). A recent structural study of HvYS1 suggested that Fe(III)-PDMA is transported in a manner very similar to Fe(III)-DMA (Yamagata et al. 2022). Thus, Fe(III)-PDMA is thought to be a highly available form as a substrate for YS1/YSL transporters, which are responsible for direct uptake of Fe(III)-chelator complexes by Strategy II in graminaceous plants, such as rice and maize.

The less effectiveness of conventional Fe(III)-chelator complexes for improving Fe chlorosis in maize and rice (Figures 2–4 and S5; Suzuki et al. 2021) suggests that these complexes are not suitable substrates for YS1/YSL transporters, in contrast to high suitability of Fe(III)-PDMA as a transporting substrate. This also suggests that Fe(III)-ligand must be exchanged to MAs secreted from graminaceous roots for Strategy II Fe uptake, and that PDMA effectively substitutes such ligands and promotes subsequent Fe uptake. This possible ligand exchange reaction is presumably affected by stability constants; Fe(III)-chelator complexes with high stability constants, such as HBED ($\log K_{\text{Fe(III)}} = 39.7$), could be less susceptible to ligand exchange than Fe(III)-chelator complexes with lower stability constants, such as EDTA ($\log K_{\text{Fe(III)}} = 25.0$) (Table S1). Consistent with this notion, application of Fe(III)-EDTA led to slight improvement of maize Fe chlorosis in calcareous soil pots, but application of Fe(III)-HBED did not (Figures 2, 3, S3, and S4). Lucena, Gárate, and Carpena (1988) suggested that the residues contained in commercial Fe(III)-EDDHA which has weaker chelating ability than *o,o*-EDDHA have positive effects on Fe uptake in graminaceous plants. Furthermore, we observed clear differences in

the recovery of maize Fe chlorosis by the chelate species in a hydroponic experiment (Figure 4), in which Fe(III)-PDMA recovered the leaf SPAD value to ~ 25 (nearly full greenness) on day 3 after treatment onset, even at a relatively low concentration (10 μM). Notably, the efficacies of other chelating agents were lower than Fe(III)-PDMA but displayed clear differences according to chelate species; the effect was relatively high for Fe(III)-EDTA, followed in order by Fe(III)-EDDHA and Fe(III)-HBED, indicating an inverse correlation with stability constants (Table S1). Fe(III)-EDTA did not show a significant effect at 10 μM from 3 days after treatment onset, but was highly effective at 100 μM (Figure 4(a)), consistent with the common use of this chelator complex at ~ 50 – 100 μM as a basal Fe source in hydroponic cultures of graminaceous plants, such as rice and maize (Kobayashi et al. 2001; Li et al. 2013; Mizuno et al. 2003). In contrast, Fe(III)-HBED showed no efficacy, even at 100 μM , on days 3 or 9 (Figure 4), consistent with the high stability constant for Fe(III) (Table S1). These results could be explained by the tendency of ligand substitution of Fe(III) from synthetic reagents to MAs secreted from Fe-deficient maize roots. Such ligand substitution is assumed to result from chemical equilibrium of Fe(III) complex formation with each chelator. Preferable uptake of Fe(III)-MAs by maize roots would shift the chemical equilibrium in favor of formation of Fe(III)-MAs in the rhizosphere, which would then decrease the concentration of free Fe(III) ions and subsequently shift the chemical equilibrium of other Fe(III) chelators in favor of Fe(III) release.

Our further experiments also suggested the possibility of ligand substitution effect of PDMA, in which supplementation of the solution of Fe(III)-EDTA with metal-free PDMA effectively improved maize and rice Fe chlorosis in hydroponic culture (Figure 5, Table S4). This effect was dose-dependent: the addition of 3 μM PDMA into the 10 μM Fe(III)-EDTA solution was highly effective, but 10 μM PDMA was more effective for full recovery of greening of maize seedlings (SPAD values of ~ 30). The efficacy of PDMA even at sub-equimolar amounts relative to Fe(III)-EDTA conforms the assumption that Fe(III)-PDMA generated by equilibrium reactions is actively taken up by maize roots through the ZmYS1 transporter. A similar effect was observed for the addition of PDMA to 10 μM Fe(III)-HBED solution, but with lower efficacy; the addition of 10 μM PDMA was only moderately effective, and the effect of 30 μM PDMA was similar to the effect of addition of 3 μM PDMA to 10 μM Fe(III)-EDTA (Figure 5). Similar results were also obtained in rice

hydroponic culture (Table S4). These results suggested that PDMA is able to chelate Fe(III) by substitution from synthetic chelators with much higher stability constants, such as HBED; however, this substitution reaction might be less prone to occur than for synthetic chelators with moderate stability constants, such as EDTA.

Our root FCR assay revealed that Fe(III)-PDMA is reducible on maize roots similarly to Fe(III)-citrate, a natural Fe(III) chelator for xylem Fe translocation (Rellán-Álvarez et al. 2010), and to much greater extents than Fe(III)-HBED and Fe(III)-EDTA (Figure 6). This finding suggests the possibility that maize plants also can utilize Fe(III)-PDMA for reduction-based Fe²⁺ uptake over conventional Fe(III)-chelators. Other graminaceous plants, such as rice and barley, take up Fe²⁺ using ZIP/IRT transporters (Ishimaru et al. 2006; Pedas et al. 2008), although it remains unclear whether maize roots take up Fe²⁺. Maize possesses 12 ZIP transporters, many of which can transport Fe²⁺ as indicated in yeast complementation assays (Ajeesh Krishna et al. 2020; Li et al. 2013; Mondal et al. 2014). The uptake of Fe²⁺ reduced from Fe(III)-PDMA may be attributable to such Fe²⁺ transporters if they are expressed on the root surface. Maize FCR activity tended to be decreased, rather than increased, under conditions of Fe deficiency using Fe(III)-PDMA as a substrate (Figure 6), in a manner similar to rice FCR activity when Fe(III)-EDTA was used as a substrate (Ishimaru et al. 2006). This finding indicated that reduction-based Fe uptake system is not activated in rice and maize under conditions of low Fe availability. When Fe(III)-PDMA is used as a substrate for FCR, metal-free PDMA could be released, which chelates sparingly soluble Fe(III) in the rhizosphere; this mechanism could increase bioavailable Fe by a shuttling effect.

Recent studies showed that PDMA also can effectively improve Fe nutrition in dicot plants, including cucumber and pumpkin, in calcareous soil pots (Ueno et al. 2021) and peanut in calcareous soil pots and in the field (Wang et al. 2023). In contrast to our observations in graminaceous plants, other synthetic chelates are also effective, although their effects are often weaker than the effect of Fe(III)-PDMA (Ueno et al. 2021; Wang et al. 2023). These results indicate the potential utility of PDMA as an alternative Fe nutrition supplement for dicot plants, in addition to other conventional chelators utilized in field applications for some dicot and tree species. Cucumber roots showed higher FCR activity when Fe(III)-PDMA was used as a substrate, compared with Fe(III)-EDTA or Fe(III)-citrate (Ueno et al. 2021), suggesting that Fe chelated to PDMA is readily taken up through the Strategy I mechanism. Additionally, recent reports suggested the presence of DMA in some nongraminaceous plants (Ariga et al. 2014; Suzuki et al. 2016), and possible uptake of Fe(III)-DMA by YSL transporters in peanut (Xiong et al. 2013), indicating that nongraminaceous plants may also take up Fe(III)-PDMA through a partial Strategy II mechanism. In peanut, application of PDMA or Fe(III)-PDMA upregulated the expression of *AhYSL1* (Wang et al. 2023), encoding a Fe(III)-DMA transporter (Xiong et al. 2013). This result suggests that PDMA can act like a biostimulant, a product improving nutrient use efficiency. Future application of PDMA will contribute to effective improvement of plant productivity, depending on the practical situation such as plant species, soil types, and climates.

In conclusion, our results indicated that PDMA is an effective Fe(III)-chelating agent for maize, as well as rice. Other synthetic chelates were less effective, consistent with the importance of Fe(III)-PDMA as a substrate for the YS1/YSL transport system (Strategy II), similar to natural Fe(III)-MAs. PDMA has great potential as a growth stimulant and Fe supplier, thus enhancing plant growth for food and biomass production in alkaline soils.

Acknowledgments

We thank Ms. Kazuko Arai, Ms. Rieko Umeno and Mr. Shigeki Terashima (Ishikawa Prefectural University) for assistance with plant culture and analysis. We also thank Dr. Satoshi Mori (NPO WINEP) and Dr. Naoko K. Nishizawa (Ishikawa Prefectural University) for valuable discussion.

Disclosure statement

MS and KH are employed by the AICHI STEEL CORPORATION. The remaining authors declare no conflicts of interest.

Funding

This research was supported by the Japan Science and Technology Agency (JST) program Adaptable and Seamless Technology Transfer Program (A-STEP) grant number JPMJTR214D (to MS, KN and TK), and was partially supported by JSPS KAKENHI Grant Number JP22H00352 (to KN and TK).

ORCID

Motofumi Suzuki  <http://orcid.org/0000-0003-4832-2829>
Kosuke Namba  <http://orcid.org/0000-0003-1548-5656>
Takanori Kobayashi  <http://orcid.org/0000-0001-7118-6955>

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