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Effect of Light-Emitting Diode (Led) Light on the Gene Expression Related With Ascorbate Biosynthesis and Metabolism in Broccoli Florets

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Abstract

Ascorbate is one of the most abundant soluble antioxidants in the plant. Multiple functions of ascorbate in photo protection have been proposed, including scavenging of reactive oxygen species generated by oxygen photoreduction and photorespiration. There is still unclear information relation to LED light with Ascorbate biosynthesis and metabolism, yellowing, chlorophyll content, and ethylene production in broccoli florets. The effect of light-emitting diodes (LED) light on ascorbate (AsA) biosynthesis and metabolism in broccoli (Brassica oleracea L. var. Italica) cultivar "Ryokurei" were studied using red (660 nm), blue (470 nm) and white LED lights as the light source and also no light treatment as the control. Gene expression involved in the biosynthesis and metabolism of AsA, AsA content, color, chlorophyll content and ethylene production rate on the postharvest broccoli were observed in 4 days. The result showed that after two days, red light treatment significantly ($p < 0,05$) delayed the decrease of ascorbate content. The result was supported by observations using Real-Time Quantitative RT-PCR showed that red light treatment can suppress mRNA level of BO-APX1, BO-APX2, and BO-sAPX on the third day. Observation of BO-GLDH mRNA level was increased in the third-day exposure of red LED light. Therefore red LED light showed up-regulated AsA biosynthesis transcriptional level. Enzymes which possibility responsible for AsA metabolism and biosynthesis in a row were Ascorbate Peroxide (APX) and L-Galactono-1,4-Lactone Dehydrogenase (GLDH). The regulation of this gene expression might contribute to the suppression of AsA reduction by red LED light treatment in broccoli. Red LED also showed suppression of yellowing and decline the chlorophyll content in postharvest broccoli florets.

Keywords: ascorbate, LED; broccoli; gene expression; real-time quantitative RT-PCR.

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1. Introduction

Broccoli (*Brassica oleracea* L *var.italica*) is the horticultural vegetables contain with high vitamin C. The highest content of ascorbic acid (AsA) was found in broccoli floret [1]. In plants, the AsA act as an antioxidant, enzyme cofactor, and also a precursor in the synthesis of tartrate and oxalate. AsA also participates in various processes including photosynthesis, photoprotection, growth and expansion of the cell wall, resistance to environmental stress and synthesis of ethylene, gibberellins, anthocyanin, and hydroxyproline. AsA were easily oxidized, so that makes the reduction of AsA content after harvesting, especially because of ASA was used as a protection agent from the side effects of photosynthesis (photoprotection) by plants tissue [2]. The complex process on AsA biosynthesis and metabolism can keep AsA availability on plant tissue.

In a previous study, the Ascorbate-Glutathione Pathway plays a role in the antioxidant defense system in plants [3–5]. On the biosynthesis and metabolism pathway, AsA occurs. The enzymes involved in this pathway are Ascorbate peroxide (APX), monodehydroascorbate reductase (MDAR), dehydroascorbic reductase (DHAR), glutathione reductase (GR) and two substrates, reducing ascorbate (DHA) and glutathione (GSH) [3]. Broccoli florets will be rapidly senescence during room temperature storage. The previous study shows that the largest loss of ascorbic acid occur in high APX activity and occurs continuously [1].

Light plays a key role as a source of energy and element that affecting plant growth also pigment formation [6–8] and AsA [9]. Light Emitting Diode (LED) is an environment lighting system that converting electrical energy into infrared energy or visible light using a semiconductor character to decrease power consumption [8]. The advantages of using LED as artificial light are high energy conversion, more efficient, smaller size, longer lasting, a specific wavelength, improve intensity and quality of light and low heat released [10].

Research on the effects of irradiation with the LED on the post-harvest broccoli, biosynthesis, and metabolism of AsA is still limited. Irradiation is not only stimulating the formation of antioxidant content in the leaves but also as a treatment to maintain or even improve the content of antioxidants in vegetables during storage [8]. Therefore, research is needed on the effect of LED irradiation on post-harvest broccoli, biosynthesis and metabolism of AsA based on gene expression were detected using Real-Time Quantitative RT-PCR. This study aims to determine the effect of the LED to yellowing, chlorophyll content, ethylene production and ascorbic acid content in the post-harvest broccoli florets, as well as to determine the genes expression and enzymes involved in the metabolism and biosynthesis of ascorbic acid on post-harvest broccoli florets.

2. Materials and methods

2.1. Plant materials and treatments

Broccoli (*Brassica oleracea* L. var. Italica) cultivar "Ryokurei", harvested from Fujieda Farm, Shizuoka University, Japan after 105 days of planting. Broccoli was cut on the branchlets then stored at room temperature (17°C) and humidity (RH > 90%). Branchlets were treated by 4 (four) treatments, i.e. storage without irradiation; storage with irradiation using a white; red; and blue LED. LED intensity was given on 100 µmol m^2s^{-1} were measured using a light meter. Observations were done in four days. Observed branchlets were randomly selected. Florets then were cut using single-edge razor every day from the branchlets. The exposed florets were immediately frozen using liquid nitrogen and then stored in a cooler at -80 °C temperature until used.

2.2. Assessment of broccoli yellowing

The color of broccoli florets was assessed visually from green to yellow. Four people did observations. The rating scale of aging in broccoli assessed from number 5 to 0: 5 all green; 4, 20% yellowing; 3, 40% yellowing; 2, 60% yellowing; 1, 80% yellowing compared to AL 16 [3]. A median number were assigned where appropriate.

2.3. Measurement of chlorophyll content

Total chlorophyll content was determined using the method described by Jamie and Saltveit [11] modified method. 5 g of crushed broccoli were extracted in 10 ml of 80% acetone in dark conditions. The extract was centrifuged at 15,000 $\times g$ for 15 min and then filtered using four layers cheesecloth. Chlorophyll content was measured using UV-VIS spectrophotometer (U-2000, Hitachi) and total chlorophyll content was calculated by the formula [chlorophyll (mg.-1) = +7.9 × 17.95 × Abs647 Abs664.5].

2.4. Measurement of ethylene production

Total ethylene production observed by taking 1 g of broccoli floret then put on a 15 ml vial, cap vial using silicone rubber material. Samples were allowed to stand for 30 minutes, 20°C. Gasses in the bottle was taken using 1ml syringe and injected directly into a gas chromatograph (Hitachi 163) equipped with an alumina column at 70°C and the flame ionization detector [1]. The rate of ethylene production was expressed in nmol ethylene/h/g wet weight.

2.5. Extractions and assays AsA and DHA

Ascorbate content as reduced and oxidized forms were assessed using HPLC according to the method described elsewhere [12] with slightly modified. Frozen samples (0.5 grams) were crushed and homogenized with 5 ml Metaphosphoric Acid (MPA) - acetic acid. The solution was then centrifuged for 30 minutes, 14000 x g, 4°C (pink). Then, the solution was filtered two times using Miracloth (Calbiochem) and 0.2 lm cellulose acetate filter. 100 µL filtrate was added to 100 µL water and 500 µL Phosphoric Acid Buffer then directly injected into the HPLC for 10 minutes, to get the content of ascorbic acid in solution. Meanwhile, to get the total AsA (AsA + DHA), 100 µL filtrate was added with 100 µL Dithio Treithol (DTT) 30 mM and 500 µL Phosphoric Acid Buffer. The solution then reacted in the dark condition for 10 minutes at 30°C and then injected into an LC-10AD pump (Shimadzu) using solvent $NH_4H_2PO_4$15%, the flow rate of 1,0 ml/min. The absorbance at 245 nm was monitored using an SPD-10A spectrophotometric detector (Shimadzu) attached to a chart recorder (C-R6A, Shimadzu). Peak data obtained were converted into concentration by diluting the stock solution AsA to obtain a standard curve. AsA content was determined in a similar way without DTT addition. Subtracting the AsA value from AsA total amount was for calculating DHA content.

2.6. RNA extractions and Real-Time RT-PCR

Total RNA was extracted from the broccoli florets after harvested in accordance with the verified method [13] as modified by Kato [14]. Total RNA was extracted and then purified using RNeasy Mini Kit (Qiagen Hilden, Germany). After purification, the extract then diluted with Diethyl polycarbonate (DEPC) water. Reverse Transcription (RT) was performed using 2 µg of pure RNA and Random Hexamer using TaqMan Reverse Transcription Reagents (Applied Biosystems). Thermal cycle conditions on RT consisted of 25°C for 10 min, 37°C for 60 min and 95°C for 5 min [3].

TaqMan Real-Time Quantitative RT-PCR was performed using TaqMan probes, a set of primers, and TaqMan Universal PCR Master Mix (Applied Biosystems) according to the instructions. As a control we used TaqMan Ribosomal RNA Control Reagents VIC Probe (Applied Biosystems). Measurement of gene expression was using Real-Time Quantitative RT-PCR. Real-Time Quantitative RT-PCR was conditioned in two stages, denaturation by the temperature of 95°C for 10 min, followed by amplification stages as 40-50 cycles of 95°C for 15 sec and 60°C for 60 seconds. Gene expression was analyzed using the ABI PRISM 7000 Sequence Detection System Software (Applied Biosystems) and normalized using the results of 18S ribosomal RNA, so the data obtained into RNA per ribosome.

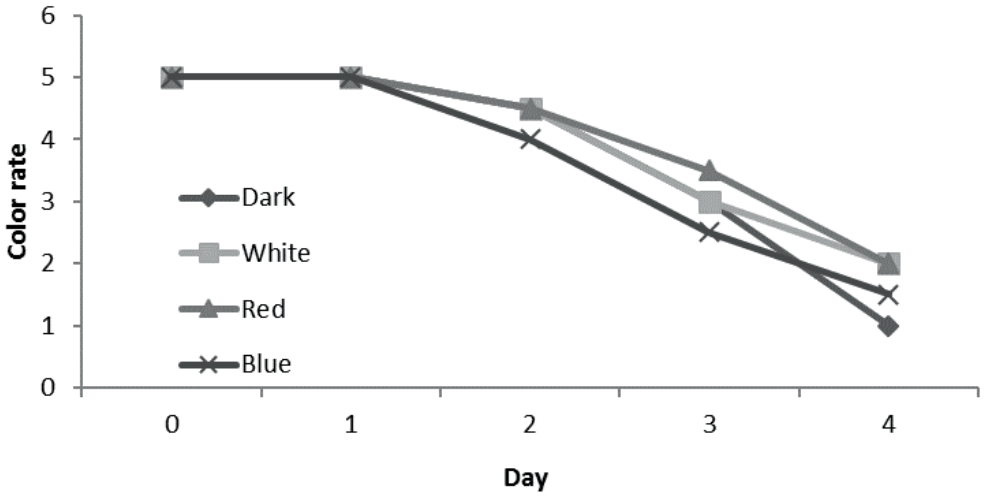

Figure 1: Changes in the green level in broccoli floret.

2.7. Statistical Analysis

All data are shown as the mean ± SE for three replicates. The data were analyzed using Tukey HSD test.

3. Results and Ddiscussions

3.1. Effect of LED lights irradiation on broccoli yellowing, chlorophyll content, and total ethylene production

Harvested broccoli florets will change its color from green to yellow (yellowing), which is one of senescence indication on broccoli. Color changes of Ryokurei varieties broccoli florets treated with irradiation using LED lights are presented in Figure 1.

It was known that on day two already occurred yellowing in broccoli florets varieties 'Ryokurei' (Figure 1). Irradiation treatment using red LED can suppress yellowing on the third day. Irradiation with blue light showed rapid yellowing process than all treatments. Yellowing in broccoli florets occurred due to the degradation of chlorophyll content.

The declining of chlorophyll content in broccoli florets occurs from the first day (Figure 2). Irradiation using red LED can suppress degradation of chlorophyll on the third day. While the treatment using blue LED degraded on the second day, though on the last day, the control treatment without irradiation showed more chlorophyll degradation. This rapid decrease by the treatment without irradiation also occurs in other research [15] which explained that fresh broccoli stored at low temperature (7°C)

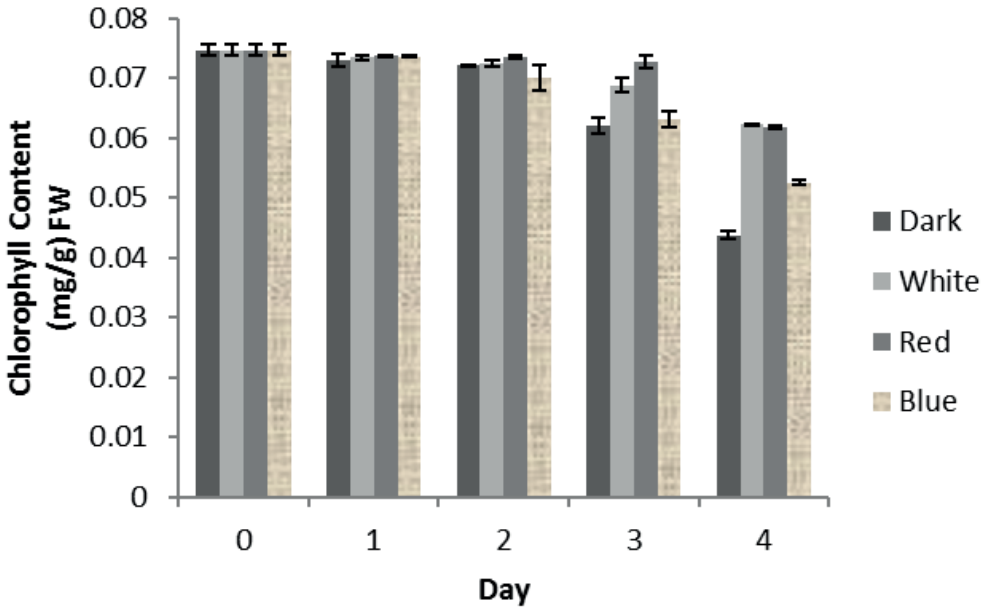

Figure 2: The decrease of chlorophyll content on Post Harvest Broccoli Florets irradiated with LED (mg/gFW).

without irradiation for ten days showed lower chlorophyll content than the samples received irradiation all the time.

Effect of light color to chlorophyll degradation on post-harvest broccoli has not been widely studied yet. Faster chlorophyll degradation on blue LED irradiation treatment compared to the red LED irradiation may be due to the massive energy photon of blue light that 1,5 times larger than the red one [16].

At a room temperature, broccoli will be rapidly senescence. Ethylene is closely linked to the yellowing of post-harvest broccoli [17]. Ethylene production of broccoli varieties Ryokurei during irradiation with LED was presented in Figure 3.

The production of ethylene on Ryokurei broccoli floret increased on the first day, and the peak was on the second day (Figure 3). Then on the third day, ethylene production decreased in all treatments. However, on the red and white LED irradiation, ethylene production increased again on the fourth day.

Effect of ethylene on chlorophyll degradation was not significantly on the blue LED irradiation treatment. The result showed that the production of ethylene on the second day was low, but the chlorophyll content rapidly declined. Suppression of ethylene production by blue light occurred because of the blue color can inhibit the activity of enzyme 1-aminocyclopropan-1-carboxylic acid oxidase (ACC oxidase) through changing the cell membrane structure and/or membrane associated with photoreceptors [18]. Red lights treatment produce quite a high ethylene. This result was because red

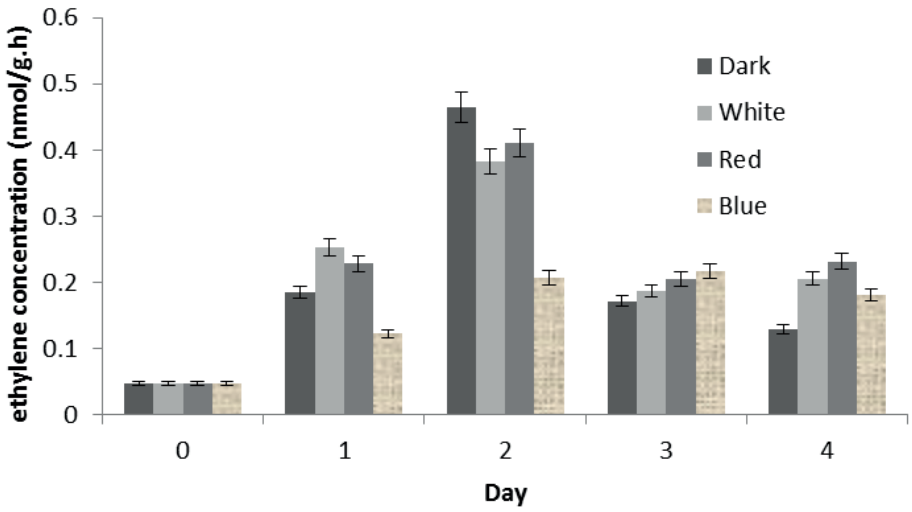

Figure 3: Ethylene concentration in Post-Harvest Broccoli Floret irradiated by LED (nmol/g.h).

light increased the activity of ACC oxidase. The red light treatment produces higher ethylene than blue and white light.

3.2. Effect of LED lights on AsA content

Ascorbic acid content in broccoli floret varieties Ryokurei rapidly declined in the first day after harvested. Red LED irradiation inhibits ascorbic acid content decline in the second day. While blue LED irradiation showed highest declining on ascorbic acid content (Figure 4). That because of the high photon energy of blue light which 1,5 times larger than red ones [16], lead to increase ascorbic acid oxidation. A high level of light energy increase the excitation energy and Reactive Oxygen Species (ROS) in lettuce, and by the mechanism of photoprotection, plants stimulate the production of antioxidants [19]. Effect of LED irradiation on dehydroascorbic (DHA) content was presented in Figure 5.

Dehydroascorbic (DHA) content in broccoli floret varieties Ryokurei was relatively small and constantly throughout the study (Figure 5). This phenomenon was similar to the research on tobacco cell cultures [20], broccoli florets [3], which reported that the unstable DHA caused the decrease of ascorbic acid which was not accompanied by an increase in DHA [20]. Increasing of DHA content in the red LED irradiation treatment on the fourth day was possible because of the rise in ethylene production [3].

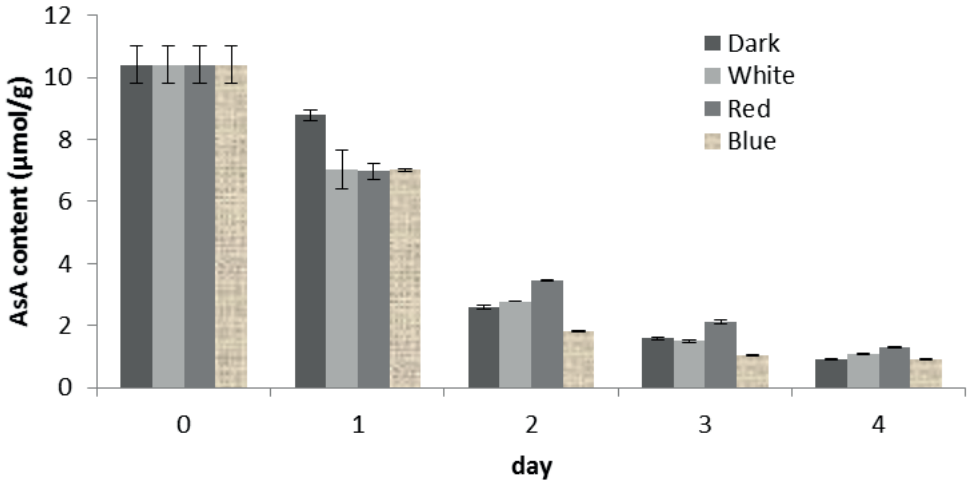

Figure 4: Ascorbic Acid Content on Post Harvest Broccoli Floret with LED irradiation (µmol/g FW).

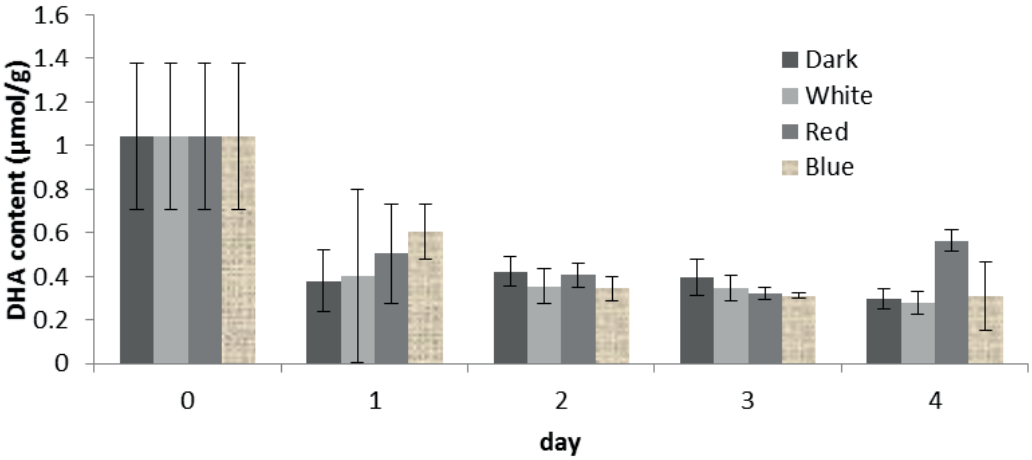

Figure 5: Dehydroascorbic content on Post Harvest Broccoli Floret with LED irradiation (µmol/gFW).

3.3. Effect of LED irradiation on gene expressions related to AsA metabolism

Total gene expression ascorbate peroxidase 1 (BO-APX 1), ascorbate peroxidase 2 (BO-APX 2), and cytosolic ascorbate peroxidise (BO-sAPX) on broccoli florets during irradiation using LED lights was presented in Figure 6, 7 and 8.

In observation of ascorbate peroxidase gene expression showed that the mRNA level of BO-APX 1 and BO-sAPX decreased after harvest and increase after the first day (Figure 6 and 8). While the mRNA level of BO-APX 2 gradually increased until the decreased on the third day (Figure 7).

Ascorbate peroxidase was the primary enzyme which responsible for the ascorbic acid breakdown [3]. In this study, there were a different trend between the three gene expressions of the gene encoding ascorbate peroxidase. It also occurs in the

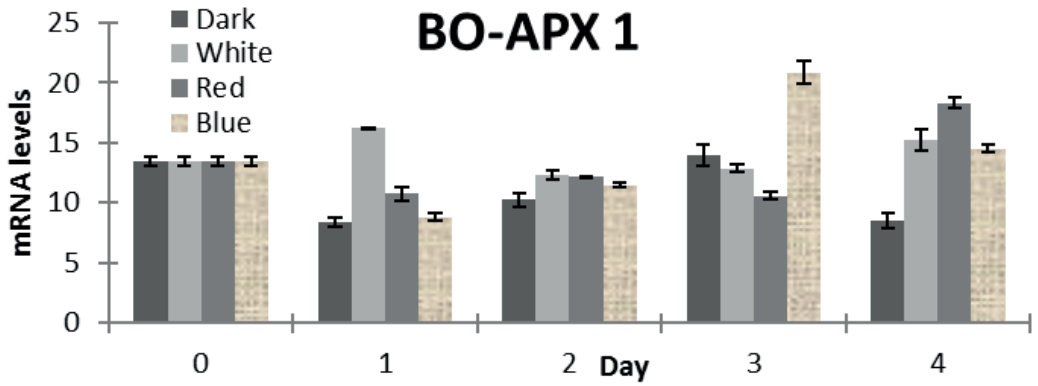

Figure 6: Ascorbate peroxidase 1 Gene Expression (BO-APX 1) on Post Harvest Broccoli Floret irradiated with LED.

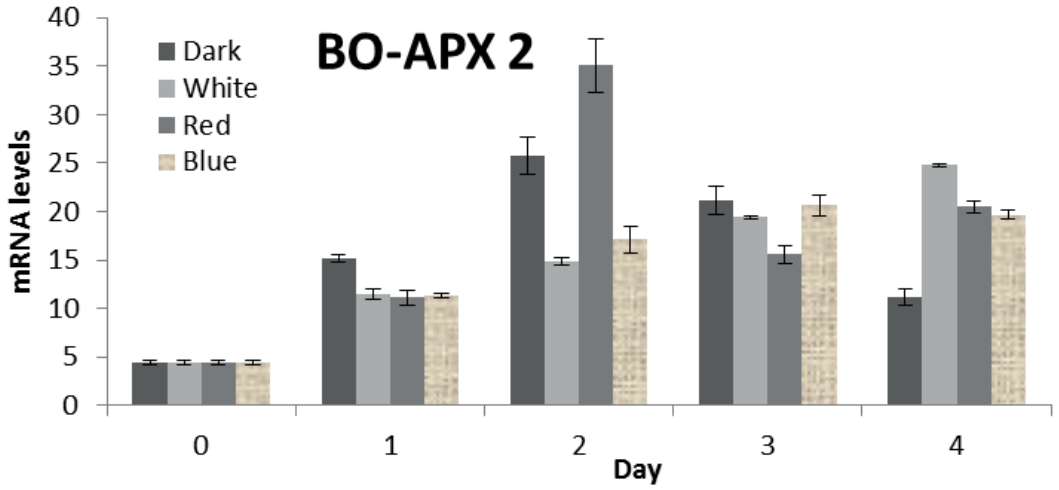

Figure 7: Ascorbate peroxidase 2 Gene Expression (BO-APX 2) on Post Harvest Broccoli Floret irradiated with LED.

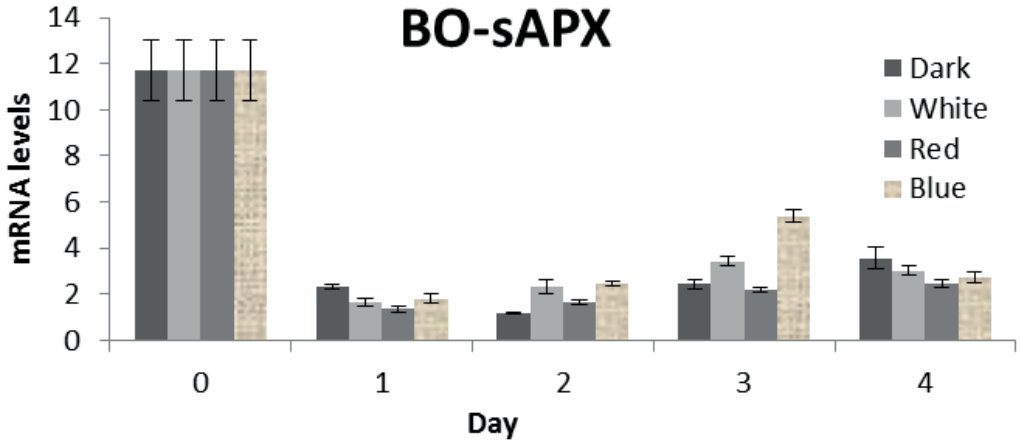

Figure 8: Cytocolic-Ascorbate Peroxidase Gene Expression (BO-sAPX) on Post Harvest Broccoli Floret irradiated with LED.

study conducted other researcher [3], where APX in the cytosol and chloroplasts have

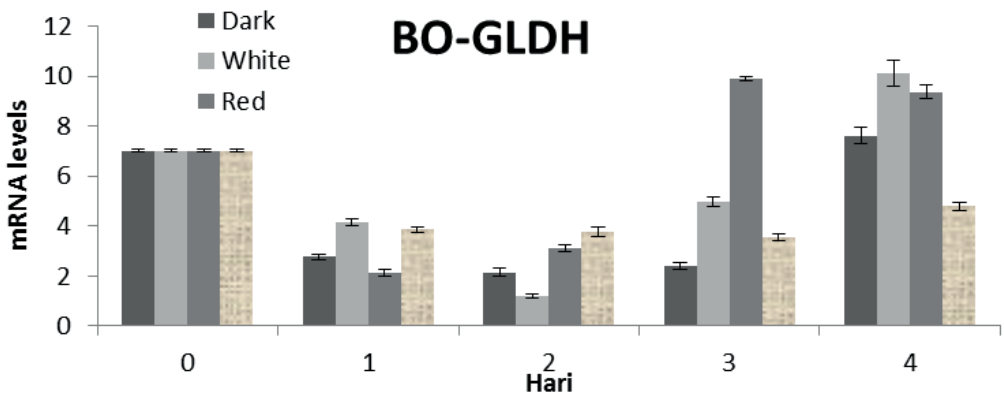

Figure 9: L-Galactono-1,4-Latone dehydrogenase gene expression (BO-GLDH) on Post Harvest Broccoli Floret Irradiated with LED.

a different trend. This was possible due to differences in cell space where these genes take affect and also the regulation of broccoli senescence. This result was also similar with previous study [21], which showed that the variation of APX isoenzymes on Arabidopsis had different tendencies at the transcription level when there was under heat stress.

This study showed that on the third day red LED irradiation had lower gene expression at the mRNA level BO-APX1, BO-APX2, and BO-sAPX. By contrast, blue LED light treatment showed higher gene expression at the mRNA level BO-APX1 and BO-sAPX. This result indicates that the effect of red LED lights irradiation had been affecting until the transcriptional level.L-Galactono-1,4-Latone Dehydrogenase gene expression in broccoli floret during LED lights irradiation was presented in Figure 9.

Figure 9 showed that the BO-GLDH mRNA levels decline after harvested on the second day, then increased to reach a peak on the fourth day. This study showed that LED light treatment on the third day had highest BO-GLDH mRNA levels than other treatments. This trend was similar with the suppression of ascorbic acid declining in broccoli floret with red LED light treatment. In contrast, the blue LED light treatment showed mRNA levels in the second day, higher than other treatments. This result indicates that biosynthesis of ascorbic acid proceeds, but the process did not significantly affect the suppression of ascorbate acid declining in broccoli.

GLDH was an enzyme located in the mitochondrial membrane directly catalyze the oxidation process of L-Galactono-1,4-Latone into ascorbic acid [3]. GLDH enzyme activity in transcription levels was reported that has a positively correlation with ascorbic acid content in tobacco and Arabidopsis [22]. However, study on tomato and wheat leaf showed that GLDH activity has no effect on the content of ascorbic acid [9].

4. Conclusions

The red LED irradiation treatments was significantly suppress the decrease of ascorbic acid content in the post-harvest broccoli. Observation of BO-GLDH mRNA level that increased on the third-day exposure indicated that a red LED light irradiation has increased the biosynthesis of ascorbic acid to the level of transcription. Ascorbate peroxidase (APX) was probably the most responsible enzyme in the metabolism of AsA. While L-Galactono-1,4-Lactone Dehydrogenase (GLDH) was an enzyme that most responsible for the biosynthesis of ascorbic acid on post-harvest broccoli irradiated with LED. Red LED also indicated inhibition of yellowing and decreased in chlorophyll content of broccoli florets.

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