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Analysis of polyethylene glycol (PEG) and proline to evaluate drought stress of double haploid new type upland rice lines

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Abstract The characterization and selection of rice were evaluated for tolerant to drought stress. The selection process of double haploid lines, especially related to drought tolerance, was done by observing the morphological features on the root system in each genotype. The polyethylene glycol (PEG) solution in the planting medium is created the stress condition because of the water availability for plants reduced. Molecular size and the concentration of PEG determined the osmotic potential. The defense mechanisms used in plants on drought stress is the accumulation of proline to adjust osmotic, production and accumulation of free amino acids like proline in plant tissues during drought stress, an adaptation response in these conditions. The result showed that PEG 6000 inhibited the germination (33.9 percent), root length (60.8 percent), and shoot length (80 percent) of upland rice lines. Drought stress treatment (60 per cent of field capacity) at the flowering period showed a non-significant reduction in the growth of doubled haploid upland rice but reduced the weight of grains per hill (52.11 percent). Drought stress decreased in total chlorophyll (20.7 μmol/cm) and increased proline content in leaves (30.3 μmol/g). The content of proline in the leaves varied in inbreds due to drought stress. The high contained proline of tolerant genotype based on PEG 6000 are P3-31, followed by P6-95, respectively 30.33, 20.82 μmol/g, and genotype moderate line P6-291 at 20.42 μmol/g. Stress drought led to a decrease in total chlorophyll, and increase the proline content in the leaves.

Keywords: Drought, Doubled haploid, Upland rice, Polyethyleneglycol, Proline

Introduction

Upland rice varieties is still very low, due to lack of availability of multi tolerant rice varieties. The problem of increased upland rice production caused by the constraints of physical, biological, and socio-economic. Land cultivation

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is generally reacted sourly with high Al saturation, in addition to the frequent droughts and nutrient deficiency. The characters of upland rice desirable for such a physical condition is early harvesting to medium, medium tillers, preferably an erect stem, blast resistance, and Al tolerant, drought and shade (Peng *et al*., 2008; Herawati *et al*., 2010 and Hairmansis *et al*., 2016). The development of upland rice faced very complex obstacles, so it needs to repair the high-yielding varieties with multi tolerant characters of the biophysical factors in dryland. Anticipate the effects of climate change on sustainable agricultural systems; various efforts are made to produce technological innovations that are expected to overcome the problem. The technology includes superior drought-tolerant varieties. The use of upland rice varieties with higher yields, as well as resistance to drought, and can adapt well to climate change, is needed to support efforts to increase yields and expansion of rice areas on dry land. The development of varieties requires time and funds are relatively large. The formation of homozygous lines can be accelerated by anther culture technique to produce inbreds in one generation. The selection process could be expressed highly efficient because the homozygous lines can be obtained immediately in the first (DH1) and second-generation (DH2) (Dewi *et al*., 1996; Herawati *et al*., 2008). In previous experiments have produced double haploid lines via anther culture as much as 348 lines (Herawati *et al*., 2008). A total of 78 lines has been through a screening test to stress the aluminum in the greenhouse with a nutrient solution, and screening blast leaves with 173 races, 033 races and 001 race in the greenhouse (Herawati *et al*., 2016). Drought stress testing is needed to determine whether these lines have a tolerance to drought stress, so long dry periods can be anticipated by planting drought-tolerant varieties.

Evaluation and characterization, as well as a selection of rice that are resistant to drought stress, is an essential stage in plant breeding. The process of selection of double haploid lines was done, especially those related to drought tolerance, by looking at the morphological features on the root system of each genotype (Herawati *et al*., 2017). Taiz and Zeiger (2002) described the plant's defenses of drought stress is hampered the development of leaf area, root development to reach a wet area, and the closing of stomata to limit transpiration. Assessment genotype trough selection is less efficient because the identification of potential high yield in drought stress is difficult obtained immediately (Clarke *et al*., 1992). Breeding purposes was done by rooting properties with tolerance to a drought that reported by Chang *et al*. (1972). Babu *et al*. (2003) revealed that the character of root positively correlated with production in drought stress. The treatment of PEG solution into the medium is expected to create conditions of stress, because of reducing the availability of water for plants Molecular size and the concentration of PEG in the solution determining the osmotic potential. According to Seshu and Sorrells (1986), 6000 PEG solution at a level of 20% has an osmotic potential -0.71 Mpa (7:06 bar). Land under conditions of osmotic potential field capacity is -0.03 Mpa (0.33 bar), and in a stage of the permanent wilting point is an osmotic potential -1.5 Mpa (15 bars) (Taiz and Zeiger, 2002). As an agent selector, PEG 6000 reported as superior to mannitol, sorbitol, or salt because it is not toxic to plants, can not be absorbed by root cells, and homogeneously lowering osmotic potential (Verslues *et al*., 1998). The use of PEG 6000 solution at a concentration of 20% is expected to create an osmotic potential that is equivalent to the soil condition between field capacity and permanent wilting point. The addition of PEG solution in germination media is expected to simulate drought stress conditions. The study aimed to determine the double haploid lines of crossbred upland rice with a new plant type (NPT) that tolerant to drought to assess the consistency of testing using PEG at the germination stage and drought stress test in the green house.

Materials and methods

The research was conducted at the laboratory and greenhouse of the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development, Indonesia, Cimanggu Bogor. The experiments were carried out two stages of early selection seeds of double haploid (DH1) with 20% polyethylene glycol (PEG) 6000 at the germination phase, and drought stress test in the greenhouse.

Test of polyethylene glycol (PEG)

The materials used in this study were 78 lines of double haploid (DH1) selected from crosses of upland rice in new plant type (PTB) Fatmawati, four elders, namely SGJT-28, SGJT-36, Way Rarem, and Fatmawati, and Jatiluhur and Cisokan as a tolerant and sensitive control. A total of 20 seeds of each line represented by treating 20% concentration of PEG 6000 on a petri dish for early selection of drought-tolerant seeds. Seeds soaked in a solution of 10 ml PEG in a petri dish.

After 24 hours, the number of seeds that germinate calculated until the age of six days. Data were collected as germination, root length, and length plumule. The Index average decline using the formula Jiang and Lafitte (2007) was measured as follows:

The average decrease (%) = [1- (Vs / Vp)] x 100

Vs = the value of the variable in drought stress conditions

Vp = value of the variable in the condition without stress

The relative root length (RRL) was used to selection of lines at 20% PEG on the germination phase. RRL data were transformed into the Z value genotype. Tolerance levels were divided into 5 groups: very sensitive if Z <-1 SD, sensitive if -1 SD> Z <-1/2 SD, moderate if -1/2 SD <Z < +1/2 SD, tolerant if +1/2 SD> Z <+1 SD, very tolerant if Z> +1 SD.

Drought stress test

The selected materials resulted from screening by PEG 6000. Varieties used for the check were Jatiluhur and Batutegi as a tolerant and Cisokan and Fatmawati were sensitive. The soil dried for one week, then sieved with four mm sieve to obtain a homogeneous soil. Soil water content was determined by weighing 3 x 100 g air-dry soil. The soil was roasted for 24 hours at t 105 ℃, then weighed and gained an average weight of oven-dry soil (ODS). Field capacity was determined by the Bouyoucos modified method. Three seeds were planted per pot. Plants were fertilized with 200 kg/ha (5 g/pot) Urea, 100 kg/ha (2.5 g/pot) SP36, and 100 kg/ha (2.5 g/pot) KCl.

Proline content analysis is referred to as the method of Bates *et al*. (1973). Three old leaves are taken as samples (Uyprasert *et al*., 2004). Standard curves were done using a proline solution at a level between 0-1.0 mmol to determine the concentrations of proline. Proline content of the material was expressed in mmol/g dry weight. Chlorophyll analysis was conducted using a spectrophotometer.

The experiments were arranged as factorial in Randomized Completely Block Design (RCBD). The first factor was the genotype, and the second factor was the drought stress with three replications. Treatment of dryness stress consists of two levels, namely: (1) Field capacity until the end of the trial, (2) 60% of field capacity was given during the critical period of the plant (three days before and three days after flowering) (Kumar *et al*., 2006; Liu *et al*., 2006; Lafitte *et al*., 2006).

The variables observation were root length, shoot length, root dry weight, shoot dry weight, shoot and root weight ratio (SRR), grain weight per hill, the content of proline and chlorophyll in the leaves. Selection of drought stress tolerance based on the ratio of grain weight per hill (GWR).

Results

Effect of polyethyleneglycol for germination of double haploid lines

The PEG 6000 treatment reduced the percentage of germination, root length, and plumule length, respectively, by 33.95 percent, 60.8 percent, and 80 percent (Figure 1).

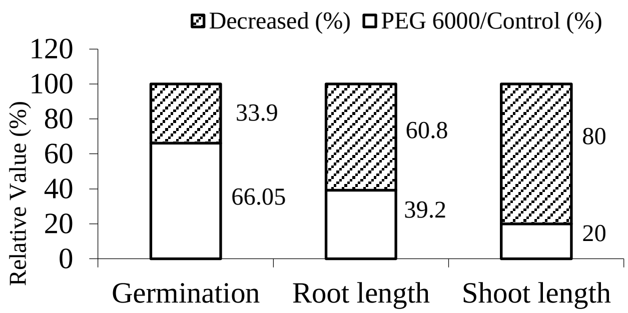

Figure 1. Effect of PEG 6000 on germination, roots length, and shoot length

PEG 6000 treatment resulted in the lowest germination in genotypes P3 and P6 (39.8 and 36.5 percent), while the genotype P4 and P5 produced the highest germination rate (77.5 and 75 percent) (Figure 2). Root length was the shortest 2.3 cm (P3), and the longest was 3.7 cm (P6) (Figure 3).

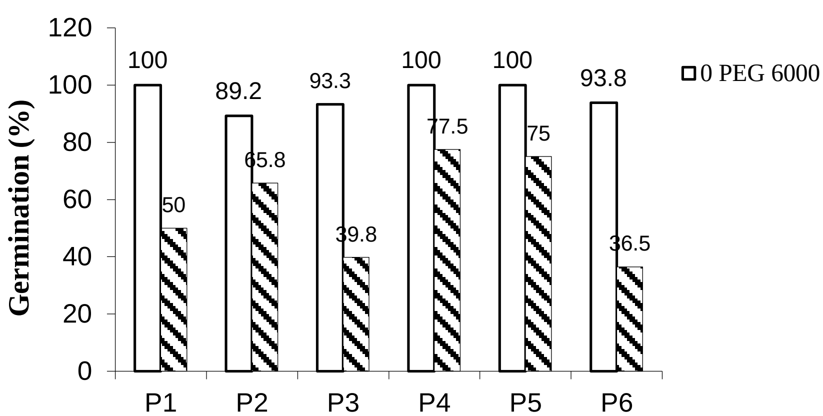

Figure 2. Effect of PEG 6000 on seed germination of double haploid lines

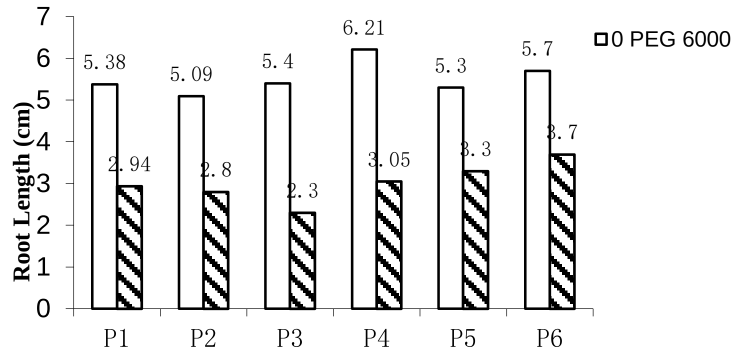

Figure 3. Effect of PEG 6000 on length root of doubled haploid lines

Shoot length varied between crosses, ranging from 0.51 - 1.22 cm. The lowest was found in P6 (0.51 cm) and P3 (0.65 cm), while the longest was in P4 and P5 (1.22 and 1.16 cm) (Figure 4).

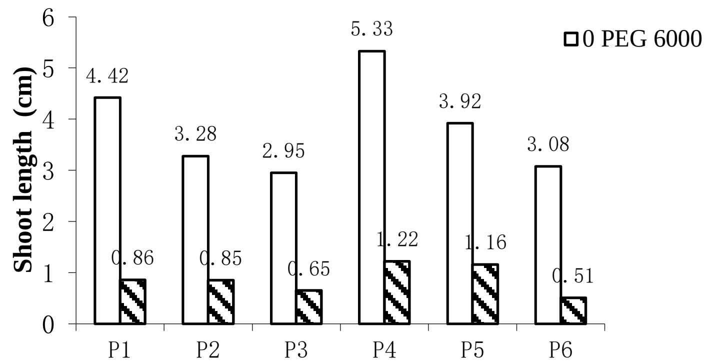

Figure 4. Effect of PEG 6000 on shoot length of doubled haploid lines

The selection of double haploid lines at 20% PEG 6000 based on the relative root length (RRL) resulted in susceptible genotype if RRL<15.46, susceptible if 15.46<RRL<28.09, moderate susceptible if 28.09 <RRL<53.35, moderately tolerant if 53.35 <RRL<65.89, and tolerant if RRL> 65.89. The results of genotype selection were ten tolerant genotypes, 13 genotypes rather tolerant, 29 moderate, eight rather susceptible, and 18 susceptible genotypes (Table 1).

Table 1. Selection of double haploid lines by relative root length (RRL) at 20% PEG 6000

Crosses	Number of lines				
	Tolerant	Rather Tolerance	Moderate	Rather susceptible	Susceptible
P1 (Fatmawati x Way Rarem)	0	1	0	0	0
P2 (Fatmawati x SGJT-28)	1	3	2	0	0
P3 (Fatmawati x SGJT-36)	7	3	14	3	7
P4 (Way Rarem x Fatmawati)	0	1	1	0	0
P5 (SGJT-28 x Fatmawati)	0	1	0	0	0
P6 (SGJT-36 x Fatmawati	2	4	12	5	11
Total	**10**	**13**	**29**	**8**	**18**

Table 2. The lines selected for drought stress testing at the greenhouse based on the PEG 6000

Lines	RLR[1]	Z	% Germ[2]	Criteria	Grains weight/hill
P6-95	66.9	1.03	100	T	43.5
P2-112	71.7	1.22	100	T	27.7
P3-190	79.0	1.51	100	T	4.77
P3-31	76.4	1.41	90	T	7.11
P4-43	42.0	0.05	80	M	18.0
P6-92	48.3	0.29	90	M	29.5
P2-2	49.8	0.35	60	M	2.71
P6-291	43.3	0.10	60	M	4.42
P6-75	0.0	-1.61	0	S	23.8
P6-64	6.0	-1.37	10	S	16.1
P6-53	8.64	-0.63	10	S	9.87
P3-221	**0.0**	**-1.61**	**0**	**S**	**3.7**

[1]/RLR= relative length root was tansform from Z values, where Z=standard values; Susceptible (S) if Z ≤ -1SD, Moderate (M) if -1SD<Z<+1SD, and Tolerance (T) if Z≥+1SD;
[2]/Germ=germination

The results of tests on polyethylene glycol (PEG) 6000 were used for drought stress testing in the greenhouses. It divided the results of genotype selection into three groups, namely sensitive genotypes, moderate genotypes, and tolerant genotypes based on their relative root length values (RRL) (Table 2).

Effect of drought stress on the growth and yield of double haploid lines

Variance analysis in drought stress experiments showed that the shoot length, root dry weight, shoot dry weight, and shoot root weight ratio (SRR) were significantly different among lines except for root length (Table 3).

Table 3. The effect of drought stress on roots length, shoot length, root dry weight, shoot dry weight, and shoot root weight ratio (SRR) of double haploid lines

Source of variance	Mean Square				
	Root length	**Shoot length**	**root dry weight**	**shoot dry weight**	**shoot root weight ratio (SRR)**
Genotipe (G)	36.41ns	1771.26**	229.23**	3749.77**	0.0067**
Drought (D)	396.09*	937.50**	256.79**	2208.57**	0.0043^{ns}
G x D	**28.99***	**64.08***	**16.94***	**129.21***	**0.0018***

**significant, ^{ns} no significant at F 0.05 test

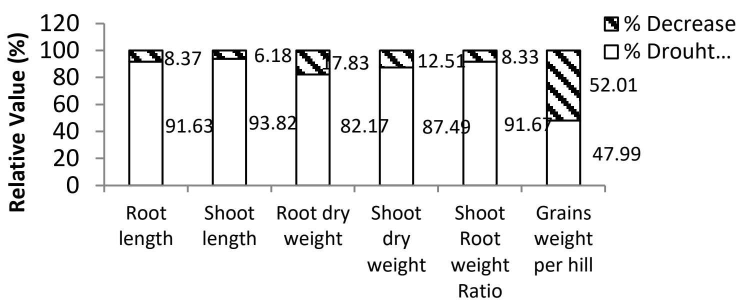

Figure 5. Effect of drought stress on growth and yield of double haploid lines derived anther culture

Drought stress treatment was significantly different in all variables except SRR. There was an interaction between genotypes on drought stress, which indicated that there were varied responses among the genotypes (Table 3). Relative values were used to know the effect of drought stress on the growth and yield of lines (Figure 5). The response of rice growth did not show a significant decrease due to drought stress. Root length, shoot length and shoot root weight ratio (SRR) decreased only by 8.37, 6.18, and 8.33 percent, respectively, because it applied the stress in a relatively short period, and plant growth was stable. Root dry weight decreased by 17.83 percent and shoot dry weight decreased by 12.51 percent; however, the decrease in grain weight/hill was up to 52.01 percent (Figure 5). Drought stress had a significant effect on yield reduction because it gave the treatment in the critical period when filling grains in the reproductive phase.

Total chlorophyll content

Total chlorophyll in the leaves varied in each genotype tested. Almost all genotypes showed a decrease in total chlorophyll, except for genotypes P6-291 and Batutegi as checks tolerant. The lowest reduction of total chlorophyll was found in genotypes P3-31, P2-2, P6-53, P6-64, and Fatmawati, while the highest decreases were seen in genotypes P6-95, P3-190, and P3-221 (Figure 6).

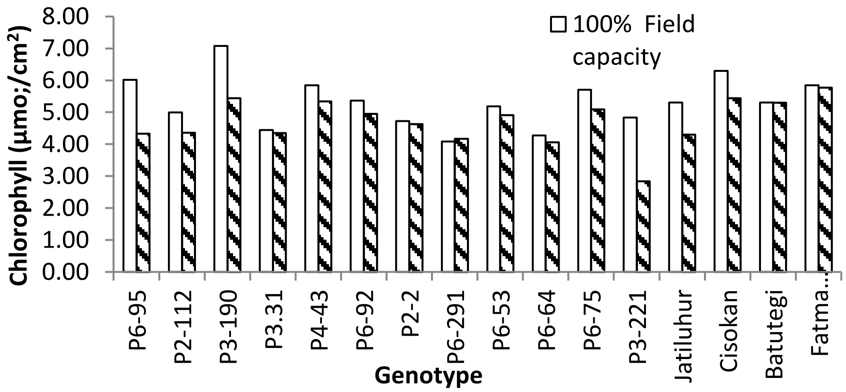

Figure 6. The total chlorophyll content of double haploid lines

The content of proline

Plants were evaluated proline accumulation to defend themselves in drought stress by osmotic adjustment, which is an adaptation response to these conditions. The content of proline in the leaves varied in the genotype tested. The content of proline was very high for tolerant genotypes based on the PEG 6000 test, which was found in P3-31 (30.33 μmol/g), and P6-95 (20.82 μmol/g), and moderate genotypes were found in P6-291(20.42 μmol/g) (Figure 7). Based on grain weight/hill ratio (GWR), the genotype was categorized as susceptible. It was due to the large amount of organic material that accumulated for osmotic adjustment, which resulted in low grain weight/hill.

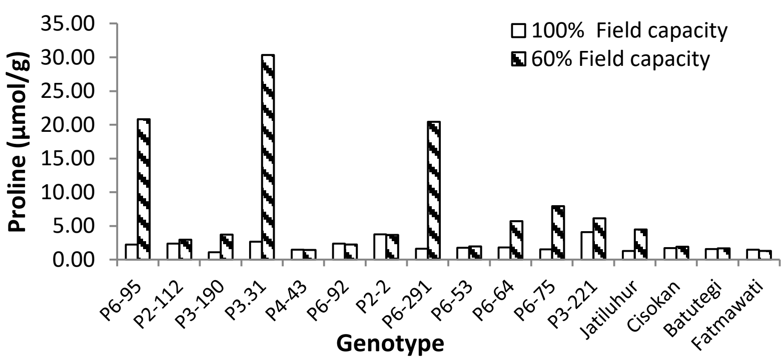

Figure 7. The content of proline of double haploid lines derived

Effect of drought stress on yield double haploid lines

The effect of drought stress on grain weight per hill was presented in Table 4. Genotypes P4-43, P6-92, and P6-53, did not show a significant decrease due to drought stress. Some lines showed significant reductions, such as genotypes P2-112, P3-90, P3-31, P2-2, P6-75, and P3-221.

Table 4. The weight of grain per hill at normal conditions and drought stress and the ratio of grain weight per panicle of doubl haploid lines

	Grain weight/hill (g)			
Lines	**100 % FC[1]**	**60 % FC**	**GWR(%)[2]**	**Criteria**
P6-95	4.31	0.28	6.49	Susceptible
P2-112	14.87	3.31	22.26	Susceptible
P3-190	28.44	19.94	70.11	Tolerant
P3-31	17.74	3.88	21.87	Susceptible
P4-43	19.16	18.22	95.09	Tolerant
P6-92	10.40	8.18	78.65	Tolerant
P2-2	17.00	3.09	18.17	Susceptible
P6-291	8.07	2.20	27.26	Susceptible
P6-53	16.98	12.20	71.85	Tolerant
P6-64	3.32	0.83	25.00	Susceptible
P6-75	24.61	2.62l	10.65	Susceptible
P3-221	8.66	1.32	15.24	Susceptible
Jatiluhur	52.34	33.01	63.07*	Tolerant
Cisokan	38.03	11.75	30.89	Moderate
Batutegi	46.77	31.37	67.07	Tolerant
Fatmawati	**38.89**	**15.55**	**39.98**	**Moderate**

[1/]FC= field capacity; [2]GWR =Grain Weght Ratio; *Based on tolerance of parental (Jatiluhur) tolerant if WGR>60%, Moderate if 30<WGR<60, and susceptible if WGR<30%

The selection of drought tolerance based on grain weight/hill ratio (GWR) showed different levels of consistency on the PEG 6000 test (Table 4). Four tolerant doubled haploid lines in the PEG 6000 test showed only one that was consistently tolerant (P3-190). In comparison, four moderate lines in the PEG test, two of them were consistent, namely P4-43 and P6-92, and four susceptible lines in the PEG test produced one was not consistent (P6-53) (Table 4).

Discussion

PEG 6000, which is equivalent to osmotic potential of -0.8 MPa, caused inhibition of germination, root elongation, and epicotyl in chickpeas (Macar *et al.*, 2009). Zapico (2008) reported that lowland rice genotypes were more sensitive than upland rice in inhibiting germination at 15% PEG 8000. PEG 6000 caused a water deficit that inhibited the entry of water molecules into plant tissue, while water was essential in the germination process. The average decrease in root length was 60.8 percent. Zapico's (2008) revealed that leaves were more inhibited than roots because of water deficits during the germination. Macar *et al*. (2009) also reported that PEG 6000 inhibited shoot rather than root elongation. Because the roots are first exposed to drought stress, causing damage to the root tissue, so it took a lot of carbohydrates to the roots, consequently the supply to the shoot decreases. In this experiment, PEG 6000 reduced shoot length by 80 percent. Li *et al*. (2006) reported that there was a decrease in chlorophyll content in barley leaves, tolerant genotypes (Tamor and Arta) decreased by 10.7, and 1.6 percent, respectively, and sensitive genotypes (Morocco9-75 and W12291) decreased by 31.3 and 30.1 percent. Refers to Pieters and Souki (2005) also reported that drought stress weakens PS II activity in rice leaf flags, resulting in reduced chlorophyll content. However, drought stress increased xanthophyll pigment in leaves, which functions to absorb excess light under high irradiation in drought stress. Furthermore, it was stated by Havaux and Lannoye (1985) that the inhibition of photosynthesis not only caused in the degradation of chlorophyll and stomatal closure but also resulted in changes in thylakoid membrane function. It has reduced quantum yields in primary photochemical reactions in PS II that direct changes in energy distribution to PS I. Herawati *et al.* (2017) proved that the composition of stomata in susceptible genotypes was denser and more numerous than tolerant genotypes. Stomatal density affected two essential processes, namely photosynthesis, and transpiration. Pirdasthi *et al*. (2009) showed that drought stress at different growth stages would increase the proline content of the leaves, and tolerant genotypes have a high proline and grain yield. Mostajeran and Rahimi-Eichi (2009) stated that the accumulation of proline varies between genotypes tested, the content of proline in young and old leaves always increases in drought stress, and the proline in young leaves was higher than older leaves in all cultivars tested. Therefore, several studies have shown that proline content can be used as an indicator of drought-tolerant genotypes.

The effect of drought stress on grain weight/hill in lines tested was discussed. Lines P4-43, P6-92, and P6-53 did not show a significant decrease due to drought stress. Some lines showed significantly decreased, such as lines P2-112, P3-90, P3-31, P2-2, P6-75, and P3-221. Sheoran and Saini (1996) and Saini (1997) have detected sensitivity to drought stress in the process of rice anther meiosis. Meiosis of the anther occurs 9-10 days before flowering depending on the position of the spikelet in panicles. The process of meiosis ends three days before the flowers come out. Therefore, stress can be given three days before or afterward to predict the effects on yield reduction. Liu *et al*. (2006) stated that water stress causes pollination failure by up to 67 percent of the total spikelet/panicle. Furthermore, if pollination occurs, the time achieved by pollen to reach the micropyle in the ovule is longer, between 1-8 days. Flowers fail to open; consequently, pollen cannot escape through the surface of the flower. Lafitte *et al*. (2005) reported that drought stress in dry land caused delays in flowering for three days in the genotype tested. However, on the contrary, genotypes were flowering faster; this is because the plant was sensitive to pollination and embryo development, so it accelerated flowering before more severe stress. Delay in flowering during drought stress will negatively affect seed filling, especially on sensitive genotypes. The assimilate partitioning from stems and leaves increased during drought stress by accelerating leaf senescence (Kumar *et al*., 2006). Yang *et al.* (2001) supported this statement that 75-92 percent during pre-anthesis, ^{14}C was stored in stems that would be relocated back to seeds when exposed to drought, 50-80 percent higher were mobilized under normal conditions. Drought stress in the grain filling period also causes faster senescence, a shorter seed filling period, but the assimilation remobilization increases (Kamoshita *et al*., 2004). Drought stress also inhibited panicle exertion due to decreased elongation at the base of the panicle, causing sterility of grain within the leaf sheath, which reduced grain yield (Ji *et al*., 2005). The results showed a decrease in grain weight/hill up to 52.01 percent because of drought stress. The six-day drought stress treatment in the flowering period significantly decreased the weight of filled grain/hill, up to 80% (Liu *et al*., 2006). Lafitte *et al*. (2005) stated that the average decreased grain yield in paddy fields was 75 percent, while Wang *et al*. (2009) reported that the IR2266 lowland rice genotype had a more significant reduction in total root length than the CT9993 upland rice genotype. The upland rice genotype (CT9993) was more adaptable to water deficit conditions in rainfed lowland areas, by avoidance strategy, which can penetrate deep roots and strong root systems. Furthermore, Kumar *et al.* (2009) reported that decreased biomass could reduce rice yield due to drought stress in sensitive genotypes, so that selection of high biomass and harvest index can be applied to obtain drought tolerant genotypes.

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