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Analysis of performance and gene *OsFER1* expression of six rice varieties with $FeSO_4$ stress treatment

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Abstract. *Khairatunnisa F, Salamah A, Maryanto AE. 2023. Analysis of performance and gene OsFER1 expression of six rice varieties with $FeSO_4$ stress treatment. Biodiversitas 24: 1391-1399.* Efforts to meet rice needs in Indonesia can be made through an extensification program. New paddy fields generally have a high $FeSO_4$ content, so rice varieties resistant to these conditions are needed. This study aims to evaluate the growth of lowland rice varieties under $FeSO_4$ stress and to analyze the expression of a gene that plays a role in iron storage, namely the *OsFER1*. Six lowland rice varieties, namely Ciherang, Inpari 42, Inpari 30, Sunggal, Logawa, and Cibogo were grown on media with 0 and 400 ppm $FeSO_4$ applications. The plant height, leaf bronzing score, and the number of filled grains produced were observed to determine the effect of $FeSO_4$ administration on the agronomic performance of rice. The *OsFER1* gene expression ratio was analyzed using real-time PCR. The best plant height at 400 ppm $FeSO_4$ was found in the Inpari 30 variety (40.3 ± 0.49 cm), with a leaf bronzing score was 1. The Inpari 30 also produced grain at 400 ppm $FeSO_4$. Molecular analysis showed that the highest *OsFER1* expression was found in the Inpari 30 grown at 400 ppm $FeSO_4$. Based on morphological and molecular observations, Inpari 30 is tolerant to $FeSO_4$ stress.

Keywords: Agronomic performance, $FeSO_4$ stress, Inpari 30, *OsFER1* gene expression, rice

INTRODUCTION

Rice (*Oryza sativa* L.) 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 (GRiSP 2013). As the food needs of the Indonesian population, rice in Indonesia has never subsided in production and consumption is increasing from year to year in accordance with the increase in population (Nizar and Abbas 2019). The total rice production in Indonesia is 32.07 million tons which have not been able to cover the needs of the Indonesian people, so Indonesia still imports 326.450 tons of rice (BPS 2022). To meet the food needs of a population whose numbers continue to increase, efforts to increase rice production are a top priority in agricultural development in Indonesia (Suwarno 2016). An effort to meet the rice demand in Indonesia is needed, such as extensification, one of which is by utilizing new paddy fields. Newly opened paddy fields have the characteristics of high levels of $FeSO_4$, which can inhibit rice growth. So rice varieties that are resistant to these conditions are needed (Ihsan 2016).

Iron (Fe) toxicity is one of the major mineral disorders affecting rice production (Rakotoson et al. 2019). High levels of Fe cause oxidative stress that can cause damage to several biological macromolecules (Galaris et al. 2019). Homeostasis of Fe must be tightly regulated in plants. Too much Fe causes cell death and is toxic to cells. It is therefore, mandatory for plants to overcome the often-restricted availability of soil iron through strategies that increase the mobility of Fe (Schmidt et al. 2020).

Rice genotypes varied in their resistance to excess Fe. Some rice cultivars rely on evasion mechanisms, limiting Fe uptake and especially Fe translocation to shoots, while others are quite tolerant of high leaf Fe concentrations. Therefore, plants have evolved mechanisms to maintain Fe homeostasis when Fe concentration is high (Sperotto et al. 2012; Ricachenevsky et al. 2018). One of the proteins that can maintain homeostasis in this condition is ferritin protein, an iron storage protein that acts as a buffer for iron in cells by storing this ion in a non-toxic and bioavailable form. Furthermore, regulation of ferritin gene expression in response to iron excess occurs at the transcriptional level in plants (Kobayashi et al. 2019).

Ferritin contents in rice grains are different for each variety because genes responsible for ferritin protein synthesis have a very large family (Nugraha et al. 2016; Pandey et al. 2018). Ferritin expression is different in each rice genotype, and the resistance to iron toxicity is related to Fe translocation and depends on the Fe contained (Carmona et al. 2021). The differences in the ability of rice genotypes to express ferritin protein at the molecular level can be detected by looking at the quantitative frequency of ferritin gene expression by analysis using qPCR. Previous research on ferritin genes has only analyzed ferritin gene sequences of tidal wetlands rice varieties, such as Bakung,

Siputih, and Serei (Roslim et al. 2013). However, no research has been found linking the lowland rice genotype performance for ferritin gene expression on $FeSO_4$ resistance. Hence, the present research aims to evaluate six lowland rice growth performances (Ciherang, Inpari 42, Inpari 30, Sunggal, Logawa, and Cibogo) under $FeSO_4$ stress and to analyze their *OsFER1* gene expression.

MATERIALS AND METHODS

Time and location of research

The research was conducted from August 2021 until June 2022 in the Greenhouse, Molecular Biology Preparation Laboratory, and Integrated Instrumentation Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok, West Java, Indonesia.

Plant materials

The materials used in this study were seeds from 6 rice varieties, namely Ciherang, Inpari 42, Inpari 30, Sunggal, Logawa, and Cibogo, which were obtained from the Seed Source Service Unit (UPBS) of the Agricultural Research and Development Agency (BPTP), East Java, Indonesia.

Research design

The research design used was a completely randomized design (CRD) consisting of 2 $FeSO_4$ treatments, i.e. 0 and 400 ppm on six rice varieties (12 treatment combinations). Each treatment consisted of 3 replications, and each replication consisted of 2 rice plants, with a total of 72 plants used.

Procedures

$FeSO_4$-stress treatment and growth performance observation

Rice seeds were sown in containers containing Lembang soil media. The rice seedling was transferred to the prepared bucket container after 21 days, then treated with $FeSO_4$. There were 2 rice plants in each bucket for each treatment. Rice plants were maintained in a greenhouse by controlling the inundation volume to keep it constant at 1 cm from the surface of the growing media by adding aquadest. Routine control was carried out to keep plants pest free. Inorganic fertilizers of NPK 16:16:16 were given 21 and 42 days after planting (DAP). The growing media's light intensity, humidity, and pH were measured every weekend during the growing period. There were 3 agronomic characteristics observed, namely: (i) the plant height per week was measured since the rice was two weeks old. The final plant height was evaluated at week 10 after planting by measuring the plant height from the planting boundary of the soil to the tip, (ii) the leaf bronzing symptoms were scored according to Shimizu et al. (2005) (Table 1), and (iii) the number of grains per panicle started to be counted at 143 DAP.

Total RNA Isolation, cDNA synthesis, and real-time PCR

Total RNA isolation and cDNA synthesis from rice leaf samples were done in the Molecular Biology Preparation Laboratory and Integrated Instrumentation Laboratory at the Department of Biology Universitas Indonesia, Depok. Rice leaves of 10 weeks of plant (30-60 mg) from each treatment were isolated using the Plant Total RNA Extraction Miniprep System Kit (Viogene) following the manufacturer's manual kit. The purity of total RNA and its concentration were measured with a NanoPhotometer® and spectrophotometer (Implen). The integrity of total RNA was checked with 1% agarose gel electrophoresis stained with GelRed (1 µL/20 mL agarose). The agarose gel was then visualized under trans-UV light using a UV light box (Analytikjena).

The cDNA synthesis was carried out according to the Revertra-Ace qPCR-RT Master Mix protocol with gDNA Remover [Toyobo] kit by adding 2 µL RNA template, NFW 6 µL, and 5X RT Master Mix II until a total volume of 10 µL. The cDNA of 0.5 µL was then used in real-time PCR reaction by adding KAPA SYBR FAST qPCR Master Mix, 2X of 10 µL, forward primer (10 µM) of 0.4 µL, reverse primer (10 µM) of 0.4 µL, and NFW until a total mixing volume of 20 µL. Real-time PCR amplification with *OsFER* primers forward 5'TCACTCTTCACCCGCCGCG'3 and reverse 5' TCGACGAACTTTTGCCTAGC '3 (Stein et al. 2009) was performed using MyGo mini real-time PCR machines (IT-IS) by applying enzyme activation for 3 minutes at 95°C and 40-45 cycles of denaturation, annealing, and extension (data acquisition) for 3 seconds at 95°C, 20 seconds at 53°C, and 20 seconds at 72°C, respectively. The appropriate number of PCR cycles was determined for each gene to ensure that amplification occurred in the linear range. Amplification of *α-TUB* cDNA was used to normalize the data (Yang et al. 2015).

Table 1. Classification of 7 levels of leaf bronzing scores

Score	Symptoms	Percentage of poisoned leaves (%)	Stress level
1	No symptoms	0	None
2	Growth and formation of tillers are normal, at the tips and old leaves there are reddish or orange spots	1-9	Very light
3	Growth and tiller formation is almost normal, old leaves are reddish brown, purple, or yellow-orange	10-29	Light
4	Slightly stunted growth and tillering, some leaves are brown or reddish or yellow-orange	20-49	Medium
5	Growth and tillering is stunted or stopped and many leaves (almost all leaves) are reddish brown or yellow-orange	50-69	Slightly heavy
6	Almost all plants (leaves) dry up and die	70-89	Heavy
7	All plants (leaves) dry and die	90-100	Very heavy

Data analysis

Both morphological (plant height and delta growth) data and molecular (the relative gene expression) levels were analyzed using two-way ANOVA with Sidak multiple comparison test at □=0.05 confidence level to determine the difference in the treatment of 0 ppm $FeSO_4$ and 400 ppm $FeSO_4$ or between the two $FeSO_4$ treatments. Statistical analysis was done using PAD-PRISM ver 8. The average value of amplification efficiencies (E) of all real-time PCR reactions was used to determine the E for each target and reference gene. Q-Rex Software 1.1.0 (QIAGEN) calculated the amplification efficiency of each reaction based on the 4 data points following the take-off point. The take-off point is the cycle where the run transitions into the exponential phase (Qiagen 2018). Calculating quantification cycle (Cq) values start from the first cycle. Cq threshold fluorescence at 0.1 was determined to calculate Cq values. The cDNA quantity of each gene was normalized to *α-TUB* (reference gene). Levels of relative gene expression (R) were analyzed using the Pfaffl method. The ratio of changes in target gene expression relative (R) to the reference/housekeeping gene in the method was calculated based on the following formula (Pfaffl 2001) as follows:

$$R = \frac{(E_{target})^{\Delta C_{target}}}{(E_{reference})^{\Delta C_{reference}}}$$

$$\Delta C_{q\ target} = C_{q\ control,\ target} - C_{q\ treatment,\ target}$$

$$\Delta C_{q\ reference} = C_{q\ control,\ reference} - C_{q\ treatment,\ reference}$$

Where R is the value of the relative gene expression ratio, E_{target} shows the amplification efficiency value of the target gene, and $E_{reference}$ shows the value of the efficiency of reference gene amplification.

RESULTS AND DISCUSSION

Effect of $FeSO_4$ on plant growth and height

Referring to the weekly rice growth, all 5 varieties at 0 ppm $FeSO_4$ treatment showed an increase in growth based on plant height measurements up to week 6, except for Ciherang up to week 7 (Figure 1). In the following week, plant growth in all rice varieties began to decline, probably due to the lack of nutrients in the growing media. Rice treated with 400 ppm $FeSO_4$ showed normal growth like control plants until the third week, then growth decreased in the fourth week due to the administration of 400 ppm $FeSO_4$ to the growing media carried out in the third week. This decrease in growth indicates that $FeSO_4$ has begun to be transported into plant tissues and causes toxic effects. The application of $FeSO_4$ made plant growth in each rice variety slower. There was a significant difference in the growth height of control plants compared to treatment plants in 5 varieties except in Logawa, where there was no significant difference in delta growth of plant height between 0 and 400 ppm $FeSO_4$ (Table 2 and Figure 1e).

The final plant height was evaluated at week 10 after planting by measuring the plant height from the planting boundary of the soil to the tip. The data obtained were calculated on average in each treatment and seen in the standard deviation listed in Table 2, and the data is represented in Figure 1. The results of plant height analysis showed that the Ciherang variety was the best-growing variety under control conditions (68.11 cm), while the 400 ppm $FeSO_4$ treatment was Inpari 30 (40.3 cm). The multiple comparison tests also confirmed that the delta plant height of 0-400 ppm $FeSO_4$ of Logawa was insignificant between control and $FeSO_4$ treated plants, indicating that the application of $FeSO_4$ did not affect the growth of the Logawa variety.

Effect of $FeSO_4$ on plant leaf bronzing score

The results of leaf bronzing scores of the 6 rice varieties used are presented in Table 3, which showed in control plants, the Ciherang variety did not show symptoms of stress with a score of 1, while the other 5 varieties showed very mild stress with a score of 2. In plants treated with 400 ppm $FeSO_4$, the Inpari 30 variety did not show any stress with a score of 1, Ciherang, Inpari 42, and Sunggal varieties showed very mild stress with a score of 2. Varieties with a score of 5 were Cibogo which showed almost all leaves are yellow-orange. The highest leaf bronzing was observed in Logawa, with a score of 6 where all leaves dried up and died. Cibogo and Logawa, respectively, indicated the presence of moderate and severe stresses of Fe in the soil.

Effect of $FeSO_4$ on the number of grains per panicle

The effect of $FeSO_4$ application during the generative phase is shown in Table 4 and plants that produce grain are shown in Figure 2. Based on our observations, the Ciherang variety begins to flower and produces grain on day 143 after planting at 0 ppm $FeSO_4$, while the Inpari 30 variety, on the 155th day. Inpari 30 variety flowered and produced grains on the 171st day after planting at 400 ppm $FeSO_4$ treated plants.

The plants still produce rice grains even though 70 DAP receive no more water. The Ciherang variety produced grain at 0 ppm $FeSO_4$, but could not produce grain at 400 $FeSO_4$, making the variety sensitive. The Inpari 30 variety produced grains at conditions of 0 ppm FeSO4 and conditions of 400 ppm $FeSO_4$, the so-called tolerant variety. The Inpari 42, Sunggal, Logawa, and Cibogo varieties had not yet produced grains at the final observation, so the varieties were called sensitive to $FeSO_4$ stress. In the generative phase, the Ciherang variety produced 72 rice grains consisting of 44 filled grains and 28 empty grains in the control treatment. In the treatment of 400 ppm $FeSO_4$, Inpari 30 variety produced 80 grains consisting of 32 filled and 55 empty grains.

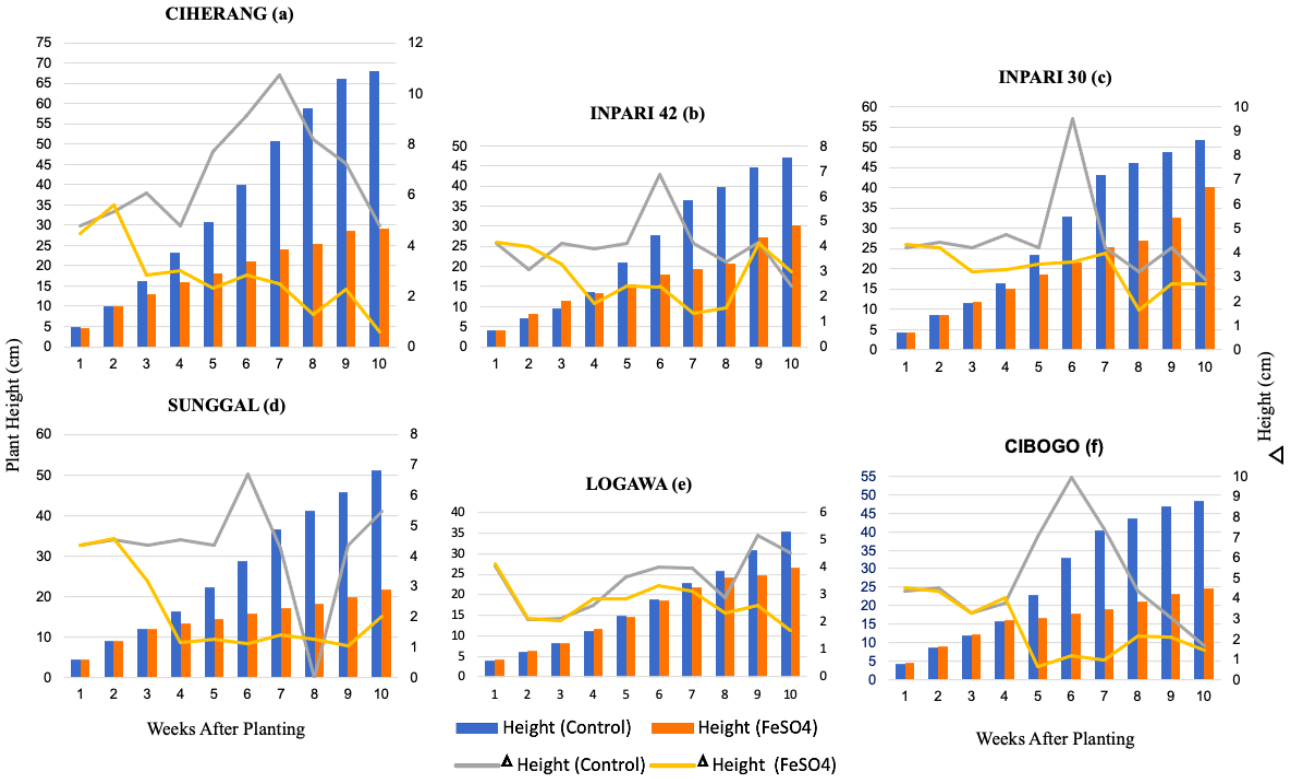

Figure 1. Plant growth rate per week and delta growth rate per week of six rice varieties

Table 2. Effect of $FeSO_4$ on plant height at 10 weeks after planting

Varieties	Plant height (cm)		delta plant height	Significance level
	0 ppm	400 ppm	0 - 400 ppm (%)	
Ciherang	$68.11^{a} \pm 0.68$	$29.16^{b} \pm 0.20$	57.19	****
Inpari 42	$47^{c} \pm 0.69$	$30.2^{b} \pm 0.23$	35.74	**
Inpari 30	$51.71^{b} \pm 1.83$	$40.3^{a} \pm 18.24$	22.07	**
Sunggal	$51.23^{b} \pm 4.04$	$21.8^{e} \pm 0.42$	57.45	****
Logawa	$35.56^{d} \pm 0.1$	$26.6^{c} \pm 0.1$	25.20	ns
Cibogo	$48.4^{c} \pm 2.76$	$24.4^{d} \pm 1.26$	49.59	****

Note: Data is the average of 6 replications. Numbers of * showed the significant difference between treatments using two-way ANOVA followed by the Sidak multiple comparison test (α=0.05), ns = not significant is used to distinguish the height of the same variety between 0 ppm and 400 ppm. Numbers followed by letters compare plant heights between varieties in each treatment

Table 3. Effect of $FeSO_4$ on the bronzing score in rice varieties

Varieties	Score leaf bronzing	
	$FeSO_4$ 0 ppm	$FeSO_4$ 400 ppm
Ciherang	1	2
Inpari 42	2	2
Inpari 30	2	1
Sunggal	2	2
Logawa	2	6
Cibogo	2	5

Table 4. Effect of $FeSO_4$ on the amount of grain produced

Varieties	Number of grains produced			
	Filled grains		Empty grains	
	0 ppm $FeSO_4$	400 ppm $FeSO_4$	0 ppm $FeSO_4$	400 ppm $FeSO_4$
Ciherang	44	0	28	0
Inpari 42	0	0	0	0
Inpari 30	22	35	19	55
Sunggal	0	0	0	0
Logawa	0	0	0	0
Cibogo	0	0	0	0

Figure 2. Grains formed during the generative period: A. Ciherang (0 ppm $FeSO_4$), B. Inpari 30 (0 ppm $FeSO_4$), C. Inpari 30 (400 ppm $FeSO_4$)

Molecular analysis of *OsFER1* gene

RNA Isolation and cDNA amplification

Molecular analysis was done to determine gene expression related to rice resistance to $FeSO_4$ stress to support agronomic character data observed in this study. The quantification of the total RNA of the rice leaves sample is shown in Table 5. The isolated RNA samples' concentration varied, ranging from 121-218 ng/µL to 121-218 ng/µL. The 260/280 nm ratio ranged from 2.061-2.217, while the 260/230 nm ratio was 1.860 -2.265, and commonly ratios of A260/280 and A260/230 as indicators of RNA purity are ~2.0 and between 1.8-2.2, respectively (Thermo Scientific 2010). The Quality and purity indices of all samples together were above 2 (2.138 ± 0.04472) for A280/260 and 2.070 ± 0.1358 for A260/230, which were greater than 1.8, are accepted as suitable indicators of RNA quality, which shows that the isolated RNA was not contaminated with protein (Kuang et al. 2018). Cibogo 400 ppm sample has a ratio of A260/230 above 2.2, but when it was amplified, it remained expressed as a single band (Figure 3).

The RNA from the six rice varieties was reverse-transcribed into cDNA. The cDNA was then amplified with *OsFER1* and *α-TUB* primers, and PCR product results were separated on agarose gel electrophoresis (Figure 3). The *OsFER1* and *α-TUB* genes in all varieties were successfully amplified in control and FeSO4 400 ppm treated plants. The size of *OsFER1* and *α-TUB*, approximately 309 bp and 215 bp, respectively, were detected in all samples. No differences between the control and treated $FeSO_4$ samples.

Table 5. Total RNA concentration and purity of six rice varieties

Rice variety	$FeSO_4$ treatment (ppm)	RNA concentrations (ng/µL)	Absorbance ratio	
			A_{260}/A_{280}	$A_{260}/_{230}$
Ciherang	0	186	2.217	1.980
	400	218	2.112	2.206
Inpari 30	0	121	2.061	1.968
	400	154	2.133	2.000
Inpari 42	0	123	2.154	1.890
	400	169	2.120	2.202
Sunggal	0	194	2.104	2.184
	400	128	2.113	2.163
Logawa	0	127	2.163	1.860
	400	177	2.202	2.000
Cibogo	0	129	2.105	2.121
	400	185	2.169	2.265

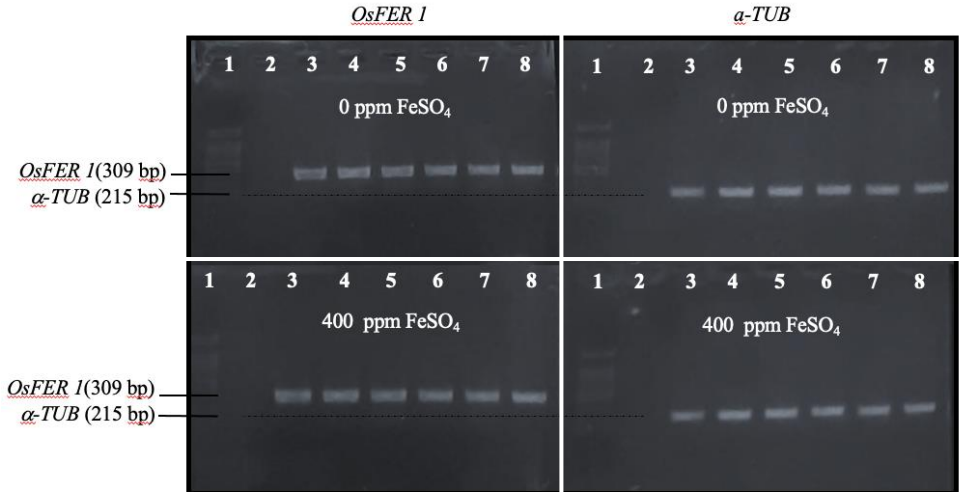

Figure 3. PCR amplification of *OsFER1 gene* (309 bp) and *α-TUB* (215 bp) of six rice varieties in control and $FeSO_4$ treated plants. Note: 1. DNA ladder 1 kbp; 2. Negative control; 3. Ciherang; 4. Inpari 42; 5. Inpari 30; 6. Sunggal; 7. Logawa; 8. Cibogo

Table 6. The mean Cq value of the *OsFER1* and *α-TUB* genes

Rice variety	Concentration of $FeSO_4$	Average Cq value of *OsFER1*	Average Cq value of *a-TUB*
Ciherang	0 ppm	$25.97^{a} \pm 0.21$	$25.80^{ab} \pm 0.22$
	400 ppm	$26.71^{b} \pm 0.25$	$27.1^{b} \pm 0.14$
Inpari 42	0 ppm	$31.38^{c} \pm 0.12$	$27.30^{c} \pm 0.13$
	400 ppm	$33.20^{c} \pm 0.44$	$26.29^{b} \pm 0.13$
Inpari 30	0 ppm	$28.65^{b} \pm 0.07$	$25.34^{ab} \pm 0.32$
	400 ppm	$25.29^{a} \pm 0.04$	$26.19^{b} \pm 0.54$
Sunggal	0 ppm	$34.78^{d} \pm 0.36$	$26.96^{c} \pm 0.11$
	400 ppm	$34.30^{d} \pm 0.13$	$26.42^{b} \pm 0.30$
Logawa	0 ppm	$30.70^{c} \pm 0.16$	$24.58^{a} \pm 0.05$
	400 ppm	$29.28^{b} \pm 0.43$	$26.71^{b} \pm 0.16$
Cibogo	0 ppm	$30.07^{c} \pm 0.48$	$23.64^{a} \pm 0.49$
	400 ppm	$32.56^{c} \pm 0.12$	$24.56^{a} \pm 0.13$

Note: Letters in a column indicate significant differences based on the LSD test ($\alpha < 0.05$) for each variety at the same concentration of $FeSO_4$

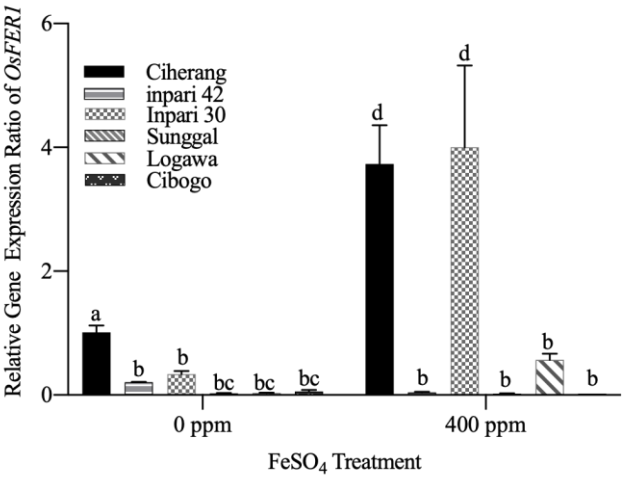

Figure 4. Relative gene expression ratio of *OsFER1* at a concentration of 0 ppm and 400 $FeSO_4$ in each variety. Note: Data is the average of 3 replications. Means and SEs were calculated from three independent samples. Letters ^{abcd} indicate significant differences between varieties treated with the same concentration of $FeSO_4$ using one-way ANOVA test with Tukey's multiple comparison tests (a=0.05), and/or between $FeSO_4$ treatments using a two-way ANOVA with the Sidak multiple comparison tests (a=0.05)

Quantitative RT-PCR analysis

Quantitative real-time PCR results are obtained in the form of quantification cycle (Cq) values presented in Table 6. The highest values of Cq *OsFER1* at concentrations of 0 ppm and 400 ppm $FeSO_4$ were obtained by the Sunggal (34.78 ± 0.36) and (34.30 ± 0.13), respectively. The lowest value of Cq *OsFER1* at a concentration of 0 ppm $FeSO_4$ was the Ciherang (25.97 ± 0.21), and at a concentration of 400 ppm, $FeSO_4$ was the Inpari 30 (25.29 ± 0.04). The highest Cq housekeeping gene *α-TUB* value at 0 ppm $FeSO_4$ was the Inpari 42 (27.30 ± 0.13), and at 400 ppm, $FeSO_4$ was the Ciherang (27.1 ± 0.14). In comparison, the lowest Cq value of the *α-TUB* gene (housekeeping) at 0 ppm $FeSO_4$ was the Cibogo (23.64 ± 0.49) which was not significantly different from the Logawa, Inpari 30, and Ciherang varieties, while at 400 $FeSO_4$ was the Logawa variety (24.56 ± 0.13).

The amplification efficiency value (E) calculation for all real-time PCR reactions was obtained by dilution 10 times with 5 repetitions for each PCR product in each gene. PCR efficiency measures the success rate of amplifying the target DNA molecule fraction in the PCR cycle. The PCR efficiency was calculated by plotting the correlation (standard curve) of the Cq value with the logarithmic concentration of the cDNA template. The E value of the *OsFER1* and *α-TUB* genes primer were 1.81 and 1.91, respectively.

OsFER1 gene expression

The ratio of target gene expression (*OsFER1*) to reference genes (*α-TUB*) was calculated using the Pfaffl formula (2001). The ratio values for each variety and replication were calculated, then analyzed using one-way ANOVA with the Tukey multiple comparison tests to determine the differences between the varieties tested at the same $FeSO_4$ concentration. The effect of $FeSO_4$ concentration was analyzed using a two-way ANOVA test followed by the Sidak multiple comparison test to see the significance (Figure 4). It was found that at a concentration of 0 ppm $FeSO_4$, the Ciherang variety significantly had the highest *OsFER1* gene expression value (1.01 ± 0.113), while at a concentration of 400 ppm $FeSO_4$, the variety Inpari 30 had significant *OsFER1* gene expression (4.00 ± 1.321). The lowest value of the *OsFER1* gene expression ratio at a concentration of 0 ppm $FeSO_4$ was observed in the Sunggal variety (0.03 ± 0.003) and the Logawa variety (0.03 ± 0.003). In comparison, at a concentration of 400 ppm $FeSO_4$, the Cibogo variety had the lowest value *OsFER1* gene expression (0.02 ± 0.0008). Based on the average *OsFER1* gene expression ratio of all varieties, rice plants that grew at 0 ppm $FeSO_4$ had lower values (0.28 ± 0.086) than plants grown at 400 ppm $FeSO_4$ (1.40 ± 0.473).

Discussion

The results of plant growth and height analysis showed that control plant growth was better than plants treated with 400 ppm $FeSO_4$. All varieties treated with 400 ppm $FeSO_4$ showed shorter plant heights than control plants (0 ppm $FeSO_4$). Research on the resistance of rice varieties under iron stress conditions has been carried out by Noor et al. (2012) at a concentration of 200-600 ppm $FeSO_4$. In general, the higher concentration of FeSO4, the more inhibited plant growth. The concentration of $FeSO_4$ > 200 ppm significantly decreased plant height compared to plants at concentrations < 100 ppm. The results supported Mehraban et al. (2008) that high levels of Fe in plants were negatively correlated with plant growth. The inhibition of growth and development of rice plants due to iron stress, which is caused by disruption of cell division at growing points covered in iron rust, reduces root spacing, resulting in the ability of roots to absorb and translocate nutrients to plant parts. Absorption of excess Fe by the rice plant has

also been reported to reduce root and shoot growth (Wu et al. 2014). These effects interfere with metabolic activity and, ultimately, plant growth shorter, which then plants will grow stunted (Bidi et al. 2021).

The highest leaf bronzing score, with a score of 6 (almost all leaves were reddish) was observed in Logawa, while a score of 1 (no leaf reddish) was observed in Inpari 30 of 400 ppm $FeSO_4$ treated plants. Bronzing in rice plants was associated with melanin pigment and chlorophyll degradation was associated with senescence. The melanin pigment produced in the bronzing process is caused by the oxidation of phenolic compounds in the leaf tissue (Taranto et al. 2017). The tolerant varieties with a low leaf bronzing score indicated that the leaves could carry out the normal photosynthesis process. In contrast, the varieties with a higher leaf bronzing score indicated that the presence of dissolved Fe affected the ability to carry out the photosynthesis process. Fe poisoning causes the photosynthesis process to decrease, which causes the amount of chlorophyll also decreases (Rout and Sahoo 2015).

Symptoms of Fe toxicity vary in each rice variety (Turhadi et al. 2019). Generally, purplish brown spots will appear on the leaves, followed by drying. Typical visual symptoms are related to Fe toxicity, especially the accumulation of oxidized polyphenols called bronzing or yellowing in rice (Noor et al. 2012). Bronzing in rice plants is associated with melanin pigment and chlorophyll degradation is associated with senescence. The melanin pigment produced in the bronzing process is caused by the oxidation of phenolic compounds in the leaf tissue (Taranto et al. 2017). The tolerant varieties with a low leaf bronzing score showed that the leaves could carry out the normal photosynthesis process. In contrast, in the varieties with a higher leaf bronzing score, the presence of the dissolved Fe affected the ability to carry out the photosynthesis process. Hence, the rate of the photosynthesis process decreased, and the amount of chlorophyll also decreased (Pereira et al. 2013; Liu et al. 2020).

In this study, the provision of $FeSO_4$ caused the growth of rice plants to be stunted, and only Ciherang and Inpari 30 produced grains. These results supported Audebert and Fofana (2009) that rice plants that experienced iron poisoning from the vegetative phase would become stunted, causing the development in the generative phase to be also hampered. This poisoning effect causes plants not to grow normally, resulting in a small number of panicles and empty grains. These generative phase results are similar to Sahrawat (2000) that high rice yields were positively correlated with tolerance to Fe toxicity. The level of Fe toxicity and grain yield is not only influenced by environmental conditions but also influenced by the sensitivity or tolerance of the varieties planted (Noor et al. 2012). The inhibition of growth and development of rice plants due to iron stress is caused by the disruption of cell division at the growing point. Iron plaque covers the tips of the roots, thus affecting changes in root morphology (Gruber et al. 2013), which causes a decrease in the ability of roots to absorb and translocate nutrients to plant parts (Wu et al. 2014). Furthermore, this impact will interfere with metabolic activities and plant growth, which in turn causes plants to grow stunted, and the number of tillers becomes small (Sahrawat 2000).

Based on molecular analysis, all varieties evaluated in this study expressed a size band of about 309 bp in the electrophoresis results (Figure 3). The results showed that the *OsFER1* gene was expressed in all rice varieties without and with 400 ppm FeSO4 treatment. The Cq value in the housekeeping gene *α-TUB* was not significantly different because the gene was constitutively expressed and rarely modulated (Farrell 2017). The difference in Cq values can be caused by many things, such as the preparation of the PCR reaction in the reaction to be carried out, such as the conditions of the master mix preparation, the quality of the reference dyes, the degradation of RNA or cDNA templates used, contamination of the reaction, and PCR efficiency (E) (Bustin et al. 2009). The Cq value cannot be directly used to analyze the gene expression level in each variety (Vermeulen et al. 2011). Since the E values of *α-TUB* (1.91) and *OsFER1* (1.81) were different, therefore Pfaffl's method (2001) was chosen to measure relative gene expression. The analysis of gene expression levels without considering the E value causes a bias in the target gene quantity data and the ratio of target gene expression to reference genes (Ruiz-Villalba et al. 2021).

The PCR amplification efficiency, E value using the primer for the *OsFER1* gene was 1.81, while for the *α-TUB* gene, 1.91. Both E values meet the requirement of the (MIQE) Minimum Information for Publication of Quantitative Real-Time PCR Experiments (Bustin et al. 2009). The accepted range for E value is generally 80 to 110% (1.8-2.05), which indicates a good PCR efficiency value because it is still in the normal range of 1.80-2.05 (Ruijter et al. 2009; Artarini et al. 2016). The E value of the qPCR obtained in the study indicates that the primer used was good. Based on Svec et al. (2015), the efficiency of PCR depends on many factors, one of which is the sequence and structure of the primer and template.

The mean value of *OsFER1* gene expression is in accordance with Majerus et al. (2007), where there was an increase in ferritin mRNA and protein levels in *Oryza glaberrima* exposed to a nutrient solution with a high Fe concentration for 72 hours compared to those not exposed. The iron storage protein ferritin plays a crucial role in iron metabolism. Its ability to absorb elements gives ferritin the dual function of detoxifying iron and storing iron stores. An oxidative stress of Fe is the accumulation of ferritin transcripts (Singh and Bhatla 2022). Rice genotypes varied in their resistance to excess Fe. Iron homeostasis in plants must be tightly regulated. The overexpressed ferritin gene is considered a potential source of iron in grain biofortification studies (Zhu et al. 2017). Overexpression of these genes also increases plant tolerance to various abiotic stresses such as temperature and high acidity (Wang et al. 2013) and other abiotic stresses associated with ROS scavenging (Zang et al. 2017). According to Noor and Khairudin (2013), tolerant rice varieties are more efficient for planting in paddy fields than sensitive varieties because they can hold more Fe in the roots.

In conclusion, based on the three agronomic characteristics of plant height, leaf Bronzing score, and the number of filled grains produced; and the molecular analysis of *OsFER1* expression, Inpari 30 is a rice variety that is tolerant to $FeSO_4$ stress compared to the Ciherang, Inpari 42, Sungal, Logawa, and Cibogo varieties.

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