Analysis of Flower Color Variations at Different Developmental Stages in Two Honeysuckle (Lonicera Japonica Thunb.) Cultivars

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Abstract. Lonicera japonica Thunb., known as Japanese honeysuckle or golden-and-silver honeysuckle, belongs to the honeysuckle family and is native to eastern Asia, including China, Japan, and Korea. Microscopy, spectrophotometry, colorimetry, and the Royal Horticulture Society of Colorimetric Card (RHSCC) were used to compare and analyze the pigment distribution, content, and color variations in the Yujin 2 and Damaohua cultivars at different developmental stages. There were notable differences in the corolla color and the cross-section color between different developmental stages and different varieties. The lightness (L*), redness (a*), and yellowness (b*) values were calculated for each period for the two cultivars to observe variation trends. The chlorophyll content in the corollas of both cultivars showed declining trends with different rates. The chlorophyll content decreased rapidly from the young period to the silver period and rose sharply during the golden period. Moreover, the carotenoid content declined slightly from the young period to the silver period and rose sharply during the golden period. The ratio of these two pigment contents increased dramatically during the golden period: by 11.51 and 6.53 times in ‘Yujin 2’ and ‘Damaohua’, respectively. There were significant differences in corolla color, cross-section color, and the content of three pigments between the two varieties of honeysuckle. Distribution and variation of pigments were the key factors affecting the flower color of honeysuckle. This study provides a basis for the identification and breeding of honeysuckle varieties and lays a foundation for further studies on the function and molecular mechanisms of pigments.

Lonicera japonica Thunb., known as Japanese honeysuckle and golden-and-silver honeysuckle, belongs to the honeysuckle family and is native to eastern Asia, including China, Japan, and Korea (He et al., 2011). L. japonica has been used as traditional medicine for the treatment of exopathogens, wind-heat, epidemic febrile diseases, sores, carbuncles, furuncles, and some infectious diseases. Currently, L. japonica Thunb. is an ornamental plant distributed naturally throughout Argentina, Brazil, Mexico, Australia, New Zealand, and the United States (Shang et al., 2011). The color is considered to be an important indicator in measuring ornamental value and offers a basis for plant classification (Zhang et al., 2012). The flower color is affected by the composition and content of pigments in the corolla, physical and chemical properties, pH value in the vacuoles, and the shape of the epidermal cells in the corolla; the main critical factors are pigment composition and content variation (Zhu et al., 2012). Many important compounds, including organic acids, flavonoids, iridoid glycosides, and saponins, have been isolated from Lonicera species, and they have both medicinal and economic value (Shang et al., 2011). L. japonica Thunb. can bloom many times in 1 year, and the flowering period is very long. The development of a single flower can be divided into the young bud stage, three green stage, two white stage, white stage, silver stage, golden stage, and fade stage, appearing as greenish off-white or yellow (Shang et al., 2011). There are few reports on the flower color in L. japonica Thunb.

In this study, the distribution and content variation of pigments in two varieties of L. japonica Thunb. were studied. The results provide a basis for the identification and breeding of new varieties, and lay a foundation for the further study of pigment function and molecular mechanisms in honeysuckle.

Materials and Methods

Plant material. The fresh flower buds or flowers of ‘Damaohua’ and ‘Yujin 2’ at six developmental stages were collected from the resource garden of the College of Life Sciences, Henan Normal University.

Determination of chloroplast pigment. Temporary slides were prepared for the observation of pigments from the plant material. A spectrophotometer was used to determine chlorophyll a, chlorophyll b, and carotenoid content according to the wavelengths of their maximum absorption peaks, which were 665, 649, and 470 nm, respectively. Determination of the three pigment contents was performed according to the following (Ren et al., 2015):

1. One gram of fresh corolla in each developmental stage was cut into pieces and put into a mortar. A small amount of quartz sand and calcium carbonate powder were added, and the mixture was ground into a homogenate with 2 to 3 mL 95% ethanol and treated for 10 min in the dark.
2. The extract was transferred into a 25-mL volumetric flask with 95% ethanol.
3. The pigment ethanol extract with a light diameter of 1 cm and 95% ethanol as a blank control were injected into the same colorimetric cup. Absorbance measurements at wavelengths of 665, 649, and 470 nm were repeated three times. The following formulas were used for analysis:

\[
\begin{align*}
\text{Concentration of chlorophyll a:} & \quad \text{Ca} = 13.95A_{665} - 6.8A_{649} \\
\text{Concentration of chlorophyll b:} & \quad \text{Cb} = 24.96A_{649} - 7.32A_{665} \\
\text{Concentration of chlorophyll:} & \quad \text{Ca} + \text{Cb} \\
\text{Content of chlorophyll} & = \frac{C \times V}{M} \\
\text{where} \quad C & \quad \text{is the chlorophyll concentration,} \quad V \quad \text{is the constant volume (measured in milliliters)} \quad \text{of chlorophyll extraction solution, and} \quad M \quad \text{is the bud weight (measured in grams)}.
\end{align*}
\]

\[
\begin{align*}
\text{Concentration of carotenoid} & = 1000A_{470} - 2.05Ca - 114.8Cb \\
\text{Content of carotenoid} & = \frac{C \times V}{M}.
\end{align*}
\]

Determination of flavonoids in pigments was performed according to the following (Fu et al., 2013):

1. Seven stages of flowering buds were collected and placed in at –80°C, frozen for 24 h, then processed by a vacuum freeze-drying machine for 72 h, milled into powder, and finally placed in a dry, dark location until further analysis.
2. To prepare the methanol extract, 1.0 g of the freeze-dried powder was added to 10 mL methanol extraction solution. Ultrasonic extraction (20 Hz, 3 h) was carried out, followed by rotary evaporation with

Received for publication 13 Dec. 2018. Accepted for publication 24 Jan. 2019.
Special subsidies were provided from the Public Health Services of Traditional Chinese Medicine in 2017 [2017]66 and Henan Province Enterprise Technology Innovation Guidance Special Project (17210700031).
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10 mL methanol until a constant volume was obtained. Samples were stored at 4 °C.

3. A standard solution of rutin (2 mg mL\(^{-1}\)) was obtained by accurately weighing and dissolving 20 mg rutin (constant weight) in a 10-mL volumetric flask with ethanol to a constant concentration.

4. Standard samples of rutin with a concentration of 2 mg mL\(^{-1}\) were placed in a 10-mL centrifuge tube at volumes of 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mL. Then, 0.2 mL NaNO\(_2\) was added at a concentration of 5% and was shaken for 6 min, followed by 0.2 mL 10% Al(NO\(_3\))\(_3\), which was also and shaken and set for 6 min. Next, 1 mL 4% NaOH was added, shaken well, and set for 15 min at room temperature; and a 60% ethanol solution was added to a constant volume of 10 mL. The first solution was used as a blank, the absorbance value at a wavelength of 510 nm was measured, the absorbance (A) was used to regress the concentration (Y), and the standard curve was constructed.

5. Then, 0.1 mL methanol extract from each flower developmental stage was extracted to replace the standard product for analysis. This was repeated three times to obtain an average value.

6. The linear regression equation for the standard concentration of rutin Y (mg mL\(^{-1}\)) and absorbance X was obtained in step 3, and the flavonoid content in the flower buds of honey-suckle at different developmental stages was calculated according to the following regression equation:

\[
Y = \frac{10 \times X \times V}{M \times 0.1},
\]

where Y is the concentration of flavonoids in the sample solution (measured in milligrams per milliliter), M is the dry weight of the sample (measured in grams), V is the final volume (measured in milliliters) of the sample, 0.1 is the amount of extract (measured in milliliters) added at the time of determination, and 10 is the constant volume (measured in milliliters) of the extract at the time of determination.

**Determination of flower color.** The buds or flowers of the young stage, three green period, two white period, great white period, silver period, and golden period of two cultivars were collected at 8 am and stored at 4 °C. We used the RHSCC to determine the color of the corolla, and measurements were repeated five times. A colorimeter was used to determine L*, a*, and b* (Yang et al., 2015). L* represents brightness; a greater value of L* indicates a brighter intensity. The value of a* represents the difference between red and green. With a greater value of a*, the color red is darker; with a smaller value of a*, the color green is darker. b* is the difference between yellow and blue. With a greater value of b*, the color yellow is darker; with a smaller value of b*, the color blue is darker.

The outside corolla color was determined by measuring directly the upper half of the bud during the first four stages, then measuring the upper petals during the silver period and the golden period. The inside corolla color was determined by sticking the unfolded corollas of the first four stages onto slides with double-sided tape and measuring the upper petals with a colorimeter during the silver and golden periods.

The L* value increased first and then clearly, reaching its maximum value during the golden period. The L* value first increased and then decreased, reaching its maximum value during the silver period in two cultivars. From the young period to the golden period, the value showed an upward trend, which was consistent with the color fading in ‘Damaohua’. The a* value in ‘Yujin 2’ went through an up-down-up process, and the greatest value appeared during the three green period, when the color was deep red. The a* value of the outside of the corolla was greater in ‘Yujin 2’ than in ‘Damaohua’ in six stages. The color variations are shown in Figs. 1 and 2.

**Chlorophyll, carotenoid, and flavonoid content.** The chlorophyll content in the corolla of both cultivars showed a decreasing trend with different rates. The chlorophyll content declined rapidly from the young period to the two white period, and changed gradually from the two white period to the golden period (Fig. 3A). However, the content of carotenoids declined slightly from the young period to the silver period and rose sharply during the golden period (Fig. 3B).

The total flavonoid content was calculated by the linear regression equation of rutin, which was produced using the standard curve Y = 12.95X – 0.0287, \(R^2 = 0.999\). The flavonoid content of ‘Damaohua’ and ‘Yujin 2’ showed a decreasing trend, with values of 128.61 and 188.19 mg g\(^{-1}\) during the young period, and87.87 and 126.03 mg g\(^{-1}\) during the golden period.

**Discussion**

**Effect of pigment distribution on flower color.** Microscopy, spectrophotometry, colorimetry, and the RHSCC were used to compare and analyze pigment distribution, content, and color variations in the two L. japonica Thunb. cultivars at different developmental stage.
The color of plants is codetermined mainly by the different distributions of chlorophyll, carotenoid, flavonoid, and betaine, which exist in the cytoplasm or vacuoles of plants. These compounds present different colors by reflecting sunlight. Carotenoids affect primarily the formation of yellow, orange, and red; flavonoids affect the formation of red, pink, purple, and blue colors in plants (Lee and Gould, 2002; Williams and Grayer, 2004). The constituents of the pigments were always chlorophyll and carotenoids in the developmental process of the two cultivars, but variation existed in the flower color because the content of each pigment changed during the growth process. The chlorophyll content was greater in the young period to three green period than in the other periods (Fig. 3). Moreover, it was not pure white but off-white in the great white period (Fig. 1), because there was a small amount of carotenoids during this stage. From the silver to golden periods, the color changed from off-white to yellow. During this process, the content of flavonoids declines throughout the developmental stage of the flower. Therefore, flavonoids may play an auxiliary role in the yellow formation of golden flowers, although it is not the main reason why honeysuckle changes from white to golden flowers. The rapid increase in carotenoid content was the main reason for the change in flower color to yellow during the golden stage. The carotenoid content was $\approx$10 times that during the silver period, whereas the chlorophyll content declined to the lowest amount, and the ratio of carotenoids to chlorophyll peaked (Fig. 3C). The carotenoids and total flavonoids in the ‘Yujin 2’ flower were greater than those in the ‘Damaohua’ flower from the young bud stage to the golden flower stage, which was why the ‘Yujin 2’ flower was red. The content of carotenoids in the golden period was $\approx$10 times greater than that in the silver period, and the transition from the silver to the golden periods only took 2 to 3 d. The sharp

Table 1. Variations in flower color inside the corolla during the six developmental stages of ‘Damaohua’ and ‘Yujin 2’.

| Cultivar | Stage            | RHSCC | L*      | a*       | b*       |
|----------|------------------|-------|---------|----------|----------|
| Damaohua | Young period     | 144B green | $-21.33 \pm 0.38$ b | $-5.03 \pm 0.43$ c | $6.67 \pm 0.47$ c |
|          | Three green period | 144D green | $-17.55 \pm 0.47$ b | $-4.83 \pm 0.58$ c | $7.69 \pm 1.53$ bc |
|          | Two white period  | 145C green | $-13.48 \pm 0.96$ a | $-2.92 \pm 1.14$ d | $9.52 \pm 1.29$ b |
|          | Great white period | 155C white | $-9.63 \pm 0.53$ a | $0.11 \pm 0.24$ c | $4.4 \pm 1.55$ d |
|          | Silver period    | NN155C white | $-9.58 \pm 0.18$ a | $1.03 \pm 0.36$ b | $9.13 \pm 0.19$ b |
|          | Golden period    | 14D yellow | $-11.93 \pm 0.09$ a | $1.69 \pm 0.40$ a | $15.12 \pm 0.50$ a |
| Yujin 2  | Young period     | 145A green | $-19.68 \pm 0.21$ f | $-4.54 \pm 0.19$ d | $6.10 \pm 0.15$ c |
|          | Three green period | 145B green | $-18.98 \pm 0.46$ c | $-4.46 \pm 0.15$ d | $6.43 \pm 0.18$ c |
|          | Two white period  | 145D Green | $-16.19 \pm 0.11$ d | $-1.69 \pm 0.29$ c | $6.50 \pm 0.48$ c |
|          | Great white period | NN155D white | $-9.61 \pm 0.34$ b | $0.80 \pm 0.25$ b | $1.1 \pm 0.47$ d |
|          | Silver period    | NN155B white | $-8.76 \pm 0.29$ a | $0.85 \pm 0.04$ b | $8.9 \pm 0.49$ b |
|          | Golden period    | 8C yellow | $-11.99 \pm 0.71$ c | $2.15 \pm 0.40$ a | $16.01 \pm 0.66$ d |

The different lowercase letters after the same cultivar represent significant differences at $P = 0.05$ by Duncan’s test ($n = 3$). RHSCC = Royal Horticulture Society of Colorimetric Card; L* = lightness; a* = redness; b* = yellowness.

Fig. 2. The morphology of corolla cross-sections and glandularia during the six developmental stages in two Lonicera japonica cultivars. The morphology of corolla cross-sections during the six developmental stages of ‘Damaohua’: (A) Young period. (B) Three green period. (C) Two white period. (D) Great white period. (E) Silver period. (F) Golden period. The morphology of glandularia during the six developmental stages of ‘Damaohua’: (G) Young period. (H) Three green period. (I) Two white period. (J) Great white period. (K) Silver period. (L) Golden period. The morphology of glandularia during the six developmental stages of ‘Yujin 2’: (g) Young period. (h) Three green period. (i) Two white period. (j) Great white period. (k) Silver period. (l) Golden period.
increase in carotenoid content over a short period of time was the main reason for the color change from white to yellow.

The corolla is purple in ‘Yujin 2’ because of abundant anthocyanins (Yu, 2013), which have very important physiologic roles, such as resisting oxidation, reducing blood fat, preventing tumors, preventing allergies, protecting gastric mucosa, and lowering blood sugar levels (Lv, 2001). Anthocyanins from the two L. japonica Thunb. cultivars, as natural edible pigments and functional food ingredients, have broad application prospects. Luteolin is a flavonoid that is highly presented at a higher level than that of ‘Yujin 2’ that have a high ornamental value and may play a role in the function and molecular mechanisms of pigments.

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**Table 2. Variations in flower color outside the corolla during the six developmental stages of ‘Damaohua’ and ‘Yujin 2’**

| Cultivar | Stage          | RHSCC | L*    | a*    | b*    |
|----------|----------------|-------|-------|-------|-------|
| Damaohua | Young period   | 144C  | -2.52 ± 0.48 e | -6.32 ± 0.31 c | 6.89 ± 1.24 c |
|          | Three green period | 145A | -18.48 ± 0.34 d | -4.50 ± 0.18 d | 12.16 ± 0.26 b |
|          | Two white period | 150C | -11.93 ± 0.38 c | -3.72 ± 0.51 c | 12.55 ± 0.02 b |
|          | Great white period | 155B | -10.41 ± 0.05 b | -1.19 ± 0.06 b | 8.49 ± 0.77 c |
|          | Silver period   | 155A | -6.78 ± 1.7 a   | 1.41 ± 0.14 a  | 9.09 ± 0.08 c |
|          | Golden period   | 11A  | -12.07 ± 0.31 c | 3.68 ± 0.32 a  | 14.05 ± 0.74 a |
| Yujin 2  | Young period   | 71B  | -23.18 ± 1.02 e | 1.01 ± 0.54 b  | 4.18 ± 1.38 c |
|          | Three green period | 72C  | -21.87 ± 0.88 d | 5.36 ± 5.57 c  | 7.46 ± 0.55 b |
|          | Two white period | 72D  | -19.12 ± 0.34 c | 4.03 ± 0.46 a  | 4.33 ± 0.31 c |
|          | Great white period | 73B | -12.23 ± 0.76 b | 3.10 ± 0.74 a  | 0.58 ± 0.19 d |
|          | Silver period   | 73C  | -10.79 ± 0.49 a | 2.07 ± 0.12 ab | 5.57 ± 0.66 c |
|          | Golden period   | 10B  | -13.29 ± 0.08 b | 3.83 ± 0.66 a  | 11.91 ± 1.37 a |

The different lowercase letters after the same cultivar represent significant differences at P = 0.05 by Duncan’s test (n = 3). RHSCC = Royal Horticulture Society of Colorimetric Card; L* = lightness; a* = redness; b* = yellowness.

**Fig. 3.** (A) Chlorophyll content in flowers or buds, (B) carotenoid content, and (C) total flavonoid content in ‘Damaohua’ and ‘Yujin 2’. The x-axis represents the six developmental stages of flowers; 1, young period; 2, three green period; 3, two white period; 4, great white period; 5, silver period; 6, golden period.

**Conclusion**

There were significant differences in corolla color, cross-section color, and the content of the three pigments between the two varieties of honeysuckle. Distribution and variation of pigments were the key factors affecting the flower color of honeysuckle. Our study provided a basis for the identification and breeding of honeysuckle varieties, and laid a foundation for further study on the function and molecular mechanisms of pigments.