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UV-Induced Changes of Active Components and Antioxidant Activity in Postharvest Pigeon Pea [*Cajanus cajan* (L.) Millsp.] Leaves

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ABSTRACT: In this study, the effect of UV irradiation (UV-A, UV-B, and UV-C) on phytochemicals, total phenolics, and antioxidant activity of postharvest pigeon pea leaves was evaluated. The response of pigeon pea leaves to UV irradiation was phytochemical specific. UV-B and UV-C induced higher levels of phytochemicals, total phenolics, and antioxidant activity in pigeon pea leaves compared with UV-A. Furthermore, UV-B irradiation proved to possess a long-lasting effect on the levels of phenolics and antioxidant activity. After adapting for 48 h at 4 °C following 4 h UV-B irradiation, total phenolics and antioxidant activity were approximately 1.5-fold and 2.2-fold increased from 39.4 mg GAE/g DM and 15.0 μmol GAE/g DM to 59.1 mg GAE/g DM and 32.5 μmol GAE/g DM, respectively. These results indicate that UV irradiation of pigeon pea leaves can be beneficial in terms of increasing active components and antioxidant activity.

KEYWORDS: *flavanoids, stilbenes, antioxidant activity, ultraviolet (UV), pigeon pea leaves*

INTRODUCTION

Pigeon pea [*Cajanus cajan* (L.) Millsp.] is a famous and multiuse grain legume crop. It is a valuable perennial or annual woody plant widely distributed in semitropical and tropical regions. Pigeon pea is a good source of dietary essential minerals and protein in the human diet. Moreover, pigeon pea possesses outstanding medicinal value. It has been used to treat measles, sores, coughs, diabetes, hepatitis, cancers, dysentery, and superficial infection and stabilize menstrual period.[1−3] Most of these uses are attributed to pigeon pea leaves. Chemical investigations indicate the main active components in pigeon pea leaves are divided into two groups of compounds, i.e., flavanoids and stilbenes, which exhibit various biological activities, such as antioxidant, antitumor, antivirus, and antibacterial activities.[4−7] In China, pigeon pea leaves have been a traditional Chinese medicine for the therapy of ischemic necrosis of femoral head.

The physiological functions of medicinal plants are strongly dependent on the composition of active components and their contents. The biosynthesis of secondary metabolites in plants depends on climatic conditions, agricultural and environmental factors, harvest period, and postharvest treatments. It is known that most plants are immobile so that they cannot escape environmental stresses.[8] Most environmental stresses are known to result in the increase of reactive oxygen species (ROS) in plant cells and cause oxidative stress.[9] To prevent the injuries caused by oxidative stress, plants develop different mechanisms to eliminate these toxic ROS. It is evidence that these defense mechanisms are based on metabolic compounds (carotenoids, flavonoids, etc.) and enzymes (superoxide dismutases, ascorbate-peroxidase).[10] Recently, many studies indicate that ultraviolet (UV) irradiation may induce photobiological stress and activate the plant defense system, leading to accumulation of secondary metabolites in plant tissues. The secondary metabolites may possess a sunscreening effect and protect cells from the irradiation.[11] Flavonoids and other phenolics are considered to possess a UV protective effectiveness because they are potent UV-absorbing compounds[12,13] and can effectively reduce the impact of free radicals.[14,15] Wang et al.[16] reported proper use of UV-C irradiation was capable of modifying flavonoids content of blueberries. UV-B could also induce the changes of volatile metabolites and phenolic compounds in blueberries.[17] Schreiner et al.[18] found that short-term and moderate UV-B irradiation significantly affected the level of glucotropaeolin in inflorescences, leaves, and unripe green seeds of nasturtium. Zheng et al.[19] reported that resveratrol content in UV-C-treated grape berry skin and leaves increased rapidly. Thus, UV irradiation might be regarded as an effective tool for enhancing the health properties of plants.

In this study, we aimed to investigate the effect of UV irradiation (UV-A, UV-B, and UV-C) on phytochemicals, total phenolics, and antioxidant activity of pigeon pea leaves. The present work might contribute to an assessment of UV application on the dynamics of health beneficial metabolites.

MATERIALS AND METHODS

Chemicals. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Folin−Ciocalteu reagent, gallic acid, orientin, naringenin, luteolin, and apigenin were purchased from Sigma-Aldrich (Steinheim, Germany). Apigenin-6,8-di-*C*-α-L-arabinopyranoside, pinostrobin chalcone, pinostrobin, longistyline C, and cajaninstilbene acid (3-hydroxy-4-prenyl-5-methoxystilbene-2-carboxylic acid) were separated and purified from pigeon pea leaves in our laboratory. Their structures were confirmed by ESI-MS, ^{1}H NMR, and ^{13}C NMR in comparison with literature data.[20−24] HPLC-grade methanol and formic acid were purchased from J & K Chemical Ltd.

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(Beijing, China) and Dima Technology Inc. (Muskegon, MI, USA), respectively.

Plant Material and Experimental Design. Pigeon pea plants were grown for 4 months in the greenhouse (daily mean temperature 18−23 °C). Leaves with uniform size were picked, and 10 g of fresh leaves per treatment was weighted. At once, preweighted leaves were irradiated by a UV-B lamp (313 nm, 40 W, Beijing Institute of Electric Light Source, China) for 2, 4, 6, and 8 h (relative humidity of 90−95%) at 50 cm distance. Meanwhile, similar treatment was performed for UV-C and UV-A irradiation using a UV-C lamp (254 nm, 40 W, Beijing Institute of Electric Light Source, China) and UV-A lamp (365 nm, 40 W, Beijing Institute of Electric Light Source, China), respectively. After irradiation, the leaves were shock frozen in liquid nitrogen and kept at −80 °C until analysis. Additionally, the leaves irradiated by UV-B for 4 h were kept at 20 or 4 °C for different adaptation times of 4, 24, 36, 48, and 72 h to evaluate the residual effect of UV-B irradiation. During the adaptation period, samples were stored in perforated plastic bags at a relative humidity of 90−95% to avoid dehydration and decay. After scheduled adaptation time, the leaves were shock frozen in liquid nitrogen and kept at −80 °C until analysis. Nonirradiated leaves were considered as control. The experiment was conducted in triplicate per treatment.

Sample Preparation. Ten grams of fresh leaves per treatment was extracted with 30 mL of 80% ethanol by homogenate extraction for 5 min. The residue was extracted with 30 mL of 80% ethanol by ultrasonic-assisted extraction for 30 min twice. The filtered solution was collected and pooled for analysis of phytochemicals, total phenolics, and antioxidant activity.

HPLC Analysis of Individual Flavanoids and Stilbenes. HPLC analysis of flavanoids and stilbenes was carried out on Agilent 1200 series HPLC system (Agilent, San Jose, CA, USA) equipped with a G1311A quaternary pump, a G1322A degasser, a G1365B MWD UV detector, and a G1328B manual injector. Chromatographic separation was achieved on a Luna C18 reversed-phase column (250 × 4.6 mm i.d., 5 μm, Phenomenex, USA). The mobile phase consisted of 0.1% formic acid aqueous solution (A) and methanol (B) using the following elution program for separation: 0−25 min, 31−38% (B); 25−45 min, 38−80% (B); 45−50 min, 80−87% (B); 50−55 min, 87−90% (B); 55−60 min, 90−90% (B); 60−65 min, 90−31% (B). The column temperature was kept at 35 °C, the flow rate was 1.0 mL/min, and the injection volume was 10 μL. The detection wavelength was 330 nm for orientin, apigenin-6,8-di-*C*-α-L-arabinopyranoside, and pinostrobin chalcone, 347 nm for apigenin and luteolin, 280 nm for pinostrobin, naringenin, and longistyline C, and 259 nm for cajaninstilbene acid. Representative chromatograms of standards mixture and a sample of pigeon pea extracts are shown in Figure 1.

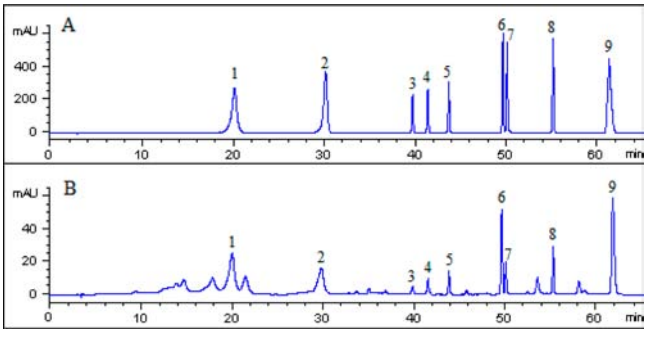

Figure 1. Representative chromatograms of standards mixture (A) and sample of pigeon pea extracts (B): (1) orientin, (2) apigenin-6,8-di-*C*-α-L-arabinopyranoside, (3) naringenin, (4) luteolin, (5) apigenin, (6) pinostrobin chalcone, (7) pinostrobin, (8) longistyline C, and (9) cajaninstilbene acid.

Determination of Total Phenolics. Total phenolics content in extracts was determined using the Folin−Ciocalteu method according to Oktay et al.[25] with some modification. In brief, 40 μL of sample solution was mixed with 1.8 mL of 0.1 N Folin−Ciocalteu reagent. After standing for 5 min at room temperature, the reaction was neutralized with 1.2 mL of 7.5% sodium carbonate aqueous solution. Then, the absorbance of solution was measured at 765 nm against a blank of 80% ethanol using a UV−vis spectrophotometer (UNICO, Shanghai, China) after incubation for 90 min at room temperature. Results were expressed as milligrams of gallic acid equivalents per gram of dry material (mg GAE/g DM).

DPPH Radical-Scavenging Activity. DPPH radical was used to determine the antioxidant activity of different extracts. The assay was performed according to the method reported by Wang et al.[16] DPPH values were expressed as micromoles of gallic acid equivalents per gram of dry material (μmol GAE/g DM).

Statistical Analysis. All statistical analysis was performed using Statistica 6.0 software (Statsoft Inc.). All results were expressed as mean values ± standard deviations (SDs). The significance of difference was conducted by one-way analysis of variance, and differences at $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Effect of Various UV Irradiations on Contents of Individual Flavanoids and Stilbenes. The components investigated include seven flavanoids, orientin, apigenin-6,8-di-*C*-α-L-arabinopyranoside, naringenin, luteolin, apigenin, pinostrobin chalcone, and pinostrobin, and two stilbenes, longistyline C and cajaninstilbene acid. Orientin and cajaninstilbene acid were present as predominant phenolics (Figure 2). As shown in Figure 2, the contents of individual flavanoids and stilbenes in nonirradiated pigeon pea leaves (control) remained constant or slightly fluctuated, but these fluctuations were statistically thoroughly nonsignificant ($P > 0.30$). In contrast, some remarkable changes in contents of individual flavanoids and stilbenes were observed in irradiated pigeon pea leaves.

Treatment with UV-C for 2 h significantly increased the contents of orientin, apigenin-6,8-di-*C*-α-L-arabinopyranoside, pinostrobin chalcone, pinostrobin, longistyline C, and cajaninstilbene acid ($P < 0.05$). Exposure to UV-C for 4 h increased the contents of all flavanoids and stilbenes detected except pinostrobin chalcone ($P < 0.02$). It was also observed that all individual compounds except naringenin, luteolin, and apigenin were affected in a similar way by UV-C irradiation. The contents of these compounds showed a notable increase first, and then a decline occurred with prolonged irradiation. UV-C irradiation for 2 or 4 h induced comparable quantitative increases in these compounds. However, for naringenin, luteolin, and apigenin, the longer the UV-C irradiation time was, the higher their contents became within tested irradiation time. The contents of naringenin, luteolin, and apigenin in pigeon pea leaves irradiated by UV-C for 8 h were 3.49-, 4.33-, and 4.82-fold increased as compared to those in freshly sampled leaves, respectively. In a word, temperate UV-C irradiation induced a considerable increase of flavanoids and stilbenes in pigeon pea leaves.

In general, the trends observed here in orientin, pinostrobin chalcone, pinostrobin, longistyline C, and cajaninstilbene acid triggered by UV-B were similar to those induced by UV-C. Similarly, 2 h UV-B treatment was enough to induce obvious increases in orientin, pinostrobin chalcone, pinostrobin, longistyline C, and cajaninstilbene acid ($P < 0.03$) (Figure 2). Moreover, exposure to UV-B for 2 or 4 h resulted in comparably quantitative increases in these compounds. However, the content peaks of these compounds in pigeon pea leaves induced by UV-B were earlier in some cases (orientin and longistyline C) and sometimes later (pinostrobin chalcone) than those induced by UV-C. In addition, the peak values induced by UV-B

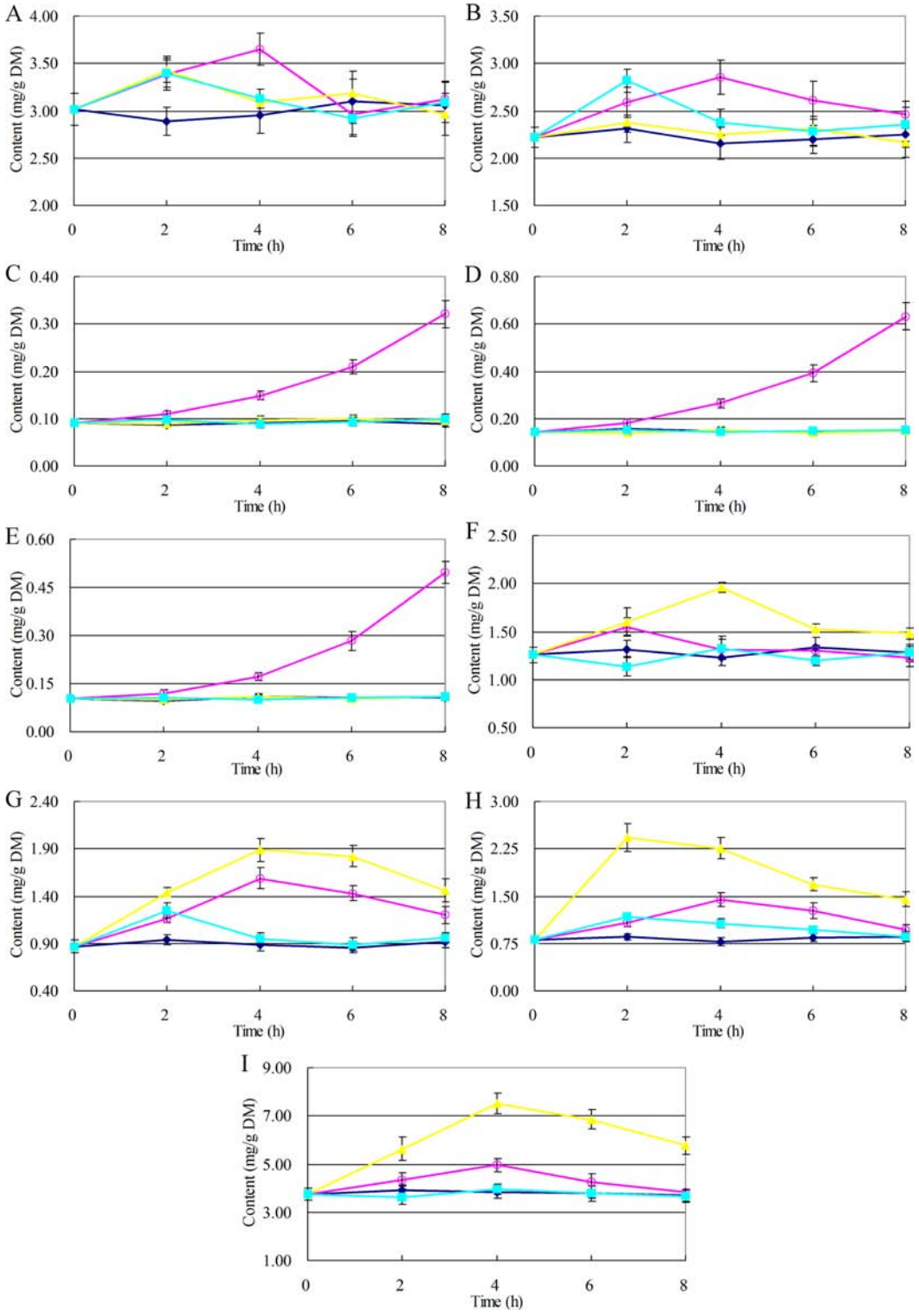

Figure 2. Effects of UV irradiations on contents of (A) orientin, (B) apigenin-6,8-di-C-α-L-arabinopyranoside, (C) naringenin, (D) luteolin, (E) apigenin, (F) pinostrobin chalcone, (G) pinostrobin, (H) longistyline C, and (I) cajaninstilbene acid in pigeon pea leaves: (♦) control, (○) UV-C, (▲) UV-B, (■) UV-A.

were much higher in all five compounds except orientin than those induced by UV-C. Meanwhile, a remarkable difference was observed between UV-B- and UV-C-irradiated pigeon pea leaves for the other four compounds (apigenin-6,8-di-C-α-L-arabinopyranoside, naringenin, luteolin, and apigenin). UV-B irradiation did not induce any significant changes in their contents ($P > 0.25$), whereas UV-C irradiation had a significant influence on their contents ($P < 0.005$) (Figure 2). Antognoni et al.[26] studied induction of flavonoid production by UV-B irradiation in *Passiflora quadrangularis* callus cultures. According to their results, UV-B irradiation was able to notably increase the production of all four flavonoids (isoorientin, orientin, isovitexin, vitexin). Besides the structures of compounds, different species might account for this difference in response to UV-B irradiation.

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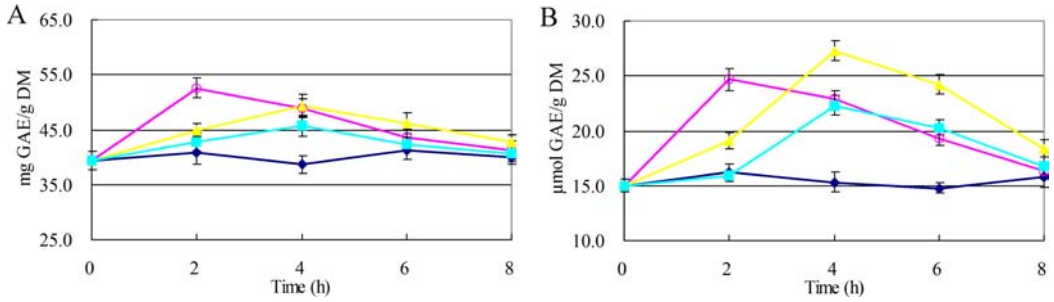

Figure 3. Effects of UV irradiation on (A) total phenolics and (B) antioxidant activity in pigeon pea leaves: (♦) control, (○) UV-C, (▲) UV-B, (■) UV-A.

The trends observed here in 9 compounds triggered by UV-A were similar to those induced by UV-B, although the changes were less marked (Figure 2). Compared with UV-C and UV-B, UV-A had a relatively weak influence on the contents of flavanoids and stilbenes in pigeon pea leaves.

Effect of Various UV Irradiations on Total Phenolics and Antioxidant Activity. UV irradiation induced the increases of total phenolics and antioxidant activity in pigeon pea leaves (Figure 3). Total phenolics and antioxidant activity in nonirradiated pigeon pea leaves (control) slightly fluctuated ($P > 0.40$), whereas significant changes in the levels of total phenolics and antioxidant activity were observed in irradiated pigeon pea leaves ($P < 0.01$).

As shown in Figure 3A, the trends observed in total phenolics triggered by UV-C, -B, and -A were the same. However, various UV irradiations induced different degrees of response. UV-C irradiation induced the highest increase in total phenolics compared with UV-B and -A. It was also observed that short-term UV irradiation induced a distinct increase in total phenolics ($P < 0.02$), and the increases seemed to be irradiation time dependent for 2 and 4 h. However, a UV irradiation time of more than 4 h did not further enhance the levels of total phenolics. In contrast, a severe decline in total phenolics was observed in pigeon pea leaves irradiated for 8 h. This trend was consistent with the changes in contents of most individual phenolics during UV irradiation.

The antioxidant activity had a similar response to UV irradiation (Figure 3B). The DPPH radical-scavenging activity was increased by UV treatment. UV irradiation for 2 or 4 h was most effective in promoting antioxidant activity. However, UV-B irradiation induced the highest increase in antioxidant activity compared with UV-C and -A. These results indicated that UV-B was more conducive to the accumulation of stronger antioxidants, although it induced less increase in total phenolics compared with UV-C. It was also found that callus cultures irradiated by UV-B showed a higher antioxidant activity compared with nonirradiated calluses, with an increase of as much as 76%.[26]

In our study, we found that UV irradiation, especially UV-B and UV-C, enhanced levels of phenolics (flavonoids and stilbenes) and antioxidant activity in pigeon pea leaves. Previous studies indicated that UV irradiation might act as abiotic stress and mediated the increases of secondary metabolism enzymes, such as phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), and stilbene synthase (STS), accelerating the biosynthesis of phenolic compounds.[27,28] The increases in phenolics and antioxidant activity as described in this study might be part of the defense mechanism of pigeon pea leaves to UV-induced stress. Flavonoids, especially anthocyanins, possess a variety of potent properties, such as antioxidant as well as UV protective effects.[17] Stilbenes are considered to be a health-promoting substance and biosynthesized in response to biotic and abiotic stresses.[29] The biosynthesis of phenolics and the increase of antioxidant activity induced by UV-B irradiation have been reported in apple.[30] The increase of resveratrol in UV-C-irradiated grape has been described.[31] All these findings agree with the hypothesis that UV irradiation can be regarded as an abiotic stress favorable for enhancing the synthesis of secondary metabolites.[32]

The increases of main phenolics and antioxidant activity appeared to be time dependent at shorter irradiation times, while longer irradiation times resulted in gradual declines. This phenomenon has also been observed in blueberries and grapes where a longer irradiation time was considered to cause too much stress and possibly led to injury.[16,19] Moreover, our results also revealed that the plant's response to UV irradiation was phytochemical specific. This was also reported for Cv. Napoleon Table Grapes.[33]

Residual Effect of UV-B Irradiation on Contents of Individual Flavanoids and Stilbenes in Pigeon Pea Laves after UV-B Irradiation. The above tests revealed that UV-B irradiation for 4 h induced the highest antioxidant activity and higher total phenolics in pigeon pea leaves. Hence, the residual effect of UV-B irradiation was investigated after the leaves had been exposed to UV-B for 4 h.

In our study, we measured the changes of individual flavanoids and stilbenes in pigeon pea leaves at adaptation temperatures of 20 (room temperature) and 4 °C (chilling temperature) as well as adaptation times of 4, 24, 36, 48, and 72 h after the leaves had been exposed to UV-B for 4 h. The results are shown in Figure 4. During the adaptation period, the contents of individual flavanoids and stilbenes in nonirradiated leaves (control) only slightly fluctuated ($P > 0.30$). The contents of orientin and apigenin-6,8-di-*C*-α-L-arabinopyranoside in irradiated leaves had no any significant changes at two adaptation temperatures 20 and 4 °C ($P > 0.30$). However, as observed for naringenin, luteolin, and apigenin, a remarkable difference was observed between 20 and 4 °C. The contents of them in irradiated leaves gradually increased with time at an adaptation temperature of 20 °C ($P < 0.001$), whereas an insignificant change was observed at an adaptation temperature of 4 °C ($P > 0.25$). After the irradiated leaves were held for 72 h at 20 °C, the contents of these compounds were at least 2-fold increased as compared to those in nonirradiated leaves (control). With regard to the other two flavanoids, pinostrobin chalcone and pinostrobin, there were significant changes at two adaptation temperatures ($P < 0.001$) and the increases at 4 °C were higher than those at 20 °C.

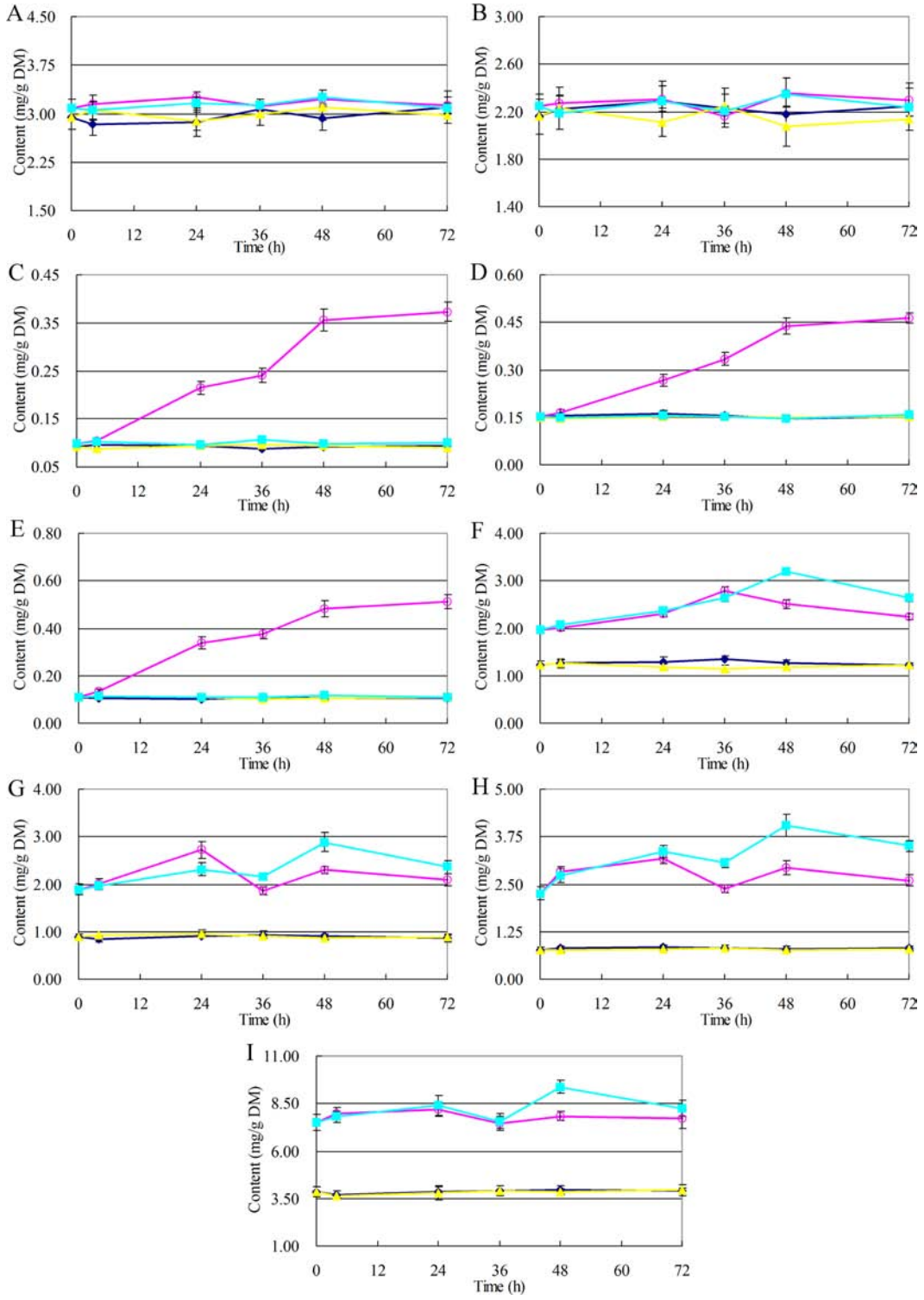

Figure 4. Changes in contents of (A) orientin, (B) apigenin-6,8-di-C-α-L-arabinopyranoside, (C) naringenin, (D) luteolin, (E) apigenin, (F) pinostrobin chalcone, (G) pinostrobin, (H) longistyline C, and (I) cajaninstilbene acid in pigeon pea leaves after being irradiated with UV-B for 4 h and held for various adaptation times at 20 and 4 °C: (♦) 20 °C control, (○) 20 °C, (▲) 4 °C control, (■) 4 °C.

Besides, it was noticed that the contents of stilbenes (longistyline C and cajaninstilbene acid) in irradiated leaves changed with adaptation time and presented two peaks at adaptation temperatures of 20 and 4 °C, occurring at adaptation times of 24 and 48 h, respectively. Similar results had been reported in young grape plants where the accumulation of resveratrol induced by UV proved to be closely related to the level of STS mRNA.[34] Previous studies revealed that the STS gene family might be classified into two sorts: some are expressed early but the producing mRNA degrades rapidly, and others are expressed later and slowly activated but produce more stable mRNA.[35,36] Thus, we presumed that the accumulation of longistyline C and cajaninstilbene acid induced by UV-B fluctuated with the level of STS mRNA and presented two peaks. It was also observed that the higher peaks of longistyline C

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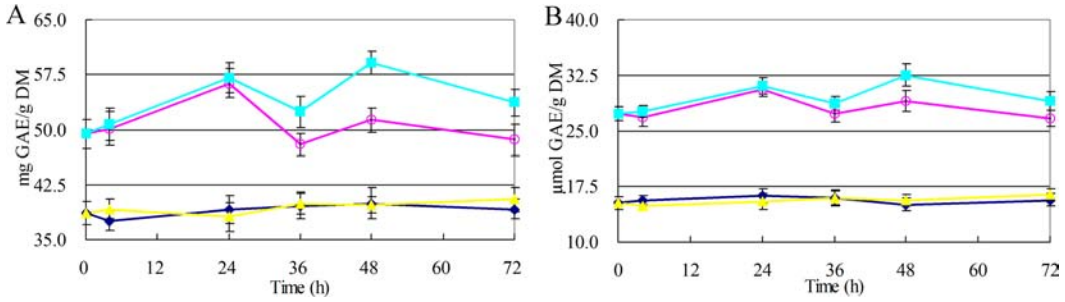

Figure 5. Changes in (A) total phenolics and (B) antioxidant activity in pigeon pea leaves after being irradiated with UV-B for 4 h and held for various adaptation times at 20 and 4 °C: (♦) 20 °C control, (○) 20 °C, (▲) 4 °C control, (■) 4 °C.

and cajaninstilbene acid at 20 °C were reached earlier (24 h) than those at 4 °C (48 h), whereas the higher peaks at 4 °C were higher than those at 20 °C.

Residual Effect of UV-B Irradiation on Total Phenolics and Antioxidant Activity in Pigeon Pea Leaves after UV-B Irradiation. The results are shown in Figure 5. During the adaptation period, the total phenolics and antioxidant activity in nonirradiated leaves (control) had no any significant changes ($P > 0.40$). The trend observed in total phenolics at 20 °C after UV-B irradiation was consistent with that at 4 °C. Total phenolics in irradiated leaves had obvious changes ($P < 0.01$) and presented two peaks at two adaptation temperatures of 20 and 4 °C, occurring at adaptation times of 24 and 48 h, respectively. Higher peaks appeared at an adaptation time of 24 h for 20 °C and 48 h for 4 °C, respectively. The trends of antioxidant activity after UV-B irradiation were very similar to those of total phenolics, and the changes of antioxidant activity were also very significant at two adaptation temperatures ($P < 0.01$). Schreiner et al.[18] studied the effects of UV-B irradiation on total phenolics in different organs of nasturtium (*Tropaeolum majus* L.). It was found that UV-B irradiation induced a significant increase in total phenolics in inflorescences after an adaptation time of just 2 h, which tended to decrease after a longer adaptation time of 22 h. However, total phenolics in leaves decreased after an adaptation time of 2 h but increased to the level of control after a longer adaptation time of 22 h. In conclusion, different species, even different organs, might show different response to UV-B irradiation.

Our results clearly demonstrated that the plant's response to UV irradiation was phytochemical specific. Higher levels of flavanoids and stilbenes, total phenolics, and antioxidant activity were induced by UV-B and UV-C irradiation. Moreover, UV-B irradiation possessed a long-lasting effect on the levels of phenolics and antioxidant activity. These results indicate that UV irradiation of pigeon pea leaves can be beneficial in terms of increasing active components and antioxidant activity.

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Notes

The authors declare no competing financial interest.

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