Evaluation of rice wild relatives as a source of traits for adaptation to iron toxicity and enhanced grain quality

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Abstract

Rice wild relatives (RWR) constitute an extended gene pool that can be tapped for the breeding of novel rice varieties adapted to abiotic stresses such as iron (Fe) toxicity. Therefore, we screened 75 *Oryza* genotypes including 16 domesticated *O. sativas*, one *O. glaberrima*, and 58 RWR representing 21 species, for tolerance to Fe toxicity. Plants were grown in a semi-artificial greenhouse setup, in which they were exposed either to control conditions, an Fe shock during the vegetative growth stage (acute treatment), or to a continuous moderately high Fe level (chronic treatment). In both stress treatments, foliar Fe concentrations were characteristic of Fe toxicity, and plants developed foliar stress symptoms, which were more pronounced in the chronic Fe stress especially toward the end of the growing season. Among the genotypes that produced seeds, only the chronic stress treatment significantly reduced yields due to increases in spikelet sterility. Moreover, a moderate but non-significant increase in grain Fe concentrations, and a significant increase in grain Zn concentrations were seen in chronic stress. Both domesticated rice and RWR exhibited substantial genotypic variation in their responses to Fe toxicity. Although no RWR strikingly outperformed domesticated rice in Fe toxic conditions, some genotypes scored highly in individual traits. Two *O. meridionalis* accessions were best in avoiding foliar symptom formation in acute Fe stress, while an *O. rufipogon* accession produced the highest grain yields in both chronic and acute Fe stress. In conclusion, this study provides the basis for using interspecific crosses for adapting rice to Fe toxicity.

Keywords: Biofortification, breeding, crop wild relatives, food security, mineral toxicity, *Oryza*

Introduction

Crop wild relatives (CWR) are widely regarded as a rich source of genetic variation for the improvement of domesticated crops, which can be tapped more effectively than ever before due to the progress in CWR genomics [1,2]. Rice is one of the most important cereal crops in the world in terms of annual production and provides the staple food for around half of the world's population [3]. Two domesticated rice species are widely grown: *Oryza sativa* L., which was domesticated from the wild progenitors *O. rufipogon* and *O. nivara* in Asia around 10, 000 years ago [4,5] and *Oryza glaberrima* Steud., which was independently domesticated from the wild progenitor *Oryza barthii* in West Africa around 3000 years ago [6]. In addition, archeological evidence suggest that rice wild relatives (RWR) originating from South America, such as *O. glumaepatula* underwent a process of domestication in the mid-Holocene by Native South Americans, but were abandoned later on when immigrants from Europe changed the fate of the continent and its inhabitants to a great extent [7]. The whole *Oryza* genus, which can be regarded as a primary, secondary and tertiary gene pool for rice breeding, contains 24 species [8]. It can be further classified into four complexes of closely related species with the same genome groups, between which fertile crossing is possible [9,10]. These are the *O. sativa* complex comprising the diploid AA species (Supplementary Table S1), the *O. officinalis* complex, the *O. granulata* complex and the *O. ridleyi* complex [11]. In addition *O. sativa f. spontanea* or 'weedy rice' can be found in rice growing regions all over the world [12] and likely represents a hybrid between cultivated rice varieties and the closely related RWR *O. rufipogon* or *O. nivara,* but it can also result from spontaneous mutations of *O. sativa* varieties or crosses between them [13].

Often, RWR are seen as weeds and thus as a major problem for rice cultivation, but they also bear a lot of potential, as *Oryza* species are distributed around the globe in very different environments, and their genome therefore might harbor adaptive genes to several biotic and abiotic stresses. Some of these

62 adaptive traits were previously used to develop commercial varieties. Breeding of male sterile rice lines,
63 which is required for the commercial production of hybrids, was originally only possible with the
64 cytoplasm of *O. sativa* f. *spontanea* [14], and those hybrids were still planted on nearly half of the rice
65 planting area of China in 2006 [15]. In 1974 the first rice varieties containing resistance genes to the
66 grassy stunt virus from *Oryza nivara* were released [14]. Crosses with *O. officinalis* lead to cultivars with
67 resistance to brown plant hoppers, of which three were released in Vietnam [14]. Several other
68 resistances of RWR against pests, pathogens and abiotic stresses and even yield improving traits have
69 been identified but not yet been taken advantage of [8,9,14]. In some cases fertile crosses with *O. sativa*
70 were accomplished. A resistance gene against bacterial blight from *O. longistaminata* was crossed into
71 popular varieties [16], although the progenies were not established as commercial varieties. Crosses
72 with the remote relative *O. brachyantha* were successful, even though the desired resistance to yellow
73 stem borer could not be preserved [17]. Abiotic stresses constitute another category of yield constraints
74 that may be mitigated by the use of RWR in breeding. *O. officinalis* has been proven as useful to address
75 heat stress, because in a cross with *O. sativa* early-morning flowering lead to higher fertility due to the
76 avoidance of high midday temperatures [18]. A cold tolerant *O. rufipogon* genotype contained several
77 interesting resistance loci [19]. *O. coarctata* is used as a model to understand salt tolerance in rice, and
78 successful crosses with the modern rice variety IR56 exist [8,20]. Aluminum tolerance in acidic soils from
79 *O. rufipogon* was previously dissected into quantitative trait loci [21]. Other potential fields of
80 application of RWR in tolerance breeding include drought and submergence [8]. Up to date not much is
81 known about the chemical and nutritional grain composition of RWR and genetic factors that might be
82 of use for grain quality breeding, although this approach may hold great potential [22].

83 Fe toxicity is one of the major mineral disorders affecting rice production, especially in parts of West
84 Africa, Southeast Asia, Madagascar and the South of Brazil [23]. It frequently occurs because rice-
85 growing soils are inherently rich in Fe, but more importantly the flooding of paddy soils leads to

86 microbial reduction processes that release abundant soluble Fe^{2+} (ferrous Fe) [23,24]. When excessive
87 Fe is taken up into rice plants it leads to the formation of oxidative stress via the Fenton reaction [25],
88 visible leaf bronzing symptoms [26], and eventually yield losses due to reduced growth and increased
89 spikelet sterility [27,28]. Fe toxicity either occurs as an acute stress during the vegetative stage, when
90 abundant amounts of Fe are mobilized from adjacent slopes due to heavy rainfall, or as a chronic stress
91 with a more gradual build-up of high Fe concentrations in soil solution [23,28]. As farmers have very few
92 management options to address Fe toxicity, the breeding of Fe tolerant rice varieties represents the
93 most promising strategy of adapting rice production to high Fe soils. A large number of genetic
94 screening and genome mapping experiments have been conducted in the past to identify donors and
95 loci that can be used for the breeding of tolerant germplasm [26,29,30]. These studies have revealed
96 that Fe tolerance is a complex trait controlled by a large number of small and medium effect loci rather
97 than large effect quantitative trait loci [26,30]. Moreover, the progress in breeding is hampered by
98 limited consistency between different screening experiments and lack of genetic variation for certain
99 desirable traits, such as the ability to translocate large amounts of Fe to the grains despite Fe toxicity
100 [28]. Another limitation is that to date no RWR have been evaluated regarding their potential to
101 contribute to Fe tolerance breeding.

102 Therefore, the present study was undertaken to explore traits of RWR that could be exploited for the
103 breeding of Fe tolerant rice. Due to the recent report of genome sequences for thirteen RWR [5], and
104 the availability of mapping populations derived from interspecific crosses of rice (e.g. [31] this is a timely
105 approach that could lead to the fast discovery of novel genes for rice breeding and thus contribute to
106 diversification and adaptation. Specifically, we hypothesized that (i) the variation observed in RWR for
107 adaptation to chronic and acute Fe stress may exceed the variation observed in domesticated rice; (ii)
108 despite low agronomic value RWR may possess specific traits that can be used in adaptive breeding; and

(iii) when grown in high Fe conditions RWR may possess grain quality traits that differ from those observed in domesticated rice.

Materials and Methods

Plant material

Seeds of 60 rice accessions were provided by the International Rice Research Institute (IRRI) in Los Baños, the Philippines. They consisted of 20 wild species of the *Oryza* genus with different countries of origin, one cross of *O. glaberrima* and *O. barthii* and two *O. sativa* varieties, for which crosses with wild relatives have been made (IRRI 154 and Curinga). In addition, 15 accessions of cultivated rice species (*O. sativa* and one *O. glaberrima*) that had previously been screened in Fe toxicity experiments [26,32,33] were added from the seed stocks of the Institute of Crop Science and Resource Conservation (INRES).

Germination

Where necessary, seeds dormancy was broken by placing seeds in an oven at 50°C for three days. Thirty seeds of each accession were then placed on a floating mesh in a germination box with about two liters of deionized water, making sure that the grains were not fully covered by the water. The seeds were incubated in darkness at a temperature of 33°C. After three days the first seeds germinated and were transferred to a germination tray floating on deionized water containing 10 µM Ferric sodium EDTA (Fe-Na-EDTA) and 0.5 mM calcium chloride (CaCl). The box was placed in a greenhouse until the seedlings were about 8 cm high. Solutions were exchanged every two days.

Experimental setup and growing conditions

The experiment was conducted in a greenhouse at Campus Klein-Altendorf, which is an agricultural trial station of the University of Bon from May to October 2017. Six polders of 2 x 6 m dimension with a soil

depth of about 50 cm were utilized for an experimental approach simulating acute and chronic iron toxicity as described previously [28]. Two independent polders each were used for the three treatments chronic iron stress, acute iron stress and control. Within each polder four sub-replicates containing all accessions were planted. The polders were ploughed and leveled manually and then kept flooded. As complete randomization was not recommendable due to unmanageable complexity, every accession was assigned a random number from 1 to 75 and this sequence was planted in a row. In every sub-replication the sequence started with a different number leading to a semi-randomized setup. Seedlings were planted with a spacing of 20 cm to each other and 10 cm to the borders. Every planting position was marked with a wooden stick to avoid confusions because of missing plants. Therefore, one polder consisted of 300 plants. In summary, the experiment was conducted as a two-factorial design (iron-treatment and genotype) with two true replicates per treatment (independent polders) and four semi-randomized sub-replicates in every polder.

The soil type in the polders was a clay silt with about 5 % smectite, 16 % vermiculite, 69 % illite and 10 % kaolinite, originating from a Luvisol on deep loess with an organic carbon content of 1.2 % and a pH between 6 and 7 [43]. Average day and night time temperatures were 28°C and 18.4°C respectively, the average relative humidity was 76.4% and the average CO_2 concentration was 553 ppm.

All polders were watered regularly at least once a week. One hundred fifty g of potassium phosphate (K_2HPO_4) dissolved in water were applied to every polder. This corresponds to a concentration of about 6 g m^{-2} (60 kg ha^{-1}) of potassium and 2.2 g m^{-2} (22 kg ha^{-1}) of phosphorus. The same method was used to apply nitrogen in the form of urea. The application was split: 78 g were given five weeks after transplanting (WAT) together with the potassium phosphate, while another 78 g were applied nine WAT leading to a total application of 6 g m^{-2} (60 kg ha^{-1}) of nitrogen. As some accessions began to lodge in the reproductive phase the plants were supported with tonkin sticks. Perforated polypropylene bags, so called crispac-bags (305 x 450 mm, perforation diameter 0.5 mm, vendor Baumann Saatzuchtbedarf

GmbH, Waldenburg, Germany) were wrapped around ripening panicles to avoid seed shattering of wild rice accessions. The insecticide 'Perfekthion' (BASF, Ludwigshafen, Germany; active compound dimethoate) was sprayed on the plants due to an infestation with *Lissorhoptrus* ssp. 5 weeks after transplantation. Predatory mites (*Phytoseiulus persimilis* and *Amblyseiuscalifornicus*) were applied during the reproductive phase to control mite infestation.

Iron treatment

Iron treatment was started six weeks after transplanting (WAT). A total of 10 kg of iron(II) sulfate heptahydrate ($FeSO_4x7H_2O$) was applied to the iron treatment polders. Five kg were applied six and seven WAT to obtain an approximate concentration of 1500 mg l^{-1} of reduced iron in the upper soil solution in acute polders, while 1 kg was given to chronic polders every week until 16 WAT to reach a concentration of 200 to 300 mg l^{-1} [28]. An even distribution of all the chemicals was ensured by diluting the powders in water and applying it with a watering can.

Collection of data during growth

Leaf bronzing score was determined at one-week interval by visual observation of bronzing symptoms on the two youngest fully developed leaves of two tillers per plant. The percentage of leaf area affected by iron-induced symptoms was scored on a scale from 0-10 (in which 0 means no visual symptom and 10 indicates dead leaves) [26].

Leaves for iron analyses were sampled at 116 days after transplanting from genotypes selected based on contrasting symptom development at that stage. From each selected plant, the third youngest leaf was taken, dried, and subjected to Fe analyses as described below.

175 **Harvest**

176 Harvest took place 24 and 27 WAT. In the first round all plants with mature panicles were harvested.
177 Three weeks later, the remaining plants were harvested. Plants were cut close to the soil surface, the
178 height was measured, the number of tillers and panicles was counted, the panicles were put into a
179 crispac-bag and the straw biomass was folded to a bundle.

180 Straw biomass was dried at 60°C for at least 4 days and then weighed. The panicles were dried at 40°C
181 for at least 4 days, then the seeds were detached from the spikes. Grain samples were weighed, and
182 then two batches of 20 unfilled grains were counted and weighed. Unfilled grains were separated from
183 the filled ones by winnowing and blowing. The filled grains were weighed and again two batches of 20
184 filled grains were counted and weighed. With this data the number of seeds per panicle, the thousand
185 kernel weight (TKW) and the percentage of unfilled grains could be determined. In order to avoid errors
186 due to the presence of awns in some accessions, two times 20 awns of three samples of all accessions
187 with strongly developed awns were separated from grains, counted and weighed. The mean weight of
188 awns was used to correct the weight calculations of these accessions as awns were removed along with
189 the unfilled grains. The harvest index was calculated from the weight of filled grains in relation to the
190 whole plant biomass in percent.

191 **Seed analysis**

192 All RWR that produced seeds were included in the grain analyses, as well as the only *O. glaberrima*, and
193 *Oryza sativa* Dom Sofid, IR 72, IRRI 154 and Curinga. Two samples per polder equaling four samples per
194 treatment were chosen of every genotype. For dehusking of the seeds a Mixer Mill (MM 2000,
195 manufacturer RETSCH, Haan, Germany) was used. The grinding jars made of polytetrafluorethen (PTFE)
196 were filled to three quarters with sample material and different numbers of small (7 mm), medium
197 (10 mm) and large (12 mm) grinding balls made of agate were used to ensure that the samples had no

198 contact with Fe or Zn containing material. It was not possible to conduct the dehusking under
199 standardized conditions as the seed properties differed widely between species and accession.
200 Therefore, conditions were calibrated for every accession to protect the pericarp, aleurone layer and
201 embryo as much as possible to produce brown rice. For grinding the agate balls were exchanged with
202 one 20 mm PTFE Ball with a steel core, and all seeds were ground at speed 70 for one minute. All
203 samples were dried again afterwards for 2 days at 60°C to ensure complete dryness.

204 Phytate analysis followed the procedure described by [34] and was adjusted to microplate format. For
205 the Wade reagent, 18 mg of iron(III) chloride ($FeCl_3$) and 350 mg of sulfosalicylic acid dihydrate (SSA · 2
206 H_2O) were dissolved in 100 ml of deionized water. Of every sample of finely ground seeds, two times
207 50 mg were weighed into 2 mm Eppendorf-tubes to obtain two analytical replicates. After adding 1 ml of
208 3.5 % hydrochloric acid (HCl) all samples were stirred with a rotator (RS-RR 5, manufacturer Phoenix
209 Instrument, Garbsen, Germany) at 40 rpm for 1 h at room temperature. Centrifugation of samples was
210 carried out with a MIKRO 200 R (manufacturer Hettich, Buckinghamshire, England) at 21382 g for
211 10 minutes at 20°C. Two times 5 µl of every sample was each mixed with 145 µl of distilled water and
212 50 µl of wade reagent in a well-plate and the absorbance taken at 500 nm. The phytate concentration
213 was calculated with a standard curve ranging from 0 to 10 µg phytate using the microplate analysis
214 software Gen5 (developer BioTek, Winooski, VT, USA).

215 For iron and zinc determination in seeds, the ground material was first solubilized with a PDS-6 Pressure
216 Digestion System (manufactured by Loftfields Analytical Solutions, NeuEichenberg, Germany). 250 mg ±
217 9 mg of every sample were weighed into one PTFE beaker each and the precise weight was noted. One
218 blank and one standard of 250 mg MSC certified carrot reference material (NCSZC73031, China National
219 Analysis Center for Iron & Steel, Beijing, China) was integrated in every set of 24 beakers. Four ml of
220 nitric acid (65 %) was added to every sample. The samples were heated in an oven (VO400,

manufactured by Memmert, Schwabach, Germany) for 7 h at 180°C. The cooled samples were filled up with deionized water to a volume of 25 ml and filtered through ashless filter paper (MN640 m, Ø125 mm, manufactured by Macherey-Nagel, Düren, Germany). Absorption was measured at 213.8 nm for Zn and 248.3 nm for Fe with an atomic absorption spectrometer (model 11003, manufactured by PerkinElmer, Waltham, MA, USA). A blank was subtracted from the measured values and the mineral concentration of every sample was calculated by using fitting standard solutions.

For the phytate/mineral molar ratios, the measured concentrations were divided by their molar mass (phytate 660.04 g $mole^{-1}$; Fe 55,845 g $mole^{-1}$; Zn 65.38 g $mole^{-1}$).

Data analysis

Statistical analyses were conducted using the statistical software SAS 9.4 (SAS institute Inc., Cary, NC, USA). For all parameters the general linear model (GLM procedure) with least square means and Tukey-Kramer adjustment for multiple comparisons was calculated for the factors genotype, treatments and their interaction. For selected traits a comparison between the two groups 'cultivated genotypes' (*O. sativa* and *O. glaberrima*) and 'wild genotypes' (all non-cultivated species) in the different treatments was undertaken by two-way analysis of variance (ANOVA).

Results

Plants treated with Fe started to develop foliar symptoms of Fe toxicity soon after the first Fe application six and seven WAT. In the acute stress treatment, the peak of symptom formation was reached at eight WAT, while in the chronic stress treatment, the average symptom level continued to rise until 14 WAT (Table 1). From eleven WAT, the average symptom level was significantly higher in the chronic stress treatment compared to the acute stress treatment (Table 1). Significant genotypic differences in symptom formation occurred on all sampling days (Table 1) and were reflected in a broad range of responses of individual genotypes in terms of average LBS across all sampling days (Figure 1). In both stress conditions, the most sensitive genotypes were RWR, but on the other hand, some RWR also ranked among the most tolerant genotypes, especially in the acute Fe stress. Here, the two most tolerant accessions were from the species *O. meridionalis* (Figure 1a). On the other hand, in the chronic Fe stress, the most tolerant genotypes were *O. sativas*. These had previously been described as Fe tolerant *i.e.* FL483 [26] and Kitrana 508 [32]. Apart from that, there was no clear pattern regarding the tolerance of domesticated and wild rice species, as both groups exhibited a broad range of differential responses.

Figure 1: Leaf bronzing score of domesticated rice varieties and rice wild relatives in acute (a) and chronic (b) Fe toxicity stress. Mean values and standard errors across nine sampling days, two experimental and four sub-replicates are plotted. White bars represent domesticated varieties while grey bars represent wild relatives

257 Table 1: ANOVA results and treatment mean values for traits determined during the vegetative growth
258 of wild and domesticated rice species exposed to acute or chronic iron toxicity

Variable	Weeks after transplanting	ANOVA results (Pr>F)			Means (Treatment)		
		Treatment	Genotype	Interaction	Control	Acute	Chronic
Leaf Bronzing Score	8	0.7452	0.0001	0.0317	n.d.	1.8	1.6
	9	0.9057	0.0011	0.5599	n.d.	1.5	1.8
	10	0.4714	<0.0001	0.4562	n.d.	1.2	3.5
	11	<0.0001	0.0001	0.9903	n.d.	0.9^{b}	2.6^{a}
	12	0.0304	<0.0001	0.0782	n.d.	1.1^{b}	4.2^{a}
	13	0.0136	<0.0001	0.6220	n.d.	1.1^{b}	3.5^{a}
	14	0.0711	<0.0001	0.0101	n.d.	1.0	4.7
	15	0.0489	<0.0001	0.0843	n.d.	1.0^{b}	4.2^{a}
	16	0.0349	<0.0001	0.0008	n.d.	1.0^{b}	4.3^{a}
Foliar Fe concentration (mg kg^{-1})	16	<0.0001	0.0016	0.0160	272^{c}	790^{b}	1177^{a}

259 Mean values not sharing the same superscript letter within one line differ significantly from each other
260 at $P < 0.05$. n.d. = not determined.

Foliar Fe analyses of selected genotype at 16 WAT demonstrated that even in the acute Fe stress, where Fe was applied already six and seven WAT, most genotypes had values exceeding 300 mg kg^{-1}, which is widely accepted as the threshold for Fe toxicity [35]. Average values were significantly higher in the chronic stress treatment compared to the acute stress (Table 1). While the highest Fe levels in control conditions occurred in *O. sativas* (Fig. 2a), the opposite was seen in chronic Fe stress (Fig. 2c). In contrast, RWR accessions had the lowest Fe concentrations in all three conditions, suggesting efficient Fe exclusion mechanisms.

Figure 2: Foliar Fe concentrations of selected domesticated rice varieties and rice wild relatives in control conditions (a) acute (b) and chronic (c) Fe toxicity stress. Mean values and standard errors are plotted (n= 3-9). White bars represent domesticated varieties while grey bars represent wild relatives.

At harvest we determined shoot morphology and straw biomass of all plants, and analyzed grain yield and quality data for those accessions, in which a sufficient number of replicated plants produced seeds. This was true for most of the domesticated rice accessions, but only ten RWR. The Fe stress treatments did not negatively affect straw biomass and plant morphological traits, in which a rather positive effect was seen especially in the acute Fe treatment (Table 1). In contrast, grain yield was negatively affected in the chronic Fe treatment, but not in the acute Fe treatment (Table 2). In both Fe treatments, the sterility rate and the harvest index were significantly negatively affected compared to the control (Table 2). Analyses of the grain yield responses of individual genotypes revealed that most accessions had slightly enhanced grain yield in the acute Fe treatment (Figure 3a). In contrast, grain yield was negatively affected in most accessions in the chronic Fe treatment, except for two *O. sativas* (Dom Sofid and Kitrana 508) and one RWR (*O. rufipogon*), which had the highest grain yield among all accession that produced seeds. As opposed to the cultivated rice accessions, the group of RWR did not respond to Fe treatment with an increase in sterility (Figure 4).

Table 2: ANOVA results and treatment mean values for yield and grain quality traits of wild and domesticated rice species exposed to acute or chronic iron toxicity

	ANOVA results (Pr> F)			*Means (Treatment)*		
Variable	Treatment	Genotype	Interaction	Control	Acute	Chronic
Straw yield (g plant^{-1})	<0.0001	<0.0001	0.029	54.7^{b}	70.5^{a}	59.2^{b}
Plant height (cm)	<0.0001	<0.0001	<0.0001	167.6^{b}	177.4^{a}	176.6^{a}
Tiller number	<0.0001	<0.0001	0.0116	12.0^{b}	14.95^{a}	12.6^{b}
Grain yield (g plant^{-1})	<0.0001	<0.0001	0.8394	18.9^{b}	23.6^{a}	15.2^{c}
Panicle number	<0.0001	<0.0001	0.2228	9.7^{b}	12.8^{a}	10.6^{b}
Seeds per panicle	0.9189	<0.0001	<0.0001	132.5	131.3	129.2
Spikelet sterility (%)	<0.0001	<0.0001	<0.0001	50.1^{a}	55.9^{b}	62.2^{c}
Thousand kernel weight (g)	<0.0001	<0.0001	<0.0001	20.8^{a}	20.4^{a}	19.3^{b}
Harvest Index (%)	<0.0001	<0.0001	0.5422	27.9^{a}	23.3^{b}	18.4^{c}
Grain Fe concentration (mg kg^{-1})	0.2584	0.0299	0.1963	31.5	32.3	39.0
Grain Zn concentration (mg kg^{-1})	<0.0001	<0.0001	<0.0001	25.6^{b}	28.3^{ab}	30.8^{a}
Phytate (mg g^{-1})	0.007	<0.0001	<0.0001	9.7^{a}	10.4^{b}	9.6^{a}
Phytate/Fe molar ratio	0.14	0.0197	0.234	40.4	39.5	29.7
Phytate/Zn molar ratio	0.007	<0.0001	0.0003	38.0^{b}	36.3^{b}	31.6^{a}

Treatment means with different superscript letters in the same line indicate significant difference ($p < 0.05$).

Figure 3: Grain yields of selected domesticated rice varieties and rice wild relatives in acute (a) and chronic (b) Fe toxicity stress compared to control conditions. The grey bars indicate the negative (directed towards the left) or positive (directed towards the right) difference from the control. Mean values and standard errors (n=2-8) are plotted. Asterisk indicates a significant difference from the control at P<0.05.

Figure 4: Sterility rates of cultivated and wild rice species in control conditions, acute and chronic Fe toxicity. Mean values and standard errors are plotted (n=33-115). Bars not sharing the same letter differ from each other at P<0.05 by Tukey's HSD test.

Grain Fe concentrations were slightly elevated in the Fe treatment but the differences from the control were not significant (Table 2). In contrast, grains produced in the chronic Fe treatment had elevated Zn concentration and lower phytate /Zn molar ratio, and grains produced in the acute Fe treatment had elevated phytate concentrations (Table 2). Comparison of grain quality traits in selected accessions revealed that the *O. sativa* Dom Sofid had the highest Fe concentration, whereas two RWR showed the highest Zn levels (Fig. 5b), which were also associated with high phytate levels (Figure 5c). Very low phytate level occurred in an *O. spontanea* accession.

Figure 5: Brown rice mineral concentrations of selected domesticated rice varieties and rice wild relatives averaged across different treatments. (a) Fe concentration; (b) Zn concentration; (c) Phytate concentration. Mean values and standard errors (n=5-12) are plotted. Bar not sharing the same letters differ significantly from each other by Tukey HSD test.

Discussion

The treatments in the present study (acute and chronic Fe stress) were conceived in order to simulate two types of Fe stress typically occurring in the field. Acute Fe toxicity frequently occurs in inland valleys during heavy rainfall events, leading to a transient peak of interflow of reduced Fe from adjacent slopes into the rice field. This type of Fe stress is wide-spread for example in West Africa [23]. On the other hand, chronic Fe stress occurs on naturally Fe rich soils in which Fe stress builds up more gradually during the growing season upon flooding. This type of stress often occurs on acid sulfate soils or Ferralsols and is widespread in Southeast Asia, the South of Brazil or Madagascar [23]. The semi-artificial experimental setup used in this study was tested previously with six *Oryza sativa* varieties differing in Fe tolerance [28]. Similar to that previous study, acute Fe stress lead to the development of stress symptoms, but did not negatively affect biomass and grain yields (Table 2). In contrast, stress symptoms were more pronounced in chronic Fe stress, which also led to significant reductions in grain yield (Table 2), largely due to higher spikelet sterility (Figure 4). Therefore, our study confirms previous observations that plants respond differently to chronic and acute Fe stress on a physiological level [36], or in terms of yield [28], and that plants can compensate for early Fe tress comparatively well. The phenomenon of positive growth responses to mild metal stresses has been termed as 'hormesis' [37], and may occur due to physiological processes including the activation of antioxidants and complex signaling pathways. In contrast, the grain yield losses due to enhanced sterility in the chronic Fe treatment may be a consequence of oxidative stress in reproductive organs. High levels of Fe lead to the Fenton reaction, in which ferrous Fe reacts with hydrogen peroxide to form ferric Fe, hydroxide and the hydroxyl radical [25,38]. The hydroxyl radical is an extremely harmful molecule which cannot be effectively scavenged by the plants' antioxidant system [39].

335 Genetic variation in adaptive traits is the prerequisite for the breeding of new varieties with enhanced
336 tolerance to Fe toxicity. In this study, both domesticated and wild rice species showed substantial
337 variation in all observed traits. Foliar stress symptoms are often used as a secondary trait indicating Fe
338 tolerance, as it has shown relatively good correlation with important agronomical traits such as grain or
339 biomass yield in field experiments [27], and they can be scored for a large number of plants in
340 comprehensive screening experiment. For this trait, the most sensitive genotypes in both stress
341 conditions were all RWR (Figure 1). This high level of Fe sensitivity compared to domesticated rice may
342 be explained with the fact that RWR often grow in non-permanently flooded soils, therefore lacking
343 adaptive mechanisms such as Fe exclusion, which becomes relevant in permanently flooded conditions,
344 where soluble Fe is abundantly available. Rice domestication and breeding has been accompanied by
345 the development of irrigation infrastructure and the permanent flooding of rice soils, making adaptation
346 to high amounts of soluble Fe a trait that breeders may unintentionally have selected for in cultivated
347 rice varieties. Nevertheless, extremely low LBS of some RWR, especially *O. meridionalis* accessions
348 (Figure 1a) indicate that specific RWR do possess adaptive genes which can supplement the gene pool
349 for adaptive rice breeding. The high level of adaptation of this particular species may have an
350 evolutionary background as it is often found at the edges of freshwater lagoons, temporary pools and
351 swamps of the Northern territories of Australia [40], where Fe-rich soils such as Ferrosols occur [41]. As
352 a member of the *O. sativa* complex with an AA genome, *O. meridionalis* represents an easily accessible
353 gene pool, and crosses with *O. sativa* have previously been reported [31].

354 Foliar Fe levels in both Fe stress treatments were mostly above 300 mg kg^{-1}, which is considered to be
355 the threshold for Fe toxicity [35]. However, one *O. alta* accession maintained very low foliar Fe level in
356 the acute Fe stress (Figure 2b). It is doubtful whether this occurred due to an active Fe exclusion
357 mechanism such as Fe oxidation in the rhizosphere [26,42]. More likely, Fe was 'diluted' as this species
358 reached an average height of 316 cm and thus produced a lot of biomass for each of its few tillers (3.7

359 tillers on average). This plant architecture is not desirable in rice breeding, thus *O. alta* may not be a
360 suitable donor of Fe tolerance traits. The low Fe levels observed in two RWR (*O. barthii* and *O. nivara*) in
361 chronic Fe stress (Figure 2c) appear more promising, especially as these two species form part of the AA
362 genome group with lower crossing barriers towards *O. sativa* or *O. glaberrima*.

363 The analysis of grain yields was to some extent compromised by the fact that many RWR did not set
364 seeds within the experimental period. Factors contributing to this phenomenon include long vegetation
365 cycles, specific light and day length requirements, and self-incompatibility of many of the RWR [40].
366 Although experimental conditions including climate were controlled within a range that supports the
367 growth of most *Oryza* species, the large heterogeneity of screened genotypes is difficult to compensate
368 or adequately address by standardized climatic conditions. Among the ten RWR that produced seeds,
369 four showed higher yields in the chronic Fe stress than in the control, even though the overall yield
370 response across all screened genotypes was significantly negative in this treatment (Figure 3). This
371 positive yield response was not unique to RWR, as it also occurred in some *O. sativa* accession that had
372 previously been described as Fe tolerant [28,32], but the overall highest grain yield was seen in an *O.*
373 *rufipogon* accession (Figure 3b), and increases in spikelet sterility due to chronic Fe stress occurred in
374 cultivated rice varieties rather than RWR (Figure 4).

375 The concentrations of essential mineral nutrients in rice grains are a matter of concern because
376 nutritional deficiencies in Fe and Zn are very widespread [43,44]. Enriching rice grains in these elements
377 ('biofortification' [45]) is therefore seen as one out of several possible strategies to combat mineral
378 disorders in human diets. On the other hand, low phytate levels in cereal grains are desirable from a
379 nutritional perspective as this P-storage compound forms insoluble complexes with Fe and Zn thereby
380 limiting the bioavailability of these elements in human diets [46]. Our present study confirms previous
381 findings in domesticated rice varieties [28] that growing rice in high Fe conditions does not significantly
382 increase grain Fe concentrations in rice grains of most genotypes (Table 2), despite substantial increases

383 in Fe concentrations in vegetative tissue (Figure 2). This indicates that rice plants possess physiological
384 barriers protecting reproductive tissue from Fe levels that could cause oxidative stress and consequently
385 sterility. The regulation of metal transporters responsible for the loading of metals from mother plant
386 tissues such as seed coats into filial tissues [47] could be such a mechanism. On the other hand, average
387 Zn concentration was enhanced in plants grown in chronic Fe stress (Table 2), which could be due to a
388 concentration effect, which occurs when plants grown under stress form less biomass or yield with the
389 same amount of available mineral elements[48]. Irrespective of the treatment and genotype, phytate/Fe
390 and phytate/Zn molar ratios were in a range indicating relatively poor bioavailability [49], which is
391 characteristic of unrefined cereals evaluated individually, and not as part of a diverse diet. While the
392 RWR did not stand out with extraordinarily high nutritional value, positive features were seen especially
393 in terms of high Zn concentration (Figure 5b) and low phytate concentration (Figure 5c).

394 **Conclusion**

395 We screened a broad range of RWR for adaptive and grain quality traits when grown in Fe toxic
396 conditions, and compared them to domesticated varieties that had been pre-selected to include some
397 with a previously-known high level of tolerance. None of the RWR drastically outperformed the
398 domesticated varieties, which is partly due to their poor agronomic traits. Nevertheless, RWR exhibited
399 promising performance in individual traits such as low symptom formation (*O. meridionalis*), high grain
400 yield (*O. rufipogon*) or low grain phytate (*O. spontanea*). Chromosomal segment substitution lines of
401 interspecific crosses in *O. sativa* background [31] are available, facilitating future in-depth physiological
402 analyses in more uniform screening experiments due to lower morphological and phenological diversity
403 of such crosses as compared to the very diverse material screened in this study. The availability of
404 genome sequences of RWR [5] provides an additional powerful resources for the discovery of genes
405 underlying some of the adaptive traits identified in this study. Therefore, this study provided an

406 important first step in expanding the gene pool towards RWR for the breeding of rice varieties adapted
407 to Fe toxicity.

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412

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558 **Supporting Information**

559 Supplementary Table S1: List of germplasm used in the screening experiment

560

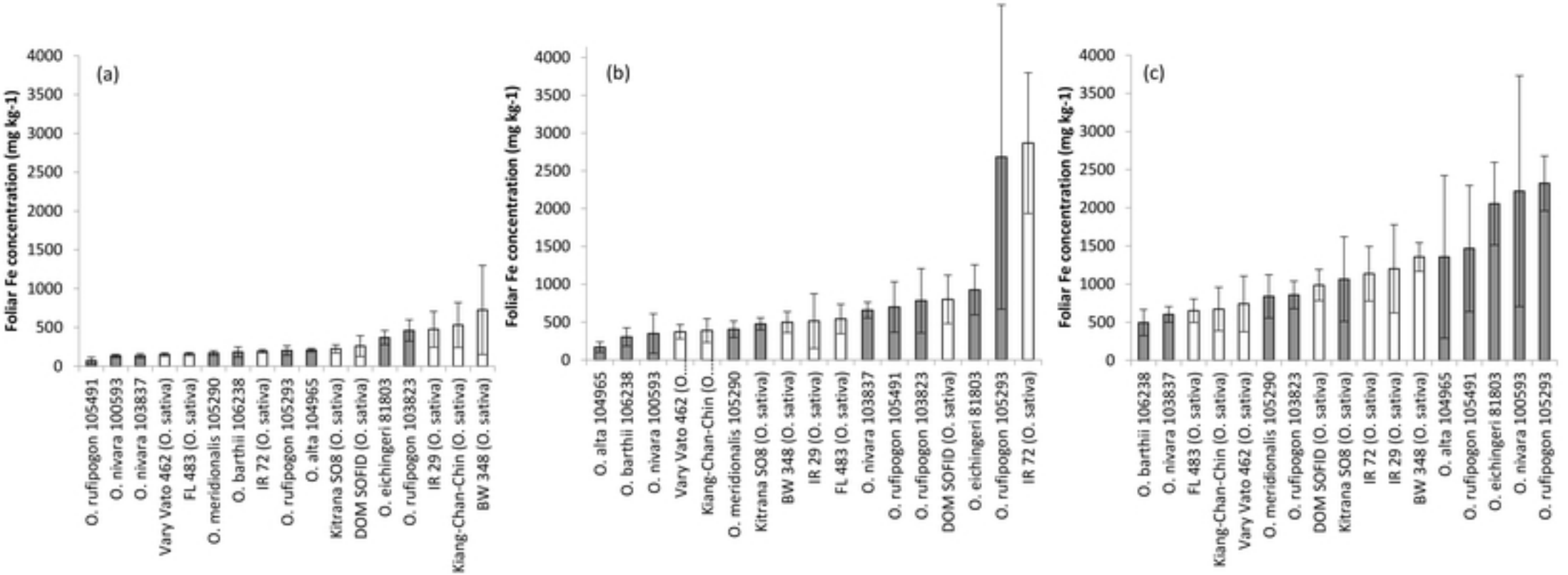
(a)
Foliar Fe concentration (mg kg-1)
0
500
1000
1500
2000
2500
3000
3500
4000
O. rufipogon 105491
O. nivara 100593
O. nivara 103837
Vary Vato 462 (O. sativa)
FL 483 (O. sativa)
O. meridionalis 105290
O. barthii 106238
IR 72 (O. sativa)
O. rufipogon 105293
O. alta 104965
Kitrana SO8 (O. sativa)
DOM SOFID (O. sativa)
O. eichingeri 81803
O. rufipogon 103823
IR 29 (O. sativa)
Kiang-Chan-Chin (O. sativa)
BW 348 (O. sativa)
(b)
Foliar Fe concentration (mg kg-1)
0
500
1000
1500
2000
2500
3000
3500
4000
O. alta 104965
O. barthii 106238
O. nivara 100593
Vary Vato 462 (O.
Kiang-Chan-Chin (O.
O. meridionalis 105290
Kitrana SO8 (O. sativa)
BW 348 (O. sativa)
IR 29 (O. sativa)
FL 483 (O. sativa)
O. nivara 103837
O. rufipogon 105491
O. rufipogon 103823
DOM SOFID (O. sativa)
O. eichingeri 81803
O. rufipogon 105293
IR 72 (O. sativa)
(c)
Foliar Fe concentration (mg kg-1)
0
500
1000
1500
2000
2500
3000
3500
4000
O. barthii 106238
O. nivara 103837
FL 483 (O. sativa)
Kiang-Chan-Chin (O. sativa)
Vary Vato 462 (O. sativa)
O. meridionalis 105290
O. rufipogon 103823
DOM SOFID (O. sativa)
Kitrana SO8 (O. sativa)
IR 72 (O. sativa)
IR 29 (O. sativa)
BW 348 (O. sativa)
O. alta 104965
O. rufipogon 105491
O. eichingeri 81803
O. nivara 100593
O. rufipogon 105293

Figure 2

(a)
Leaf bronzing score
2
1,5
1
0,5
0
O. meridionalis 82041
O. meridionalis 105290
Kitrana SO8 (O. sativa)
O. punctata 101429
IR 29 (O. sativa)
O. barthii 106238
Suakoko 8 (O. sativa)
BW 348 (O. sativa)
O. rufipogon 105293
O. nivara 102463
O. barthii x glaberrima 104137
DOM SOFID (O. sativa)
Taichung native 1 (O. sativa)
O. barthii 103581
O. punctata 101409
FL 483 (O. sativa)
O. punctata 105082
O. spontanea 88428
O. barthii 103895
O. spontanea 81805
O. nivara 101510
O. punctata 100886
O. rufipogon 126954
Tchibanga (O. sativa)
CG 14 (O. glaberrima)
O. longistaminata 89153
O. officinalis 81794
O. rufipogon 105491
O. rufipogon 106123
O. glumaepatula 105692
O. officinalis 102460
O. rufipogon 81801
Yodanya (O. sativa)
O. latifolia 105176
Guan Yin Tsan (O. sativa)
Arabi (O. sativa)
O. rufipogon 88785
O. brachyantha 104155
O. glumaepatula 82031
O. eichingeri 81803
Curinga (O. sativa)
Vary Vato 462 (O. sativa)
O. nivara 88868
O. meridionalis 105564
IR 72 (O. sativa)
O. rufipogon 106276
O. nivara 103837
O. nivara 100593
O. spontanea 104620
O. glumaepatula 100184
IRRI 154 (O. sativa)
O. barthii 105613
O. barthii 89146
O. spontanea 80612
Kiang-Chan-Chin (O. sativa)
O. malampuzhaensis 105322
O. brachyantha 101236
O. nivara 100916
O. brachyantha 101233
O. alta 100161
O. alta 104965
O. grandiglumis 101405
O. rufipogon 103823
O. longistaminata 101206
O. brachyantha 81950
(b)
Leaf bronzing score
6
5
4
3
2
1
0
FL 483 (O. sativa)
Kitrana SO8 (O. sativa)
O. rufipogon 126954
O. spontanea 88428
O. meridionalis 105290
O. barthii 106238
DOM SOFID (O. sativa)
O. nivara 103837
O. spontanea 81805
Taichung native 1 (O. sativa)
CG 14 (O. glaberrima)
BW 348 (O. sativa)
O. nivara 100593
O. rufipogon 105491
O. meridionalis 82041
Vary Vato 462 (O. sativa)
Suakoko 8 (O. sativa)
O. nivara 101510
O. punctata 101429
IR 29 (O. sativa)
O. barthii 103581
IRRI 154 (O. sativa)
O. rufipogon 103823
IR 72 (O. sativa)
Guan Yin Tsan (O. sativa)
O. glumaepatula 82031
Arabi (O. sativa)
O. rufipogon 88785
Yodanya (O. sativa)
O. spontanea 104620
O. punctata 105082
O. rufipogon 105293
O. punctata 100886
O. officinalis 81794
O. longistaminata 101206
O. officinalis 102460
O. glumaepatula 100184
O. nivara 102463
O. longistaminata 89153
O. barthii x glaberrima 104137
O. rufipogon 106123
O. meridionalis 105564
O. nivara 88868
O. alta 100161
O. malampuzhaensis 105322
O. eichingeri 81803
Tchibanga (O. sativa)
O. nivara 100916
O. latifolia 105176
Curinga (O. sativa)
O. alta 104965
O. barthii 103895
O. australiensis 86527
O. rufipogon 106276
O. barthii 89146
O. glumaepatula 105692
O. barthii 105613
O. brachyantha 101233
O. rufipogon 81801
O. grandiglumis 101405
Kiang-Chan-Chin (O. sativa)
O. brachyantha 81950
O. brachyantha 101236
O. latifolia 105145
O. eichingeri 89245
O. longiglumis 105147

Figure 1

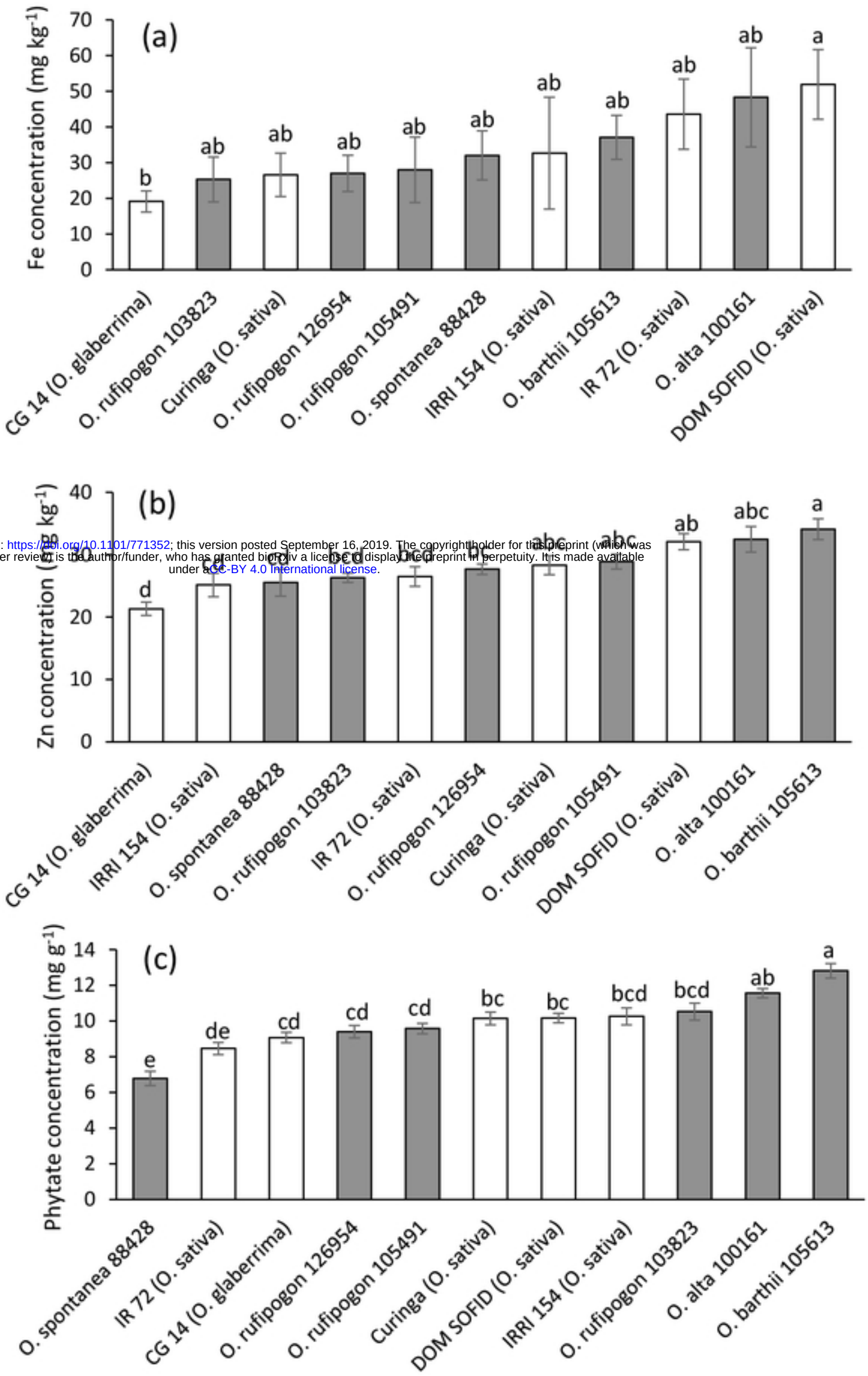

Figure 5

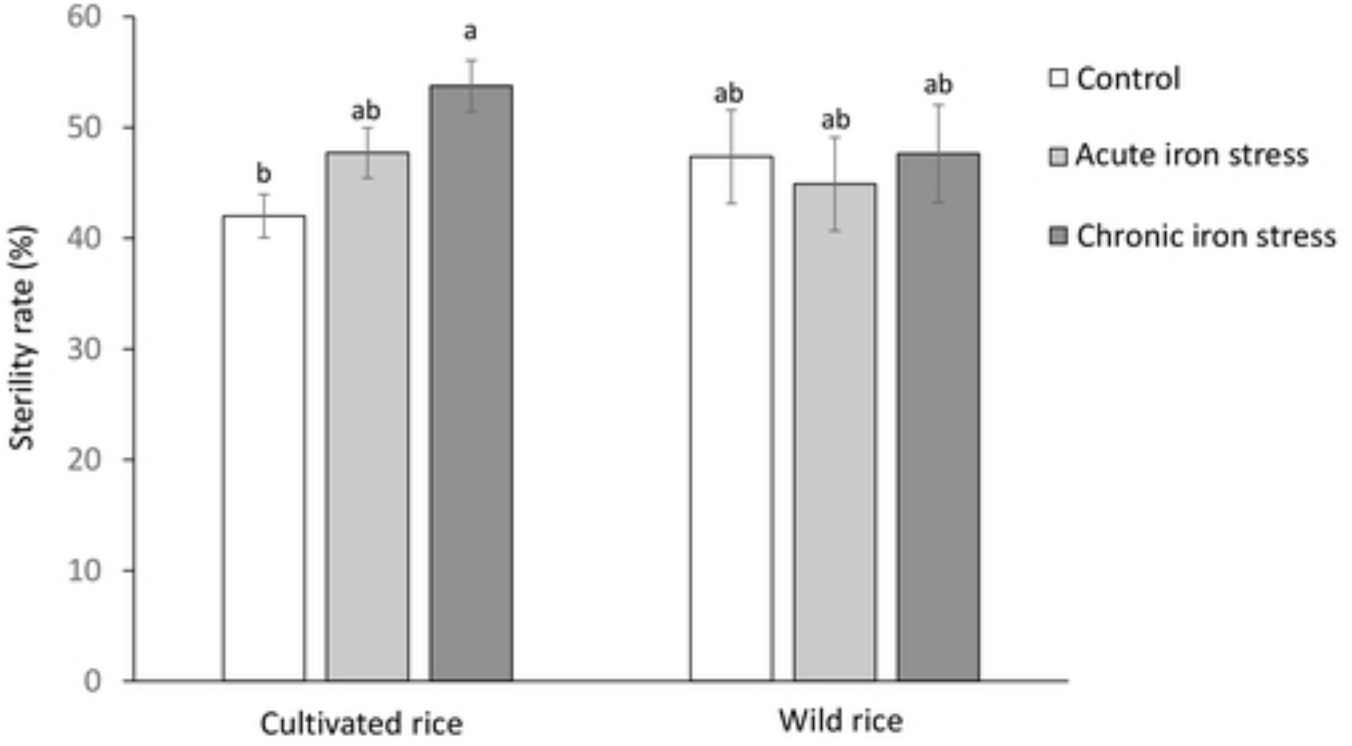
60
50
40
30
20
10
0
Sterility rate (%)
b
ab
a
ab
ab
ab
Cultivated rice
Wild rice
Control
Acute iron stress
Chronic iron stress

Figure 4

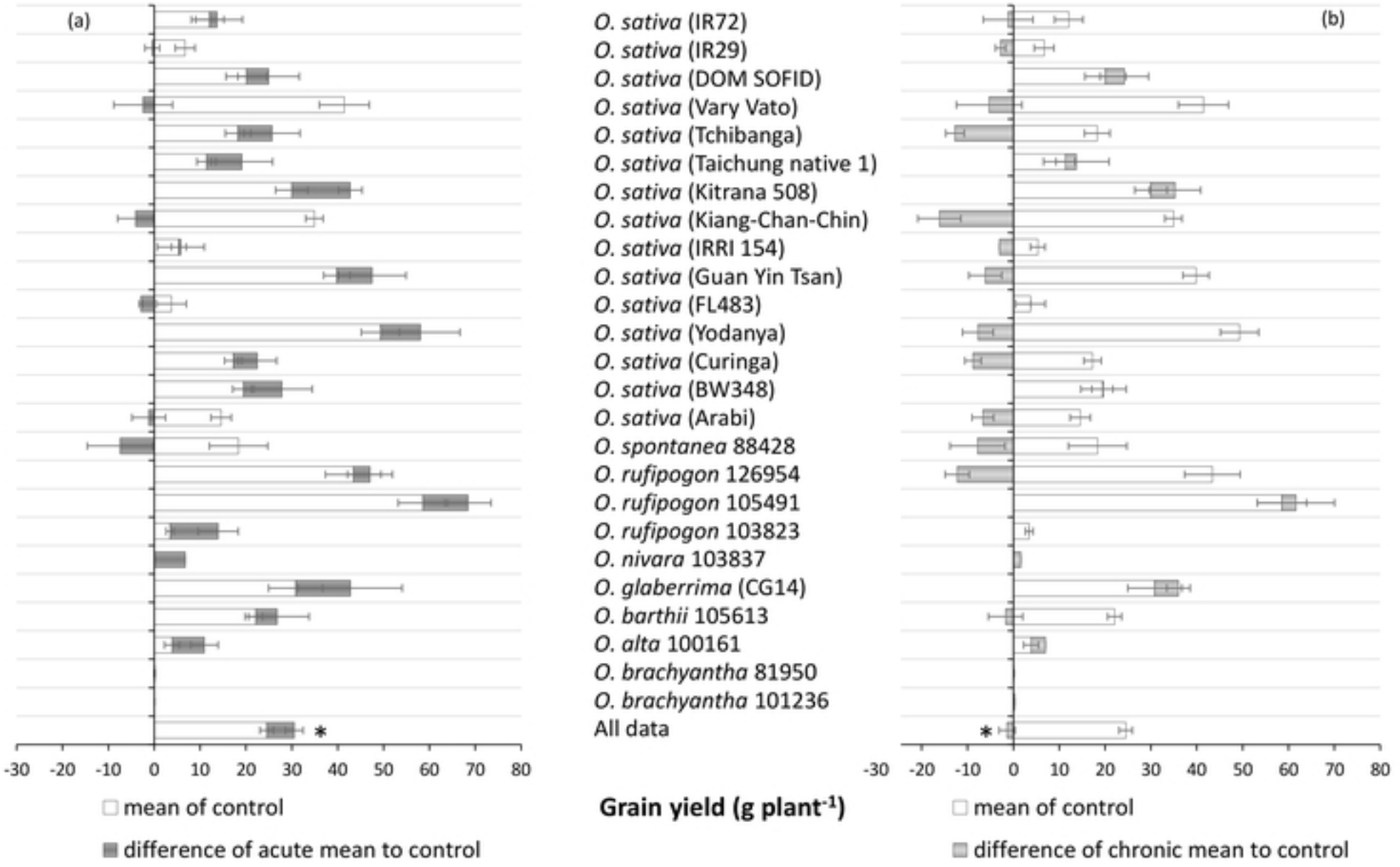
(a)
(b)
O. sativa (IR72)
O. sativa (IR29)
O. sativa (DOM SOFID)
O. sativa (Vary Vato)
O. sativa (Tchibanga)
O. sativa (Taichung native 1)
O. sativa (Kitrana 508)
O. sativa (Kiang-Chan-Chin)
O. sativa (IRRI 154)
O. sativa (Guan Yin Tsan)
O. sativa (FL483)
O. sativa (Yodanya)
O. sativa (Curinga)
O. sativa (BW348)
O. sativa (Arabi)
O. spontanea 88428
O. rufipogon 126954
O. rufipogon 105491
O. rufipogon 103823
O. nivara 103837
O. glaberrima (CG14)
O. barthii 105613
O. alta 100161
O. brachyantha 81950
O. brachyantha 101236
All data
-30 -20 -10 0 10 20 30 40 50 60 70 80
mean of control
difference of acute mean to control
Grain yield (g plant-1)
mean of control
difference of chronic mean to control

Figure 3