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Effect of UV-C treatment on chlorophyll degradation, antioxidant enzyme activities and senescence in Chinese kale (*Brassica oleracea* var. *alboglabra*)

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Abstract

The changes in chlorophyll content, antioxidant enzyme activities and senescence in Chinese kale (*Brassica oleracea* var. *alboglaba*) irradiated with different UV-C dosages (1.8, 3.6, 5.4 and 7.2 kJ m^{-2}) were determined during storage at 20°C. The irradiation dose of 3.6 and 5.4 kJ m^{-2} delayed leaf yellowing depicted as higher hue values and chlorophyll contents and lower activity of chlorophyllase, Mg-dechelatase and chlorophyll-degrading peroxidase as compared to the other treatments. Similarly, UV-C treatment delayed the decrease in activities of antioxidant enzymes, particularly peroxidase (POD) and superoxide dismutase (SOD). It also reduced ethylene production and respiration rates. The results indicate that UV-C irradiation could be a useful non-chemical treatment to delay senscence and improve shelf life of Chinese kale.

Introduction

Chinese kale (*Brassica oleraceae* var. *alboglabra*) is one of the most important and popular leafy vegetables in the Southeast Asian countries including Thailand. Major problem with storage of fresh Chinese kale is leaf yellowing due to chlorophyll degradation which occurs within few days at 20°C or higher (Burana and Srilaong, 2010). During chlorophyll degradation, chlorophyll a is transformed to chlorophyllide a through the action of the chlorophyllase enzyme (Hortensteiner, 1999; Matile *et al*., 1999). Chlorophyllide a is then acted by the enzyme Mg-dechelatase removing Mg^{2+} from the molecule and forming pheophorbide a, consequently the green color is lost (Matile, 1980; Fumamoto *et al*., 2002).

The potential of irradiation to extend the shelf life of various fruits and vegetables has been explored in previous studies (Allend *et al*., 2006; Barka *et al*., 2000). UV-C irradiation was shown to delay yellowing and retain the quality of broccoli florets (Costa *et al*., 2006). UV-C light at beneficial doses was also found to induce the accumulation of phytoalexins (Devlin and Gustine, 1992) and activate the genes encoding pathogenesis-related proteins (Green and Fluhr, 1995). At injurious level, UV-C decreased protein synthesis and increased DNA damage (Danon and Gallois, 1998; Brosche *et al*., 1999). Therefore, this study was conducted to determine the effects of different UV-C irradiation doses on chlorophyll degradation and antioxidant enzyme activities associated with yellowing and senescence of Chinese kale.

Materials and Methods

Plant material

Freshly harvested Chinese kale (*Brassica oleracea* var. *alboglaba*) cultivars 'Fang No.1' at commercial maturity (45 days from planting) were obtained from a commercial grower in Ratchaburi province, Thailand, and immediately transported to the laboratory. The samples were selected based on uniformity in color and size, and absence of visual defects. Each plant was trimmed retaining five leaves each. To remove field heat, the samples were hydrocooled for 10 min at 3°C.

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UV-C irradiation treatment

Chinese kale samples were irradiated with UV-C lamp (G20T10, 20W, Sankyo Denki, JAPAN) by placing at a distance of 30 cm from the light source to obtain dose 1.8, 3.6, 5.4 and 7.2 kJ m^{-2}. The illumination intensity was determined with a digital UVmeter (Model 8.0UVC; Solartech Inc, USA).Un-irradiated samples served as controls. After treatment, the samples were packed in 0.05 mm polyvinyl chloride film and then stored at 20°C with 93-97% RH. All treatments were replicated three times with 3 plants per replicate comprising of 10 samples each for non-destructive and destructive sampling.

Measurement of leaf yellowing

Hue angle (h°) was used as a measure of yellowing and was calculated from a* and b* readings from a colorimeter (Minolta CR 400, Japan). Color readings were taken from nine positions on each of the 10 plants sampled per replicate.

Respiration and ethylene production rate

Three plants of Chinese kale were placed in 6600 cm^3 plastic box, sealed and incubated at 20°C for 3 h. Gas samples were withdrawn with 1 mL syringe through a septum fitted in the box lid and analyzed for respiration rate and ethylene production. For measurement of respiration rate was determined by gas chromatography (Shimazu, Model GC-8A) equipped with Porapak Q (Mesh 80/100) column. Ethylene production was measured with gas chromatograph (GC 2010, Shimadzu, Japan) with a flame ionization detector (FID) and activated alumina column (1 min length) (Conway and Sams, 1987).

Chlorophyll contents and chlorophyll-degrading enzyme assays

Chlorophyll content was determined by the N, N-dimethylformamide method (Moran, 1982). For enzyme assays, acetone powder (500 mg) of the samples was suspended in 15 mL of 10 mM phosphate buffer (pH 7.0) for chlorophyll-degrading peroxidase and in 15 mL of 50 mM phosphate buffer (pH 7.0) containing 50 mM KCl and 0.12% Triton X-100 for chlorophyllase and Mg-dechelatase activities. The mixture was stirred for 1 h at 0°C and then filtered through Miracloth (Calbiochem, USA). The filtrate was centrifuged at 16000 × g for 15 min at 4°C and the supernatant was used as the crude enzyme extract.

Chlorophyllase activity was determined by the method of Amir-Shapira *et al*. (1987) with modifications. The reaction mixture contained 0.5 mL enzyme solution, 0.1 mL 1%3-[(3-chloramido-propyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.2 mL Chlorophyll a acetone solution (chlorophyll a 100 mg•L^{-1}), and 0.5mL of a 0.1M phosphate buffer (pH 7.5). The mixture was incubated in water at 25°C for 40 min, and the enzyme reaction was stopped by the addition of 4 mL of acetone. The remaining (non-degraded) chlorophyll a was extracted with 4 mL of hexane and assayed by reading the absorbance at 663 nm (Shimadzu UV-1501, Japan). The activity was based on the decrease in absorbance by chlorophyll a at 663 nm.

Mg-dechelatase activity was determined by the method of Suzuki and Shioi (2002) with modifications. The reaction mixture contained a crude enzyme solution (0.2 mL), 98 nM chlorophyllin a (0.3 mL), and 50 mM Tris-HCl buffer (pH 8.0) (0.75 mL). Activity was measured at 35°C by following the change in absorbance at 686 nm. Mg-dechelation activity was expressed as increased absorbance at 686 nm per min.

Chlorophyllin a was prepared according to the procedure of Vicentini *et al*. (1995). Thirty milliliters of a chlorophyll a acetone solution was partitioned into 20 mL petroleum ether. The ether phase was separated, washed three times with 20 mL distilled water, and mixed with 1 µL of 30% (w/v) KOH in methanol per 30 µg chlorophyll a to form chlorophyllin a. The solution in which chlorophyllin a was allowed to precipitate was centrifuged at 16000 × g at 4°C for 10 min. The precipitate was dissolved in distilled water and brought to pH 9 by adding 2M Tricine.

Chlorophyll-degrading peroxidase was determined as previously described (Yamauchi *et al*., 1997). The reaction mixture contained 0.5 mL enzyme solution, 0.1 mL 1.0% Triton X-100, 0.1 mL 5.0 mM p-coumaric acid, 0.1 mL 0.3% hydrogen peroxide, 0.2 mL chlorophyll a acetone solution (chlorophyll a-100 mg•L^{-1}), and 1.5 mL 0.1 M phosphate buffer (pH 5.5). Activity was determined spectrophotometrically by measuring the decrease of chlorophyll a. One unit of chlorophyllase and chlorophyll-degrading peroxidase was defined as a change of 1.0 µg chlorophyll a degradation per min. The enzyme protein content was assayed by the method of Bradford (1976).

Antioxidative enzyme extraction and assays

Ten gram of sample was homogenized with 30 mL extraction buffer containing 100 mM Tris-HCl buffer (pH 8.0) and 50 mM sodium phosphate buffer (pH 7.0). The homogenate was filtered through one layer of miracloth; the filtrate was centrifuged at 13000×g for 30 min at 4°C; and the supernatant was collected as a crude enzyme.

CAT activity was assayed based on H_2O_2

disappearance measured by the decrease in absorbance at 240 nm during 3.5 min time period, according to the method of Aebi (1984) with some modifications. The reaction mixture contained 1.7 mL of 50 mM sodium phosphate buffer (pH 7.0), 1 mL distilled water and 0.2 mL of crude enzyme. The mixture was activated by 0.1 mL of 100 mM H_2O_2. The results were expressed as unit mg^{-1}; one unit of enzyme activity was equivalent to a decrease of 0.1 of absorbance at 240 nm per minute at 25 °C.

POD activity was determined at 25°C by measuring the increase in absorbance at 470 nm, according to the method of Jiang *et al*. (1984) with some modifications. The reaction mixture consisted of 0.02 mL of the crude enzyme and 0.05% guaiacol in 50 mM sodium phosphate buffer (pH 7.0). It was incubated at 25°C for 15 min and 0.08% of H_2O_2 was added to assay the enzyme activity. Enzyme activity was expressed unit mg^{-1}; one unit of enzyme activity was equivalent to an increase of 0.1 of absorbance at 470 nm per minute at 25°C.

SOD activity was assayed following the method of Ukeda *et al*. (1997). The reaction mixture consisted of 50 mM carbonate buffer (pH 10.2), 3 mM xanthine, 3 mM EDTA, 0.75 mM nitroblue tetrazolium (NBT) and bovine serum albumin 15%, 0.1 mL SOD solution and 0.02 mL of crude enzyme. The reaction was started by addition of xanthine oxidase. The slope of initial reaction was adjusted to 0.02 OD_{560}/min. One unit of SOD activity was defined as the amount of the enzyme required to cause 50% inhibition of the rate of NBT reduction at 560 nm.

Statistical analysis

A completely randomized design with three replicates was used. Data were subjected to analysis of variance (ANOVA) using the statistic program. Standard error of the means was also calculated.

Results and Discussion

Respiration and ethylene production

Respiration increased with storage at a higher rate in the untreated control than in UV-C treated Chinese kale (Figure 1A). Among UV-C levels, 5.4 kJ m^{-2} was the most effective in reducing respiration rate. UV-C at 3.6 kJ m^{-2} had comparable depressing effect on respiration but only during the first 4 days of storage. Similar trend was obtained for ethylene production rates which decreased most with UV-C treatment at 5.4 kJ m^{-2} followed by that at 3.6 kJ m^{-2} (Figure 1B). Using lower UV-C level (1.8 kJ m^{-2}) or increasing the level to 7.2 kJ m^{-2} resulted in reduced efficacy in decreasing ethylene production rates. The results seem to concur with earlier findings in which

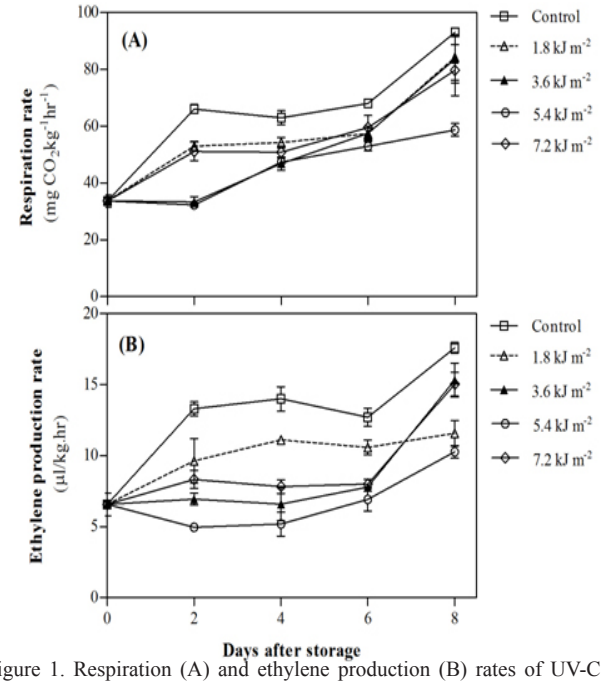

Figure 1. Respiration (A) and ethylene production (B) rates of UV-C treated and untreated Chinese kale during storage at 20°C. Vertical bars represent standard error (S.E) of the mean (n = 3)

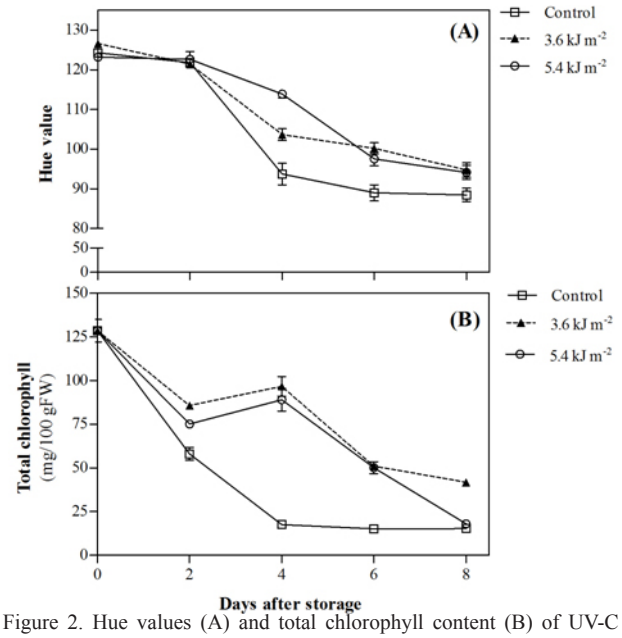

Figure 2. Hue values (A) and total chlorophyll content (B) of UV-C treated and untreated Chinese kale during storage at 20°C. Vertical bars represent standard error (S.E) of the mean (n = 3)

UV-C treatment lowered the rate of respiration of peppers (Vicente *et al*., 2005) and the rate of ethylene production of a number of fruit and vegetables (Stevens *et al*., 1996; Maharaj *et al*., 1999). In the present study, UV-C at levels of 3.6 and 5.4 kJ m^{-2} were the more promising treatments; their effects on leaf yellowing, chlorophyll metabolism and antioxidant enzyme activities are compared in the following sections.

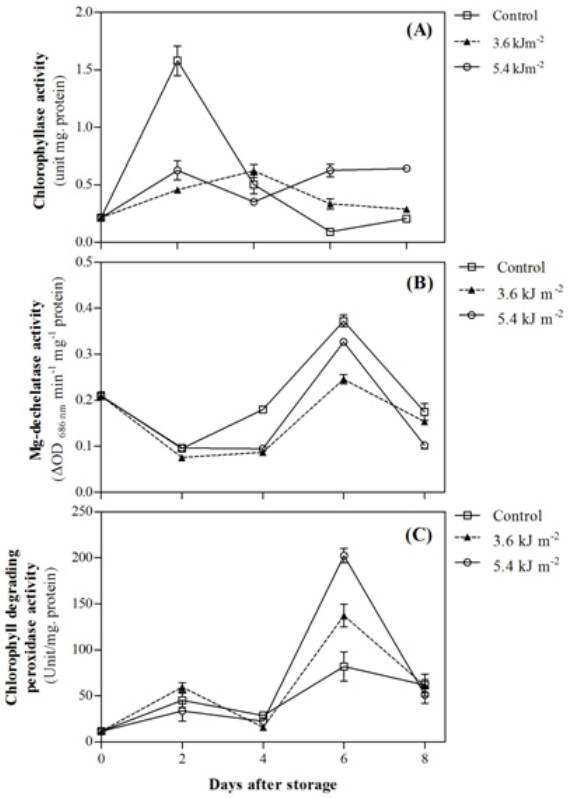

Figure 3. Chlorophyllase (A), Mg-dechelatase (B) and chlorophyll degrading peroxidase (C) activities in UV-C treated and untreated Chinese kale during storage at 20°C. Vertical bars represent standard error (S.E) of the mean (n = 3)

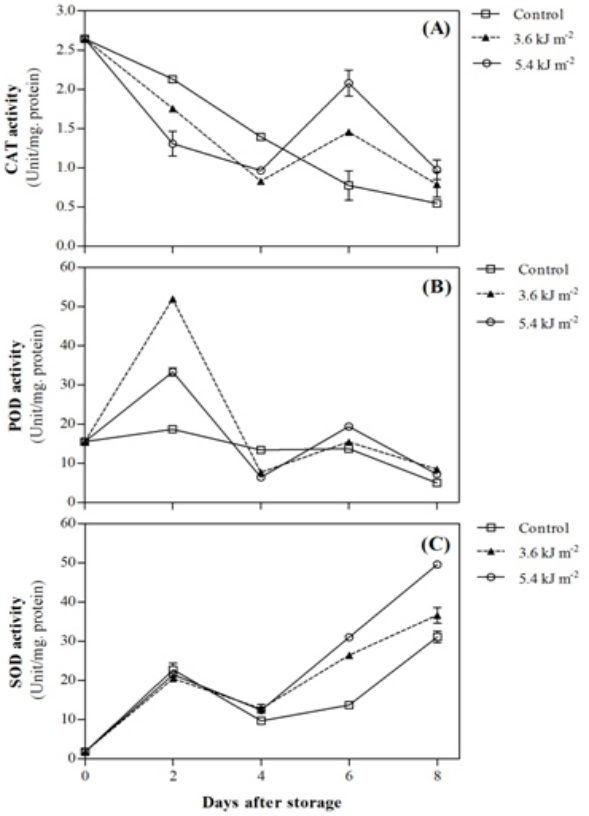

Figure 4. Catalase, CAT (A), peroxidase, POD (B) and superoxide dismutase, SOD (C) activities in UV-C treated and untreated Chinese kale during storage at 20°C. Vertical bars represent standard error (S.E) of the mean (n = 3)

Leaf yellowing and chlorophyll changes

Chinese kale leaves turned yellow with storage manifested as decreasing hue values (Figure 2A). This was accompanied by the decrease in chlorophyll content (Fig. 2B). UV-C irradiation inhibited yellowing and chlorophyll loss. The effect of the two UV-C levels did not differ much except after 2-4 days of storage. After 2 days of storage, treatment effects on yellowing were not apparent but chlorophyll content was higher in UV-C treated samples than untreated ones. Two days later, degree of yellowing was significantly lower in 5.4 kJ m^{-2} than in 3.6 kJ m^{-2} UV-C treatment but in terms of chlorophyll content, both UV-C treatments were comparably effective in maintaining higher level than the control. These results suggest that internal changes in chlorophyll content may not necessarily correlate directly with the visible symptom of yellowing.

Chlorophyllase activity in the untreated control increased sharply after 2 days of storage and then decreased to levels comparable to or lower than the pre-storage or initial level (day 0) (Figure 3A). UV-C treatment prevented such dramatic rise in chlorophyllase activity which remained almost unchanged or only slightly higher than the initial level throughout the storage period. In general, 5.4 kJ m^{-2} UV-C treatment resulted in higher chlorophyllase activity than the 3.6 kJ m^{-2} treatment after 6-8 days of storage. Mg-dechelatase activity decreased after 2 days of storage and treatment effects were not significant (Figure 3B). Later, it increased at a higher magnitude in the control than in UV-C treated samples. The effect of the two UV-C levels differed clearly after 6 days storage, with the 3.6 kJ m^{-2} UV-C level causing higher reduction of enzyme activity than 5.4 kJ m^{-2} UV-C level. On the other hand, the chlorophyll degrading peroxidase activity did not greatly change during the first 4 days of storage regardless of treatment (Figure 3C). On the 6th day of storage, peroxidase activity increased in all treatments but was highest in the control and lowest in samples treated with 5.4 kJ m^{-2} UV-C. It then decreased and treatment differences were again not significant.

The results show that not all enzymes of chlorophyll degradation correlated well with loss of chlorophyll. On the second day of storage when the control had the highest amount of chlorophyll loss, the increase in chlorophyllase activity seemed to be responsible for this. The control continued to lose more chlorophyll than the two UV-C treatments on the fourth day of storage and this response appeared to be due to the differences in Mg-dechelatase activity. Thereafter, chlorophyll loss in the control was due

to increased activities of both Mg-dechelatase and peroxidase rather than that of chlorophyllase. In the UV-C treated samples, particularly after 6 days of storage, chlorophyll loss did not differ among the two treatments which could be due to the balance in Mg-dechelatase and peroxidase activities; those treated with 5.4 kJ m^{-2} UV-C had higher Mg-dechelatase activity but lower peroxidase activity than those treated with 3.6 kJ m^{-2} UV-C. Previous studies have only demonstrated the inhibitory effect of UV-C light on chlorophyll breakdown and on the activities of Mg-dechelatase, chlorophyllase and chlorophyll degrading peroxidase in green vegetables (Costa e*t al*., 2006; Funamoto *et al*., 2002).

Antioxidant enzymes activities

CAT activity decreased with storage with no pronounced treatment effects (Fig. 4A). POD activity similarly decreased with storage but after 2 days, UV-C treatment at 3.6 kJ m^{-2} induced a marked increase while that at 5.4 kJ m^{-2} maintained the pre-storage activity in contrast to that of the control (Figure 4B). On the other hand, SOD activity did not differ with treatment during the first 2 days of storage (Figure 4C). With advancing storage period, both UV-C treatments increased SOD activities relative to that of the control (Figure 4C). This result was in agreement with previous reports of Erkan *et al*. (2008) and Jiang *et al*. (2010) who found that UV-C treatment increased antioxidant enzyme activity in strawberry fruit and shiitake (*Lentinus edodes*) mushrooms, respectively.

Conclusion

Loss of green color during Chinese kale senescence was associated with the reduction of chlorophyll content. Chlorophyllase, Mg-dechelatase, and chlorophyll degrading peroxidase activities also increased but their role in chlorophyll degradation seemed to be time bound since the loss of chlorophyll at certain period of storage could not be attributed to increased activities of these three enzymes. UV-C treatment inhibited yellowing, chlorophyll loss and the activities of chlorophyll degrading enzymes. It similarly reduced the rates of respiration and ethylene production and maintained high antioxidant enzyme activities, particularly POD and SOD. The results suggest that UV-C irradiation could be used to delay senescence of Chinese kale and a dose of 3.6 kJ m^{-2} would be sufficient.

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