Tolerance Mechanisms and Irrigation Management to Reduce Iron Stress in Irrigated Rice

Felipe de Campos Carmona
Capivari do Sul, Brazil
Janete Mariza Adamski
Universidade Federal do Rio Grande do Sul
Andriele Wairich
Universidade Federal do Rio Grande do Sul
Joseane Biso de Carvalho
Universidade Federal do Rio Grande do Sul
Gustavo Gomes Lima
Capivari do Sul, Brazil
Ibanor Anghinoni
Universidade Federal do Rio Grande do Sul. Av. Bento Gonçalves
Isadora Rodrigues Jaeger
Universidade Federal do Rio Grande do Sul. Av. Bento Gonçalves
Paulo Regis Ferreira da Silva
Cachoeirinha, Brazil
Tatiana de Freitas Terra
Universidade Federal do Rio Grande do Sul
Janette Palma Fett
Universidade Federal do Rio Grande do Sul
Filipe Carlos (filipeselaucarlos@hotmail.com)
Federal University of Pelotas https://orcid.org/0000-0002-3068-0399

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Abstract

Iron toxicity is a major nutritional disorder in rice plants, especially in flooded areas. The use of alternative crop management practices, such as soil drainage, may mitigate negative impacts of iron toxicity, since soil aeration that follows drainage can oxidize and precipitate potentially toxic Fe^{+2} into Fe^{3+}. This study aimed to evaluate the impact of alternative water management on agronomical and physiological parameters in rice plants grown in a field location with iron toxicity history. Rice cultivars BR-IRGA 409 (sensitive) and IRGA 425 (resistant to iron toxicity) were tested. Irrigation management comprised three treatments: Continuous Irrigation (CI), one cycle of water Suppression (1S) and two cycles of water Suppression (2S). Evaluations included the ionic composition of soil solution and leaf tissues, grain yield, antioxidant responses and gene expression. Permanent soil flooding resulted in higher grain yield in plants from the resistant than from the sensitive genotype, which had higher malondialdehyde (MDA) concentrations in leaves. In contrast, two cycles of alternate soil drying resulted in equivalent grain yield and MDA concentrations in both genotypes. Resistance to iron toxicity in IRGA 425 plants seems related to limited Fe translocation to shoots, increased tolerance to oxidative stress in leaves and higher expression of Ferritin, *OsGAP1*, *OsWRKY80* and *Oryzain-α* genes. Plants from the BR-IRGA 409 cultivar (sensitive to Fe toxicity) improved growth and yield under the interrupted irrigation treatments, probably due to lower Fe availability in the soil solution. Management of water irrigation successfully alleviated Fe toxicity in rice plants cultivated in field conditions.

1 Introduction

Iron (Fe) is an essential micronutrient for plants, being required for fundamental biochemical activities and cellular functions, such as photosynthesis, respiration, DNA synthesis and repair. Most Fe functions are based on its reversible redox properties and conversion reactions of Fe^{+2} (ferrous) and Fe^{3+} (ferric) ions. On the other hand, Fe excess leads to toxicity symptoms (Sperotto et al. 2010). Because of its essential role and potential toxic effects, plants must tightly regulate cellular Fe homeostasis.

Iron toxicity in lowland rice (*Oryza sativa* L.) is a nutritional disorder that affects the production of this cereal in several rice-producing regions worldwide. It occurs most frequently in acid soils rich in sulphate and iron oxide, where, due to the anoxic environment and to the chemical reactions that occur after flooding, reduced iron (Fe^{2+}) becomes soluble and available to plants at high concentrations. Under these conditions, Fe can be excessively absorbed by plants (Ponnamperuma 1972).

High concentration of ferrous iron in plant tissues is closely related to increased oxidative stress and generation of hydroxyl radicals, which are extremely toxic to cellular metabolism, leading to oxidation and degradation of macromolecules, which impairs a wide range of physiological processes (Robello et al. 2007; Sperotto et al. 2010; Adamski et al. 2012). Fe toxicity can hinder plant development, leading to plant atrophy, tillering reduction and even to sterility (Dufey et al. 2009).

Several root nutrient transporters have broad substrate specificity (Korshunova et al. 1999; Dufey et al. 2015; Mahender et al. 2019). For example, the Iron Regulated Transporter protein from Arabidopsis (IRT1) can transport not only Fe^{2+}, but also Mn^{+2}, Zn^{+2}, Cd^{+2}, Co^{+2} e Ni^{+2} (Korshunova et al. 1999; Vert et al. 2001; Connolly et al. 2002; Schaaf et al. 2006), and the rice IRT1 and IRT2 proteins were also shown to be able to transport Cd^{+2} (Nakanishi et al. 2006). Since iron transporters are not completely selective, different cations may be absorbed concomitantly (Korshunova et al. 1999; Connolly et al. 2002; Vert et al. 2002; Schaaf et al. 2006; Nakanishi et al. 2006). As a consequence, the relation between the cations available in the soil solution is one of the factors associated with the manifestation of iron toxicity symptoms. The high concentration of Fe^{2+} may exert an inhibitory effect on the uptake of other cationic nutrients, especially at low concentrations (Tadano and Tanaka 1970). It has been reported that Fe toxicity can result in altered concentrations of other essential nutrients in leaf tissue and in altered relations between nutrient concentrations (Olaleye et al. 2009). This may be also a consequence of mineral precipitation at the “iron plaque”, which may be formed in the root apoplast when root cells are able to provide enough oxygen for Fe oxidation (Ando et al. 1983).

Iron toxicity symptoms in rice plants vary according to the cultivar and are usually characterized by orange or bronze-colored lower leaves, often including the ferric “iron plaque” formation at the outer surface of roots (Sahrawat 2004). The severity of yield losses induced by iron toxicity can vary within the growing season as well as between seasons and years.

Yield losses associated with the onset of toxicity symptoms commonly reach 15 to 30%, depending on the cultivar tolerance and the severity of toxicity. However, in extreme cases, there may be losses of up to 100%, especially at the seedling stage (Audbert and Saharawat 2000).

Since the manifestation of toxicity symptoms depends on soil factors, plant nutrient status and genetic variation, it is difficult to establish a critical level of Fe^{2+} in the soil solution as a parameter that determines the possibility that the disorder may occur. The toxicity symptoms have been observed at concentrations that vary from 10 to over 2,000 mg L^{-1} (Becker and Asch 2005). Likewise, there is no consensus about the contents within plant tissues that relate to the problem. Even so, the tissue concentration of 300 mg kg^{-1} of Fe has been considered critical for irrigated rice (Becker and Asch 2005; Fageria et al. 2008).

There are rice genotypes resistant to Fe toxicity, which make use of diverse avoidance or tolerance mechanisms (Dufey et al. 2015; Mahender et al. 2019). As an exclusion strategy, under Fe excess stress, Fe uptake- and transport-related genes started to be suppressed in roots (Aung et al. 2018). Some plants exclude potentially toxic Fe^{2+} through its oxidation at the root surface, using oxygen transported via the aerenchyma, or by retaining it at the root surface or within the root tissue (Becker and Asch 2005).

Another strategy is the compartmentalization of excessive Fe^{2+} by storage in the apoplasm and vacuoles, or by Fe^{2+} sequestering by the ferritin protein in plastids (Becker and Asch 2005; Briat et al. 2010). Ferritin is a ubiquitous Fe-storage protein capable of storing up to 4,500 atoms of iron within its internal cavity

(Briat et al. 2010). Tolerance to iron toxicity can also be achieved by detoxification of oxidative molecules, which mitigates cell injury caused by reactive oxygen species (ROS). This pathway is composed of both enzymatic mechanisms, mediated by radical scavenger enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) as well as by non-enzymatic mechanism (Mittler 2002; Dufey et al. 2015). Other rice plants are inefficient in all these processes and are, therefore, sensitive to excess Fe^{2+} toxicity (Stein et al. 2009a; Sperotto et al. 2010; Stein et al. 2014). Currently, growers tend to use cultivars resistant to Fe^{2+} toxicity in affected areas.

Several studies have recently reported quantitative trait loci (QTLs) associated with tolerance to Fe overload. The QTLs were identified based on morphological, physiological and agronomic parameters, in populations grown under controlled and in iron-toxic field conditions. These QTLs explain from 6.2 to 40.5 of the phenotypic variation and were mapped on several chromosomes (Dufey et al. 2009; Wu et al. 2014; Dufey et al. 2015). The expressive number of mapped QTLs, mostly of small effects, emphasizes the concept of multiple tolerance mechanisms involved in different types of Fe toxicity (Wu et al. 2014).

Besides the use cultivars resistant to Fe^{2+} toxicity, the adoption of alternative cultivation practices can also mitigate the problem. Agronomic management practices have been proposed to reduce the Fe^{2+} concentration in the soil solution, such as alternative water irrigation management and nutrient management in lowland rice (Schimidt et al. 2013, Wu et al. 2014, Becker and Asch 2005). The utilization of an irrigation system that includes alternate suppression of irrigation may favor plant development and preserve the rice production potential in soils with high Fe^{2+} contents, due to oxygen entry into the soil and consequent oxidation of soluble Fe^{2+} to Fe^{3+} (Kongchum 2005). Monocot plants (such as rice) are known to produce and extrude iron chelators (phytosiderophores, PS) to be able to take up Fe^{3+}, which is transported as Fe-PS complexes by transporters from the Yellow Stripe family (Walker and Connolly 2008; Sperotto et al. 2010). However, rice plants are not very efficient in the production of PS (Mori et al. 1991), and cultivars adapted to flooded conditions (where Fe^{2+} is highly available) may be even less efficient in the uptake of Fe^{3+}, which is the available form of iron under dry (and aerated) conditions. In irrigation systems that alternate cycles of flooded soil with moderately drained soil, it is possible that the soil redox potential rapidly returns to values close to the original before a new irrigation, as long as the soil oxygenation is effective (Kyuma 2004). Alternate wetting and drying conditions have been recommended also to reduce water requirements in rice production and lower environmental footprints. Improved yield under those conditions has been related to rice cultivars with some important root architectural traits (Sandhu et al. 2017).

Previous work from our group has shown higher accumulation of the iron storage protein ferritin in leaves from EPAGRI 108 plants, which are tolerant to Fe excess, than in BR-IRGA 409 plants (Silveira et al. 2009). We have also shown increased levels or ferritin transcripts (*OsFER1* and *OsFER2*) in seedlings and leaves of rice Nipponbare plants treated with excess iron than in control plants (Stein et al. 2009b). These genes are reported to be highly induced in most tissues of Fe excess treated rice plants regardless of Fe excess level (Aung et al. 2018). More recently, transcriptomic analysis revealed up-regulation of both *OsFER1* and

OsFER2 in BR-IRGA 409 plants exposed to iron excess treatment (Stein et al. 2019). Using representational difference analysis (RDA), we isolated sequences up-regulated after exposure to iron excess in BR-IRGA 409 plants, including ferritin, the transcription factor *OsWRKY80*, the proteolysis enzyme *oryzain-α*, and the stress-related gene *OsGAP1* (Ricachenevsky et al. 2010). However, expression of these genes has never been evaluated in plants from the IRGA 425 cultivar, tolerant to iron excess, used in the present work, as well as in rice plants grown under alternative irrigation patterns.

The objective of this work was to evaluate the effects of irrigation water management on the mitigation of iron toxicity symptoms in rice plants and to provide physiological characterization of those effects. To evaluate this, we analyzed agronomic parameters, activity of antioxidant enzymes and the pattern of gene expression associated with tolerance mechanisms to Fe overload. Our data suggest that two cycles of water suppression alleviate iron overload in plant tissues, reduce oxidative stress and improves the production of grains in rice plants.

2 Material And Methods

2.1 Experimental design and water management

The experiment was performed on the 2011/12 crop season, on a property in the municipality of Restinga Seca, Rio Grande do Sul, Brazil, on a Planosol Dystric soil (classified according to the World Reference Base for Soil Resources - IUSS Working Group, 2015) with a history of Fe^{2+} toxicity and which had been cultivated continuously with irrigated rice since 2008.

In order to evaluate the effect of irrigation water management, the following treatments were established: **CI** – *continuous irrigation* with a water depth of 5.0 cm from stage V_{3-4} to R_6 (developmental stages according to Counce et al., 2000); **1S** – continuous irrigation with *one suppression* of irrigation between stages V_6 and V_8 and later irrigation until R_6; and **2S** – continuous irrigation with *two* cycles of irrigation *suppression* (suppression between stages V_6 and V_8, immediate re-establishment of the water depth, and a new suppression between stages V_8 and V_{10}, followed by irrigation until R_6). Two cultivars previously shown to have contrasting levels of resistance to Fe^{2+} toxicity were tested: sensitive BR-IRGA 409 and resistant IRGA 425 (SOSBAI 2010; Janick 2014). The experimental design was of randomized blocks with three repetitions in subdivided plots with the irrigation treatments on the main plots and the cultivars on the subplots.

Seeds were sown during the second fortnight of October 2011, with a density of 100 kg ha^{-1}. Soil analyses were performed as described by Gonçalves and Meurer 2010. Base fertilization with nitrogen, phosphorus and potassium was performed according to expectations of a high response to fertilization (SOSBAI 2010), considering the results of the soil analysis (Table 1), and adding 14, 59 and 94 Kg ha^{-1} of N (ammoniacal), P_2O_5 and K_2O, respectively.

Table 1
Soil characteristics in the experimental area prior to sowing.

Clay (g kg^{-1})	**210**
Organic matter (g kg^{-1})	19
pH	5.2
P ($cmol_c$ dm^{-3})	0.009
K ($cmol_c$ dm^{-3})	0.15
Ca ($cmol_c$ dm^{-3})	1.9
Mg ($cmol_c$ dm^{-3})	1.4
CEC ($cmol_c$ dm^{-3})	5.1
H^+Al ($cmol_c$ dm^{-3})	10.9
Soil samples were collected before sowing in ten points randomly distributed throughout the experimental area. CEC: cation exchange capacity; H^+Al: exchangeable acidity; $cmol_c$: centimol of charge.	

Plot size was 8 x 16 m. Each plot was isolated by mud walls with a mean height of 40 cm in order to provide individual irrigation according to the treatment. The two cultivars studied were sown side by side, making up 64 m² of seeded area per cultivar. The water used for irrigation was adducted by gravity in PVC tubes (100 mm diameter) with lateral diversions to the experimental units, so as to irrigate one plot at a time, according to the treatments proposed.

In all treatments, the beginning of the water inflow occurred in stage V_{3-4}, right after applying the herbicide and the first nitrogen fertilization coverage (80 Kg N ha^{-1}, in the form of urea). When the plants reached the stage of floral primordium differentiation (beginning of the reproductive period), the second coverage of N was applied (40 Kg ha^{-1}, in the form of urea).

2.2 Harvest and yield components

At the end of the growing cycle, when grains reached about 20% humidity, the experiment was harvested in an area of 12.56 m² (8 x 1.57 m) per treatment and cultivar. The grain yield was obtained by extrapolating the production obtained in the work area to one hectare, correcting humidity to 130 g kg^{-1}. The number of panicles was determined as number of panicles in a given area (m^2). The number of

grains per panicle was calculated by the ratio between total number of grains formed and the number of panicles harvested in the area of the sample. The spikelet sterility was calculated by counting the number of sterile spikelets, separated by air-blowing equipment, and expressed as percentage in relation to the total number of spikelets per panicle.

2.3 Analyses of soil solution, leaf concentrations of nutrients and shoot dry weight

When the plants reached the floral primordium differentiation stage (R1), triplicate samples of 20 mL of soil solution were collected at a depth of 10 cm and filtered directly on glass vials containing 2 mL HCl 1.1 M, so that the final HCl concentration in each sample was 0.1 M (Gonçalves and Meurer 2010). Concentrations of Fe, Ca and Mg were determined by atomic absorption (Perkin Elmer 403). Concentrations of P were determined in a colorimeter (Varian Series 634). Concentrations of K were determined by a flame photometric method using a Digimed NK-2000 equipment (Mehlich 1953).

On the same occasion, the third fully expanded leaf of the main stem was collected, from ten plants per cultivar. The leaves were stored for later analysis of Fe, K, P, Ca, Mg concentrations by atomic absorption, using three replicates (each composed of samples from three or four plants) per cultivar and treatment. After analysis, all concentrations were transformed to the same unit (μg g^{-1} DW) and ratios between concentrations of each element and iron concentrations were calculated.

Shoot dry weight was determined by harvesting all plants in a linear row of 1 meter, collecting all tissues above soil level, with 10 replicates per treatment. The harvested materials were dried at 60°C until reaching constant weight.

2.4 Activities of antioxidant enzymes

Four biological replicates of leaves from rice plants at the booting stage (R2) were evaluated for each treatment. Approximately 1 g of leaves previously frozen in liquid nitrogen were homogenized with sodium phosphate buffer 50 mM (pH 7.0) containing polyvinylpyrrolidone 10 g L^{-1}, ethylenediaminetetraacetic acid 0.2 mM and triton X-100 10 mL L^{-1}. The homogenate was centrifuged at 12,000 g for 20 min at 4°C. The supernatant was used for analysis of antioxidant enzymes activity. Catalase (CAT, EC 1.11.1.6) activity was determined by monitoring the disappearance of H_2O_2 at 240 nm (Aebi 1984). Superoxide dismutase (SOD, E.C 1.15.1.1) activity was determined using 15 min of illumination and recording the absorbance at 560 nm (Beyer and Fridovich 1987). The protein concentration from all samples was quantified by the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

2.5 Lipid peroxidation and hydrogen peroxide concentration

Four biological replicates of leaves from rice plants at the booting stage (R2) were evaluated per treatment. Malondialdehyde (MDA) concentration was determined as a final product of lipid peroxidation through reaction with thiobarbituric acid (TBA) (El-Moshaty et al. 1993). The lipid peroxides were

expressed as nmol MDA mg protein^{-1}, using the extinction molar coefficient of 155 L^{-1} mol^{-1} cm^{-1}. The hydrogen peroxide concentration was determined by comparing absorbance of each sample with a calibration curve at 390 nm. The H_2O_2 concentration was expressed as µmol g^{-1}fresh weight (Loreto and Velikova 2001).

2.6 RNA extraction and Gene expression by RT-qPCR

Each treatment consisted of four biological replicates of leaves from rice plants at the booting stage (R2). Total RNA from leaves was extracted with Concert RNA Plant Reagent (Life Technologies, Carlsbad, USA), quantified by Nanodrop and treated with Turbo DNAse (Life Technologies, Carlsbad, USA). First-strand cDNA synthesis was performed with OligodT, reverse transcriptase M-MLV (Life Technologies, Carlsbad, USA) and 1 µg of RNA.

The cDNA samples from each time point were diluted 100 times. Reactions were carried out in a StepOne Real-Time Cycler (Applied Biosystems). Reaction conditions included an initial denaturation step at 95°C for 5 min, followed by 40 cycles at 95°C for 15 s, 60°C for 10 s and 72°C for 15 s. For generating the denaturing curve, amplicons were denatured for 2 min at 60°C and then heated from 55 to 99°C with a ramp of 0.3°C per 1 s. RT-qPCR reactions were carried out in 20 µL final volume composed of 10 µL of cDNA samples (diluted 100 times) and PCR mix containing 0.2 µM primers (Forward plus Reverse), 0.1 mM dNTPs, 0.2 X SYBR Green (Molecular Probe, 10.000 X), 1 X PCR Buffer, 3 mM $MgCl_2$, and 0.01 U Taq Platinum Polymerase (Life Technologies, Invitrogen, Carlsbad, USA). Data were analyzed by the normalized gene expression method (Simon 2003). This process of normalization takes the different efficiencies of PCR amplification for the target (E_{target}) and the reference ($E_{reference}$) genes and transforms the logarithmic scaled raw data unit Cycle Threshold (CT) into the linear unit of normalized expressions (NE), such as NE = $(E_{reference})^{CTreference)}/(E_{target})^{CTtarget}$. The patterns of expression of rice Ferritins, *Oryzain α*, C2 domain GTPase (*OsGAP1*) and *OsWRKY80* were analyzed in this experiment because these genes were previously shown to be responsive to excess iron in BR-IRGA 409 plants (Ricachenevsky et al. 2010). We used a primer pair that amplifies transcripts from both rice Ferritin genes (*OsFER1* and *OsFER2*, Stein et al. 2009b).

2.7 Statistical analyses

Data were subjected to combined analyses of variance using the general linear model (GLM) procedure to determine the significance of the main effects and interactions. Following ANOVA, means were compared by the Duncan's Test (means from different irrigation treatments within each cultivar) and Student's T Test (means from different cultivars within each irrigation treatment) using the Statistical Analysis System Enterprise Guide (version 6.1, SAS Institute, USA). In all figures the spread of values are shown as error bars representing standard errors of the means.

3 Results

3.1 Impacts of soil drainage on crop performance

Plant visual observation revealed that IRGA 425 plants (resistant) did not suffer apparent damage due to iron toxicity, while plants from the BR-IRGA 409 cultivar (sensitive to Fe toxicity) showed clear signs of chlorosis under continuous irrigation. The chlorotic symptoms were ameliorated with interruptions of irrigation (Fig. 1A). The rice grain yield of cultivar BR-IRGA 409 was significantly increased by the treatments with interrupted irrigation: a major increase in productivity (over 3,000 Kg ha^{-1}) was observed in the 2S treatment in relation to CI (Fig. 1B), reaching productivity levels equivalent to the ones seen in the resistant cultivar, IRGA 425, which was not affected by the treatments. Under continuous irrigation, BR-IRGA 409 plants tended to have higher spikelet sterility and lower shoot dry weight than plants from the resistant cultivar (Fig. 2A and B). However, the rigor of statistical tests did not indicate significant differences, both between cultivars and treatments. No differences were seen in the number of grains per panicle within cultivars or treatments (Fig. 2C). In the resistant cultivar, IRGA 425, the number of panicles per square meter was significantly higher in the treatment with two suppressions of irrigation than in the treatment with continuous irrigation (Fig. 2D).

3.2 Concentrations of nutrients in soil solutions and leaves

Fe concentrations in the soil solution were lower with suppressions of irrigation than under continuous irrigation (Fig. 1C). Clear variation in the color of the soil solution between the different treatments was observed (Fig. 1D). Concentrations of Mg, P, K and Ca in the soil solution did not change significantly in the different treatments (Fig. 3).

Under continuous irrigation, leaf iron concentrations were about 2.3-fold higher in the iron sensitive cultivar, BR-IRGA 409, than in IRGA 425 (Fig. 1E). The irrigation suppression (1S and 2S treatments) resulted in altered concentrations of nutrients in leaves of both cultivars. In the sensitive cultivar, BR-IRGA 409, there were significant reductions in Fe, K and Ca leaf concentrations in the 2S treatment and in K and Mn concentrations in the 1S treatment (Fig. 4). In the resistant cultivar, IRGA 425, leaf concentrations of Ca and K were higher under continuous irrigation than with two irrigation suppressions (Fig. 4).

Mineral concentrations in leaves were used to calculate ratios of each mineral in relation to iron concentration when plants were at the floral primordium differentiation stage (R1). The leaf P/Fe, K/Fe, Mn/Fe, Ca/Fe, Mg/Fe and Zn/Fe relations increased with irrigation suppression only in the sensitive cultivar, BR-IRGA 409, with higher values being reached under the 2S treatment (Fig. 5).

3.3 Oxidative damage and antioxidant enzymes

To assess whether one or two cycles of irrigation could change the antioxidant response in rice leaves, we evaluated the concentrations of oxidative stress markers and the activities of enzymes involved in antioxidant protection.

Lipid peroxidation was higher in BR-IRGA 409 than in IRGA 425 leaves when plants were grown under constant irrigation. With two cycles of irrigation suppression, the MDA concentration in the sensitive cultivar decreased to levels equivalent to the ones found in the resistant cultivar (Fig. 6A). Hydrogen

peroxide concentrations were higher in IRGA 425 plants than in BR-IRGA 409 under constant irrigation and in the 1S treatment (Fig. 6B).

CAT activity was higher in the BR-IRGA 409 cultivar than in IRGA 425 in the treatment with two suppressions of irrigation (2S). The activity of this enzyme in leaves from IRGA 425 plants in the 2S treatment was lower than in the 1S treatment (Fig. 6C). The irrigation suppression treatments did not alter SOD activity in leaves of both cultivars (Fig. 6D).

3.4 Gene expression

The levels of Ferritin transcripts (*OsFER1* and *OsFER2*, evaluated together) were 7-fold higher in the cultivar IRGA 425 (resistant) than in the cultivar BR-IRGA 409 under continuous irrigation (Fig. 7A). The C2 domain-GTPase (*OsGAP1*) transcript levels were lower in BR-IRGA 409 upon continuous irrigation than under the 1S and 2S treatments, whereas in IRGA 425 there were no significant changes in the level of expression within treatments (Fig. 7B). The *OsGAP1* transcript levels under continuous irrigation were 3-fold higher in IRGA 425 plants than in BR-IRGA 409 (Fig. 7B). In BR-IRGA 409 plants, the transcript levels of the *OsWRKY80* transcription factor were about 6-fold higher under two cycles of irrigation suppression than in the other treatments, reaching expression levels equivalent to those seen in the tolerant cultivar (Fig. 7C). Expression of *Oryzain α* in BR-IRGA 409 was higher in the 2S treatment than in the other two treatments. Under continuous irrigation, expression of this gene was 3.5-fold higher in plants from the IRGA 425 cultivar than in BR-IRGA 409 (Fig. 7D).

4 Discussion

Iron toxicity is one of the main nutritional disorders which limits yield of lowland rice (Ethan et al. 2011). The main symptom of toxicity by excess Fe in rice is the bronzing of lower leaves. This nutritional stress can lead to growth retardation, low productivity, spikelet sterility and, in more severe cases, plant death (Sahrawat 2004).

In a greenhouse experiment, Schmidt et al. (2013) showed that the use of repeated drainages during the vegetative growth was efficient to control Fe toxicity in rice grown in flooded soil. In the present study, under field conditions, the sensitive cultivar (BR-IRGA 409) had lower grain yield than the resistant one when both were cultivated under continuous irrigation (Fig. 1B). Irrigation suppression allowed the sensitive cultivar to achieve higher grain yield, which, in the 2S treatment, was statistically equivalent to the yield of the resistant cultivar (Fig. 1B). This result demonstrates that the water suppression minimized the toxic effects of excess iron on grain yield, probably due to decreased iron concentration in leaf tissues (Fig. 1E). Fe toxicity in rice plants is considered to occur when iron concentrations are higher than 300 μg Fe g^{-1} leaf dry weight (Becker and Asch 2005). In this work, concentrations above this threshold were reached only in BR-IRGA 409 plants under continuous irrigation (Fig. 1E). Under the same treatment, lower concentrations of Fe were detected in IRGA 425 plants, more resistant to Fe toxicity (Fig. 1E). These plants may be resistant to excess Fe due to a combination of several characteristics, including the induction of avoidance mechanisms (which allow plants to have low Fe uptake) or the

compartmentalization of Fe within roots. In both cases, plants have low iron translocation to shoots (Sperotto et al. 2010).

Additionally, one and two cycles of water suppression resulted in higher ratios of phosphorus to iron concentrations (P/Fe) in leaves of the sensitive cultivar than under continuous irrigation (Fig. 5), at the stage of floral primordium initiation. Oxygen release from rice roots allows the formation of Fe and Mn depositions. High amounts of accumulated Fe oxides at the external surface of roots can adsorb anions such as phosphate (Zhang et al. 1999), limiting P translocation to shoots (Silveira et al. 2007). In this experiment, higher P/Fe concentration rates in leaves of BR-IRGA 409 plants under one and two cycles of water suppression (Fig. 5) may be due to decreased formation of iron plaques at the roots. The same possibility may explain the higher Mn/Fe concentration rates in leaves of BR-IRGA 409 plants grown under two cycles of water suppression (Fig. 5).

Higher availability of iron to plants can also cause nutrient imbalances through antagonistic effects on mineral uptake, including K and Zn. In rice, low K absorption under Fe excess has been reported (Sperotto et al. 2012). However, our data showed higher K concentration in BR-IRGA 409 leaves when plants were under continuous irrigation than with one or two cycles of water suppression (Fig. 4). Our results agree with Silveira et al. (2007), who detected higher K concentration in shoots of iron excess-sensitive plants (BR-IRGA 409, the same sensitive cultivar used in our study) under Fe excess than under non-toxic iron concentrations. Due to a 5.7-fold lower leaf Fe concentration in the 2S treatment than under CI (Fig. 1E), BR-IRGA 409 plants achieved a better nutritional balance, with higher P/Fe, K/Fe, Mn/Fe, Ca/Fe, Mg/Fe and Zn/Fe ratios with two cycles of water suppression than under continuous irrigation (Fig. 5), with consequent increase in grain yield (Fig. 1B).

The presence of Fe^{2+} toxicity in rice, besides being related to the level of tolerance from each genotype, also depends on factors such as the form and source of iron in the soil solution, temperature and solar radiation (Bode et al. 1995). For this reason, the critical level of Fe^{2+} in the soil solution is very variable, and symptoms may appear in soil with levels as low as 10 mg L^{-1} of Fe^{2+} (Becker and Asch 2005). The iron concentration in soil solution under continuous irrigation at our experimental site (13.3 mg L^{-1}, Fig. 1C) was above the threshold of 10 mg L^{-1}, what is consistent with the toxicity symptoms observed in plants from the sensitive genotype (BR-IRGA 409) under continuous irrigation (Fig. 1A).

The soil from the area of study was acidic and had low organic matter (Table 1), which is used as substrate for microbial metabolism. The low amount of organic matter may be related to the relatively moderate concentrations of soluble iron extracted from the soil solution after flooding (averages 13.3, 10.9 and 3.5 mg L^{-1}, Fig. 1C), considering that the concentration of iron available in the soil solution increases according to the availability of easily decomposable organic matter, temperature and redox potentials (Ponnamperuma 1972), which are amplified in acid soils (Prade et al. 1990).

Lipid peroxidation is considered a biochemical marker for free radical mediated stress (Fang et al. 2001). Under continuous irrigation, lipid peroxidation was over two times higher in plants sensitive to iron

toxicity than in the resistant plants, indicating that BR-IRGA 409 plants were suffering oxidative stress (Fig. 6A). Two cycles of irrigation suppression were apparently enough to recover those plants from stress, since lipid peroxidation reached levels as low as the ones seen in the resistant plants (Fig. 6A).

One of the consequences of stress is an increase in cellular concentrations of reactive oxygen species, including H_2O_2 (Fang et al. 2001). Excessive H_2O_2 accumulation can lead to oxidative stress in plants, which then triggers cell death (Quan et al. 2008). However, increased activity of antioxidant enzymes can detoxify H_2O_2 (Mittler 2002). H_2O_2 concentrations were higher in leaves from the resistant cultivar than in the sensitive one in the CI and 1S treatments (Fig. 6B). It is possible that higher levels of H_2O_2 in the resistant cultivar, as well as fluctuations observed within treatments, may be related to a signaling role of H_2O_2. This molecule can mediate responses to various stimuli in plants, promoting development, increasing resistance to environmental stressors, and regulating gene expression (Neill et al. 2002). In our experiment, the maximum variation observed in H_2O_2 concentration in the resistant cultivar was a 20% lower average concentration (not statistically significant) in the 2S treatment in relation to the 1S. Considering that there were no significant variations in H_2O_2 concentration within treatments in the Fe-sensitive plants as well, and that variations due to stressful conditions in plants can range from 10 to 30-fold increases (Mittler, 2002), we suggest that the fluctuations observed in the Fe-resistant plants are related to the signaling roles of hydrogen peroxide.

High activity of protective enzymes, such as calatase (CAT) and peroxidase, has been reported in an iron-tolerant rice variety (Zhang et al. 2011). In the present work, CAT activity was lower in the tolerant IRGA 425 plants than in BR-IRA 409 (Fig. 6C), suggesting lower need for this enzyme's activity in the tolerant genotype. This could be in agreement with a possible signaling role of H_2O_2 in this genotype. It is possible that modulation of both catalase activity and H_2O_2 concentrations may be involved in the responses of IRGA 425 plants to the iron availability in the environment, leading to increased tolerance.

Increased levels of superoxide dismutase (SOD) activity have been reported to result in enhanced tolerance to oxidative stress in plants (Mittler 2002; Wang et al. 2019). SOD converts superoxide radicals to H_2O_2. SOD activity was shown to be increased by excess iron in BR-IRGA 409, the same sensitive cultivar used in our study (Stein et al. 2009a). On the other hand, SOD activity decreased under conditions of excess $FeSO_4$ in detached rice leaves (Fang et al. 2001). In this experiment, SOD activity was not affected by excess iron in both cultivars, indicating that the activity of this enzyme was not induced by oxidative stress generated by the higher amounts of iron in leaves at the time point evaluated (R2 stage) (Fig. 6D). Other mechanisms of free radical detoxification could be acting in plants from both genotypes, such as the ascorbate/glutathione cycle, the peroxiredoxin system and higher activities of glutathione peroxidases (Møller et al. 2007).

Interestingly, expression of all four genes evaluated in the BR-IRGA 409 plants was higher under the 2S treatment than under continuous irrigation. Expression levels of Ferritin, OsGAP1, OsWRKY80 and *Oryzain a* under continuous irrigation in this iron-sensitive cultivar were also significantly lower than in the

tolerant cultivar (Fig. 7). At the time point evaluated (R2 stage), the BR-IRGA 409 plants cultivated under continuous irrigation had been submitted to stress for a long period, showing visual symptoms of toxicity (leaf chlorosis and bronzing of lower leaves) and high levels of lipid peroxidation (Pinto *et al*. 2016). It is possible that the capacity to regulate gene expression in response to iron excess was compromised in BR-IRGA 409 under the CI treatment. In the 2S treatment, where Fe concentrations in the soil solution were lower, these plants were able to induce higher gene expression than in the CI treatment.

High accumulation of ferritin protein under iron excess was previously shown in the iron resistant rice cultivar EPAGRI 108, suggesting a possible role of this protein in a mechanism of tolerance to iron excess (Silveira et al. 2009). The IRGA 425 cultivar, used in this study, was originated from the lineage IRGA 2911-24-3-I-1Pg, which resulted from genealogical selection performed in progeny derived from a cross between the parents IRGA 1598-3 -2F-1-4-1 and EPAGRI 108 (Lopes et al. 2009). In our study, large accumulation of Ferritin transcripts was observed in the resistant cultivar (IRGA 425) under continuous irrigation (Fig. 7A), suggesting that this protein has an important role in the mechanism of tolerance to excess iron in this cultivar. Also under continuous irrigation, IRGA 425 plants accumulated less iron in leaves than BR-IRGA 409 plants at the differentiation of floral primordium stage (Fig. 1E). Both the limitation of iron translocation and increased ferritin accumulation can be suggested as mechanisms of resistance to excess iron in the IRGA 425 cultivar.

The C2 domain protein *OsGAP1* was already characterized as related to stress in rice (Fu et al. 2007; Cheung et al. 2008), being up-regulated by Fe-excess treatment (Ricachenevsky et al. 2010). *OsGAP1* is also up-regulated by wounding and interacts with the G-protein OsYchF1, suggesting a role in signal transduction (Cheung et al. 2008). In barley, a C2 domain-containing protein has been described as responsive to heavy metal stress and is associated with leaf senescence (Ouelhadj et al. 2006). In this work, transcript levels were higher in IRGA 425 plants than in BR-IRGA 409 under the Fe-excess treatment (CI) (Fig. 7B), suggesting a possible role in tolerance to iron excess.

OsWRKY80 is a transcription factor of the WRKY superfamily, which is related to plant defense and responses to abiotic stresses. In a previous work from our group, expression of *OsWRKY80* was up-regulated in rice leaves, stems and roots after Fe-excess treatment (Ricachenevsky et al. 2010). In this work, transcript levels of this gene in BR-IRGA 409 plants were higher in the 2S treatment than in CI and 1S, reaching transcripts levels equivalent to the ones seen in the resistant cultivar, IRGA 425 (Fig. 7C). Higher expression of *OsWRKY80* in the tolerant than in the sensitive cultivar in the two conditions with highest concentrations of soluble iron in the soil solution may be related to a possible role in the tolerance mechanism.

Oryzain-α is a cysteine proteinase that can be required in several physiological and developmental process, mainly by modulating degradation of specific proteins under specified conditions such as drought, salinity, dehydration, low temperature, sucrose starvation and natural senescence in different organs and tissues, as well as in the incompatible interaction between plant and pathogen (Fu et al. 2007). Cysteine proteinases participate in various metabolic processes, replying to signaling molecules

such as methyl jasmonate, salycilic acid and ABA (Fu et al. 2007). Expression of *Oryzain α* was shown to be induced by iron excess in rice leaves (Ricachenevsky et al. 2010), and it is involved in senescence and seed germination (Fu et al. 2007; Ricachenevsky et al. 2010). Transcript levels of *Oryzain-α* were high when IRGA 425 plants were submitted to continuous irrigation (Fig. 7D), which could be associated with the stress condition induced by a high concentration of iron in the soil solution, and may be involved with tolerance to iron excess.

It is important to consider that our gene expression analyses correspond to only one time point during the plant's life. Different results might have been found in other developmental stages.

5 Conclusions

While the resistant genotype thrives well under permanent flooded condition and continued (chronic) high Fe^{+2} concentration in the soil solution, two cycles of intermittent soil drainage can temporarily alleviate iron toxicity, thereby reducing the oxidative stress to the sensitive genotype, which responds with a 87% higher grain yield. Thus, in the absence of available resistant germplasm, field drainage appears a feasible alternative for rice production in iron toxicity-affected areas. Limited Fe translocation to shoots, increased tolerance to oxidative stress in leaves and higher expression of *Ferritin*, *OsGAP1*, *OsWRKY80* and *Oryzain-α* genes seem to be important characteristics of IRGA 425 plants, possibly participating in a complex mechanism of resistance to Fe excess. Suppression of irrigation clearly resulted in lower levels of oxidative stress in the sensitive cultivar, which accumulated much lower Fe concentrations in leaves under this treatment than under continuous irrigation.

Declarations

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Figures

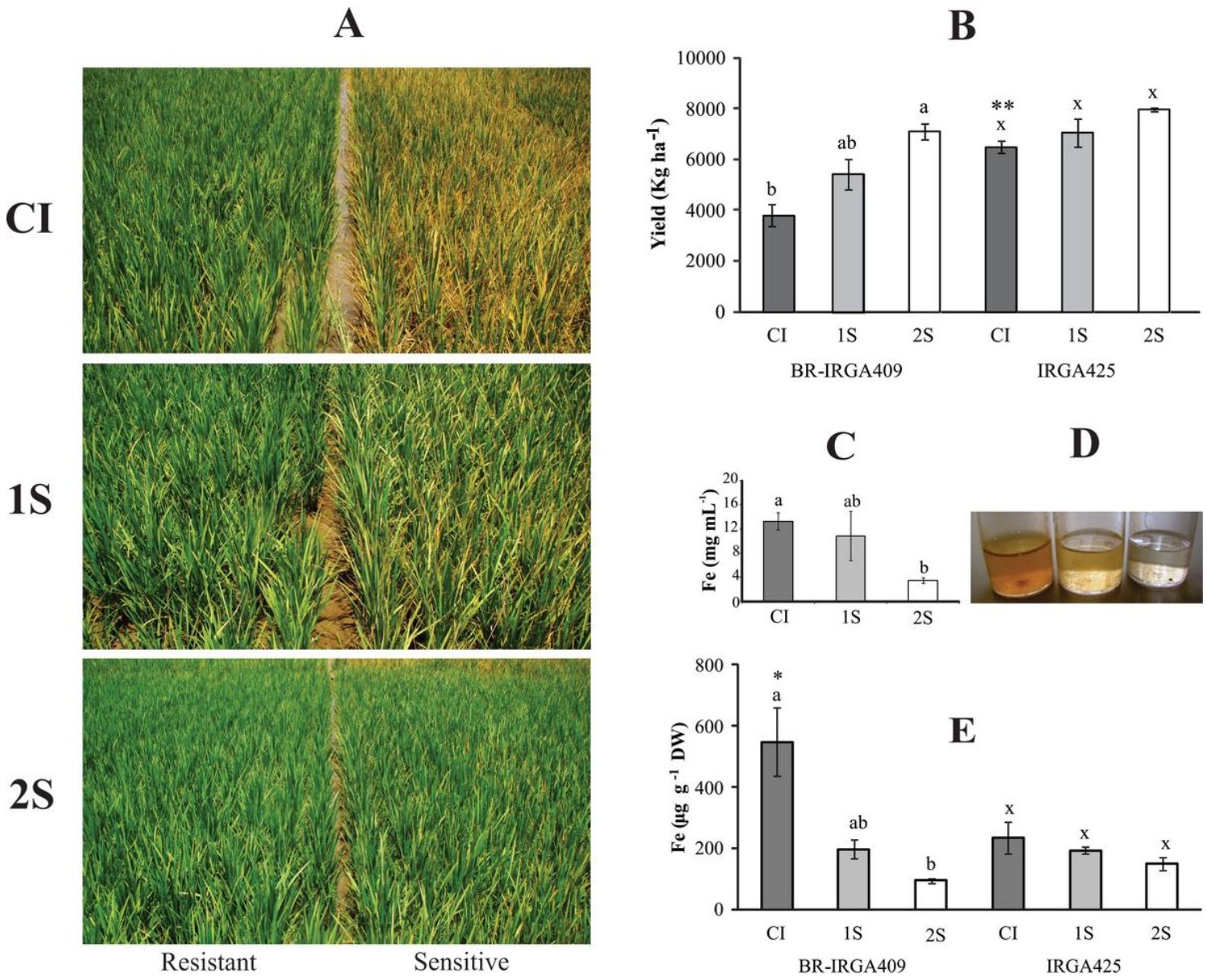

Figure 1

Impact of alternative irrigation management in two rice cultivars, IRGA 425 (resistant to Fe toxicity) and BR-IRGA 409 (sensitive to Fe toxicity). Plants were submitted to continuous irrigation (CI), one suppression of irrigation between stages V6-V8 (1S) and two cycles of irrigation suppression, between stages V6-V8 and V8-V10 (2S). A. Visual aspect of cultivars IRGA 425 (left) and BR-IRGA 409 (right) in the field at the floral primordium differentiation stage (R1). B. Grain yield, determined at the end of the growing cycle, when grains reached about 20% humidity. The values represent the means of six replicates

and the bars indicate the standard errors. C. Iron concentrations in the soil solution. Samples (n=3) were collected when plants were at the of floral primordium differentiation stage (R1). Different letters indicate significant differences by the Duncan's Test ($P < 0.05$). D. Image of representative samples of soil solution from each treatment. E. Leaf iron concentrations in rice plants at the floral primordium differentiation stage (R1). Samples (three replicates, each composed of 3 or 4 leaves) correspond to the third fully expanded leaf from the main stem of individual plants. In B and E, bars with different letters are significantly different by the Duncan's Test ($P < 0.05$) within each cultivar. Significant differences among cultivars within each irrigation treatment were tested by the Student's T Test (LSD) ($P < 0.05$) and represented by asterisks (*).

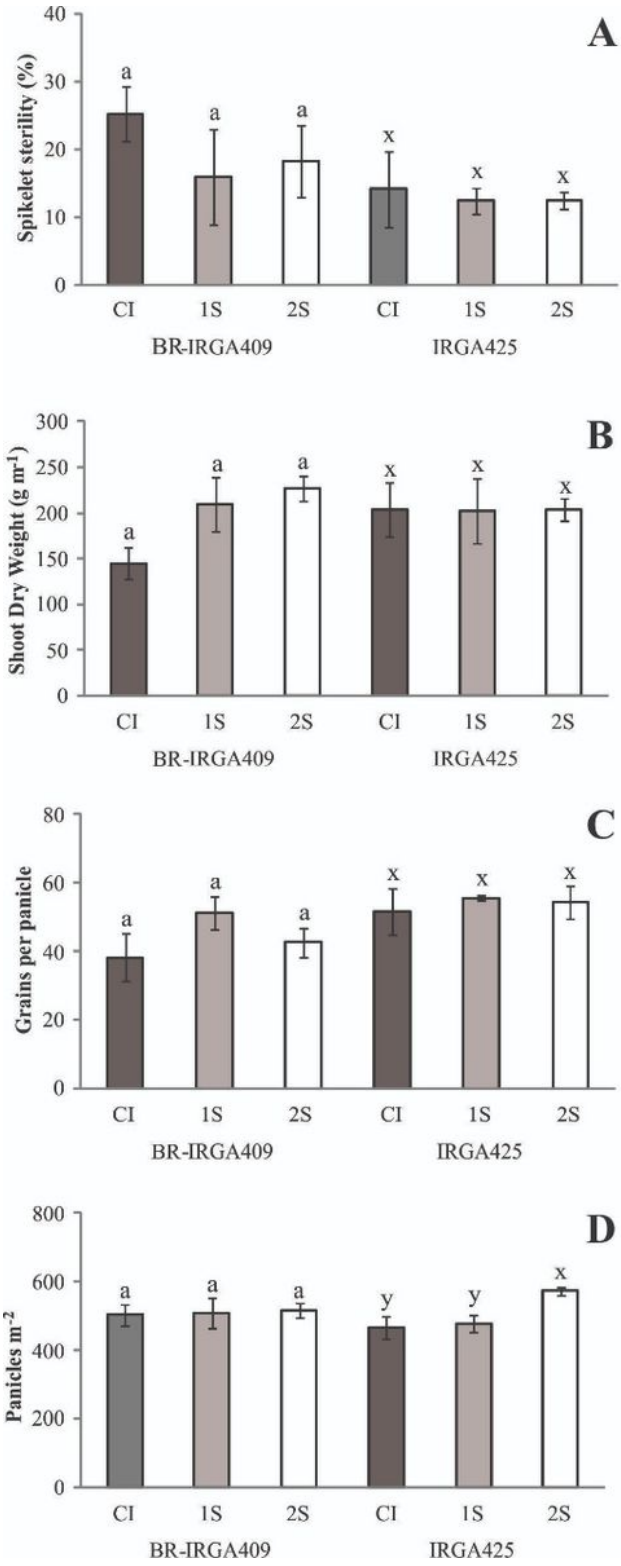

Figure 2

Yield parameters in two rice cultivars, BR-IRGA 409 (sensitive to Fe toxicity) and IRGA 425 (resistant to Fe toxicity). Plants were grown under continuous irrigation (CI); one cycle of irrigation suppression between stages V6-V8 (1S); and two cycles of irrigation suppression between stages V6-V8 and V8-V10 (2S). Spikelet sterility (A) was determined at the end of the growing cycle, when grains reached about 20% humidity. Shoot dry weight (B) was determined at the floral primordium differentiation stage (R1), by

harvesting all tissues above soil level in a linear row of 1 meter, with 10 replicates per treatment, and drying at 60 °C until constant weight. Numbers of grain per panicle (C) and of panicles per square meter (D) were determined at the end of the growing cycle, when grains reached about 20% humidity, with six replicates per treatment. Values represent the means and the bars indicate the standard errors. Different letters indicate means significantly different by the Duncan's Test ($P < 0.05$) within each cultivar. Significant differences among cultivars within each irrigation treatment were tested by the Student's T Test ($P < 0.05$) and represented by asterisk (*).

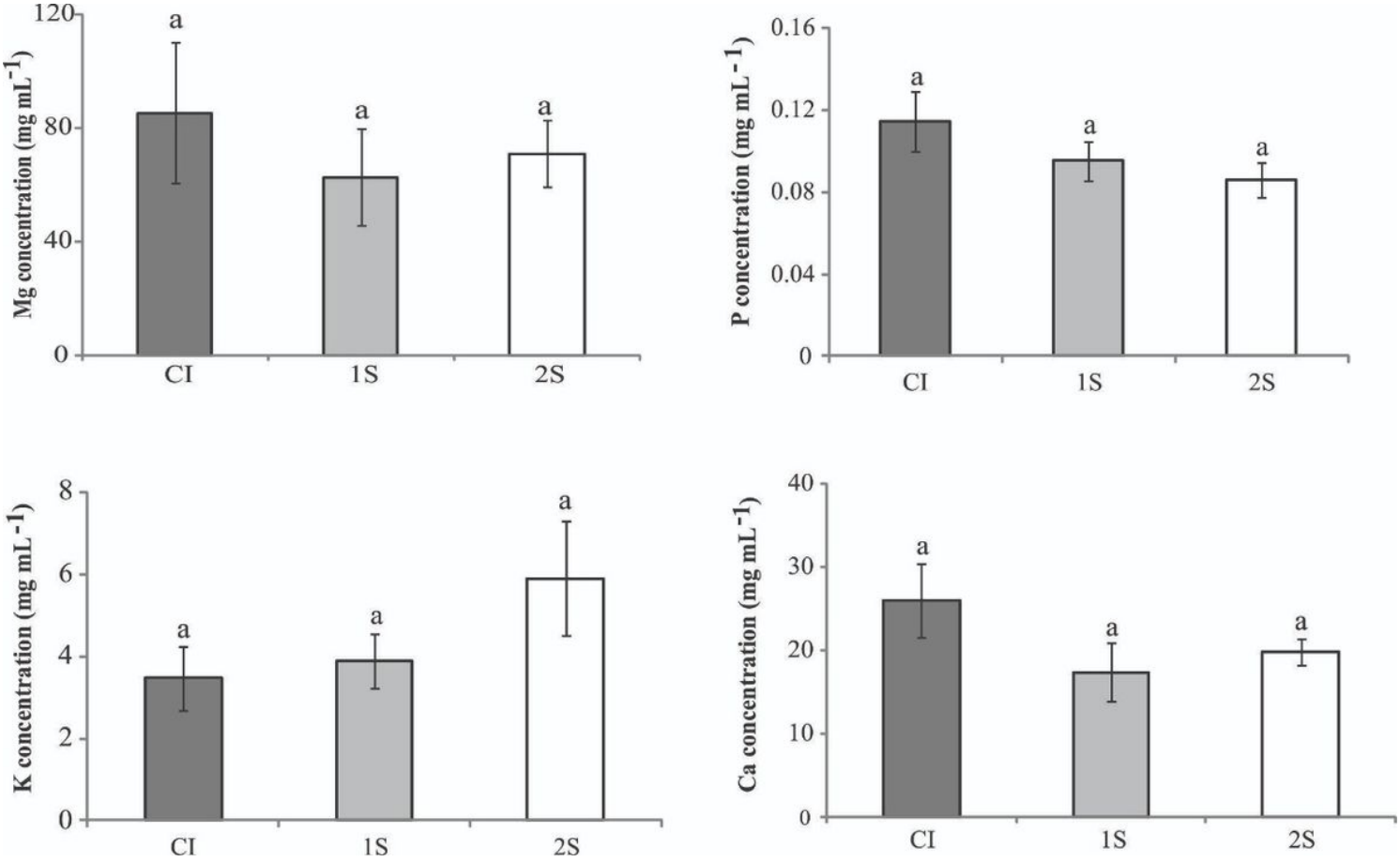

Figure 3

Concentrations of nutrients (magnesium, phosphorus, potassium and calcium) in the soil solution. Samples were collected when plants were at the floral primordium differentiation stage (R1). Water management treatments were as follows: continuous irrigation (CI); one cycle of irrigation suppression between stages V6-V8 (1S); and two cycles of irrigation suppression between stages V6-V8 and V8-V10 (2S). The values represent the means of three replicates and the bars indicate the standard errors. Different letters indicate means significantly different by the Duncan's Test ($P < 0.05$).

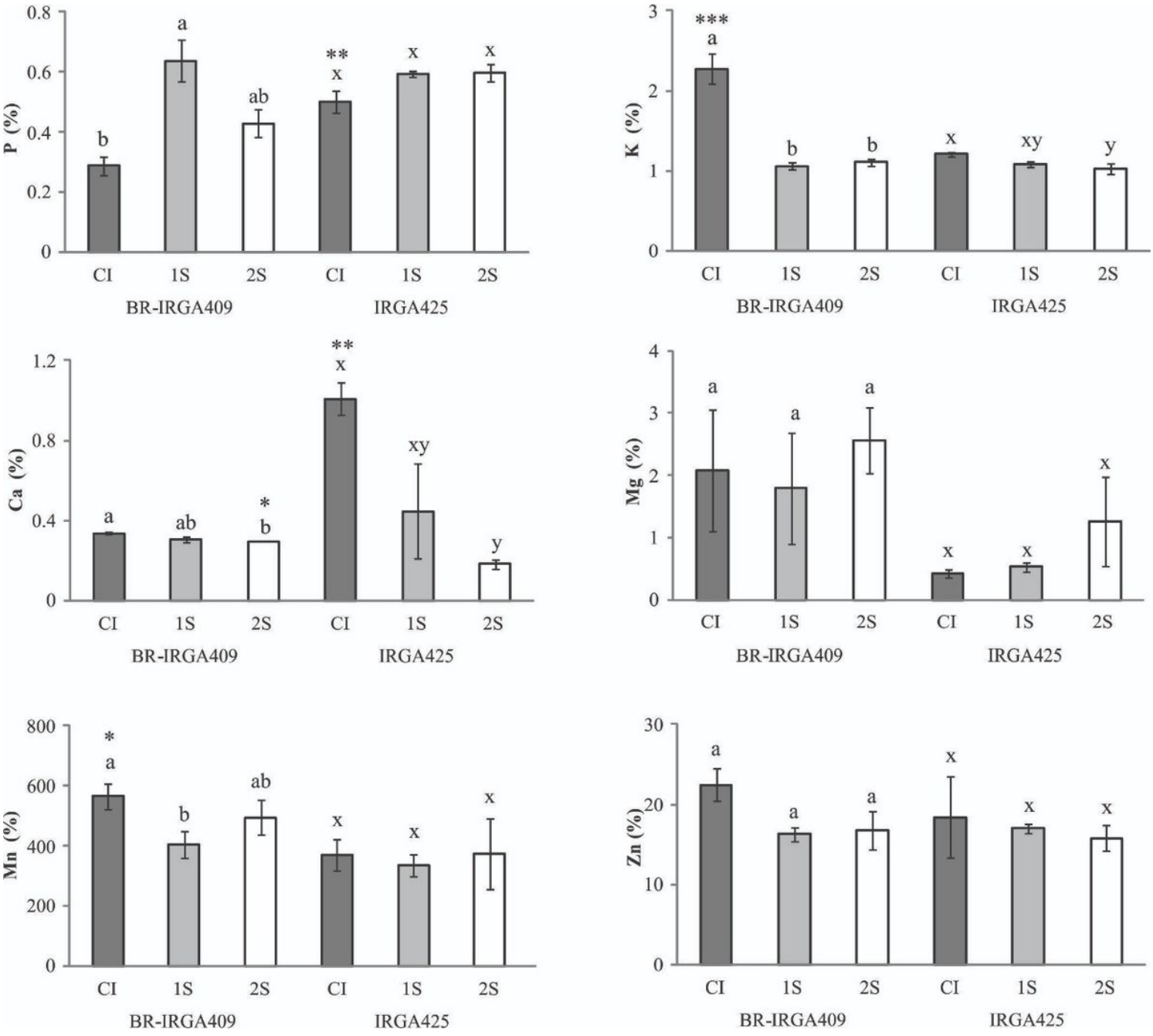

Figure 4

Nutrient concentrations (P, K, Ca, Mg, Mn and Zn) in leaves of rice plants from cultivars BR-IRGA 409 (sensitive to iron toxicity) and IRGA425 (resistant) at the floral primordium differentiation stage (R1). Samples (three replicates, each composed of 3 or 4 leaves) correspond to the third fully expanded leaf from the main stem of individual plants. Plants were grown under continuous irrigation (CI); one cycle of irrigation suppression between stages V6-V8 (1S); and two cycles of irrigation suppression between stages V6-V8 and V8-V10 (2S). The values represent the means of three replicates and the bars indicate the standard errors. Bars with different letters are significantly different by the Duncan's Test ($P < 0.05$) within each cultivar. Significant differences among cultivars within each irrigation treatment were tested by the Student's T Test ($P < 0.05$) and represented by asterisks (*).

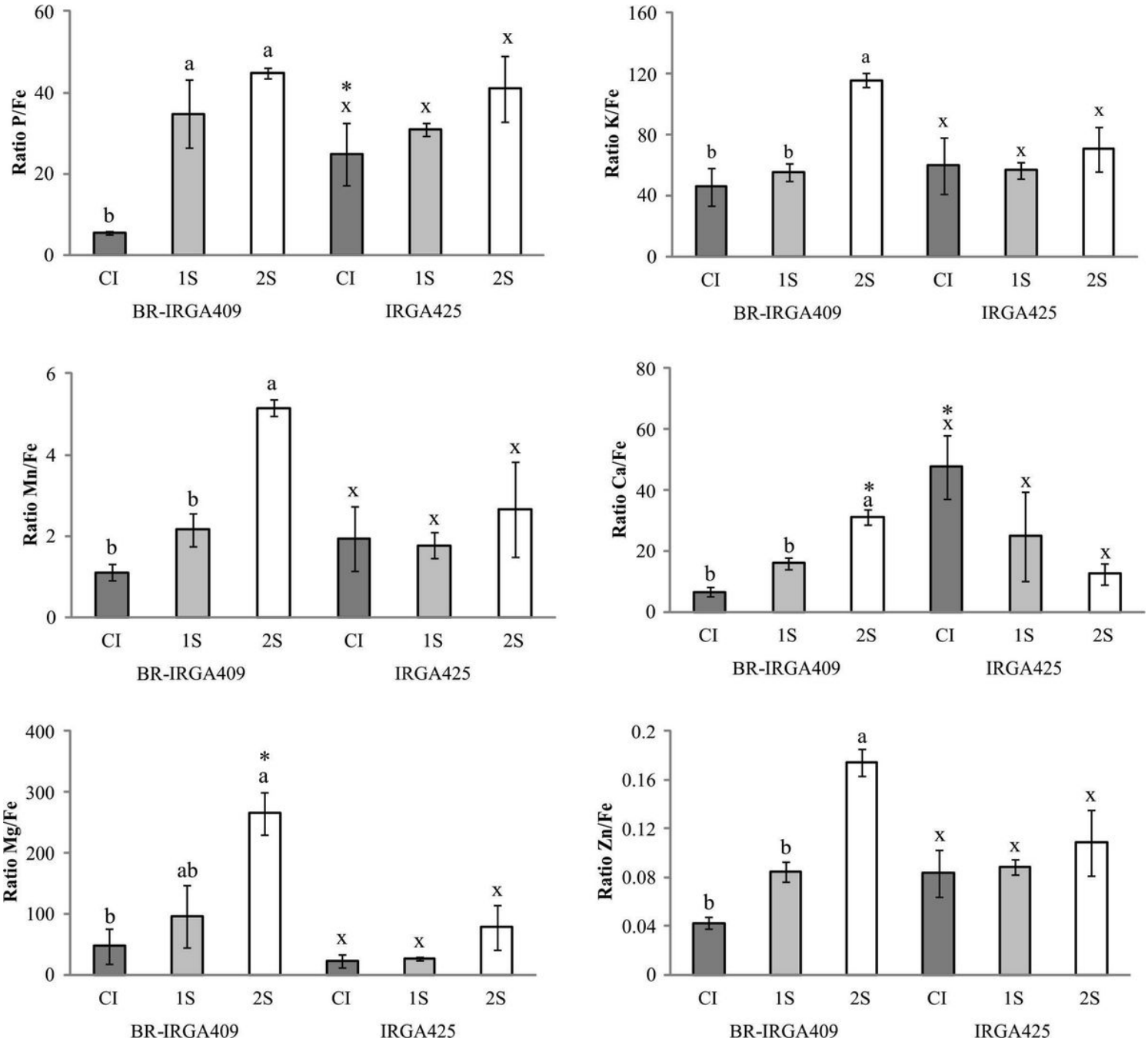

Figure 5

Nutrient concentration ratios (P/Fe, K/Fe, Mn/Fe, Ca/Fe, Mg/Fe and Zn/Fe) at the third totally expanded leaf in two rice cultivars, BR-IRGA 409 (sensitive to Fe toxicity) and IRGA 425 (resistant to Fe toxicity), at the floral primordium differentiation stage (R1). Plants were grown under continuous irrigation (CI); one cycle of irrigation suppression between stages V6-V8 (1S); and two cycles of irrigation suppression between stages V6-V8 and V8-V10 (2S). The values represent the means of three replicates and the bars indicate the standard errors. Bars with different letters are significantly different by the Duncan's Test ($P < 0.05$) within each cultivar. Significant differences among cultivars within each irrigation treatment were tested by Student's T Test ($P < 0.05$) and represented by asterisks (*).

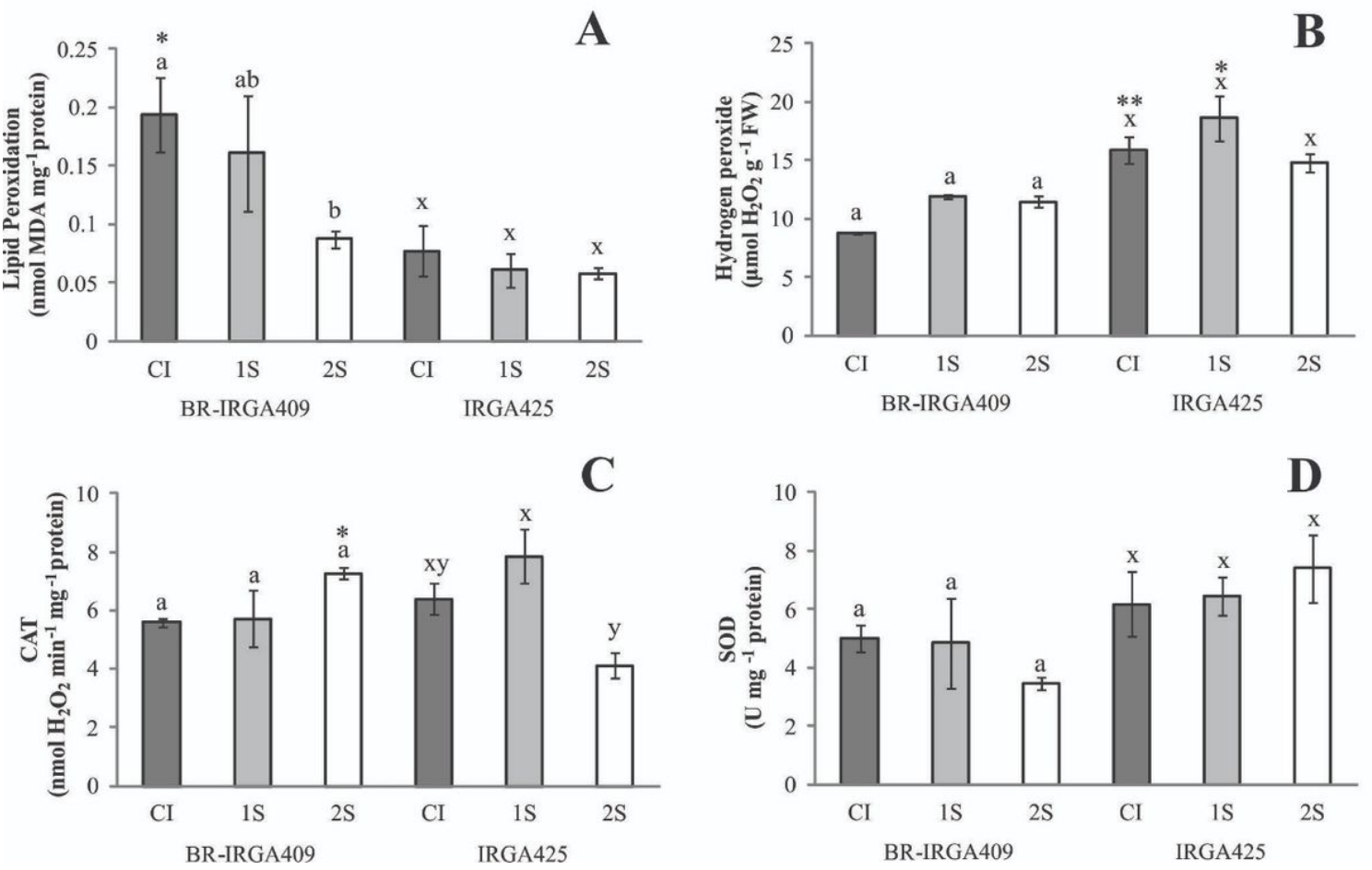

Figure 6

Oxidative stress at the third completely expanded leaf in rice plants from cultivars BR-IRGA 409 (sensitive to iron toxicity) and IRGA425 (resistant) at the booting stage (R2). Plants were grown under continuous irrigation (CI); one cycle of irrigation suppression between stages V6-V8 (1S); and two cycles of irrigation suppression between stages V6-V8 and V8-V10 (2S). Lipid peroxidation (A) was evaluated by quantifying malondialdehyde (MDA) production after reaction with thiobarbituric acid (TBA) and expressed as nmol MDA mg protein-1. Hydrogen peroxide (H2O2) concentration (B) was expressed as µmol g-1fresh weight. Enzyme activities of CAT (catalase, C) and SOD (superoxide dismutase, D) were expressed per mg of protein. The values represent the means of four replicates and the bars indicate the standard errors. Bars with different letters are significantly different by the Duncan's Test ($P < 0.05$) within each cultivar. Significant differences among cultivars within each irrigation treatment were tested by Student's T Test ($P < 0.05$) and represented by asterisk (*).

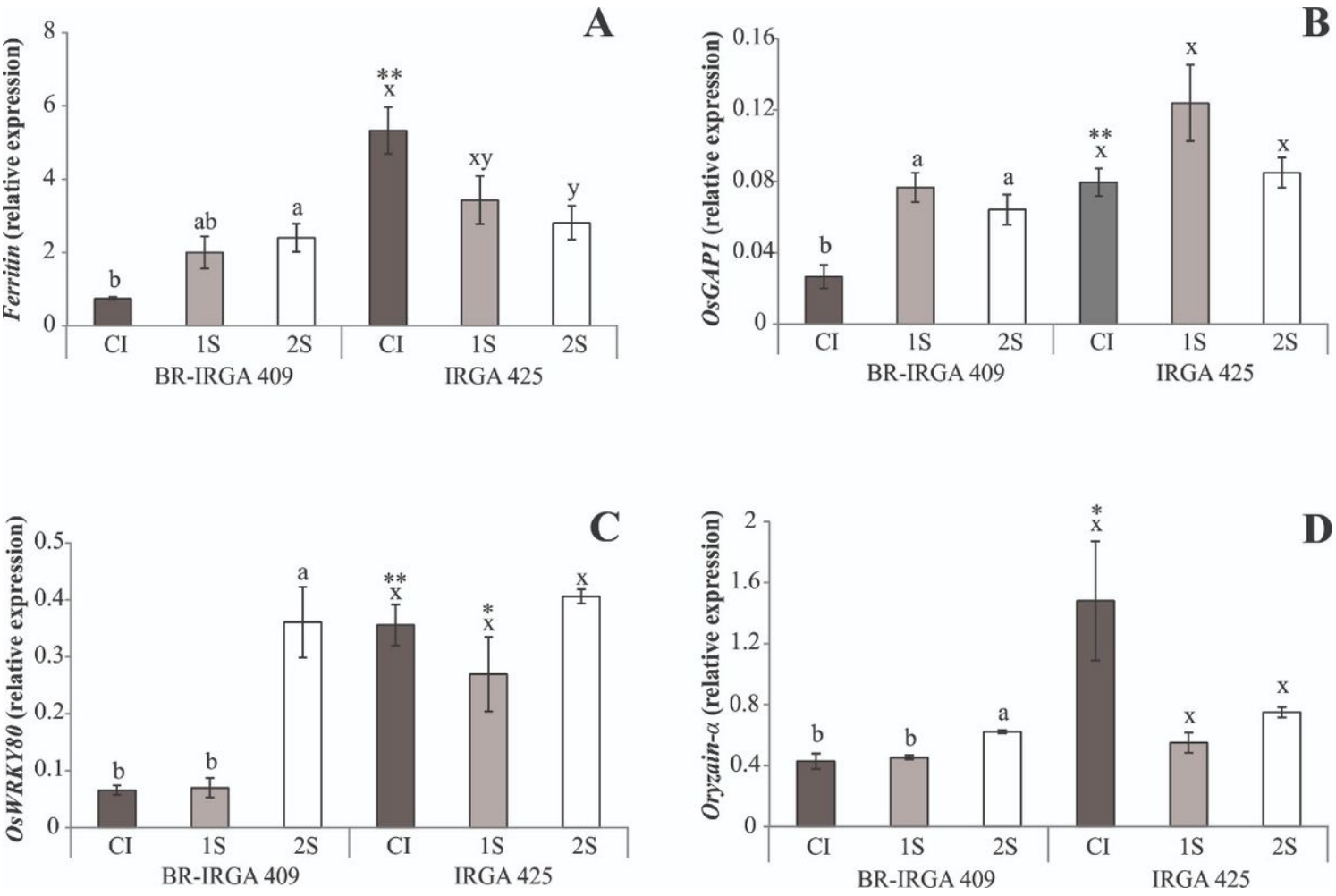

Figure 7

Relative gene expression of Ferritin, OsGAP1, OsWRKY80 and Oryzain-α at the third completely expanded leaf in rice plants from cultivars BR-IRGA 409 (sensitive to iron toxicity) and IRGA425 (resistant) at the booting stage (R2). Plants were grown under continuous irrigation (CI); one cycle of irrigation suppression between stages V6-V8 (1S); and two cycles of irrigation suppression between stages V6-V8 and V8-V10 (2S). The Ubiquitin 5 gene was used as reference gene for normalization of gene expression. The values represent means of three biological replications and four technical replications per biological sample. The bars indicate the standard errors. Different letters indicate means significantly different by the Duncan's Test ($P < 0.05$) within each cultivar. Significant differences among cultivars within each irrigation treatment were tested by the Student's T Test (LSD) ($P < 0.05$) and represented by asterisk (*).