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Full Length Article

Analysis of *Superoxide Dismutase* (*OsSOD*) Gene Expression using qRT-PCR, its Morphophysiological Characters and Path Analysis in Rice Variety IR64 Under Aluminum Stress

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Abstract

Rice treated with aluminum (Al) can produce active oxygen species (AOS). The existence of AOS can cause the rice plants to become morpho-physiologically damaged and eventually cause a decrease in productivity. The AOS reductive compounds are physiologically responded by plants using antioxidant compounds, one of which is the superoxide dismutase enzyme (SOD). Therefore, this research aimed to analyze *FeSOD*, *MnSOD* and *CuZnSOD* gene expressions in IR64 rice variety as information for selection and genetic improvement of rice to overcome national food security. We tested the root architecture, analyzed *SOD* gene expression and performed path analysis tests. The results showed that rice treated with aluminum (Al) showed dramatically increased *CuZnSOD* and *FeSOD* gene expression, while *MnSOD* gene expression was relatively the same under Al stress or normal conditions. The level of expression of the two genes positively correlated with physiological characteristics such as chlorophyll and root length. Thus, these two genes can be used as markers in studying Al tolerance in Indica cv. rice. IR64.

Keywords: Aluminum Tolerance; AOS; Path Analysis; Rice; *SOD*

Introduction

The provision of food, especially rice, in sufficient quantities and at affordable prices is the main priority for national and international development. Therefore, increasing rice production is one of the solutions to achieve food security particularly in Indonesia and universally in the world. One of the efforts that can be made to increase rice production is by utilizing suboptimal dry acid land. Acid soil is quite extensive in Indonesia especially outside Java Island (Karama and Abdurrachman 1993). There are 47.6 million hectares of suboptimal dry land, which is generally dominated by acid soils in Indonesia (Fendiyanto *et al.* 2019a; Miftahudin *et al.* 2021). Acidic soils with a pH less than 5 have very limited availability of N, P, K, Ca, Mg and Mo nutrients, as well as the presence of quite high amounts of dissolved aluminum (Al). The solubility of Al is related to the pH status of the soil. According to Kochian (1995), there are three forms of Al compounds, *i.e*., Al mononuclear (Al^{3+}), Al polynuclear and Al complex molecules in the form of $Al(OH)_4^-$. When the pH of the soil solution is low (less than 4), Al will be in the form of $Al(H_2O)_6^{3+}$ which is the most toxic form for plants (Matsumoto *et al.* 1992).

Aluminum can have a detrimental effect on plants, either directly or indirectly. The direct effect of Al-stress is to inhibit root growth and interfere with nutrient and water absorption, while the indirect effect of Al stress is to reduce plant production by 25 to 85% (Herrera *et al*. 2008). Rice production reached 82.3 million tons in Indonesia in 2019. This production decreased to 81.0 million tons in 2020. This indicates a decrease in the production of 1.3 million tonnes per year (Miftahudin *et al.* 2021). The decline in rice production is inversely proportional to the increasing Indonesian population. This

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has resulted in problems related to National food security. Therefore, high productivity rice varieties that are resistant to Al stress are the need of the hour.

One factor that has received a lot of attention is the presence of active oxygen species (AOS) which is involved in inducing cellular damage to plants. AOS compounds are formed through various metabolic reactions in plants that are induced by stress, one of which is due to aluminum stress. Naturally, plants have a mechanism to control the accumulation of the production of AOS compounds by involving reactions of peroxidase enzymes and antioxidant compounds (Niyogi 1999). Increased function of antioxidants is usually associated with the formation of oxidants including free radicals from active oxygen species. Several mechanisms involve enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and glutathione reductase (GR), or involve antioxidant compounds such as ascorbic acid, α-tocopherol, glutathione, and *β*-carotene.

The transgenic tobacco plant (*Nicotiana tabacum*) showed overexpression of the enzymes SOD and GPX under oxidative stress (Gupta *et al*. 1993; Zhang *et al*. 2017; Zhuang *et al*. 2020; Rajput *et al*. 2021). Increased activity of the antioxidant enzymes SOD, APX, GR and ASA (ascorbic acid) was shown in soybean varieties Tidar, Burangrang, Panderman and wild soybeans which were subject to drought and paraquat stress (Violita 2007; Hamim *et al*. 2017). Research on increasing the activity of the SOD enzyme against Al stress is relatively limited in rice variety. Therefore, research on the expression of *SOD* on rice cv. IR64 under Al stress was important to do. This research is expected to contribute to primary information for plant breeders and biologists particularly using *SOD* genes to transform other rice varieties to be Al-tolerant. This study aimed to analyze the expression of 3 types of genes encoding rice-antioxidant enzyme *Oryza sativa* L. superoxide dismutase (*OsSOD*) and perform morpho-physiological characterization in rice cv. IR64 that are exposed to aluminum stress.

Materials and Methods

Rice planting and aluminum stress treatment

Rice seeds were sterilized in 0.5% (v/v) NaOCl solution for 15 min, then washed three times with distilled water. The seeds were then soaked in distilled water for 24 h at room temperature. The seeds are then germinated on damp paper for 4 d at room temperature and stored in a dark place. After germinating, the rice seeds are planted on a plastic net that is floated on a minimum nutrient culture medium without Al with a pH of 4.0 which is aerated (Miftahudin *et al.* 2005; Miftahudin *et al.* 2021) for adaptation for 24 h. The Al stress treatment was simulated by administering 15 ppm of Al^{3+} in the form of $AlCl_3.6H_2O$ for 72 h in a nutrient solution. The next step is recovery, namely the provision of a minimum nutrient solution without Al for 48 h (Miftahudin *et al.* 2002; Fendiyanto *et al.* 2019a; Miftahudin *et al.* 2021). The acidity of the solution is maintained at pH 4 every day with the addition of 1 N HCl or 1 N NaOH. The adaptation, Al stress, and recovery treatments were carried out in the growth chamber at room temperature and 300 Photo Proton Flux Density (PPFD) lighting for 12 h every day. The minimum nutrient solution is maintained at pH 4.0 by changing the minimum nutrient solution every day. The composition of the minimum nutrient solution follows Table 1.

Statistical analysis

The experiment was designed and performed using a completely randomized design (CRD) with a factorial treatment design consisting of one factor covering 2 levels, *i.e*., Al stress treatment and control. Statistical testing was investigated using the R version 3.5.1 program following Miftahudin *et al.* (2021) and Fendiyanto *et al.* (2019a).

Root systems observation

Analysis of the physiological parameters of Al stress includes Root Regrowth (RRG) and root length, *i.e*., primary root length (PRL) and total root length (TRL) measurements (Fendiyanto *et al*. 2019a, b; Miftahudin *et al*. 2021). RRG measurement of plants that had been treated with Al stress was carried out by measuring the root length at the end of the Al stress treatment and the end of recovery. The difference between the root length at the end of recovery and the final measurement of Al stress treatment is the RRG parameter (Miftahudin *et al*. 2005).

Hematoxyline staining

Hematoxyline staining was performed to detect aluminum in roots qualitatively and we generated the method following Fendiyanto *et al.* (2019a) with slight modifications (Miftahudin *et al.* 2021). After the Al treatment, the roots were rinsed three times and then mixed in a 0.6% (w/v) of hematoxylin (Merck, USA) solution for 2 min. The roots are then subsequently rinsed three times with distilled water. Rice root tip was precisely observed with a Stereomicroscope (Olympus SZ51, Japan) installed with a camera (Indomicro, Indonesia).

Malondialdehyde (MDA) content

We tested malondialdehyde (MDA) content based on the method of Fendiyanto *et al*. (2019a) with slight modification (Meriga *et al.* 2010). A total of 0.5 g of rice cv. IR64 was ground and mixed with 5 mL of 0.1% (w/v) trichloroacetic acid (TCA) (Merck, USA). The mixture was then centrifuged gradually at 11,000 rpm for 3 min. 1 mL of supernatant was then mixed with 4 mL of the thiobarbituric-trichloroacetic acid (TBA-TCA) solution, particularly with

the composition [0.1% (w/v) Thiobarbituric acid (TBA) (Merck, USA) in 20% (w/v) TCA (Merck, USA)] (Fendiyanto *et al*. 2019a). The suspension was gently incubated at 80°C and measured its absorbance at 532 and 600 nm wavelengths. The MDA content was calculated by following the Heath and Packer (1968) calculation.

$$\text{MDA} = \frac{\left(\frac{A532 - A600}{\varepsilon}\right) \text{X } 10^6}{\text{Fresh weight (g)}}$$

MDA : MDA concentration (nmol/g)
ε : MDA extension coefficient value (155 mM^{-1} cm^{-1})

Analysis of total chlorophyll

We followed Fendiyanto *et al*. (2019a) to performed chlorophyll analysis using reagent 100% (v/v) acetone (Merck, USA) with slight modification. Rice cv. IR64 leaves (0.5 g) were added to liquid N_2, ground and mixed with 10 mL of acetone (Merck, USA). The mixture was gently centrifuged at 11,000 rpm/min. The supernatant was subsequently used for the measurement of absorbance at 470, 646 and 662 nm wavelengths using a spectrophotometer. Total chlorophyll, carotenoid, chlorophyll-a and chlorophyll-b contents were specifically calculated using Dere *et al*. (1998) formula:

$Ca=11.75\ A_{662}-2.350\ A_{646}$
$Cb=18.61\ A_{646}-3.960\ A_{662}$
$Cx=1000\ A_{470}-2.270\ Ca-81.4\ Cb/227$

Ca : Chlorophyll-a content (mg/g FW)
Cb : Chlorophyll-b content (mg/g FW)
Cx : Carotenoid content (mg/g FW)

Total RNA isolation of rice

Total RNA isolation was carried out using a Total RNA kit for Plant (ATP Biotech, Taiwan). The samples used for total RNA isolation came from plant roots in the control and 15 ppm Al stress treatment. The DNase treatment was carried out during the RNA isolation process and we also used RNase free water (such as DEPC) during the process. The quantification of the total RNA isolated was carried out by dissolving 1 μL of total RNA in 399 μL ddH_2O-DEPC 0.01%, then reading it with a spectrophotometer (UV-Vis, GeneQuant 1300, USA) at a wavelength of 260 and 280 nm. The purity of the total RNA isolation results was carried out by calculating the ratio value from the OD ratio at λ260/280. RNA integrity was analyzed by migrating RNA on 1% formaldehyde agarose gel for 60 min using TBE 1x buffer.

cDNA synthesis of rice cv. IR64

Synthesis of cDNA was carried out using the RevertAid FirstStrand cDNA Synthesis kit (Thermo Scientific, USA). Total RNA is used as a template for cDNA synthesis with the reverse transcriptase (RT) enzyme. The evaluation of the success of cDNA synthesis was carried out by cDNA amplification *via* PCR with primers of the *Actin* gene (Table 2) following the procedure of Satrio *et al.* (2019) and Miftahudin *et al.* (2021).

Analysis of *SOD* gene expression

Analysis of SOD gene expression was carried out by amplification of the cDNA obtained from the RT results as a template using primers from the SOD gene. The SOD genes tested included MnSOD, FeSOD and Cu/ZnSOD. Specific primers for the three genes were designed based on the mRNA sequence of the SOD enzyme coding gene obtained from NCBI particularly *MnSOD* (GeneBank: KY752530.1), *FeSOD* (GeneBank: AB014056.1) and *Cu/ZnSOD* (GeneBank: KY752531.1). For the endogenous gene, we used *Actin* (GeneBank: CA762906). We designed the primers using Primer 3 in U-Gene Program (Okonechnikov *et al.* 2012).

The success of the SOD gene PCR process was known by electrophoresis on 1% agarose gel for 60 min with 1x TBE buffer. Analysis of SOD gene expression was carried out by looking at the intensity produced by each band of PCR results using software from the Digi Doc-it program (Muzuni 2003; Mashuda 2006). Expression of each *SOD* gene from rice cv. IR64 at two treatment levels (0 and 72 h Al-stress treated) was compared with standardization. Standardization of *SOD* gene expression was carried out by comparing *SOD* gene expression with *Actin* at the same treatment levels. Therefore the expression of the *SOD* target gene was standardized using the formula:

$$\text{EBXpv} = \frac{\text{IXp}}{\text{Ip}}$$

EBXpv: Relative expression of *SOD* gene in p treatment
Ixp: The intensity of *SOD* gene qPCR results in treatment p
Iapv : The intensity of the qPCR results of the *Actin* gene in the treatment p
p : control or Al-stress

Results

Root systems architecture of rice cv. IR64 under aluminum stress

Rice cv. IR64 which received Al stress for 72 h in the germination phase showed a decrease in the root system. In the Al stress treatment, a decrease in the root system occurred both in number and length of the roots, *i.e*., in total root length, primary root length, lateral root length, seminal root length, and the number of lateral roots compared to the control conditions (Fig. 1). The main root length decreased significantly (3x fold changed) in Al stress when compared to the control conditions (Fig. 1). The results of root physiological measurements showed that the length of

Table 1: Minimum nutrient composition (Miftahudin *et al.* 2002)

Reagent (PA)	Concentration
$CaCl_2.2H_2O$	0.40 m*M*
KNO_3	0.65 m*M*
$MgCl_2.6H_2O$	0.25 m*M*
$(NH_4)_2SO_4$	0.01 m*M*
NH_4NO_3	0.04 m*M*

Table 2: Primer sequences designed for amplification of *SOD* gene sequences

No.	Primer	Sequence	Tm	%GC
1.	Cu_ZnSOD_F	5'- GCTAGCAGTGAGGGTGTCAAG-3'	60.0658	57.14
2.	Cu_ZnSOD_R	5'- CTAACCCTGGAGTCCGATGAT -3'	60.3340	52.38
3.	FeSOD_F	5'- GGGCTGTAGATCTCGAAGGTATT-3'	60.0042	47.92
4.	FeSOD_R	5'- CAGTATCCCAAGAGACAAGATGG-3'	60.0049	47.82
5.	MnSOD_F	5'- CTACGTCGCCAACTACAACAAG -3'	59.8655	50.00
6.	MnSOD_R	5'- AGTCGCATTTTCGATCACCT -3'	59.6998	45.00
7.	Actin_F	5'- GAAGGATGCCTATGTTGGTGA-3'	59.9473	47.61
8.	Actin_R	5'- CTTCATAGATTGGCACGGTGT-3'	60.0077	47.61

Legend: Primers were designed with the Primer3 application in U-gene Software

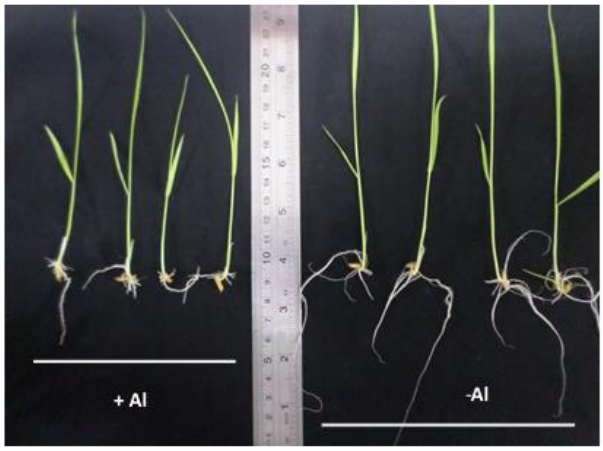

Fig. 1: Root systems architechture (RSA) of rice cv. IR64 under Aluminum (Al)-treated and normal condition. +Al: Al-treated, -Al: normal condition, without Al. Rice was stress using Al_2Cl_3 for 72 h in hyroponically minimum nutrient culture

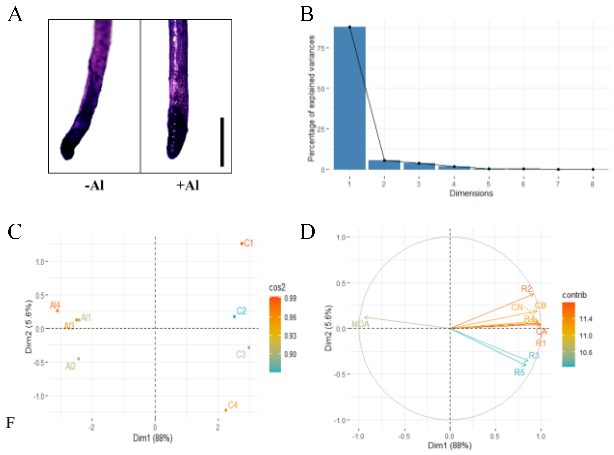

Fig. 2: Morpho-physiological characters of rice cv. IR64 under Aluminum (Al)- stress. Hematoxyline staining to detect qualitatively lipid peroxidation due to Al-stress (**A**). –Al: Normal Condition or Without Al, +Al: Al-treated. Screen plot of all morpho-physiological components (**B**). Dimensions are similar to principal components. Individuals-Principal Component Analysis (PCA) of morpho-physiological characters (**C**). Variables- PCA and the biplot of morpho-physiological characters (**D**). Dim1: Dimensions 1, Dim2: Dimensions 2, MDA: Malondialdehyde, CA: Chlorophyll-a, CB: Chlorophyll-b, CN: Carotene, R1: total root length, R2: primary root length, R3: lateral root length, R4: seminal root length, R5: number of the root

the main roots of rice that was treated with aluminum showed shorter roots than those that were not treated with aluminum (Fig. 1).

Morphophysiological analysis

Rice cv. IR64 demonstrated significantly different nine morpho-physiological characters between Al-stressed and control conditions particularly in malondialdehyde, chlorophyll-a, chlorophyll-b, carotene, total root length, primary root length, lateral root length, seminal root length, and number of the root (Fig. 2). Based on the hematoxyline staining test, rice cv. IR64 treated with Al has a dark purple color when compared to control, indicating rice cv. Al-treated IR64 suffered membrane damage and Al accumulation occurred in the rhizosphere (Fig. 2A). Based on the nine morpho-physiological characters tested, the percentage of variation in the principal components (PCA) analysis was relatively large, namely 88% for PC1 and 5.6% for PC2 (Fig. 2B). Individuals-PCA showed that rice cv. IR64 in the control conditions had a morpho-physiological character that was dramatically different from that of the Al treatment (Fig. 2C). Analysis of Variables-PCA and the biplot showed that malondialdehyde (MDA) characters have different vectors with chlorophyll characters and root lengths (Fig. 2D). Therefore, MDA characters have a negative correlation to other characters.

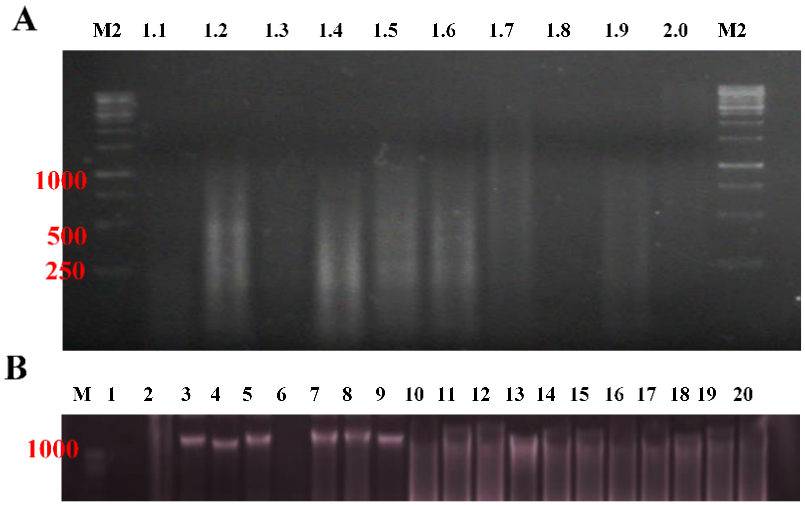

Fig. 3: Total RNA isolation of rice cv. IR64 on Al (**A**) and control (**B**) stress treatment. RNA isolation was carried out using the Trizol method and isolated when the rice was treated for 72 hours on minimum nutrient culture media. 1.1-2.0: Total RNA from rice treated with Al stress. 1: negative control (ddH_2O), 2-9: rice DNA cv.IR64 as positive control, 10-20: RNA rice cv. IR64 on control (non Al). M: marker 1000 bp, M2: marker 1 kb. 250, 500, 1000 are size in base pair (bp). RNA is isolated from root tissue

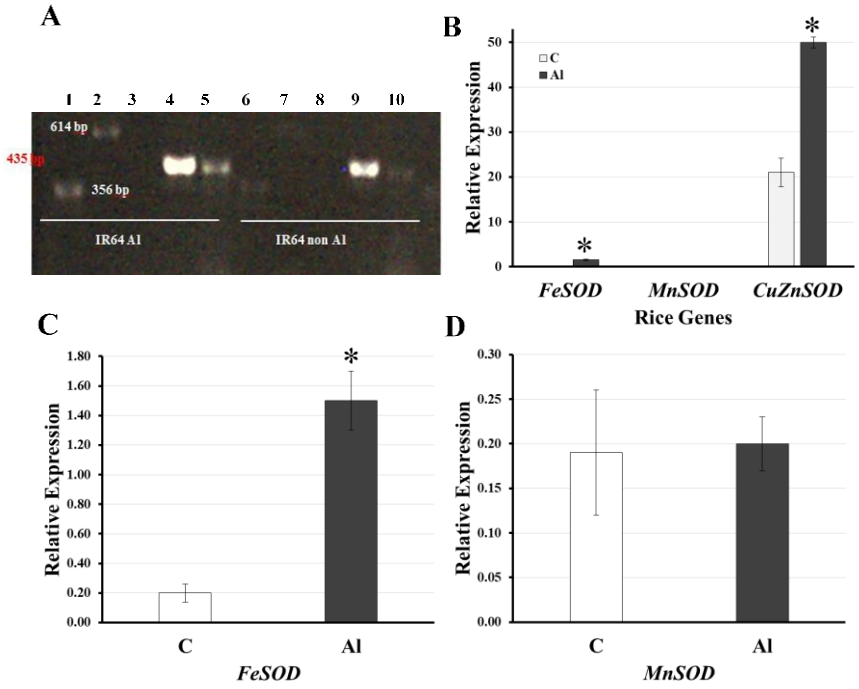

Fig. 4: Reverse transcriptase-PCR (RT-PCR) (**A**) and quantitatively RT-PCR (realtime PCR) expression analysis of SOD genes (FeSOD, MnSOD, and CuZnSOD) (**B**-**D**). Each experiment was replicated three times (3 biological and 3 technical repeated). Values are the mean ± SE, t-Student, **P* value < 0.05 to control (C), respectively. C or non Al: control condition, Al: Al-treated

Total RNA isolation of rice cv. IR64

Total RNA isolation was successfully carried out and two bands were obtained. This can be shown in Fig. 3. In the total RNA isolation from ten sample replications, six samples showed positive results. The six samples from total RNA isolation were thought to be successful because they showed two firm bands, namely 28S rRNA and 18S rRNA (Fig. 3). The purity level of RNA also showed good results where the A260 / A280 values ranged from 1.8–2.0.

Based on the results of electrophoresis, the two bands indicated the 28S rRNA and 18S rRNA bands. It also shows that plant RNA (eukaryotic) isolation was successful. Apart from RNA isolation, plant genomic DNA isolation was also

Table 3: Path Analysis of Morpho-physiological Characters to *FeSOD* gene expression

	MDA	CA	CB	CN	R1	R2	R3	R4	R5	Direct Effect	Total Effect (Correlation)
MDA		-0.209	0.732	-0.421	0.742	-0.132	0.228	0.102	0.097	-0.208	0.931**
CA	0.190		-0.843	0.491	-0.774	0.148	-0.226	-0.104	-0.102	0.230	-0.990**
CB	0.177	0.225		0.491	-0.734	0.150	-0.194	-0.100	-0.096	-0.861	-0.942**
CN	0.175	0.225	-0.843		-0.742	0.145	-0.210	-0.100	-0.102	0.501	-0.951**
R1	0.194	0.223	-0.792	0.466		0.152	-0.237	-0.105	-0.093	-0.798	-0.990**
R2	0.171	0.211	-0.800	0.451	-0.750		-0.180	-0.100	-0.075	0.161	-0.911**
R3	0.177	0.193	-0.620	0.391	-0.702	0.108		-0.087	-0.092	-0.269	-0.901**
R4	0.196	0.221	-0.792	0.461	-0.774	0.148	-0.215		-0.097	-0.108	-0.960**
R5	0.163	0.188	-0.663	0.411	-0.598	0.097	-0.199	-0.085		-0.124	-0.810*
									Residual Effect	-0.001	

Legends: MDA: Malondialdehyde, CA: Chlorophyll-a, CB: Chlorophyll-b, CN: Carotene, R1: total root length, R2: primary root length, R3: lateral root length, R4: seminal root length, R5: number of the root. Significantly different in $^{**}P < 0.01$ and $^{*}P < 0.05$, respectively

Table 4: Path Analysis of Morpho-physiological Characters to *CuZnSOD* gene expression

	MDA	CA	CB	CN	R1	R2	R3	R4	R5	Direct Effect	Total Effect (Correlation)
MDA		-0.910	0.679	-0.039	0.456	-0.135	0.156	0.127	0.040	-0.045	0.329**
CA	0.041		-0.783	0.046	-0.475	0.151	-0.154	-0.130	-0.042	1.000	-0.346**
CB	0.039	0.980		0.046	-0.451	0.153	-0.132	-0.124	-0.040	-0.798	-0.327**
CN	0.038	0.980	-0.783		-0.456	0.148	-0.143	-0.124	-0.042	0.046	-0.336**
R1	0.042	0.970	-0.735	0.043		0.155	-0.162	-0.131	-0.039	-0.490	-0.347**
R2	0.037	0.920	-0.743	0.042	-0.461		-0.123	-0.124	-0.031	0.164	-0.319**
R3	0.039	0.840	-0.575	0.036	-0.431	0.110		-0.108	-0.038	-0.184	-0.311**
R4	0.043	0.960	-0.735	0.043	-0.475	0.151	-0.147		-0.040	-0.135	-0.335**
R5	0.035	0.820	-0.615	0.038	-0.368	0.099	-0.136	-0.105		-0.052	-0.284*
									Residual Effect	0.006	

Legends: MDA: Malondialdehyde, CA: Chlorophyll-a, CB: Chlorophyll-b, CN: Carotene, R1: total root length, R2: primary root length, R3: lateral root length, R4: seminal root length, R5: number of the root. Significantly different in $^{**}P < 0.01$ and $^{*}P < 0.05$, respectively

carried out. This can be seen in Fig. 3. Isolation of genomic DNA was carried out to ensure that the unamplified amplicon did not come from a faulty primary design. The primary design has also been carried out and the sequence can be seen in Table 2.

Analysis of *SOD* gene expression

The expression analysis on the three SOD genes showed different results in rice cv. IR64 treated aluminum (Al) and under control conditions (Fig. 4). Based on the results of the RT-PCR analysis showed that the *CuZnSOD* gene had the highest expression, while the *MnSOD* gene had the lowest expression (Fig. 4A). *CuZnSOD* gene had a dramatically high expression when treated with Al (50x fold change) as compared to controls (Fig. 4B). In addition, the *FeSOD* gene also had a significantly high expression under Al stress (Fig. 4C). Based on the results of this study, the *MnSOD* gene had relatively the same level of expression among control and Al-stress conditions (Fig. 4D).

Path analysis among gene expression and morphophysiological characters

Path Analysis is a correlation study that examines the direct and indirect effects of one character on other characters. Path analysis test is very well tested on several abiotic stresses such as salinity stress and drought stress, but it has never been analyzed on Al stress. Therefore, in this study, we tested several characters of Al tolerance with path analysis.

Two genes that express significantly when choking Al and under normal conditions are *FeSOD*and *CuZnSOD*. The two genes were further studied using path analysis. The characters that have direct effect on *FeSOD* gene expression in rice cv. IR64 when treated with Al are Carotene (CN), chlorophyll-a (CA) and primary root length (R2) (Table 3). Similar to the *FeSOD* gene, the characters that have direct effect on *CuZnSOD* gene expression are CA, R2, and CN (Table 4). The indirect effect on path analysis of the *FeSOD* gene occurred in the R1-CN and R2-CN correlations. On the contrary, the *CuZnSOD* gene correlated with CB-CA and CN-CA (Tables 3 and 4).

Discussion

Rice is the most tolerant crop compared to other crops in the Gramineae family (Ma *et al.* 2014; Kochian *et al*. 2015). However, very little is known about the morphophysiological properties and tolerance of aluminum (Al) in Indica subspecies, especially the IR64 variety. In general, Japonica subspecies rice is more tolerant than Indica rice (Ma *et al*. 2014). This is thought to be related to the morphophysiological and genetic characteristics of the two subspecies. IR64 rice is reported to be included in rice that is sensitive to Al (Fendiyanto *et al.* 2019a). The sensitivity mechanism to Al is also influenced by the presence of morphophysiological factors, SOD expression, and the correlation between the two parameters.

Root systems architecture (RSA) is one of the root characters that can be used as a marker for the level of Al stress in rice (Fendiyanto *et al.* 2019a, b; Miftahudin *et al*. 2021). This study showed that Al stress influenced all root

length parameters in rice cv. IR64. The results in this study are in accordance with those reported by Fendiyanto *et al*. (2019a) and Satrio *et al*. (2019) that rice cv. IR64 is sensitive to abiotic stresses such as Al and drought. Thus, rice cv. IR64 showed decreased root length in Al-stress treated.

Physiological characters in a plant are related to the level of adaptation in dealing with stress both abiotic and biotic, growth and development, hormone levels, important metabolites, and productivity (Fendiyanto *et al.* 2019a; Fendiyanto *et al.* 2020). Specifically, to Al-stress (abiotic stress), lipid peroxidation is a crucial physiological parameter in rice (Miftahudin *et al.* 2021). The parameter could be determined using hematoxyline staining as well as qualitative method or malondialdehyde measurement as well as quantitative method (Fendiyanto *et al.* 2019a). Hematoxyline staining is a qualitative test for the presence of Al accumulation at the root tip and this is commonly tested to determine the level of sensitivity to Al stress (Fendiyanto *et al.* 2019a; Miftahudin *et al.* 2021). The hematoxyline test can be confirmed by testing for membrane damage in quantitative MDA testing. Both hematoxyline and MDA staining showed that Al-treated IR64 rice had higher membrane damage and Al accumulation at root tips compared to the control (Fig. 2). These results support the research conducted by Siska *et al*. (2017) and Fendiyanto *et al*. (2019b). Apart from MDA and hematoxyline staining, chlorophyll character and root length also showed significant inhibition of IR64 rice during Al stress (Fig. 1 and 2). The antenna complex and photosystem proteins that are mediated through the presence of reactive oxygen species (ROS) will be damaged (Ohki 1986). This damage *via* ROS occurs due to the response of plants when stressed by Al (Kochian *et al.* 2015).

Several genes related to the regulation of transcription factors, transport proteins, activators, regulators, and repressors on the Al tolerance mechanism have been widely reported in plants, especially rice (Ma *et al*. 2014; Kochian *et al*. 2015). Genes related to Al tolerance are *ART1* (Yamaji *et al.* 2009), *ART2* (Che *et al.* 2018), *STAR1* and *STAR2* (Huang *et al.* 2009) and *OsFRDL4* (Li *et al.* 2018), *OsASR5* (Arenhart *et al.* 2014), *OsASR1* (Arenhart *et al.* 2016), *B11* (Fendiyanto *et al.* 2019a), and *OsGERLP* (Miftahudin *et al.* 2021). However, there have been no reports on the expression of three types of *SOD* genes related to the regulation of Al tolerance in cv. rice. IR64 (Indica subspecies). So that in this study, analysis of SOD expression is important to do.

It is known that three types of *SOD* genes can respond to ROS due to stress in plants, *i.e*., *FeSOD, MnSOD* and *CuZnSOD* (Gupta *et al*. 1993; Zhuang *et al.* 2020; Rajput *et al*. 2021). In IR64 rice, it is not known which SOD gene has high expression when stressed by Al. Therefore, RNA isolation is an important expression analysis stage in examining the role of a gene in abiotic stress, especially Al stress (Miftahudin *et al.* 2021). This study confirmed that *FeSOD* and *CuZnSOD* genes were highly expressed in Al treatment but do not in the control condition (Fig. 3 and 4). Those genes are highly correlated with chlorophyll content characters (Table 3 and 4). The FeSOD and CuZnSOD proteins were also thought to have a high expression to overcome the high number of ROS in Al-stressed particularly in rice cv. IR64 (Gupta *et al*. 1993). However, *MnSOD* genes expressed higher in 24 h after oxidative stress treatment (Gupta *et al*. 1993). In this study, *MnSOD* did not express higher in Al-stress condition because we checked the expression after 72 h Al-treatment. *MnSOD* might be expressed higher before 72 h Al-treatment and act as early response genes. In the root of *Pisum sativum*, *MnSOD* gene expression showed lower in 48 h compared to 24 h after Al treatment (Panda and Matsumoto 2010). These fundings support that *MnSOD* has a mode of action as early response genes in Al-stress conditions. In addition to analysis, MnSOD protein is located abundantly in the mitochondrion and few in peroxisomes (Alscher *et al.* 2002). When rice experienced Al-stress in early response might increase *MnSOD* expression, however, in middle or late response, *MnSOD* may have a normal or lower gene expression again. However, it needs to be confirmed again in the next studies.

Based on the path analysis test, these two genes are directly positively affected by chlorophyll A and carotene. In addition, both genes are also indirectly correlated with chlorophyll A and carotene. These findings support Alscher *et al.* (2002) that Fe and CuZn-SOD are located in chloroplasts. Therefore, it is possible Fe and CuZnSOD influenced chlorophyll content parameters. Based on PCA analysis, there is a positive correlation among parameters of chlorophyll-a, chlorophyll-b, carotene, total root length, primary root length, lateral root length, seminal root length, and the number of the root in rice cv. IR64 under Al-stressed. Conversely, only the MDA parameter has a negative correlation with other morphophysiological characters. A similar result was reported by Fendiyanto *et al*. (2019a) that MDA characters have a negative correlation with Al tolerance level in rice cv. Inpago. It was suggested that rice cv. IR64 and Inpago have a similar morphophysiological response to Al stress.

The high number of ROS will damage the components of the photosynthetic device. However, IR64 rice when Al-stressed has a defense mechanism by increasing the expression of *FeSOD* and *CuZnSOD* genes and further enhancing photosynthetic devices such as chlorophyll and carotene.

Conclusion

Two SOD genes (*CuZnSOD* and *FeSOD*) have a high expression when stressed by Al in rice subspecies Indica cv. IR64. Conversely, the *MnSOD* gene has the same expression in either the Al stress treatment or under normal conditions. Based on path analysis, the characteristics of chlorophyll (chlorophyll A and carotene), and root lengths have a direct and indirect effect with the expression of both

CuZnSOD and *FeSOD* genes. Rice cv. IR64 has a significant difference in physiological response when treated by Al compared to the control. This discovery is important to become the *SOD* gene as a molecular marker concerning the Al stress tolerance mechanism for plant breeders and biological researchers.

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Author Contributions

MHF and MPP wrote the manuscript, designed the experiment, and conducted path analysis and morphophysiological tests. RDS and IAN edited the manuscript and conducted expression analysis. IDK, NIP, and DDR edited the manuscript and conducted chlorophyll content analysis.

Conflict of Interest

The authors declare that they have no conflict of interest.

Data Availability

We hereby declare that all data reported in this paper are available and will be produced on demand.

Ethics Approval

Not applicable.

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