GROWTH AND PROLINE CONTENT OF IRRADIATED IN VITRO SHOOTS OF *UBI KUNING* CASSAVA GENOTYPE CULTURED AT DIFFERENT TEMPERATURES

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Abstract

Cassava (*Manihot esculenta*) is an important crop to food security under climate change due to its various tolerance mechanism under stress conditions. However, the sustainable growth of cassava in the field depends on many factors especially temperature. The objective of the research was to investigate the growth performances and proline contents of irradiated *Ubi Kuning* at dosage of 10 Gy, cultured in Murashige Skoog (MS) hormone-free solid medium for 4 weeks at three different temperature treatments i.e 25ºC, 28ºC and 30ºC. Each treatment consisted of 3 clone explants with 5 replicates. Results show that growth performances of irradiated plantlets were better compared to that of non-irradiated plantlets in terms of plant height and number of leaves at all temperature tested. The best growth performances were obtained from irradiated plantlets grown under 30ºC. The proline content of irradiated *Ubi Kuning* was high when they were grown under 25 ºC and 30ºC, implying that these plantlets had the possibility to tolerant to lower and higher-temperature condition. This study is initially useful to find out the growth ability of irradiated *Ubi Kuning* in response to lower and higher temperature.

Key words: *Ubi Kuning*, gamma irradiation, temperatures, proline , growth performances, in vitro

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Introduction

Cassava (*Manihot esculenta*) is a staple food for over 1 billion people worldwide due to its ability to grow in marginal land. It is also known as the third most important crops for calorie sources in the tropics including sub-Saharan Africa, Asia and South America (Knox *et al*., 2012). Due to its various tolerance mechanism under stress conditions, cassava has been promoted as an important crop to food security under climate change (Wheeler & Von Braun, 2013). The production of cassava used for food or for industrial purposes has increased significantly per year. In Indonesia, it is predicted that the need of fresh cassava will increase about 30 million tons by 2025 (Karni *et al*., 2015). Many efforts to cope the cassava demand have been done using conventional breeding and vegetative propagation. However, this conventional technique had many limitations regarding with the production and utilization of the crop.

The combination of conventional breeding with mutation induction through gamma ray irradiation is useful approaches to produce superior cassava with the desired traits and sustainable availability (Suprasanna*et al*., 2015; Oladosu *et al*., 2016). Many studies on the in vitro propagation combined with gamma ray irradiation had been successfully reported in Caper (Al-Safadi & Elias, 2011), Cassava (Supatmi & Sudarmonowati, 2012), Potato (Yaycili, & Alikamanoğlu, 2012) and Banana (Datta *et al*., 2018). However, the sustainable growth of those plants in the field depends on many factors i.e drought, water and temperature.

Temperature affects the growth and photosynthetic plasticity in plants (Brown *et al*., 2016). In an appropriate temperature treatment, plants can grow and produce a high yield. Normally, cassava in Asia regions grows well at the temperature of 26-27 °C throughout

the year (Onwueme, 2002). Several cassava genotypes can also grow on drier regions which gives cassava an ecological comparative advantage with other crops. Cassava grown in the controlled glasshouse at temperature of 23 °C and 34 °C exhibited differences in plant height and plant biomass (Brown *et al*., 2016). Generally, the growth of plants will be inhibited under stress temperature (low and high). Furthermore, they will accumulate large quantities of compatible solutes i.e proline, sucrose, polyols and trehalose, which usually non-toxic at high cellular concentrations (Hayat *et al*., 2012). These solutes have a function to protect plants from stress by involving in ROS detoxification, cellular osmotic adjustment and protein stabilization (Kishor *et al*., 2014).

Proline is one of compatible solutes, which act as an osmolyte for osmotic adjustment and also contributes to stabilize subcellular structures and to scavenge free radicals under stress conditions (Filippou *et al*., 2014; Kishor *et al*., 2014). In many plants, the accumulation of proline occurs when plants grow under water deficit (Pirzad *et al*., 2011; Planchet *et al*., 2014), low temperature (Liu *et al*., 2013), salinity (Filippou *et al*., 2014) and UV radiations (Salama *et al*., 2011). This accumulation has been also correlated with stress tolerance by generally accumulating higher in tolerant plants than in sensitive plants. Proline is normally accumulated in the cytoplasm, which has a function as molecular chaperons by stabilizing the structure of proteins and maintain cell redox status (Kishor *et al*., 2014). The accumulation of proline may be a mechanism of signal stress to trigger adaptive responses.

Ubi Kuning is one of local genotype cassava, which has been identified as a potential genotype with high beta-carotene contents (Hartati *et al*., 2012). It is also known as a local genotype, which could be growing well in drought stress although it is quite susceptible to disease (Hartati *et al*., 2013). Therefore, the objective of this study was to investigate the effect of different temperature treatments on growth and proline content of irradiated *Ubi Kuning* grown in vitro. This study will be initially useful to know the growth ability of irradiated *Ubi Kuning* in response to different temperatures.

Materials and Methods

Plant materials and temperature treatments:

The 3 clones of irradiated in vitro shoots of *Ubi Kuning* cassava at dosage of 10 Gy and non-irradiated *Ubi Kuning* propagated until seven times every 3 months in Murashige Skoog (MS) hormone – free solid medium were used in this study (Figure 1). These clones were selected since the irradiated plantlets with the dosage of 10 Gy were the most survived plantlets and had the normal growth appearances compared to other dosages of irradiated plantlets (Rahman *et al*., 2013). Those plants were cut into 0.6-1.5 cm long with 1 node. Explants were then cultured in MS hormone – free medium with the pH medium adjusted to 5.8. Medium were solidified with 0.8% agar before autoclaving at 121°C for 15 minutes. Irradiated plantlets and non irradiated plantlets were then incubated in culture rooms with three different temperature treatments i.e 25°C, 28°C and 30°C under continuous light. The temperature treatments has been set stably in three different controlled incubators. Each treatment consisted of 3 explant clones with 5 replicates. Every week, the growth parameters i.e the plant height, number of roots and number of leaves were recorded until 4 weeks of culture. All data were then analyzed using variance analysis (ANOVA), Independent T-test analysis, followed by Duncan's Multiple Range Test (DMRT) at 5% of significant probability from the mean comparison and Correlation analysis using SPSS program 16.0.

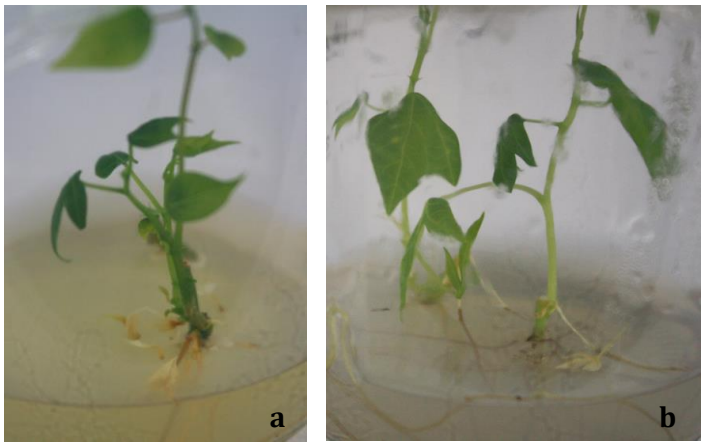

Figure 1. In vitro *Ubi Kuning* propagated in Murashige Skoog (MS) hormone - freesolid medium for 3 months. a. Non-irradiated *Ubi Kuning*; b. Irradiated *Ubi Kuning* at 10 Gray

Determination of Proline Content

After four weeks of culture, whole plantlets in treatment media were used for proline analysis. Proline was measured as described by Bates *et al* (1973) and Khedr *et al* (2003). Purified proline was used as a standard for proline quantification. About 0.5 grams of samples were homogenized in 1.5 ml of 3% Sulphosalicylic acid and the remained residue was removed by centrifugation at 12000 rpm for 10 minutes. 100 µl of the extract was reacted with 2 ml glacial acetic acid and 2 ml acid ninhydrin. The ninhydrin was prepared by warming 1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml of 6 M phosphoric acid until dissolved for 1 h at 100 ℃. The reaction was terminated in an ice bath. The reaction mixture was then extracted with 1 ml toluene. The chromophore-containing toluene (1 ml, upper phase) was warmed to room temperature and its optical density was measured at 520 nm with toluene used as a blank. The proline content was determined by comparing the results of samples with a standard curve and calculated on a fresh weight basis with the equation as follows: [(µg proline/mL x mL toluene)/115.5 µg/µmole]/[(g sample)/5]= µ moles proline / g of fresh weight material. 5 (denominator) derived from total volume (100 µl extract + 2 ml glacial acetic acid +2 ml acid ninhydrin +1 ml toluene) divided with 1 ml sample volume.

Results

The differences of growth responses of non irradiated and irradiated plantlets at different temperatures based on T-test analysis

In order to know the different response of plant growth between irradiated and non-irradiated plants, T-test analysis was performed in each different temperatures. This is important to know the effect of irradiated and non-irradiated plants in plant growth. In vitro *Ubi Kuning* of non-irradiated plantlets and irradiated of 10 Gy plantlets grown under different temperatures (25˚C, 28˚C, 30˚C) for 4 weeks showed that the irradiation treatment effect on growth of *Ubi Kuning* in vitro in terms of number of leaves at 28˚C and the height of plants at 25˚C and 30˚C (Table 1). Interestingly, the increase values of growth parameters occurred on irradiated plantlets, although the significant increase of those growth parameters was different in various growth temperature. In terms of plant height parameter, it showed that plant height of the irradiated plants grown both under 25˚C and 30˚C were higher than those control plants with the difference value of 0.6 cm and 1 cm, respectively ($P \leq 0.05$). At a temperature of 28˚C, the plant height and the number of root of irradiated plants at a dosage of 10 Gy were higher than those control plants although the value of both parameters did not significantly different ($P \geq 0.05$). In terms of the number of

leaves, irradiated plantlets had lower their number of leaves significantly at P≤ 0.05 (Table 1). In comparison with non-irradiated treatment, the irradiated *Ubi Kuning* at dosage of 10Gy apparently had been well-developed at all three different temperature treatments, which could be observed from the number of root and plant height except for the number of leaves of irradiated plants grown at temperature of 28 ˚C showing the decreasing trend significantly.

Table 1. The growth of control and irradiated Ubi Kuning grown at different temperatures of 4 week old cultures.

Temperatures	Growth Parameters	Plant condition	
		Non-irradiated plantlets (control)	**Irradiated plantlets After irradiation**
25˚C	Number of roots	0.6±0.2	1.7±0.6
	Number of leaves	1.4±0.2	1.5±0.2
	Plant height (*)	1±0	1.6±0.1
28˚C	Number of roots	1.2±0.4	2.2±0.5
	Number of leaves (*)	2.6±1	1.4±0.2
	Plant height	0.8±0.2	1.6±0.2
30˚C	Number of roots	1.1±0.3	1.9±0.5
	Number of leaves	0.8±0.2	1.6±0.4
	Plant height (*)	1.07±0.07	2±0.2

Note: mean±standard error followed by asterisk (*) represent a significant differences of plant growth parameters.

Growth performances of non-irradiated and irradiated plantlets grown under various temperatures based on ANOVA analysis

The growth response of irradiated and non-irradited *Ubi Kuning* was observed prior to assessing the best growth performances of plants under three different temperatures. In this study, ANOVA analysis has been used to know the best response of plant growth treated under three different temperatures. Results showed that the non-irradiated *Ubi Kuning* could grow in culture media under various temperature treatments. This could be observed from the growth parameters including the number of roots, the number of leaves and the plant height (Table 2). Interestingly, the number of leaves of these plants was significantly different when plantlets were cultured in media at the temperature of 25˚C compared with other temperature treatments (p ≤0.05) (Table 2). Meanwhile, another growth parameters including the number of roots and the plant height were similar in all those temperature treatments (p ≥0.05). Apparently, the non-irradiated *Ubi Kuning* showed best performances when they were cultured under 25˚C which could be observed from the significant number of leaves, although the number of roots and plant height apparently were similar to the condition of other explants at 28˚C and 30˚C.

Of the growth performances of irradiated *Ubi Kuning* with dosage of 10 Gy, resluts showed that most of irradiated plants had a significant growth parameters when they were cultured on media under 30˚C. It could be observed from the plant height of irradiated *Ubi Kuning* showing a significant difference compared to that of other temperature treatments (p ≤0.05) (Table 2). Another growth parameters including number of roots and number of leaves in all various temperatures were not significantly different. Of all temperature treatments, it shows that irradiated *Ubi Kuning* could survive in all temperature treatments. Interestingly, under higher temperature at 30˚C, the growth of irradiated *Ubi Kuning* was significantly well-developed, which could be observed from the plant height,

the number of roots and the number of leaves (Table 2 & Figure 2).

Table 2. The growth response ofnon-irradiated and irradiated *Ubi Kuning*in vitro grown under different temperature .

Temperature treatments	The growth of non-irradiated *Ubi Kuning* (control)			The growth of irradiated *Ubi Kuning*		
	The number of roots (ns)	The number of leaves (*)	The plant height (ns)	The number of roots (ns)	The number of leaves (ns)	The plant height (*)
25˚C	0.4±0.2	1.2±0.2[a]	0.9±0.07	1.6±0.5	1.3±0.3	1.5±0.08[b]
28˚C	1.2±0.4	0.8±0.2[b]	0.8±0.1	2.2±0.4	1.3±0.3	1.5±0.1[b]
30˚C	1.1±0.3	0.8±0.2[b]	1.07±0.07	1.9±0.5	1.6±0.4	2±0.2[a]

Note: mean ± standard error followed by different letter in the each column was significantly different at P<0.05 by DMRT; ns and * denote non significance and significance at P<0.05 levels.

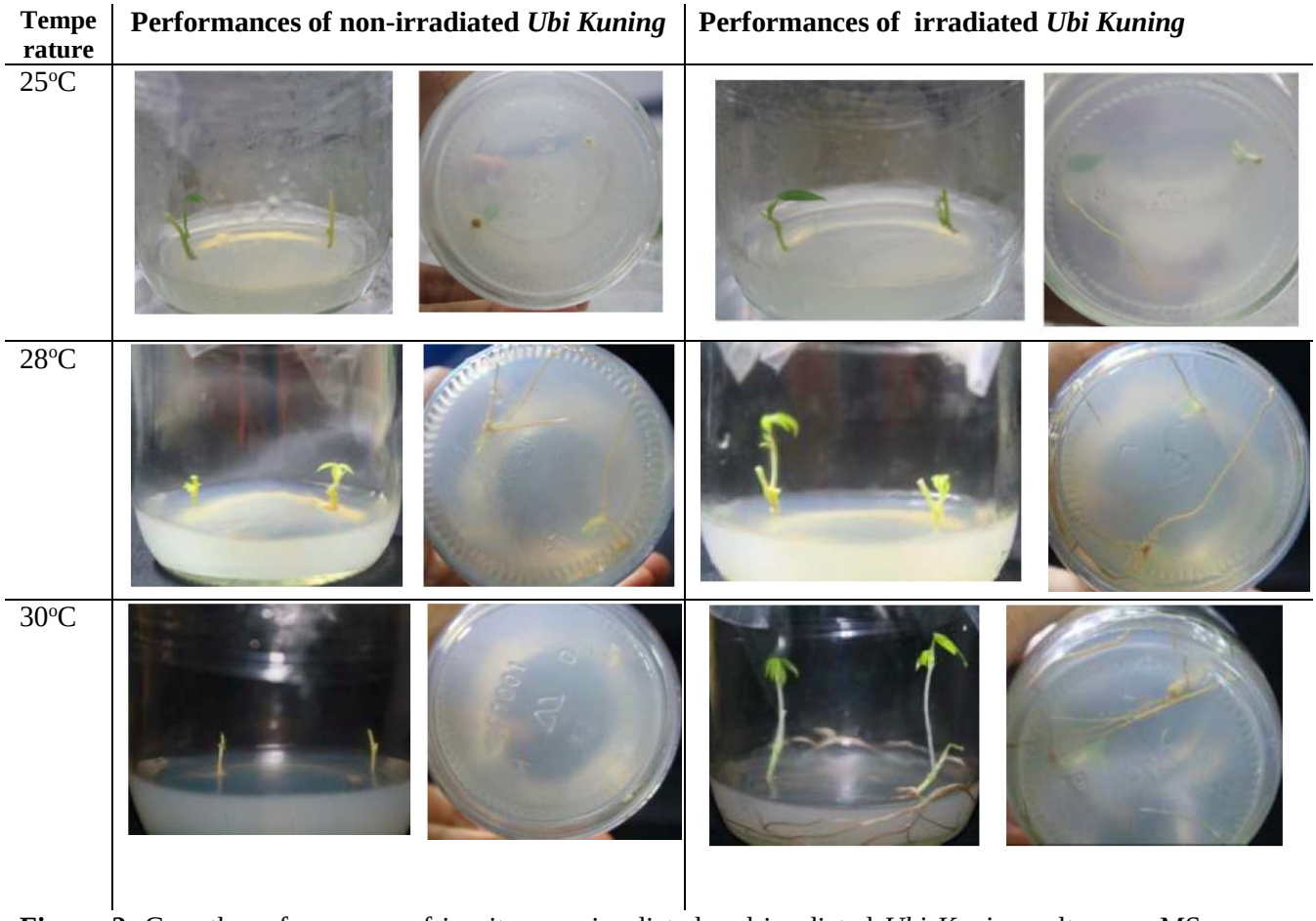

Figure 2. Growth performances of in vitro non-irradiated and irradiated *Ubi Kuning* culture on MS hormone – free medium of for 4 weeks old cultures grown under different temperature. The right figures in each columns shows the whole performance of plantlets; the left figures in each columns shows the root growth of plantlets.

The correlation between growth parameters and different temperature treatments of in vitro irradiated *Ubi Kuning*.

Correlation analysis between the growth parameters and treatment of various growth temperatures showed that in vitro irradiated *Ubi Kuning* grown under 25˚C had a negative correlation especially on the number of roots and the number of leaves although the value was not significant. While under the temperature of 28˚C, the number of root and the number of leaves had a positive

correlation($P \leq 0.05$). It means that the increase number of roots was in line with the increase number of leaves and vice versa. Interestingly, another parameters i.e the number of leaves also had a significant positive correlation with the plant height ($P \leq 0.01$). Of the in vitro irradiated *Ubi Kuning* grown under 30˚C, it showed that all parameters including the number of root, the number of leaves and plant height had a positive correlation. Interestingly, those correlations were highly significant ($P \leq 0.01$ and $P \leq 0.05$). It indicates that irradiated *Ubi Kuning* had a better growth development after it was cultured at the temperature of 30˚C and 28˚C, which could be observed from all the observed growth parameters (Table 3). It shows that the temperature of 30˚C is the optimum temperature for growing in vitro irradiated *Ubi Kuning* followed by that at 28 ˚C. Although the other factors such as the effect of irradiation treatment may influence the ability of plant to tolerance to different temperature treatments since the control showed a limit growth performance grown under temperature of 30˚C.

Table 3. The correlation of each growth parameters of in vitro irradiated Ubi Kuning after 4 weeks grown under different temperature treatments.

Temperature treatments	Growth Parameter	25˚C			28˚C			30˚C		
		JA	**JD**	**TT**	**JA**	**JD**	**TT**	**JA**	**JD**	**TT**
25˚C	JA	1	-.273	.000						
	JD		1	.298						
	TT			1						
28˚C	JA				1	.739*	.628			
	JD					1	.737*			
	TT						1			
30˚C	JA							1	.772**	.794**
	JD								1	.740*
	TT									1

Note:Values followed by *, and ** represent significance differences at $P \leq 0.05$ and $P \leq 0.01$. Means followed by *ns* do not represent significance differences at $P \geq 0.05$. JA : Number of roots, JD : Number of leaves, TT: Plant height

Proline content analysis

The proline content of irradiated and non irradiated *Ubi Kuning* plantlets grown under different temperature are presented in Figure 3. Results show that non irradiated plantlets had the increased trend of proline contents along with the increased level of temperature treatments. Meanwhile, the proline contents of irradiated plantlets showed higher after grown under 25ºC followed by those under 30ºC and 28ºC. However, proline contents of non-irradiated plants were lower compared to non-irradiated plants grown under different temperature treatments. Of the variety of temperature treatments, irradiated plants possessed the highest proline contents (0.027 µmoles/g FW) at temperature of 25ºC followed by 30ºC and 28ºC with the proline contents were 0.015 µmoles/g FW and 0.011 µmoles/g FW, respectively (Figure 3). Interestingly, the different results had been achieved in non-irradiated plants, showing that the temperature of 30ºC led to the higher production of proline. This indicates that in vitro *Ubi Kuning* grown under 30ºC might suffer to stress conditions since proline accumulation is related to the response of stressed plants. Meanwhile, the irradiated in vitro *Ubi Kuning* grown under 25ºC was more likely to suffer followed by those under 30 ºC and 28ºC, respectively. It is apparent that the irradiated in vitro *Ubi Kuning* had a less stress when they were grown at 28ºC and 30 ºC, which may lead to the optimum plant growth.

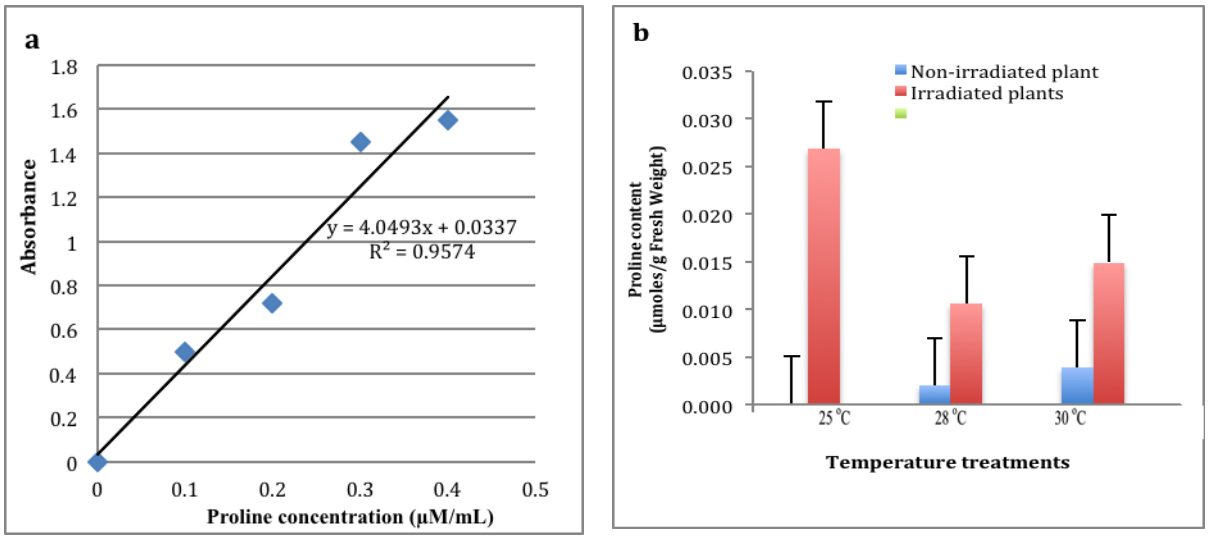

Figure 3. The proline content of in vitro *Ubi Kuning* grown under various temperature treatments under temperature of 25 ºC, 28 ºC and 30 ºC . a) The standard curve of proline, b) The proline concentration of control and irradiated plants.

Discussion

The in vitro propagation combined with the irradiation treatment is one of the best technique to generate plant mutant. In this study, the differences of growth responses of non-irradiated (control) and irradiated in vitro *Ubi Kuning* at 10 Gy as well as the different temperature treatments had been investigated. It shows that two growth parameters i.e plant height and number of leaves of irradiated plantlets at a dosage of 10 Gy grown in all of temperature treatments were significantly different compared to those control plants. It shows that the irradiation treatment affected the growth performances of *Ubi Kuning* plantlets. Some reports stated that the irradiation treatment at the appropriate dosage can trigger the desired mutation of plants (Kole *et al*., 2012; Chaudhary, 2014; Das *et al*., 2014). In this case, it is possible that after irradiation treatments, the growth performances of in vitro *Ubi Kuning* at a dosage of 10 Gy, changed the ability of Ubi Kuning to grow at different temperatures, which may lead to the new potential mutant generation, which is tolerant to different temperature treatments. Although the phenotypic performances, stability character and molecular characterization need to be further assessed.

Temperature has a great effect on the plant metabolism. At ambient temperatures or suitable temperatures, the photosynthesis process of plants can occur fluently because the water evaporation is limited. This causes the plant metabolism can be proceeded to plant growth purposes including leaf and root development, and plant height. In vitro plantlets also perform metabolic processes such as the process of formation and decomposition of food into organic elements used for plant growth so that the plantlet becomes higher, the number of leaves becomes more and wider (Jan *et al*., 2012). Several studies related to higher or lower temperature treatments in in vitro plants had been conducted. Truong *et al*., (2017) reported that treatments of high temperature at 45ºC at 4-day–old seedling of several wheat cultivars for 20 h had the highest survival rates, which means that those cultivars might be candidates of heat tolerant cultivars. An and Zhang (2012) also reported that different low temperature-treated cassava apical shoots for 0, 4 and 9 h had different performances and genetic profiling changes, which means they responded to cold stress. In this study, it shows that the number of leaves of non-irradiated *Ubi Kuning* grown under 25ºC was higher compared to those grown under 28ºC and 30ºC. However, the number of leaves of irradiated *Ubi Kuning* shows indifferent amongst treated temperatures. This may relate to many factors including genetic mutation and environment. Brown (2016) reported that in

vitro plants grown under more than 30˚C can lead to photorespiration and tend to reduce the results of photosynthesis. As a result, when photosynthesis is disturbed, the growth of other plant organs will also be disrupted. This was also in line with Pertamawati (2012) who reported that the leaf of potato plantlets cultured at low temperatures was seemly wider and thinner, although the width (leaf area) and the number of leaves were large and many to capture more light for photosynthesis purposes. Moreover, Fitter and Hay (2012) reported that the ability of plants to absorb nutrients during growth and development, especially taking or absorption is different. This may lead to the different growth response of plants. This could be observed not only from the number of leaves but also from the plant height. Normally, most of cassava can grow well at 27 °C in the field (Onwueme, 2002). Of the several treatments, the temperature of 30°C was the highest temperature treatment and had a significant impact on plant growth compared to those grown at 28°C and 25°C. In this study, the plant height of irradiated *Ubi Kuning* was significantly higher when it was grown under 30ºC. This might relate to the changes of plant metabolism especially endogenous auxin in plants leading to the cell lengthening process (Yamori *et al*., 2014). This implies that under temperature of 30ºC, the irradiated *Ubi Kuning* apparently showed optimum growth.

In order to confirm the correlation in each growth parameters i.e the number of leaves, the number of roots and the plant height of in vitro irradiated *Ubi Kuning* grown under different temperature treatments, the correlation analysis was performed. It shows that the number of roots of in vitro irradiated *Ubi Kuning* significantly increased, which was in line with the increase of the number of leaves. This might relate to the plant metabolism. The temperature of 30°C is the highest temperature among other temperature treatments; however, in vitro irradiated *Ubi Kuning* did not experience the temperature stress instead of growing well. It can be interpreted that the metabolism of irradiated *Ubi Kuning* is controlled very well at a higher temperature. This might be due to the activity of enzymes is triggered to catalyze the metabolism changes i.e cell lengthening caused by the production of endogen hormones i.e gibberellin or auxin during the optimum temperature (Bita & Gerats, 2013; Yamori *et al.,* 2014). The enzyme can work optimally at optimum temperature, while at a high temperature enzyme work will be disrupted (Yamori *et al.,* 2014). Therefore, it is possible that temperature of 30°C is still the optimal temperature for the growth of in vitro irradiated *Ubi Kuning*. This also corresponds to Bita & Gerats (2013) who reported that basically all metabolic activity in plant body is controlled by enzyme, and the activity of this enzyme is greatly influenced by temperature.

Plant cells exposed to unsuitable environmental conditions such as drought, salinity, low and high temperature, and shading produce proline in order to maintain the osmotic balance of cells. The osmotic adjustment in a stress condition will result in the accumulation of soluble compounds such as sugar and proline amino acid (Kishor *et al*., 2014). The accumulation of proline in stress-treated plants acts as osmolyte to keep the organelle and the remain leaf in still green condition (Hayat *et al*., 2012). Proline is able to act as a protective agent for cytoplasmic enzymes and membrane enzymes or as a storage material for growth after stressful plants (Filippou *et al*, 2014). The level of proline content in the irradiated *Ubi Kuning* grown under temperature variation of 25ºC, 28ºC and 30ºC had been tested using spectrophotometer at 520 nm wavelength. In this study, it shows that the proline contents of irradiated *Ubi Kuning* were higher compared to that of non-irradiated *Ubi Kuning*. Moreover, under the temperature of 25ºC, the proline content of irradiated *Ubi Kuning* was higher followed by that under 30 ºC and 28 ºC. This might happen due to plants must respond to stress. Furthermore, plants must balance between growth and stress defence either by reducing the growth and yield or producing soluble compound such as proline (Sharma*et al.,* 2012). In this case, beside the proline content of irradiated *Ubi Kuning* was higher, the plant height of this plantlet was also higher but the number of leaves and roots were lower compared to that in other temperature treatments. This implies that, in vitro irradiated *Ubi Kuning* had a well-balance resource allocation between the growth and stress defence. This might also correlate with the irradiation treatments, which trigger the gene mutation especially genes involved in heat

stress, although the further molecular analysis must be done to confirm this possibility.

This study highlighted the growth performances and proline contents of *Ubi Kuning* derived from in vitro radiation, grown under different temperature treatments i.e 25ºC, 28ºC and 30ºC. It shows that the growth performances of in vitro irradiated *Ubi Kuning* was better compared to that of non-irradiated *Ubi Kuning* in terms of plant height and number of leaves. The best growth performances were obtained in irradiated *Ubi Kuning* grown under 30ºC, which could be observed from the plant height. The proline content of in vitro irradiated *Ubi Kuning* was also higher when it was grown under 25 ºC and 30ºC implying that these plantlets had the possibility to tolerant to lower and higher stress temperatures. Although, the further analysis on the molecular characterization and genes involved in the high-stress temperatures must be further done.

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