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I am looking forward to hear from you soon.

Best regards

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Dear editor,

I have revised the manuscript, please kindly find the attached file

Best regards,
Dr. Reny Herawati

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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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Herawati, R. Purwoko, B.S. Dewi, IS., Romeida, A., Ganefianti, D.W. and Marlin (2019). Analysis of polyethene glycol (PEG) and proline to evaluate drought stress of double haploid new type upland rice lines. International Journal of Agricultural Technology X(X): XX-XX

Abstract Evaluation, ~~and~~ characterisation ~~as well as a~~ and selection of rice were conducted for ~~that are~~ tolerant to drought stress. ~~is an essential stage in plant breeding~~. ~~To make~~ The process of selection of double haploid lines, especially those related to drought tolerance ~~can be~~ was done by ~~looking~~ observation ~~at~~ the morphological features on the root system in each genotype. The treatment of PEG solution in the planting medium is ~~expected to~~ created the stress condition ~~of stress~~ because of the water availability ~~of water~~ for plants ~~to be~~ reduced. Molecular size and ~~the~~ concentration of PEG in the solution determined ~~ing~~ the osmotic potential. ~~that occurs~~. The defense mechanisms used in ~~by~~ plants ~~to defend~~ on drought stress is ~~through~~ the accumulation of proline to ~~for~~ adjust ~~ment~~ osmotic, production and accumulation of free amino acids like proline in plant tissues during drought stress, an adaptation response in these conditions. ~~In this research,~~ Result showed that PEG 6000 inhibited the germination (33.9 per cent), root length (60.8 per cent), and shoot length (80 per cent) of upland rice lines. Drought stress treatment (60 per cent of field capacity) at the flowering period showed non-significant reduction in the growth of doubled haploid upland rice but reduced the weight of grains per hill (52.11 per cent). 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, and 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 decrease in total chlorophyll and increased the proline content in the leave.

Keywords: drought, doubled haploid, upland rice, polyethyleneglycol, proline
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Introduction

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

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 more 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 aluminium in the greenhouse with a nutrient solution, and screening blast leaves with 173 race, 033 races and 001 races 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 characterisation, as well as a selection of rice that are resistant to drought stress, is an essential stage in plant breeding. To make the process of selection of double haploid lines, 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) describe the plant's defences 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). Selection for breeding purposes by connecting between rooting properties with tolerance to drought have conducted by Chang *et al*. (1972). Results of research Babu *et al*. (2003) revealed that the character of root positively correlated with production in drought stress.

The treatment of PEG solution into the planting medium is expected to create conditions of stress because of the availability of water for plants to be reduced. Molecular size and the concentration of PEG in the solution determining the osmotic potential that occurs. According to Seshu and Sorrells (1986), 6000 PEG solution with a level of 20% has an osmotic potential -0.71 Mpa (7:06 bar). Land under conditions of osmotic potential field capacity have -0.03 Mpa (0.33 bar) and in a state of the permanent wilting point has an osmotic potential -1.5 Mpa (15 bars) (Taiz and Zeiger 2002). As an agent selector, PEG 6000 reportedly 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 with a concentration of 20% is expected to create an osmotic potential that is equivalent to the condition of the soil between field capacity and permanent wilting point. The addition of PEG solution in germination media is expected to simulate drought stress conditions. This study aims to determine the double haploid lines of crossbred upland rice with a new plant type (NPT) that are tolerant to drought, as well as to assess the consistency of testing using PEG at germination stage and the drought stress test in the greenhouse.

Materials and methods

The research ~~had~~ 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% polyethene glycol (PEG) 6000 at the germination phase, and drought stress test in the greenhouse.

Test of 20% polyethene glycol (PEG) 6000

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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 collected for germination, root length, and length plumule. Measuring the Index average decline using the formula Jiang and Lafitte (2007) 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

Selection of lines at 20% PEG on germination phase based on the relative root length (RRL), follows the normal distribution pattern. RLR data transformed into raw value genotype Z. Tolerance levels grouped 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

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The selected materials result from screening with PEG 6000. Varieties used for comparison is Jatiluhur and Batu Tegi as a comparison tolerant and varieties Cisokan and Fatmawati as a sensitive comparison. The soil dried for one week, then sieved with four mm sieve to obtain a homogeneous soil. Determination of soil water content by weighing 3 x 100 g air-dry soil. The soil roasted for 24 hours at t 105 ° C, then weighed and gained an average weight of oven-dry soil (ODS). Determination of field capacity with Bouyoucos modified method. Three seeds planted per pot. Having grown been two of the best. Plants 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 refers to Bates *et al*. (1973). Three old leaf samples were taken at each genotype that has opened full, during the day (± 1 am) (Uyprasert *et al*. 2004). Standard curves using proline solution with a level of between 0-1.0 mmol to determine the concentrations of proline. Proline content of the material expressed in mmol/g dry weight. Chlorophyll analysis was conducted using a spectrophotometer.

The experiments arranged into factorial Randomized Block Design (RBD) factorial, the first factor is the genotype, and the second factor is the drought with three replications. Treatment of dryness stress consists of two levels, namely: (1) the provision of water commonly at field capacity until the

end of the trial, (2) the provision of water to 60% of field capacity given during the critical period of plant that is over six days (3 days before and 3 days after flowering) (Kumar *et al*., 2006; Liu *et al*., 2006; Lafitte *et al*., 2005).

The variables observation are 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 (RGW), which is the ratio between grain weight/hill on drought stress and grain weight/hill without drought stress.

RESULTS AND DISCUSSION

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Effect of Polyethyleneglycol (PEG) 6000 to Germination of Doubles Haploid lines Derived Anther Culture

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PEG 6000 treatment affects the germination percentage, root length and length plumule. Germination percentage decreased by 33.95 per cent, followed by a decrease in root length (60.8 per cent), and long-plumule (80 per cent) (Figure 1). Research by Macar *et al*. (2009) showed that PEG 6000 is equivalent to the osmotic pressure of -0.8 MPa causes inhibition of germination, root elongation and epicotyl on chickpea plants.

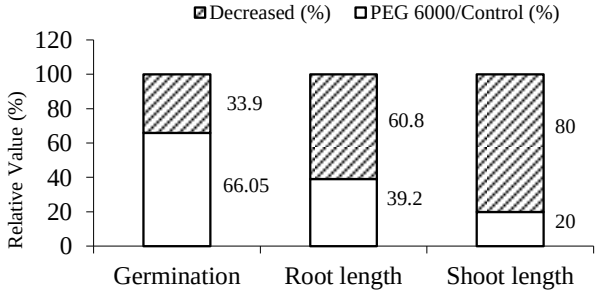

Figure 1. Effect of PEG 6000 on germination, roots length and shoot length

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PEG 6000 treatment resulted in the lowest germination in genotypes P3 and P6 (39.8 and 36.5 per cent), while the genotype P4 and P5 produced the highest germination rate (77.5 and 75 per cent) (Figure 2). Zapico (2008) reported that the lowland rice genotypes are more sensitive than upland rice on

the inhibition of germination in treatment 15% PEG 8000. PEG 6000 led to a water deficit that will inhibit the entry of water molecules into plant tissues, whereas water is indispensable in the process of germination. An average reduction of 60.8 per cent root length (Figure 1). Long roots lowest average in treatment 20% PEG 6000 is P3 (2.3 cm), and the highest average length root is P6 (3.7 cm) (Figure 3).

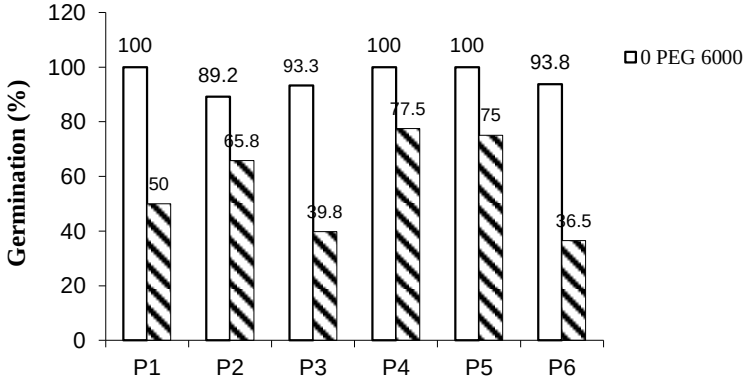

Figure 2. Effect of PEG 6000 on seed germination of double haploid lines derived anther culture

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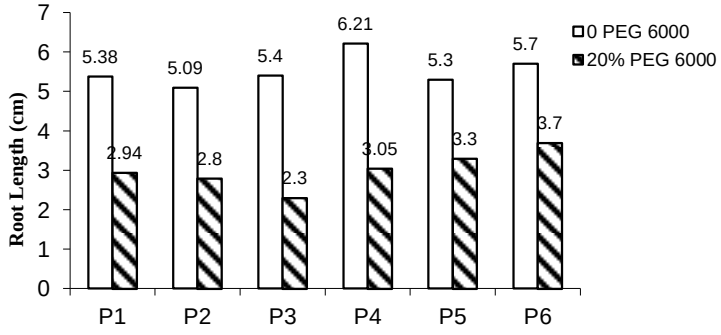

Figure 3. Effect of PEG 6000 on length root of doubled haploid lines derived anther culture

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Results Zapico (2008) showed that the leaf is more constrained than the roots of the water deficit during the germination process. The same reported by Macar *et al*. (2009) revealed that PEG 6000 inhibits the shoots and the root elongation. The former of the roots are more exposed to conditions of drought stress and caused damage to the root tissue, so it requires much supply of carbohydrates to the roots, consequently supply to the shoots reduced.

PEG 6000 treatment caused a reduction in the shoot length by 80 per cent (Figure 2). Shoot length varies between the crossing ranging from 0.51 to 1.22 cm. The lowest average of shoot length is P6 (0.51 cm) and P3 (0.65 cm), while the longest in P4 and P5 is 1.22 and 1.16 cm, respectively (Figure 4).

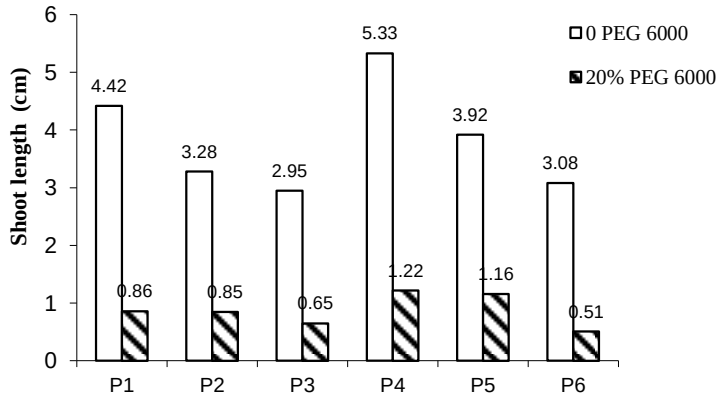

Figure 4. Effect of PEG 6000 on shoot length of double haploid lines derived anther culture

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The selection of double haploid lines at 20% PEG 6000 in the phase of germination based on the value of relative root length (RRL) results in very sensitive genotype if RRL<15.46, sensitive if 15.46<RRL<28.09, moderate sensitive if 28.09 <RRL<53.35, moderately tolerant if 53.35 <RRL<65.89, and tolerant if RRL> 65.89. Double haploid lines selection results in the treatment of 20% PEG 6000 in various crosses produced ten genotypes tolerant, 13 somewhat tolerant genotypes, 29 genotypes moderate, eight genotypes rather sensitive and 18 sensitive genotypes (Table 1).

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

Crosses	Number of lines				
	Tolerant	Rather Tolerance	Moderate	rather sensitive	sensitive
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 were chosen to test the consistency of drought stress by 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

Test results with polyethyleneglycol (PEG) 6000 is used for testing drought in the greenhouse. The resulting genotype grouped into three criteria,

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namely the sensitive genotypes, genotypes moderate and tolerant genotypes based on the relative root length (RRL) (Table 2).

Effect of Drought Stress on the Growth and Yield of Doubled Haploid Lines Results from Anther Culture

Effect of Drought Stress on Growth

The results of variance in drought stress experiments showed that the shoot length, root dry weight, shoot dry weight, and shoot root weight ratio (SRR) was significantly different among lines except for variables 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 derived anther culture

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

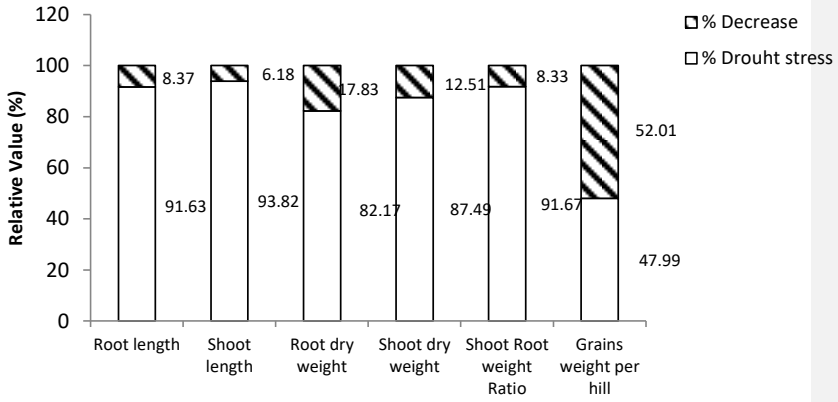

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Figure 5. Effect of drought stress on growth and yield of doubled haploid lines derived anther culture

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Drought stress treatment significantly different at all variables except for SRR, and there are interactions between genotypes to drought stress, which indicates that their response to drought stress to the difference between genotypes (Table 3). Relative values were used to know the effect of drought stress on the growth and yield of rice (Figure 5).

Drought stress treatment did not show a substantial decrease in growth response. Root length, shoot length and shoot root weight ratio (SRR) only decreased respectively by 8.37, 6.18, and 8.33 per cent because stress give in a relatively short period and the plant growth has stabilised. The variables root dry weight decreased by 17.83 per cent and shoot dry weight decreased by 12.51 per cent; however, a decrease in grain weight/hill up 52.01 per cent (Figure 5). Drought stress treatment significantly affects the yield reduction due to drought stress treatment is given at a critical period when filling seed in the reproductive phase.

Total Chlorophyll content

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The total chlorophyll content in leaves varies in genotype tested to drought stress. Almost all genotypes showed a decrease in total chlorophyll, except genotypes P6-291 and Batutegi as tolerant check variety. The reduction in total chlorophyll lowest in genotype P3-31, P2-2, P6-53, P6-64, and Fatmawati, while the highest decline in genotype P6-95, P3-190 and P3-221 (Figure 6).

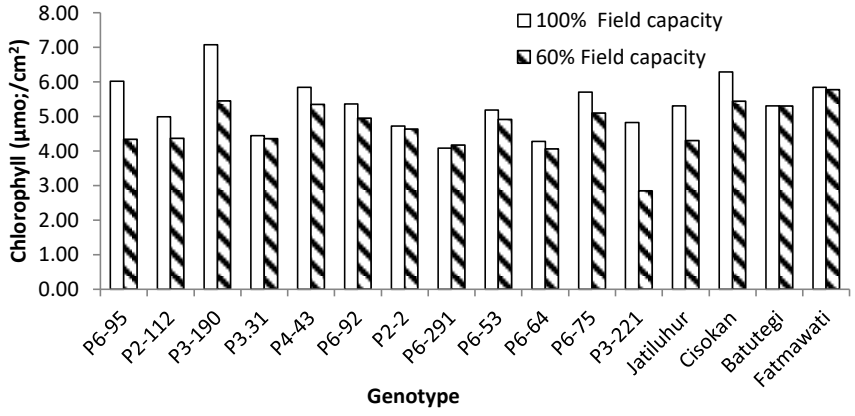

Figure 6. The total chlorophyll content of doubled haploid lines derived anther culture on drought stress

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Li et al. (2006) reported that there is a decrease in chlorophyll content in the leaves of barley, the tolerant genotype (Tamor and Arta) chlorophyll content dropped respectively by 10.7, and 1.6 per cent and the sensitive genotype (Morocco9-75 and W12291) dropped respectively by 31.3 and 30.1 per cent. Refers to Pieters and Souki (2005), the drought stress weakens the activity of PS II in the flag leaf, reduced chlorophyll, and increased the pigment content xanthophyll, which serves to absorb excess light under irradiation higher in drought conditions. Further proposed by Havaux and Lannoye (1985) that the inhibition of photosynthesis due to drought stress not only causes degradation of chlorophyll and stomatal closure but also resulted in changes in the function of the thylakoid membrane. It reduces quantum photochemical reaction primer on PS II which directs changes in the distribution of energy to PS I. Herawati *et al*. (2017) revealed that the stomatal composition and density in the susceptible genotype being denser and number full than the tolerant genotype. The stomatal density could affect two essential processes, photosynthesis, and transpiration,

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The content of Proline

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One of the mechanisms used by plants to defend on drought stress is through the accumulation of proline for adjustment osmotic, production and accumulation of free amino acids like proline in plant tissues during drought stress, an adaptation response in these conditions (Cattivelli *et al*., 2008; Vendruscolo *et al*. 2007). Further Vajrabhaya *et al*. (2001) revealed that the high proline content not only plays a role in the osmotic adjustment in water stress, proline accumulation was also thought to be involved in the protection of the structure of enzymes and cells from free radicals.

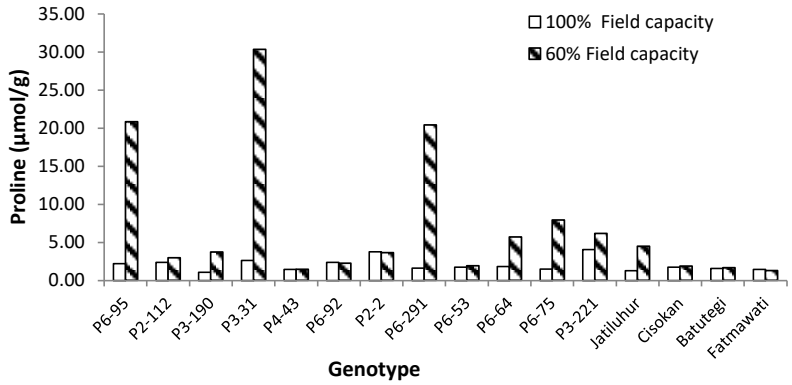

Figure 7. The content of proline of doubled haploid lines derived anther culture on drought stress

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The content of proline in the leaves varies in inbreds due to drought stress. The content is very high proline contained in tolerant genotype test criteria based on genotype PEG 6000, i.e. P3-31, followed by P6-95 respectively 30.33, 20.82 µmol/g, and genotype moderate line P6-291 at 20:42 µmol/g (Figure 7). Based on criteria of grain weight/hill ratio (GWR), the genotype categorised as sensitive. The amount of organic material that has been accumulated for osmotic adjustment, resulting in grain weight/hill is low. Results of research Pirdasthi *et al.* (2009) showed that drought stress at different growth stages would increase the proline content in the leaves, and the tolerant genotype contains proline and high rice yield. The experimental results Mostajeran and Rahimi-Eichi (2009) states that the accumulation of proline varies among genotypes tested, the content of proline in young leaves and old leaves always increased in drought stress, and the content of proline in young leaves was higher than leaves parents in all cultivars tested. Therefore, some studies suggest that the proline content used for the selection of drought-tolerant genotypes.

Effect of Drought Stress on Yield Double Haploid Lines

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The effect of drought on the grain weight per hill on some lines tested presented in Table 4. The line P4-43, P6-92, and P6-53 do not show significant declines due to drought stress. Some lines showed substantial reductions due to drought stress as P2-112, P3-90, P3-31, P2-2, P6-75, and P3-221. Sheoran and Saini (1996) and Saini (1997) has detected a sensitivity to drought stress at meiosis anther rice plants. Meiosis in the anther occurs in 9-10 days before flowering depending on the position of the panicle rice. The process of meiosis

ends three days before the flowers come out. Therefore stress can be given three days before the flowers come out or afterwards to see the effects on yield reduction.

Liu *et al*. (2006) found that water stress causes the failure of pollination by pollen up to 67 per cent of total grain/ panicle. Furthermore, in the case of pollination, the time to achieve the pollen to reach the ovule micropyle longer is 1-8 days. Flowers failed to open. Consequently, pollen can not get out through the surface of interest due to drought stress (Liu *et al*. 2006). Lafitte *et al*. (2005) reported that drought stress on dryland cause delays flowering an average of 3 days in some genotypes tested, but on the contrary there is a genotype faster flowering phase, it is presumably because these plants are sensitive to the process of pollination and development of the embryo, so flowering accelerated before more severe stress.

Delayed flowering during drought stress will negatively affect the filling of grains, especially in sensitive genotype, assimilate partitioning of the stems and leaves to the grain filling increases during drought stress by speeding senescence on the leaves (Kumar *et al.* 2006). This statement is supported by Yang *et al*. (2001) that 75-92 per cent during the pre-anthesis, ^{14}C deposited on the trunk that will be relocated back to the seed when there is drought, 50-80 per cent higher than the amount remobilisation on condition without stress. Drought stress on grain filling period also led to senescence faster, shorter grain filling period, but increased assimilate remobilisation (Kamoshita *et al*. 2004).

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

Lines	Grain weight/hill (g)			Criteria
	100 % FC[1]	60 % FC	GWR(%)[2]	
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

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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= kapasitas lapang;
[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%

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Drought stress also causing delays exertion of panicle elongation resulting in a reduction in the base of the panicle, causing sterility of grain inside the leaf sheath, which can decrease grain yield (Ji *et al.*, 2005).

The selection of drought stress tolerance based on the ratio of grain weight per hill (GWR) indicates that the level of consistency varies with test PEG 6000 (Table 4). A total of four double haploid lines tolerant to testing PEG 6000 was only one consistent is the line P3-190, while four moderate lines on the trial of PEG 6000, two of which are compatible based on GWR i.e line P4-43 and P6-92, and four lines sensitive on the trial of PEG 6000 only one inconsistent and otherwise intolerant based on GWR namely line P6-53 (Table 4). When viewed from the relative decline in grain weight/hill due to drought stress decreased to 52.11 per cent (Figure 6). Drought stress six days before flowering significant causes a decrease in grain weight/hill, the most significant impact when pressure is at the time of flowering can cause a reduction in fill grain of up to 80% (Liu *et al*. 2006).

The experimental results Lafitte *et al*. (2005) states that the average reduction in grain yield in lowland rice by 75 per cent due to drought stress. Wang *et al*. 2009 reported that the lowland rice genotypes IR2266 more significant reduction in total root length as a result of drought stress than upland rice genotypes CT9993. Upland rice genotypes (CT9993) more adapted to the conditions of water deficit in the rainfed areas, by way of avoidance (avoidance strategy) that can penetrate deep roots and reliable root system. Furthermore, Kumar *et al.* (2009) reported that the decrease in plant biomass could reduce rice yields due to drought stress in sensitive genotype, so the selection of high biomass and harvest index can be used to obtain drought tolerant genotypes.

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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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Herawati, R. Purwoko, B.S. Dewi, IS., Romeida, A., Ganefianti, D.W., and Marlin (2020). Analysis of polyethylene glycol (PEG) and proline to evaluate drought stress of double haploid new type upland rice lines. International Journal of Agricultural Technology X(X): XX-XX

Abstract Evaluation, characterization, and selection of rice were conducted to be tolerant to drought stress. The process of selection of double haploid lines, especially those related to drought tolerance, was done by observing the morphological features on the root system in each genotype. The treatment of 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
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Introduction

Using of 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 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; 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 20% polyethylene glycol (PEG) 6000

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°C, 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 (PEG) 6000 to Germination of Doubles 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).

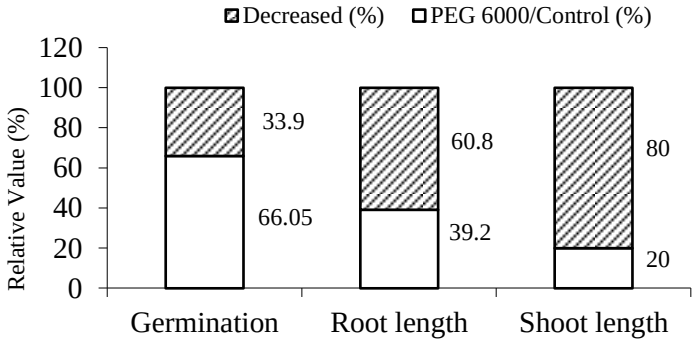

Figure 1. Effect of PEG 6000 on germination, roots length, and shoot length

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).

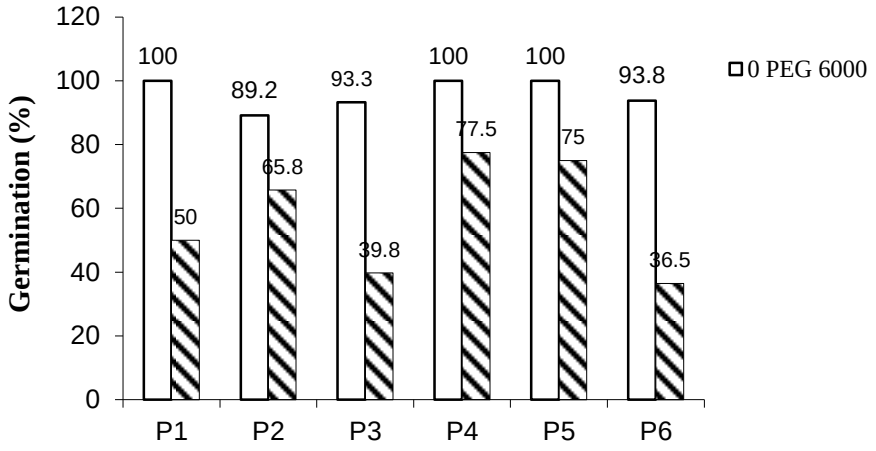

Figure 2. Effect of PEG 6000 on seed germination of double haploid lines

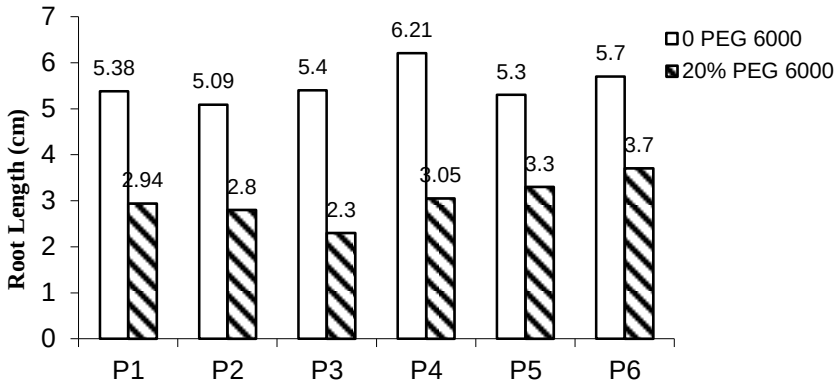

Figure 3. Effect of PEG 6000 on length root of doubled haploid lines

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).

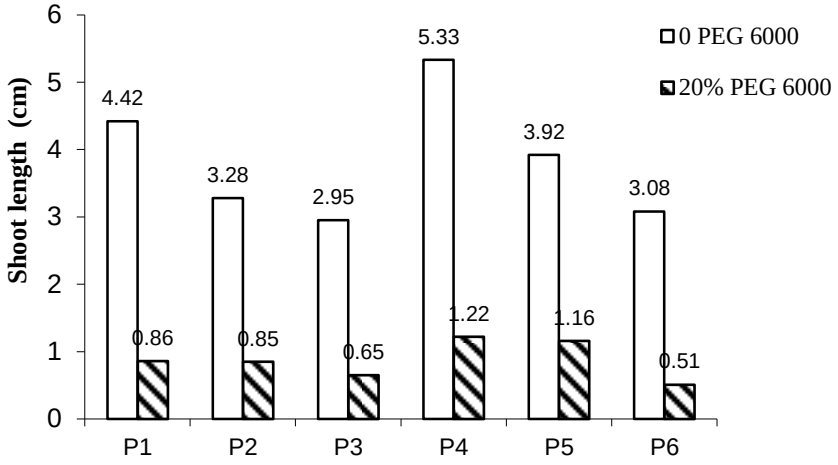

Figure 4. Effect of PEG 6000 on shoot length of doubled haploid lines

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, rather 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 susceptible 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 Doubled Haploid Lines

Effect of Drought Stress on Growth

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

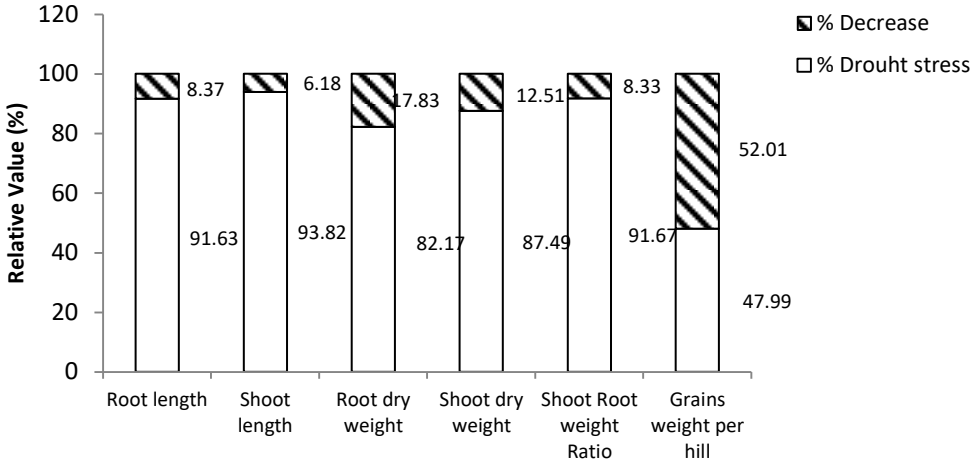

Figure 5. Effect of drought stress on growth and yield of doubled haploid lines derived anther culture

Drought stress treatment was significantly different in all variables except SRR, and 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).

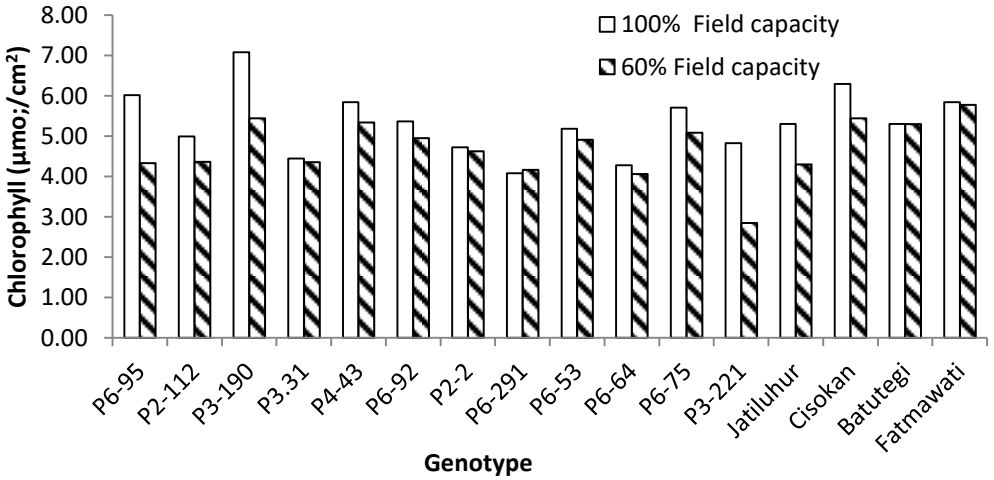

Figure 6. The total chlorophyll content of doubled haploid lines

The content of Proline

Plants use 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 has accumulated for osmotic adjustment, which resulted in low grain weight/hill.

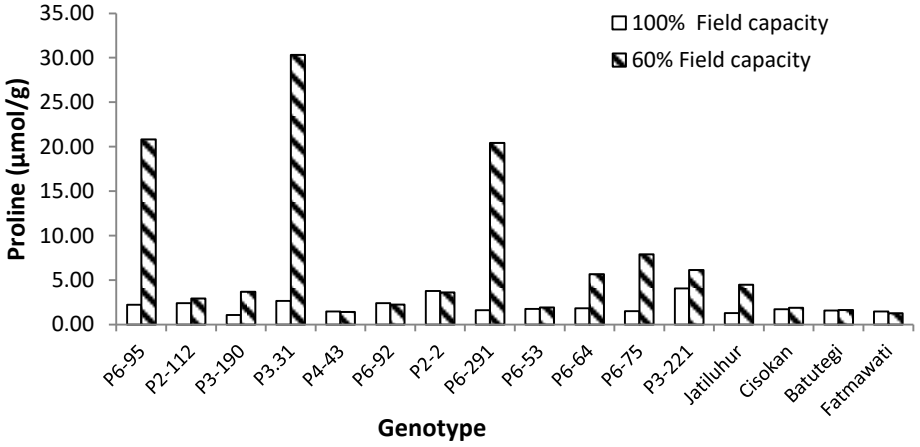

Figure 7. The content of proline of doubled haploid lines derived

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 doubled 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 (Figure 1).

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 (Figure 2).

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 presented in Table 4. 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 (Table 4). 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 (Figure 5). 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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Analysis of polyethylene glycol (PEG) and proline to evaluate drought stress of double haploid new type upland rice lines

Herawati, R.[1*], Purwoko, B.S.[2], Dewi, I.S.[3], Romeida, A.[1], Ganefianti, D.W.[1] and Marlin[1]

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Abstract Evaluation, characterisation and selection of rice were conducted to be tolerant to drought stress. The process of selection of double haploid lines, especially those related to drought tolerance was done by observation the morphological features on the root system in each genotype. The treatment of polyethene 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 ~~in the solution~~ determined the osmotic potential. The defense mechanisms used in plants on drought stress is the accumulation of proline to adjust ~~ment~~ osmotic, production and accumulation of free amino acids like proline in plant tissues during drought stress, an adaptation response in these conditions. Result showed that PEG 6000 inhibited the germination (33.9 per cent), root length (60.8 per cent), and shoot length (80 per cent) of upland rice lines. Drought stress treatment (60 per cent of field capacity) at the flowering period showed non-significant reduction in the growth of doubled haploid upland rice but reduced the weight of grains per hill (52.11 per cent). 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. ~~Stress drought led to decrease in total chlorophyll and increased the proline content in the leave.~~

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

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Introduction

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

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 ~~more~~ 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 aluminium in the greenhouse with a nutrient solution, and screening blast leaves with 173 races, 033 races and 001 race~~s~~ 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 characterisation, as well as a selection of rice that are resistant to drought stress, is an essential stage in plant breeding. ~~To make~~ The process of selection of double haploid lines were 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) describe~~d~~ the plant's defences 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). ~~Selection for~~ Breeding purposes was done by ~~connecting between~~ rooting properties with tolerance to drought that reported ~~conducted~~ by Chang *et al*. (1972). ~~Results of research~~ Babu *et al*. (2003) revealed that the character of root positively correlated with production in drought stress.

The treatment of PEG solution into the planting medium is expected to create conditions of stress because of the availability of water for plants to be reduced. Molecular size and the concentration of PEG in the solution determining the osmotic potential that occurs. According to Seshu and Sorrells (1986), 6000 PEG solution with a level of 20% has an osmotic potential -0.71 Mpa (7:06 bar). Land under conditions of osmotic potential field capacity is ~~have~~ -0.03 Mpa (0.33 bar), and in a ~~state~~ stage of the permanent wilting point is ~~has~~ an osmotic potential -1.5 Mpa (15 bars) (Taiz and Zeiger, 2002). As an agent selector, PEG 6000 reported~~ly~~ 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 with a concentration of 20% is expected to create an osmotic potential that is equivalent to the soil condition ~~of the soil~~ 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 ~~s~~ to determine the double haploid lines of crossbred upland rice with a new plant type (NPT) that ~~are~~ tolerant to drought, ~~as well as~~ and to assess the consistency of testing using PEG at germination stage and ~~the~~ drought stress test in ~~the~~ greenhouse.

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% polyethene glycol (PEG) 6000 at the germination phase, and drought stress test in the greenhouse.

Test of 20% polyethene glycol (PEG) 6000

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 ~~for~~ as germination, root length, and length plumule. ~~Measuring~~ 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

Selection of lines at 20% PEG on germination phase is based on the relative root length (RRL), and followed by the normal distribution pattern. RLR data were transformed into raw value genotype Z. Tolerance levels were ~~grouped~~ 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 with PEG 6000. Varieties used for comparison is Jatiluhur and Batu Tegi as a comparison tolerant and varieties Cisokan and Fatmawati were a sensitive comparison. The soil dried for one week, then sieved with four mm sieve to obtain a homogeneous soil. ~~Determination of~~ Soil water content was determined by weighing 3 x 100 g air-dry soil. The soil was roasted for 24 hours at t 105 ° C, then weighed and gained an average weight of oven-dry soil (ODS). ~~Determination of~~ Field capacity was determined with Bouyoucos modified method. Three seeds were planted per pot. Having grown been two of the best. 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 refered ~~s~~ to the mehod of Bates *et al*. (1973). Three old leaf samples were taken ~~at~~ in ~~each genotype that has opened full, during the day (± 1 am)~~ (Uyprasert *et al*. 2004). Standard curves was done using proline solution with a level of 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.

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The experiments were arranged ~~into~~ as factorial in Randomized Completely Block Design (RCBD). ~~factorial,~~ The first factor ~~is~~ was the genotype, and the second factor was ~~is~~ the drought with three replications. Treatment of dryness stress consists of two levels, namely: (1) the provision of water commonly at field capacity until the end of the trial, (2) the provision of water to 60% of field capacity given during the critical period of plant that is over six days (3 days before and 3 days after flowering) (Kumar *et al*., 2006; Liu *et al*., 2006; Lafitte *et al*., 2006).

The variables observation ~~are~~ 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 (RGW), which is the ratio between grain weight/hill on drought stress and grain weight/hill without drought stress.

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RESULTS

Effect of Polyethyleneglycol (PEG) 6000 to Germination of Doubles Haploid lines Derived Anther Culture

PEG 6000 treatment affects the germination percentage, root length and length plumule. Germination percentage decreased by 33.95 per cent, followed by a decrease in root length (60.8 per cent), and long-plumule (80 per cent) (Figure 1).

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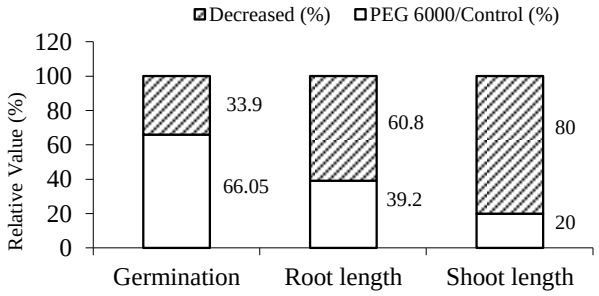

Figure 1. Effect of PEG 6000 on germination, roots length and shoot length

PEG 6000 treatment resulted in the lowest germination in genotypes P3 and P6 (39.8 and 36.5 per cent), while the genotype P4 and P5 produced the highest germination rate (77.5 and 75 per cent) (Figure 2). Long roots lowest average in treatment 20% PEG 6000 is P3 (2.3 cm), and the highest average length root is P6 (3.7 cm) (Figure 3).

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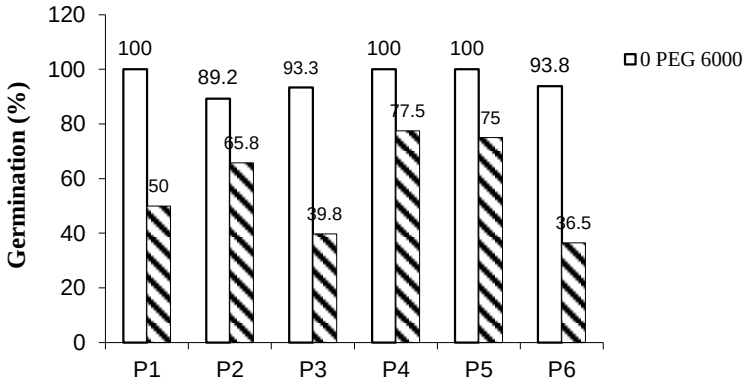

Figure 2. Effect of PEG 6000 on seed germination of double haploid lines derived anther culture

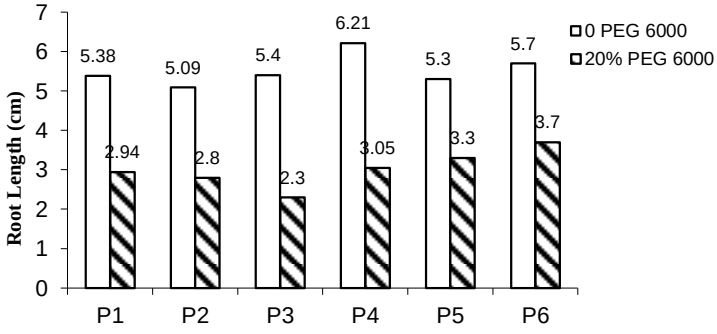

Figure 3. Effect of PEG 6000 on length root of doubled haploid lines derived anther culture

Shoot length varies between the crossing ranging from 0.51 to 1.22 cm. The lowest average of shoot length is P6 (0.51 cm) and P3 (0.65 cm), while the longest in P4 and P5 is 1.22 and 1.16 cm, respectively (Figure 4).

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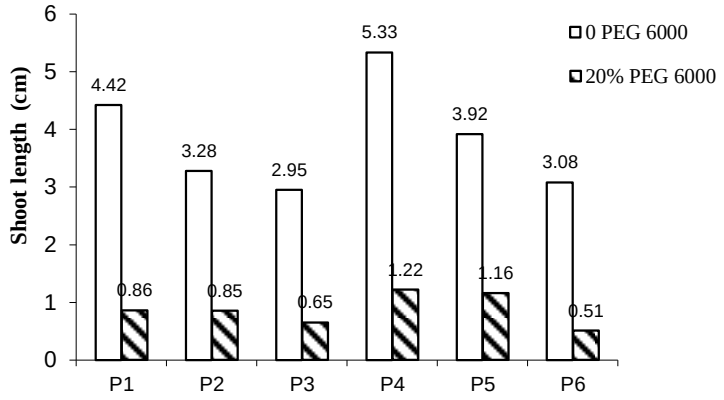

Figure 4. Effect of PEG 6000 on shoot length of double haploid lines derived anther culture

The selection of double haploid lines at 20% PEG 6000 in the phase of germination based on the value of relative root length (RRL) results in very sensitive genotype if RRL<15.46, sensitive if 15.46<RRL<28.09, moderate sensitive if 28.09 <RRL<53.35, moderately tolerant if 53.35 <RRL<65.89, and tolerant if RRL> 65.89. Double haploid lines selection results in the treatment of 20% PEG 6000 in various crosses produced ten genotypes tolerant, 13 somewhat tolerant genotypes, 29 genotypes moderate, eight genotypes rather sensitive and 18 sensitive genotypes (Table 1).

Comment [IJAT8]: it must be changed to be Past tense.

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

Crosses	Number of lines				
	Tolerant	Rather Tolerance	Moderate	rather sensitive	sensitive
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 were chosen to test the consistency of drought stress by 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

Test results with polyethyleneglycol (PEG) 6000 is used for testing drought in the greenhouse. The resulting genotype grouped into three criteria, namely the sensitive genotypes, genotypes moderate and tolerant genotypes based on the relative root length (RRL) (Table 2).

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Effect of Drought Stress on the Growth and Yield of Doubled Haploid Lines Results from Anther Culture

Effect of Drought Stress on Growth

The results of variance in drought stress experiments showed that the shoot length, root dry weight, shoot dry weight, and shoot root weight ratio (SRR) was significantly different among lines except for variables 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 derived anther culture

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

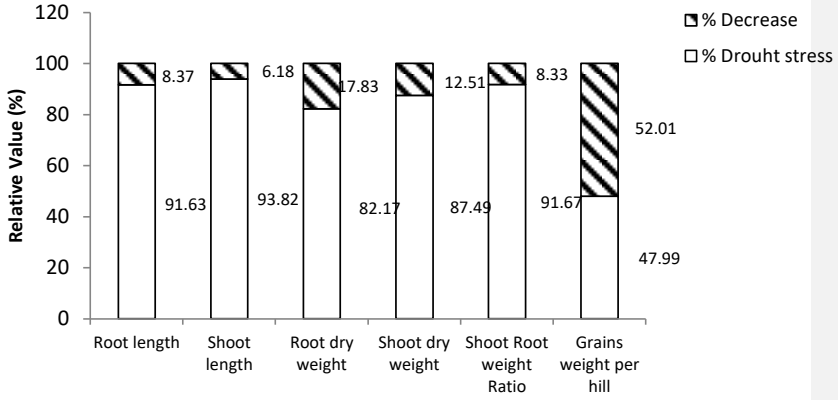

Figure 5. Effect of drought stress on growth and yield of doubled haploid lines derived anther culture

Drought stress treatment significantly different at all variables except for SRR, and there are interactions between genotypes to drought stress, which indicates that their response to drought stress to the difference between genotypes (Table 3). Relative values were used to know the effect of drought stress on the growth and yield of rice (Figure 5).

Drought stress treatment did not show a substantial decrease in growth response. Root length, shoot length and shoot root weight ratio (SRR) only decreased respectively by 8.37, 6.18, and 8.33 per cent because stress give in a relatively short period and the plant growth has stabilised. The variables root

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dry weight decreased by 17.83 per cent and shoot dry weight decreased by 12.51 per cent; however, a decrease in grain weight/hill up 52.01 per cent (Figure 5). Drought stress treatment significantly affects the yield reduction due to drought stress treatment is given at a critical period when filling seed in the reproductive phase.

Total Chlorophyll content

The total chlorophyll content in leaves varies in genotype tested to drought stress. Almost all genotypes showed a decrease in total chlorophyll, except genotypes P6-291 and Batutegi as tolerant check variety. The reduction in total chlorophyll lowest in genotype P3-31, P2-2, P6-53, P6-64, and Fatmawati, while the highest decline in genotype P6-95, P3-190 and P3-221 (Figure 6).

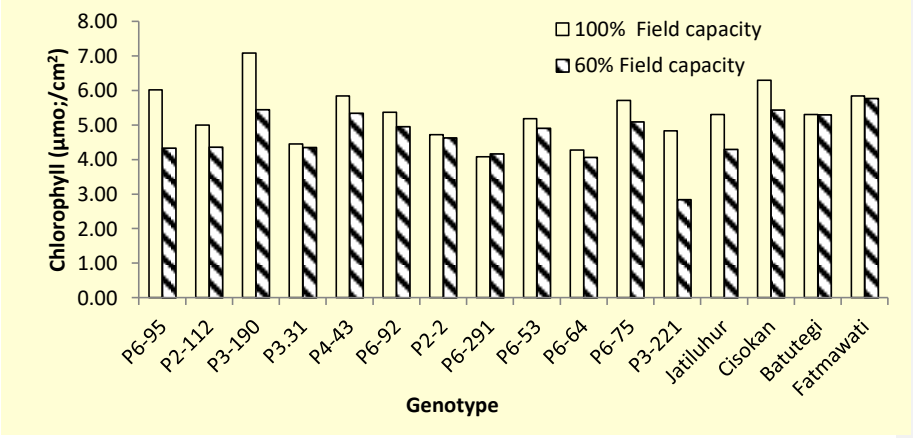

Figure 6. The total chlorophyll content of doubled haploid lines derived anther culture on drought stress

The content of Proline

The content of proline in the leaves varies in inbreds due to drought stress. The content is very high proline contained in tolerant genotype test criteria based on genotype PEG 6000, i.e. P3-31, followed by P6-95 respectively 30.33, 20.82 µmol/g, and genotype moderate line P6-291 at 20:42 µmol/g (Figure 7). Based on criteria of grain weight/hill ratio (GWR), the genotype categorised as sensitive. The amount of organic material that has been accumulated for osmotic adjustment, resulting in grain weight/hill is low.

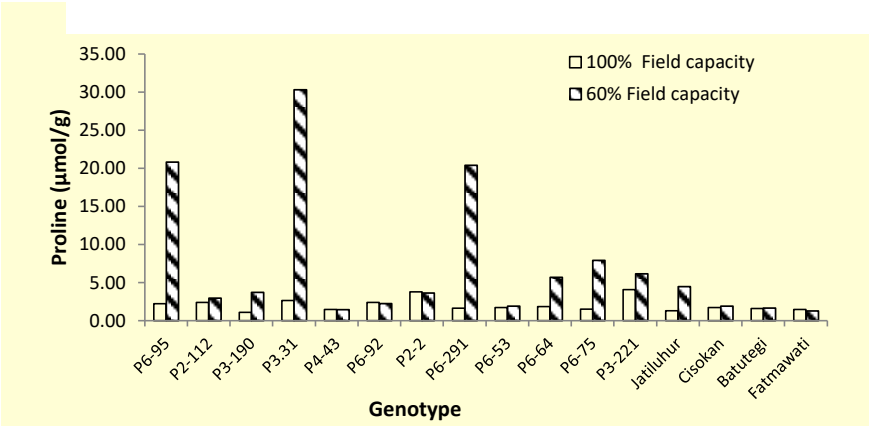

Figure 7. The content of proline of doubled haploid lines derived anther culture on drought stress

Effect of Drought Stress on Yield Double Haploid Lines

The effect of drought on the grain weight per hill on some lines tested presented in Table 4. The line P4-43, P6-92, and P6-53 do not show significant declines due to drought stress. Some lines showed substantial reductions due to drought stress as 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 doubled haploid lines derived anther culture

Lines	Grain weight/hill (g)		GWR(%)[2]	Criteria
	100 % FC[1]	60 % FC		
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 stress tolerance based on the ratio of grain weight per hill (GWR) indicates that the level of consistency varies with test PEG 6000 (Table 4). A total of four double haploid lines tolerant to testing PEG 6000 was only one consistent is the line P3-190, while four moderate lines on the trial of PEG 6000, two of which are compatible based on GWR i.e line P4-43 and P6-92, and four lines sensitive on the trial of PEG 6000 only one inconsistent and otherwise intolerant based on GWR namely line P6-53 (Table 4).

DISCUSSION

Comment [MS12]: MUST SEPARATEV RESULT AND DISCUSSION

PEG 6000 is equivalent to the osmotic pressure of -0.8 MPa causes inhibition of germination, root elongation and epicotyl on chickpea plants (Macar *et al*., 2009). Zapico (2008) reported that the lowland rice genotypes are more sensitive than upland rice on the inhibition of germination in treatment 15% PEG 8000. PEG 6000 led to a water deficit that will inhibit the entry of water molecules into plant tissues, whereas water is indispensable in the process of germination. An average reduction of 60.8 per cent root length (Figure 1). Results Zapico (2008) showed that the leaf is more constrained than the roots of the water deficit during the germination process. The same reported by Macar *et al*. (2009) revealed that PEG 6000 inhibits the shoots and the root elongation. The former of the roots are more exposed to conditions of drought stress and caused damage to the root tissue, so it requires much supply of carbohydrates to the roots, consequently supply to the shoots reduced. PEG 6000 treatment caused a reduction in the shoot length by 80 per cent (Figure 2).

Li *et al*. (2006) reported that there is a decrease in chlorophyll content in the leaves of barley, the tolerant genotype (Tamor and Arta) chlorophyll content dropped respectively by 10.7, and 1.6 per cent and the sensitive genotype (Morocco9-75 and W12291) dropped respectively by 31.3 and 30.1

per cent. Refers to Pieters and Souki (2005), the drought stress weakens the activity of PS II in the flag leaf, reduced chlorophyll, and increased the pigment content xanthophyll, which serves to absorb excess light under irradiation higher in drought conditions. Further proposed by Havaux and Lannoye (1985) that the inhibition of photosynthesis due to drought stress not only causes degradation of chlorophyll and stomatal closure but also resulted in changes in the function of the thylakoid membrane. It reduces quantum photochemical reaction primer on PS II which directs changes in the distribution of energy to PS I. Herawati *et al.* (2017) revealed that the stomatal composition and density in the susceptible genotype being denser and number full than the tolerant genotype. The stomatal density could affect two essential processes, photosynthesis, and transpiration.

Results of research Pirdasthi *et al*. (2009) showed that drought stress at different growth stages would increase the proline content in the leaves, and the tolerant genotype contains proline and high rice yield. The experimental results Mostajeran and Rahimi-Eichi (2009) states that the accumulation of proline varies among genotypes tested, the content of proline in young leaves and old leaves always increased in drought stress, and the content of proline in young leaves was higher than leaves parents in all cultivars tested. Therefore, some studies suggest that the proline content used for the selection of drought-tolerant genotypes.

The effect of drought on the grain weight per hill on some lines tested presented in Table 4. The line P4-43, P6-92, and P6-53 do not show significant declines due to drought stress. Some lines showed substantial reductions due to drought stress as P2-112, P3-90, P3-31, P2-2, P6-75, and P3-221. Sheoran and Saini (1996) and Saini (1997) has detected a sensitivity to drought stress at meiosis anther rice plants. Meiosis in the anther occurs in 9-10 days before flowering depending on the position of the panicle rice. The process of meiosis ends three days before the flowers come out. Therefore stress can be given three days before the flowers come out or afterwards to see the effects on yield reduction.

Liu *et al*. (2006) found that water stress causes the failure of pollination by pollen up to 67 per cent of total grain/ panicle. Furthermore, in the case of pollination, the time to achieve the pollen to reach the ovule micropyle longer is 1-8 days. Flowers failed to open. Consequently, pollen can not get out through the surface of interest due to drought stress (Liu *et al*. 2006). Lafitte *et al*. (2005) reported that drought stress on dryland cause delays flowering an average of 3 days in some genotypes tested, but on the contrary there is a genotype faster flowering phase, it is presumably because these plants are

sensitive to the process of pollination and development of the embryo, so flowering accelerated before more severe stress.

Delayed flowering during drought stress will negatively affect the filling of grains, especially in sensitive genotype, assimilate partitioning of the stems and leaves to the grain filling increases during drought stress by speeding senescence on the leaves (Kumar *et al.* 2006). This statement is supported by Yang *et al*. (2001) that 75-92 per cent during the pre-anthesis, ^{14}C deposited on the trunk that will be relocated back to the seed when there is drought, 50-80 per cent higher than the amount remobilisation on condition without stress. Drought stress on grain filling period also led to senescence faster, shorter grain filling period, but increased assimilate remobilisation (Kamoshita *et al*. 2004).

Drought stress also causing delays exertion of panicle elongation resulting in a reduction in the base of the panicle, causing sterility of grain inside the leaf sheath, which can decrease grain yield (Ji *et al.*, 2005). When viewed from the relative decline in grain weight/hill due to drought stress decreased to 52.11 per cent (Figure 6). Drought stress six days before flowering significant causes a decrease in grain weight/hill, the most significant impact when pressure is at the time of flowering can cause a reduction in fill grain of up to 80% (Liu *et al*. 2006).

The experimental results Lafitte *et al*. (2005) states that the average reduction in grain yield in lowland rice by 75 per cent due to drought stress. Wang *et al.* 2009 reported that the lowland rice genotypes IR2266 more significant reduction in total root length as a result of drought stress than upland rice genotypes CT9993. Upland rice genotypes (CT9993) more adapted to the conditions of water deficit in the rainfed areas, by way of avoidance (avoidance strategy) that can penetrate deep roots and reliable root system. Furthermore, Kumar *et al.* (2009) reported that the decrease in plant biomass could reduce rice yields due to drought stress in sensitive genotype, so the selection of high biomass and harvest index can be used to obtain drought tolerant genotypes.

One of the mechanisms used by plants to defend on drought stress is through the accumulation of proline for adjustment osmotic, production and accumulation of free amino acids like proline in plant tissues during drought stress, an adaptation response in these conditions (Cattivelli *et al.*, 2008; Vendruscolo *et al*. 2007). Further Vajrabhaya *et al*. (2001) revealed that the high proline content not only plays a role in the osmotic adjustment in water stress, proline accumulation was also thought to be involved in the protection of the structure of enzymes and cells from free radicals.

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Reviewers suggested to edit English by native English speaker.

Acknowledgement

We would like to thank Yenni and Imam (staff at the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development) for their assistance in the Laboratorium and field works.

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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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Herawati, R., Purwoko, B. S., Dewi, I. S., Romeida, A., Ganefianti, D. W. and Marlin (2020). Analysis of polyethylene glycol (PEG) and proline to evaluate drought stress of double haploid new type upland rice lines. International Journal of Agricultural Technology X(X): XX-XX

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

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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 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°C, 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).

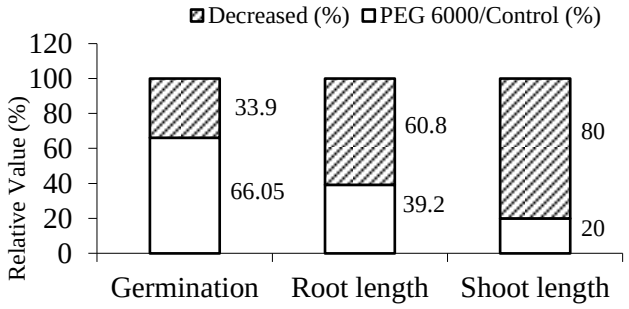

Figure 1. Effect of PEG 6000 on germination, roots length, and shoot length

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).

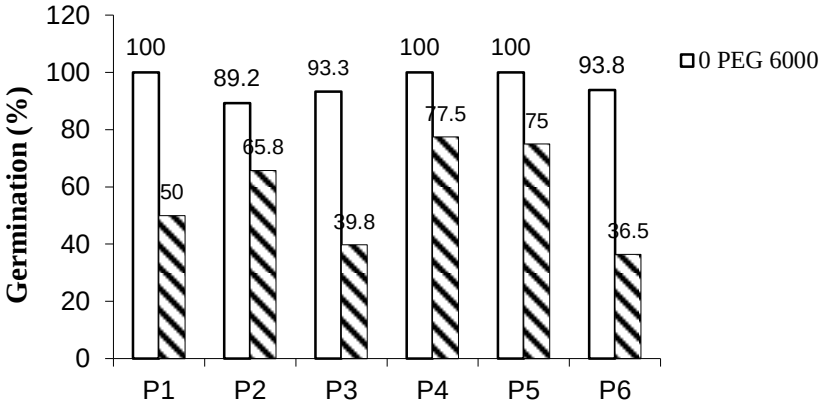

Figure 2. Effect of PEG 6000 on seed germination of double haploid lines

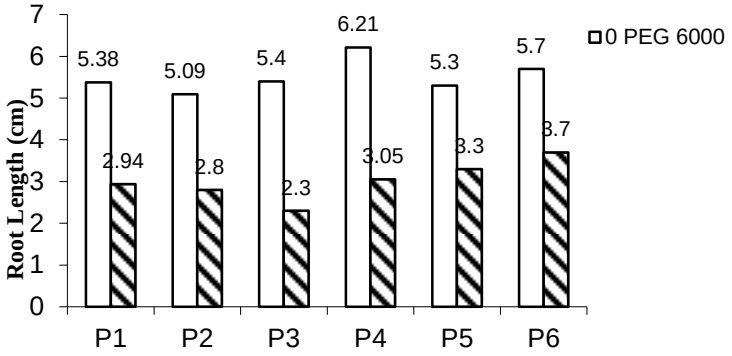

Figure 3. Effect of PEG 6000 on length root of doubled haploid lines

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).

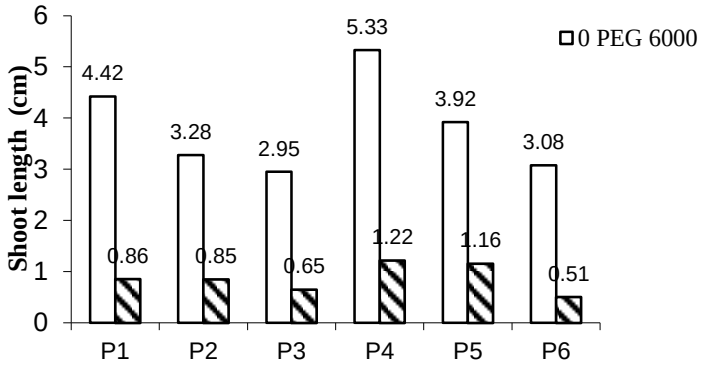

Figure 4. Effect of PEG 6000 on shoot length of doubled haploid lines

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

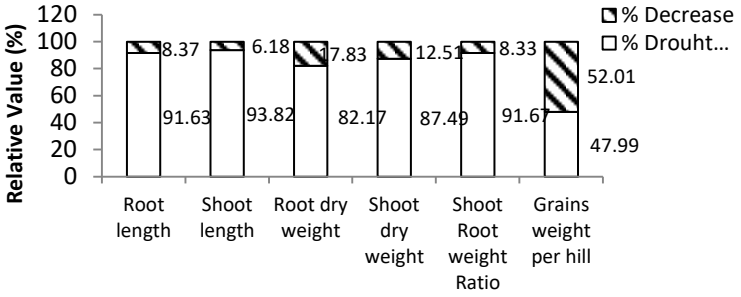

Figure 5. Effect of drought stress on growth and yield of double haploid lines derived anther culture

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).

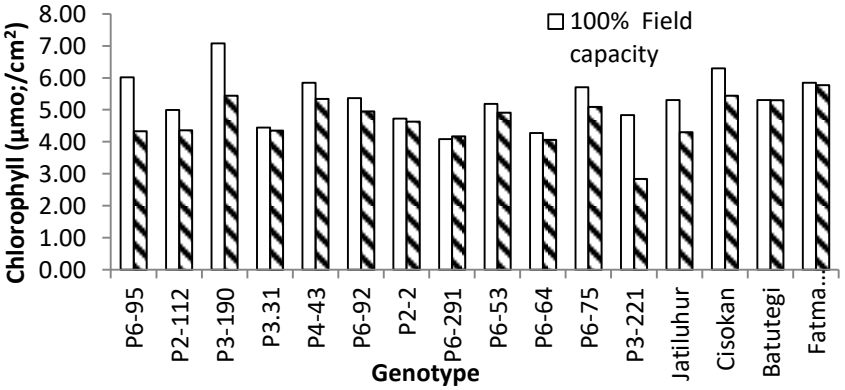

Figure 6. The total chlorophyll content of double haploid lines

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.

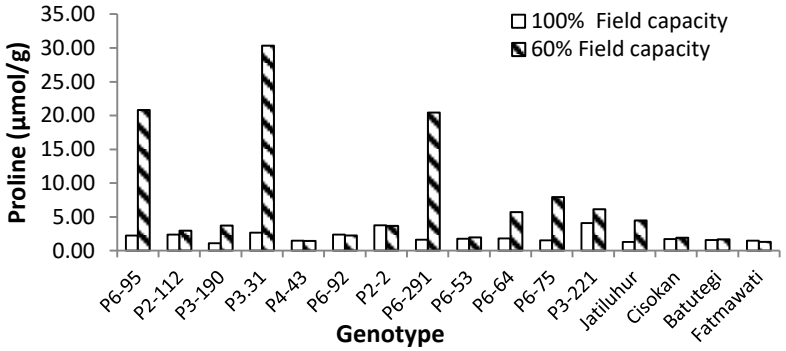

Figure 7. The content of proline of double haploid lines derived

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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