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The morphology, anatomy and physiology characterization of mutant wheat (*Triticum aestivum* L.) "Alibey" in tropical lowland area

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Abstract

The performance information necessary to determine mutant wheat mutant traits in the breeding program of activities to increase production. The aim of this research was to obtain variability that could be used as selection criteria and obtain an adaptive mutant wheat in area of tropical. This research was conducted in the Experimental Garden of SEAMEO-BIOTROP in Bogor with approximately 250 m. above sea level, in the period of April - November 2013. Mutants wheat "Alibey" used were 16 mutants M3 derivative resulteds from treatments using the EMS. LC_{50} was at 0,1% EMS for 60 minutes. Data were analyzed using a variety of methods of Augmented Design. The results showed that the mutant was affected the morphology signifantly indicated by four characters i.e. long stem panicle, grain weight/panicle, weight of 100 seeds and seed weight/plant. While there was no significant effect in the other nine characters of morphology i.e., flowering time, cooking, harvest, panicle length, plant height, number of tillers, panicle number, and leaf area. Anatomical character appearance on leaf thickness and the size of stomata showed different levels of tolerance between mutant plant (AB-0,1.60-1-7-1) and controls. As for the physiological character there were significant differences in the amount of proline and glucose levels. Proline level in control was 4.15 ug/g BB, while that in mutant "AB-0.1.60-3-16-1" was 263.47 µg/g BB, and that in "AB-0.1.60-3-3-2" was 235.90 µ/g BB. Likewise, glucose level in control was 132.88 mg/ml, while in mutant "AB-0.1.60-3-16-1" was 181.48 mg/ml, and that in "AB-0.1.60-3-3-2" was 287.41 mg/ml.

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Introduction

Wheat (*Triticum aestivum* L.) is a subtropic plant, but the possibility can attempt to be adapted in the tropical area such as Indonesia. Wheat was introduced and planted in 1784 in Indonesia, especially in Java and Timor island. The different climate and limited effort of wheat promotion to the farmer by the government bring the unsuccessful plantation and low production of wheat in this country (Wiyono 1980; Sastrosumarjo 1987). Therefore, the genetic variety of wheat that has been developed in Indonesia are still very low. Recently, wheat has been promoted and planted in Tosari (East Java), Banjarnegara (Central Java), Salatiga (Central Java), Malino (South Sulawesi), Sinjai (South Sulawesi) and Padang (West Sumatera) at an altitude >1000 m above sea level, with relatively high productivity. But to expand the acreage of wheat development in the region is less prospective, as the wheat crop is only used as a crop plant hortikuktura the sidelines of economic value is much higher. So the long-term program of grain development in tropical environments Indonesia needs continuous research and directed at lowland area. Assembly wheat varieties that are able to adapt well to the environment in Indonesia, high yield (± 2 tons / ha in lowland) and worth market is very urgent demands to reduce import activities and anticipate greater demand increases with the increasing population. Wheat research is currently directed at lowland area.

Before developing wheat in Indonesian tropical environment, need an understanding of the types and characteristics of common wheat which has been developed in the world. Because only a small portion of the grain that has been growing in the world that can grow and produce well in tropical environments, special Indonesian tropical environment is very diverse. According Sleeper and Poehlman (2006) Genetic diversity and planting wheat in general can divided into two, namely:

1. physiological characteristics of wheat plants adapt to different wheat cultivars for production on a wide range of climatic environments. Physiological characteristics that are essential to understand and limit the adaptability of wheat to climate change / different agro-ecosystem is generally associated with vernalization, winter and response to photoperiodisitas.

2. Chemical and physical characteristics of wheat glutein that contribute to the wide use of wheat seeds for various types of different foods. The diversity of end products where wheat is used requires the production of cultivars with a range of characteristics that affect seed quality. Genetic differences in seed quality that varies with the class of grain market.

Wheat genetic improvement through breeding programs in Indonesia is always at the start by introducing strains of elite from various countries who are considered suitable to area tropical, especially in Indonesia. This is because in Indonesia does not have a local germplasm so as to run the breeding program forward. Extensive genetic diversity is needed to get adaptive tropical wheat varieties in the Indonesian tropical, especially the lowland area. Increased genetic diversity of wheat which has been running is through cross breeding and mutation (seeds and somaclonal variation). Not all types of wheat in the world can be planted in Indonesia's tropical environment, just kind of wheat from Triticum aestivum types of groups of spring wheat that can be developed in the tropical environment of Indonesia (> 1000 m asl). Development of tropical wheat <1000 m asl must be supported breeding programs ranging from the early formation of the population until the release of varieties in a sustainable manner with the method of Augmented Design.

A consortium program has been established in 2009 to enlarged the wheat plantation area and to promote research in increasing the number of wheat variants that can be planted in lowland areas in Indonesia.

One of wheat consortium research project is the introduction of physical or chemical mutants to establish a new strain that can produce higher harvest. Physical mutants cause changes at the chromosomal level, while chemical mutants cause changes on the gene or bases level (Aisyah 2006). Both physical and chemical mutants are causing changes on the morphology and anatomy characteristics.

The morphology, anatomy and physiology characteristics can also being changed by the enviromental factor. The environmental factor can detain the growth of the plant itself and reduce the speed of photosynthesis, due to the closure of stomata that controlled by water retention on the leaves and the dehydration of cuticula (Pennypacker *et al.* 1990, Watanabe *et al.* 1991). The influenced morphology characteristic such as the height of the plant, number of segment, as well as pod per segments (Hoogenboom *et al.* 1987), and number of flower and seed (Korte *et al.*1983).

The plant metabolism and physiologic factor need to be adjusted in the high temperature environment. One of the mechanism that was found in plant during adaptation process is the accumulation of prolin (stable amino acid), absisic acid, dehydrin protein, complex carbohydrates, sorbitol, vitamin C, organic acids, glycine-betaine, superoksida dismutase and K+ for reducing the cell potential osmotic pressure without limiting the function of enzymes (Yoshida *et al.* 1997). Mechanism wheat crop tolerance to high temperature stress is necessary to be studied and understood to support genetic improvement through tropical wheat breeding program. According Dolferus *et al* (2011) that the most critical phase due to abiotic stresses experienced by the wheat crop is the reproductive phase.

The performance information necessary to determine the mutant wheat mutant traits in the breeding program of activities to increase production. This research aims to obtain superior mutant wheat that can adapt in the lowlands and to identify was performed to characterize the morphology, anatomy and physiology of Alibey M3 wheat mutant variant that were planted in the tropical lowland area.

Materials and methods

This study was performed in the SEAMEO-BIOTROP experimental planting area (± 250 m asl), started on April up to December 2013. Sixteen mutant of Alibey wheat as the third generation (M3) of EMS treatment result was used in this study. The LC_{50} of the Alibey wheat was 0,1% EMS for 60 minutes. This study was consists of three stages, *i.e* 1) morphology characteristic observation in the tropical lowland area, 2) anatomy characteristic observation by measure density and the size of stomata and 3) physiology characteristic observation by prolin and glucose concentration analysis.

Morphology characteristic observation in the tropical lowland area

The selected third generation (M3) was planted in the tropical lowland area (<400 m asl). Selected seed was desinfected using Sevin® insecticide. The insecticide Carbofuran® was added into the planting hole. The distance between the plants within a line was 20 x 25 cm. Compost and husk (2:1) was added to the planting media before used. The fertilizer, 200 kg.ha^{-1} Urea, 150 kg.ha^{-1} SP36 and KCl 100 kg.ha^{-1}, was added 14 days after planting. The second fertilizer, 150 kg.ha^{-1} Urea, was added on day 30 after planting. The weeds were removed twice, during fertilizer addition and generatif growth period.

The parameters observed for morphology characterization were the length of tassel (TL), the height of plant (PH), stem length panicle (PTM), seed weight/plant (BBT), panicle number (JM), seed weight/panicle (BBM), dan weight of 100 grains (BBM100). The genotype of putatif mutant was well adapted in the tropical lowland area if the morphological characteristic is fine and harvest more than the control plant.

Data was analyzed using Augmented Design that refer to Petersen (1994). Error standard was using mean squared error (MSE), that calculated using variant analysis. Error standard was used to estimate the Least Significant Increase (LSI). LSI is important to compare the M3 and control population.

Anatomy characteristic observation: measurement of density and the size of stomata

The density of stomata was calculated using the number of stomata in certain area of surveillance on the leaves. Leave samples were marked using nail polish on the bottom to get the pattern of the stomata on the surface (refer to Capellades *et al.* 1990). Then, it was glued to release the epidermis part. The density of stomata was observed randomly under the stereo microscope. About two stomatas were observed per each plant. The observartion was perform at 7 to 9 am every morning.

The formulation to calculate the density of stomata *) : Ǿok = Ǿol x pl /pk

The diameter of surveillance area (10 x 40) = 5 x 10^{-1}mm = 0.5 mm**.**

Note :

Ǿ ok = the diameter of surveillance area, strong objective magnificient

Ǿ ol = the diameter of surveillance area, weak objective magnificient

pk = strong objective magnificient

pl = weak objective magnificient

The wide of surveillance area = ¼ πd^2

= ¼ (3.14) $(0.5)^2$

= 0.19625 mm^2

$$\text{Density of stomata} = \frac{\text{Number of stomata}}{\text{The wide of surveillance area}}$$

(∑ stomata/mm^2)

Physiology characteristic observation: prolin and glucose concentration analysis

Prolin concentration was analyzed using Bates method (Bates *et al.,* 1973). About 0.5 gr flag leaves from mutant and control groups were crushed and homogenized using 9 ml of 3% sulphosalisilat acid. Moreover, samples were adjusted up to 10 ml using sulphosalisilat acid and centrifugated in 5000 rpm for 5 minutes. About 2 ml of ninhidrin acid and glacial acid were mixed with 2 ml of supernatan and heated at 100˚C for 60 minutes. Next, the mixed solution was incubated in the ice bath for 5 minutes. The reaction was extracted using 4 ml Toluene (as the standard) and mixed for 15-20 seconds to perform the chromoform. The free chromoform was separated from the solution in the room temperature. The chromoform concentration was measured using 520 nm of spectrophotometer. Proline concentration (µg/g) was measured using standard curve and calculated as follow:

[(proline (µg/ml) × toluene (ml)) / 115.5 µg/µmole]/[(sample (g))/5] = proline (µmoles) / fresh leaves weight (g)

Glucose concentration was analyzed refer to Smogy Nelson method (1982). About 2 to 2.5 g of flag leaves were heated and dried in the oven at 40-45˚C for 2 days. All dried leaves were milled. About 200mg of milled leaves centrifuged with 20 ml of 80% absolute ethanol for 20 minutes at 60-70˚C. Supernatan was removed and 20 ml of ethanol was added to the residu (extract). These procedures were repeated three times.

Absolute fluid in flat evaporated in a water bath until the remaining 1-2 ml. The remaining liquid was filtered with a filter paper in a 100 ml volumetric flask approximately 50ml + 5ml of distilled water + 5 ml Ba $(OH)_2$ 5% + 5% $ZnSO_4$ 5ml, resulting in the deposition of protein. The solution was added aquadest up to 100ml, shaken and then filtered again using filter paper. Total sugar analysis performed by the procedure: 5ml pipette extract solution in a test tube 5ml H_2SO_4 + 1,4N then heated (10 min) in a water bath, then cooled. The solution was neutralized with 1N NaOH, to form a pink color. The solution was added aquadest uo to 20ml and shaken (extract II).

The process of reduction / coloring: Take II 2ml ethanol in a test tube 25ml + 2ml reagent Cu, and heated for 10 minutes in a water bath. (Create a standard sequence 5, 10, 15, 20, 25 ppm), then cooled and added with 2 ml Nelson reagent, shake up the CO2 that is lost and the color changed to transparent. The solution was added aquadest up to 25ml, and shaken until blended and allowed to stand for 30 minutes. The solution was measured with a spectrophotometer wavelength of 500 nm.

Results and discussion

The average rainfall at Seameo-Biotrop plantation area during this study was 360 mm, with temperature and humidity were around 26.4°C and 85%. The total rainy days during this study were 15.25 days, while the lenght of sunlight was 63% with sunlight radiation intensity was 237.5 Cal/Cm² (BMG, 2013). All of those parameters met the classification as the tropical lowland area as the requirement in this study. The optimum temperature for wheat refer to Wiyono (1980) and Van Ginkel and Villareal (1996) is 15 to 25°C. The increase of 1°C of temperature could inhibit the growth of the wheat. Temperature is the most important factor to grow the wheat, because this plant is very sensitive to temperature changes (Handoko 2007).

The augmented design analysis in Table 1 and 2 showed that there was significant different in stem length panicle (8 mutants), seed weight/plant (9 mutants), seed weight/plant (1 mutant) dan weight of 100 graints (4 mutants). The different of temperature in the location for control versus mutants did not indicate a significant different of height of plant (TT) and panicle number (JM). Paralell to our results, Setyowati *et al.* (2009) reported significant different in three characterics, *i.e* number of tillers, productive tillers and panicles stem length (PTM). Based on the result obtained "Alibey" mutants could be selected mainly based on the two characters i.e. stem panicle length and seed weight/plant because more mutants with both characters as compared to the other characters, could be generated.

Table 1. The augmented design of the panicles stem length (PTM), seed weight/plant (BBT), panicle number (JM) in the mutant Alibey in the tropical lowland area.

Genotipe	ADJ	LSI	PTM	ADJ	LSI	BBT	ADJ	LSI	JM
Ab.0,1.60-1-7-1	3.00	5.26	ns	14.48	11.76	*	2.70	6.08	ns
Ab.0,1.60-1-7-2	5.72	5.26	*	3.298	11.76	ns	1.70	6.08	ns
Ab.0,1.60-1-11-1	5.82	5.26	*	10.678	11.76	ns	1.03	6.08	ns
Ab.0,1.60-1-11-2	4.82	5.26	ns	11.208	11.76	ns	2.37	6.08	ns
Ab.0,1.60-2-14-1	6.22	5.26	*	12.398	11.76	*	1.70	6.08	ns
Ab.0,1.60-2-14-2	5.52	5.26	*	5.568	11.76	ns	1.70	6.08	ns
Ab.0,1.60-2-20-1	4.82	5.26	ns	11.408	11.76	ns	2.37	6.08	ns
Ab.0,1.60-2-20-2	3.72	5.26	ns	16.118	11.76	*	3.03	6.08	ns
Ab.0,1.60-3-3-1	8.02	5.26	*	17.048	11.76	*	3.03	6.08	ns
Ab.0,1.60-3-3-2	6.52	5.26	*	17.218	11.76	*	2.37	6.08	ns
Ab.0,1.60-3-16-1	3.82	5.26	ns	15.268	11.76	*	5.03	6.08	ns
Ab.0,1.60-3-16-2	3.82	5.26	ns	9.048	11.76	ns	3.03	6.08	ns
Ab.0,1.60-4-13-1	5.22	5.26	ns	12.418	11.76	*	4.03	6.08	ns
Ab.0,1.60-4-19-1	6.82	5.26	*	12.298	11.76	*	2.37	6.08	ns
Ab.0,1.60-5-4-1	5.32	5.26	*	8.178	11.76	ns	2.70	6.08	ns
Ab.0,1.60-5-11-1	4.52	5.26	ns	17.588	11.76	*	3.03	6.08	ns

Note. *= statistically significant (higher than control(LSI)/different compare to control).

Table 2. The augmented design of the seed weight / panicle (BBM), weight of 100 grains (BBM100), height of plant (TT) in the mutant Alibey in the tropical lowland area.

Genotipe	ADJ	LSI	BBM	ADJ	LSI	BBM100	ADJ	LSI	TT
Ab.0,1.60-1-7-1	1.03	0.93	*	103.28	92.81	*	46.60	66.51	ns
Ab.0,1.60-1-7-2	0.503	0.93	ns	60.722	92.81	ns	43.37	66.51	ns
Ab.0,1.60-1-11-1	0.653	0.93	ns	75.722	92.81	ns	52.37	66.51	ns
Ab.0,1.60-1-11-2	0.763	0.93	ns	86.722	92.81	ns	51.07	66.51	ns
Ab.0,1.60-2-14-1	0.873	0.93	ns	97.722	92.81	*	49.77	66.51	ns
Ab.0,1.60-2-14-2	0.573	0.93	ns	67.722	92.81	ns	51.37	66.51	ns
Ab.0,1.60-2-20-1	0.473	0.93	ns	57.722	92.81	ns	51.77	66.51	ns
Ab.0,1.60-2-20-2	0.613	0.93	ns	71.722	92.81	ns	50.77	66.51	ns
Ab.0,1.60-3-3-1	0.683	0.93	ns	78.722	92.81	ns	60.37	66.51	ns
Ab.0,1.60-3-3-2	0.753	0.93	ns	85.722	92.81	ns	55.07	66.51	ns
Ab.0,1.60-3-16-1	0.833	0.93	ns	93.722	92.81	*	52.77	66.51	ns
Ab.0,1.60-3-16-2	0.693	0.93	ns	79.722	92.81	ns	49.77	66.51	ns
Ab.0,1.60-4-13-1	0.863	0.93	ns	96.722	92.81	*	50.77	66.51	ns
Ab.0,1.60-4-19-1	0.483	0.93	ns	58.722	92.81	ns	46.37	66.51	ns
Ab.0,1.60-5-4-1	0.563	0.93	ns	66.722	92.81	ns	49.77	66.51	ns
Ab.0,1.60-5-11-1	0.773	0.93	ns	87.722	92.81	ns	53.07	66.51	ns

Note. *= statistically significant (higher than control(LSI)/different compare to control).

The significant different of moprhology characteristic indicated the high variety among control and mutant group. Thus, the mutant group in this study is strongly indicated as a new variant of Alibey (Fig. 1). The mutant group showed positive response and tolerant to the extremely different temperature between its normal condition in subtropical area. It gave positive indication that the M4 (generated from M3) can be planted and growth in the tropical lowland area. It is expected that the M4 can handle the different of temperature, humidity, sunlight intensity, as well as the rainfall, in the tropical lowland area. Moreover, the M4 is expected can produce harvest as the control wheat production in the subtropic area.

Ivory (1989) reported that elevation influence the vegetative growth phase, flower and seed formation as well as grain filling, cumulatively. As the response to the elevation, usually the plant change its physiological activity. High temperature and rainfall usually cause the shortening of pollen anthesis lenght and reduce the number of viable pollen. Huan *et al.* (2000), Kakani *et al.* (2002) dan Thuzar *et al.* (2010) reported that high temperature obstruct the germination and the growth of pollen tube, reduce the number of flower and sensitivity, as well as fruit set among plant variety.

Fig. 1. The different of plant in the control compare to mutant Alibey in the tropical lowland area.

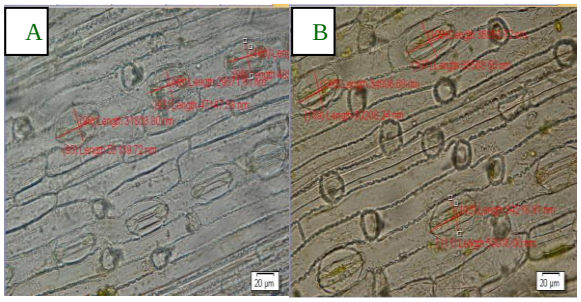

Fig. 2. The stomata of Alibey. A) Control, B) Mutant (AB-0.1.60- 1-7-1).

The density of stomata and the thickness of leaves were significantly different among control versus mutant (Fig. 2 and 3). The average of stomata density was 35.66mm² (control) versus 25.47 mm² (mutant). Meanwhile, the lenght and width of stomata were 47147.30-53139.72nm and 29671.81–31803.80nm in control, versus 50208.24–69508.56nm and 34996.69–36953.72nm in mutant Alibey. The different of stomata density and leaves thickness in control versus mutant group observed due to the differences in ability to absorb the nutrient, water evaporation and sunlight intensity to the palisade cells. High intensity of sunlight and temperature cause the inrease of palise cells length. Moreover, it also becomes multi-layered. The increasing of CO_2 concentration is also causing limited anatomical characteristic changes (Taiz & Zeiger 2002; Sopandie 2014).

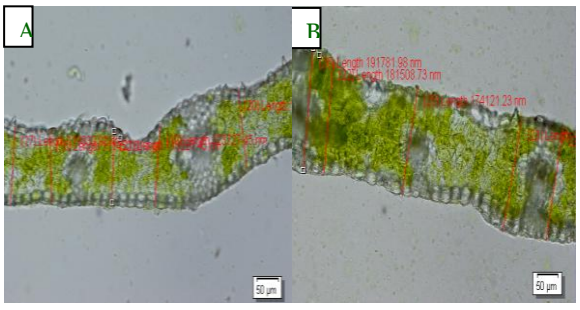

Fig. 3. The thickness of the leaves using microtechnique method.
A) Control, B) Mutant (AB-0.1.60-1-7-1).

Physiologic characteristic can be used as a valid parameter to compare the mutant versus control. Proline and glucose analysis shown in the Fig. 4. Proline accumulation in the mutant was shown as a compensation to the increase of temperature in the tropical lowland area. That mechanism is called as osmotic adjustment.

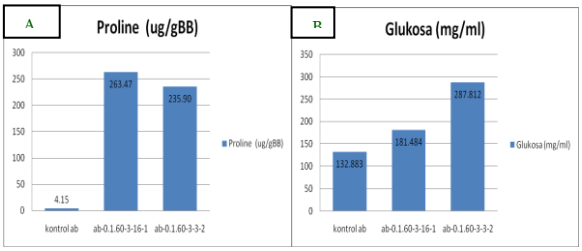

Fig. 4. Proline and Glucose analysis in control and mutant plant.
A). Stands for Proline analysis, while B). stands for Glucose analysis.

The Proline concentration analysis showed significant different among control (4.15µg/gBB) versus mutant ab-0.1.60-3-3-2 (235.90µg/gBB) and ab-0.1.60-3-16-1 (263.47µg/gBB). Moreover, the Glucose concentration analysis was also differ among control (132.883mg/ml) versus mutant ab-0.1.60-3-3-2 (287.812 mg/ml) and ab-0.1.60-3-16-1 (181.484mg.ml). These results showed that two mutant groups (ab-0.1.60-3-3-2 and ab-0.1.60-3-16-1) were response the temperature changes with osmotic adjustion. Patil (2008) and Kadir (2011) reported that the Proline concentration in sugar cane and patchouli clone was significantly increase at extremely dry environment. The lowest proline concentration occur at sugar cane in normal irigation environment, while the lowest nitrate reductase enzyme activity occur at extremely dry environment. In Barley plant, Proline accumulation also occur at 10 times drier environment (Bandurska 2000).

Conclusion

In conclusion, mutant Alibey has significant different of the morphology, anatomy and physiology characteristic compare to control one. We expect that all differences in observed parameters were useful to

adjust the productivity of mutant Alibey in the tropical lowland area. Moreover, the new variant of Alibey would increase the number of biodiversity in Indonesia.

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