Drought-induced production of reactive oxygen species and antioxidants activity of four local upland rice cultivars in Central Sulawesi, Indonesia

RUSLAN BOY[1,2,♥], DIDIK INDRADEWA[2], EKA TARWACA SUSILA PUTRA[2], BUDIASTUTI KURNIASIH[2]

[1]Assessment Institute for Agricultural Technology of Central Sulawesi. Jl. Palu-Kulawi Street Km.15, Watubula, Regency of Sigi, Central Sulawesi, Indonesia. Tel.: +62-451-482546, Fax.: +62-451-482549, ♥email: boyruslan73@yahoo.co.id
[2]Department of Agronomy, Faculty of Agriculture, Universitas Gadjah Mada. Jl. Flora, Bulaksumur, Sleman 55281, Yogyakarta, Indonesia

Abstract. *Boy R, Indradewa D, Putra ETS, Kurniasih B. 2020. Drought-induced production of reactive oxygen species and antioxidants activity of four local upland rice cultivars in Central Sulawesi, Indonesia. Biodiversitas 21: 2555-2565.* Drought can be the most severe threat to the production of crop in the world. This water deficit condition may cause a considerable decrease in yield grain of upland rice. Present study was conducted to figure out the level of ROS production and antioxidant activity on upland rice cultivars under drought stress. The experiment was performed using randomized completely block design with two factors, i.e. four cultivars of upland rice (Habo, Hiwanggu, Sunggul, and Lambara) and watering interval (once in one, two, four and eight days). The results showed that there was interaction between cultivars and watering intervals. Drought tolerant cultivars of Habo and Sunggul had higher activities of SOD, POD, AAred as well as α-Toch with lower content on free radicals of O_2^- and H_2O_2; whereas non-drought tolerant cultivars of Hiwanggu and Lambara exhibited higher production of O_2^- and H_2O_2 and lower level of SOD, POD, AAred as well as α-Toch. It could be concluded that the optimum soil moisture affecting maximum activities of SOD, POD, AAred as well as α-Toch and minimum content of O_2^- and H_2O_2 was around 15.88-27.56% in range which was equal to 50.88-87.38% of field capacity.

Keywords: Antioxidant, drought, *Oryza sativa*, reactive oxygen species

INTRODUCTION

Paddy is one of important cereal crops for human main food worldwide (Kumar et al. 2014). It may be planted in dryland. agroecosystem depending on water supply from rainfall and extremely susceptible to drought (Basu et al. 2017). Drought becomes one of the most serious constraints for crop production throughout the world (Kumar et al. 2018). This water deficit situation causes a considerable decline in yield grain of upland rice (Guimaraes et al. 2013). Drought stress interrupts the balance in the formation of reactive oxygen species (ROS) and activity of oxidative defense. When the formation of ROS exceeds the activity of cell oxidative defense, peroxidation rate of lipid membrane increases (Anjum et al. 2011). ROS is abundantly produced by crops under biotic and abiotic pressures (Atkinson and Urwin 2012). The effect of ROS accumulation can interrupt the enzymatic metabolism and cell structure causing plant death (Phung et al. 2011). ROS affecting oxidative stress are triplet ($3O_2$), singlet ($^1O_2$), anion superoxide (O_2^-), hydroxyl radical (^-OH), and hydrogen peroxide (H_2O_2) (Choudhury et al. 2017).

Drought-tolerant rice plants are characterized by the capability to adapt and tolerate the structure and biochemical traits through activation of oxidative defense mechanisms (Swapna and Shylaraj 2017). Unfavorable environmental conditions may enable the plant cell to activate the antioxidant protection systems (Jovicic et al. 2018). A set of mechanisms is found in plants to minimize the dangerous impacts (Hirayama and Shinozaki 2010; Hu and Xiong 2014; Xiong et al. 2018). Defense mechanism in the crop for restricting the damage caused by ROS is by increasing antioxidants, either enzymatic or non-enzymatic antioxidants. These antioxidants have a role in protecting the crop from damage due to oxidative stress. Enzymatic antioxidants are superoxide dismutase (SOD) and peroxide dismutase (POD), while non-enzymatic ones are ascorbic acid and alpha-tocopherol (Miller et al. 2010).

Central Sulawesi is one of provinces in Indonesia where most farmers cultivate local upland rice. Some cultivars can grow well in areas with limited rainfall. The developed upland rice is the genetic resource of the local area that has been cultivated for generations as a source of economic activity. It is also an important food crop to meet the need for rice as well as to support food security and sustainable agriculture. There is lack of information regarding enzymatic and non-enzymatic antioxidants on local cultivar upland rice of Central Sulawesi under drought stress. Therefore, this research was conducted to study drought defense mechanism on upland rice cultivars from Central Sulawesi correlating with the accumulation of ROS activity (such as O_2^- and H_2O_2), antioxidant of SOD and POD, reduced form of ascorbic acid (AAred) as well as alpha-tocopherol (α-Toch).

MATERIALS AND METHODS

Plant material

This study used four local upland rice cultivars from Palu, Central Sulawesi. The local upland rice cultivars were Habo, Sunggul, Hiwanggu, and Lambara. Habo cultivar representing high yield drought-tolerant, Sunggul for low yield drought-tolerant, Hiwanggu for high yield non-drought tolerant, and Lambara for low yield non-drought tolerant. These cultivars were commonly cultivated by local farmers in Central Sulawesi. They were selected based on screening of 20 cultivars generating those categories which were determined using stress sensitivity index (SSI).

Field experiment

The field experiment was performed in randomized completely block design in polyethylene house at Faculty of Agriculture, Universitas Gadjah Mada, from May until September 2018 (one season experiment). The altitude of research location was about 100 m asl. During growth period, climate condition was dry season, average temperature of 25.75°C as well as average humidity of 79.82%.

One seed of each local rice cultivars was directly sown in a polybag of 35×35 cm^2 in size containing 10 kg of entisol soil and fertilizer. The inorganic fertilizers dose was SP-36 with dose of 25 kg ha^{-1} (0.10 g P polybag^{-1}), Urea 280 kg ha^{-1} (1.07 g N polybag^{-1}), KCl 30 kg ha^{-1} (0.11 g K polybag^{-1}), and organic fertilizer were cattle dung about 2000 kg ha^{-1} (7.69 g polybag^{-1}).

Drought stress treatment

Treatment of drought stress was started from 16 days after sowing (das) to one week prior to harvest. The drought stress was applied by managing watering interval treatment consisted of once in one, two, four, and eight days which equal to soil moisture of 31.54%, 27.85%, 23.36%, and 13.41%, respectively. Soil moisture under field capacity was 33.34% and permanent wilting point was 8.14%.

Soil moisture was measured using gravimetric method with oven drying (ISRIC 1993). Five kilograms of air-dried soil samples were dried in the oven at 105°C for 3 hours. After cooled in desiccator the weight of oven-dried soil samples was measured. The loss of weight was noted as soil water content. The analysis was conducted at 12 weeks-old after planting in Laboratory of Soil Science, Universitas Gadjah Mada, Yogyakarta. The soil moisture was calculated with the following formula:

Water content (%) = (soil water content/weight of soil sample) $\times$ 100

Correction factor of water content (fk) = 100/(100 − water content)

Drought stress sensitivity index analysis

Tolerant indicator against drought stress was determined using drought sensitivity index:

Index of stress sensitivity (SSI) = $\dfrac{1 - Ys/Yp}{1 - Y\bar{s}/Y\bar{p}}$

Ys is yield of a cultivar on stress condition (watering in every eight days), Yp is yield of a cultivar under non-stress condition (watering every day), $Y\bar{s}$ is the average yield of all cultivars under stress condition, $Y\bar{p}$ is of the average yield of all cultivars under non-stress condition (Fernandez 1992). Criteria for a drought-tolerant cultivar is indicated by SSI value < 0.5 and moderate with 0.5 < SSI < 1 and sensitive with SSI > 1 (Fischer and Maurer 1978).

Free radicals and antioxidants analysis

Plants free radicals and antioxidants analysis were determined at early generative stage, i.e. 12 weeks after sowing (84 das). This analysis was carried out in Laboratory of Joined Research, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta. Free radicals and antioxidants were observed on the first leaf under rice flag leaf. The free radical groups observed in this study were O_2^- and H_2O_2 activities. Antioxidants content analyzed was superoxide dismutase (SOD), peroxide dismutase POD, reduced form of ascorbic acid (AAred), and alpha-tocopherol (α-Toch).

Superoxide

Superoxide was measured using method of Malecka et al. (2014); with some modifications. A 0.5 g of fresh leaves of paddy was ground and transferred into test tubes and added with 7 mL of 50 mM phosphate buffer (pH 7.8) containing 0.05% NBT and 10 mM NaN3. The mixture was incubated under dark conditions at room temperature for 5 min. Two milliliters of solution was heated in water bath at 85°C for 10 min and then cooled in ice for 5 min. The absorbance value of solution was analyzed at λ 589/580 nm. Level of O_2 was notified in a λ 580 g^{-1} fresh weight of samples.

Hydrogen peroxide

This analysis used method of Bouazizi et al. (2007); with some modifications. A 0.5 g of fresh leaves of paddy was ground, homogenized into cooled 5 mL 0.1% TCA and centrifuged under 12,000 rpm and 4°C for 15 min. Supernatant was collected to determine H_2O_2 content. Reaction mixture consisted of 0.5 mL of supernatant, 0.5 mL of 10 mM phosphate buffer (pH 7.0) and 1 mL of 1 M KI solution. Its value was measured at λ 390 nm. The H_2O_2 content was determined according to its standard curve (100-500 nmol mL^{-1}).

Superoxide dismutase

This parameter was analyzed using modified NBT method of Beyer and Fridovich (1987). A 0.2 g of fresh leaves of paddy was ground, homogenized with 2 mL of 50 mM phosphate buffer (pH 7.8) containing 2 mM EDTA, 9.9 mM L-methionine, 55 μM NBT and 0.025% Triton-X100. Twenty microliters of sample extract were collected and added with 20 μL of 1 mM riboflavin into reaction mixture, wrapped with aluminum foil, and then illuminated with 15 W fluorescent lamp at approximately 20 cm distance for 10 min. Similar sample extract was placed under dark condition and used as Blanco sample. Absorbance sample was immediately observed using

spectronic 21 D at 560 nm wavelength. The activity of SOD enzymes was notified with unit/g protein. Sample was determined using standard curve of pure SOD.

Peroxide dismutase

It was measured using method of Saravanan et al. (2004); with several modifications. Five grams of fresh leaves of paddy was ground and homogenized for 2 min in vortex with 0.1 M Na-phosphate buffer (pH 6.5) solution and the filtrate was filtered using filter paper. The collected filtrate was centrifuge at 5000 rpm and 4°C for 5 min. Five hundred microliter of supernatant was collected and reacted with 1.5 mL reagent (consisting of 10 mL of 0.5 M pyrogallol added with 15.2 mL of 0.066 M phosphate buffer and finalized with aquadest for final volume of 1000 mL). For cuvet sample, 500 µL of supernatant was reacted with 1.5 mL of reagent and 0.5 mL of 1% H_2O_2. All sample mixtures were vortexed, and their absorbance was analyzed using spectrophotometer at 420 nm wavelength.

Reduced form of ascorbic acid

This parameter was analyzed using method of Ribeiro et al. (2012); with required modifications. One gram of fresh leaves of paddy was ground and homogenized with 2 mL of cooled 5% TCA (w/v). Homogenate was centrifuged at 4,000 rpm and 4°C for 10 min. Supernatant was transferred into microtube and centrifuged at 12'000 rpm and 4°C for 20 min. One milliliter of extract was transferred into test tubes containing 1 mL of 200 nM phosphate buffer (pH 7.4), and then incubated in water bath at 42°C for 15 min. After incubation, 200 µL of 2% TCA (w/v), 200 µL of 8.4% H_3PO_4 (w/v), 200 µL of 0.8% 2.2 dipyridyl (w/v), and 0.3% $FeCl_3$ (w/v) were sequentially added into same test tubes. The mixture was homogenized using vortex for 5 s and its absorbance was observed at λ525 nm. The preparation of Blanco solution used a similar procedure to test solutions without any addition of leaves. The concentration of AAred was calculated using linear equation of standard curve which was created with the concentration of 0-5 nM AAred in 5% TCA 5% (w/v). The solution was injected with HPLC.

Alpha-tocopherol

This analysis used method of Baker et al. (1980); with required modifications. One gram of freeze-liquid nitrogen of paddy leaves was gently ground and homogenized with 20 mL of mixture of petroleum, ether, and ethanol (2: 1: 6). Homogenate was centrifuged twice at 4,000 rpm and 4°C for 20 min. One milliliter of supernatant was mixed with 200 µL of 0.8% 2.2 dipyridyl (w/v) in ethanol. The mixture was incubated under darkroom for 5 min. Its absorbance was viewed at λ525 nm. The preparation of Blanco solution used a similar procedure to test solutions without any addition of leaf extract. Concentration of α-Toch was calculated with linear equation of created standard curve. The solution was injected with HPLC.

Data analysis

The normality test of data was analyzed using the Kolmogorov test and Q-Q plot (Mocanda et al. 2014) if the data was not homogenous yet. Analysis of covariance was conducted to evaluate the effect of plant genotypes (local rice cultivars) and drought stress treatment on the reactive oxygen species (ROS) and antioxidants (Hinkelman and Kempthorne 2008). The relationship between ROS and antioxidants was analyzed using Pearson correlation (Bhattacharyya and Johnson 1977). All analysis was performed using the PROC GLM and PROC CORR in SAS 9.4 (SAS Institute 2013).

RESULTS AND DISCUSSION

Soil moisture

The correlation between watering interval and soil moisture indicated that the soil field capacity condition (pF 2.54) in this study had soil moisture content of 33.34% and permanent wilting point (pF 4.2) at soil moisture of 8.14% equal to field capacity of 24.42% (Figure 1).

The treatments of watering intervals affected the soil moisture content and field capacity in the experimental sites (Table 1). The available soil moisture and field capacity tended to decrease following the reduction of watering interval, while the decrease of soil moisture was getting higher from treatment of once in a day to once in eight days watering intervals.

Soil moisture is affected by some processes occurring in the land-atmosphere interface, including water infiltration, water flow, evaporation, heat and gas exchange, infiltration of soluble substances, erosion as well as soil texture (Garnaud et al. 2017). Soil moisture has important role in the cycle of terrestrial water and agriculture, under main applications such as the monitoring of crop growth and management of hydrogeology (Palombo et al. 2019).
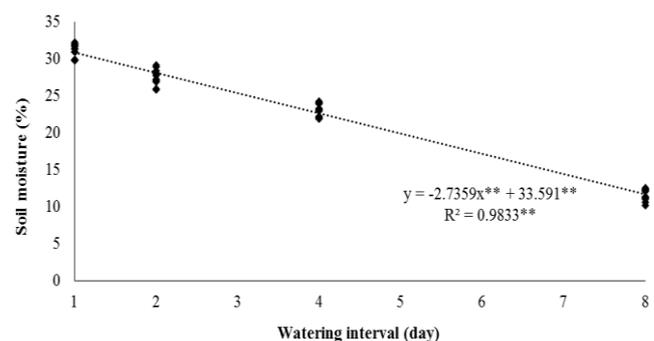

$$y = -2.7359x^{**} + 33.591^{**}$$
$$R^2 = 0.9833^{**}$$

Figure 1. Relationship between watering interval and soil moisture content

Table 1. Soil moisture contents with corresponding field capacity under various watering intervals

Treatment of watering interval	Available soil moisture (%)	Field capacity (%)	Decrease of soil moisture (%)
Once in one day	31.54	94.60	5.40
Once in two days	27.85	83.53	11.70
Once in four days	23.36	70.07	16.12
Once in eight days	13.41	40.22	42.59

Table 2. Index of stress sensitivity (SSI) of local upland rice cultivars based on yield parameter

Cultivar	Yield Hasil (g) per grove under four levels of soil moisture				SSI value	Category
	31.54 %	27.85 %	23.36 %	13.41 %		
Habo	27.83	29.52	31.54	25.69	0.30	Drought tolerant
Sunggul	14.57	15.92	17.03	13.78	0.48	Drought tolerant
Hiwanggu	24.41	24.84	26.14	9.50	1.51	Non-drought tolerant
Lambara	13.51	13.78	14.11	5.43	1.84	Non-drought tolerant

Stress sensitivity index

Analysis of stress sensitivity index (SSI) using yield parameter revealed that Habo and Sunggul cultivars had SSI value less than 0.5, referring to drought-tolerant cultivars (Table 2). Meanwhile, Hiwanggu and Lambara cultivars were categorized into non-drought tolerant cultivars since their SSI values were more than 1. Habo and Hiwanggu were considered as high-yield drought and non-drought tolerant cultivars with range of yield approximately 25.69-31.54 g per grove and 9.50-26.14 g per grove, respectively; while Sunggul and Lambara were grouped into low-yield drought and non-drought cultivars with the yield of 13.78-17.03 g per grove and 5.43-14.11 g per grove in range, respectively. Overall, the minimum and maximum yields were found on soil moisture levels of 13.41 and 23.36%, respectively. Various responses between drought and non-drought tolerant cultivars indicated the diversity in sensitivity level against drought stress.

Impact of drought on superoxide

There was an interaction between cultivar and soil moisture on the activity of O_2^- (p<0.01**) (Figure 2). In general, Habo and Sunggul cultivars belonging to drought-tolerant category formed lower O_2^- than that of Hiwanggu and Lambara categorized into non-drought tolerant cultivars under all soil moisture contents. On the early stage of stress treatment with soil moisture of 31.54-13.41%, drought-tolerant cultivars of Habo and Sunggul did not show the significant alteration in the O_2^- activity. The shift in O_2^- activity was not found on non-drought tolerant cultivars of Hiwanggu and Lambara under decrease of soil moisture around 31.54-23.36%. but it was recorded on the soil moisture of 13.41%. The increasing of O_2^- activity was 10.26, 8.00, 30.77, and 23.08% in Habo, Sunggul, Hiwanggu, and Lambara cultivars, respectively.

The results exhibited that the activities of O_2^- on drought-tolerant cultivars of Habo and Sunggul cultivar were lower than those on non-drought tolerant cultivars of Hiwanggu and Lambara, either under optimum or minimum soil moisture conditions (Table 3). The field capacity was also recorded higher on non-drought tolerant cultivars of Hiwanggu and Lambara than that on drought cultivars of Habo and Sunggul.
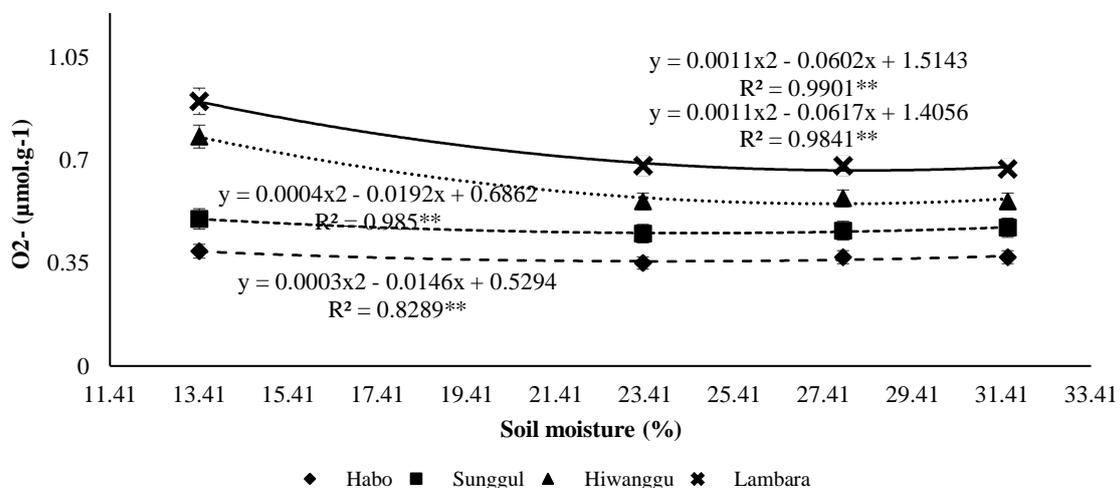

Figure 2. Relationship between soil moisture with activity of O_2^- on four cultivars of upland rice

Table 3. Activity of O_2^- on four local upland rice cultivars under optimum and minimum soil moisture conditions
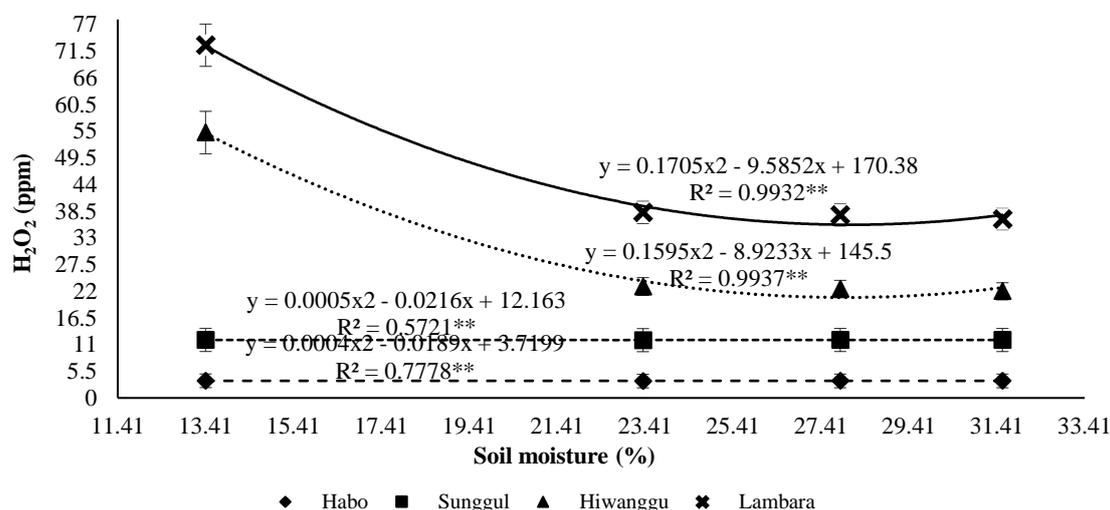
Cultivar	Optimum soil moisture (%)	Minimum soil moisture (%)	Field capacity (%)
Habo	13.41 (O_2^- maximum 0.39 $\mu mol.g^{-1}$)	24.34 (O_2^- minimum 0.35 $\mu mol.g^{-1}$)	77.17
Sunggul	13.41 (O_2^- maximum 0.50 $\mu mol.g^{-1}$)	24.00 (O_2^- minimum 0.46 $\mu mol.g^{-1}$)	76.09
Hiwanggu	13.41 (O_2^- maximum 0.54 $\mu mol.g^{-1}$)	28.04 (O_2^- minimum 0.78 $\mu mol.g^{-1}$)	88.90
Lambara	13.41 (O_2^- maximum 0.70 $\mu mol.g^{-1}$)	27.27 (O_2^- minimum 0.91 $\mu mol.g^{-1}$)	86.46

Different responses between drought-tolerant and non-drought tolerant cultivars on O_2^- activity under drought stress treatment due to the tolerant cultivars had capability in developing tolerant mechanism by suppressing O_2^- activity at all soil moisture level compared to drought-sensitive cultivars. The decrease of O_2^- activity on Hiwanggu and Lambara, the drought-sensitive cultivars, at soil moisture of 13.41% level was probably caused by the accumulation of toxic O_2^- in target cells so that the rolling enzymes in antioxidative system was failure to control the rate of O_2^- activity under secure level. Previous studies documented that plants had antioxidative defense mechanisms to control number of ROS in safe range, consisting of antioxidant enzymatic and antioxidant non-enzymatic systems (Atkinson and Urwin 2012). Drought stress would induce oxidative stress in plant cells due to high electron-leakage reducing O_2 during photosynthesis and respiration process so that ROS was increasing (Murshed et al. 2013).

Effects of drought on hydrogen peroxide

Generally, drought-tolerant cultivars, Habo, and Sunggul, formed a lower H_2O_2 than those of non-drought tolerant cultivars, Hiwanggu and Lambara, at all soil moisture levels (p<0.01**) (Figure 3). In Habo and Sunggul cultivars, the alteration of soil moisture at all treatment levels did not cause a significant shift in H_2O_2 activity. Contrarily, the reduction of soil moisture from 31.54% to 13.41% affected the significant increase of H_2O_2 activity on Hiwanggu and Lambara cultivars. The increasing of H_2O_2 activity was 1.13, 0.25, 62.03, and 50.81% in Habo, Sunggul, Hiwanggu, and Lambara cultivars, respectively.

The results recorded higher H_2O_2 activities on non-drought tolerant cultivars of Hiwanggu and Lambara than drought-tolerant cultivars of Habo and Siwanggu, either under optimum or minimum soil moisture conditions (Table 4). Higher field capacity was documented on non-drought tolerant cultivars of Hiwanggu and Lambara than drought-tolerant cultivars of Habo and Siwanggu.

Figure 3. Relationship between soil moisture and activity of H_2O_2 on four cultivars of upland rice

Table 4. Activity of H_2O_2 on four local upland rice cultivars under optimum and minimum soil moisture conditions

Cultivar	Optimum soil moisture (%)	Minimum soil moisture (%)	Field capacity (%)
Habo	13.41 (H_2O_2 maximum 3.54 ppm)	23.63 (H_2O_2 minimum 3.50 ppm)	74.92
Sunggul	13.41 (H_2O_2 maximum 11.96 ppm)	21.60 (H_2O_2 minimum 11.93 ppm)	64.48
Hiwanggu	13.41 (H_2O_2 maximum 54.52 ppm)	27.97 (H_2O_2 minimum 20.70 ppm)	88.68
Lambara	13.41 (H_2O_2 maximum 72.50 ppm)	28.11 (H_2O_2 minimum 35.66 ppm)	89.12

The increment of H_2O_2 activity on Hiwanggu and Lambara under reduction of soil moisture was caused by low capability of antioxidant defense system in controlling ROS activity at secure level under drought stress so that ROS activity in the cell was getting higher and the availability of antioxidant was getting lower due to its oxidized form. Prior works explained that higher rate of ROS formation than its degradation rate generated oxidative stress conditions (Miller et al. 2010). Hydrogen peroxide (H_2O_2) in the plant cells can have a role as signaling molecules i.e. the defense against the stress, hormonal responses, and growth, as well as development. When exposed to environmental stress conditions, H_2O_2 production will increase. However, excessive accumulation of H_2O_2 will be toxic, thus its activity must be strictly controlled by the plants (Sharma et al. 2012). Kalanamak 3131 cultivar with drought treatment shows 47% increase of H_2O_2 concentration in comparison to without drought treatment (Shukla et al. 2012).

Role of superoxide dismutase under drought

There was interaction between drought stress treatment/soil moisture and cultivar in SOD activity (p<0.01**) (Figure 4). The activities of SOD were found higher on drought-tolerant cultivars of Habo and Sunggul than those on non-drought tolerant cultivars of Hiwanggu and Lambara under all soil moisture levels.

The reduction of soil moisture from 31.54% to 23.36% on Habo and Sunggul cultivars exhibited significant increase in SOD activity. There was no change in SOD activity under further soil moisture decrease. In contrast, the decrease of soil moisture from 31.54% to 23.26% on Hiwanggu and Lambara cultivars did not indicate the alteration in SOD activity yet. The significant decrease in SOD activity was found under further reduction of soil moisture. The activities of SOD subsequently decreased in the Habo, Sunggul, Hiwanggu, and Lambara cultivars approximately 4.48%, 2.79%, 73.13%, and 81.45%, respectively.

The results revealed that activity of SOD was higher on drought-tolerant cultivars of Habo and Sunggul than that on non-drought tolerant cultivars of Hiwanggu and Lambara, either under optimum or minimum soil moisture conditions (Table 5). Meanwhile, higher field capacity was recorded on non-drought tolerant cultivars of Hiwanggu and Lambara than that on drought-tolerant cultivars of Habo and Sunggul.
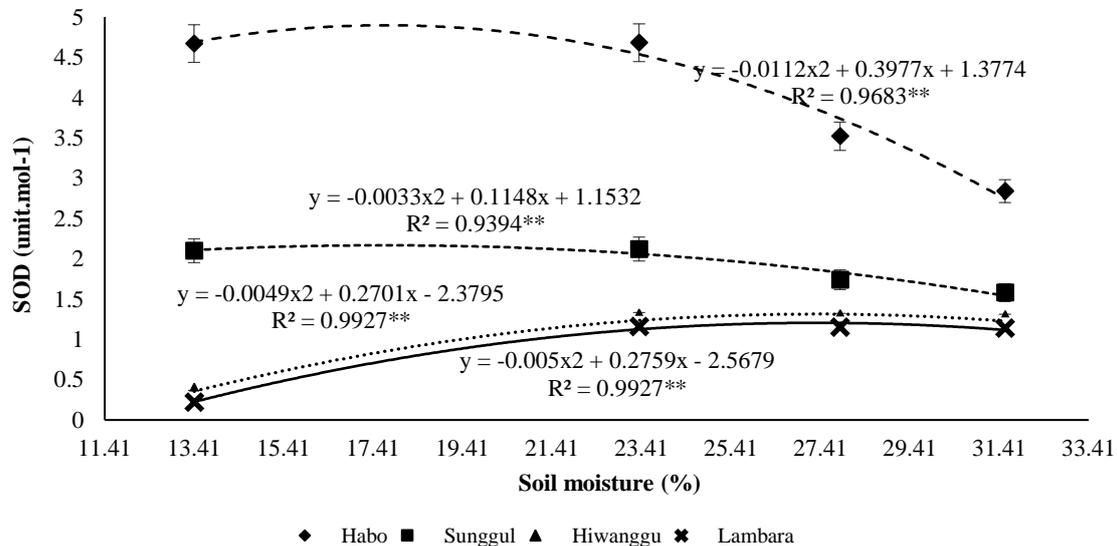

Figure 4. Relationship between soil moisture with activity of SOD on four cultivars of upland rice

Table 5. Activity of SOD on foul local upland rice cultivars under optimum and minimum soil moisture conditions

Cultivar	Optimum soil moisture (%)	Minimum soil moisture (%)	Field capacity (%)
Habo	17.76 (SOD maximum 4.91 unit.mL^{-1})	13.41 (SOD minimum 4.69 unit.mL^{-1})	56.31
Sunggul	17.40 (SOD maximum 2.15 unit.mL^{-1})	13.41 (SOD minimum 2.09 unit.mL^{-1})	55.17
Hiwanggu	27.56 (SOD maximum 1.34 unit.mL^{-1})	13.41 (SOD minimum 0.36 unit.mL^{-1})	87.38
Lambara	27.59 (SOD maximum 1.24 unit.mL^{-1})	13.41 (SOD minimum 0.23 unit.mL^{-1})	87.47

Various responses in SOD activity on drought-tolerant cultivars of Habo and Sunggul as well as non-drought tolerant cultivars of Hiwanggu and Lambara under established soil moisture due to the different mechanisms in oxidative defense systems developed by each cultivar to anticipate negative impact generated by ROS during drought stress period. Therefore, different patterns in alteration of SOD activity among cultivars indicated that their tolerance level against drought stress was determined by the increment in SOD activity. Other investigations summarized that the increase of antioxidant synthesis under stress conditions was extremely varied among species and even cultivars within one plant species (Shao et al. 2008). The SOD activity of drought-tolerant Jin23B rice powder increases after six days of drought stress treatment in comparison to the control plants. On the other hand, drought intolerant 97B rice shows earlier increasing of SOD activity after the third day of drought stress and decreasing activity on the sixth day of drought treatment in comparison to the control plant (Fu et al. 2010). The sensitivity of each cultivar can be different in drought stress depending on the resilience character of each cultivar (Cabello et al. 2013).

Role of peroxide dismutase under drought

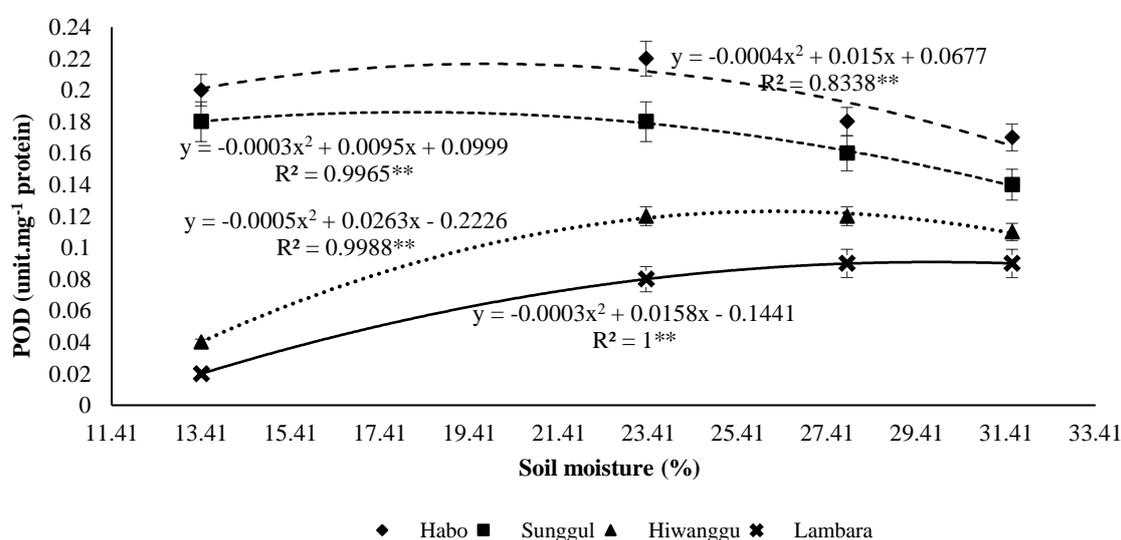Interaction between different rice cultivars and soil moisture has occurred against POD activity (p<0.01**)

(Figure 5). The drought-tolerant cultivars, Habo and Sunggul, formed higher POD than those of non-drought tolerant cultivars of Hiwanggu and Lambara under all soil moisture levels.

The reduction of soil moisture from 31.54% to 23.36% on drought-tolerant cultivars of Habo and Sunggul generated a significant increment in POD activity. However, there was no significant alteration in POD activity under further reduction of soil moisture at 13.41% level. Contrarily, there was significant alteration in POD activity on non-drought tolerant cultivars of Hiwanggu and Lambara under reduction of soil moisture from 31.54% to 23.36%: however further decrease of soil moisture at 13.41% level caused significant reduction in POD activity. The decreases in POD activity in the Habo, Sunggul, Hiwanggu, and Lambara cultivars were 9.52%, 5.56%, 66.67%, and 83.33%, respectively.

The results found that drought-tolerant cultivars of Habo and Sunggul had higher activities of POD than those on non-drought cultivars of Hiwanggu and Lambara, either under optimum or minimum soil moisture conditions (Table 6). However, the higher field capacity was documented on non-drought cultivars of Hiwanggu and Lambara under such conditions.

In the figure, the following regression equations are shown:

$y = -0.0004x^2 + 0.015x + 0.0677$
$R^2 = 0.8338**$

$y = -0.0003x^2 + 0.0095x + 0.0999$
$R^2 = 0.9965**$

$y = -0.0005x^2 + 0.0263x - 0.2226$
$R^2 = 0.9988**$

$y = -0.0003x^2 + 0.0158x - 0.1441$
$R^2 = 1**$

Figure 5. Relationship between soil moisture with POD activity on four cultivars of upland rice

Table 6. Activity of POD on four local upland rice cultivars under optimum and minimum soil moisture conditions

Cultivar	Optimum soil moisture (%)	Minimum soil moisture (%)	Field capacity (%)
Habo	18.78 (POD maximum 0.21 unit.mg^{-1}.protein)	13.41 (POD minimum 0.19 unit.mg^{-1}.protein)	59.54
Sunggul	15.88 (POD maximum 0.18 unit.mg^{-1}.protein)	13.41 (POD minimum 0.17 unit.mg^{-1}.protein)	50.35
Hiwanggu	26.30 (POD maximum 0.12 unit.mg^{-1}.protein)	13.41 (POD minimum 0.04 unit.mg^{-1}.protein)	83.39
Lambara	26.34 (POD maximum 0.06 unit.mg^{-1}.protein)	13.41 (POD minimum 0.01 unit.mg^{-1}.protein)	85.51

At the early treatment until 23.36%, drought-tolerant cultivars were stimulated to develop metabolic activity since the presence of ROS activity compared to non-tolerant cultivars. Following further reduction of soil moisture at 13.41%, the decrease POD activity on non-drought tolerant cultivars was caused by higher required antioxidant POD enzymatic to reduce over-expression of ROS activity. Former work noted that POD enzyme was widely allocated in plant cells and as important protein in ROS detoxification in chloroplast (Foyer and Shigeoka 2011). POD enzymes play a major role in reducing the H_2O_2 accumulated activity in cells (Ahmad and Haddad 2011). Research on drought-treated potato plants showed higher POD activity in comparison to the control plants (Wegener and Jansen 2013).

Role of AAred under drought

Figure 6 showed the occurrence of interaction between cultivar with soil moisture against activity of AAred ($p<0.01$**). The results revealed that drought-tolerant cultivars of Habo and Sunggul produced higher AAred than those of non-drought tolerant cultivars of Hiwanggu and Lambara under all soil moisture levels.

The reduction of soil moisture from 31.54% to 23.36% on Habo and Sunggul cultivars resulted in a significant increase of AAred activity. In contrast, there was no alteration in AAred activity on Hiwanggu and Lambara cultivars under similar conditions of soil moisture. The

decrease of AAred activity was documented on all cultivars under further reduction of soil moisture at 13.41% level. The activities of AAred in Habo, Sunggul, Hiwanggu, and Lambara reduced by 10.02%, 14.32%, 56.44%, and 54.81%, respectively.

The results exhibited that the activities of AAred were higher on drought-tolerant cultivars of Habo and Sunggul than those on non-drought tolerant cultivars of Hiwanggu and Lambara either under optimum or minimum soil moisture conditions (Table 7). Meanwhile, the higher field capacity was found on non-drought cultivars of Hiwanggu and Lambara.

AAred activity under reduction of soil moisture up to 23.26% in Habo and Sunggul cultivars indicating that these cultivars had better tolerance capability against drought rather than Hiwanggu and Lambara cultivars. The activity of AAred was one of developed mechanisms to overcome the prevalence of oxidative stress. Meanwhile, the reduction of AAred activity on all cultivars following the decline of soil moisture at 13.41% was presumed due to there was increment in antioxidant requirement in reducing the increasing activity of free radicals of cell during drought stress period. Previous study reported that the Ascorbic acid could directly neutralize O^- dan H_2O_2 or as substrate for APX enzyme in AA-GSH cycle (Szarka et al. 2012). AAred is an abundant antioxidant in the plant functionating to prevent cell damage due to ROS by donating electrons during drought stress (Gill and Tuteja 2010).
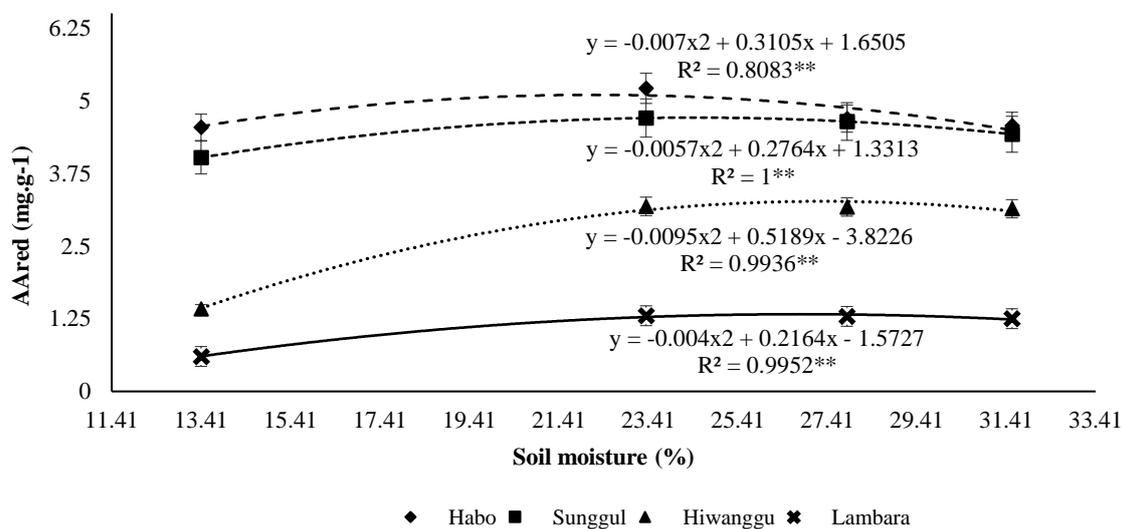

Figure 6. Relationship between soil moisture and activity of AAred on four cultivars of upland rice

Table 7. Activity of AAred on four local upland rice cultivars under optimum and minimum soil moisture conditions

Cultivar	Optimum soil moisture (%)	Minimum soil moisture (%)	Field capacity (%)
Habo	22.17 (AAred maximum 5.09 mg.g⁻¹)	13.41 (AAred minimum 4.56 mg.g⁻¹)	70.29
Sunggul	24.25 (AAred maximum 4.68 mg.g⁻¹)	13.41 (AAred minimum 4.01 mg.g⁻¹)	76.89
Hiwanggu	27.31 (AAred maximum 3.26 mg.g⁻¹)	13.41 (AAred minimum 1.42 mg.g⁻¹)	86.59
Lambara	27.06 (AAred maximum 1.35 mg.g⁻¹)	13.41 (AAred minimum 0.61 mg.g⁻¹)	85.80

Role of alpha-tocopherol under drought

There was interaction between cultivar with soil moisture on the activity of α-Toch (p<0.01**) (Figure 7). The results revealed that drought-tolerant cultivars of Habo and Sunggul formed higher α-Toch than those of non-drought tolerant cultivars of Hiwanggu and Lambara under all soil moisture levels.

The decrease of soil moisture from 31.54% to 23.36% on Habo and Sunggul cultivars showed a significant increase in α-Toch activity. Contrarily, there was no alteration in α-Toch activity under similar soil moisture levels on Hiwanggu and Lambara cultivars. The reduction of α-Toch activity was recorded on all cultivars under further decrease of soil moisture at 13.41% level. The decreases of α-Toch activity were recorded in Habo, Sunggul, Hiwanggu, and Lambara cultivars around 24.41%, 39.68%, 67.62%, and 76.29%, respectively.

The results showed that higher activities of α-Toch were found on drought-tolerant cultivars of Habo and Sunggul (Table 8). Meanwhile, field capacity was higher on non-drought tolerant cultivars of Hiwanggu and Lambara.
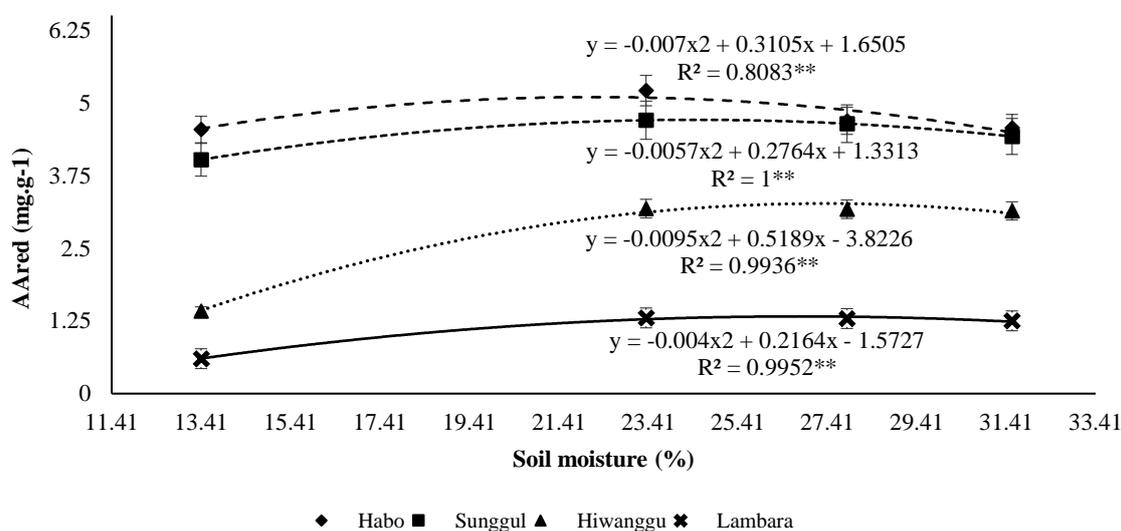

Figure 7. Relationship between soil moisture and activity of α-Toch on four cultivars of upland rice

Table 8. Activity of α-Toch on four local upland rice cultivars under optimum and minimum soil moisture conditions

Cultivar	Optimum soil moisture (%)	Minimum soil moisture (%)	Field capacity (%)
Habo	22.56 (α-Toch maximum 1.70 mg.g^{-1})	13.41 (α-Toch minimum 1.20 mg.g^{-1})	71.53
Sunggul	24.70 (α-Toch maximum 1.26 mg.g^{-1})	13.41 (α-Toch minimum 0.76 mg.g^{-1})	78.31
Hiwanggu	27.09 (α-Toch maximum 1.05 mg.g^{-1})	13.41 (α-Toch minimum 0.34 mg.g^{-1})	85.89
Lambara	27.56 (α-Toch maximum 0.97 mg.g^{-1})	13.41 (α-Toch minimum 0.23 mg.g^{-1})	87.38

Table 9. Correlation between free radical groups with antioxidants

	O_2^-	H_2O_2	SOD	POD	AAred	α-Toch
O_2^-	1					
H_2O_2	0.985**	1				
SOD	-0.838**	-0.789**	1			
POD	-0.943**	-0.941**	0.867**	1		
AAred	-0.949**	-0.932**	0.765**	0.921**	1	
α-Toch	-0.897**	-0.874**	0.807**	0.849**	0.801**	1

Note: ** correlation at α level = 0.01%.

The local drought-tolerant cultivars, Habo and Sunggul, had higher tolerance level against drought stress than Hiwanggu and Lambara cultivars due to the formation of higher α-Toch activity. The similar responses revealed by all cultivars under reduction of soil moisture at 13.41% and causing the decrease of α-Toch activity illustrated high use of that antioxidant to support functions in oxidative defense system under drought stress condition and the decrease of biosynthesis rate in α-Toch during drought stress due to ROS accumulation exceeding tolerance level of oxidative defense in a cultivar under given soil moisture level. The role of α-Toch under drought stress had been proven on previous research that furthermore, α-Toch could functionate to reduce ROS molecules, particularly O_2^- and OH, under stress condition as well as might inhibit the process of lipid peroxidation process on photosynthetic membrane (Li et al. 2008). α-tocopherol functioned in maintaining homeostasis of cellular Na^+/K^+ and hormonal balance in the plant (Ellouzi et al. 2013). Accumulation of α-tocopherol indicated the presence of increment in relative water content, photosynthesis rate, and the reduction in membrane damage under oxidative stress (Ezpinosa et al. 2013).

Correlation among variables

There were significant negative correlations between antioxidant groups activity with ROS activity (Table 9). Generally, the increment of antioxidant activity caused a decrease in ROS activity. The increase in the SOD and POD enzyme activities as well as AAred and α-Toch significantly reduced O_2^- and H_2O_2. Drought tolerant cultivars of Habo and Sunggul were characterized with high antioxidant activities and low free radicals, whereas those of non-drought tolerant cultivars, Hiwanggu and Lambara, were indicated by low antioxidant and high free radicals.

Reactive oxygen species production will disturb the homeostasis of cell growth, depending on the quantity of ROS production. Exceeding ROS production antioxidant capacity leads cell to oxidative stress. Conversely, lower or balance ROS production with antioxidant capacity will enable cell growth. Pandev and Shukla (2015) stated that the increased activity of antioxidant defensive enzymes can represent the protective activities of the rice plants to cope with oxidative damage caused by drought conditions.

Drought tolerant cultivars of Habo and Sunggul had higher activities of SOD, POD, AAred as well as α-Toch than those in Hiwanggu and Lambara, causing lower content of free radicals such as O_2^- and H_2O_2 in the tolerant cultivars than those of non-drought tolerant cultivars.

In conclusion, the developed drought resistance of tested four upland rice cultivars was tolerance mechanism through the improvement of antioxidant defense system consisting of SOD, POD, AAred, and α-Toch to prevent the reaction of free radicals of O_2^- and H_2O_2 under drought stress condition. The optimum soil moisture causing maximum activities of SOD, POD, AAred, and α-Toch as well as minimum content of O_2^- and H_2O_2 was the range of 15.88-27.56% equal to field capacity of 50.88-87.38%.

REFERENCES

Ahmad ST, Haddad R. 2011. Study of silicon effects on antioxidant enzyme activities and osmotic adjustment of wheat under drought stress. Czech J Genet Plant Breed 47: 17-27.

Anjum SA, Saleem MF, Wang L, Bilal FM, Saeed A. 2012. Protective role of glycine betaine in maize against drought-induced lipid peroxidation by enhancing capacity of antioxidative system. Aust J Crop Sci 6: 576-583.

Atkinson NJ, Urwin PE. 2012. The interaction of plant biotic and abiotic stress: from genes to the field. J Exp Bot 63: 3523-3554.

Bhattacharyya GK, Johnson RA. 1977. Statistical Concepts and Methods. John Wiley and Sons Inc, USA.

Baker H, Frank O, De-Angelis B, Feingold S. 1980. Plasma tocopherol in man at various times after ingesting free or acetylated tocopherol. Nutr Rep Intl 21: 531-536.

Basu S, Giri RK, Benazir I, Kumar S, Rajwanshi R, Dwivedi SK, Kumar G. 2017. Comprehensive physiological analyses and reactive oxygen species profiling in drought-tolerant rice genotypes under salinity stress. Physiol Mol Biol Plants 23 (4): 837-850.

Beyer WF, Fridovich I. 1987. Assaying for superoxide dismutase activity: some large consequences of minor changes of conditions. Anal Biochem 161: 559-566.

Bouazizi H, Jouili H, Ferjani EE. 2007. Effects of copper excess on growth, H_2O_2 production, and peroxidase activities in maize seedlings (*Zea mays* L.). Pak J Biol Sci 10 (5): 751-756.

Cabello JV, Lodeyro AF, Zurbriggen MD. 2013. Novel perspectives for the engineering of abiotic stress tolerance in plants. Curr Opin Biotechnol 26: 62-70.

Choudhury FK, Rivero RM, Blumwald E, Mittler R. 2017. Reactive oxygen species, abiotic stress and stress combination. Plant J 90: 856-867.

Ellouzi H, Hamed KB, Cela J, Müller M, Abdelly C, Munné-Bosch S. 2013. Increased sensitivity to salt stress in tocopherol deficient Arabidopsis mutants growing in a hydroponic system. Plant Signal Behav 8 (2): e23136-1 - e23136-13.

Ezpinoza A, Martín AS, López-Climent M, Ruiz-Lara S, Gómez-Cadenas A, Casaretto JA. 2013. Engineered drought-induced biosynthesis of α-tocopherol alleviates stress-induced leaf damage in tobacco. J Plant Physiol 170: 1285-1294.

Fernandez GCJ. 1992. Effective selection criteria for assessing stress tolerance. In: Kuo C.G (Ed.) Proceedings of the international symposium on adaptation of vegetables and other food crops in temperature and water stress. Publication Tainan Taiwan.

Fischer RA, Maurer R. 1978. Drought resistance in spring wheat cultivars. I grain yield responses. Aust J Agric Res 29: 897-912.

Foyer CH, Shigeoka S. 2011. Understanding oxidative stress and antioxidant functions to enhance photosynthesis. Plant Physiol 155: 93-100.

Fu GF, Song J, Li YR, Yue MK, Xiang J, Tao LX. 2010. Alterations of panicle antioxidant metabolism and carbohydrate content and pistil water potential involved in spikelet sterility in rice under water-deficit stress. Rice Sci 17 (4): 303-310.

Garnaud C, Bélair S, Carrera M, McNairn H, Pacheco A. 2017. Field-scale spatial variability of soil moisture and L-band brightness temperature from land surface modeling. J Hydrometeorol 18: 573-589.

Gill SS, Tuteja N. 2010. Reactive oxygen species and antioxidant machinery in abiotic stress. Plant Physiol Biochemist 48: 909-930.

Guimarães CM, Stone LF, Rangel PHN, Silva ACL. 2013. Tolerance of upland rice genotypes to water deficit. Revista Brasileira de Engenharia Agrícola e Ambiental 17 (8): 805-810.

Hinkelman K, Kempthorne O. 2008. Design and Analysis of Experiments. 2nd eds. John Wiley and Sons, USA.

Hirayama T, Shinozaki K. 2010. Research on plant abiotic stress responses in the post-genome era: past, present and future. Plant J 61: 1041-1052.

Hu H, Xiong L. 2014. Genetic engineering and breeding of drought-resistant crops. Annu Rev Plant Biol 65: 715-741.

ISRIC. 1993. Procedures for Soil Analysis. In: Van Reeuwijk LP (eds) Technical paper, international soil reference and information centre 4th ed. Wageningen The Netherlands.

Jovicic D, Nikolic Z, Mikic A, Milosevic B, Milojevic D, Ignjatov M, Marinkovic D. 2018. Antioxidant enzymes activities in Vicia seedlings during drought stress. Ratar Povrt 55 (1): 1-5.

Kumar A, Dixit S, Ram T, Yadaw RB, Mishra KK, Mandal NP. 2014. Breeding high-yielding drought-tolerant rice: genetic variations and conventional and molecular approaches. J Exp Bot 65 (21): 6265-6278.

Kumar S, Sachdeva S, Bhat KV, Vats S. 2018. Plant responses to drought stress: Physiological, biochemical and molecular basis. In: Sharad V (eds) Biotic and abiotic stress tolerance in plants. Springer Nature Singapore Pte Ltd, Singapore.

Li Y, Wang Z, Sun X, Tang K. 2008. Current opinions on the functions of tocopherol based on the genetic manipulation of tocopherol biosynthesis in plants. J Integr Plant Biol 50 (9): 1057-1069.

Malecka A, Piechalak A, Zielinska B, Kutrowska A, Tomaszewska B. 2014. Response of the pea roots defense systems to the two element combinations of metals (Cu, Zn, Cd, Pb). Acta Biochimica Polonica 61 (91): 23-28.

Miller G, Suzuki N, Ciftci-Yilmaz S, Mittler R. 2010. Reactive oxygen species homeostatic and signalling during drought and salinity stresses. Plant Cell Environ 33: 453-467.

Mocanda MP, Gabriels D, Cornelis WM. 2014. Data-driven analysis of soil quality parameters using limited data. Geoderma 235-236: 271-278.

Murshed R, Lopez-Lauri F, Sallanon H. 2013. Effect of water stress on antioxidant systems and oxidative parameters in fruits of tomato (*Solanum lycopersicon* L, cv. Micro-tom). Physiol Mol Biol Plants 19 (3): 363-378.

Palombo A, Pascucci S, Loperte A, Lettino A, Castaldi F, Muolo MR, Santini F. 2019. Soil moisture retrieval by integrating TASI-600 airborne thermal data, worldview 2 satellite data and field measurements: Petacciato case study. J Sensors 19 (1515): 1-19.

Pandey V, Shukla A. 2015. Acclimation and tolerance strategies of rice under drought stress. Rice Sci 22 (4): 147-161.

Phung TH, Jung HI, Park JH, Kim JG, Back K, Jung S. 2011. Porphyrin biosynthesis control under water stress: sustained porphyrin status correlates with drought tolerance in transgenic rice. Plant Physiol 157: 1746-1764.

Ribeiro C, Cambraia J, Henrique P, Peixoto PHP, Meira E, Júnior F. 2012. Antioxidant system response induced by aluminium in two rice cultivars. Braz J Plant Physiol 24 (2): 107-116.

Saravanan T, Bhaskaran R, Muthusamy M. 2004. Pseudomonas fluorescens induced enzymological changes in banana roots (Cv. Rasthali) against *fusarium* wilt disease. Plant Pathol J 3 (2): 72-80.

SAS Institute. 2013. SAS System for Windows 9.4. United States North Carolina SAS Institute Inc.

Shao HB, Chu LY, Shao MA, Jaleel CA, Mi HM. 2008. Higher plant antioxidants and redox signalling under environmental stresses. Comp Rend Biol 331: 433-441.

Sharma P, Jha AB, Dubey RS, Pessarakli M. 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. J Bot 1-26.

Shukla N, Awasthi RP, Rawat L, Kumar J. 2012. Biochemical and physiological responses of rice (*Oryza sativa* L.) as influenced by *Trichoderma harzianum* under drought stress. Plant Physiol Biochemist 54: 78-88.

Swapna S, Shylaraj KS, 2017. Screening for osmotic stress responses in rice cultivars under drought conditions. Rice Sci 24 (5): 253-263.

Szarka A, Tomasskovics B, Benhegyi G. 2012. The ascorbate-glutathione-α-tocopherol triad in abiotic stress response. Int J Mol Sci 13: 4458-4483.

Wegener CB, Jansen G. 2013. Antioxidants in different potato genotypes: Effect of drought and wounding stress. Agriculture 3: 131-146.

Xiong H, Yu J, Miao J, Li J, Zhang H, Wang X, Liu P, Zhao Y, Jiang C, Yin Z, Li Y, Guo Y, Fu B, Wang W, Li Z, Ali J, Lia Z. 2018. Natural variation in *OsLG3* increases drought tolerance in rice by inducing ROS scavenging. Plant Physiol 178: 451-467.