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Effects of Foliar Iron Application on Iron Concentration in Polished Rice Grain and Its Bioavailability

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ABSTRACT: Iron (Fe) deficiency in humans caused by inadequate dietary intake is a global nutritional problem. A glass house pot experiment was conducted to evaluate the effect of foliar $FeSO_4$ containing applications on concentrations of Fe, Zn, and Fe bioavailability in polished rice among five rice cultivars. The results showed that foliar application of $FeSO_4$, $FeSO_4$, plus nicotianamine (NA), and $FeSO_4$ plus NA with $ZnSO_4$ increased the grain Fe concentration by 16.97%, 29.9%, and 27.08%, respectively. The grain Fe bioavailability also increased by foliar application of $FeSO_4$, $FeSO_4$ plus NA, and $FeSO_4$ plus NA with $ZnSO_4$; these represent increases of 12.63%, 20.86%, and 18.75%, respectively. Foliar $FeSO_4$ containing applications improved the Fe bioavailability and might be attributed to the reduction of phytic acid and the increase of Fe concentration in polished rice. Addition of $ZnSO_4$ to foliar Fe application increased both Fe and Zn content without altering Fe content and bioavailability. In addition, the cultivar difference in Fe and Zn concentration was observed and may be due to the genetic control of leaf absorption and seed deposition of foliar application. Furthermore, the cultivar difference in Fe bioavailability observed might be attributed to the variation of grain Fe, phytic acid, and total phenolics contents among the five rice cultivars. The results suggested that foliar $FeSO_4$ containing applications represent a promising agricultural approach to reduce Fe deficiency in countries where polished rice is extensively consumed.

KEYWORDS: *rice, iron, foliar fertilization, bioavailability*

INTRODUCTION

Iron (Fe) is an essential micronutrient in biological systems and is receiving growing concern worldwide because of increasing reports about Fe deficiencies in humans.[1] According to a report by the World Health Organization in 2002, Fe deficiency affects over 3 billion people in the world, especially in developing countries.[2] Iron deficiency causes impairments in mental and psychomotor development in children and diminished productivity in adults, and represents the most common cause of anemia.[3]

Inadequate intake with poor bioavailability of Fe in foods is in general the main cause of global Fe deficiency in humans. As the most dominant staple crop, rice (*Oryza stavia* L.) is the main source of energy and essential minerals for almost half of the world's population.[4] Because of the poor taste and color of brown rice, nutrient rich aleurone layers and embryos are traditionally removed during the polishing process, leaving only the starch rich endosperm (polished rice) as the edible part.[5] The resulting polished rice is very poor in Fe, Zinc (Zn), and other essential minerals, and also contains several antinutrients, such as phytic acid, and phenolic compounds, which could complex with Fe and reduce Fe absorption in the human body.[6,7] Thus, improved Fe concentration as well as bioavailability in polished rice will generate major health benefits for a large number of susceptible people.

In response to this problem, many potential approaches have been proposed and applied to increase bioavailable Fe concentrations in rice endosperm. Biofortification of rice endosperm with Fe and Zn is receiving much global attention. Three key tools for biofortification are the agronomic approach, breeding, and genetic engineering.[1,8] However, yield factor, interactions between genotype and environment, lack of sufficient genetic diversity in current cultivars for the breeding program, consumer resistance, and safety of genetically modified crops are the main bottlenecks of breeding and genetic engineering.[1,9]Alternatively, the agronomic approach, especially Fe fertilization is, therefore, an urgent and essential solution for improving Fe concentration and bioavailability in rice grain to address ongoing human Fe deficiency.[10]

Crop nutrients could be provided through different application methods including soil amendment, seed priming, and foliar application.[11] Foliar application of Fe was considered as a short time tool for biofortification of rice with Fe because soil application of most Fe sources are generally ineffective because of the rapid conversion of soluble Fe into plant-unavailable solid Fe(III) forms.[11,12] Furthermore, foliar applied Fe caused greater increases in brown rice Fe concentration than soil application.[13] Foliar applied Fe can be absorbed by the leaf epidermis, remobilized, and transferred into the grain via the phloem, and the loading of Fe into the phloem was likely limited by the availability of endogenous chelates nicotianamine (NA), which is a key ligand involved in metals such as Fe, and Zn transport and homeostasis in plants.[12,14−16] Moreover, the time of foliar application may differentially influence grain Fe concentration. It is well established that foliar Fe application after the flowering stage more distinctly increases the grain Fe concentration.[17] $FeSO_4$ is a widely used Fe application form,

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and some works have reported positive responses to foliar application of $FeSO_4$, either alone or combined with NA, on Fe enrichment in plants.[16,18,19] In rice, Yuan et al.[18] reported that addition of NA to foliar Fe application accelerated Fe accumulation in brown rice, but till now, studies evaluating the effectiveness of foliar application of $FeSO_4$ alone or addition of NA to $FeSO_4$ on Fe accumulation in polished rice are still rare. We hypothesized that addition of NA to $FeSO_4$ spray could promote more Fe accumulation in polished rice than that of applied $FeSO_4$ alone. In addition, foliar application of complex micronutrients containing Fe and Zn has been recommended recently, it is superior to foliar single micronutrient application for yield increase and grain mineral.[19,20] Thus, we hypothesized that addition of NA to $FeSO_4$ application combined with $ZnSO_4$ could simultaneously increase the Fe and Zn concentrations in polished rice.

The metabolizable Fe from biofortified rice grain not only depends on net Fe concentration but also a large extent on the bioavailability of Fe. Although, it is assumed that foliar application of Fe could improve grain Fe bioavailability,[19] till now there were rarely any studies on the Fe bioavailability of rice grain by foliar applications. Therefore, whether the Fe bioavailability of biofortified grain is fundamental to the successful implementation of any biofortification strategy should be assessed. Given the high cost of quantifying Fe bioavailability via in vivo studies for screening large numbers of samples, a high-throughput in vitro methodology to assess Fe bioavailability has been developed in recent years, which mimics the gastric and intestinal digestion of humans coupled with the human intentional epithelial cells (Caco-2). [21] Moreover, a comparison study using both human subjects and the Caco-2 cell model concluded that the Caco-2 cell model predicts Fe bioavailability quite well.[22] Recently, the Caco-2 cell model has been successfully applied to assume the Fe bioavailability in cereal grains.[6,23,24] In the current study, we have used this model to evaluate Fe bioavailability in polished rice as affected by foliar Fe fertilizer applications among five rice cultivars.

The aim of this study was as follows: (1) to investigate the effects of foliar application of $FeSO_4$, either alone or combined with NA on Fe accumulation in polished rice, (2) to investigate the effects of foliar application of $FeSO_4$ combined with NA and $ZnSO_4$ on Fe and Zn accumulation in polished rice, (3) to investigate the effects of foliar Fe containing application on the Fe bioavailability of polished rice, and (4) to investigate the effects of cultivars on Fe concentration and bioavailability in polished rice.

■ MATERIALS AND METHODS

Chemicals and Reagents. Dulbecco's modified Eagle's medium (DMEM-high glucose), fetal bovine serum, glutamine, nonessential amino acids, penicillin, and streptomycin were obtained from Gibco Life Technologies (Grand Island, NY). Porcine pepsin, pancreatin, and bile extract were purchased from Sigma-Aldrich (St. Louis, MO). All of the other chemicals used in this study were of analytical grade purchased from local chemical suppliers. All reagents were prepared with deionized water ($\leq 0.1\ \mu S/cm$) using a Milli-Q system (Millipore, Billerica, MA). All laboratory glassware used in the experiments were soaked in 10% nitric acid for 24 h and subsequently rinsed with deionized water and air-dried.

Glasshouse Experiment. Surface soil (0−30 cm depth) was collected from the agricultural farm of Zhejiang University (Zhejiang, Hangzhou, China; 30°15′19″ N, 120° 10′8″ E). The air-dried soil was ground to pass through a 2 mm sieve. The soil was analyzed for various physicochemical properties. The soil had a pH of 5.86, alkali-hydrolyzable N was 67.41 mg/kg, available P was 63.95 mg/kg, and exchangeable K was 85.36 mg/kg. The concentration of diethylenetriamine pentaacetic acid (DTPA)-Fe was 15.48 mg/kg, and DTPA-Zn was 4.6 mg/kg.

Thirty day old seedlings of each cultivar were transplanted to a plastic pot containing 8.5 kg of sieved soil. Each pot had three hills, and two seedlings were transplanted in each hill. Before transplanting, the standard recommended dose of NPK fertilizer was applied to each plot (per kg dry soil) at rates of 180 mg of N (70% applied as the basal dose and 30% as the topdressing at the panicle initiation stage), 60 mg of P_2O_5, and 100 mg of K_2O.

Distilled water was used to maintain the soil water content throughout the experimental period. During the experiment, the average temperature in the glasshouse was 25 ± 5 °C during the day and 20 ± 5 °C during the night, and natural daylight was used in this experiment.

Foliar treatments included (1) foliar application of deionized water as the control, (2) $FeSO_4$ (w/v, 0.2%), (3) $FeSO_4$ (w/v, 0.2%) plus NA (w/v, 1%), and (4) $FeSO_4$ (w/v, 0.2%) plus NA (w/v, 1%) with $ZnSO_4$ (w/v, 0.2%). All foliar applications contained 0.01% (v/v) Tween80 as a surfactant. Five rice cultivars, namely, LYP9, XS110, Hai31, Bing91185, and X0117 were used in this experiment. The experiment has four replicates. The foliar applications were applied three times, one time at the panicle initiation stage, two times at 7 days after the flowering stage. Spray was applied after sunset. During foliar applications, pot soil was shielded in order to minimize the contamination of soil with foliar applications.

Plants were harvested from each pot at maturity and were manually threshed to separate grain. Straw and grains were washed with distilled water and briefly blotted with tissue papers and dried at 60 °C for 48h. They were weighed in order to determine the dry matter. Brown rice was prepared by removing the husk using a laboratory dehusker (JLGJ4.5, Taizhou Cereal and Oil Instrument Co. Ltd., Zhejiang, China), and the polished rice was prepared by a laboratory polishing machine (JNMJ3, Taizhou Cereal and Oil Instrument Co. Ltd., Zhejiang, China). The rice samples were powdered to make flour by using a ball mill (Retsch, MM-301, Germany), then put into the plastic bag, and keep at −20 °C until analysis. A part of the rice was cooked for 15 min with 1:2 rice/deionized water (w/v). The cooked rice samples were then homogenized in a polytron homogenizer, and then the homogenates were frozen and lyophilized before testing via the in vitro digestion/Caco-2 cell model.

Mineral Concentration Determination. The rice flour samples of each treatment were placed in a digestion tube and digested with nitric acid and hydrogen peroxide (v/v, 4:1). The concentrations of Fe and Zn in the sample were determined by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7500a, Agilent Technologies, California). In the experiment, the standard reference material, rice flour (SRM 1568a), from National Institute of Standards and Technology (Gaithersburg, MD, USA) was used to check the accuracy of Fe and Zn analysis. The measured value was 7.1 ± 0.5 mg/kg for Fe and 18.9 ± 0.3 mg/kg for Zn, which were in accordance with the certified range of 7.4 ± 0.9 mg/kg for Fe and 19.4 ± 0.5 mg/kg for Zn.

Phytic Acid Content Determination. Grain phytic acid was extracted by 0.2 M HCl and measured by the Fe-precipitation method as detailed by our previous report.[25] The Fe concentration in solution was measured by ICP-MS (Agilent 7500a, Agilent Technologies, California). The phytic acid content was calculated by multiplying Fe content by the factor 4.2.

Protein Content Determination. Protein content in rice samples was analyzed by the determination of total nitrogen. The Kjeldahl method was used to determine total nitrogen. Rice samples were digested by H_2SO_4 and then distilled in KjelFlex K-360 (Buchi, Flawil, Switzerland) with 40% (w/v) NaOH and 2% (w/v) boric acid (methyl red and bromcresol green were used as an indicator solution), then titrated with 0.02 mM H_2SO_4. Protein content was calculated by multiplying nitrogen content by 5.95.[26]

Total Phenolics Content Determination. Rice flour samples were extracted with an acidic solution of methanol and water (50:50,

Table 1. Analysis of Variance for Straw Dry Weight, Grain Yield, Fe Concentration, Zn Concentration, Protein, Phytic Acid, Total Phenolics, and Fe Bioavailability of Five Cultivars under Different Foliar Fe Applications[a]

source of variation	df	straw dry weight	grain yield	brown rice Fe	polished rice Fe	brown rice Zn
foliar application (F)	3	1.231	0.300	106.896[b]	63.934[b]	38.706[b]
cultivar (C)	4	25.503[b]	28.179[b]	207.664[b]	286.316[b]	137.727[b]
F × C	12	0.524	0.872	2.333[b]	2.054[b]	0.822
source of variation	df	polished rice Zn	phytic acid	total phenolics	protein	Fe bioavailability
foliar application (F)	3	53.473[b]	19.316[b]	3.476	0.799	71.463[b]
cultivar (C)	4	98.051[b]	89.141[b]	24.152[b]	13.418[b]	379.379[b]
F × C	12	0.339	1.037	0.572	0.367	3.997[b]

[a] Data presented are F-test values. [b] Significant at the 0.05 and 0.01 probability levels.

v/v, pH 2) for 60 min and then centrifuged at 3500 rpm for 15 min. The supernatants were pooled for analysis of total phenolics using the Folin−Ciocalteu's reagent (Sigma-Aldrich, St Louis, MO, USA) as reported.[27] The absorbance of each sample was measured against a blank at 765 nm using an UV/vis spectrophotometer (Lambda 35, UV/vis spectrophotometer, PerkinElmer Ltd., Shelton, CT, USA). Using gallic acid as a standard, the total phenolics was expressed as mg gallic acid equivalents (GAE)/kg of rice sample.

Iron Bioavailability Determined by an In Vitro Digestion/Caco-2 Cell Model. *In Vitro Digestion of Samples.* The cooked rice samples (1 g) of polished rice were used for in vitro digestion. The preparation of digestion solutions including pepsin, pancreatin, and bile extract, and in vitro digestion procedures were performed according to a previously described method.[23] Total Fe content in digested solutions was determined by ICP-MS (Agilent 7500a, Agilent Technologies, California, USA).

Caco-2 Cell Culture. Caco-2 cells were collected from the Institute of Biochemistry and Cell Biology (SIBS, CAS, Shanghai, China) and used in assays at passage between 25 and 32. The cells were seeded in collagen-treated six-well plates (Costar Crop, NY, USA) at a seeding density of 50000 cells/cm^2. The cells were grown in the Dulbecco's minimal essential medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1% (v/v) nonessential amino acids, and 2% (v/v) penicillin−streptomycin solution. The culture medium was changed every 2 days. The cells were maintained at 37 °C in an incubator (Heraeus, BB15, Germany) with 5% CO_2 and 95% air atmosphere at constant humidity. Confluent cultures of differentiated cells were used in the Fe bioavailability experiments at 13 days postseeding.

Iron Bioavailability. Bioavailabililty of Fe from polished rice grain was determined via subsequent exposure of the in vitro digested solution to the Caco-2 cells. The process has been previously described by Glahn et al.[23] At the end of the Fe bioavailability experiment, the cells were harvested, and the Caco-2 cell protein was determined using a semimicro adaptation of the Bio-Rad DC protein (colorimetric) assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The sonicated Caco-2 cell monolayer sample (10 μL) was harvested in 2 mL of water and used for each ferritin measurement. Caco-2 cell ferritin content was determined by the immunoradiometric assay (FER-Iron II Ferritin Assay, RAMCO Laboratories, Houston, TX, USA), according to the manufacturer's instructions. Therefore, the ratio of ferritin/total protein (expressed as ng ferritin/mg protein) was as an index of Fe bioavailability.

Statistical Analysis. Data were analyzed using SPSS software (SPSS 16.0, Chicago, USA). Analysis of variance (ANOVA) was performed with the least significant difference (LSD) to compare the various means of each series of experiments. Means were considered to be significantly different if p values were <0.05.

■ RESULTS

Straw and Grain Yield. As shown in Table 1, the cultivar showed significant effect on straw dry weight and grain yield, but foliar application had no effect on straw dry weight and grain yield. Variability in straw dry weight and grain yield was noted among five test rice cultivars, and no difference was found among the foliar applications (Table 2).

Table 2. Effects of Foliar Fe-Containing Fertilizers on Straw Dry Weight and Grain Yield in Different Rice Cultivars[a]

	straw dry weight (g/pot)[b]			
cultivars	control	$FeSO_4$	$FeSO_4$ + NA	$FeSO_4$ + NA + Zn
LYP9	63.57 aA	65.88 aA	66.13 aA	65.12 aA
XS110	58.36 aAB	57.83 aB	58.51 aCD	58.10 aB
Hai31	54.98 aB	54.79 aB	54.83 aD	53.04 aB
Bing91185	55.20 aB	58.59 aB	58.32 aBC	59.24 aB
X0117	62.81 aA	63.95 aA	68.14 aAB	63.43 aA
	grain yield (g/pot)			
	control	$FeSO_4$	$FeSO_4$ + NA	$FeSO_4$ + NA + Zn
LYP9	44.27 aA	44.48 aA	45.20 aA	45.33 aA
XS110	41.64 aBC	42.06 aB	41.79 aB	42.56 aB
Hai31	40.18 aC	40.74 aBC	40.44 aC	39.48 aC
Bing91185	41.34 aBC	40.11 aC	39.87 aC	41.79 aB
X0117	42.15 aB	42.63 aAB	43.25 aB	42.62 aB

[a] Values are the means, n = 4. [b] Rows with different lowercase letters showed significance at $p < 0.05$. Columns with different capital letters showed significance at $p < 0.05$.

Effect of Foliar Applications on Fe Concentration in Rice Grain among Five Cultivars. The Fe concentration of brown rice and polished rice was significantly affected by foliar application, cultivar, and their interaction (Table 1). Generally, Fe concentration in brown rice was increased by foliar Fe containing applications (Figure 1A). Compared to the control, Fe concentration in brown rice was increased by 13.87%, 22.95%, and 21.12% via foliar application of $FeSO_4$, addition of NA to $FeSO_4$, and $FeSO_4$ plus NA with $ZnSO_4$, respectively.

Moreover, the results showed that the response of Fe in polished rice was highly similar to those of brown rice (Figure 1B) and that polished rice Fe concentration was increased from 3.91 mg/kg in the control to 4.57 mg/kg by foliar $FeSO_4$, to 5.08 mg/kg by foliar $FeSO_4$ plus NA, and to 4.96 mg/kg by foliar $FeSO_4$ plus NA with $ZnSO_4$. These represented increases of 16.97%, 29.91%, and 27.08%, respectively. In the current study, a significant positive correlation in Fe concentration between polished rice and brown rice was found (R^2 = 0.886, p < 0.01). Furthermore, the response of grain Fe concentration to foliar applications was cultivar dependent (Figure 1). In the current study, cultivar X0117 contains the higher Fe concentration of polished rice than other cultivars in all treatments.

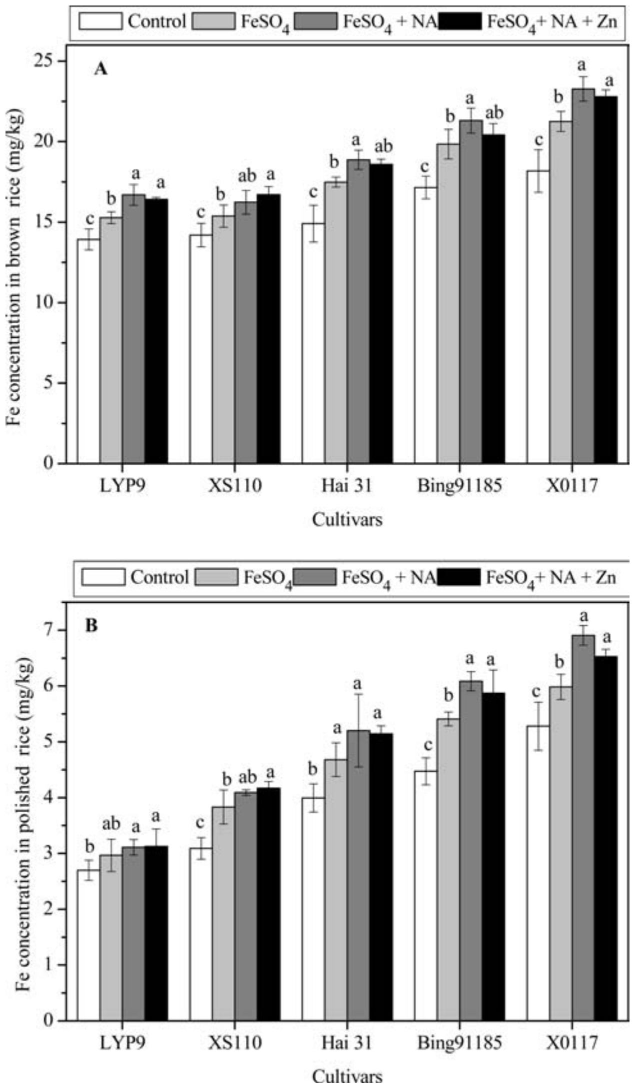

Figure 1. Iron concentration in brown rice (A) and polished rice (B) affected by foliar Fe fertilizer applications. Error bars show standard errors of the means (n = 4). Different letters indicate significant differences among the foliar Fe containing applications according to the LSD test.

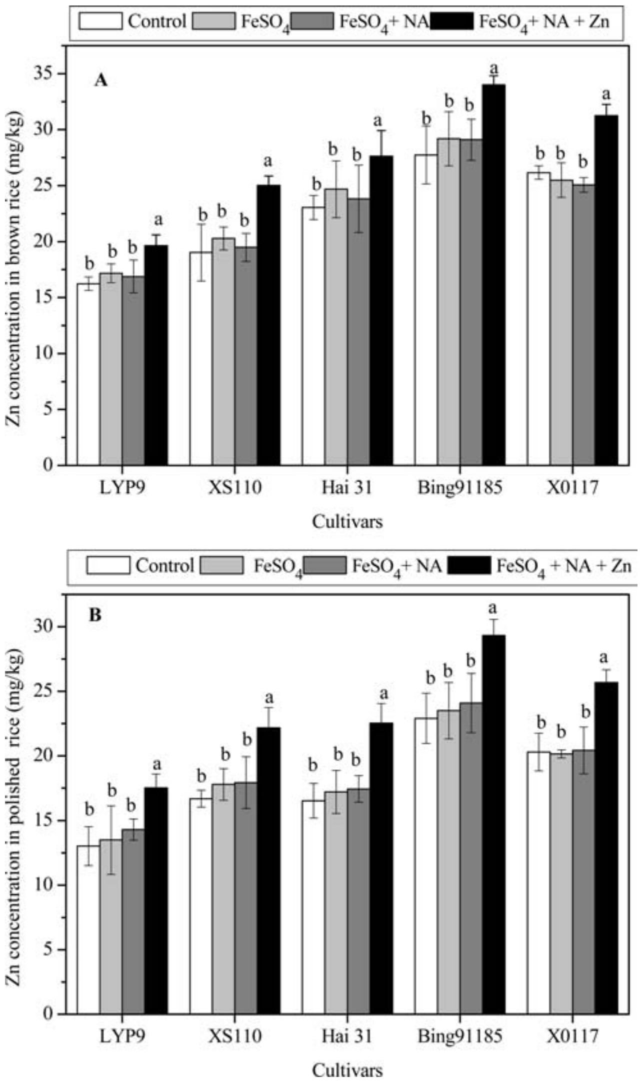

Figure 2. Zinc concentration of brown rice (A) and polished rice (B) affected by foliar Fe fertilizer applications. Error bars show standard errors of the means (n = 4). Different letters indicate significant differences among the foliar Fe containing applications according to the LSD test.

Effect of Foliar Applications on Zn Concentration in Rice Grain among Five Cultivars. In the current study, we tested whether foliar application of both Fe and Zn could increase the Fe and Zn in rice grain together. Both foliar application and cultivar have significant effect on Zn concentration in brown rice and polished rice (Table 1). Regardless of the five cultivars, compared to the control, the foliar Fe application containing $ZnSO_4$ increased the Zn concentration in brown rice and polished rice by 22.59% and 31.10%, respectively (Figure 2A and B). No significant difference on Zn concentration was found among the control, $FeSO_4$, and $FeSO_4$ plus NA, whereas foliar application of Fe combined with $ZnSO_4$ simultaneously increased the Fe and Zn concentration in rice grain. In the present study, grain Zn concentration was cultivar dependent, with cultivar Bing91185 containing the highest Zn concentration in both brown and polished rice.

Effect of Foliar Applications on Protein, Phytic Acid, and Total Phenol Content in Rice Grain among Five Cultivars. The current results showed phytic acid content was significantly affected by both foliar application and cultivar (Table 1). Phytic acid content ranged from 1.67 to 2.89 mg/g in different rice cultivars. Regardless of the five cultivars, phytic acid content in polished rice was significantly decreased by foliar applications of $FeSO_4$ alone or by the addition of NA to $FeSO_4$, or with $ZnSO_4$, by 17.54%, 17.03%, and 15.69%, respectively (Table 3). In the current studies, total phenolics content was not affected by foliar Fe containing applications but varied by cultivar (Table 1), which ranged from 97.19 to 115.22 mg GAE/kg (Table 3). Grain protein content ranged from 8 to 9.44%, which was not affected by foliar application but varied by cultivar (Tables 1 and 3).

Effect of Foliar Applications on Bioavailability of Fe among Five Cultivars. To assess the enhancement of Fe bioavailability in polished rice obtained from foliar Fe applications, in vitro digested polished rice was used to carry out the Fe bioavailability experiment in Caco-2 cells. The results showed that foliar application, cultivar, and their interaction had significant effects on Fe bioavailability in polished rice (Table 1). In general, Fe bioavailability was

Table 3. Effects of Foliar Fe Containing Fertilizers on Protein, Phytic Acid, and Total Phenolics in Polished Rice[a]

	phytic acid (mg/g)[b]			
cultivars	control	$FeSO_4$	$FeSO_4$ + NA	$FeSO_4$ + NA + Zn
LYP9	3.26aA	2.88abA	2.73bA	2.71bA
XS110	1.94aD	1.51bD	1.55bC	1.68abD
Hai31	2.63aB	2.24bB	2.17bB	2.25bB
Bing91185	2.34aC	1.73cCD	2.06bB	2.01bBC
X0117	2.18aCD	1.97bBC	1.90bB	1.92bCD
	total phenolics (mg/GAE kg)			
	control	$FeSO_4$	$FeSO_4$ + NA	$FeSO_4$ + NA + Zn
LYP9	117.01aA	116.49aA	117.80aA	109.56aAB
XS110	97.14aC	95.27aC	103.50aB	92.87aC
Hai31	106.58aABC	109.03aAB	111.28aAB	100.37aBC
Bing91185	99.50aBC	103.76aBC	101.72aB	99.93aBC
X0117	109.85aAB	112.35aAB	108.58aAB	111.81aA
	protein (%)			
	control	$FeSO_4$	$FeSO_4$ + NA	$FeSO_4$ + NA + Zn
LYP9	7.88aB	8.50aB	8.22aB	7.95aB
XS110	9.12aA	9.47aA	9.58aA	9.61aA
Hai31	7.86aB	8.25aB	7.96aB	7.95aB
Bing91185	8.39aAB	8.43aB	8.79aAB	8.81aAB
X0117	8.70aAB	8.62aAB	8.68aB	8.73aB

[a]Values are the means, $n = 4$. [b]Rows with different lowercase letters show significance at $p < 0.05$; columns with different captial letters show significance at $p < 0.05$.

increased by the foliar Fe containing applications (Figure 3). Regardless of the five cultivars, Fe bioavailability in polished rice was increased from 8.45 ng ferritin/mg protein in the control to 9.52 ng ferritin/mg protein by foliar $FeSO_4$, to 10.21 ng ferritin/mg protein by foliar $FeSO_4$ plus NA, and to 10.03 ng ferritin/mg protein by foliar $FeSO_4$ plus NA with $ZnSO_4$. These represented increases of 12.63%, 20.86%, and 18.75%, respectively. Addition of NA to $FeSO_4$ application could obtain relatively higher Fe bioavailability from polished rice in some cultivars such as X0117, but poor in LYP9. In addition, no significant difference was found in Fe bioavailability in polished rice obtained from foliar Fe application combined with or without $ZnSO_4$ (Figure 3).

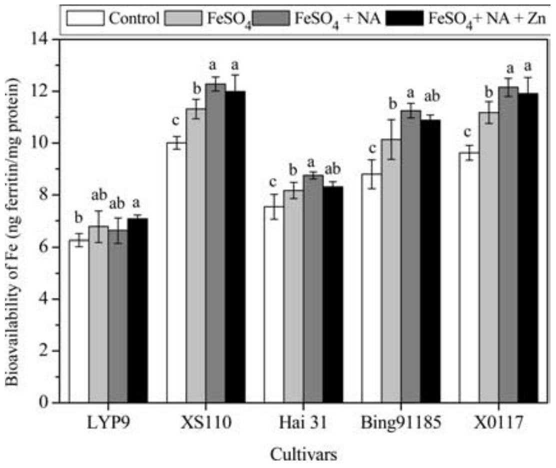

Figure 3. Bioavailability of Fe in polished rice affected by foliar Fe fertilizer applications. Error bars show standard errors of the means ($n = 4$). Different letters indicate significant differences among foliar Fe containing applications according to the LSD test.

Furthermore, averaged Fe bioavailability across foliar Fe treatments ranged from 6.69 to 11.40 ng ferritin/mg protein, and the difference in Fe bioavailability was observed among the cultivars in this study (Figure 3). Cultivar LYP9 contains the lowest Fe bioavailability compared to that of other cultivars in foliar Fe containing applications; meanwhile, cultivars XS110 and X0117 contain higher Fe bioavailability than the other cultivars in all the Fe containing applications.

DISCUSSION

Straw dry weight and grain yield of rice plants were not affected by foliar applications and only varied among five test rice cultivars (Table 2), consistent with the results from previous studies.[17,19] This might be due to relatively high DTPA-Fe and DTPA-Zn concentrations in the experimental soil, and thus, the plant was in the sufficient Fe and Zn nutritional status.

In contrast to grain yield, foliar application of the Fe containing fertilizer had a positive impact on the Fe concentration of brown rice (Figure 1A), which was confirmed in earlier literature where it was reported that foliar Fe fertilizers could improve Fe concentration in cereal grains.[17−19,28] As polished rice is the main consumable portion, we have given particular attention to Fe concentration in polished rice, which is more relevant than brown rice in terms of human nutrition. In the current study, we observed that regardless of cultivar, although the polishing process decreases a substantial amount Fe from the mature grain, the Fe concentration in polished rice was increased 16.97−29.91% compared to that of the control (Figure 1B), and a significant positive correlation in Fe concentration between polished rice and brown rice was found ($R^2 = 0.886$, $p < 0.01$), indicating that Fe concentration in polished rice probably improved by increasing the Fe concentration in brown rice. This agreed with a previous study[19] suggesting that excess foliar applied Fe could penetrate into the inner layers of the rice endosperm. Especially, the cases of the addition of NA to $FeSO_4$ with or without $ZnSO_4$ application were more effective than $FeSO_4$ alone on enrichment of Fe in rice endosperm, which was in agreement with previous study.[18] The possible reason might be that NA acts as a mobile binding partner for Fe translocation from cell to cell after being absorbed by the leaves and that NA also might be chelated with Fe in the plant, which would promote the mobility of Fe and transported into the grain.[16,29] In addition, the results showed that rice cultivars differ greatly in their response to foliar Fe applications in terms of increase in grain Fe (Figure 1); the variation might come from the genetic control of the ability of leaf absorption and seed deposition of foliar applied Fe.

Zinc is another important micronutrient for crops and humans. Foliar Fe application containing Zn could significantly increase the Zn concentration in brown rice and polished rice (Figure 2). No significant difference on Zn concentration was found among the control, $FeSO_4$, and $FeSO_4$ plus NA. The results indicated that foliar application of Fe had no negative effect on Zn accumulation, whereas foliar application of Fe combined with $ZnSO_4$ simultaneously increases the Fe and Zn concentration in rice grains. These results agreed with previous studies.[19,30] In the current study, accumulation of Zn in brown rice and polished rice was cultivar dependent, which can be explained by the genetic variability between varieties.

Information about changes of antinutrients and other nutrients in polished rice during foliar applications is important because it related to the Fe bioavailability and nutritional quality of rice, which impact global human health. Phytic acid has long been known as a form of stored phosphorus in seeds, which was considered as an inhibitor of Fe bioavailability, even when the presence of phytic acid is only in relatively small amounts.[6,31]

The present study revealed a negative effect of foliar Fe containing fertilizations on phytic acid concentration (Table 3). Phenolic compounds occurring in cereal grain were considered as another important inhibitor of Fe bioavailability.[6] In the present study, total phenolics content was not affected by foliar Fe containing applications but varied by cultivar (Table 3). A similar result was also found in grain protein content, which was not affected by foliar application, but varied by cultivar (Table 3). The above results indicated that foliar Fe containing application could reduce the phytic acid content, while contents of protein and phenolics in polished rice were unchanged and varied by cultivar, which are consistent with the results of previous studies.[18,19,28]

The final goal of increased Fe concentration in rice grains is to better satisfy human demands; therefore, the bioavailability of increased Fe in polished rice should also be of concern. Bioavailability of Fe is defined as the proportion of the total amount of Fe that is potentially absorbable in a metabolically active form.[32] To assess the enhancement of Fe bioavailability in polished rice obtained from foliar Fe applications, in vitro digested polished rice was used to carry out the Fe bioavailability experiment in Caco-2 cells, which offer a more physiological tool for screening Fe bioavailability in food matrices.[6,21,24] In the study presented here, Fe bioavailability in polished rice averaged at 9.54 ng ferritin/mg protein, falling within the previously reported Fe bioavailability in the Caco-2 cell model from polished rice.[6] Compared to the control, Fe bioavailability in polished rice was increased by 12.63%, 20.86%, and 18.75% via foliar applications of $FeSO_4$ alone or the addition of NA to $FeSO_4$, or with $ZnSO_4$, respectively. To the best of our knowledge, no literature data regarding foliar Fe containing applications on Fe bioavailability in rice grain are yet available. The possible explanation of these results was that reduction of the phytic acid in polished rice by foliar Fe containing application, as well as the increased total amount of grain Fe, improved the Fe bioavailability.[6,14,19] Addition of NA effectively enhance grain mineral bioavailability has been reported in previous studies,[33,34] here, addition of NA to $FeSO_4$ application could accelerate Fe bioavailability in polished rice to a small extent. However, the mechanism is still not clear and requires more research (e.g., grain Fe speciation) for clarification in the future. In addition, $ZnSO_4$ combined with foliar Fe containing application had no negative effect on Fe bioavailability in polished rice (Figure 3), suggesting improved Fe bioavailability and Zn level in rice grains through an extra supply of $ZnSO_4$ to Fe fertilization. Furthermore, Fe bioavailability in polished rice obtained from foliar Fe applications was significantly different among cultivars (Figure 3). The difference in Fe bioavailability among cultivars may be attributed to the combined effect of phytic acid, total phenolics, or Fe accumulated in the rice grain, consistent with previous studies.[6,23] In the current study, we also found that some genotypes showed very poor response to foliar Fe application such as LYP9. It seems that the impact of foliar Fe applications on Fe bioavailability in polished rice can be maximized by selecting genotypes with higher Fe bioavailability combined with foliar Fe containing applications. The cultivar X0117 had the highest Fe concentration and higher bioavailability of Fe in all foliar Fe applications; thus, it was identified as the most promising cultivar for the Fe biofortification program in this study.

In conclusion, foliar Fe fertilization is an effective agricultural approach for promoting grain Fe concentration and bioavailability. Foliar Fe applications could increase total amounts of grain Fe and reduce the phytic acid content, and as a result enhance the higher bioavailability of Fe from the polished rice. NA added to foliar application of $FeSO_4$ could accelerate Fe accumulation and bioavailability in polished rice. Addition of NA to foliar application $FeSO_4$ could accelerate Fe accumulation and bioavailability in polished rice in some extent. The cultivar difference on the amount of Fe bioavailability in polished rice observed might be attributed to the variation of phytic acid, total phenolics, and the level of Fe concentration in polished rice among the five tested cultivars. The cultivar X0117 was identified as the most promising cultivar for the Fe biofortification program. Foliar application might be a rapid method for improving the amount of Fe intake and alleviating human Fe deficiency.

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