New Citrate-Bicarbonate-Ethylenediaminetetraacetate (CBE) Method for Chemical Extraction of Hydrous Iron Oxides from Plant Root Surfaces

A New Citrate-Bicarbonate-Ethylenediaminetetraacetate (CBE) Method for Chemical Extraction of Hydrous Iron Oxides from Plant Root Surfaces

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Abstract

In the present study, sodium citrate, sodium bicarbonate and ethylenediaminetetraacetate (CBE) method was evaluated for Fe extraction from plant root surfaces and compared with dithionite-citrate-bicarbonate (DCB) method. Fe-plaque on root surfaces was induced by growing rice seedlings in soil with 1.8 mM Fe^{2+}, and Fe-plaque was extracted following CBE and DCB methods. The effects of pH, temperature and incubation time of these methods on Fe extraction from root surfaces were also examined. Iron extraction of CBE and DCB methods did not differ significantly ($p < 0.05$) at pH between 6 and 8, while Fe extraction decreased substantially for further increase of the pH of CBE and DCB solution. In some instances, there were significant differences between CBE and DCB methods in extracellular Fe extraction for temperature and incubation time. The average Fe extraction of CBE and DCB methods were 94% and 81%, respectively, indicating that CBE method would be a better choice for Fe extraction from plant roots. The recommended optimal conditions for CBE method are: pH 8, volume of the solution 30 mL, incubation time 30 min, and solution temperature 22±2 °C.

Keywords: DCB, CBE, Fe extraction, Fe-plaque, Plant roots

Introduction

Hydrous iron oxide (Fe-plaque) is commonly formed on the surfaces of roots of wetland plants, such as rice (*Oryza sativa* L.), *Typha latifolia,* and *Phragmites communis*, and is mainly caused by the oxidation of ferrous iron and the precipitation of iron oxide on the root surface and rhizosphere (Taylor and Crowder 1983; Taylor, Crowder, and Rodden 1984; Zhou, Shi, and Zhang 2007). In anaerobic conditions, rice roots release oxygen to the rhizosphere that results in the formation of Fe-plaque on the surface of the roots (Armstrong 1967; Chen, Dixon, and Turner 1980). The formation of Fe-plaque can prevent the rice plant from excessive uptake of ions such as Fe^{2+}, Mn^{2+} and other reduced phytotoxins formed under waterlogged conditions (Armstrong 1967;

Taylor and Crowder 1983; Iremonger and Kelly 1988). The main form of Fe-plaque is ferric hydroxides (63%) (Hansel et al. 2001), and the functional groups of Fe-hydroxides may sequester certain metals and metalloids (Deng, Ye, and Wong 2009; Blute et al. 2004; Liu et al. 2006; Zhou, Shi, and Zhang 2007; Kuo 1986), and influence their uptake in plants (Rahman et al. 2008; Chen et al. 2005; Greipsson 1995). Fe-plaque can reduce the nutrient uptake by causing nutrient deficiency (Howeler 1973), while others suggest that Fe-plaque could act as a sink of nutrients during moderate supply conditions (Bienfait, Van den Briel, and Mesland-Mul 1984; Conlin and Crowder 1989; Greipsson 1994).

In addition to wetland plants, there is currently a great interest in Fe and other trace metal nutrition of marine phytoplankton in laboratory cultures and in the field. Fe is an important nutrient for phytoplankton growth. In cultures and in nature, ferric (oxyhydro-)oxides (FeO*x*) precipitate and become associated with phytoplankton surfaces (Tang and Morel 2006). Other trace elements adsorb on FeO*x*, making it difficult to differentiate between cellular- and oxide-associated concentrations of both Fe and these elements. Therefore, washing method for selective dissolution of FeO*x* attached to wetland plant's roots and phytoplankton cell surfaces is critically important to distinguish between intracellular and oxide-associated concentrations of both Fe and other elements to understand the role of FeO*x*/Fe-plaque on nutrient and other element uptake in the cell.

Several washing methods have been used to selectively dissolve the FeO*x* attached to wetland plant's root and phytoplankton cell surfaces. These methods typically involve single chelating agent, such as ethylenediaminetetraacetate (EDTA) and diethylenetriaminepentaacetate (DTPA) (Knauer, Behra, and Sigg 1997; Hutchins et al. 1999; Chang and Reinfelder 2000), or reductants, such as Ti(III) or ascorbic acid (Anderson and Morel 1982; Hudson and Morel 1990). Although widely used, wash solutions containing a single chelating agent have been reported to be less-effective to dissolve FeO*x* and associated trace metals (Hutchins et al. 1999). However, washing solutions containing tertiary complex of washing reagents, such as dithionate-citrate-bicarbonate (DCB) (Taylor and Crowder 1983), citrate-Ti(III)-EDTA (Hudson and Morel 1989), oxalate-EDTA-citrate

(Tovar-Sanchez et al. 2003), have been found quite effective for dissolving FeO*x*/Fe-plaque with no release of intracellular contents (Table 1).

The effectiveness of various washing solutions containing tertiary complex of reagents have been studied in removing the extracellular FeO*x* and associated trace elements with marine phytoplankton (Hassler, Slaveykova, and Wilkinson 2004; Hudson and Morel 1989; Tang and Morel 2006; Tovar-Sanchez et al. 2003). The DCB solution of Taylor and Crowder (1983) has been widely used for many years to extract Fe-plaque from root surface of wetland plants. Citrate-Ti(III)-EDTA solution has also been used for the extraction of extracellular Fe fractions from plant roots (e.g., (Hasegawa et al. 2011). Recently, we used a citrate-bicarbonate-ethylenediaminetetraacetate (CBE) solution, a modified solution of DCB, for the first time to extract Fe-plaque and arsenic from the root surface of an aquatic macrophyte (*Spirodela polyrhiza* L.) (Rahman et al. 2008), and wetland plant (*Oryza sativa* L.) (Rahman et al. 2009). The chemical composition of CBE and DCB washing solutions are similar except that CBE solution contains EDTA instead of dithionate in DCB (Table 1). Since EDTA has a strong binding affinity for metal cations, this chelating ligand has been used to dissolve Fe(III) (hydr-)oxides (goethite and hydrous ferric oxide) (Nowack and Sigg 1997). EDTA has also been used for metal extraction from biological surfaces to discriminate between intra- and extracellular metals (Hassler, Slaveykova, and Wilkinson 2004). Therefore, we assumed that CBE solution would be a better washing solution for extracellular Fe extraction from plant root surfaces than DCB solution. However, there was no experimental evidence on this assumption. The objectives of the study were to – (i) investigate extracellular Fe extraction from plant root surfaces using CBE method, and (ii) set the optimum conditions for CBE method for Fe extraction. We also compared the effect of EDTA and dithionate concentrations in CBE and DCB solutions, respectively, on extracellular Fe extraction of these methods.

Materials and Methods

Seed sterilization and germination

Rice seeds of BRRI dhan28 verity were collected from the Bangladesh Rice Research Institute, Gazipur, Bangladesh. The seeds were surface-sterilized before use in the experiment. For surface sterilization, about 100 g seeds were soaked in 200 mL of 1% methyl-1-butylcarbamoyl-2-benzimidazole carbonate solution for 10 min. Seeds were then washed with deionized (DI) water (using an E-pure system (Barnstead)) and soaked in DI water at 20, 45, and 52 ºC for 24 h, 2 min, and 10 min, respectively. Sterilized rice seeds were then soaked in DI water for 48 h, and were germinated on moistened filter paper placed in petri dishes.

Fe-plaque induction on rice roots

When the germinated seeds produced enough roots to grow in soil and about 2 cm of shoots, plants were transplanted in 500-mL polystyrene test vessels (130mm×90mm×70mm) containing 200 g soil. About 20 germinated seeds were transplanted in each vessel, and the seedlings were grown in a plant growth chamber with conditions of 14:10 h light/dark schedule, 100-125 µE m^{-2} s^{-1} light intensity, 22(±2) ºC.

Soil was purchased from supermarket and the chemical composition of the soil was: SiO_2 (95.5%), Al_2O_3 (2.3%), Fe_2O_3 (0.2%), CaO (0.02%), MgO (0.08%). The particle size of the soil was 0.42-0.60 mm (24%) and 0.30-0.42 mm (60%). Before seedling transplantation, the dry soil was flooded with modified Murashige and Skoog (MS) nutrient solution (Murashige and Skoog 1962) (Table 2). The background concentration of Fe in the soil was 0.03 mM, which was calculated from the per cent content of Fe_2O_3 (0.2%). Fe-plaque on rice roots was induced by growing rice with 1.8 mM Fe^{2+}, in addition to its background concentration in soil. Additional Fe was added to the soil by dissolving $FeSO_4 \cdot 7H_2O$ in the modified MS solution. In order to induce Fe-plaque on rice root surfaces, pH of the soil was modified to 8.0 by using 0.1 M HCl and NaOH and this pH was maintained throughout the experiment using buffer solution. After 10 days of rice seedling cultivation, roots of rice seedling were coated by thick and physically visible brown-colored layer of iron oxide.

Fe extraction from rice root surfaces

Rice seedlings were uprooted by hand and washed three times with DI water to remove sand from the root surface. Fe-plaque from rice root surfaces was extracted using both DCB (Taylor and Crowder 1983) and CBE methods (Table 1).

To study the effects of solution pH and temperature, rice roots were treated with 30 mL of DCB or CBE solutions for 60 min at room temperature (22±2 °C). To investigate the effect of incubation time on Fe extraction, rice roots were incubated in 30 mL of standard DCB or CBE solutions (Table 1) for 20 – 120 min using 20-min intervals. The roots were then rinsed three times with DI water, the rinsed water was added to the extracts, and made to a total volume of 50 mL using DI water.

Chemical analysis

Chemical extracts and rice roots were analyzed to distinguish between extra- and intracellular concentration of Fe in rice roots. Fe concentrations in chemical extracts were analyzed directly, while rice roots were subjected to a chemical digestion process before analysis. For chemical digestion, rice roots were rinsed three times with DI water and kept on clean absorbent paper to remove the water from the plant surfaces. The samples were dried at 65 °C until they reached a constant weight. After measuring dry weight (d. wt.) of roots, the samples were taken into 50-mL polyethylene tubes (*DigiTubes*, SCP Science, Canada) for digestion. Five mL of 65% nitric acid (HNO_3) were added to the sample and then, left to incubate for 12 h. The samples were heated on a heating block (*DigiPREP*, SCP Science, Canada) at 95 °C for 2 h. After cooling to room temperature, 3 mL of 30% hydrogen peroxide (H_2O_2) were added and the samples were heated again at 105 °C for 30 min. Then, the digests were diluted to 30 mL with DI water and taken into 50-mL polyethylene bottles (LDPE, NALGENE®, Nalge Nunc International, Rochester, NY) in readiness for analysis. The samples were analyzed for Fe concentration by graphite-furnace atomic absorption spectrometer (AAnalyst 600, Perkin Elmer, Germany).

All chemical reagents used in this experiment were of analytical grade or higher. Glassware and dishes were washed with detergent and then 5 M HCl solution, and rinsed with deionized water before use.

Statistical analysis

One-way ANOVA was performed to analyze the variance of Fe concentrations in roots and root extracts of rice seedlings using CBE or DCB solution for different treatments using SPSS (v15.0 for windows). Linear regression analysis was performed to justify the correlation between Fe concentrations in roots/root extracts of rice seedlings and different variables. GraphPad Prism (v 5.02 for windows) was used for regression analysis of the data.

Results and discussion

After growing rice (*Oryza sativa* L.) seedlings with 1.8 mM Fe^{+2}, extractable Fe concentrations on rice roots surfaces using CBE or DCB wash solution were compared at different pH, temperature and incubation/exposure time. Fe extraction from rice root surfaces was influenced substantially by the pH, temperature, and incubation time of the CBE and DCB solutions. Irrespective of pH of the washing solutions, concentrations of extractable Fe on rice roots were substantially higher for CBE extraction than for DCB extraction (Fig. 1B) indicating that CBE solution is more effective for extracellular Fe extraction. The highest Fe extraction (about 87%) of CBE and DCB was obtained at pH 8 (Table 3). Regression equations indicate significant correlation between CBE- or DCB-extractable Fe on roots of rice seedlings and pH of the CBE or DCB solutions at the $p > 0.05$ level (Fig. 1B). Furthermore, Fe extraction of either method did not differ significantly ($p < 0.05$) at pH between 6 and 8, while further increase of the pH of the washing solutions decreased Fe extraction significantly ($p < 0.05$; Table 3). Thus, pH of the washing solutions has significant influence on extracellular Fe extraction on plant roots, and we proposed pH 8 as the most effective for CBE method, while Taylor and Crowder (1983) proposed pH 6.5 for

DCB method. The pH 8 has been proposed for TiCE (Hudson and Morel 1989) and OEC (Tovar-Sanchez et al. 2003) methods to extract the FeO*x* associated with phytoplankton.

Temperature of CBE or DCB solution also influenced Fe extraction from rice root surfaces. Fe concentrations in rice roots increased gradually ($p > 0.05$) with increasing temperature of both DCB and CBE solutions (Fig. 2A). Regression equations indicate significant correlation between Fe concentrations of the roots and temperature of CBE solution ($p > 0.05$), while correlation between Fe concentrations of the roots and temperature of DCB solution was not significant ($p < 0.05$). Fe concentrations of the roots were substantially higher for DCB method than for CBE method (Fig. 2A). Concentrations of extractable Fe on rice roots decreased gradually with increasing temperature of the washing solutions (Table 3; Fig. 2B). Regression equations indicate significant correlation between CBE- or DCB-extractable Fe concentrations on rice roots and temperature of CBE or DCB solutions at the $p > 0.05$ level. However, there was no significant dereferences in extractable Fe concentrations on rice roots for CBE and DCB methods (Fig. 2B). From this result we proposed normal room temperature (22±2 °C) as optimal for CBE method, while 21 °C was suggested for DCB method (Taylor and Crowder 1983).

The chemical composition of CBE and DCB washing solutions are similar except that CBE solution contains EDTA instead of dithionate in DCB (Table 1). However, the concentrations of EDTA and dithionate in CBE and DCB solutions, respectively, did not affect Fe extraction of the methods significantly ($p < 0.05$; Fig. 3). In addition, per cent extraction of Fe from rice root surfaces did not differ significantly ($p < 0.05$) for CBE and DCB methods; however, CBE solution had slightly increased Fe extraction than DCB solution (Fig. 3). Based on this result, 0.05 M of EDTA has been recommended for CBE solution.

Per cent extraction of Fe was estimated for exposure time between 10 and 120 min, and the result showed that exposure time did influence Fe extraction of CBE and DCB methods (Fig. 4). Regression equations indicate that correlation between extractable Fe (%) on rice roots and incubation time was not significant ($p < 0.05$) for CBE solution, while significant correlation existed between extractable Fe (%) on rice roots and incubation time ($p > 0.05$) for DCB solution. Between 10 and 120 min exposure time, the average Fe extraction ability of CBE and DCB methods were 94% and 81%, respectively, indicating that CBE method can extract about 13% more Fe than DCB method (Fig. 4). From this result, we recommend 30 min of exposure time for CBE method, while the recommended exposure time for DCB method has been 15 min (Taylor and Crowder 1983).

Conclusion

This experiment was conducted to investigate whether the CBE method can be used for the extraction of FeO*x* and associated metals from plant root surfaces by comparing the results of this method with those of more commonly used DCB method. The results reveal that in some instances, there was significant difference in extracellular Fe extraction ability depending on pH, temperature and incubation time with both CBE and DCB methods. Irrespective of these parameters, extracellular Fe extraction ability of CBE method from plant root surfaces is comparable to that of DCB method. However, the average per cent Fe extraction of CBE and DCB methods were 94% and 81%, respectively, indicating that the CBE method may have slightly higher Fe extraction ability than the DCB method. From the results of the present study, the proposed recommended optimal conditions for CBE method for extracellular Fe extraction on plant roots are: pH – 8, volume of the solution – 30 mL, exposure/incubation time – 30 min, and solution temperature – 22±2 °C.

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Table 1: The commonly used washing methods for the extraction of FeOx/Fe-plaque from biological surfaces (phytoplankton and plant roots) to distinguish between extra- and intra-cellular Fe and associated metals/metalloids

Washing methods	Chemical composition of the washing solutions		Conditions	Target organism	References
	Chemicals	Concentration			
DCB	Sodium dithionate ($Na_2S_2O_4$)	0.06 M	pH – 6.5	Plant roots	(Taylor and Crowder 1983)
	Sodium citrate ($Na_3C_6H_5O_7{\cdot}2H_2O$)	0.03 M	Volume – 40 mL		
	Sodium bicarbonate ($NaHCO_3$)	0.125 M	Incubation – 15 min.		
			Temperature – 21 °C		
TiCE	Titanium chloride ($TiCl_3$)	0.047 M	pH – 8.0	Phytoplankton	(Hudson and Morel 1989)
	Sodium citrate ($Na_3C_6H_5O_7{\cdot}2H_2O$)	0.047 M			
	Na_2EDTA ($Na_2C_{10}H_{14}N_2O_8{\cdot}2H_2O$)	0.047 M			
OEC	Oxalate ($C_2H_2O_4{\cdot}2H_2O$)	0.10 M	pH – 8.0	Phytoplankton	(Tovar-Sanchez et al. 2003)
	Na_2EDTA ($Na_2C_{10}H_{14}N_2O_8{\cdot}2H_2O$)	0.05 M			
	Sodium citrate ($Na_3C_6H_5O_7{\cdot}2H_2O$)	0.05 M			
CBE	Sodium citrate ($Na_3C_6H_5O_7{\cdot}2H_2O$)	0.03 M	pH – 8.0	Plant roots	(Rahman et al. 2008)
	Sodium bicarbonate ($NaHCO_3$)	0.125 M	Volume – 30 mL		
	Na_2EDTA ($Na_2C_{10}H_{14}N_2O_8{\cdot}2H_2O$)	0.05 M	Incubation – 30 min.		
			Temperature – 22±2 °C		

DCB = Dithionate-Citrate-Bicarbonate; TiCE = Titanium-Citrate-EDTA; OEC = Oxalate-EDTA-Citrate; CBE = Citrate-Bicarbonate-EDTA.

Table 2: Composition of modified Murashige and Skoog (MS) nutrient solutions used for growing rice seedlings (*Oryza sativa* L.) in soil for the induction of FeOx/Fe-plaque on rice roots.

Nutrient elements	**Concentration (mg L^{-1})**
Macroelements	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2 \cdot 2H_2O$	440
$MgSO_4 \cdot 7H_2O$	370
KH_2PO_4	27.8
Micronutrients	
KI	0.83
H_3BO_3	6.2
$MnSO_4 \cdot 4H_2O$	22.3
$ZnSO_4 \cdot 7H_2O$	8.6
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
Iron source	
$FeSO_4 \cdot 7H_2O$	1.8 mM

Table 3: Fe extraction from rice root surfaces using CBE and DCB methods at different pH, temperature, and incubation time. For each variable, any two values which share the same letter within a column do not differ at the $p > 0.05$ probability level according to the least significant difference (LSD).

Parameters	Total Fe (µmol g^{-1} d. wt.)		% Extraction of Fe from root surface	
	CBE extract + intracellular	DCB extract + intracellular	CBE-method	DCB-method
pH				
5	0.19±0.05c	0.15±0.03b	75.70±3.52ab	86.72±4.15a
6	0.19±0.03c	0.15±0.01b	83.86±2.20a	87.54±5.45a
7	0.18±0.06c	0.13±0.03c	80.50±5.33a	84.63±7.95a
8	0.23±0.07b	0.22±0.04a	87.98±5.86a	87.87±3.83a
9	0.21±0.06bc	0.22±0.06a	67.32±6.82b	84.92±3.58a
10	0.26±0.04a	0.16±0.03b	70.45±4.43b	71.38±4.05b
11	0.23±0.02b	0.16±0.03b	72.88±3.71b	72.50±6.82b
12	0.18±0.04c	0.15±0.01b	68.01±5.78b	77.81±3.04ab
p values	0.39	0.78	0.10	0.02
Temperature (°C)				
8	0.39±0.03a	0.37±0.03a	94.20±2.88a	88.76±2.13a
16	0.39±0.04a	0.34±0.02a	92.80±2.14a	85.03±3.40a
24	0.41±0.01a	0.37±0.01a	90.29±0.91a	85.19±0.28a
32	0.42±0.05a	0.37±0.01a	89.23±0.28a	83.20±4.22a
40	0.39±0.07a	0.36±0.03a	88.02±2.80a	80.06±6.27a
48	0.36±0.06a	0.34±0.03a	85.81±2.71a	78.91±5.79a
p values	0.48	0.53	0.48	0.53
Exposure time (min.)				
10	0.14±0.00a	0.09±0.02a	94.48±2.94a	80.50±6.40a
20	0.14±0.02a	0.09±0.01a	95.05±2.40a	81.64±3.72a
30	0.13±0.02a	0.11±0.01a	92.29±3.51a	80.40±8.58a
40	0.12±0.04a	0.11±0.01a	95.16±2.85a	80.96±9.73a
60	0.14±0.02a	0.09±0.00a	91.84±2.80a	81.99±4.27a
80	0.13±0.00a	0.09±0.01a	93.62±3.06a	84.53±7.35a
100	0.13±0.01a	0.09±0.00a	93.58±3.58a	84.20±4.88a
120	0.13±0.01a	0.09±0.01a	95.39±2.65a	83.31±8.42a
p values	0.95	0.88	0.90	0.01

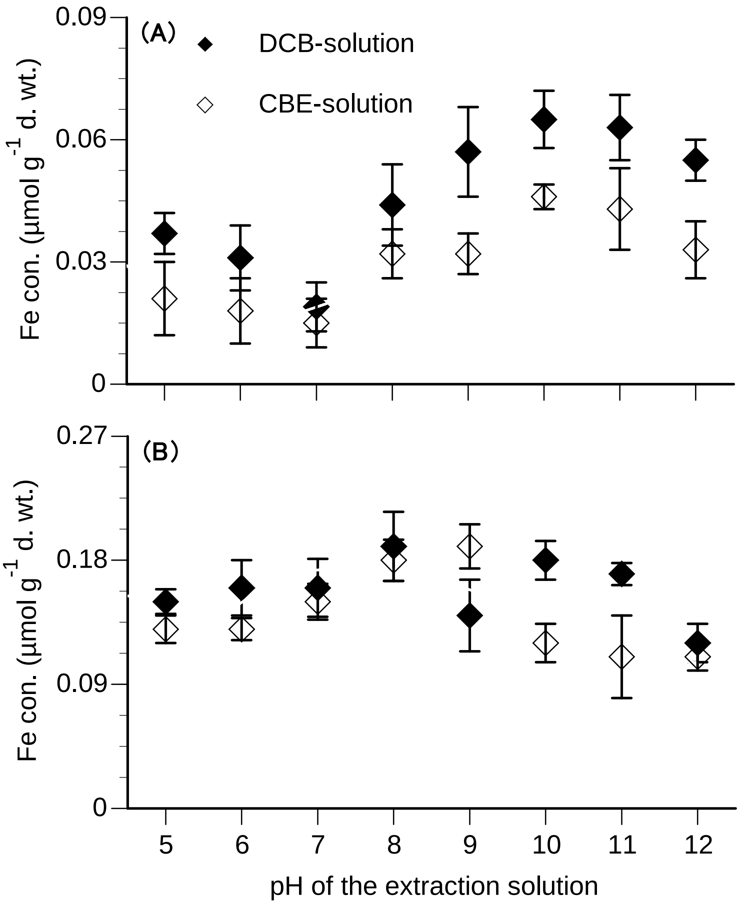

Fig. 1: Effect of pH on Fe extraction from rice roots surfaces using CBE and DCB methods. **(A)** Relationship between intracellular Fe concentrations of rice roots after CBE or DCB extraction and pH of the CBE or DCB solutions. Regression equations indicate that the correlation was not significant at the $p > 0.05$ level. **(B)** Relationship between CBE- or DCB-extractable Fe on roots of rice seedlings and pH of the CBE or DCB solutions. Regression equations indicate significant correlation at the $p > 0.05$ level. Results are mean ± SD ($n = 3$).

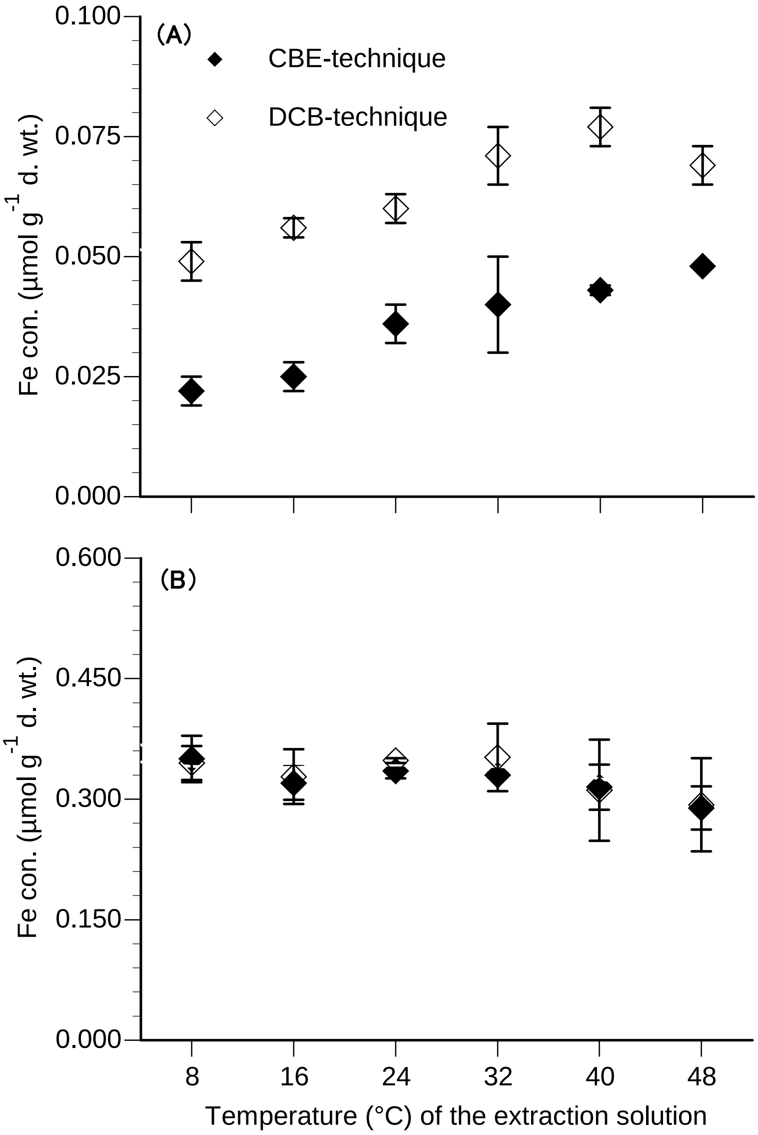

Fig. 2: Effect of temperature on Fe extraction from rice roots surfaces using CBE and DCB methods. **(A)** Relationship between intracellular Fe concentrations of rice roots and temperature of the CBE or DCB solutions. Regression equations indicate significant correlation between root Fe concentrations and temperature of CBE solution ($p > 0.05$), while correlation between root Fe concentrations and temperature of DCB solution was not significant ($p < 0.05$). **(B)** Relationship between CBE- or DCB-extractable Fe on roots of rice seedlings and temperature of the CBE or DCB solutions. Regression equations indicate significant correlation at the $p > 0.05$ level. Results are mean ± SD ($n = 3$).

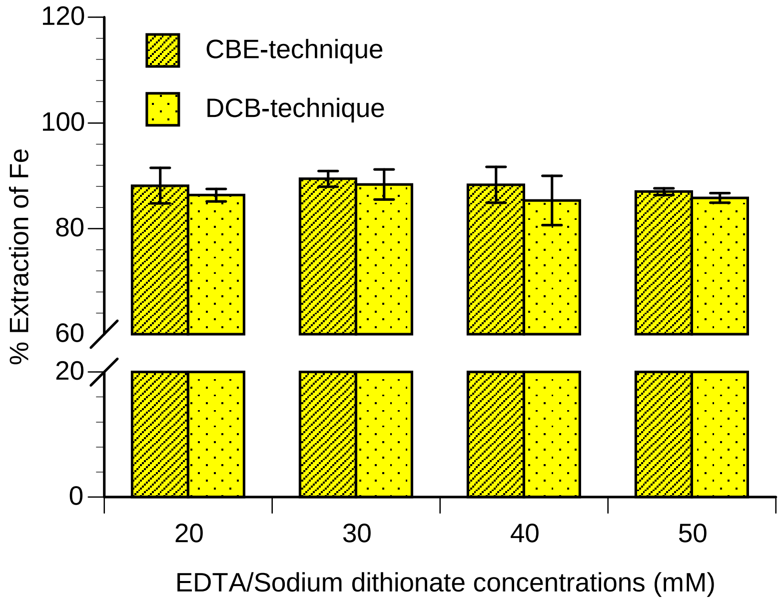

Fig. 3: Effect of EDTA and sodium dithionate concentrations in CBE- and DCB-solutions, respectively, on Fe extraction by CBE and DCB methods from rice root surfaces. Results are mean ± SD (n = 3).

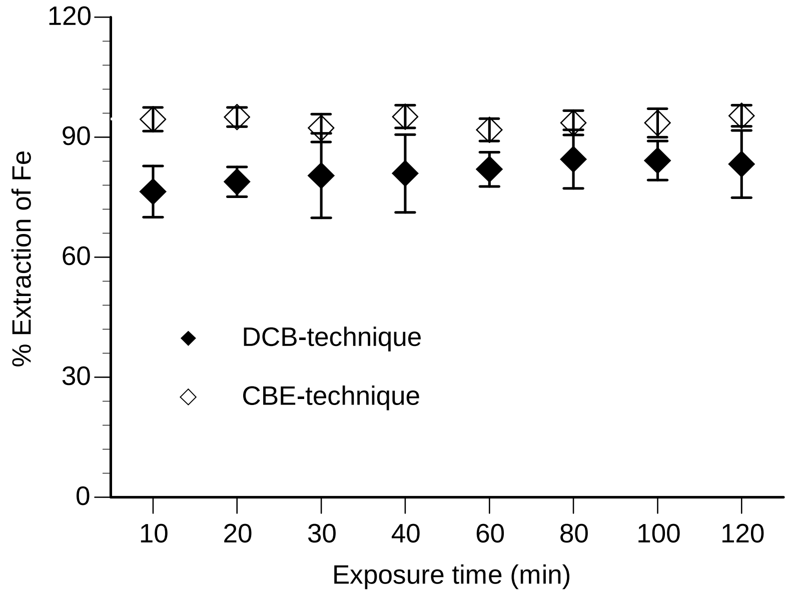

Fig. 4: Relationship between incubation time and Fe extraction from rice root surfaces using CBE or DCB method. Regression equations indicate that correlation between extractable Fe (%) on rice roots and incubation time was not significant ($p < 0.05$) for CBE solution, while significant correlation exists between extractable Fe (%) on rice roots and incubation time ($p > 0.05$) for DCB solution. Results are mean ± SD ($n = 3$).