

Original Research Paper

# Morpho-Physiological and Genetic Responses of Bali Local Rice Cultivars to Drought Stress at Seedling Stage

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**Abstract:** Rice is the main food source in many countries in the world. One of the major limiting factors for rice growth and productivity is drought stress. In Bali there are several local rice cultivars, two of them are 'Mansur' and 'Putih Cempaka'. The cultivar 'IR 64' is one rice cultivar released by IRRI. The responses of those rice cultivars to drought stress were tested. This study aimed to analyze growth responses and expression of *DREB* and *SOD* genes under drought stress induced by PEG. Rice seedlings were grown in a hydroponic system. Two weeks old seedlings were treated with PEG 20% (-0.49 MPa) and 30% (-1.03 MPa) for three days. Root to shoot ratio was calculated and chlorophyll content was measured. RNA was extracted from the shoot. Semi-quantitative real-time PCR was conducted and the products were visualized using 1% agarose gel electrophoresis. The result showed that root to shoot ratio increased as PEG concentration increased in all rice cultivars tested. The chlorophyll content decrease under PEG treatments in all rice cultivars. The expression of Superoxide Dismutase (*Cu/Zn-SOD*) gene increased under drought treatments in 'Mansur' and 'Putih Cempaka', while in 'IR64' using *SOD* primer tested, the gene was not expressed. The expression of Dehydration-Responsive Element Binding (*DREB1*) gene increased under PEG treatments both in 'Mansur' and 'Putih Cempaka'.

**Keywords:** Gene Expression, Morpho-Physiology, Rice, Drought

## Introduction

Rice (*Oryza sativa* L.) is a primary source of food for people in many countries including Indonesia. Rice productivity is affected by several factors such as high temperature, salinity and drought stress. Among various abiotic stresses, the main yield producing factor is drought stress (Karim and Rahman, 2015). Drought stress in plant is a condition of inadequate water availability in plant environment. This can be due to lack of water supply in the root areas and the need for excessive water by leaves because of high transpiration rate that exceeding the rate of water absorption (Farooq *et al.*, 2012). Drought leads to the decrease of photosynthesis and disturbance of enzymes activity especially those involved in ATP synthesis (Farooq *et al.*, 2012). Drought stress conditions change the structure of roots such as increased root branch and density (Eghball and Maranville, 1993).

In Indonesia, El Niño/Southern Oscillation (ENSO) event induced early season droughts, delayed onset of the monsoon and delayed planting of rice which in the end leads to rice production shortfall (Naylor *et al.*, 2007). Between 1979-2004, ENSO events caused rice production reduced by 11% in East Java/Bali during the main rice harvest season between January and April, while in West/Central Java the reduction was 6.5% (Naylor *et al.*, 2007).

Plants develop a variety of mechanisms to adapt to environmental stresses including reprogramming of gene expression (Romo *et al.*, 2001) such as the decrease of mRNA level related to photosynthesis (Bartels and Nelson, 2004). Certain genes will be induced if plants under drought stress. The product of those genes involved in cellular protection to damage caused by stress. Changes in expression of genes responsive to stress are an important mechanism in plant adaptation. Among several

genes that are known to be responsive to drought stress, two of them are Dehydration-Responsive Element Binding (*DREB*) and Superoxide Dismutase (*SOD*).

Superoxide dismutase genes responsible for antioxidant enzymes that block oxidative stress (Aydin *et al.*, 2014). Drought condition induces the presence of Reactive Oxygen Species (ROS) including superoxide radicals ( $O_2^-$ ), singlet oxygen ( $1O_2$ ), hydroxyl radicals ( $OH^-$ ) and hydrogen peroxide ( $H_2O_2$ ) (You and Chan, 2015). Activation of antioxidant enzymes is required to protect plant cell from oxidative damage. *DREB* regulates protection of plant to abiotic stress. *DREB1* is involved in the mechanism of plant tolerance to drought, cold and salinity (Jadhao *et al.*, 2014).

Drought stress can be stimulated by the addition of Polyethylene Glycol (PEG). Polyethylene glycol with high molecular weight is an osmotic regulator that decrease water potential in nutrient without being absorbed by (Roy *et al.*, 2009). The solution of PEG 6000 has been used in many studies of drought stress in plant (Aydin *et al.*, 2014).

Bali local rice 'Mansur' and 'Putih Cempaka' are two among several bali rice cultivars grown in Bali, Indonesia. 'Mansur' is a member of subspecies *Indica*, while 'Putih Cempaka' is *Javanica* rice (Budiwati, 2016). Bali local rice has long stem and tassel. The harvesting time is approximately six months. 'IR64' is one of rice superior quality rice cultivar released by Philippine Seed-board in 1985 (Khush and Virk, 2005). This cultivar is widely planted in many countries. 'IR64' has an early maturity, approximately 117 days (Khush and Virk, 2005). This study aimed to evaluate respond of Bali local rice 'Mansur' and 'Putih Cempaka' to drought stress at seedling stage and compare it to 'IR64' including root length, fresh weight root to shoot ratio, chlorophyll content and *DREB* and *SOD* gene expression.

## Materials and Methods

### *Plant Materials, Planting and Treatments*

Seeds of Bali local rice ('Mansur' and 'Putih Cempaka') and 'IR 64' were collected from a farmer in Tabanan Regency, Bali. The cultivar of 'IR64' was included as a comparison between Bali local rice and the superior rice quality. Seedlings were grown in Rockwool cubes as growing media in seedling trays for one week. After that, seedlings were transferred to static hydroponic system supplemented by 1/4 Murashige and Skoog (MS) nutrient (Pardo *et al.*, 1998). Five seedlings were grown in each pot. The seedlings were grown for another week and PEG 6000 were added at the concentration of 20% (-0.49 MPa) and 30% (-1.03 MPa) as severe drought stress for three days, following Lima *et al.*

(2015) with modification. The experiment was conducted in a factorial design with three replicates.

### *Measurement of Morpho-Physiological Characters*

At the end of treatment period, seedlings were harvested and root length, fresh weight of shoot and root were measured. The ratio of the fresh weight of root to shoot was calculated. Chlorophyll content was extracted using 80% acetone and calculated according to Arnon (1949).

### *Gene Expression*

The analyses of *DREB* and *SOD* expression were done using semi-quantitative real-time PCR. The shoot was collected three days after treatments and RNA were extracted using RNeasy Plant Mini Kit (Qiagen) according to manufacturer instruction. RNA concentration was measured using spectrophotometer at 260 nm and 280 nm (Sambrook and Russell, 2001). Synthesis of cDNA was done using Transcriptor First Strand cDNA Kit (Roche) following manufacturer protocol. PCR was done using cDNA as template. Amplification of *SOD* gene was conducted using forward primer GTCCATCGTAGATGAACCGTC and CGATCCATCTGAGATCCACAC as reverse primer (Singh and Sadasivam, 2003). *DREB* amplification was conducted using forward primer sequence CCGTTGATTGCTGATAGCCTCCTTGA and reverse primer sequence TGAAATATTCCTATTGACCCGCAGCA (Wang *et al.*, 2004). Actin was used as control gene and amplified using primer CTCCTCATGCCATCCTGC (forward) and GCAAGCTTCTCCTTGATGTCC (reverse) (Yu *et al.*, 2007) in a thermal cycler (Infinigen Biotech, CA, USA). PCR consisted of 1  $\mu$ L cDNA, 1 $\times$  PCR buffer (MDBio, USA), 200  $\mu$ M dNTP (Promega, USA), 2.5 mM  $MgCl_2$ , 0.5  $\mu$ M of each forward and reverse primers, 1 U Taq polymerase (MDBio, USA) and  $H_2O$  to reach 25  $\mu$ L reaction. The cycles of PCR were 1 $\times$  denaturation at 95°C for 5 min, followed by 35 $\times$  denaturation at 95°C for 1 min, annealing at 52°C (*DREB*), 60°C (*SOD*) and 56°C (Actin) for 1 min and extension at 72°C for 1 min. This cycle was followed by one cycle of final extension at 72°C for 10 min.

Electrophoresis was done in 1.5% agarose gel in 1 $\times$  TAE buffer (Tris Acetic Acid, EDTA) at 100 volts for 45 min. The gel was stained using ethidium bromide and visualized using GelDoc UV transilluminator (Uvitec, UK). Quantification of gene expression was performed using ImageJ software (NIH; <http://rsb.info.nih.gov/ij/>).

### *Statistical Analysis*

The root length, fresh weight root to shoot ratio and chlorophyll content were analyzed using one-way ANOVA and significant between means were analyzed

using Duncan's test at 5% probability levels ( $p \leq 0.05$ ). Two-way factorial ANOVA was used to determine the interaction between treatment and cultivar.

## Results

### Morpho-Physiological Responses to Drought

There was a reduction in shoot length in the three rice cultivars after PEG treatments, however, the values were not significantly different (data not shown). Statistical analysis showed that there was no interaction between root length and cultivars.

Root length of rice cultivar 'Mansur' increased significantly under PEG treatments (Table 1). There were no changes in root length of 'Putih Cempaka' between control and PEG-treated seedling, while in 'IR64' root length was reduced at higher concentration of PEG (Table 1).

The ratio of root to shoot fresh weight of increased as the concentration of PEG increased (Table 2). The rice cultivars responded differently in root to shoot ratio under drought stress. There was an interaction between treatments and cultivars in root to shoot ratio. The maximum increase of root to shoot ratio (0.57g) was observed at rice cultivar 'Putih Cempaka' at 30% PEG, while the minimum increase was at 'Mansur' with 20% PEG treatment (0.049g).

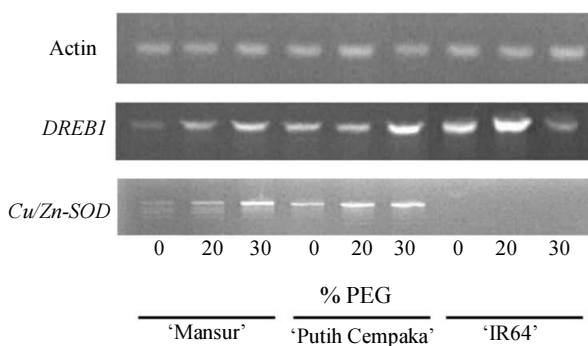
Table 3 showed chlorophyll content in 'Mansur', 'Putih Cempaka' and 'IR64' under drought stress. Statistical analysis showed that chlorophyll content under drought differed in rice cultivars. Plants treated with PEG have lower chlorophyll content than control plants. Treatment with 30% PEG resulted in the lowest chlorophyll content in all rice cultivars. The maximum

reduction of chlorophyll content was at 'Putih Cempaka' treated with 30% PEG (-7.277 mg/mL). Rice cultivar 'IR64' with PEG treatment showed the minimum reduction of chlorophyll both at 20% PEG and 30% PEG i.e., -1.768 and -2.929 mg/mL respectively.

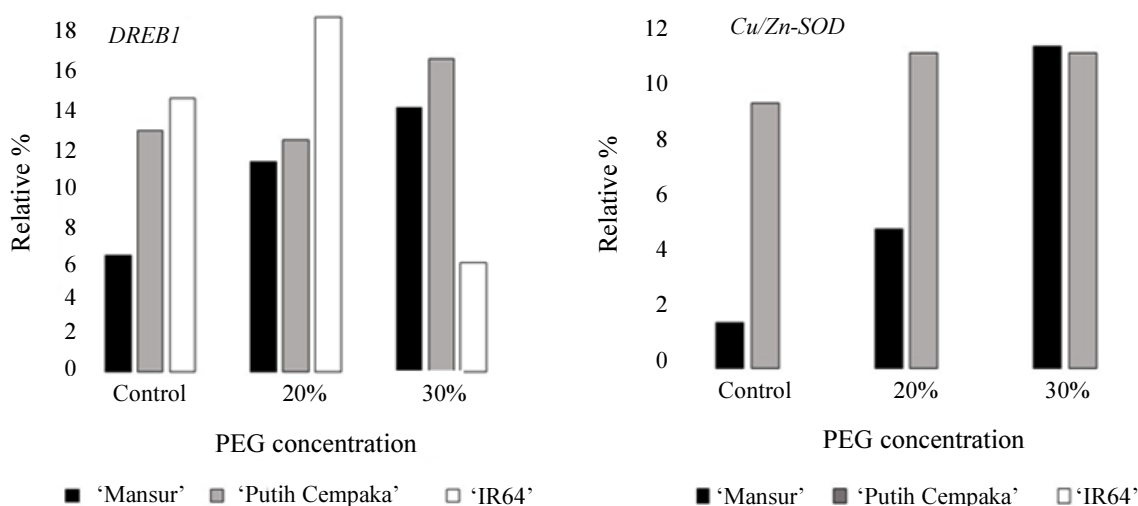
### Gene Expression under Drought

The expression of *SOD* gene increased under PEG stressed in 'Mansur' and 'Putih Cempaka', while in 'IR64', *SOD* was not expressed both in untreated and PEG-treated seedling (Fig. 1).

Both concentrations of PEG treatments increased expression of *DREB* gene in 'Mansur' and 'Putih Cempaka'. On the contrary, in 'IR64', the expression of *DREB* increased only at 20% PEG and decreased at 30% PEG. The histograms in Fig. 2 were the quantification of *SOD* and *DREB* expression using the ImageJ software.



**Fig. 1:** Expression of *Cu/Zn-SOD* and *DREB1* in Bali local rice cultivars 'Mansur', 'Putih Cempaka' and 'IR64' after PEG treatment. Actin was used as a transcriptional control gene



**Fig. 2:** Quantification of *DREB1* and *Cu/Zn-SOD* and Actin expression calculated using ImageJ software

**Table 1:** Root length after three days treatment with PEG

Treatment	Length (cm)		
	'Mansur'	'Putih Cempaka'	'IR64'
Control	10.93±0.176 <sup>a</sup>	12.43±0.426 <sup>a</sup>	8.23±0.426 <sup>a</sup>
PEG 20%	12.03±0.797 <sup>b</sup>	12.27±0.267 <sup>a</sup>	8.50±0.289 <sup>a</sup>
PEG 30%	12.50±0.252 <sup>b</sup>	12.03±0.77 <sup>a</sup>	6.83±0.167 <sup>b</sup>

Numbers are average from 3 replicates ± SD. Means with same letters in the same column are not significantly different

**Table 2:** Fresh weight root to shoot ratio after three days treatment with PEG

Treatment	Root to shoot Ratio (g)			
	'Mansur'	'Putih Cempaka'	'IR64'	Average
Control	0.438±0.017 <sup>a</sup>	0.407±0.030 <sup>a</sup>	0.254±0.042 <sup>c</sup>	0.366 <sup>A</sup>
PEG 20%	0.487±0.018 <sup>a</sup>	0.563±0.058 <sup>ab</sup>	0.316±0.020 <sup>c</sup>	0.455 <sup>B</sup>
PEG 30%	0.816±0.082 <sup>b</sup>	0.977±0.125 <sup>b</sup>	0.382±0.087 <sup>ac</sup>	0.725 <sup>C</sup>
Average	0.580 <sup>A</sup>	0.649 <sup>B</sup>	0.317 <sup>C</sup>	

Numbers are average from 3 replicates ± SD. Means with same letters are not significantly different

**Table 3:** Total chlorophyll content of rice cultivars after three days PEG treatments.

Treatment	Chlorophyll Content (mg/mL)			
	'Mansur'	'Putih Cempaka'	'IR64'	Average
Control	10.931±0.853 <sup>a</sup>	13.251±0.576 <sup>a</sup>	9.384±1.321 <sup>a</sup>	11.189 <sup>A</sup>
PEG 20%	8.355±1.258 <sup>ab</sup>	8.995±0.386 <sup>a</sup>	7.616±0.679 <sup>ab</sup>	8.322 <sup>B</sup>
PEG 30%	4.775±0.537 <sup>b</sup>	5.974±0.347 <sup>b</sup>	6.465±0.636 <sup>b</sup>	5.671 <sup>C</sup>
Average	8.020 <sup>A</sup>	9.41 <sup>B</sup>	7.821 <sup>A</sup>	

Numbers are average from 3 replicates ± SD. Means with same letters mean are not significantly different

## Discussion

Drought stress inhibits cell division and cell growth, which lead to inhibition of plant growth and development (Sarvestani *et al.*, 2008). Plant under drought stress develops physiological and genetic adaptation. The ratio of root to shoot increased in rice seedling that undergone drought stress. Bali local rice cultivars tested in this study had higher fresh weight root to shoot ratio as compared to 'IR64' both at PEG20% and PEG 30%. 'IR64' has the lowest root to shoot ratio, almost 1/3 of that in 'Putih Cempaka'. 'IR64' has been known as drought sensitive rice cultivar (Prakash *et al.*, 2016). The high root to shoot ratio is to increase water uptake (Xu *et al.*, 2015). Root characteristics become important for selection of rice cultivars (Baloch *et al.*, 2012). Thick and deep root is root characters that present in plant tolerant to drought stress.

Leaf colour in plant treated with PEG turned to be yellowish. Chlorophyll content decreased significantly under drought stress induced by PEG. Reduced chlorophyll content in drought stress rice seedling has been reported earlier (Dalal and Tripathy, 2012; Kadhimi *et al.*, 2016). The decrease was caused by lack of chlorophyll biosynthetic intermediates, due to down-regulation of gene expression of enzymes for chlorophyll biosynthesis (Dalal and Tripathy, 2012). Another explanation for

reduced chlorophyll content was chlorophyll degradation which leads to senescence (Chen *et al.*, 2015).

In this study, under 30% PEG, 'IR64' had the highest chlorophyll content (6.465 mg/ml) followed by 'Putih Cempaka' (5.974 mg/mL). It was demonstrated that high chlorophyll content is a desirable trait for screening drought tolerant due to low photosynthesis inhibition (Moaveni, 2011). Drought stress enhances the production of Reactive Oxygen Species (ROS) that cause stress oxidative in chloroplast and decrease chlorophyll content (de Carvalho, 2008). High ROS production functions as a signal for activation of a system for protection from oxidative damage (You and Chan, 2015).

The *DREB* gene used for this study was classified as the *DREB1* transcription factor (Pharmawati and Kurniasih, 2016). In this study, there was an increase in gene expression of *DREB1* as a response to drought stress. However, in 'IR64' the *DREB1* expression only increased at 20% PEG but decreased at 30% PEG indicating low oxidative defense at high concentration of PEG. The decrease of expression may be related to the characteristics of 'IR64' as drought susceptible cultivar. The increase of *DREB1* expression shows better tolerance to drought in several crop species including rice (Joshi *et al.*, 2016). Studies have shown that transgenic over-expression of *DREB1F* improve tolerance to drought stress induced by PEG 6000 and air-drying in rice (Wang *et al.*, 2008) and transgenic rice

that over-expressed *DREB1G* and *DREB1E* have a better rate of survival under drought (Chen *et al.*, 2008).

DNA sequence analysis of *SOD* gene used in this study revealed that the sequence had high similarity with *SODCc1* (cytosolic copper/zinc superoxide dismutase) gene (Pharmawati and Kurniasih, 2016). The increase of *SODCc1* expression was detected in 'Mansur' and 'Putih Cempaka', while in 'IR64' this gene was not detected. In this experiment, the *SOD* primer pair used was designed by Singh and Sadasivam (2003). The primer pair was reported to amplify a new isomer of *SOD* which does not present in drought-sensitive rice plant. Singh and Sadasivam (2003) shown that this isoform was absent in 'IR64'.

The induction of *SOD* expression indicates its important role in the defense mechanism of the plant (Ashraf and Ali, 2008). It was reported that in the vegetative stage of rice, the expression of *Cu/Zn-SOD* increased under drought both in drought-sensitive and drought-tolerant cultivar, however, the dominant expression was found in drought tolerant cultivar (de Deus *et al.*, 2015). The increase of *SOD* expression under drought has been reported in 'Situ Bagendit', a local rice cultivar from Indonesia (Refli *et al.*, 2014).

Morpho-physiological, as well as genetic responses of rice cultivar 'Mansur', 'Putih Cempaka' and 'IR64' evaluated in this study, provide information for the future breeding program to develop cultivar tolerant to water deficit. Based on morpho-physiological data, among two local rice cultivars tested, 'Putih Cempaka' showed the highest fresh weight root to shoot ratio and higher chlorophyll content. The expression of *DREB1* and *SOD* in 'Putih Cempaka' increased at 30% PEG treatment indicating the involvement of transcription factor to withstand drought stress as well as showing oxidative defense.

## Conclusion

Drought stress increased fresh weight root to shoot ratio and reduced chlorophyll content in rice cultivars 'Mansur', 'Putih Cempaka' and 'IR64'. The expression of *DREB1* and *SOD* increased in 'Mansur' and 'Putih Cempaka'. Further study in the field, need to be done to evaluate 'Mansur' and 'Putih Cempaka' production under drought stress. Furthermore, based on the root to shoot ratio as well as *DREB1* and *SOD*, 'Mansur' and 'Putih Cempaka' can be used as materials in rice breeding to develop drought-tolerant cultivars.

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## Author Contributions

**Made Pharmawati:** Designed the experiments, performed gene expression analyses and prepared manuscript.

**Luh Putu Wrsiati:** Performed morpho-physiological analyses and prepared manuscript.

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