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Variation in Contents of Main Active Components and Antioxidant Activity in Leaves of Different Pigeon Pea Cultivars during Growth

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Supporting Information

ABSTRACT: Pigeon pea is an important and multiuse grain legume crop, and its leaves are a very valuable natural resource. To obtain a high-quality biological resource, it is necessary to choose the excellent cultivar and determine the appropriate harvest time. In this study, the variation in contents of main active components and antioxidant activity in leaves of six pigeon pea cultivars during growth were investigated. The level of each individual active component significantly varied during growth, but with a different pattern, and this variation was different among cultivars. Flavonoid glycosides orientin, vitexin, and apigenin-6,8-di-*C*-α-L-arabinopyranoside showed two peak values at mid-late and final stages of growth in most cases. Pinostrobin chalcone, longistyline C, and cajaninstilbene acid showed remarkablely higher values at the mid-late stage of growth than at other stages. Pinostrobin had an extremely different variation pattern compared to other active components. Its content was the highest at the earlier stage of growth. Principal component analysis (PCA) revealed that vitexin and apigenin-6,8-di-*C*-α-L-arabinopyranoside were mainly responsible for distinguishing cultivars analyzed. In a comprehensive consideration, the leaves should preferentially be harvested at the 135th day after sowing when the level of active components and antioxidant activity reached higher values. Cultivars ICP 13092, ICPL 87091, and ICPL 96053 were considered to be excellent cultivars with high antioxidant activity. Our findings can provide valuable information for producing a high-quality pigeon pea resource.

KEYWORDS: *Flavanoids, stilbenes, antioxidant activity, cultivar, harvest time, pigeon pea leaves, Cajanus cajan*

INTRODUCTION

Pigeon pea [*Cajanus cajan* (L.) Millsp.], also called congo pea, red gram, non-eye pea, etc., is a multipurpose plant of the Leguminosae family (genus *Cajanus*). It ranks sixth in area and production in grain legumes worldwide. Pigeon pea is a good source of dietary essential minerals, vitamin B, and protein in the human diet, especially in the vegetarian population.[1] Pigeon pea leaves has many uses in traditional folk medicine. It has been used widely to treat bedsores, bladder stones, jaundice, dysentery, diabetes, reproductive system infection, etc.[2−4] At present, in China, pigeon pea leaves have been exploited in traditional Chinese medicine (TCM) for the therapy of ischemic necrosis of the femoral head. Pharmacological investigations found pigeon pea leaves possessed many bioactivities, such as antitumor, antibacterial, hypotriglycerimic, hypoglycemic, insecticidal, antiplasmodial, antioxidant activities, etc.[3,5−11] Chemical studies revealed that flavonoids and stilbenes represented the main active components found in pigeon pea leaves.[3,10,12−15] Orientin, pinostrobin chalcone, and pinostrobin, which belonged to flavone *C*-glycosides, chalcone, and flavanone, respectively, were representative flavonoids of pigeon pea leaves. Orientin has been reported to possess antiviral, antimicrobial, antioxidant, and radiation protection activities.[16,17] Pinostrobin chalcone showed significant activity *in vitro* against *Leishmania amazonensis*, antioxidant activity, and neuroprotective effects.[18−20] Pinostrobin was known as an antispasmodic agent, possesses anti-aromatase activity in human placental microsomes, and inhibits the growth of MCF-7 cells induced by dehydroepiandrosterone sulfate and 17β-estradiol.[21,22] It also showed antiviral activity toward herpes simplex virus-1 (HSV-1) with EC_{50} of 22.71 ± 1.72 μg/mL.[23] As representative stilbenes in pigeon pea leaves, longistyline C and cajaninstilbene acid were rarely present in plants and had estrogenic, hypoglycemic, hypotriglyceridemic, and hypocholesterolemic activities as well as a potential effect in the therapy of postmenopausal osteoporosis.[7,24,25] Longistyline C also exhibited excellent inhibitory activity on various cancer cells.[5]

The content of phytochemicals in plants depends upon numerous factors, including genotype, cultivation techniques, climatic conditions, harvest time, postharvest treatments, etc.[26,27] Bernaert et al.[28] found there was a remarkable effect of the cultivar on the amount of isoalliin and methiin in leek and isoalliin and methiin changed significantly during the growth season. Pérez-Balibrea et al.[29] reported that the edible sprouts of different broccoli cultivars showed significant differences for individual glucosinolates, total phenolic content, and antioxidant capacity. Significant differences were also found among harvest times and varieties for sensory quality and

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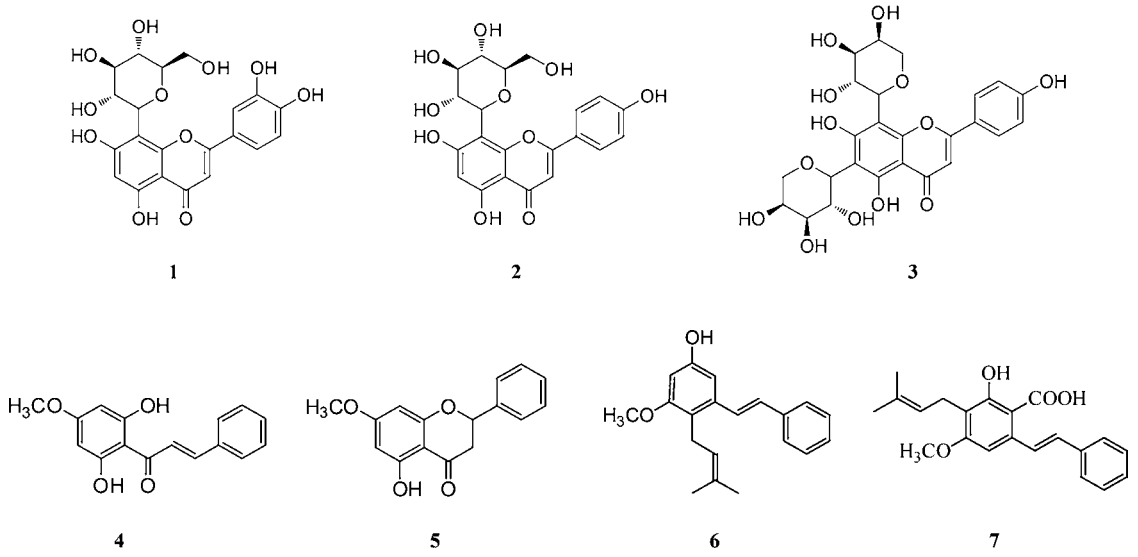

Figure 1. Chemical structures of (1) orientin, (2) vitexin, (3) apigenin-6,8-di-*C*-α-L-arabinopyranoside, (4) pinostrobin chalcone, (5) pinostrobin, (6) longistyline C, and (7) cajaninstilbene acid.

chemical composition of Jerusalem artichoke (*Helianthus tuberosus* L.) tuber.[30] In a word, the level of phytochemicals might vary among different cultivars and harvest times, and variation in bioactivity was also often encountered. Hence, to obtain a high-quality biological resource, it was necessary to choose the excellent cultivar and determine appropriate harvest time.

Although metabolite profiles of pigeon pea leaves have been reported extensively, literature with regard to the effect of cultivar and harvest time was scarce. Hence, the objective of this study was to investigate the variation in contents of seven active components (Figure 1) in leaves of six cultivars during growth. Meanwhile, the variation in antioxidant activity was also assessed by means of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical-scavenging assay. The present work might contribute to produce a high-quality pigeon pea resource.

MATERIALS AND METHODS

Reagents and Chemicals. Gallic acid, orientin, and vitexin 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.[26] Their structures were confirmed by electrospray ionization mass spectrometry (ESI−MS) and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) (see Figures S1−S5 of the Supporting Information) in comparison to literature data.[6,31−34] A total antioxidant capacity assay kit with the ABTS method was purchased from Beyotime Institute of Biotechnology (China). High-performance liquid chromatography (HPLC)-grade methanol and formic acid were obtained from J&K Chemical, Ltd. (Beijing, China) and Dima Technology, Inc. (Muskegon, MI), respectively. Deionized water was prepared using a Milli-Q water purification system from Millipore (Bedford, MA). Ethanol was of analytical grade and purchased from Beijing Chemical Reagents Co. (Beijing, China).

Plant Material and Experimental Design. Pigeon pea leaves from six different cultivars (ICP 13092, ICPL 87051, ICPL 87091, ICPL 87119 Asha, ICPL 96053, and ICPL 80563 Lakshmi) were studied. The seeds of all cultivars were obtained from the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT). The seeds were immersed in 10% sodium hypochlorite solution for 5 min, rinsed 3 times with distilled water, and then immersed in aerated deionized water overnight. Immersed seeds of six cultivars were sown in triplicate in a non-heated greenhouse in April 2011. In May 2011, the six cultivars were planted in the field, with each repetition in a different row. At eight different times after sowing (60, 90, 105, 120, 135, 150, 165, and 180 days), plants of each repetition were harvested from each cultivar. Representative changes of pigeon pea plants during growth were shown in Figure 2. Monthly average temperature and

Figure 2. Representative changes of pigeon pea plants during growth after sowing: (A) 60th day, (B) 120th day, (C) 135th day, and (D) 165th day.

monthly average radiation intensity were given in Figure 3. After harvesting, the leaves were shock-frozen in liquid nitrogen and kept at −80 °C prior to freeze-drying. The freeze-dried leaves were pulverized to pass through a sieve (40 mesh) and stored in a desiccator until extraction.

Sample Preparation. The dried powder of pigeon pea leaves (5.00 g) was extracted with 70 mL of aqueous ethanol (30:70, v/v) solution in an ultrasonic bath (Kunshan Ultrasonic Instrument, China) for 30 min. After vacuum filtration, the obtained residue was again extracted using the same conditions, repeated twice. The filtered solution was collected and pooled for analysis of active components and antioxidant activity.

HPLC Determination of Individual Flavanoids and Stilbenes. Individual flavanoids and stilbenes were quantitated using an Agilent

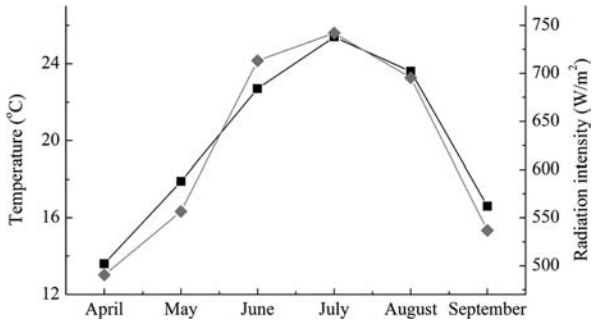

Figure 3. Monthly average temperature (black curve) and monthly average radiation intensity (gray curve) during experiment.

1200 series liquid chromatography system (Agilent, San Jose, CA) consisting of a G1311A quaternary pump, a G1322A degasser, a G1365B MWD UV detector, and a G1328B manual injector. Separation of these active components was performed on a HIQ sil C18 V reversed-phase column (250 × 4.6 mm inner diameter, 5 μm, KYA Technologies Corporation, Tokyo, Japan) protected with a guard column. The mobile phase was a gradient of 0.1% formic acid aqueous solution (A) and methanol (B). The gradient was as follows: 0–25 min, 31–38% B; 25–45 min, 38–80% B; 45–50 min, 80–86% B; 50–55 min, 86–90% B; 55–60 min, 90% B; and 60–65 min, 90–31% B. The flow rate was set at 1 mL/min, and the injection volume was 10 μL. The column temperature was held at 30 °C for chromatographic separation. After 10 min of re-equilibration, the column was ready for a new injection.

The detection wavelength was 330 nm for orientin, vitexin, apigenin-6,8-di-*C*-α-L-arabinopyranoside, and pinostrobin chalcone, 280 nm for pinostrobin and longistyline C, and 259 nm for cajaninstilbene acid. Representative chromatograms of the standard mixture and extracts of pigeon pea leaves are shown in Figure 4. For quantitation of active components, an external standard method was used. The working calibration curves showed good linearity over the tested ranges (see Table S1 of the Supporting Information). Results were expressed as milligrams of individual components per gram of dry material (mg/g of dm).

Analysis of Antioxidant Activity. The total antioxidant activity was measured by the ABTS$^{\bullet+}$ radical-scavenging assay. A blue/green ABTS$^{\bullet+}$ radical was prepared by reacting ABTS water solution with potassium persulfate, and the mixture was held in the dark at room temperature for 12–16 h prior to use. The resulting ABTS$^{\bullet+}$ stock solution was diluted with 80% ethanol to the absorbance of 0.70 ± 0.05 at 734 nm to obtain working solution. For the spectrophotometric assay, 10 μL of sample and 200 μL of ABTS$^{\bullet+}$ working solution were mixed, and after 6 min, the absorbance of the mixture was measured at 734 nm. The blank was measured using 70% ethanol. Trolox was used as the reference standard to prepare a calibration curve for a concentration range of 0.10–1.5 mM. Results were expressed micromoles of trolox equivalents per gram of dry material (μmol of TE/g of dm).

Statistical Analysis. All results were presented as the mean value ± standard deviation (SD) of three measurements. Analysis of variance (ANOVA) was carried out by SPSS 19.0 statistical software (SPSS, Inc., Chicago, IL) with cultivar and harvest time as factors, taking their interaction into account. In the case of significant differences, multiple comparisons of means were performed with Duncan's multiple range test. Statistical significance was set at the level of $p < 0.05$. Moreover, principal component analysis (PCA) was performed on active components and antioxidant activity to describe the variation among varieties and harvest times using the same statistical software.

RESULTS AND DISCUSSION

Variation in Contents of Flavanoids and Stilbenes in Leaves of Six Pigeon Pea Cultivars during Growth. To evaluate the changes in active components of the studied cultivars, five flavonoids and two stilbenes were quantified at eight different harvest times (from June to September). The results were shown in Figure 5. Significant differences in contents of seven active components were observed among cultivars and harvest times (Table 1). In addition, there were also statistically significant interactions between cultivar and harvest time.

In cultivars ICP 13092, ICPL 87119 Asha, ICPL 96053, and ICPL 80563 Lakshmi, the content of orientin significantly fluctuated with harvest time ($p < 0.01$) and showed two peak values (Figure 5A). In these cases, the contents of orientin were relatively stable from 60 to 105 days ($p > 0.50$) and sharply increased at the 120th day and then a severe decrease occurred followed by a significant rebound after 150 days. For these four

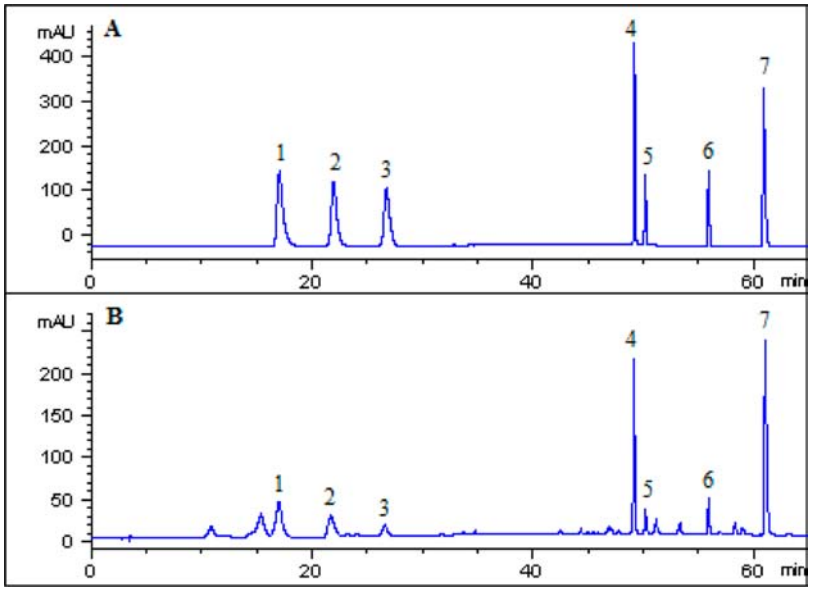

Figure 4. Representative chromatograms of (A) standard mixture and (B) extracts of pigeon pea leaves: (1) orientin, (2) vitexin, (3) apigenin-6,8-di-*C*-α-L-arabinopyranoside, (4) pinostrobin chalcone, (5) pinostrobin, (6) longistyline C, and (7) cajaninstilbene acid.

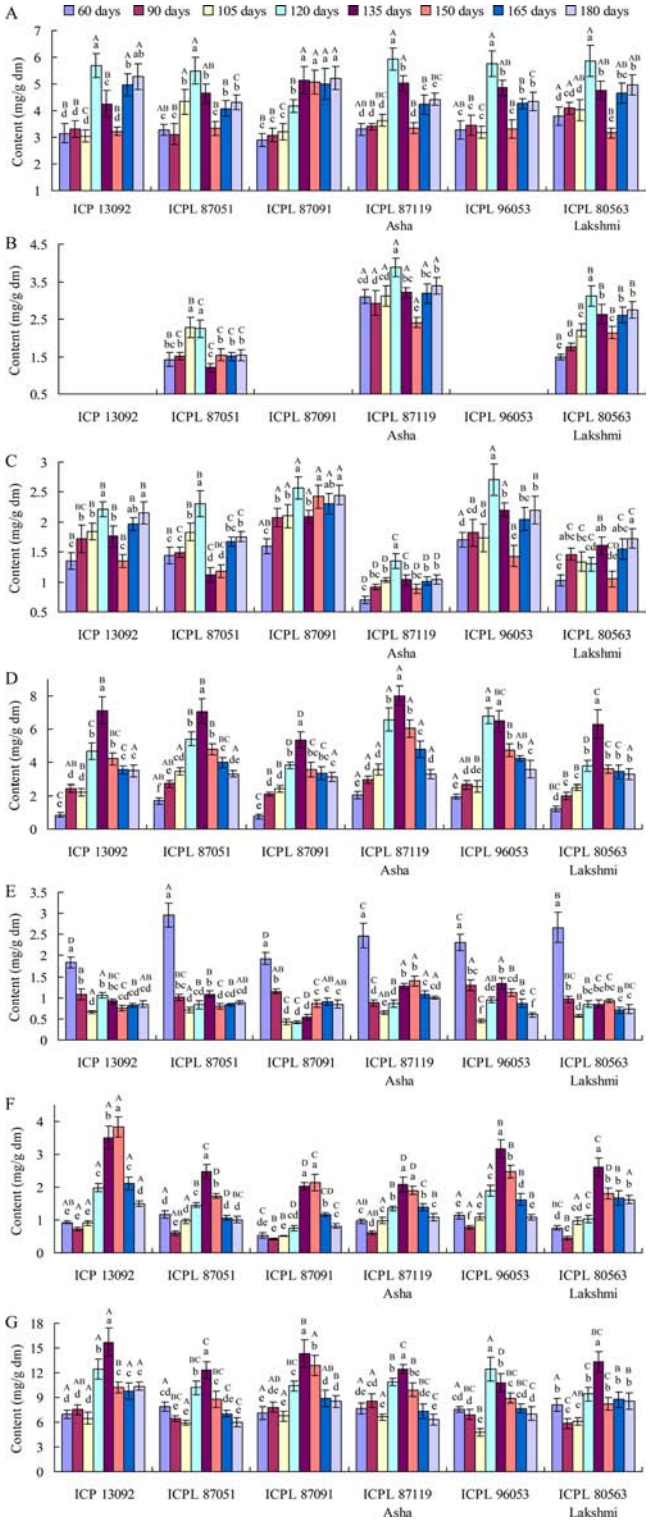

Figure 5. Variation in contents of (A) orientin, (B) vitexin, (C) apigenin-6,8-di-C-α-L-arabinopyranoside, (D) pinostrobin chalcone, (E) pinostrobin, (F) longistyline C, and (G) cajaninstilbene acid in leaves of six pigeon pea cultivars during growth. Significant differences between harvest times within variety (lowercase letters) and between varieties within harvest time (capital letters) as determined by Duncan's multiple range test ($p < 0.05$) are indicated by different letters.

cultivars, the highest contents of orientin appeared at the 120th day, which were 5.69, 5.94, 5.76, and 5.85 mg/g of dm,

Table 1. Two-Way ANOVA for Individual Flavanoids and Stilbenes and Antioxidant Activity of the Leaves of Six Pigeon Pea Cultivars Harvested at Eight Harvest Times and the Interaction Cultivar × Harvest Time

	cultivar		harvest time		cultivar × harvest time	
	F value	*P* value	*F* value	*P* value	*F* value	*P* value
OR[a]	3.40	*b*	96.88	*c*	6.18	*c*
VI	29.42	*c*	2224.63	*c*	12.08	*c*
AAA	191.32	*c*	48.29	*c*	5.67	*c*
PIC	55.16	*c*	323.76	*c*	4.59	*c*
PI	24.14	*c*	417.70	*c*	11.60	*c*
LLC	113.35	*c*	444.43	*c*	16.08	*c*
CSA	18.79	*c*	137.65	*c*	5.13	*c*
ABTS	22.42	*c*	175.77	*c*	5.27	*c*

[a]OR, orientin; VI, vitexin; AAA, apigenin-6,8-di-C-α-L-arabinopyranoside; PIC, pinostrobin chalcone; PI, pinostrobin; LLC, longistyline C; and CSA, cajaninstilbene acid. [b]Statistically significant differences at $p < 0.01$. [c]Statistically significant differences at $p < 0.001$.

respectively. In cultivar ICPL 87051, the contents of orientin were relatively stable from 60 to 90 days ($p > 0.50$) and sharply increased from 105 to 120 days and then a severe decrease occurred followed by a significant rebound after 150 days. However, in cultivar ICPL 87091, the variation pattern of orientin was very different from the other cultivars. In this case, the contents of orientin were relatively stable from 60 to 105 days ($p > 0.35$), sharply increased at the 120th day, and then reached a stable level after 180 days.

For vitexin, it was only detected in ICPL 87051, ICPL 87119 Asha, and ICPL 80563 Lakshmi. In these cultivars, the content of vitexin significantly changed with harvest time ($p < 0.01$) and also showed two peak values (Figure 5B). The higher peak values for ICPL 87119 Asha and ICPL 80563 Lakshmi appeared at the 120th day, which were 3.88 and 3.12 mg/g of dm, respectively, while the higher peak value for ICPL 87051 appeared at the 105th day, which was 2.28 mg/g of dm. In general, the level of vitexin in ICPL 87119 Asha was higher than those in the other two cultivars during growth. In ICP 13092, ICPL 87091, and ICPL 96053, the lack of vitexin-related gene expression may be responsible for the absence of vitexin. A similar result has also been observed in rice cultivar, where the lack of phytoene synthase (PSY) gene expression led to the absence of secondary metabolite carotenoids.[35]

In all cultivars, except ICPL 87119 Asha, the variation pattern of apigenin-6,8-di-C-α-L-arabinopyranoside was similar during growth (Figure 5C). The content of apigenin-6,8-di-C-α-L-arabinopyranoside in the leaves of these five cultivars significantly changed with harvest time ($p < 0.01$) and showed two peak values. In these cases, the content of apigenin-6,8-di-C-α-L-arabinopyranoside statistically significantly increased from 60 to 120 or 135 days, declined, then notably increased. The higher peak values for ICP 13092, ICPL 87051, ICPL 87091, and ICPL 96053 appeared at the 120th day, which were 2.22, 2.31, 2.57, and 2.70 mg/g of dm, respectively, while the higher peak value for ICPL 80563 Lakshmi appeared at the 180th day, which was 1.73 mg/g of dm. In ICPL 87119 Asha, the content of apigenin-6,8-di-C-α-L-arabinopyranoside statistically significantly increased from 60 to 120 days ($p < 0.01$), declined, then reached a stable level after 180 days ($p > 0.10$). The highest content in ICPL 87119 Asha was determined to be 1.35 mg/g of dm. In general, the level of apigenin-6,8-di-C-α-L-

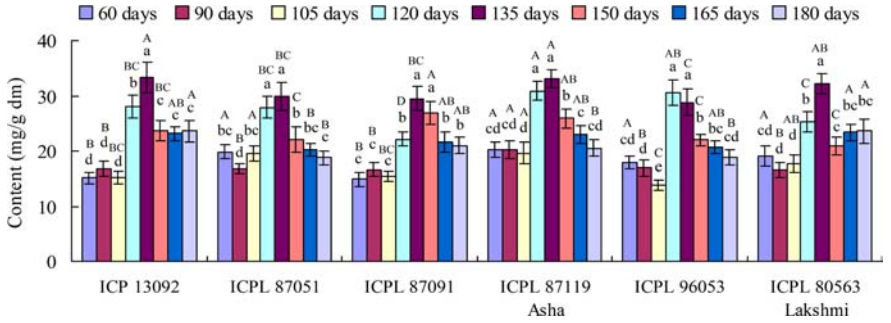

Figure 6. Variation in the level of active components (flavanoids and stilbenes) in leaves of six pigeon pea cultivars during growth. Significant differences between harvest times within variety (lowercase letters) and between varieties within harvest time (capital letters) as determined by Duncan's multiple range test ($p < 0.05$) are indicated by different letters.

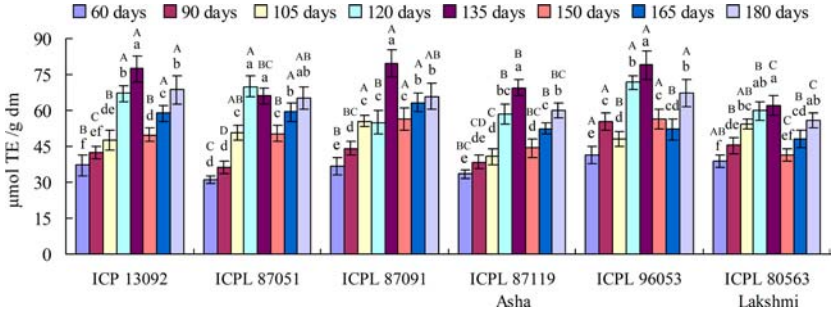

Figure 7. Variation in $ABTS^{\bullet+}$ radical-scavenging capacity of the leaves of six pigeon pea cultivars during growth. Significant differences between harvest times within variety (lowercase letters) and between varieties within harvest time (capital letters) as determined by Duncan's multiple range test ($p < 0.05$) are indicated by different letters.

arabinopyranoside in ICPL 87119 Asha and ICPL 80563 Lakshmi was lower than those in the other four cultivars during growth.

For pinostrobin chalcone, the variation pattern was similar in all cultivars (Figure 5D). The level of pinostrobin chalcone significantly increased first and then sharply decreased. For all cultivars, except ICPL 96053, harvest at the 135th day resulted in the highest pinostrobin chalcone level, as compared to the leaves harvested at the other times. In cultivar ICPL 96053, pinostrobin chalcone reached the highest level at the 120th day. At the first harvest time (60th day), the content of pinostrobin chalcone in six cultivars was 0.86−2.06 mg/g of dm. The level increased 3.30−8.31-fold at the 135th day. At the 180th day, the level of pinostrobin chalcone fell down to 3.11−3.57 mg/g of dm. These indicated that there was extremely intense fluctuation of the pinostrobin chalcone level in pigeon pea leaves during growth. It was also observed that the level of pinostrobin chalcone in ICPL 87091 was relatively lower than those in the other five cultivars.

In comparison to other analyzed components, pinostrobin showed a very different variation pattern in all cultivars (Figure 5E). The highest peak was measured at the first harvest (60th day) for all cultivars, which was 1.84−2.96 mg/g of dm. In general, harvest at the 105th day resulted in the lowest pinostrobin level (0.43−0.72 mg/g of dm), regardless of the cultivar. Although the pinostrobin level significantly rebounded after the 105th day, it was not yet comparable to that at the 60th day. The highest content of 2.96 mg/g of dm was determined in the leaves of ICPL 87051 harvested at the 60th day.

Longistyline C showed a very homogeneous variation pattern in the analyzed six cultivars during growth (Figure 5F). Harvest at the 90th day resulted in the lowest longistyline C level, as compared to the leaves harvested at other times. After 105 days, the longistyline C level remarkablely increased ($p < 0.01$) and reached the highest values at the 150th day for ICP 13092 (3.83 mg/g of dm) and ICPL 87091 (2.14 mg/g of dm) and at the 135th day for ICPL 87051 (2.48 mg/g of dm), ICPL 87119 Asha (2.08 mg/g of dm), ICPL 96053 (3.17 mg/g of dm), and ICPL 80563 Lakshmi (2.61 mg/g of dm), followed by a gradual decrease after 180 days. The leaves of cultivar ICP 13092 contained, in general, the higher amount of longistyline C, regardless of the time of harvest.

Cajaninstilbene acid was present as the most predominant phytochemical in pigeon pea leaves (Figure 5G). In six studied cultivars, cajaninstilbene acid also showed a very homogeneous variation pattern during growth. The content of cajaninstilbene acid significantly changed with harvest time ($p < 0.01$). In general, harvest at the 105th day resulted in the lowest cajaninstilbene acid level, as compared to the leaves harvested at other times. After 105 days, the cajaninstilbene acid level dramatically increased ($p < 0.01$) and reached the highest values at the 135th day for ICP 13092 (15.70 mg/g of dm), ICPL 87051 (12.31 mg/g of dm), ICPL 87091 (14.34 mg/g of dm), ICPL 87119 Asha (12.44 mg/g of dm), and ICPL 80563 Lakshmi (13.37 mg/g of dm), and at the 120th day for ICPL 96053 (12.49 mg/g of dm), followed by a significant decrease after 180 days. The same variation pattern was also found for stilbene kobophenol A in *Caragana sinica* leaves, where its level increased with the growth of the leaves from March to August and then decreased with the senescence from September to October.[36]

In our study, we found that the level of each individual active component had a marked variation during growth but with a different pattern, and this variation was different among cultivars. Because assimilation is usually more intensive than

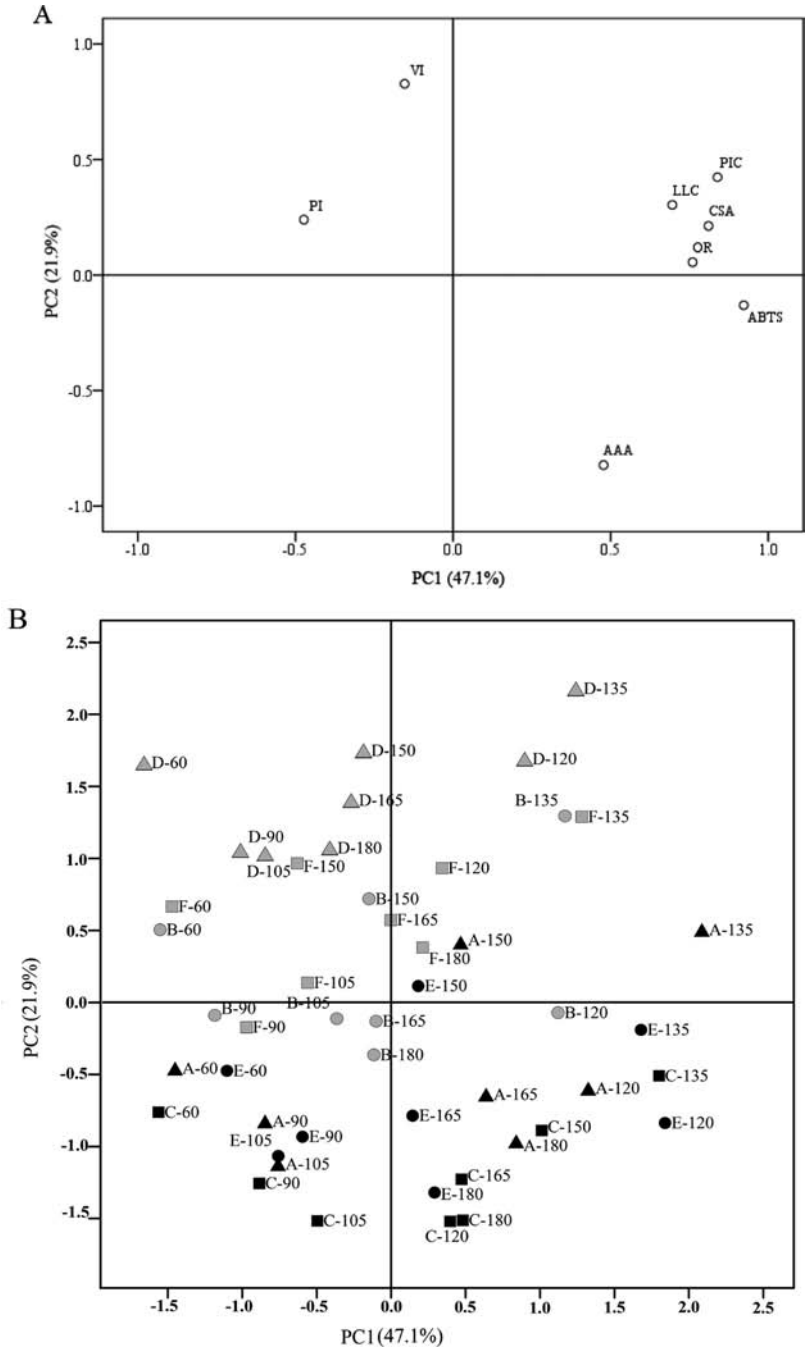

Figure 8. PCA (PC2 versus PC1): (A) distribution of variables on the loading plot and (B) distribution of samples on the score plot. OR, orientin; VI, vitexin; AAA, apigenin-6,8-di-C-α-L-arabinopyranoside; PIC, pinostrobin chalcone; PI, pinostrobin; LLC, longistyline C; CSA, cajaninstilbene acid; A, ICP 13092; B, ICPL 87051; C, ICPL 87091; D, ICPL 87119 Asha; E, ICPL 96053; and F, ICPL 80563 Lakshmi. The values of 60, 90, 105, 120, 135, 150, 165, and 180 represent the harvest time.

disassimilation during vegetative growth, the secondary metabolites increase continually in the plants during this course. However, the data provided by this work showed the contents of some active components insignificantly increased, even declined, during vegetative growth. This might be due to the rapid growth of plants during this period with the increase of the temperature (Figure 3), suggesting a dilution effect of active components. The leaves harvested at the 120th or 135th day (in the summer) contained, in general, the highest amount of all analyzed active components, except pinostrobin. The activities of secondary metabolism enzymes, such as phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), and stilbene synthase (STS), are light-dependent.[37] Therefore, the activities of these enzymes were enhanced because of very intensive solar radiation in the summer, accelerating the biosynthesis of flavanoids and stilbenes. After reaching the highest amounts, the contents of most individual flavanoids and stilbenes in the leaves underwent a severe decrease, regardless of the cultivar. This might be due to their transfer to other tissues or organs, such as the stem, flower, and fruit, or degradation with plant development.[38] However, the levels of three flavonoid glycosides had a significant rebound at the final stage of the growth season. This phenomenon has also been

observed in a dynamic change of the flavonoid content in *Cyclocarya paliurus* and bamboo leaves.[39,40]

As shown in Figure 6, the highest levels of active components (flavanoids and stilbenes) for ICP 13092, ICPL 87051, ICPL 87091, ICPL 87119 Asha, and ICPL 80563 Lakshmi appeared at the 135th day, which were 33.27, 29.91, 29.49, 33.08, and 32.15 mg/g of dm, respectively. For ICPL 96053, the highest level of active components appeared at the 120th day (30.59 mg/g of dm). Therefore, in terms of the levels of active components, the optimum harvesting time is the 120th day for ICPL 96053 and the 135th day for other five cultivars after sowing. Moreover, it was also found that, for ICPL 87051, ICPL 87119 Asha, and ICPL 96053, there were no significant differences in the levels of active components in leaves harvested at the 120th and 135th days. All cultivars presented significantly higher levels of active components at the 135th day (in the summer), which agrees with the hypothesis that flavanoids and stilbenes were related to solar radiation because of their function as sun filters.

Variation in Antioxidant Activity in Leaves of Six Pigeon Pea Cultivars during Growth. The total antioxidant capacity of the pigeon pea leaves was determined by the ABTS method. A significant difference in antioxidant activity was observed among cultivars and harvest times (Table 1). In addition, there was also a statistically significant interaction between cultivar and harvest time.

As shown in Figure 7, in all cultivars, the antioxidant activity significantly changed with harvest time ($p < 0.01$) and also showed two peak values. The higher peak values for ICP 13092, ICPL 87091, ICPL 87119 Asha, ICPL 96053, and ICPL 80563 Lakshmi appeared at the 135th day, which were 77.47, 79.70, 69.51, 78.98, and 62.04 μmol of TE/g of dm, respectively, while the higher peak values for ICPL 87051 appeared at the 120th day, which was 70.04 μmol of TE/g of dm. Therefore, in terms of antioxidant activity, the optimum harvesting time is the 120th day for ICPL 87051 and the 135th day for the other five cultivars after sowing. Moreover, it was also found that, for ICPL 87051, ICPL 96053, and ICPL 80563 Lakshmi, there were no significant differences in antioxidant activity of the leaves harvested at the 120th and 135th days. At optimum harvesting time, cultivars ICP 13092, ICPL 87091, and ICPL 96053 had obvious higher antioxidant activity.

PCA. In this study, because of the large amount of data obtained, PCA was used to identify the variables that exhibited the greatest variance within a population and determine closely related variables. All data were analyzed by PCA, and the results were visualized by the principal component score and loading plots (Figure 8). Each point on the loading plot represented the contribution of a variable (individual flavanoids and stilbenes or antioxidant activity) to the score, while each point on the score plot represented a tested sample. Principal component 1 (PC1) and principal component 2 (PC2) could explain 47.1 and 21.9% of the variation in the analyzed data, respectively. As shown in Figure 8A, PC1 was mainly correlated with the level of orientin, pinostrobin chalcone, longistyline C, cajaninstilbene acid, and antioxidant activity (positively) and pinostrobin (negatively). These variables had high loading values in PC1. PC2 was mainly correlated with the level of vitexin (positively) and apigenin-6,8-di-*C*-α-L-arabinopyranoside (negatively). These two variables also had high loading values in PC2. The loading values of individual flavanoids and stilbenes and antioxidant activity can be used to calculate the score values for the six cultivars.

PCA can separate samples in the score plot on the basis of the cumulative correlation of all component data, outlining the differences among samples. As seen from Figure 8B, PC1 explained the difference in harvest time, with the harvest at 135 days located in the rightmost position of the plot, related to the high level of orientin, pinostrobin chalcone, longistyline C, cajaninstilbene acid, and antioxidant activity, and the harvest at 60 days placed in the leftmost position of the plot, related to high level of pinostrobin. PC2 spanned the difference between the varieties ICPL 87119 Asha and ICPL 80563 Lakshmi, which were associated with the high level of vitexin, and ICP 13092, ICPL 87091, and ICPL 96053 on the other side, being associated with the high level of apigenin-6,8-di-*C*-α-L-arabinopyranoside.

Our results clearly indicated that pigeon pea leaves at different developmental stages had different levels of active components for all studied cultivars and pointed out different metabolite profiles among cultivars. In a comprehensive consideration, the leaves should preferentially be harvested at the 135th day after sowing when the level of active components and antioxidant activity reached higher values. Moreover, ICP 13092, ICPL 87091, and ICPL 96053 were regarded as excellent cultivars because of their relatively higher antioxidant activity.

■ ASSOCIATED CONTENT

Supporting Information

ESI−MS and CID−ESI−MS/MS (A), ^{1}H NMR (B), and ^{13}C NMR (C) spectra of longistyline C (Figure S1), pinostrbin (Figure S2), apigenin-6,8-di-*C*-α-L-arabinopyranoside (Figure S3), pinostrbin chalcone (Figure S4), and cajaninstilbene acid (Figure S5) and regression data, LODs, and LOQs for seven active components (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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