The Biochemical Basis for Flower Color in *Calibrachoa*

Rebecca A. Waterworth¹ and Robert J. Griesbach²

Floral and Nursery Plant Research, U.S. National Arboretum, U.S. Department of Agriculture, Agricultural Research Service, BARC-West, BLDG. 010-A, Beltsville, MD 20705-2350

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Abstract. Recently, several new *Calibrachoa* La Llave & Lexarza (Solanaceae Juss.) cultivars have been developed with novel red and blue flowers. Most wild species of *Calibrachoa* have purple flowers. The differences in color were not due to anthocyanin composition, but rather to vacuolar pH. The pH of the red-flowered cultivar was 4.8 while that of the blue-flowered cultivar was 5.6. The wild purple-flowered species had an intermediate pH of 5.0. These data suggest that different pH and pigment genes may be introgressed into other *Calibrachoa* species to increase cultivar diversity.

Calibrachoa La Llave & Lexarza (Solanaceae Juss.) is becoming an important new landscape plant, the first cultivars having been released in the early 1990s (Poehlman, 1997). This genus is very closely related to the well-known genus *Petunia* Jussieu. Historically, *Calibrachoa* species were included in *Petunia* because of their morphological similarity. Wijsman and Jong (1985) first recognized that there were two distinct groups of species based upon chromosome number. Therefore, they transferred 15 *Petunia* species into the genus *Calibrachoa* (Wijsman, 1990). Recently, an additional seven species have been added (Stehman and Semir, 1997). *Calibrachoa* species are characterized by a $2n=18$ chromosome number, woody stems, conduplicate aestivation with two petals covering the other three, and seedcoat cells with straight anticlinal walls. *Petunia* species are characterized by a $2n=14$ chromosome number, nonwoody stems, imbricate aestivation, and seedcoat cells with wavy anticlinal walls (Stehman and Semir, 1997).

Most wild species of *Calibrachoa* have purple flowers. Recently, several new cultivars have been developed with novel flower colors. The objective of this study was to determine the biochemical basis of these novel blue and red flower colors.

Materials and Methods

Plant material. Plants of hybrid *Calibrachoa* 'Million Bells Trailing Blue' and 'Million Bells Cherry' (Suntory, Tokyo) were obtained from a local retail nursery. Plants of the wild species *C. sellowiana* 'Rose' (Sendtr.)

Wijsman were obtained from K.C. Sink, Michigan State Univ., under the name *P. alpicola* Smith & Downs. A seedling population was created from the cross of 'Million Bells Trailing Blue' and 'Million Bells Cherry'. All plants were maintained in a greenhouse under standard cultural practices for *Petunia* (Dole and Wilkins, 1999). The daylength was 16 h at 25 °C day/20 °C night. Supplemental HID lighting with incandescent light was used to extend the daylength. The maximum light intensity was 1050 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The Royal Horticultural Society (RHS) Colour Charts (RHS, 1986) were used to denote flower color using only the first chip as suggested by Voss and Hale (1998).

Flavonoid analysis. Anthocyanins and flavonols were isolated and analyzed by high resolution, high performance liquid chromatography (HPLC) as described by Griesbach et al. (1991). Individual anthocyanins were purified by preparatory HPLC and then hydrolyzed (Griesbach et al., 1991). The hydrolyzation products were characterized by diagnostic visible and ultraviolet spectrophotometry and by thin layer chromatography (TLC) with known standards. Individual anthocyanins are reported as the percentage of total anthocyanin present. Each value is the mean of three single-flower replicates from the same plant harvested on different days. The Student's *t* test was used to compare the means.

pH Determination. The pH of petal cell sap was determined 1 day after anthesis. Griesbach (1998) reported that the pH of the whole petal tissue was the same as the pH of just the upper epidermis. Therefore, the whole tissue was homogenized and the pH measured with a Sentron 1001 pH meter (Sentron, Federal Way, Wash.). The pH measurements are reported as the mean of 10 replicates with each replicate representing a different flower. The Student's *t* test was used to compare the means.

Anthocyanin-flavonol complex. An anthocyanin-flavonol complex was created as previously described by Asen et al. (1986). The absorption of this complex was measured at pH 5.6 and 4.8, which were based upon the pH of the cultivars.

Results and Discussion

The HPLC profiles of the flavonols were the same for 'Rose', 'Million Bells Trailing Blue' and 'Million Bells Cherry' (data not shown). Upon hydrolysis, a single flavonol (kaempferol) was identified. Based upon the anthocyanin and flavonol composition of the cultivars, the anthocyanin to flavonol ratio was 1:1.7.

Both malvidin and petunidin were found in 'Rose', 'Million Bells Cherry' and 'Million Bells Trailing Blue' (Table 1). 'Million Bells Cherry' had a higher concentration ($t=30.575$, $P\leq 0.001$) of petunidin than malvidin, whereas the reverse was true for 'Rose' and 'Million Bells Trailing Blue'. Four anthocyanins were detected in these cultivars—petunidin-3-caffeoylrutinoside-5-glucoside (Pt-ca), petunidin-3-coumarylrutinoside-5-glucoside (Pt-co), malvidin-3-caffeoylrutinoside-5-glucoside (Mv-ca), and malvidin-3-coumarylrutinoside-5-glucoside (Mv-co). In 'Million Bells Trailing Blue' and 'Rose', Mv-ca was the major anthocyanin and comprised nearly 70% of the total anthocyanin content. However, three anthocyanins were present in about equal concentrations in 'Million Bells Cherry'.

These results might lead one to conclude that the anthocyanin composition was responsible for the differences in flower color among 'Rose' (RHS 81A), 'Million Bells Trailing Blue' (RHS 88A), and 'Million Bells Cherry' (RHS 67A). However, this is incorrect (the differences in anthocyanin composition cannot explain the variation in flower color), because the acylated 3-rutinoside-5-glucosides

Table 1. The color, petal pH, and anthocyanin composition of *Calibrachoa sellowiana* 'Rose' (Rose) and *C. hybrids* 'Million Bells Trailing Blue' (Blue), 'Million Bells Cherry' (Cherry), and the hybrid between 'Million Bells Trailing Blue' and 'Million Bells Cherry' (Hybrid). Abbreviations: Pt-ca = petunidin-3-caffeoylrutinoside-5-glucoside; Pt-co = petunidin-3-coumarylrutinoside-5-glucoside; Mv-ca = malvidin-3-caffeoylrutinoside-5-glucoside; and Mv-co = malvidin-3-coumarylrutinoside-5-glucoside.

Plant	Color ^a	pH	Pt-ca	Pt-co	Mv-ca	Mv-co
Rose	81A	5.0 (0.1) ^b	8 (2)	9 (1)	67 (9)	16 (5)
Blue	88A	5.6 (0.3)	8 (2)	20 (1)	68 (1)	7 (2)
Cherry	67A	4.8 (0.1)	34 (1)	30 (1)	24 (1)	12 (1)
Hybrid	74A	5.3 (0.2)	14 (2)	31 (1)	23 (1)	32 (1)

^aRHS Colour Chart (Royal Horticultural Society, 1986)

^bMean (standard deviation)

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¹Student Intern.

²Research Geneticist. To whom requests for reprints should be addressed.

Fig 1. Absorption profile of mixtures of 1 part anthocyanin : 1.7 parts kaempferol-3-sophoroside. Absorption is in arbitrary units. A, petunidin-3-caffeoylrutinoside-5-glucoside at pH 4.8; B, malvidin-3-caffeoylrutinoside-5-glucoside at pH 4.8; and C, malvidin-3-caffeoylrutinoside-5-glucoside at pH 5.6.

of petunidin and malvidin have nearly the same visible absorption spectra. The absorption profile at pH 4.8 of a mixture of 1 part Mv-ca : 1.7 parts kaempferol-3-sophoroside (Km-so) was the same as a mixture of 1 part Pt-ca : 1.7 parts Km-so (Fig. 1). Although the $\lambda_{\max}$ was different for the two solutions, they had the same visible color, for the percentage of absorption over 555 nm was nearly the same.

The pH of the vacuole can effect the color by modifying the physical interaction between the electrons in the pigments (Brouillard, 1988). 'Rose', 'Million Bells Cherry', and 'Million Bells Trailing Blue' differed in petal pH (Table 1). These values being 5.0, 4.8, and 5.6, respectively. The difference in all comparisons was statistically significant ($t = 55.00$, $P \leq 0.001$). The pH can influence the color of an anthocyanin-flavonol complex (Griesbach, 1996 and 1998; Stewart et. al., 1975). The absorption spectra of a mixture of 1 part Mv-ca and 1.7 parts Km-so was measured at 4.8 and 5.6 (Fig. 1). At pH 5.6, a peak of absorption at ≈ 550 nm occurred that was not present at the lower pH. In addition, the percentage of absorption over 550 nm was greater at the higher pH. These data sug-

gest that the pH, and not the anthocyanin composition, was responsible for the differences in color among the three cultivars.

Preliminary experiments suggested that both the anthocyanin composition and pH were co-dominantly inherited. The 'Million Bells Trailing Blue' x 'Million Bells Cherry' hybrid had a flower color, petal pH, and anthocyanin composition that were intermediate between its parents (Table 1). These data suggest that introgressing different pH and pigment genes into the other *Calibrachoa* species would increase cultivar diversity.

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