# Identification of Anthocyanins and Phenolic Acid in the Flowers of Three Lungwort (*Pulmonaria*) Cultivars and Their Comparisons during Flower Developmental Stage

**Takayuki Mizuno[1, *], Yusuke Akita[2], Ayumi Uehara[3] and Tsukasa Iwashina[1]**

[1] Department of Botany, National Museum of Nature and Science,
4–1–1 Amakubo Tsukuba, Ibaraki 305–0005, Japan
[2] Department of Life Science and Green Chemistry, Graduate School of Engineering, Saitama Institute of
Technology, 1690 Fusaiji, Fukaya, Saitama 369–0293, Japan
[3] Department of Agri-Environmental Sciences, College of Agriculture, Tamagawa University,
6–1–1 Tamagawa-gakuen, Machida, Tokyo 194–0041, Japan
*E-mail address: tmizuno@kahaku.go.jp

**Abstract** Three anthocyanins, delphinidin 3-*O*-glucoside, petunidin 3,5-di-*O*-glucoside, and petunidin 3-*O*-glucoside, and rosmarinic acid were isolated and identified from the flowers of three lungwort (*Pulmonaria*) cultivars. Composition of these compounds were quantitatively compared during two developmental stages, i.e. violet color in early flowering stage and blue color in later flowering stage. In addition, pH values of petal juices and flower color value were measured in both stages. Qualitative differences among three cultivars were observed in anthocyanins. However, qualitative and quantitative changes during development was not observed. Increase of rosmarinic acid was observed in one cultivar during development of the flowers. Notable alkalization was not revealed in vacuoles. These results mean that the flower bluing in these lungwort cultivars is not mediated by intramolecular copigmentation and alkalization of vacuole. Furthermore, we performed *in vitro* examination using the isolated anthocyanin, delphinidin 3-*O*-glucoside or petunidin 3-*O*-glucoside, and rosmarinic acid. As the results, it was shown that the absorption curves of the mixture solutions and intact petal did not match. Thus, it was suggested that the petal bluing after flowering may be due to intermolecular interaction which was influenced by metal ions.

**Keywords :** delphinidin, floral color change, petunidin, rosmarinic acid.

## Introduction

Lungwort (*Pulmonaria*) is a rhizomatous perennial in the Boraginaceae and was known as an ornamental plant in the shaded garden. The funnel-shaped flowers are bloomed in late winter to spring, and a various patterned leaf is developed. Flower corolla has been known as to change from violet to blue throughout flowering time, and it has been suggested to function as signals of the directing pollinators by some eco-logical investigation (Müller, 1883; Oberrath *et al.*, 1995; Oberrath and Böhning-Gaese, 1999).

The blue color expression in some flowers has been reviewed (Honda and Saito, 2002; Yoshida *et al.*, 2009; Iwashina, 2015). The well-known bluing factors are intermolecular interaction between anthocyanin and other compounds or/ and metal ions, the poly-acylation of the antho-cyanin, and the alkalization of the petal vacuole. In the case of the floral color change of *Lathyrus hirsutus* L., the standard is bluish red, and it fades from blue to green blue, and the pale blue

wing petals are fade to green blue during senescence (Pecket, 1966). The occurrence of the color change has been suggested that the increase of the cell sap pH, and copigmentation by decrease of the amount of anthocyanin (Pecket, 1966). Petal color change from red to blue in the morning glory (*Ipomoea tricolor*) 'Heavenly Blue' during flower opening is induced by increase of cell sap pH (Asen *et al.*, 1977; Yoshida *et al.*, 2005). Although the floral color change is an ecologically important trait in lungwort, the physiological mechanism has not been investigated.

In the present study, we isolated and identified three anthocyanins and one phenolic acid from the corollas of lungwort cultivars. Moreover, their color values, absorption spectra, and pH values were compared among three cultivars. Furthermore, to understand the mechanism of color change in the lungwort flowers, we performed *in vitro* reconstruction using authentic compounds.

## Materials and Methods

### Plant Materials

Lungwort (*Pulmonaria*) cultivars, 'Blue Ensign' (fresh flowers, 18.3 g), 'Bertram Anderson' (known as 'E.B. Anderson', 61.0 g) and 'Diana Clare' (22.0 g) were cultivated in Tsukuba Botanical Garden, National Museum of Nature and Science, Ibaraki Pref., Japan, and their corollas were collected (Fig. 1A–C). We divided the development of these flowers into 3 stages. Flower color change of the lungwort cultivars was taking place in approximately three to four days after flowering. It has been reported that color change occurs with the proceeding the age of the flower and does not be triggered by pollinators (Oberrath *et al.*, 1995). We defined as stage 1: the corollas become open, stage 2: the tip side of corolla has reflexed outwards (next day of stage 1), and stage 3: the petals fully opened (after one or two days of stage 2) (Fig. 1D). It was observed that these corollas are ini-
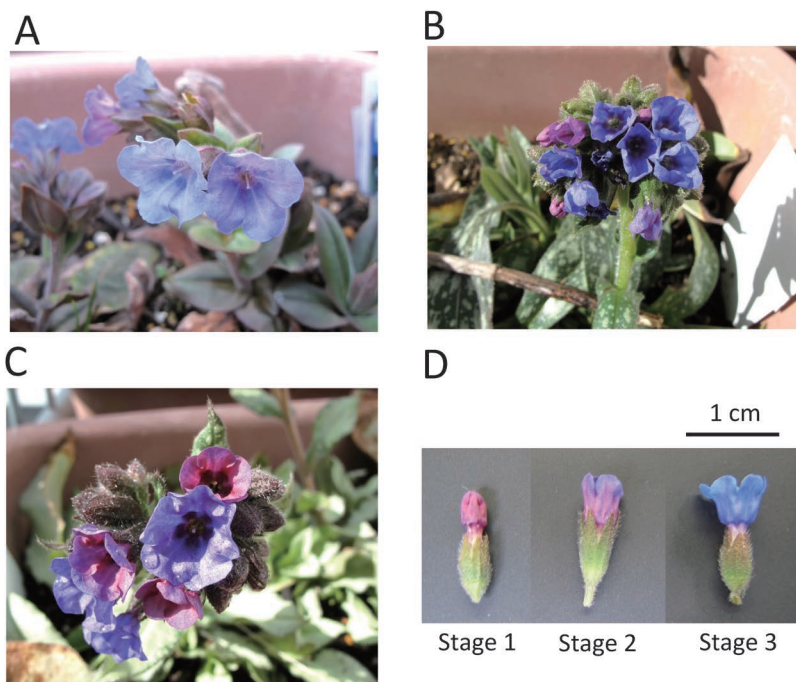


Fig. 1.   The flower of lungwort cultivars 'Blue Ensign'(A), 'Bertram Anderson' (B) and 'Diana Clare'(C). Flower development of 'Bertram Anderson' (D). Stage 1: The petals become open. Stage 2: the tip side of petals has reflexed outwards (next day of stage 1). Stage 3: The petals fully opened (after one or two days of stage 2).

tially purple violet (stages 1 and 2) and turn to blue (stage 3) according to flower development. The violet and blue corollas were collected in the winter to spring season in 2014 and 2015, and were stored at $-20°C$ in the refrigerator.

*Extraction and isolation*

Anthocyanins and phenolic acid were extracted from the 'Diana Clare' flowers with MeOH containing 8% HCOOH. The extracts were evaporated and applied to Amberlite XAD-7 column (I.D. $2.5 \times 18$ cm). The extract was eluted with MeOH containing 5% HOAc after washed with 5% aq.HOAc. The eluted fractions were applied to preparative paper chromatography using BAW (*n*-BuOH/HOAc/ $H_2O = 4:1:5$, upper phase) and a Sephadex LH-20 column chromatography using MeOH/ $H_2O$/HOAc (70:25:5). Finally, anthocyanins and phenolic acid were separated by preparative HPLC, which was performed with Tosoh HPLC systems using an Inertsil ODS-4 (I.D. $10 \times 250$ mm, GL Sciences, Japan), at a flow rate of 3.0–3.5 mL min$^{-1}$, injection of 300–350 $\mu$L, detection wavelength at 530 nm for anthocyanins and 340 nm for phenolic acid, using solvent system, FMW (HCOOH/MeCN/$H_2O = 5:10:85$).

*Identification of anthocyanins and phenolic acid*

Three anthocyanins (**A1–3**) and one phenolic acid (**O1**) were identified by LC-MS, and comparisons with authentic samples using HPLC and TLC (BAW, BuHCl (n-BuOH/2N HCl = 1:1, upper phase), 1% HCl and AHW (HOAc/HCl/ $H_2O = 15:3:82$)).

LC-MS (Shimadzu, Japan) was performed using an Inertsil ODS-4 column (I.D. $2.1 \times 100$ mm, GL Sciences, Japan) for **A1–3**, and *L*-column2 ODS (I.D. $2.1 \times 100$ mm, Chemicals Evaluation and Research Institute, Japan) for **O1**, at a flow rate of 0.2 mL min$^{-1}$, detection wavelength at 530 and 525 nm for **A1–3** and 340 and 280 nm for **O1**, and eluents were FMW for anthocyanins, and FMW2 (HCOOH/MeCN/ $H_2O = 5:22:73$), ESI$^+$ 4.5 kV, ESI$^-$ 3.5 kV, 250°C.

Authentic samples of delphinidin 3-*O*-glucoside, and petunidin 3-*O*-glucoside were purchased from the Tokiwa Phytochemical, Japan) and rosmarinic acid was purchased from the Cayman Chemical Company (USA). Petunidin 3,5-di-*O*-glucoside was synthesized from petunidin 3-*O*-glucoside using an enzyme isolated from *Cyclamen purpurascens* (Cpur5GT, Kang *et al.*, 2021). TLC, HPLC, UV, and LC-MS data of the isolated compounds (**A1–3**, **O1**) were as follows.

Delphinidin 3-*O*-glucoside (**A1**, myrtillin, Fig. 2). TLC: Rf 0.09 (BAW), 0.03 (BuHCl), 0.03 (1%HCl), 0.19 (AHW); UV-Vis: $\lambda$max (nm) 0.01% HCl-MeOH 277, 541; $E_{440}/E_{max}$ 16%; + AlCl$_3$ bathochromic shift; LC-MS: *m/z* 465 [M]$^+$, 303 [M-162]$^+$; HPLC: *t*R (min) 8.3 (mobile phase A).

Petunidin 3,5-di-*O*-glucoside (**A2**, petunin, Fig. 3). TLC: Rf 0.12 (BAW), 0.06 (BuHCl), 0.01 (1%HCl), 0.07 (AHW); UV-Vis: $\lambda$max (nm) 0.01% HCl-MeOH 276, 540; $E_{440}/E_{max}$ 11%; + AlCl$_3$ bathochromic shift; LC-MS: *m/z* 641 [M]$^+$; HPLC: *t*R (min) 9.0 (mobile phase A).
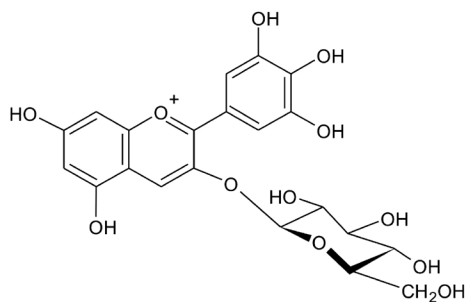


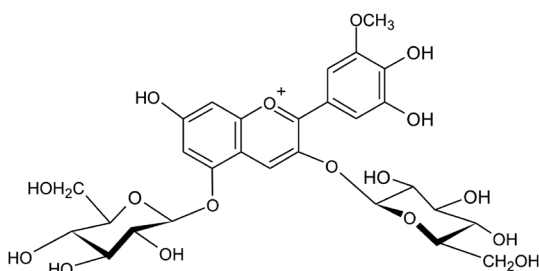Fig. 2.    delphinidin 3-*O*-glucoside (**A1**).

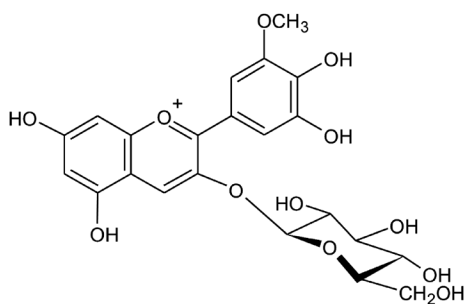

Fig. 3.    Petunidin 3,5-di-*O*-glucoside (**A2**).

Fig. 4.   Petunidin 3-*O*-glucoside (**A3**).
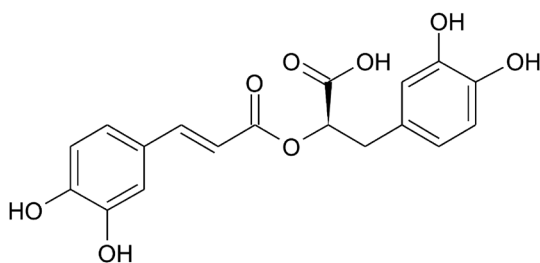


Fig. 5.   Rosmarinic acid (**O1**).

Petunidin 3-*O*-glucoside (**A3**, Fig. 4). TLC: Rf 0.16 (BAW), 0.06 (BuHCl), 0.01 (1%HCl), 0.10 (AHW); UV-Vis: $\lambda$max (nm) 0.01% HCl-MeOH 278, 540; $E_{440}/E_{max}$ 17%; +AlCl$_3$ bathochromic shift; LC-MS: *m/z* 479 [M]$^+$, 317 [M-162]$^+$; HPLC: *t*R (min) 15.8 (mobile phase A).

Rosmarinic acid (**O1**, Fig. 5). UV $\lambda$max (nm) MeOH 292, 331; LC-MS: *m/z* 359 [M–H]$^-$, HPLC: *t*R (min) 23.6 (mobile phase B).

### Color measurement

The color values of purple violet (stage 2) and blue (stage 3) corollas were measured by the Royal Horticultural Society Color Chart (RHSCC) and Color Reader (CR-10, Konica Minolta Sensing, Japan). The colorimetric values were calculated as Commission Inter-national d'Eclairage (CIE) $L^*a^*b^*$ color space, which consists of lightness ($L^*$), and two chromatic compositions, redness ($a^*$) and yellowness ($b^*$). The values of chroma ($C$) and hue angle ($h$) were also calculated based on the equations: $C = (a^{*2} + b^{*2})^{1/2}$ and $h = \tan^{-1} (b^*/a^*)$ (Gonnet, 1998; Mizuno *et al.*, 2015). The measurement

was performed in triplicate.

### Quantitative analyses

The extracted solutions (1 mL per fresh corolla) with FMW3 (HCOOH/MeCN/H$_2$O = 8 : 20 : 72) were prepared by soaking overnight and used for estimation of their concentration. It was performed on Shimadzu HPLC systems using Inertsil ODS-4 column (I.D. 6.0 × 150 mm, GL Sciences, Japan) for anthocyanins and *L*-column2 ODS (I.D. 6.0 × 150 mm, Chemicals Evaluation and Research Institute) for rosmarinic acid, at 40°C. Eluents were mobile phase A (MeCN/HOAc/H$_2$O/H$_3$PO$_4$ = 6 : 8 : 83 : 3) for anthocyanins and mobile phase B (MeCN/H$_2$O/H$_3$PO$_4$ = 18 : 82 : 0.2) for rosmarinic acid at a flow rate of 1.0 mL min$^{-1}$. Concentration was calculated as the total peak area (mAU) at 530 nm (anthocyanins) and the main peak area of 330 nm (rosmarinic acid) using LC solution software (Shimadzu, Japan).

### pH measurement

The pH of pressed juices from the corollas were immediately measured using a twin pH meter AS-212 (Horiba Ltd., Japan). The measurements were performed in triplicate.

### Absorption spectra of fresh flowers

The violet (stage 2) and blue (stage 3) corollas were used for the measurement of visible absorption spectra (400–700 nm) with UV-2600 spectrophotometer (Shimadzu, Japan) which attached integrated sphere ISR-2600 (Shimadzu, Japan).

### In vitro examination

Authentic anthocyanins, delphinidin 3-*O*-glucoside (**A1**), Petunidin 3-*O*-glucoside (**A3**), and rosmarinic acid (**O1**) were used for flower color reconstruction. The 1 mM anthocyanin (**A1** or **A3**) and mixture of 1 mM anthocyanin and 1 mM or 2 mM **O1** were prepared as an acetate buffer solution (pH 5.0). The visible spectra of the mixture were recorded on a UV-2600 spectrophotometer (Shimadzu, Japan) using Nano Stick-S (Scinco, Korea).

*Statistical analysis*

The mean value and standard errors (SE) were revealed in the color values and quantity of anthocyanins and phenolic acid. An *t*-test was used to compare the mean values between two developmental stages 2 and 3.

## Results and Discussion

Three anthocyanins (**A1–3**) and one phenolic acid (**O1**) are isolated from the flowers of lungwort cultivar 'Diana Clare' as principal pigments. Three anthocyanins were revealed the bathochromic shift of absorption maxima on visible-UV spectra by addition of $AlCl_3$, meaning that the compounds have adjacent hydroxyl group in B-ring. LC-MS survey of **A1** showed the protonated ion peaks at *m/z* 465 $[M]^+$, and fragment ion peak at *m/z* 303 $[M-162]^+$, meaning the attachment of 1 mol hexose to delphinidin. Compound **A2** showed the protonated ion peaks at *m/z* 641 $[M]^+$. On the other hand, **A3** showed the protonated ion peaks at *m/z* 479 $[M]^+$. Thus, it was shown that **A2** and **A3** are petunidin which attached 2 and 1 mol hexoses, respectively. Finally, they were identified as delphinidin 3-*O*-glucoside (**A1**), petunidin 3,5-di-*O*-glucoside (**A2**) and petunidin 3-*O*-glucoside (**A3**) by HPLC and TLC comparisons with authentic

samples.

The main phenolic acid (**O1**) was identified as rosmarinic acid by UV spectra, LC-MS, and HPLC and TLC comparison with authentic sample. Rosmarinic acid is commonly found in the Boraginaceae and the subfamily Nepetoideae of the Lamiaceae (Petersen and Simmonds, 2003). Furthermore, it has been reported that rosmarinic acid is distributed not only in the leaves but also in the flowers such as *Rosmarinus officinalis* L. (del Bañño *et al.*, 2003).

Color values in lungwort cultivar flowers were revealed in Table 1. The value of RHSCC showed that stage 2 is "Purple Violet" to "Violet" and stage 3 is "Violet Blue" to "Blue". The significant differences between stages 2 and 3 were revealed in the $CIEL^*a^*b^*$ values, and $a^*$ and $b^*$ values of 'Blue Ensign', the $L^*$ and $b^*$ values of 'Bertram Anderson' and the $a^*$ and $b^*$ values of 'Diana Clare' (Table 1). The highest chroma (*C*) value, 46.4, and the lowest hue angle (*h*), 287° was shown in stage 3 of 'Blue Ensign'. It means that the full bloomed 'Blue Ensign' is the most vivid and blue. On the other hand, the lowest *C* value was shown in stage 2 of 'Bertram Anderson', and the highest *h* was shown in stage 2 of 'Blue Ensign'. The degree of floral color changes ($\Delta h$) from stage 2 to stage 3 were calculated as 45.8 in 'Blue Ensign', 11 in

Table 1. Chromaticity and their comparisons with two different development stages from the corolla of three lungwort cultivars

| Cultivars | Stage | RHSCC[x] | Chromaticity | | | | |
|---|---|---|---|---|---|---|---|
| | | | $L^*$ | $a^*$ | $b^*$ | *C* | *h* (°) |
| 'Blue Ensign' | 2 | Purple Violet N81A | $41.0 \pm 1.8$ | $32.0 \pm 1.5$ | $-16.2 \pm 3.5$ | 35.9 | 333 |
| | 3 | Blue 100A | $42.7 \pm 0.4$ | $13.8 \pm 1.5$ | $-44.3 \pm 1.8$ | 46.4 | 287 |
| | | *t*-test | NS | *** | ** | — | — |
| 'Bertram Anderson' | 2 | PurpleViolet N80 | $29.0 \pm 0.5$ | $13.7 \pm 2.4$ | $-23.2 \pm 3.0$ | 26.9 | 301 |
| | 3 | Violet Blue 96A | $38.5 \pm 1.5$ | $13.8 \pm 0.8$ | $-38.8 \pm 1.9$ | 41.2 | 290 |
| | | *t*-test | * | NS | * | — | — |
| 'Diana Clare' | 2 | Violet 83C | $37.5 \pm 0.7$ | $23.0 \pm 2.2$ | $-18.8 \pm 1.3$ | 29.7 | 321 |
| | 3 | Violet Blue 96C | $36.5 \pm 1.9$ | $15.5 \pm 0.8$ | $-37.8 \pm 1.6$ | 40.9 | 292 |
| | | *t*-test | NS | * | * | — | — |

[x] RHSCC = Royal Horticultural Society Color Chart.

The measurment of chromaticity value was performed in triplicate.

The $L^*a^*b^*$ values were showed as average $\pm$ SE.

The result of *t*-test was revealed as NS = No significance, * = 5%, ** = 1% and *** = 0.1%

Table 2.　HPLC analysis of the development stages from the corollas of three lungwort cultivars

| Cultivars | Stage | Detected at 530 nm[*] (Rt, min) | | | | | Detected at 330 nm[**] (Rt, min) |
|---|---|---|---|---|---|---|---|
| | | 5.0 | 5.4 | 8.3 (**A1**) | 9.0 (**A2**) | 15.8 (**A3**) | 23.6 (**O1**) |
| 'Blue Ensign' | 2 | N.D. | + | t | + + + | N.D. | + + + |
| | 3 | N.D. | + | t | + + + | t | + + + |
| 'Bertram Anderson' | 2 | + | + | t | + + + | N.D. | + + + |
| | 3 | + | + | t | + + + | t | + + + |
| 'Diana Clare' | 2 | t | t | + + | + | + + | + + |
| | 3 | t | t | + + | + | + + | + + |

HPLC analysis were performed in triplicate.

**A1**＝delphinidin 3-*O*-glucoside, **A2**＝petunidin 3,5-di-*O*-glucoside, **A3**＝petunidin 3-*O*-glucoside, **O1**＝rosmarinic acid

\* Mobile phase A (MeCN/HOAc/$H_2O$/$H_3PO_4$＝6:8:83:3), \*\* Mobile phase B (MeCN/$H_2O$/$H_3PO_4$＝18:82:0.2).

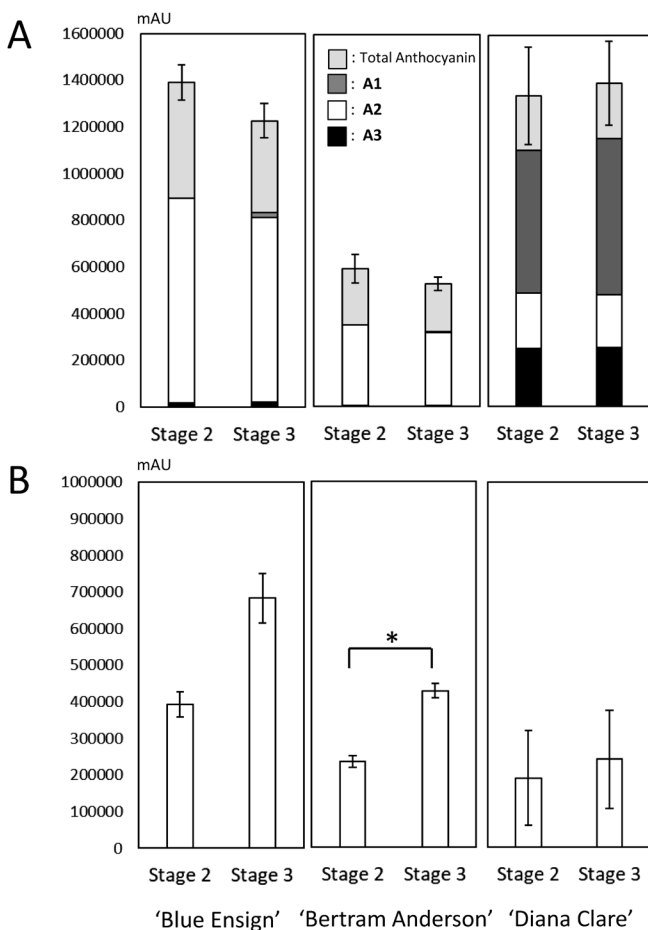t＜10%, ＋ ＜20%, ＋ ＋ ＜50%, ＋ ＋ ＋ ＞ 50%, N.D.＝no detected.



Fig. 6.　Quantitative HPLC analysis of anthocyanins (A) and rosmarinic acid (B) from the corollas of three lung-wort cultivars. **A1**＝delphinidin 3-*O*-glucoside, **A2**＝petunidin 3,5-di-*O*-glucoside, **A3**＝petunidin 3-*O*-glucoside. The extracts (1 mL per corolla) were prepared and analyzed triplicate. \*Significantly difference in *t*-test (5%). Error bars are standard errors for total anthocyanins (A) and rosmarinic acid (B).
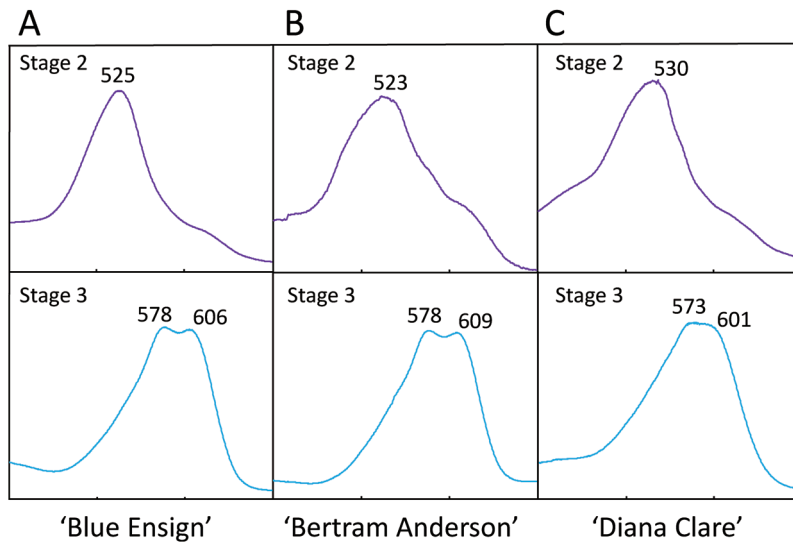
Fig. 7. Absorption spectral curves from the violet (stage 2, upper) and blue (stage 3, lower) corollas of three lungwort cultivars. A = 'Blue Ensign', B = 'Bertram Anderson', C = 'Diana Clare.'



| Sample | λ max |
|---|---|
| 1 mM **A1** | 527 |
| 1 mM **A1** + 1 mM **O1** | 528 |
| 1 mM **A1** + 2 mM **O1** | 528 |

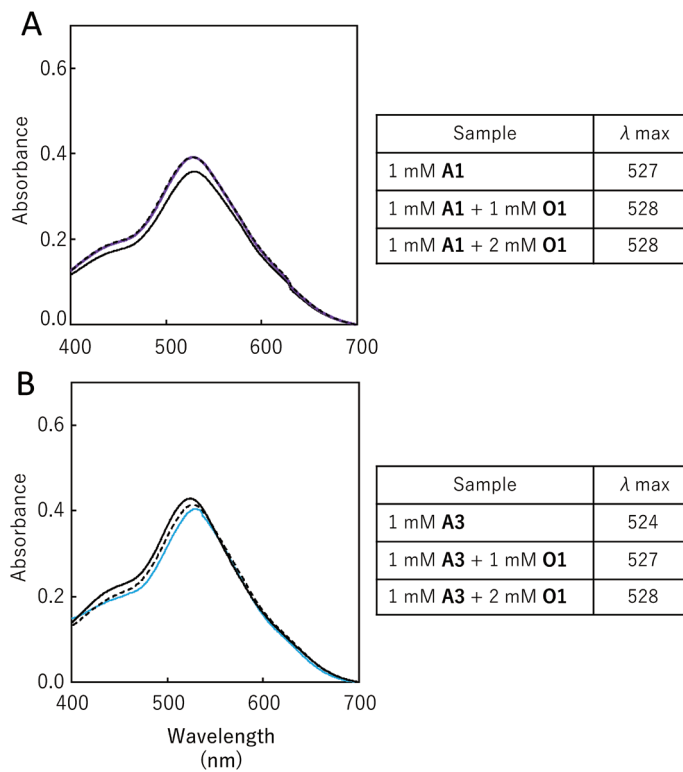| Sample | λ max |
|---|---|
| 1 mM **A3** | 524 |
| 1 mM **A3** + 1 mM **O1** | 527 |
| 1 mM **A3** + 2 mM **O1** | 528 |

Fig. 8. *In vitro* examination with delphinidin 3-*O*-glucoside (**A1**) or petunidin 3-*O*-glucoside (**A3**) and rosmarinic acid (**O1**) (pH 5.0). Absorption spectral curves of 1 mM **A1** and mixture of 1 mM **A1** and **O1**, and 1 mM **A1** and 2 mM **O1** were revealed as black solid line, black dashed line and purple solid line, respectively (A). Absorption spectral curves of 1 mM **A3** and mixture of 1 mM **A3** and **O1**, and 1 mM **A3** and 2 mM **O1** were revealed as black solid line, black dashed line and blue solid line, respectively (B).

'Bertram Anderson', and 28.5 in 'Diana Clare'. It means that the color change of 'Blue Ensign' is the largest (Table 1).

Anthocyanin and organic acid composition in violet (stage 2) and blue corolla (stage 3) were shown in Table 2 and Fig. 6. In 'Blue Ensign' and 'Bertram Anderson', the largest peak was **A2**. On the other hand, **A1** and **A3** were revealed as major anthocyanins in 'Diana Clare'. It was revealed that rosmarinic acid (**O1**) is the largest peak in each cultivar, and its relative amount is 62.8% to 80.5% in 'Blue Ensign' and 'Bertram Anderson', and 42.7% to 49.1% in 'Diana Clare'. Significant differences were not recognized between developmental stages. However, **A3** occurred as a minor peak in stage 3 of 'Blue Ensign' and 'Bertram Anderson'. We performed the quantitative HPLC comparison of the total anthocyanins and rosmarinic acid (Fig. 6). The amount of **O1** increased in 'Bertram Anderson' during stage 2 to 3, but the other two cultivars did not show the significant difference.

The pH values of corolla juice were estimated to be 4.3 (stage 2) and 4.3 (stage 3) in 'Blue Ensign', 3.9 (stage 2) and 4.7 (stage 3) in 'Bertram Anderson', and 5.0 (stage 2) and 5.2 (stage 3) in 'Diana Clare'. These values indicated weak acidity. The vacuole pH of the flower is generally slightly acidic (Shibata *et al.*, 1949; Stewart *et al.*, 1975). Exceptionally, some species having bluish flowers show relatively high pH values such as *Veronica persica* Poir. (6.2) (Mori *et al.*, 2009; Ono *et al.*, 2010) and morning glory (*I. tricolor*) 'Heavenly Blue' (7.7) (Yoshida *et al.*, 2005). The results of this study suggested that the pH change is not important for floral color change, because relatively high vacuole of pH was not detected.

Visible absorption spectra of stages 2 and 3 in lungwort cultivars are shown in Fig. 7. It was revealed that the absorption maxima of violet corollas, stage 2, was single maxima, i.e. 525 nm in 'Blue Ensign', 523 nm in 'Bertram Anderson' and 530 nm in 'Diana Clare'. On the other hand, the blue corollas showed two absorption maxima, 578 and 606 nm in 'Blue Ensign', 578

and 609 nm in 'Bertram Anderson' and 573 and 601 nm in 'Diana Clare'. Furthermore, these values were larger than those of stage 2.

The results of *in vitro* examination using delphinidin 3-*O*-glucoside (**A1**) and rosmarinic acid (**O1**) is shown in Fig. 8A. Absorption curves of three solutions, 1 mM **A1** and mixture of 1 mM **A1** and 1 or 2 mM **O1** (pH 5.0), showed one absorption maxima at 527–528 nm, respectively. These absorption curves were clearly different from that of intact blue corollas (Fig. 7). The result means that intermolecular copigmentation between **A1** and **O1** do not occur in lungwort cultivars. On the other hand, absorption maxima of the mixture of 1 mM petunidin 3-*O*-glucoside (**A3**) and 1 or 2 mM rosmarinic acid (**O1**) showed the bathochromic shift (3–4 nm) (Fig. 8B). However, the spectral curve in the fresh blue corollas and the mixture solution were clearly different, as well as those of **A1** and mixture of **A1** and **O1**. In the case of intermolecular copigmentation, e.g. Japanese garden iris (*Iris ensata* Thunb.) (Yabuya *et al.*, 1997), blue Dutch iris (*Iris × hollandica* Hort. ex Todd.) (Mizuno *et al.*, 2013) and viola (*Viola cornuta* L.) (Sugahara *et al.*, 2018), additional peaks were observed on the long wavelength side with increase of accessory pigment. In these research, *in vitro* reconstruction has been performed as a higher proportion i.e. 1:1~70 (anthocyanin: *C*-glycosylflavone) of accessory pigments. We performed *in vitro* examination in lower proportion, such as 1:1~2 (anthocyanin: rosmarinic acid), because it was indicated that the proportion of rosmarinic acid is lower from the result of quantitative analysis (Fig. 6). These results suggested that the blue color expression in lungwort cultivar flowers is not developed by the presence of rosmarinic acid, as well as the results of the quantitative analysis. In this experiment, we were not able to perform *in vitro* experiments using petunidin 3,5-di-*O*-glucoside due to its small amount. More detailed reconstruction needs for elucidation of blue coloration in lungwort flowers.

Blue color expression in some flowers has been reviewed (Honda and Saito, 2002; Yoshida

*et al.*, 2009; Iwashina, 2015). The blue coloration factors are metal anthocyanin, intermolecular copigmentation, intramoleculer copigmentation and higher pH. From these results, we presumed that intermolecular copigmentation and the effect of higher pH are unlikely. In addition, there is no possibility of intramolecular copigmentation, because the isolated anthocyanins are non-acylated anthocyanins. Moreover, it is suggested that the possibility of the formation of metalloanthocyanin in the blue flowers of lungwort. We will conduct further research to elucidate the mechanism of the blue coloration.

## Acknowledgments

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