

# Identification, separation and component analysis of reddish brown and non-reddish brown iron plaque on rice (*Oryza sativa*) root surface

You-Qiang Fu · Xu-Jian Yang · Zhi-Hong Ye · Hong Shen

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## Abstract

**Background and aim** Iron plaque (IP) on rice (*Oryza sativa*) root surface consists of reddish brown IP (RIP) and non-reddish brown IP (NRIP), however, their extraction and characterization need further studies.

**Methods** A reliable method combining chemical and X-ray diffraction (XRD) analysis was introduced to discriminate RIP and NRIP on root surface of rice plants subjected to different phosphate (P) treatments.

**Results** RIP appeared only on P-deficient rice root surface, and NRIP occurred on both P-deficient and P-sufficient rice root surface. Both RIP and NRIP could be extracted by dithionite-citrate-bicarbonate while only NRIP could be extracted by 0.1 M tetrasodium pyrophosphate ( $\text{Na}_4\text{P}_2\text{O}_7$ ) specifically. NRIP on P-sufficient root surface was 2.42 times of that on P-deficient plants. Iron speciation analysis showed that the total, amorphous and crystalline iron on P-deficient root surface

were 1.47-, 1.50- and 1.35-times of those of P-sufficient plants, respectively. XRD analysis further confirmed that IP on both P-sufficient and P-deficient root surface presented as amorphous features. Dominant minerals of NRIP were iron phosphates, while those of RIP were ferric (hydr)oxides. The specific removal effect of 0.1 M  $\text{Na}_4\text{P}_2\text{O}_7$  for NRIP was also verified by XRD.

**Conclusion** In this study, phosphate levels in nutrient solution significantly influence the formation of RIP and NRIP on rice root surface. Main components of RIP and NRIP are ferric (hydr)oxides and iron phosphates, respectively. They can be separated by 0.1 M  $\text{Na}_4\text{P}_2\text{O}_7$ .

**Keywords** Non-reddish brown Iron Plaque (NRIP) · Reddish brown Iron Plaque (RIP) · Rice (*Oryza sativa*) · Tetrasodium pyrophosphate ( $\text{Na}_4\text{P}_2\text{O}_7$ ) · X-ray Diffraction (XRD)

Y-Q Fu and X-J Yang made equal contribution to this article

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Y.-Q. Fu · X.-J. Yang · H. Shen (✉)  
College of Agriculture, South China Agricultural University,  
Guangzhou 510642, People's Republic of China  
e-mail: hshen@scau.edu.cn

Z.-H. Ye  
State Key Laboratory for Bio-control, and School of Life Sciences,  
Sun Yat-sen University, Guangzhou 510006, People's Republic of  
China

## Introduction

Formation of iron plaque (IP) on root surface is a characteristic phenomenon for helophytes growing under submerging conditions. IP is usually described as a layer of amorphous or crystalline ferric or ferrous compounds precipitating on helophytes root surface (Chen et al. 1980; Wang and John 1999). Previous studies reported that goethite ( $\alpha\text{-FeOOH}$ ), amorphous  $\text{Fe}(\text{OH})_3$ , ferrihydrite ( $\text{Fe}_{10}\text{O}_{15}\cdot 9\text{H}_2\text{O}$ ) and minor siderite ( $\text{FeCO}_3$ ) were the components of IP (Bacha and Hossner 1977; Chen et al. 1980; Chabbi 1999; Wang and John 1999; Hansel et al. 2001). However, Patrick and Khalid (1974)

indicated that under the reductive submerging conditions, ferrous (hydr)oxides were the predominant minerals in soil. Fu et al. (2011) also found that a certain amount of ferrous ions existed on rice root surface, however these ferrous (hydr)oxides were not identified as IP. The reason is probably associated with strong root oxidizing capacity of rice (Mei et al. 2009, 2012). In addition, Hossain et al. (2009) reported that low phosphate treatment could raise DCB-extractable Fe concentration and induced formation of reddish-brown IP on root surface in the pot-culture experiment. While under the high phosphate condition, DCB-extractable Fe could be detected, but reddish-brown IP could not be observed on rice root surface in a hydroponic experiment (Fu et al. 2014a). These results suggest phosphorus levels influence the quantity and visualization of IP on root surface significantly. The invisible DCB-Fe is little reported and never separated from IP on root surface, which should not be regarded as IP. Therefore, an accurate definition of IP is still lacking.

The sodium dithionite ( $\text{Na}_2\text{S}_2\text{O}_4$ )-sodium citrate ( $\text{Na}_3\text{C}_6\text{O}_7\text{H}_5$ )-sodium bicarbonate ( $\text{NaHCO}_3$ ) (DCB) mixture solution is an extraction reagent for IP on root surface, which has been widely used and regarded as a classical method (Lee et al. 2013). But in fact, the initial function of DCB solution is used for extraction of free irons from soil. These free irons include haematite ( $\alpha\text{-Fe}_2\text{O}_3$ ), maghaemite ( $\gamma\text{-Fe}_2\text{O}_3$ ), goethite, lepidocrocite ( $\gamma\text{-FeOOH}$ ) and amorphous  $\text{Fe}(\text{OH})_3$  gel (Kitagawa 2005). Since some of haematite, maghaemite, goethite, lepidocrocite and amorphous  $\text{Fe}(\text{OH})_3$  gel also belong to the components of IP (Zhang et al. 1998; Liang et al. 2006), DCB solution is applied for IP extraction (Taylor and Crowder 1983).

Based on the color and visibility, IP was divided into reddish brown iron plaque (RIP) and non-reddish brown iron plaque (NRIP) (Fu et al. 2014a). It is worthy to note that goethite, amorphous  $\text{Fe}(\text{OH})_3$  and ferrihydrites have yellow, brown or orange colors respectively. They may be classified into RIP. Anyways, it needs reliable proofs to support. On the other hand, Fu et al. (2014a) found that colorless iron compounds could be also extracted by DCB solution. This DCB-extractable but colorless iron-containing compounds are NRIP. Therefore, the results from DCB extraction can not reflect RIP accurately. Accordingly, it was greatly disputed whether the high concentration of DCB-Fe including RIP and NRIP on root surface could block the plant uptake of heavy metals or not (Liu et al. 2008;

Zhou et al. 2015). Our previous studies suggested that RIP possesses more dense spatial structure and stronger adsorption capacity of nutrient than NRIP (Fu et al. 2014a). For these reasons, the underlying differences of physical-chemical property between RIP and NRIP might influence the plant uptake of heavy metals and nutrient elements. A more specific extraction method for IP that can discriminate RIP and NRIP is necessary and meaningful.

X-ray crystal diffraction spectroscopy is a widely-used qualitative and quantitative method to study various substances including minerals and sediments. It identifies crystals by characteristic peaks of diffraction pattern, which is only determined by the nature of crystals. Therefore, the sample requirement for X-ray crystal diffraction spectroscopy is only fine-ground powder. A series of advanced instrumental analytic methods including energy dispersive absorption X-ray (fluorescence) spectrometric microanalysis [EDA (F)X], X-ray absorption near edge structure spectrometry (XANES) and (Extended) X-ray absorption fine structure spectrometry [(E)XAFS] have been applied for IP component analysis and make great contribution in amorphous substance identification and quantitative composition calculation (Hansel et al. 2001; Franco et al. 2013; Mi et al. 2013; Syu et al. 2014; de Araujo et al. 2015). Based the questions mentioned above, the objective of this study was to explore the exact components of IP, especially RIP, by chemical and spectroscopic methods. Meanwhile a reliable method was provided to discriminate RIP and NRIP on root surface.

## Materials and methods

### Plant cultivation

Seeds of rice cultivar (*Oryza sativa* cv. Tianyou 998) were sterilized by  $3 \text{ g L}^{-1} \text{ H}_2\text{O}_2$  for 30 min and then rinsed thoroughly with deionized water over three times. Sterilized seeds germinated in Petri dishes with moist filter paper. The Petri dishes were sealed by porous polyethylene film and placed in an incubation chamber set at  $30^\circ\text{C}$ . Germinated seeds were transplanted on 3-mm plastic mesh and cultivated with 1/4 to 1/2 strength complete nutrient solution progressively for a week. When the second euphylla expanded completely, seedlings were transplanted to plastic boxes. Seedlings were fixed into the holes on plantation plates with sponge above plastic

box for treatments. Each box contained 9 L of full strength nutrient solution and was planted with 48 seedlings.

### Nutrient solution

The formula of nutrient solution was modified slightly according to Yoshida et al. (1976) as follows:  $\text{NH}_4\text{NO}_3$  0.429 mM,  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  1 mM,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  1.667 mM,  $\text{KH}_2\text{PO}_4$  1 mM,  $\text{K}_2\text{SO}_4$  0.513 mM,  $\text{FeNa-EDTA}$  50  $\mu\text{M}$ ,  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  9.1  $\mu\text{M}$ ,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  0.15  $\mu\text{M}$ ,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  0.16  $\mu\text{M}$ ,  $(\text{NH}_4)_4\text{MoO}_{24} \cdot 4\text{H}_2\text{O}$  0.52  $\mu\text{M}$ ,  $\text{H}_3\text{BO}_3$  19  $\mu\text{M}$ . The pH of nutrient solution was adjusted at 5.5 with diluted  $\text{H}_2\text{SO}_4$  or  $\text{NaOH}$ .

### Experiment design

To explore the influence of P and Fe on the formation of RIP, four treatments with different Fe and P concentrations were set as follows: 1) CK, P-sufficient (complete) nutrient solution; 2) -P, P-deficient nutrient solution; 3) CK + Fe, P-sufficient nutrient solution +0.1 mM  $\text{Fe}^{2+}$ ; 4) -P + Fe, P-deficient nutrient solution +0.1 mM  $\text{Fe}^{2+}$ . Four replicates were set in each treatment. Rice seedlings were treated in plastic cups with fixed sponges. Each seedling was cultivated in a cup containing 350 mL of nutrient solution. After growing in P-sufficient nutrient solution for 21 d, rice seedlings were then transplanted in corresponding nutrient solution according to experiment design, and  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  solution was not added into nutrient solution until 2 d later for eliminating P interference. Treatments lasted another 2 d after  $\text{Fe}^{2+}$  had been added. In the P-deficient treatments,  $\text{KH}_2\text{PO}_4$  was replaced by  $\text{KCl}$ . The pH of all treatments was adjusted to 5.5 with diluted  $\text{H}_2\text{SO}_4$  or  $\text{NaOH}$ .

### $\text{Fe}^{3+}$ staining

Rice roots were soaked in fresh staining solution containing 0.24 M  $\text{HCl}$  and 20 g  $\text{L}^{-1}$   $\text{K}_4[\text{Fe}(\text{CN})_6]$  in vacuum condition for 15 min, photos were taken after roots were rinsed with deionized water.  $\text{Fe}^{3+}$  concentration on root surface can be observed by eye or by microscopy, more blue  $\text{Fe}_4[\text{Fe}(\text{CN})_6]_3$  precipitation on root surface means higher levels of  $\text{Fe}^{3+}$  in root tissue. Juvenile roots with 100- to 130-mm length and uniform size were selected for freehand cross section in the 10- to 25-mm region from root-shoot junction. Cross sections

were observed in optical microscope (Model BX43, Olympus, Japan) and taken photos (Yokosho et al. 2009).

### DCB extraction

DCB-extractable Fe included Fe-containing substances adsorbed or precipitated on root surface. The extraction method was modified according to Lee et al. (2013). The whole rice roots, around 1.0 g, were rinsed with deionized water and soaked in 150-mL flask containing extraction solution. The extraction solution was the mixtures of 40 mL of 0.3 M  $\text{Na}_3\text{C}_6\text{O}_7\text{H}$ , 5 mL of 1 M  $\text{NaHCO}_3$  and 1 g of  $\text{Na}_2\text{S}_2\text{O}_4$ . Horizontal shake was conducted at 25 °C with the speed of 220 rpm for 3 h. The extract solution was transferred to volumetric flask and made up to 100 mL with deionized water. Fe concentration in extract solution was measured by Zeeman polarized atomic absorption spectrometer (Model Z-5300, Hitachi, Japan) and the DCB-Fe concentration on root surface was represented as g per kg of rice roots dry weight basis.

### Determination of $\text{Fe}^{2+}$ and $\text{Fe}^{3+}$ content on root surface

Ethylenediamine tetraacetic acid disodium ( $\text{Na}_2\text{EDTA}$ )-bathophenanthroline disulfonate (BPDS) chelation extraction method was applied to determine  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  contents on root surface (Montas-Ramirez et al. 2003). After treatment, intact rice plants were transferred to deionized water for 6 h. Water on roots was absorbed by filter paper. Plant roots were cut and added into aluminum foil-coated test tube containing 10 mL of 1.0 M  $\text{Na}_2\text{EDTA}$  - 0.3 M BPDS mixing extraction solution. Test tubes were shaken in horizontal shaker with the speed of 125 rpm for 5 h at room temperature. After being left to stand for 15 h, extraction solution was transferred to 25 mL tubes with volume mark and made up to 25 mL with deionized water. The absorbance of solution at 535 nm of  $\text{Fe}^{2+}$ -BPDS complex after 30 min was measured with a spectrophotometer (Model 754, Shunyu Hengping, China). The concentration of  $\text{Fe}^{2+}$  was calculated according to a standard curve. The extraction system for total Fe consisted of 2 mL of 30 mM  $\text{Na}_2\text{S}_2\text{O}_6$  and 2 mL of fresh  $\text{EDTA}$ -BPDS. The extraction solution was completely mixed and left to stand for 1 h to reduce  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  thoroughly. Absorbance of solution at 535 nm was measured and the difference of two measurements was the concentration of  $\text{Fe}^{3+}$ ,  $\text{Fe}^{2+}$  or  $\text{Fe}^{3+}$ .

concentration on root surface was represented as mg per kg of fresh rice roots.

#### NaOH treatment

Petersen and Corey (1966) reported that the hydroxyl ions of sodium hydroxide (NaOH) could replace the phosphate species of iron-phosphate and formed sodium phosphate. Sodium phosphate was dissolved in extraction solution and iron ion under alkaline condition existed as reddish-brown precipitation of iron hydroxide. In order to observe the existence of NRIP on root surface (namely most iron precipitation) under CK + Fe treatment, we ingeniously designed a NaOH experiment to show invisible iron precipitation to prove the existence of NRIP as follows: Roots with CK + Fe and -P + Fe treatments were rinsed with deionized water thoroughly and then soaked in 0.1 M NaOH for 20 min at room temperature. Roots were used for observation after rinsed with deionized water twice.

#### Extraction reagents screening for NRIP removal

Rice roots were rinsed with deionized water and soaked in 150-mL flask containing 50 mL of extract reagent candidates: 0.05 M  $\text{Na}_2\text{SO}_4$  + 0.4 M  $\text{H}_2\text{SO}_4$  (extracting minerals of which solubilities were sensitive to low pH according to Woolson et al. 1971), 1 M  $\text{CH}_3\text{COONa}$  (extracting metals from the carbonate fraction; Tessier et al. 1979), 0.3 M  $\text{Na}_3\text{C}_6\text{O}_7\text{H}_5$  (a complexing agent; Turonova et al. 2008), 0.5 mM ethylenediamine tetraacetic acid disodium ( $\text{Na}_2\text{EDTA}$ ; metal-chelating agents) (Tandy et al. 2004), and 0.1 M  $\text{Na}_4\text{P}_2\text{O}_7$  (extracting metals from the organic fraction) (Kaiser and Zech 1996) respectively, for 2-h vibration in the speed of 100 rpm. Candidate extraction reagents were subjected to a further 4-h vibration in the speed of 180 rpm if they performed well in the previous screening. After extraction, the roots were rinsed with deionized water and stained in 0.24 M  $\text{HCl}$ -20  $\text{g L}^{-1}$   $\text{K}_4[\text{Fe}(\text{CN})_6]$  solution for 10 min to assess removal effects.

#### NRIP and RIP extraction

NRIP extraction: Firstly, rice roots were rinsed with deionized water, and then soaked in deionized water for 1 h to reduce residual Fe adsorbed on root surface. Finally, rice roots were placed in 150-mL flask

containing 50 mL of 0.1 M sodium pyrophosphate ( $\text{Na}_4\text{P}_2\text{O}_7$ ) for 4-h vibration in the speed of 180 rpm.

RIP extraction: After NRIP extraction, rice roots were extracted by DCB extraction solution with the speed of 220 rpm for 3 h. Total DCB-Fe indicated the sum of RIP and NRIP.

#### Iron speciation analysis

Since IP covered root surface, and the severe extraction conditions, such as strong acid/alkali, and high temperature, might damage plant roots and posed root iron outflow. A mild sequential extraction procedure was selected according to the methods of Poulton and Canfield (2005) and Claff et al. (2010) to measure the iron speciation on rice root surface as follows. Fraction I-Fe (Exchangeable Fe) was extracted by 1 M magnesium chloride ( $\text{MgCl}_2$ ) in the speed of 180 rpm for 1 h at room temperature. Fraction II-Fe (Carbonate Fe) was extracted by pH 4.5, 1 M sodium acetate (adjusted with acetic acid) in the speed of 180 rpm for 1 h at room temperature. Fraction III-Fe (Poorly crystalline Fe) was extracted by pH 3.2, 0.2 M ammonium oxalate in the speed of 180 rpm for 1 h at room temperature. Fraction IV-Fe (Crystalline Fe) was extracted by 0.2 M sodium citrate buffer with 50  $\text{g L}^{-1}$  sodium dithionite in the speed of 180 rpm for 1 h. All Fe fractions of the extract solution were measured with an atomic absorption spectrometry (Model Z-5300, Hitachi, Japan).

#### X-ray powder diffraction analysis of IP

To identify exact components of IP, fresh root samples with both -P + Fe and CK + Fe treatments as well as those after NRIP removal were subjected to X-ray powder diffraction analysis. Rice roots were rinsed with deionized water to remove residual extraction reagents. Water on root surface was absorbed by filter paper. After speed freezing to  $-80^\circ\text{C}$  in ultra-low temperature freezer for 8 h and freezing drying in  $-80^\circ\text{C}$  and 0.8 Pa condition in vacuum freezing dryer (FreezeZone 2.5 Plus, Labconco, USA) for 36 h, root samples with IP were ground into powder in agate mortar containing liquid nitrogen. Samples were stored in desiccator with adequate silica gel. X-ray diffraction analysis was conducted by an X-ray diffractometer (Model XD-2, Purkinje General, China). Sample powder was compacted in glass slide with 1 cm  $\times$  1 cm groove. X-ray tube with Cu target worked in conditions of

36 kV accelerative voltage and 30 mA tube current. A graphite monochromator was used. The diffraction patterns were scanned within the  $2\theta$  angle ranging from 5 to  $60^\circ$  in continuous scanning mode with the scanning speed of  $8^\circ \text{ min}^{-1}$  and step length of  $0.02^\circ$ . The curves of X-ray diffraction were analyzed with Jade 5.0 (Materials Data Inc., USA) for peak comparison of multiple patterns and phase identification by profile-base search and match function.

### Statistical analysis

Means and standard errors of all data were calculated by Microsoft Excel 2003, and one-way ANOVA multiple comparisons were conducted with Duncan's new multiple range methods in SPSS 12.0.

## Result

### Distribution of RIP on root surface

Figure 1 showed that RIP did not distribute uniformly on root surface. Aged and thin roots had less RIP on their surface while juvenile and thick roots had more RIP (Fig. 1a, b). Interestingly, RIP with different quantities precipitated on the similar regions of different roots and different regions in the same roots (Fig. 1b–d). Tri-valence Fe was the main valence of iron in RIP

(Fig. 1f). Due to the formation of large amounts of aerenchyma, root base secreted more oxidative substances into root surface, and thus formed more RIP on basal root. On the contrary, little RIP precipitated on the surface of root tips due to less formation of aerenchyma. It was out of our expectation that root tips, the most active areas of metabolism, were covered by a large amount of RIP (called “iron cap”) (Fig. 1b). Due to rapid growth speed and stronger oxidizing capacity, RIP on root surface of root tips was easily ignored since RIP was wrapped by gluey substances and the iron cap was penetrated, peeled and slipped over on root tips.

### Discrimination of RIP and NRIP

As shown in Fig. 2, RIP occurred only on -P + Fe treated root surface, but not on CK + Fe treated root surface (Fig. 2a). A higher amount of DCB-extractable  $\text{Fe}^{3+}$  was observed on root surface with CK + Fe treatment than that with CK treatment (Fig. 2b and f). The results suggested that CK + Fe treatment enhanced  $\text{Fe}^{2+}$  oxidation and  $\text{Fe}^{3+}$  precipitation on root surface. Iron ion measurement showed that the concentration of  $\text{Fe}^{3+}$  in IP was over twice of that of  $\text{Fe}^{2+}$  (Fig. 2g). On other hand, pH decline occurred in both CK + Fe and -P + Fe treatments (Fig. 2e). This was due to  $\text{H}^+$  release accompanying with  $\text{Fe}^{2+}$  oxidation in the reaction of  $4\text{Fe}^{2+} + \text{O}_2 + 10\text{H}_2\text{O} = 4\text{Fe}(\text{OH})_3 + 8\text{H}^+$ . The results further

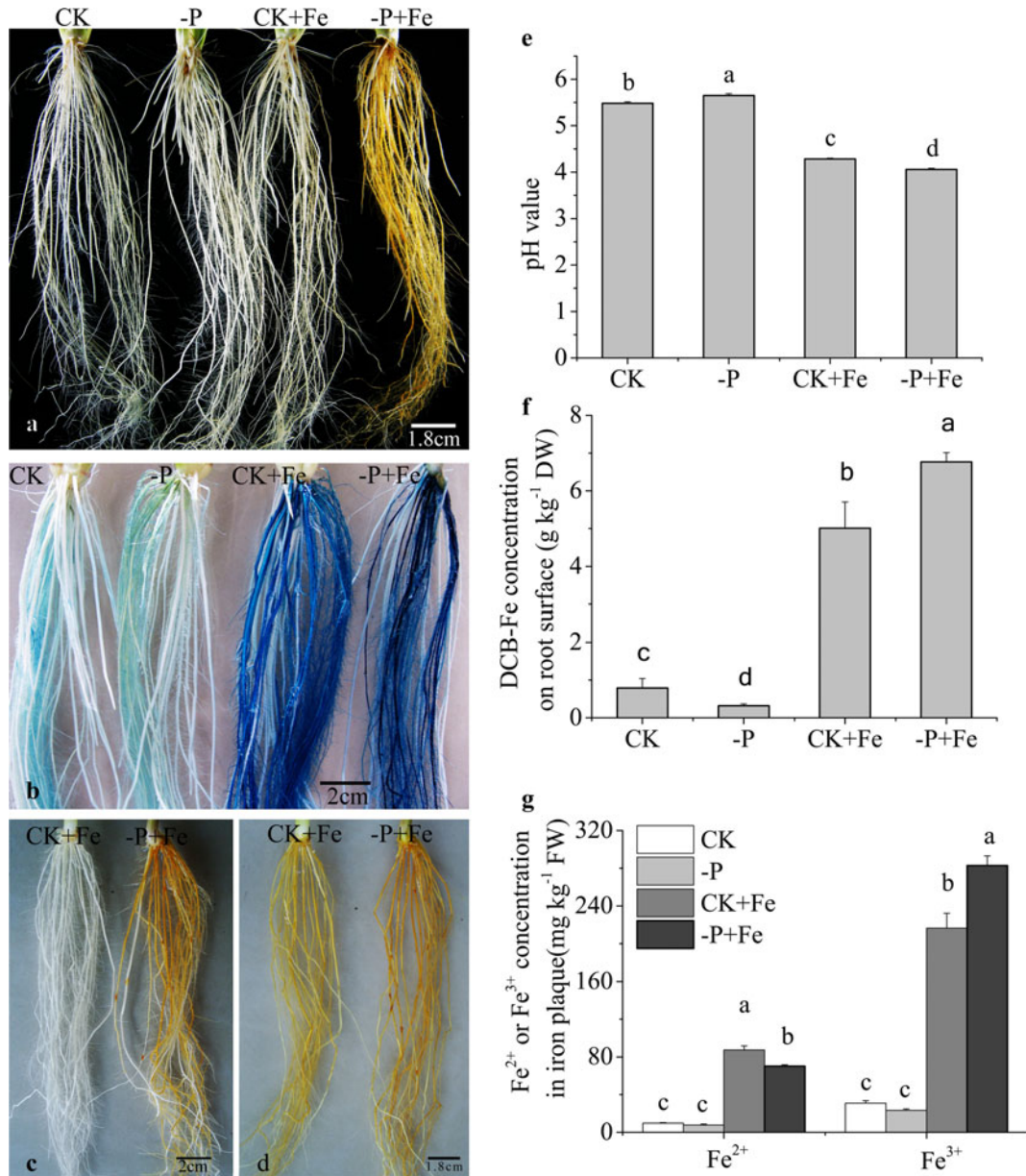
**Fig. 1** Distribution of RIP on rice root surface. **A**, intact rice roots with RIP; **B**, reddish brown “iron cap” and RIP on root with different root activities, roots in **a** to **c** had higher root activity than those in **d**; **C** and **D**, RIP on the same region in two roots; **e** and **f**, RIP and  $\text{Fe}^{3+}$  staining on cross section of root base



indicated  $\text{Fe}^{2+}$  oxidation in two treatments, which was consistent with the results of Fig. 2b and g.

A higher  $\text{Fe}^{3+}$  concentration of IP occurred on  $-\text{P} + \text{Fe}$  treated root surface than that of  $\text{CK} + \text{Fe}$ , while  $\text{Fe}^{2+}$  concentration of IP was lower on  $-\text{P} + \text{Fe}$  treated root surface than that of  $\text{CK} + \text{Fe}$  treated roots (Fig. 2g). The results indicated that  $-\text{P} + \text{Fe}$  treated rice roots elevated

the oxidation of  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$ . Since the phosphate existed on the root surface of  $\text{CK} + \text{Fe}$  treatment and not on the surface of  $-\text{P} + \text{Fe}$  treated rice roots (data not shown), colorless iron phosphates might be the main compounds on the root surface of  $\text{CK} + \text{Fe}$  treatment. The results explained why no RIP appeared on  $\text{CK} + \text{Fe}$  treated root surface, even with a large amount of  $\text{Fe}^{3+}$  sediment.



**Fig. 2** Discrimination of RIP and NRIP. **a**, roots with different treatments; **b**, root  $\text{Fe}^{3+}$  staining with different treatments; **c**, magnified roots with  $\text{CK} + \text{Fe}$  and  $-\text{P} + \text{Fe}$  treatments; **d**, magnified roots from **c** after  $\text{NaOH}$  treatment; **e**, nutrient solution pH after  $\text{Fe}$

treatment for 2 d; **f**, DCB-Fe content on root surface; **g**,  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  content in IP. The data indicated means  $\pm$  standard errors ( $n = 4$ ), and the data with the same letter in the same item meant no significant difference between treatments ( $p < 0.05$ )

Additional experiment of NaOH treatment to CK + Fe and -P + Fe treated roots (Fig. 2c and d) showed that no significant changes of IP were observed on -P + Fe treated root surface after NaOH treatment, while RIP appeared on root surface with CK + Fe after NaOH treatment. Since the solubility constant ( $K_{sp}$ ) of iron phosphate was larger than that of ferric hydroxides [ $\text{Fe}(\text{OH})_3$ ], phosphate was replaced by hydroxyl and then reddish brown ferric hydroxides appeared on root surface with CK + Fe after NaOH treatment.

### Separation of RIP and NRIP

The results from Figs. 1 and 2 suggested that phosphate level was an important factor influencing the formation of RIP on root surface. Non-reddish brown but DCB-extractable IP (i.e. NRIP) precipitated on root surface of rice seedlings with CK + Fe or -P + Fe treatment. It is necessary to find a method to separate RIP and NRIP. Figure 3a–d showed the NRIP removal effects of different extraction solution. Removing duration was 2 h and vibration frequency was 100 rpm.  $\text{Fe}^{3+}$  in both CK + Fe and -P + Fe treatments could not be removed by 0.3 M  $\text{Na}_3\text{C}_6\text{O}_7\text{H}$  or 1 M  $\text{CH}_3\text{COONa}$  significantly (Fig. 3c' and d'), while 0.05 M  $\text{Na}_2\text{SO}_4$  + 0.4 M  $\text{H}_2\text{SO}_4$  could remove  $\text{Fe}^{3+}$  in both treatments efficiently (Fig. 3b'). 0.1 M  $\text{Na}_4\text{P}_2\text{O}_7$  and 0.5 M  $\text{Na}_2\text{EDTA}$  seemed to be an ideal extraction solution since they removed  $\text{Fe}^{3+}$  on CK + Fe treated root surface only, but not on -P + Fe treated root surface (Fig. 3e' and f'). However,  $\text{Na}_2\text{EDTA}$  extraction with a longer extraction (4 h) and a faster vibration frequency (180 rpm) (Fig. 3e) could remove both NRIP (h' of Fig. 3e and f) and RIP (g' of Fig. 3e and f). Meanwhile,  $\text{Na}_4\text{P}_2\text{O}_7$  extraction could only remove almost of NRIP (h' of Fig. 3e and f) and little of RIP (g' of Fig. 3e and f). The results were also verified by  $\text{Fe}^{3+}$  staining assay (Fig. 3f).

### IP fraction analysis

According to extraction procedures, RIP and NRIP on root surface were extracted and determined. The result from Fig. 4 indicated that under the CK or -P treatment condition, RIP, NRIP and DCB-Fe were low due to without ferrous ( $\text{Fe}^{2+}$ ) supply. NRIP on CK-treated root surface was more than that of -P treatment. This was probably associated with Fe translocation and uptake. P starvation induced a higher Fe uptake in comparison to P supply. Fe supply increased the concentrations of RIP,

NRIP and DCB-Fe significantly. The concentrations of both RIP and DCB-Fe on root surface with -P + Fe treatment were obviously higher than those of CK + Fe. RIP occupied 94.2 % of total IP in -P + Fe treatment while the corresponding value was 53.7 % in CK + Fe treatment.

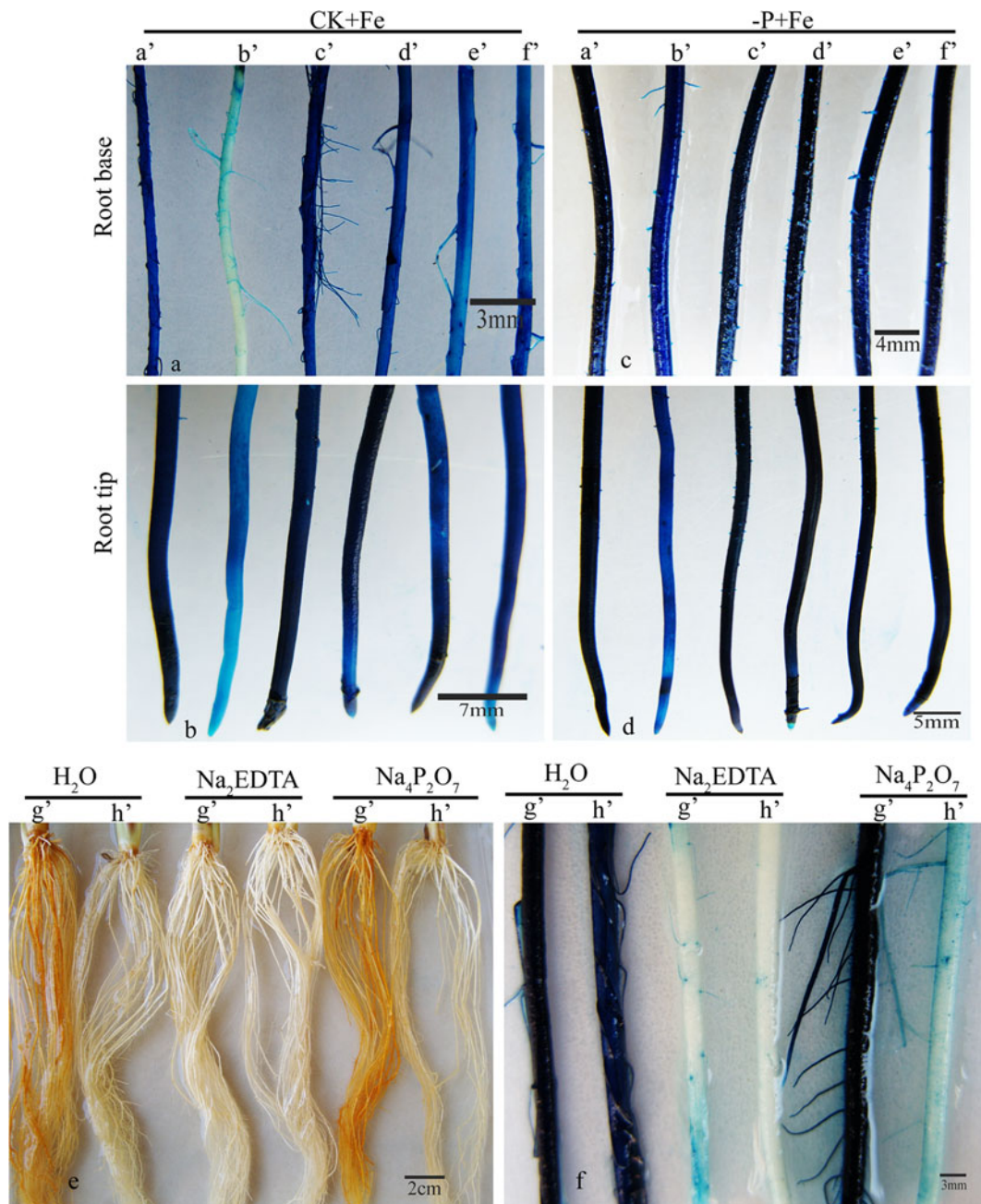
### Iron speciation in IP

To explore the difference of RIP and NRIP, the iron speciation was investigated according to the methods described by Poulton and Canfield (2005) and Claff et al. (2010). Results from Table 1 indicated that amorphous and poor crystalline iron (fraction III) were the major fraction in IP, which occupied over 80 % of the total iron-containing compounds in those treatments with Fe supply. Other iron concentrations except for fraction II on -P treated root surface were lower than the corresponding values of CK treatment. Iron concentration in fraction I treated with CK + Fe was significantly higher than that of -P + Fe treatment, but opposite results occurred in fractions III and IV. The above results indicated that phosphate deficiency could promote the formation of amorphous, poor crystalline and fine crystalline iron (fractions III and IV) as well as reduce exchangeable iron (fraction I).

### X-ray diffraction analysis of IP

Results from Fig. 2 and Table 1 gave an indirect explanation of IP components. To obtain a convincing and direct result, X-ray diffraction analysis was performed to examine IP components. Results from Fig. 5 indicated that XRD spectrum of IP showed similar amorphous characteristic patterns, though some peaks of iron compounds could be identified. The results were consistent with those of iron speciation analysis (Table 1).

Figure of merit (FOM) was calculated based on the similarity of pattern and characteristic peaks of powder diffraction file. Most possible mineral values indicated by FOM were less than 20. The full data of mineral phase report of each pattern was shown in Supplemental Materials (phase report). The dominant minerals with CK + Fe and -P + Fe treatments were significantly different. The main minerals in IP with CK + Fe treatment were tincite [ $\text{Fe}_6(\text{PO}_4)_4(\text{OH})_6 \cdot 7\text{H}_2\text{O}$ ], ludiamite [ $\text{Fe}_3(\text{PO}_4)_2 \cdot 4\text{H}_2\text{O}$ ], graftonite [ $\text{Fe}_3(\text{PO}_4)_2$ ], wolfeite [ $\text{Fe}_2\text{PO}_4(\text{OH})$ ] and goethite (Fig. 5a), while those with -P + Fe treatment were goethite, lepidocrocite,



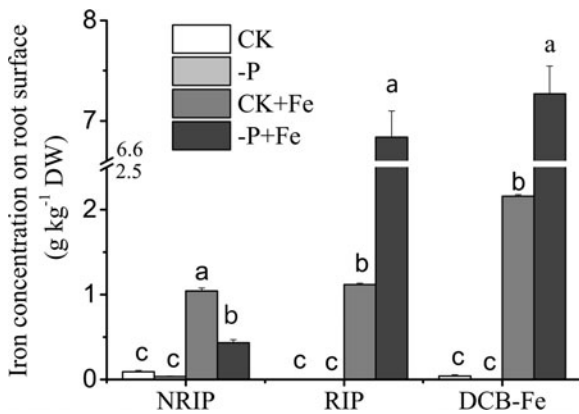
**Fig. 3** Iron removal effects with different extraction solutions. **a - d**, root tip and base surface  $\text{Fe}^{3+}$  staining of CK + Fe and -P + Fe treatments after 2-h vibration in the speed of 100 rpm; **e - f**, iron plaque and  $\text{Fe}^{3+}$  staining on root surface with CK + Fe and -P + Fe treatments after 4-h vibration in the speed of 180 rpm. **a** and **c**, root base with CK + Fe and -P + Fe treatments; **b** and **d**, root tip with

CK + Fe and -P + Fe treatments. **a'** to **f'** treatments in Panel **a** to **d**: **a'**, deionized water; **b'**, 0.05 M  $\text{Na}_2\text{SO}_4$  + 0.4 M  $\text{H}_2\text{SO}_4$ , **c'**, 1 M  $\text{CH}_3\text{COONa}$ , **d'**, 0.3 M  $\text{Na}_3\text{C}_6\text{O}_7\text{H}_5$ , **e'**, 0.5 M  $\text{Na}_2\text{EDTA}$ , **f'**, 0.1 M  $\text{Na}_4\text{P}_2\text{O}_7$ ; **g'** and **h'** in Panel **e** and **f**: **g'**, -P + Fe treatment; **h'**, CK + Fe treatment

magnetite ( $\text{Fe}_3\text{O}_4$ ) and haematite (Fig. 5b) respectively. These results were consistent with the previous results that more  $\text{Fe}^{2+}$  presented in NRIP than in RIP (Fig. 2g).

After  $\text{Na}_4\text{P}_2\text{O}_7$  treatment, lepidocrocite became the dominant mineral of IP with -P + Fe and CK + Fe treatments (Fig. 6).





**Fig. 4** Effects of different treatments on RIP (reddish brown iron plaque), NRIP (non-reddish brown iron plaque) and DCB-Fe on root surface. The concentration of DCB-Fe was the sum of NRIP and RIP. Data indicated means  $\pm$  standard errors ( $n = 4$ ), and different letters above the column indicated significant differences ( $p < 0.05$ )

## Discussion

In early days, reddish sediments appeared on helophyte root surface are named as “iron plaque” (Chen et al. 1980). So the original definition of IP indicated RIP. However, the quantitative method measuring RIP is still lacking. Since minerals in IP are similar to free iron in soil, and soil free iron can be extracted by DCB solution, some researchers used DCB solution to extract IP, and thus DCB method was used for IP measurement (Lee et al. 2013). The components of IP include both DCB-extractable substances and other compounds. In other words, DCB extraction is not specific for RIP. Many studies indicated that IP precipitated mainly on root surface in low phosphorous cultivation condition (Hu et al. 2005; Liang et al. 2006; Hossain et al. 2009).

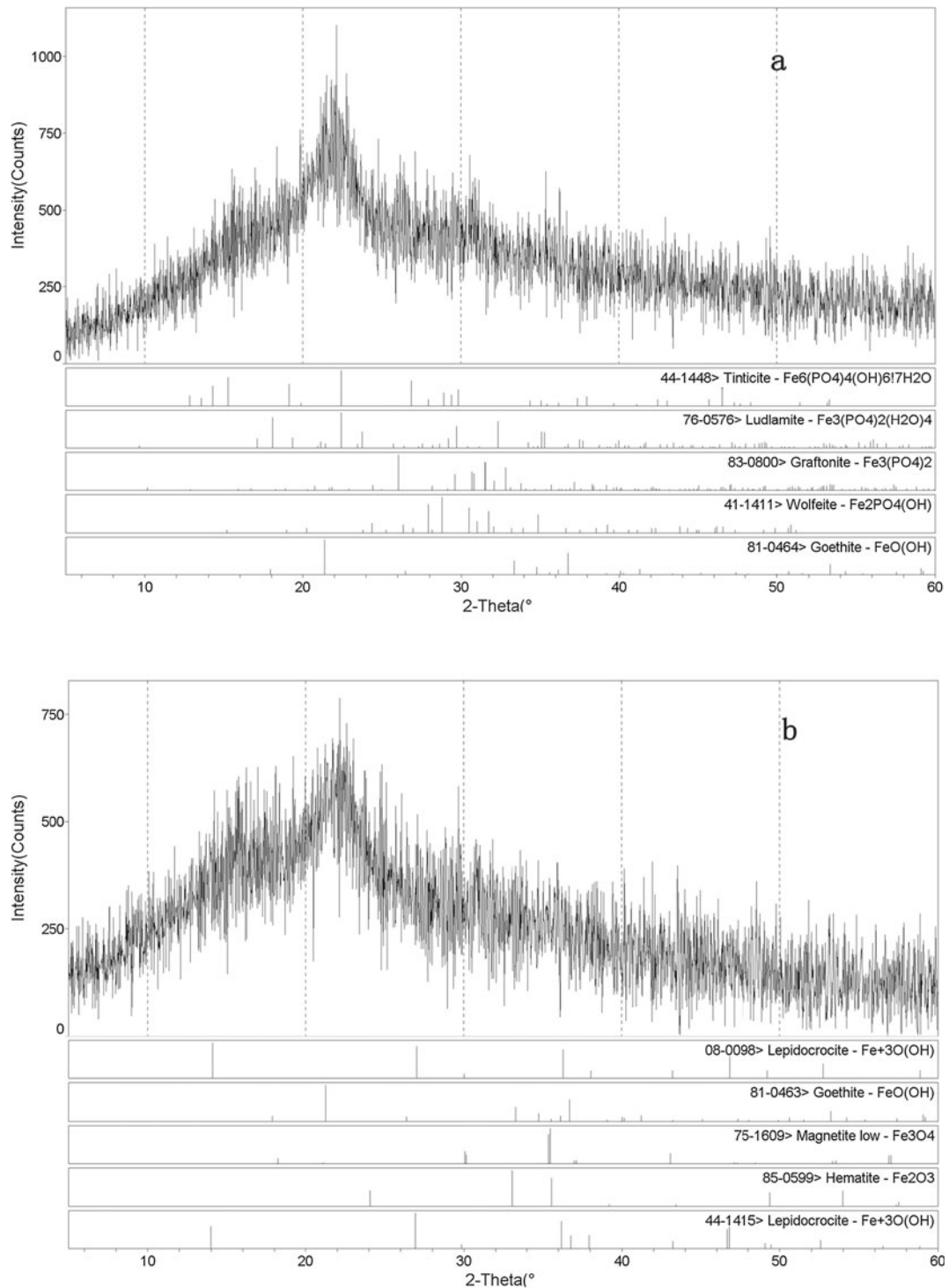
Our previous studies also found that low phosphate, especially low phosphate ferrous ratio (P:Fe = 1:3) could increase IP especially RIP precipitation on root surface (Fu et al. 2014a, b). In this experiment, the concentration of phosphate was higher than that of hydroxyl in nutrient solution under CK + Fe treatment (1 mM versus 3.16 mM), so white ferric phosphates formation took precedence than ferric hydroxides, which could be proved by white fluffy precipitation in nutrient solution, and consumed ferrous for IP formation. Voegelin et al. (2013) suggested that when phosphate ferrous ratio over 1:2, no iron hydroxide but iron phosphates formed in dilute solution system. Our results from Fig. 2 indicated that  $\text{Fe}^{3+}$ -containing compounds could be extracted by DCB, though RIP was absented (Fig. 2a, b and f). Transformation from NRIP to substances similar to RIP in CK + Fe treatment after NaOH demonstrated that the differences between RIP and NRIP did exist (Fig. 2c and d). Seyfferth (2015) indicated that the addition of phosphate, an oxyanion ligand, significantly transforms crystalline type of ferric hydroxide in IP, from goethite ( $\alpha\text{-FeOOH}$ ) when phosphate is absent to lepidocrocite ( $\gamma\text{-FeOOH}$ ) when phosphate presents in hydroponic condition. However, goethite was also a dominant phase in CK + Fe treatment in our study (Figs. 5 and 6). In fact, it has been proved that phosphate has a high affinity to ferric hydroxides (e.g. goethite and lepidocrocite), and concomitant precipitation of amorphous iron phosphates retards polymerization of ferric hydroxides, as a result, the proportion of RIP main components (goethite and lepidocrocite) is low in IP (Voegelin et al. 2013, Senn et al. 2015). However, we still found goethite in  $-\text{P} + \text{Fe}$  treated IP, which was different from the one from Senn et al. (2015)

**Table 1** Iron speciation of IP on root surface of rice seedlings

	Iron concentration ( $\text{g kg}^{-1}$ DW)				Total Fe content ( $\text{g kg}^{-1}$ DW)
	Fraction I <sup>a)</sup>	Fraction II	Fraction III	Fraction IV	
CK	0.029 $\pm$ 0.008 c <sup>b)</sup>	0.226 $\pm$ 0.011 b	0.363 $\pm$ 0.032 c	0.013 $\pm$ 0.004 c	0.670 $\pm$ 0.04 c
-P	0.025 $\pm$ 0.002 c	0.257 $\pm$ 0.024 b	0.316 $\pm$ 0.013 c	0.006 $\pm$ 0.003 c	0.636 $\pm$ 0.03 c
CK + Fe	0.075 $\pm$ 0.002 a	0.933 $\pm$ 0.097 a	4.972 $\pm$ 0.616 b	0.354 $\pm$ 0.027 b	6.083 $\pm$ 0.75 b
-P + Fe	0.051 $\pm$ 0.000 b	0.945 $\pm$ 0.039 a	7.461 $\pm$ 0.276 a	0.478 $\pm$ 0.009 a	8.935 $\pm$ 0.31 a

<sup>a)</sup> Fraction I, exchangeable Fe; Fraction II, carbonate Fe; Fraction III, amorphous and poor crystalline; Fraction IV, crystalline Fe. CK: P-sufficient nutrient solution treatment; -P: P-deficient nutrient solution treatment; CK + Fe: P-sufficient nutrient solution +0.1 mM  $\text{Fe}^{2+}$  treatment; -P + Fe: P-deficient nutrient solution +0.1 mM  $\text{Fe}^{2+}$  treatment

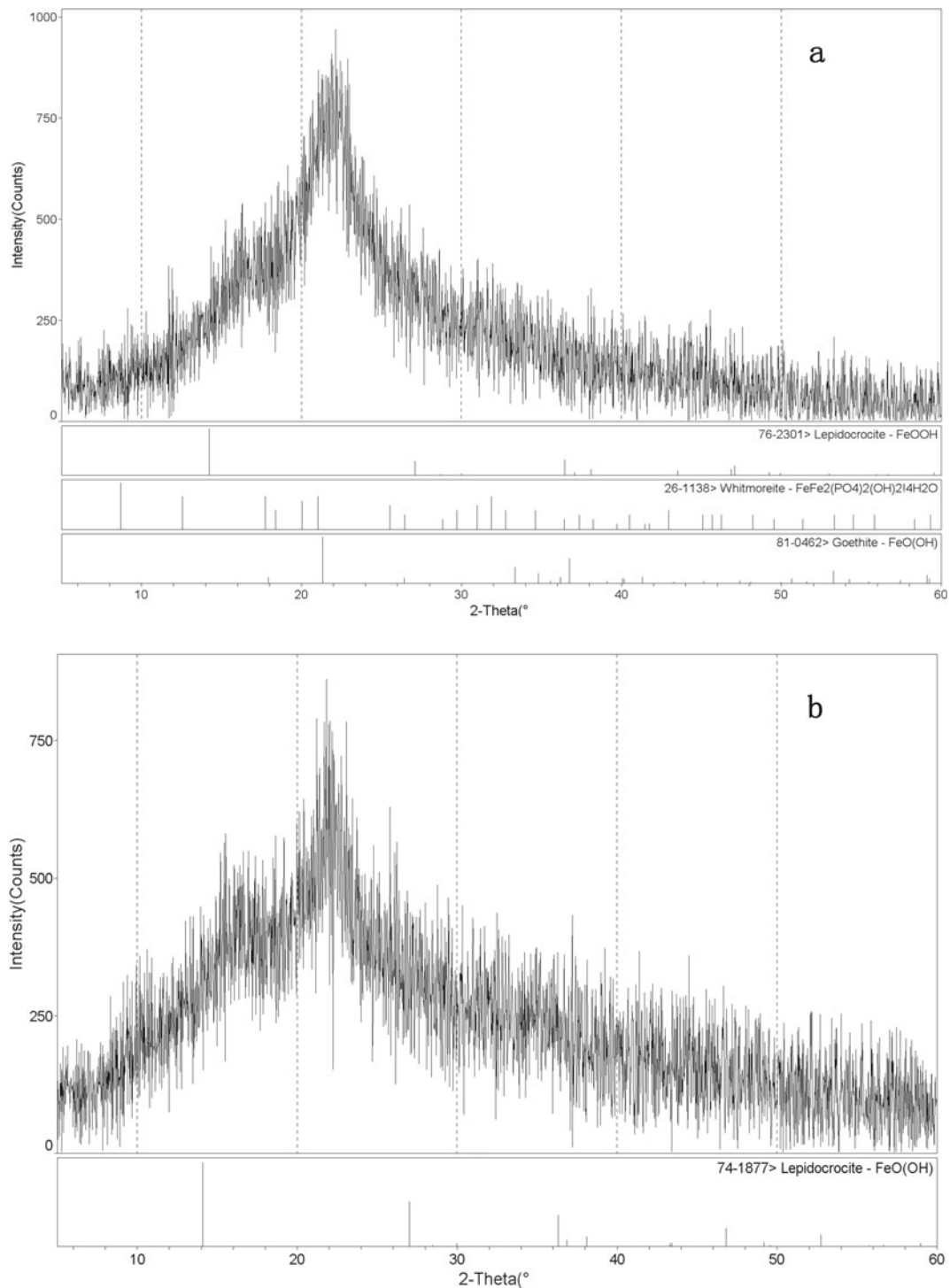
<sup>b)</sup> Data indicated means  $\pm$  standard errors ( $n = 4$ ), and data with the different letters within the same column indicated significant differences ( $p < 0.05$ )



**Fig. 5** Effects of different treatments on XRD pattern of IP on root surface. **a**, CK + Fe treatment, **b**, -P + Fe treatment

and Seyfferth (2015). The reasons resulting in above divergences might be: (1) this study excluded biotic factors in IP formation process by precipitating and

collecting IP with solution filtrates and silicone tubes; (2) this study was performed in ultrapure water rather than nutrient solution, and the pH of water was different



**Fig. 6** XRD pattern of IP on root surface with different treatments after  $\text{Na}_4\text{P}_2\text{O}_7$  treatment **a**, CK + Fe treatment, **b**, -P + Fe treatment

from ours. Anyways, a large of reaction occurs during ferrous oxidation in IP formation process rather than a simply competition between phosphate and hydroxyl

(or water and dissolved oxygen in water) (Voegelin et al. 2013). Further studies are needed for more detail elucidations.

Phosphate deficiency seems to be unnecessary for RIP formation under soil condition since IP forms ubiquitously on the root surface of helophytes in soils with various phosphate supply levels. Regional phosphate deficiency still exists in rhizosphere despite that it's abundant in soil in general due to low mobility of phosphate in soil (Mengel et al. 2001). So, ferrous ion in rhizosphere soil solution is not easy to react with phosphate as in hydroponics. Besides, soil is an iron pool that can supply abundant ferrous in continuous submerging condition. As a result, RIP can be easily formed on root surface under soil conditions. In addition, sufficient ferrous ions and high oxidizing capacity are two essential factors for visible iron plaque formation. The relationship among RIP, NRIP and root oxidizing capacity needs further studies.

The results of NRIP extraction solution screening can be explained by characteristics of extraction solution: Respectively, 0.3 M  $\text{Na}_3\text{C}_6\text{O}_7\text{H}_5$  and 1 M  $\text{CH}_3\text{COONa}$  mainly extracted free irons and carbonate-adsorbing irons (Tessier et al. 1979; Turonova et al. 2008). RIP and NRIP consisted of amorphous and crystalline iron (hydr)oxides (Table 1), thus two kinds of extraction solutions were not suitable for RIP and NRIP separation (c' and d' in Fig. 3a–d).  $\text{Na}_2\text{SO}_4$  and  $\text{H}_2\text{SO}_4$  could remove iron-phosphate compounds, but iron hydroxides could also be dissolved in 0.4 M  $\text{H}_2\text{SO}_4$  due to neutralization reaction (Woolson et al. 1971). 0.05 M  $\text{Na}_2\text{SO}_4$  + 0.4 M  $\text{H}_2\text{SO}_4$  mixing solution were not specific for RIP or NRIP extraction (b' in Fig. 3a–d).  $\text{Na}_4\text{P}_2\text{O}_7$  and EDTA could extract iron complexes and chelated iron, respectively (Kaiser and Zech, 1996; Tandy et al. 2004). More speaking accurately,  $\text{Na}_4\text{P}_2\text{O}_7$  was a specific extractant for organic matter-binding iron. But results from the screening assay (e' and f' in Fig. 3a–d) indicated that it could extract NRIP. 0.1 M  $\text{Na}_4\text{P}_2\text{O}_7$  performed better than 0.05 M  $\text{Na}_2\text{EDTA}$  according to 4-h assay.

The mechanism of  $\text{Na}_4\text{P}_2\text{O}_7$  to remove NRIP can be partly explained as below: the pH of 0.1 M  $\text{Na}_4\text{P}_2\text{O}_7$  is around 10. At this pH environment, the dominant species of phosphate ions exist as  $\text{HPO}_4^{2-}$ , and  $\text{Fe}^{3+}$  reacts with  $\text{HPO}_4^{2-}$  into  $\text{Fe}_4(\text{P}_2\text{O}_7)_3$  and  $\text{Fe}_2(\text{HPO}_4)_3$ . Due to various pH and different humidities, many types of iron (hydroxyl) phosphate minerals are formed. For example, the  $K_{\text{sp}}$  value of strengite [ $\text{FePO}_4 \cdot 2\text{H}_2\text{O}$ , also presented as  $\text{Fe}(\text{OH})_2\text{H}_2\text{PO}_4$ ] varies greatly in different literature, ranging from  $1.41 \times 10^{-7}$  (McDowell and Sharpley 2003; Iuliano et al. 2007) to  $10^{-33}$  (Li 2006). Nevertheless, the solubility of  $\text{Fe}_4(\text{P}_2\text{O}_7)_3$  is  $3.7 \text{ g kg}^{-1}$ ,

which is extremely higher than those of iron (hydroxyl) phosphates. Therefore, iron phosphates can be removed from root surface and dissolved into extraction solution with high concentration of pyrophosphate and long-term continuous vibration. However iron phosphates are not all of NRIP, the further studies are needed. Because nearly a half of DCB-Fe in CK + Fe treatment was  $\text{Na}_4\text{P}_2\text{O}_7$ -extractable and invisible RIP (Fig. 4), it is better to define NRIP as iron-containing compounds that could be extracted by 0.1 M  $\text{Na}_4\text{P}_2\text{O}_7$ . Correspondingly, RIP was defined as DCB-extractable iron-containing compounds after NRIP being removed.

The results that iron plaque mainly consisted of amorphous iron hydroxides (Table 1) agreed with the views of Chong et al. (2013) and Xu and Yu (2013), but it is opposite to the results reported by the others (Chen et al. 1980; Chabbi 1999; Hansel et al. 2001). X-ray diffraction is unable to produce meaningful pattern to identify amorphous compounds since it has not regular lattice and crystalline planar face arrangement. Therefore, amorphous  $\text{Fe}(\text{OH})_3$  is often ignored though it belongs to IP and is the dominant component of RIP (Wang and John 1999). The reason leading to this difference might be associated with plant growth environment factors including *Eh* variation, microbial activity, ferrous and ferric iron concentrations (Syu et al. 2014; Yamaguchi et al. 2014). IP samples in early years are collected from plants growing in solid aggregates e. g. soil or sand cultivation while some of current studies are collected from those on hydroponics. It is reasonable to assume that crystallization occurs quickly with the assistance of solid aggregates as crystalline core due to the adsorption property of these aggregates. On the contrary, there was little solid matrix for initial crystallization except root surface under hydroponic condition. The dissolved oxygen in nutrient solution might effectively consume ferrous ion and made it precipitate outside rhizosphere (Xu and Yu 2013). Obviously, this sediment could not be assumed as IP. Zhang et al. (1998) demonstrated the beneficial effect of pre-added  $\text{Fe}(\text{OH})_3$  in nutrient solution for IP precipitation on rice roots. IP precipitation is difficult to build uniform crystal without crystalline core. What's more, a portion of crystalline iron (hydr)oxides in soil can be transformed into amorphous ones after flooding (Zhang et al. 2003), it is reasonable to assume that this process also occurs in IP on root surface due to the similarities of components and surrounding conditions. These results suggest that it's better to collect IP sample from plants in soil or sand

cultivation when studying IP components by traditional X-ray diffraction spectroscopy.

## Conclusion

In this study, phosphate concentrations in nutrient solution remarkably influenced the formation of reddish brown iron plaque (RIP) and non-reddish brown iron plaque (NRIP) on rice root surface. Dominant components of RIP and NRIP were ferric hydroxides and iron (ferric or ferrous) phosphates, respectively. All of these substances could be reduced by dithionite, and then they could form into soluble complexes with citrate in pH condition maintained by bicarbonate. 0.1 M  $\text{Na}_4\text{P}_2\text{O}_7$  removed NRIP only. RIP content could be determined by DCB extraction solution after the removal of NRIP.

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