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https://doi.org/10.5109/1564078

出版情報：九州大学大学院農学研究院紀要. 61 (1), pp.37-40, 2016-02-29. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **61** (1), 37–40 (2016)

Distribution and Composition of Flavonols in the Flowers of *Rhododendron oldhamii* Maxim.

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(*Received November 9, 2015 and accepted November 19, 2015*)

*Rhododendron oldhami*i Maxim. is an endemic evergreen azalea in Taiwan and recently planted in Japan. This species is distributed from 150 m to 2,700 m above sea level. Red–flowered species with petal color ranging from orange to red normally contain only cyanidin series anthocyanins and no flavonols in their petals. However, the color of blotches on the upper lobe of the petals or at the base of the funnel is slightly reddish–purple that suggested the presence of flavonols. In *R. oldhamii*, cyanidin 3–arabinoside and cyanidin 3–galactoside are two major anthocyanins in both upper and lower petals but flavonols were only detected in the upper petals, specifically in the blotches areas, by HPLC analysis. Two major flavonols were identified as quercetin glycosides. The pH value of reddish–purple blotches was slightly higher than that of surrounding areas and lower petals. These results indicated that co–pigmentation between cyanidin glycosides and quercetin glycosides pigments associated with higher pH condition, causing reddish–purple color for blotches in *R. oldhamii* flowers.

Key words: anthocyanin, copigmentation, flavonol, *Rhododendron oldhamii*

INTRODUCTION

Rhododendron oldhamii Maxim., classified in the subgenus Tsutsusi, is a subtropical broadleaf species endemic to Taiwan and was scientifically described for the first time in 1870 (Hsieh *et al.*, 2013). This species is distributed from 150 m to 2,700 m above sea level, and the population size of it is the largest among all *Rhododendrons* in Taiwan. It is commonly used as important ornamental plants for gardens, street plantings or flowerpots because of the beauty of flowers and multiple flowering seasons per year.

Rhododendron oldhamii is semideciduous shrubs with red, brick–red or orange five–petal–lobe flowers and reddish–purple blotches on the upper insides of the petals (Fig. 1). Pigment components of some red flowered species such as *R. simsii, R. indicum* and *R. oldhamii* flowers have been reported to be cyanidin 3–galactoside and cyanidin 3–arabinoside as two major anthocyanins in whole flower petals (Hang *et al.*, 2011). However, the development of the reddish–purple color of blotches in *R. oldhamii* has not been well analyzed and explained. In azaleas, it is well–known that co–pigmentation between anthocyanins and flavonols has a bluing effect for flower color (Asen *et al.*, 1971; De Loose, 1978). Reddish–purple blotches of *R. oldhamii* suggest the existence of kind of co–pigments, which should be revealed.

The purpose of this study is to clarify the distribution and composition of pigments in reddish–purple blotches of *R. oldhamii* petals by anatomical and chromatographic methods.

Fig. 1. Full opened *Rhododendron oldhamii* flowers. Bar indicates 1 cm.

MATERIALS AND METHODS

Plant materials

Full–opened *R. oldhamii* flowers were collected at Kyushu University greenhouse. A part of fresh petals was used for microscopic observation of the cross–sections. The remaining was separated into upper and lower petals, and each part of petals was boiled at 100˚C for 5 seconds and immediately cooled in water. Then the samples were dried in the forced convention oven overnight at 50˚C . The dried samples were stored in a desiccator at 4˚C until using for pigment analysis.

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Microscopic observation of pigment distribution and absorption spectra of fresh petals

Upper and lower petals of fresh flowers of *R. oldhamii* were cut into small squares at the center positions and fixed using 5% agar in the petri dish. Then they were sliced using a microslicer (DTK–1000, Dosaka EM; Kyoto, Japan) at a thickness of 150 μm. After slicing, cross–sections were observed under an optical microscope (Leica DM–2500; Leica Microsystems GmbH, Wetzlar, Germany).

HPLC analysis

Dried petals (ca. 50 mg) of *R. oldhamii* were soaked overnight with 50% HOAc–H_2O. After filtration, analytical HPLC was conducted on a LC–20AD pump (Shimadzu, Kyoto, Japan), using a Cosmosil 5C_{18} MS–II column (4.6 ϕ × 250 mm; Nakalai Tesque, Kyoto, Japan) at 40°C with a flow rate of 1 mL·min^{-1}, and monitoring at 520 nm and 360 nm for anthocyanins and flavonols, respectively. A linear gradient elution was applied for 40 min from 20 to 85% solvent B (1.5% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) in solvent A (1.5% H_3PO_4 in H_2O).

Identification of pigments

Two–dimensional thin layer chromatography (TLC) was carried out on cellulose–coated glass plates (Merck, Darmstadt, Germany) using two mobile phases: BAW (*1*–BuOH/HOAc/H_2O, 4:1:2, v/v/v) and 10% HOAc. The plates were observed under the UV light (365 nm), and the color of all spots was recorded. Subsequently, each spot was collected from the TLC plates, and dissolved using MeOH for HPLC analysis.

Isolation of major flavonols

Dried petals (ca. 0.7 g) of *R. oldhamii* were soaked overnight with 100% MeOH. After filtration, preparative HPLC was performed on an LC–6AD system (Shimadzu, Kyoto, Japan), using a Cosmosil 5C_{18} AR column (20 ϕ × 250 mm; Nakalai Tesque, Kyoto, Japan) at 40°C with a flow rate of 9 mL·min^{-1}, and monitoring at 360 nm for isolation of major flavonol peaks. A linear gradient elution was applied for 40 min from 50 to 85% solvent B (10% formic acid, 40% MeCN in H_2O) in solvent A (10% formic acid in H_2O). Major peaks were obtained using a fraction collector.

Identification of flavonol aglycones

Each purified flavonol was acid hydrolyzed by 2N HCl at 100°C for 90 min. The flavonol aglycones were co–chromatographed with authentic standard flavonols aglycones such as myricetin, quercetin and kaempferol, by HPLC with constant flow of 75% solvent A (0.1 M HOAc): 25% solvent B (MeCN). The HPLC system, column, and flow rate were the same as mentioned above (*HPLC analysis*). Wavelength was set at 360 nm.

pH measurement

Fresh flowers of *R. oldhamii* were separated into blotches, surrounding areas of botches and lower petals. The pH value of squeezed juice of each part was determined using a compact pH meter (TWIN pH waterproof B–212; Horiba Ltd., Kyoto, Japan). Five flowers were measured as replications.

RESULTS AND DISCUSSION

Distribution of pigmented cells in the petals of *R. oldhamii* flowers

Blotches of *R. oldhamii* flowers showed numerous reddish–purple pigmented cells, which accumulated in the adaxial epidermis of upper petals (Fig. 2B). On the other hand, red colored cells were distributed in the epidermis of both upper and lower petals (Fig. 2B, C). The upper epidermis of blotch areas was flat, and the shape of reddish–purple pigmented cells was not different from that of red pigmented cells. Generally, blotches in flowers of *Rhododendron* species contain stronger colored cells in the adaxial subepidermis rather than in epidermis. (Pecherer, 1992). The same results were observed in the transverse section of red blotches in *R. schlippenbachii* flowers. While light pink colored cells accumulated in the upper epidermis, red pigments were distributed in subepidermal cells (Yamagishi and Akagi, 2013). In *R. oldhamii* flowers, reddish–purple pigmented cells are only distributed in adaxial subepidermis of blotches areas.

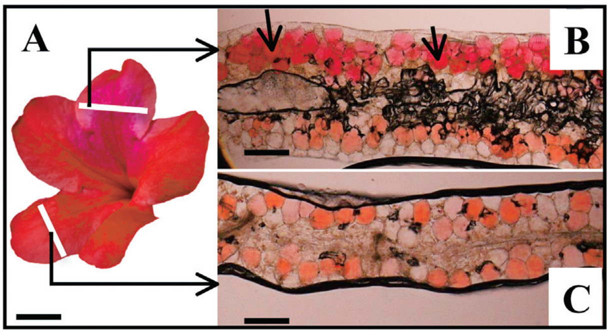

Fig. 2. Cross–sections of flower petals of *R. oldhamii*. A: Whole flower, B: Cross–section of upper petal, C: Cross–section of lower petal.
White lines in the photo A indicate sliced positions. Arrows in the photo B indicate reddish–purple cells. Bars: photo A= 1 cm, photo B and C= 20 μm.

Pigment composition in flower petals

Both upper and lower petals contained two major anthocyanins (Fig. 3A). These two anthocyanin pigments were reexamined and confirmed to be cyanidin 3–galactoside (Cy 3Ga) and cyanidin 3–arabinoside (Cy 3Ar) as previously reported in red flowered *R. simsii* and *R. oldhamii* as well (Hang *et al.*, 2011). At the absorbance of 360 nm, two major peaks were detected in upper petals, while no major peak was detected in lower petals (Fig. 3B). Thus, these two flavonoids seem to be present only in upper petals of *R. oldhamii* flowers.

To identify these two major flavonoids in upper petals, two–dimensional TLC was carried out. Eight discrete spots appeared in the TLC plates (Fig. 4) and color properties were recorded under visible and UV light

Table 1. Rf values and color properties of spots of pigments extracted from *R. oldhamii* flowers and separated by two–dimensional thin layer chromatography

Spot No.	Rf value (×100)		Color in	
	BAW [1]	10% AcOH	Visible light	UV–light
1	17	22	Red–lilac	Violet
2	48	14	Red–lilac	Violet
3	65	20	Pale brown	Yellow F. [3]
4	76	30	Pale brown	Yellow F.
5	75	48	– [2]	Blue F.
6	39	50	–	Blue F.
7	64	66	–	Blue F.
8	37	70	–	Blue F.

[1] *1*–BuOH/HOAc/H_2O = 4:1:2, v/v/v
[2] colorless.
[3] fluorescent.

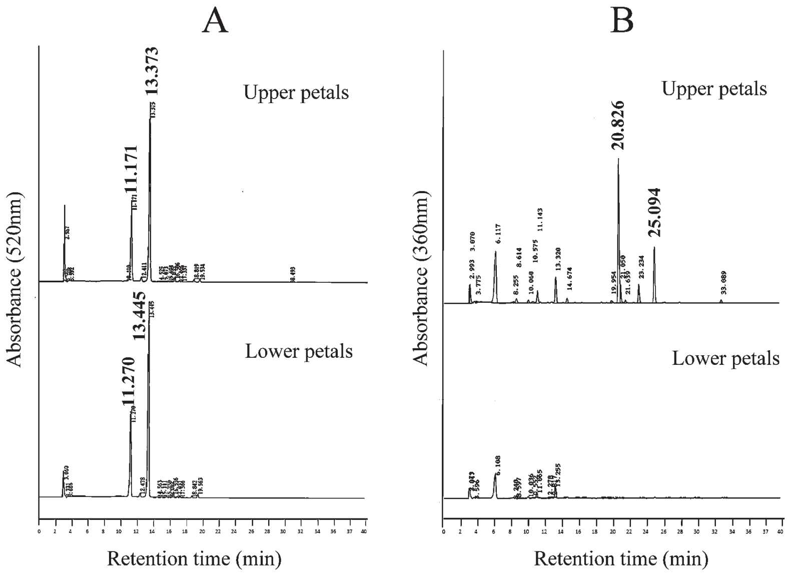

Fig. 3. HPLC profiles of the extracts from upper and lower petals of *R. oldhamii* at 520 nm (A) and 360 nm (B).

(Table 1). Spot numbers 1 and 2 expressed red–lilac color under visible light and violet under UV light. This result suggested that they were two major anthocyanins (Cy 3Ga and Cy 3Ar). In addition, spots 3 and 4 appeared pale brown under visible light, and showed yellow florescence under UV light. Spethmann (1980) investigated flavonoids of Rhododendron flowers, and reported that some flavonol glycosides, such as quercetin 3–arabinoside, quercetin 3–rhamnoside or kaempferol 5–methylether, appeared brown or pale brown under visible light and colored greenish–yellow to yellow florescence under UV light on TLC plate. These findings suggested that spots 3 and 4 are flavonols. The HPLC analysis of spots 3 and 4 was carried out after collection and extraction from TLC plate. From HPLC analysis, spots 3 and 4 coincided with two major peaks in upper petals (Fig. 3B).

Next, these two major peaks in upper petals were isolated by preparative HPLC and acid hydrolyzed using 2N HCl. HPLC analysis showed that two major peaks

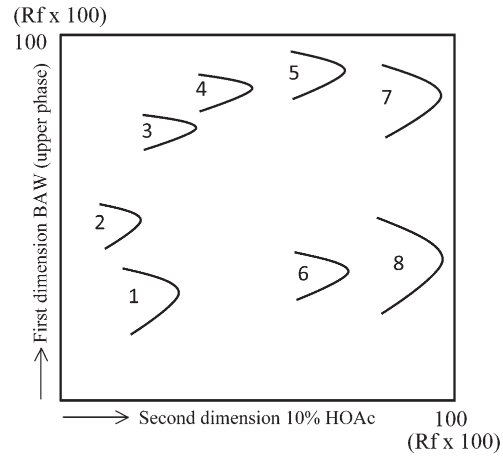

Fig. 4. Two–dimensional thin layer chromatogram of MeOH extract from upper petals of *R. oldhamii* flowers. BAW (*1*–BuOH/HOAc/H_2O, 4:1:2, v/v/v). See Table 1 for color properties of spots.

Table 2. HPLC retention time of standard flavonols and flavonol aglycones of *R. oldhamii* flowers

Aglycones	Retention times (min)
Myricetin	8.0
Quercetin	15.2
Kaempferol	28.8
Peak 1	15.2
Peak 2	15.2

Table 3. pH value of the flower parts of *R. oldhamii*

Flower parts	pH value
Blotches	3.14 a[1)]
Surrounding areas of blotches	2.18 b
Lower petal	2.26 b

[1)] Values with different letters are significantly different at $P<0.05$ by Tukey's test.

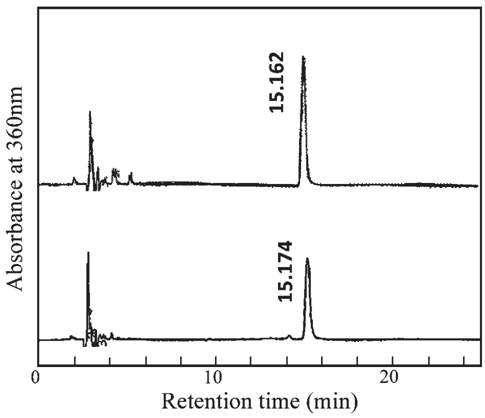

Fig. 5. HPLC tracing of acid hydrolysates of two major flavonols isolated from the petals of *R. oldhamii*.

have same flavonol aglycones at the retention time of 15 min (Fig. 4). In comparison to authentic standard samples, such as myricetin, quercetin and kaempferol, the aglycones of two major peaks were identified as quercetin (Table 2).

Asen *et al*. (1971) determined the cause of the difference in color expression between 'Red Wing' azalea and an orange sport of this cultivar. The orange color of the mutant was due to cyanidin glycosides, whereas the color of 'Red Wing' azalea was due to the same cyanidin glycosides co–pigmented with quercetin glycosides. Carmine red color of some cultivar of azalea species, such as 'Vuyk's Scarlet', is in effect produced by the addition of flavonol (quercetin–glycosides) to the red pigments cyanidin or peonidin (Heursel, 1987). Thus, quercetin glycosides co–pigmented with cyanidin glycosides to develop 'bluing effect' causing reddish–purple blotches in *R. oldhamii* flowers.

pH value of petal parts

The pH value of the pressed juice from various positions of same flowers was quite different. The pH of reddish–purple blotches was 3.14. On the other hand, that value of surrounding areas of blotches and lower petals were 2.18 and 2.26, respectively. The same results were also reported by Stewart *et al*. (1975) in fuchsia cv. Fanfare flowers. The pH of pink calyx was 4.1, while that of the strong red corolla was 3.7. The pH of reddish–purple blotch areas was higher than that of red petal parts. However, petal color is not only determined by pH value, but also by co–pigments or other inorganic substances, such as Al^{3+} (Yoshida *et al*., 2003).

In conclusion, these results indicated that co–pigmentation between cyanidin glycosides and quercetin glycosides pigments associated with higher pH condition, causing reddish–purple color for blotches in *R. oldhamii* flowers.

REFERENCES

Asen, S., R. N. Stewart and K. H. Norris 1971 Co–pigmentation effect of quercetin glycosides on absorption characteristics of cyanidin glycosides and color of Red Wing azalea. *Phytochem*. **10**: 171–175

De Loose, R. 1978 *Azalea indica* flower color as related to the parameters pH, anthocyanins and flavonol co–pigments. *Scientia Hortic*. **9**: 285–290

Hang, T. T. N., I. Miyajima, K. Ureshino, N. Kobayashi, Y. Kurashige, T. Matsui and H. Okubo 2011 Anthocyanins of wild *Rhododendron simsii*. Planch. flowers in Vietnam and Japan. *J. Japan. Soc. Hort. Sci*. **80**: 206–213

Hsieh, Y. C., J. D. Chung, C. N. Wang, C. T Chang, C.Y Chen and S. Y. Hwang 2013 Historical connectivity, contemporary isolation and local adaptation in a widespread but discontinuously distributed species endemic to Taiwan, *Rhododendron oldhamii* (Ericaceae). *Heredity* **111**: 147–156

Heursel, J. 1987 Inheritance of flower colors and breeding of evergreen azaleas. *J. Amer. Rhododendron Soc*. **41**(3) <http://scholar.lib.vt.edu/ejournals/JARS/v41n3/v41n3–heursel.htm>.

Pecherer, B. 1992 The color of *Rhododendron* flowers. *J. Amer. Rhododendron Soc*. **46**(4) <http://scholar.lib.vt.edu/ejournals/JARS/v46n4/v46n4–pecherer.htm>.

Spethmann, W. 1980 Flavonoids and carotenoids of *Rhododendron* flowers and their significance for the classification of the genus *Rhododendron*. pp. 247–276. In: J. L. Luteyn and M. E. O'Brien (eds.). Contributions toward a classification of *Rhododendron*. New York Bot. Garden, New York

Stewart, R. N., K. H. Norris and S. Asen 1975 Microspectrophotometric measurement of pH and pH effect on color of petal epidermal cells. *Phytochem*. **14**: 937–942

Yamagishi, M and K. Akagi 2013 Morphological and heredity of tepal spots in Asiatic and Oriental hybrid lilies (*Lilium* spp.). *Euphytica* **194**: 325–334

Yoshida, K., Y. Toyama–Kato, K. Kameda and T. Kondo 2003 S epal color variation of *Hydrangea macrophylla* and vacuolar pH measured with a proton–selective microelectrode. *Plant Cell Physiol*. **44**(3): 262–268