Studies on the Flower Coloration and Heat Stress Tolerance of Rhododendron simsii Planch. Distributed in Vietnam

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Studies on the Flower Coloration and Heat Stress Tolerance of *Rhododendron simsii* Planch. Distributed in Vietnam

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CONTENTS

Chapter I

INTRODUCTION

The genus *Rhododendron* has about a thousand different species and grows in the wild in many parts of the world. They range in size from tiny, mat-like plants to trees up to 30 m tall. The greatest natural gene center, with more than 300 species, is in an area of Asia ranging from Nepal along the line of the Himalayas into northern Myanmar and the provinces of Yunnan and Szechwan in South-West China (Leach, 1969). Almost the same number of species is found in South-East Asia from Thailand and Vietnam to Malaysia, Indonesia and New Guinea. In Japan and North America, number of native species was recorded as more than 50 and 30 species, respectively (Chamberlain et al., 1996).

Taxonomically, all azaleas and rhododendrons are classified as two subgenus of the genus *Rhododendron*. In which, rhododendrons are evergreen with larger and bolder foliage while the azaleas can be either evergreen or deciduous with smaller leaves (Chamberlain et al., 1996). Leaf size and shape, plant height and flower color are varied among different species. The flower colors range from white through pink to brilliant red, from yellow to orange, from pale lavender through harsh magenta shades to almost blue (Leach, 1961). The two subgenus contributed many valuable species for landscaping of around the world.

Among *Rhododendron* species, *R. simsii* Planch. is one of the most important species, and considered as mother species of Belgian cultivars and hybrids. *Rhododendron simsii* is native to East Asia such as Ryukyu Archipelago of Japan, Taiwan, southern China and Vietnam. This species grows from sea level to ca. 3000 m height. It is a kind of shrub

or small tree with leaves that are ovate, elliptic-ovate or obovate to oblanceolate. Flowers are funnel shaped or campaniform with single or double type. *Rhododendron simsii* is rich in colors such as white, red, pink, purple with spots, blotches or stripes (Bu et al., 2010). Due to wide variation of color, this species was introduced from China to Europe in the end of 18th century for breeding, and up to now Belgium is considered as the world's leading producer of pot azaleas (Van Huylenbroeck et al., 2015).

In Vietnam, some accessions of wild *R. simsii* were found in northern and central parts, where plants grow near riverside or streamside at 800- 1400 m altitudes (Hang et al., 2010; Ho, 1991). All of these accessions show only red colored funnel shaped flowers with reddish-purple blotches in the inside of three upper petals. Flowers are broadly funnel shape containing 10 stamens. Leaves are narrow obovate-elliptic or obovate-oblong (Figure I-1).

Recently, Vietnamese people prefer to buy potted azaleas for decorating their houses during lunar New Year ceremony in spring because of its happy and lucky meanings. The available potted azaleas with pink, white or purple color flowers, however, are regrettably imported from China, and the domestic breeding program using wild *R. simsii* has not been conducted yet in Vietnam. Moreover, the color variation of Vietnamese species is very limited. Thus, it is necessary to introduce new cultivars by breeding with prospective evergreen azalea materials using Vietnamese germplasm for domestic potted azalea production.

Before starting the breeding program, it is important to consider several physiological characteristics of *R. simsii*. In ornamental plants, flower color is a very important feature, and it can enhance ornamental value of floral species. The breeders always think about how to improve and modify the color expression of flowers. Flavonoids

Figure I-1. Flowers of wild *Rhododendron simsii* Planch. in Than Uyen, Lai Chau, Vietnam.

account for the pigmentation in azalea (De loose, 1968 and 1969). Wild evergreen azaleas contain anthocyanin pigments, such as cyanidin type pigments (cyanidin and peonidin) in red-colored flowers or cyanindin and dephinidin type pigments (delphinidin, malvidin and petunidin) in purple flowers (De Loose, 1969; Kunishige and Kobayahsi, 1980; Umeki and Inazu, 1989; Mizuta et al., 2009). Anthocyanins in the petals of *R. simsii* in Vietnam has been identified and reported by Hang et al. (2011). However, reddish purple blotch areas in upper petals suggest additional component besides anthocyanins. Thus, at first this study focuses on elucidation of the pigment composition and cause of reddish-purple colored blotches in three upper petals of *R. simsii* flowers.

In the breeding of ornamentals, the role of interspecific hybridization for creating more variation is very important. When using interspecific hybrids in new cross combinations hybrid vigor can be captured and traits that do not occur in the crosses within a single species can be combined (Van Tuyl and De Jeu, 1997). Interspecific hybridization is common among plants and is a significant evolutionary mechanism that can give rise to new evolutionary lineages and species (Arnold, 1997 and 2006; Bartier et al., 1999; Grant et al., 2005, Rieseberg and Carney, 1998; Soltis et al., 2009). In azaleas, the variation in flower color of cultivated varieties is considered to be the results of the combination of genes controlling flavonoid biosynthesis through natural or artificial interspecific hybridization (Kunishige and Kobayashi, 1980). In fact, Vietnamese *R. simsii* is a very important genetic resource for breeding. However, numbers of breeders as well as hybridization partners are limited. On the other hand, Japanese and Taiwanese evergreen azaleas are very famous for wide color and pigment constitution variation in flowers as well as good tolerance to heat and cold stress conditions (Mizuta et al., 2009; Arisumi et al.,

1985). Furthermore, evergreen azaleas are not only interesting in its considerable horticultural importance and in the wide geographic distribution, but also interesting in its feasibility to hybridize among species. Therefore, interspecific hybridization between Vietnamese *R. simsii* and Japanese and Taiwanese evergreen azalea species should be carried out, since the crosses will reveal cross compatibility in this breeding research.

Desirable traits have been obtained in many cultivars and varieties of genus *Rhododendron* through breeding such as cold hardiness, disease resistance or heat and drought stress tolerance (Kehr, 1987). Numbers of research have been carried out in breeding of the heat tolerant hybrid rhododendrons in Japan (Arisumi et al., 1979, 1986 and 1988), and in evaluating heat tolerance ability of some *Rhododendron* species (Davidian, 1982b; Ranney et al., 1995; Thornton, 1989 and 1990). There is limited information about heat tolerance in evergreen azaleas even though they are probably the most commercially successful members of the genus *Rhododendron*. Because *R. simsii* distributed along eastern Asia to southwestern China and Vietnam, each accession has its own growing habitat. Plants distributed in different regions with unlike climatic condition may perform different morphological and physiological characteristics. Therefore, in following part of this study, heat stress tolerance of *R. simsii* and other evergreen azalea species was evaluated and discussed to obtain useful information for cultivation and breeding of evergreen azaleas.

For these, the purpose of this study is to elucidate physiological characteristics of Vietnamese *R. simsii* and establish the breeding system to obtain the vigorous interspecific F_1 hybrids with desirable characteristics from their parents.

This study was conducted as following steps:

1. Flower coloration and co-pigmentation of anthocyanin-flavonol in reddish-purple blotch areas were investigated in Chapter II, section II-1.

2. Pigment constitution of *R. simsii* flowers was analyzed by chromatographic analysis in Chapter II, section II-2.

3. To investigate the cross compatibility between *R. simsii* and several Japanese and Taiwanese evergreen azaleas, interspecific hybridization was carried out in Chapter III.

4. Evaluation of the heat stress tolerance in *R. simsii* was also conducted in Chapter IV. Then general discussion about these topics is performed in Chapter V.

Chapter II

FLOWER COLORATION AND PIGMENT CONSTITUTION IN *RHODODENDRON SIMSII*

Section II-1

FLOWER COLORATION AND CO-PIGMENTATION OF ANTHOCYANIN-FLAVONOL IN THE BLOTCH AREAS OF *RHODODENDRON SIMSII* FLOWERS

Introduction

Some plant species have petal blotches or spots on their flowers. These blotches or spots may generate variation of flower color and pigmentation patterns, which are valuable for ornamental species. Some aspects of blotches were interested and revealed in orchids (Griesbach, 2008; Liu et al., 2012) and lily flowers (Yamagishi and Akagi, 2013). In flowers of some species, morphologies of blotches is region-specific and much different from normal region (Thomas et al., 2009); or the same with the remain parts of the petals (van Houwelingen et al., 1999; Itoh et al., 2002; Iida et al., 2004; Fujino et al., 2011). Normally, anthocyanins are main pigments accumulated in the blotches or spots (Zhang et al., 2007; Cooley and Willis, 2009; Thomas et al., 2009).

In *R. simsii* flowers, blotch areas are distributed only in upper inside of three upper petals of the flowers, and express reddish-purple color. Hang et al. (2011) reported that two major anthocyanins found in *R. simsii* accessions of Vietnam and Japan were identified as cyanidin 3-galactoside (Cy 3Ga) and cyanidin 3-arabinoside (Cy 3Ar). However, no report

indicated the cause of the different color expressions between reddish-purple blotch areas and red petal lobes in wild *R. simsii* flowers.

In this section, microscopic observation and plant pigment analysis were conducted to clarify the coloration of reddish-purple blotches in the three upper petals of *R. simsii* flowers.

Materials and Methods

Plant materials

Fresh flowers of wild *R. simsii* distributed in Than Uyen district, Lai Chau province, Vietnam were collected at anthesis. A part of fresh petals was used for color property measurement, microscopic observation and absorption spectra measurement. The remaining flowers were teared to separate into three upper and two lower petals, and each of the petals was boiled at 100^{o}C for 5 seconds, and immediately cooled in water. Then, the samples were dried in a forced convention oven overnight at 50^{o}C. The dried samples were stored in a desiccator at 4^{o}C until pigment analysis.

Color property measurement

The flowers of each accession were teared to separate into three upper and two lower petals. Each of the separated petals was measured for color property by CIE L^* a^* b^* color coordinate using a colormeter NF333 (Nippon Denshoku Ind.Co.Ltd). L^* value indicated lightness (0 = black, 100 = white) and parameters a^* (from green to red) and b^* (from blue to yellow) are the two chromatic components, which range from −120 to 120.

Microscopic observation of pigment distribution

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

Absorption spectra of fresh petals

Full opened fresh petals were cut into squares in the center of the lobes of upper and lower petals. Then, the samples were attached to the glass cell using transparent tape, and immediately measured from 370 nm to 700 nm using a UV spectrophotometer (U-2910, Hitachi, Kyoto, Japan). Five flower petals were used as replication samples.

HPLC analysis

Dried petals (ca. 50 mg) of *R. simsii* 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 x 250 mm; Nacalai Tesque, Kyoto, Japan) at 40^oC with a flow rate of 1 $mL \cdot min^{-1}$, 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% AcOH, 25% MeCN in H_2O) in solvent A (1.5% H_3PO_4 in H_2O).

Isolation of anthocyanins and flavonols

Approximately 20 g dried upper petals of *R. simsii* was immersed with 1 L of 10% formic acid in H_2O for 24 hours under 5^{o}C. After filtration, the filtrate was diluted with the same amount of water (1 L), and pass through Diaion HP-20 resin (350 mm x 60 mm ϕ; Mitsubishi Chemical, Tokyo, Japan). The column was thoroughly washed with 5 L of water, and pigments were eluted using 5% formic acid in MeOH. After concentration of the eluent, it was again purified, and fractionated using Sephadex LH-20 (200 mm x 40 mm ϕ; Sigma-Aldrich, St. Louis, Missouri, U.S.A.) with 5% formic acid in 50% EtOH solution. The absorption spectra of the obtained fractions were measured using a UV spectrophotometer (U-2910, Hitachi, Kyoto, Japan) at wavelengths from 300 nm to 700 nm to avoid contamination between anthocyanins and flavonols. Then separated anthocyanin and flavonol fractions were concentrated below 40^{o}C *in vacuo* to almost dryness using an evaporator for powdering.

In vitro co-pigmentation test

To examine anthocyanin-flavonol co-pigmentation, powder of anthocyanins and flavonols were dissolved in 0.1 M Glycine-HCl buffer (pH = 2.5), and mixed together at various ratios from 1:0 to 1:10 (anthocyanin: flavonol, w/w). Since the pH value of squeezed juice of fresh petals was determined to be 2.52 using a compact pH meter (TWIN pH waterproof B-212; Horiba Ltd., Kyoto, Japan), and that value was similar in both upper and lower petals of *R. simsii*, I decided to prepare the buffer of co-pigmentation test with the same pH value. The absorbance of each mixed solution was measured using a UV spectrophotometer (U-2910; Hitachi).

Quantification of total anthocyanins and flavonols of blotch areas

In this experiment, blotch areas were separated using razor blades, frozen with liquid nitrogen and grinded well to be powder. Anthocyanins and flavonols were extracted from 100 mg and 25 mg dried petals by 5 mL 1% HCl in MeOH and 10 mL warm MeOH for five times, respectively. Obtained extractions were measured for absorbance at 520 nm and 360 nm by a UV spectrophotometer (U-2910; Hitachi) for anthocyanins and flavonols, respectively. For each sample, three replications were carried out.

Total anthocyanin and flavonol contents were calculated from the equation of linear regression lines using cyanidin-3-glucoside (Cy 3Gl) and quercetin-3-glucoside (Qu 3Gl) as standards. One mg of Cy 3Gl and 1 mg Qu 3Gl were dissolved in 25mL 1% HCl-MeOH and 25mL MeOH, respectively. Each solution was measured for the absorption spectra at 520nm for Cy 3Gl and 360nm for Qu 3Gl by UV spectrophotometer. Then the dissolved solutions were diluted 5 times by the same solvents and absorption spectra were also measured. The data of absorption spectra from standard samples were used to establish linear regression lines.

Results and Discussion

Figure II-1 showed the color property of upper and lower fresh petals from three *R. simsii* accessions. Based on the lightness (*L**) of upper petals which have reddish-purple blotch area showed lower values than that of lower petals. These differences were significant in all three accessions of Vietnamese *R. simsii*. Even though *b** value of upper petals was obviously lower (7.17-19.42) compared to that of lower petals (18.86-27.86), there was no appreciable difference in *a** values between upper and lower petals. From this

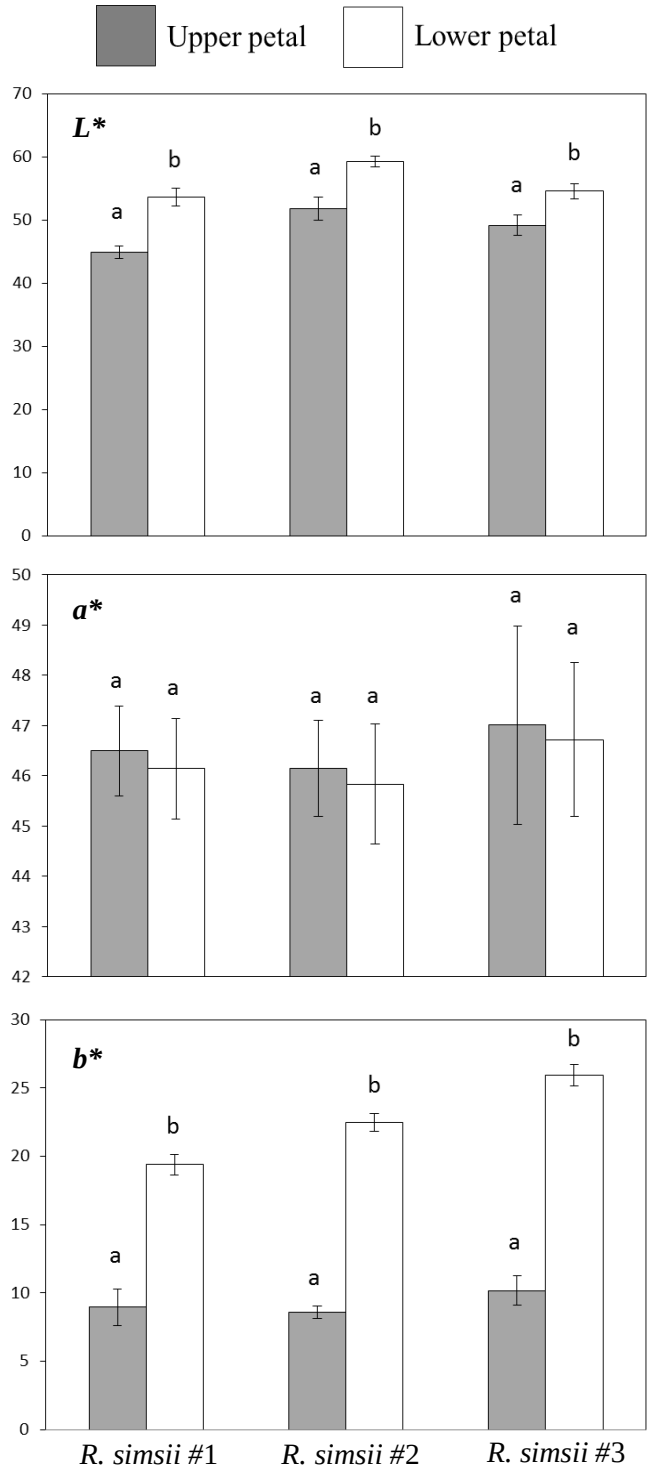


Figure II-1. *L**, *a** and *b** values of upper and lower petals from three accessions of *R. simsii* flowers. Vertical bars are standard errors. Means with different letters present statistical difference by T-test ($P < 0.05$).

point of view, upper petals showed darker color than lower petals, and *b** values of upper petals indicated that they have bluer expression in color compared to lower petals.

Pigments accumulate in the epidermis of petals of many flower species. However, the cross-sections of blotches from fresh upper petals of *R. simsii* showed that reddish-purple pigment cells accumulated in the adaxial subepidermis of upper petals, while red colored cells were numerously distributed in the epidermis of both upper and lower petals (Figure II-2 A, B, 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, in blotches of *Rhododendron* species flowers, the upper and lower epidermal cells have weaker color intensity compared with adaxial subepidermal cells, which are strongly colored by pigments (Pecherer, 1992). The same results were observed in the transverse section of red blotches in *R. schlippenbachii* flowers. In this species, red pigments were distributed in subepidermal cells while light pink colored cells accumulated in the upper epidermis (Yamagishi et al., 2013). The current results indicated that reddish-purple blotches arise from pigment accumulation in adaxial subepidermal cells of upper petals. The clear difference in higher *L** and lower *b** is due to the presence of reddish-purple colored cell formed by anthocyanins and flavonols in blotch areas.

The absorption spectra of fresh petal samples showed the λ_{max} in the visible region at 514.9 nm and 505.7 nm for upper and lower petals, respectively (Figure II-3). The wavelength of upper petals bathochromically shifted 9.2 nm longer than that of lower petals.

The HPLC results showed that both upper and lower petals contained two major anthocyanins (Figure II-4A), which have been reported as Cy 3Ga and Cy 3Ar (Hang et al.,

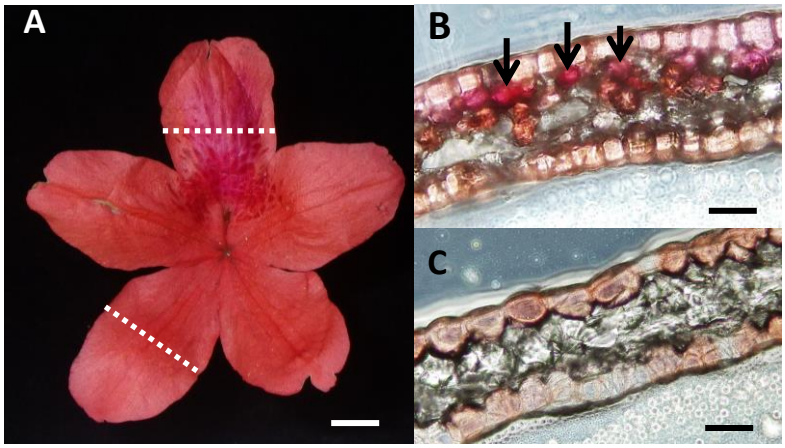


Figure II-2. Cross-sections of flower petals of *R. simsii*. A: A whole flower, B: Cross-section of upper petal, C: Cross-section of lower petal.
Broken lines in photo A indicate sliced positions. Arrows in photo B indicate reddish-purple cells. Bars: photo A= 1 cm, photo B and C= 20 µm.

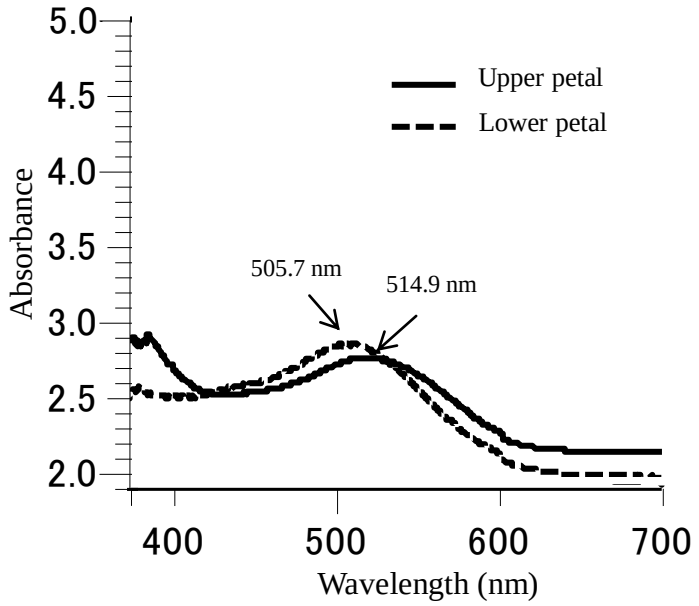


Figure II-3. Absorption spectra of intact petals of *R. simsii*.

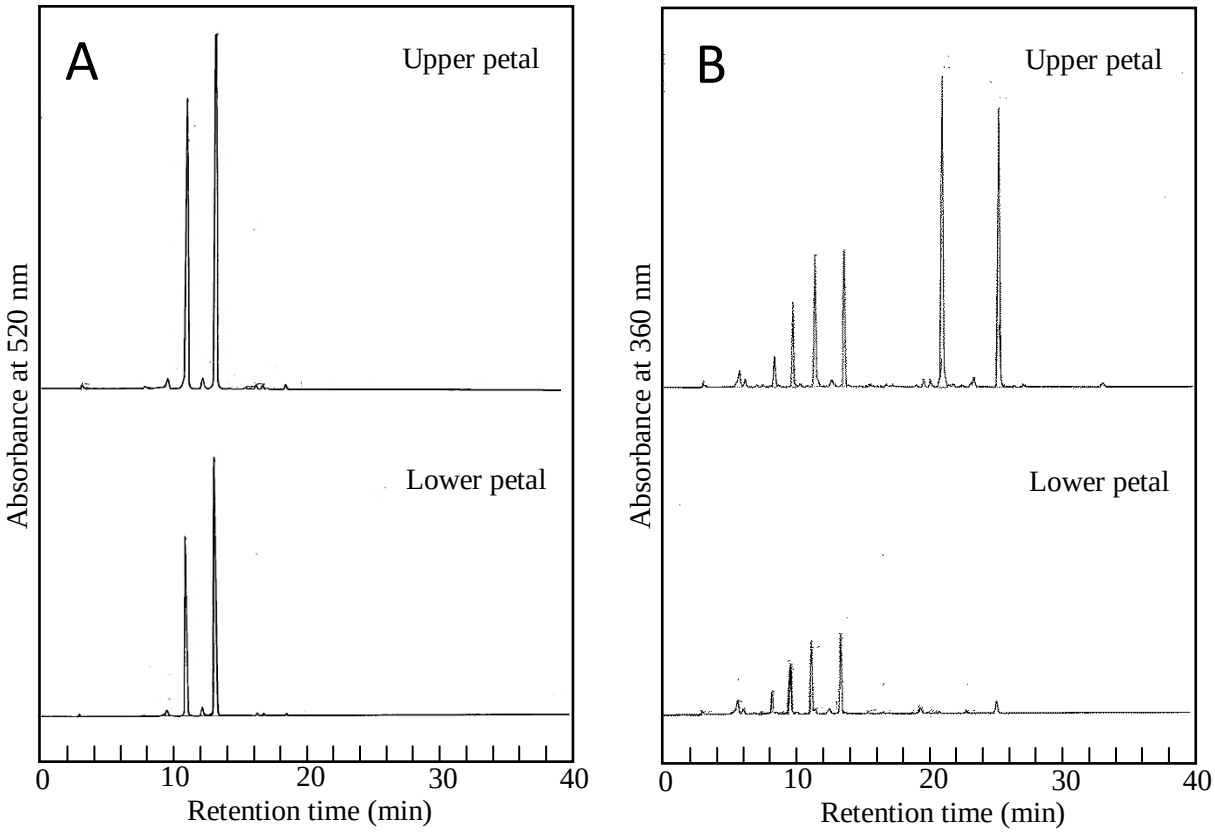


Figure II-4. HPLC analysis of extracts from upper and lower petals of *R. simsii* under 520 nm (A) and 360 nm (B).

2011). At 360 nm, two major peaks were detected in upper petals, while there was only trace amount available in lower petals (Figure II-4B).Thus, these two flavonoids seem to be present only in upper petals, and to be flavonols from the preliminary experiment (data not shown). Then, anthocyanins and flavonols were isolated and purified by column chromatography.

In total, 11 fractions of pigment were obtained (Figure II-5). By the measurement of absorption spectra of each fraction, absolute purified anthocyanin fractions (Fr. 1 to Fr. 4) and flavonol fractions (Fr. 8 to Fr. 11) were obtained, and used as materials for the co-pigmentation test. Since fractions 5, 6, and 7 were mixtures of anthocyanins and flavonols, they were discarded.

The result of the *in vitro* co-pigmentation test showed that the λ_{max} increased gradually as the concentration of flavonol increased in the mixed solution (Figure II-6). The λ_{max} of mixed solution was 510 nm when it contained only anthocyanins (1.0 $mg \cdot mL^{-1}$), and a 15.2 nm bathochromic shift was obtained from the mixed solution at the ratio of 1:10 equivalent of anthocyanins to flavonols (Table II-1). In comparison, the *in vitro* co-pigmentation test showed that a 9.7 nm bathochromic shift was obtained from mixed solution of anthocyanin and flavonol at the ratio of concentration of 1:7.5. These results were similar to the bathochromic shift obtained between upper and lower petals of intact flowers (9.2 nm) (Figure II- 3).

For the measurement of qualification of total anthocyanins and flavonols of the blotch areas of *R. simsii*, Cy 3Gl and Qu 3Gl were used as standard substances for establishment of calibration linear regression lines (Figure II-7). Based on this result, the amount of anthocyanins (5.87 μg/mg dry weight) was almost two times higher than that of

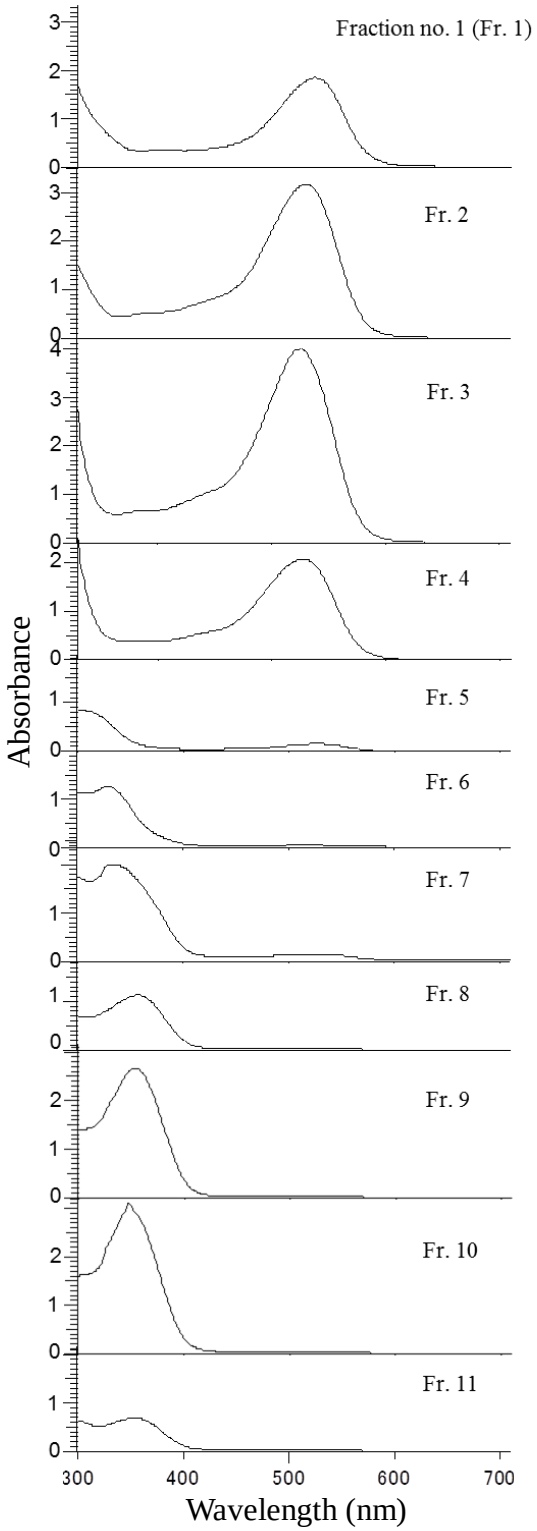


Figure II-5. Absorption spectra of fractions from *R. simsii* petal extraction purified and isolated using Sephadex LH-20 column. Fractions (Fr.) were numbered from 1 to 11. Fr.1 - Fr.4: anthocyanin fractions; Fr.5 - Fr.7: mixture of anthocyanin and flavonol fractions; Fr.8 - Fr.11: flavonol fractions.

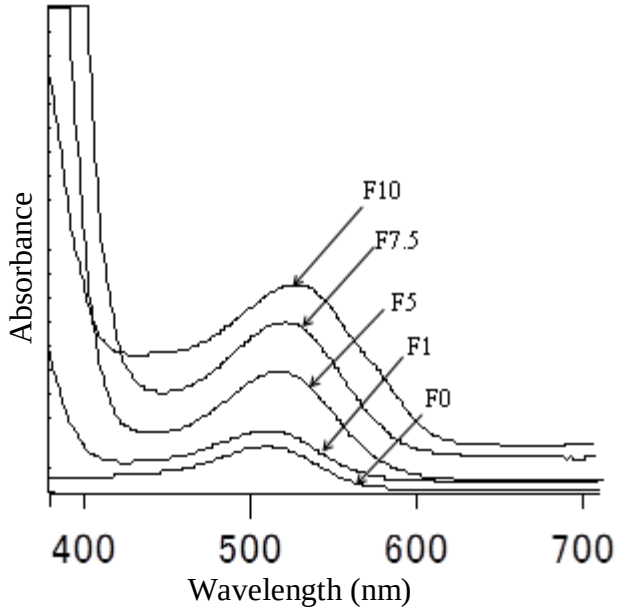
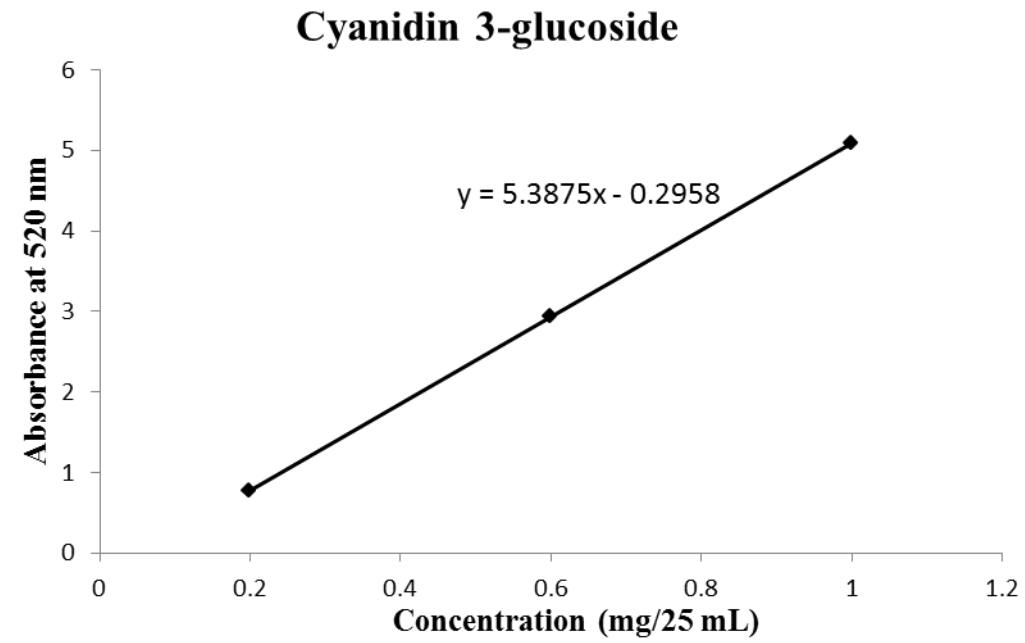


Figure II-6. *In vitro* co-pigmentation test with anthocyanins and flavonols. The anthocyanin and flavonol fractions extracted from upper petals of *R. simsii* flowers were dissolved in 0.1M Glycine-HCl buffer (pH= 2.5), and mixed together at different ratios. All samples include 1.0 mg·mL^{-1} anthocyanin. F0; flavonol 0 mg·mL^{-1}, F1; flavonol 1.0 mg·mL^{-1}, F5; flavonol 5.0 mg·mL^{-1}, F7.5; flavonol 7.5 mg·mL^{-1}, F10; flavonol 10.0 mg·mL^{-1}.

Table II-1**.** Effect of flavonols on the wavelength of maximum absorbance of anthocyanins in *in vitro* co-pigmentation test.

Concentration ($mg \cdot mL^{-1}$)		λ_{max} in 0.1M glycine-HCl buffer[z] (nm)	$\Delta\lambda_{max}$
Anthocyanin	Flavonol		
1.0	0.0	510.0	-
1.0	0.1	511.3	1.3
1.0	0.5	511.5	1.5
1.0	1.0	513.3	3.3
1.0	2.0	514.7	4.7
1.0	5.0	516.3	6.3
1.0	7.5	519.7	9.7
1.0	10.0	525.2	15.2

[z] 0.1M Glycine: 0.1M HCl: H_2O (50:2.5:47.5, v/v/v), pH= 2.5.



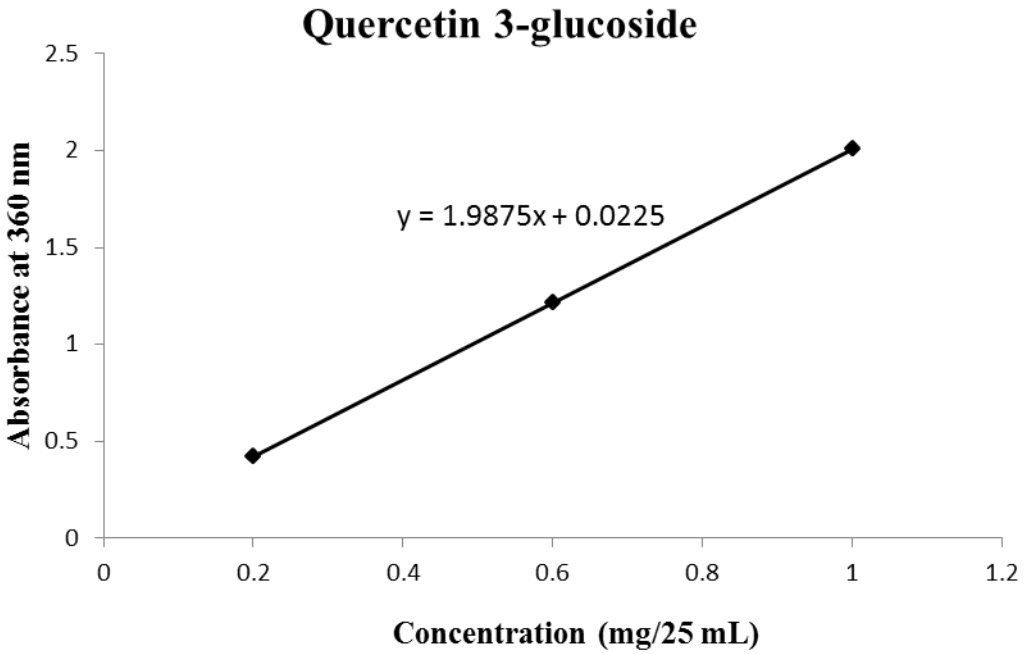


Figure II-7. Calibration linear regression lines of anthocyanin standard (cyanidin 3-glucoside) and flavonol standard (quercetin 3-glucoside). The equations were established based on absorbance data of two standard substances.

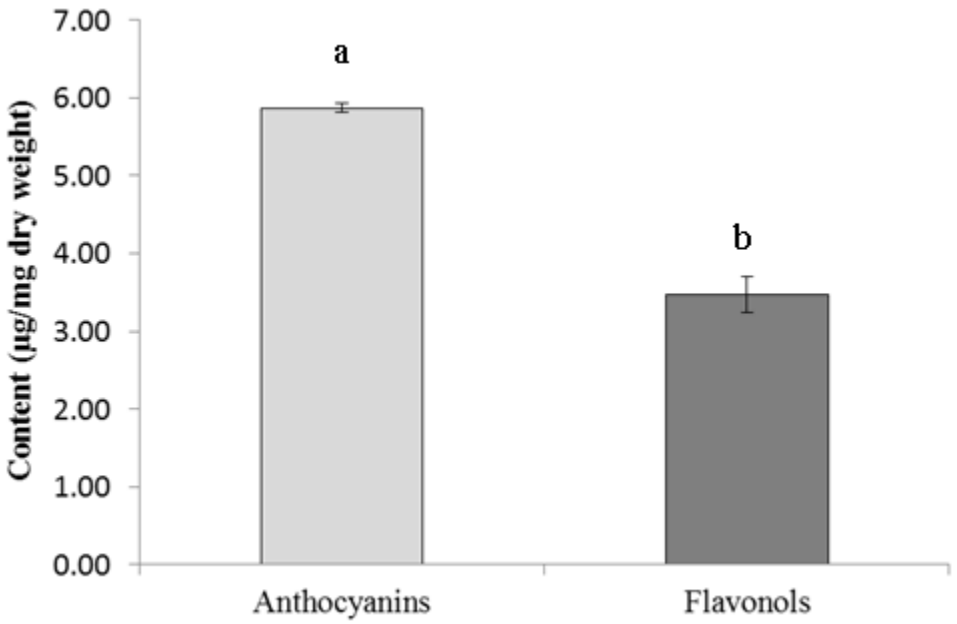


Figure II-8. Anthocyanin and flavonol content in reddish-purple blotch areas of *R. simsii* flowers. Vertical bars indicate standard errors. Means with different letters present statistical difference by T-test ($P< 0.05$).

flavonols (3.47 μg/mg dry weight) (Figure II-8). In the blotch areas, red pigmented cells abundantly distributed both in abaxial and adaxial epidermis while reddish-purple pigmented cells were only in the subepidemis of adaxial surface. Thus, the amount of anthocyanins should be higher than that of flavonols. Furthermore, the ratio of 1:7.5, obtained in *in vitro* co-pigmentation test, can be understood as the ratio of anthocyanins to flavonols within the reddish-purple pigmented cells, not counted for whole area of blotches.

In purple, red-purple or blue flower colors due to anthocyanins are well known to be influenced by co-pigments and other factors such as metal complexation or vacuolar pH values of epidermal flower cells (Takeda, 1980, 1994; Brouillard, 1988; Goto, 1987; Goto and Kondo, 1991). This phenomenon is due to molecular associations between pigments and other (usually non-colored) organic molecules in solution. These associations cause the pigments to exhibit far greater color than would be expected from their concentration (Boulton, 2001). Among the factors influencing flower color, flavonols represent another key subgroup which play pivotal roles in the pigmentation of flowers (Czemmel et al., 2009), and the flavonol derivatives have been shown to influence anthocyanin-mediated coloration by co-pigmentation effects.

Previously, co-pigmentation tests were done in some flower species, such as Japanese garden iris (Yabuya et al., 1997), torenia (Aida et al., 2000), or blue *Veronica persica* flowers (Ono et al., 2010). In these flowers, blue flower color is expressed by co-pigmentation between anthocyanin and flavone. 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. These results suggested that the co-pigmentation of anthocyanin-flavonol occurs in the blotches in upper petals of *R. simsii* flowers.

In nature, plants and pollinators have co-evolved phenotypic characters that make them more likely to interact successfully. The plants benefit from attracting a particular type of pollinator to their flower by color signal, ensuring that pollen will be carried to another flowers of the same species, and resulting in successful reproduction. Hirota and Nitta (2012) investigated that swallowtail butterflies preferentially visited reddish or orange-colored flowers and hawkmoths frequently visited yellowish flowers of daylily and nightlily F_1 and F_2 hybrids. More previous studies showed that swallowtail butterflies have an innate color preference toward yellow and red than blue and green (Kinoshita et al.,1999; Weiss, 2003), while hawkmoths have an innate preference for blue and weaker innate preferences for violet and yellow (Kelber, 1997). A study of flower morphology of *Clarkia gracilis* species has demonstrated that insect pollinators have the ability to distinguish between spotted and non-spotted flowers, and seed number of plants having spots in flower petals was 32% higher than that in non-spotted flower plants (Jones, 1996), suggesting that color spots on flower petals may significantly influence pollinator-mediated pollen transfer and seed production. In some *Rhododendron* species, swallowtail butterflies and lasioglossums frequency visited reddish flower, while bees and flower flies preferred to visit light-purplish flowers (Tagane, 2008). Asen (1972) also indicated that flavones/flavonols seems to be a key modulators of anthocyanins for pollinator attraction. Thus, the reddish-purple color of blotches in *R. simsii* flowers, which is caused by the co-pigmentation between anthocyanins and flavonols, might attract pollinators due to the wider color range perception.

When breeding program using *R. simsii* will be done, not only anthocyanin inheritance, but also flavonol inheritance should be considered because the co-pigmentation effect is one of the most important factors of flower color expression in azaleas. Heursel and Horn (1977) identified the gene Q that is responsible for the presence of quercetin glycoside in *R. simsii* flowers. De Loose (1969) noted that scarlet and salmon flowers of the Belgian hybrids of *R. simsii* have low flavonol content, while the petals of deep red, magenta and blue red flowers contain high flavonol content. Red flowered *R. simsii* is believed to have a small prospect for gaining bluish flower color due to the low flavonol content and epidermal pH (Heursel, 1975). However, interspecific crossing with blue tone flowered species may be a promising mean to produce bluer shade flowers by increasing the amount of flavonols sufficient to form full co-pigmentation in the hybrids. Interspecific crossing should be carried out in further studies to confirm the color inheritance, and produce wide color variation in *R. simsii* in Vietnam.

Summary

In this experiment, pigmentation of reddish-purple blotch areas in the three upper petals of *R. simsii* flowers was investigated. Reddish-purple color expression of blotch areas in *R. simsii* flowers was due to the presence of two cyanidin glycosides and flavonol glycosides in the cells, which were distributed in the subepidermis of upper petals. Furthermore, when anthocyanins and flavonols co-pigmented at the ratio of 1:7.5, respectively, reddish-purple color would be formed. Within the reddish-purple cells, flavonols amount was much higher than that of anthocyanins. In term of whole blotch areas, however, anthocyanins were almost double in the content compared to flavonols.

Section II-2

PIGMENT CONSTITUTION OF *RHODODENDRON SIMSII* FLOWERS

Introduction

As for all flowering plants, flower color is one of the most important features for breeding. Flavonoids mainly account for coloration of flowers. They are a group of secondary metabolites belonging to the class of phenylpropanoids, have the widest color range, from pale-yellow to blue. In azalea flowers, flower color ranges from purple through carmine red, red, pink and white. Anthocyanins tend to occur mainly as cyanidin, and azaleatin is the most common flavonol (De Loose, 1969). Mizuta et al. (2009) and Nakatsuka et al. (2015) investigated and suggested that red-flowered azaleas such as *R. indicum, R. kaempferi, R. olhamii* and *R. scarbrum* contain two major anthocyanins in their petals. As mentioned in the former section, these two major anthocyanins were identified as Cy 3Ga and Cy 3Ar (Hang et al., 2011).

In addition to anthocyanins, flavonols are biologically important because they are involved in flower color as co-pigments and have insect attractant properties due to their UV-light absorbing properties. Flavonol glycosides are frequently found together with anthocyanins but the occurrence of common flavonols (quercetin, kaempferol and myricetin) is much more wide spread (Harborn, 1965, 1967b; Swain, 1976; Wollenweber and Jay, 1988). In previous section, co-pigmentation of anthocyanin-flavonol was carried out, and the effect on flavonol on coloration of reddish-purple blotch areas of *R. simsii* flowers was revealed. However, the flavonols isolated from reddish-purple blotches of *R.*

simsii flowers have not been yet identified.

Flavonols of some wild evergreen azalea species were effectively analyzed by HPLC and TLC analysis (Sakata et al., 1991, 1993; Miyajima et al., 1995, 1997, 2001). In this section, flavonol constitution in the reddish-purple blotch areas was detected and identified using chromatographic analyses, and the flavonol properties in the flowers of several red flowered azalea species were also discussed.

Materials and Methods

Plant materials

Plant materials were the same as described in Section II-1.

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 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. simsii* 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 x 250 mm; Nacalai Tesque, Kyoto, Japan) at 40^{o}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 collected using a fraction collector.

Identification of flavonol aglycones

Each purified flavonol was acid hydrolyzed using 2N HCl at 100^{o}C for 90 min. The flavonol aglycones were co-chromatographed with authentic standard flavonol aglycones, such as myricetin, quercetin and kaempferol, by HPLC with constant flow of 75% solvent A (0.1M HOAc): 25% solvent B (MeCN). Other HPLC conditions (pump, column, column oven temperature and flow rate) were same as mentioned in the section II-1 (HPLC analysis). Wavelength was set at 360 nm.

Co-chromatography by TLC analysis

After isolating from preparative HPLC, each purified flavonol fraction was dried by evaporator and diluted by drops of MeOH. Each pigment was spotted on 10 cm × 20 cm cellulose–coated glass plates (Merck, Darmstadt, Germany). In addition, four authentic standard quercetin glycosides: quercetin 3-glucoside (isoquercetin), quercetin 3-galactoside (hyperoside), quercetin 3-rutinoside (rutin) and quercetin 3-rhamnoside (quercitrin) were also spotted in order to compare with two major flavonols. Thin layer chromatography analyses were performed by three mobile phases according to the method of Harborne (1959): BAW (*1*-BuOH/AcOH/H_2O, 4:1:2, v/v/v), 100% H_2O and 15% AcOH. Then all spots were observed under UV light (365 nm) and Rf values were also recorded.

Co-chromatography by HPLC analysis

In order to compare the retention times of two major flavonols and four authentic standard quercetin glycosides (same as above), HPLC analyses were conducted in three different solvent systems, i.e., solvent system I: A (1.5% H_3PO_4) and B (1.5% H_3PO_4: 20% AcOH: 25% CH_3CN), B conc.: 20%-85% at 40 min; solvent system II: A (4% H_3PO_4) and B (CH_3CN), B conc.: 15%-30% in 30min; solvent system III: A (0.1M AcOH) and B (CH_3CN) with constant flow. The wavelength of the analyses was 360 nm. Other HPLC conditions (pump, column, column oven temperature and flow rate) were same as mentioned in Section II-1.

LC-MS analysis

Two purified major flavonols were analyzed using LC-MS by TSK gel ODS-80Ts QA 2.0 ϕ × 150 mm column. A constant flow was applied for 30 min at 20% solvent B (MeCN) in solvent A (0.1 M AcOH) with a flow rate of 0.2 $mL \cdot min^{-1}$ and 40^{o}C of column temperature.

Results and Discussion

Eight discrete spots appeared in the TLC plates (Figure II-9) and color properties were recorded under visible and UV light (Table II-2). Spot numbers 1 and 2 expressed red-lilac color under visible light and violet under UV light. These two spots were considered to be 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

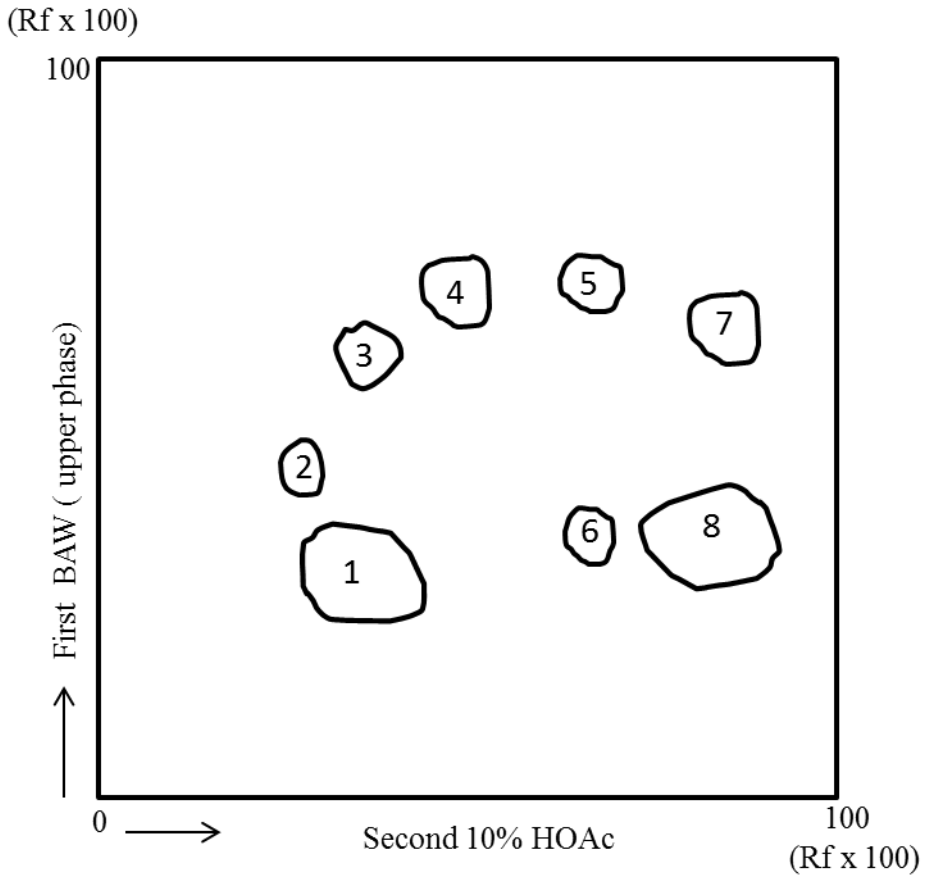


Figure II-9. Two-dimensional thin layer chromatogram of MeOH extract from upper petals of *R. simsii* flowers. BAW (*1*-BuOH/AcOH/H_2O, 4:1:2, v/v/v). See Table II-2 for color properties of spots.

Table II-2. Color properties of pigments extracted from the petals of *R. simsii* flowers and separated by two-dimensional thin layer chromatography.

Spot no.	Color in	
	Visible light	UV-light
1	Red-lilac	Violet
2	Red-lilac	Violet
3	Pale brown	F.[y] yellow
4	Pale brown	F. yellow
5	-[z]	F. blue
6	-	F. blue
7	-	F. blue
8	-	F. blue

[z] without color.

[y] fluorescent.

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 P2 and P1, respectively (Figure II-10).

These two major pigments in upper petals were isolated by preparative HPLC, and acid hydrolyzed using 2N HCl. Acid hydrolyzed products showed only one peak for each major pigment, and both appeared at 15 min of retention time (Figure II-11). In comparison to authentic standard samples, such as myricetin, quercetin and kaempferol, the aglycones of two major peaks were identified as quercetin (Table II-3).

The result of co-chromatography by TLC with four authentic quercetin glycosides showed that the R_f value of pigment 1 (P1) was almost same as Qu 3Gl or Qu 3Ga in three different solvent systems and could not be distinguished. Pigment 2 (P2) seemed to be quercetin 3-rhamnoside (Table II-4). However, HPLC analysis results indicated the differences in retention times between two standard flavonols Qu 3Gl and Qu 3Ga, and the retention time of P1 was close to that of Qu 3Gl. Thus, P1 and P2 were almost coincided with quercetin 3-glucoside and quercetin 3-rhamnoside, respectively (Table II-5). The molecular ion peak of P1 [*m/z* 465.09 ($M + H^+$)] and P2 [*m/z* 449.10 ($M + H^+$)] by LC-MS analysis also supported the results of TLC and HPLC analyses (Table II-6). Chemical structures of P1 and P2 were shown in Figure II-12.

De Loose (1969) reported that quercetin glycosides (3-rhamnoside or 3-galactoside) seem to be popular in the flower of *R. simsii* hybrids. However, this is the first

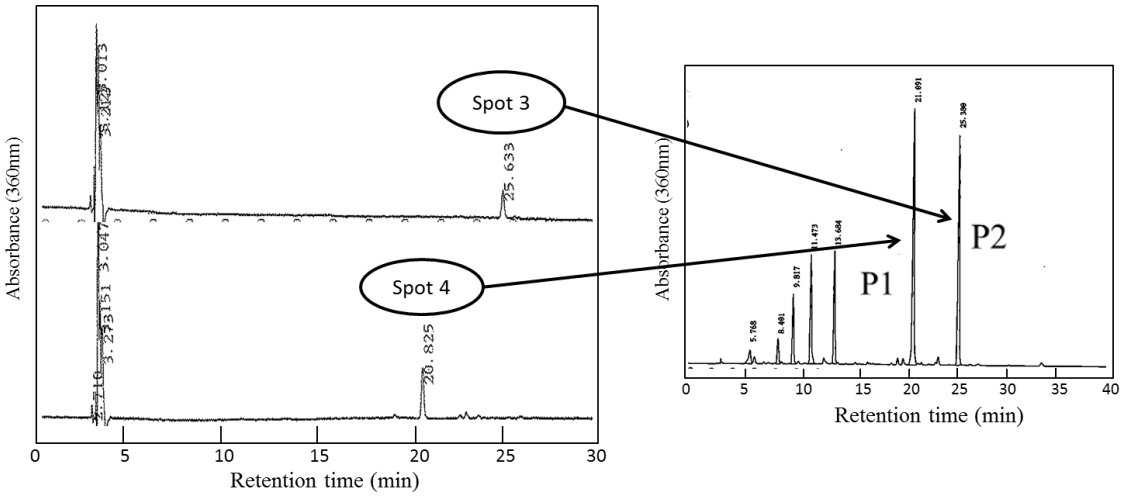


Figure II-10. HPLC analysis of spots 3 and 4 collected from two dimensional TLC plate.

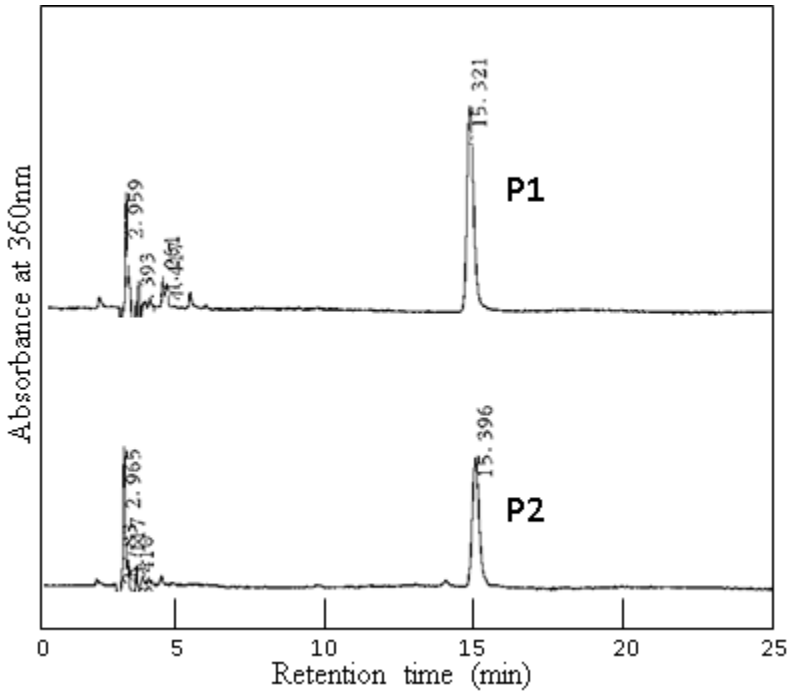


Figure II-11. HPLC tracing of acid hydrolysates of P1 and P2 with 2N HCl.

Table II-3. Retention time of standard flavonols and flavonol aglycones of *R. simsii* flowers.

Aglycones	Retention time (min)
Myricetin	8.0
Quercetin	15.2
Kaempferol	28.8
Pigment 1	15.3
Pigment 2	15.3

Table II-4. TLC analysis of P1, P2 and four authentic standard quercetin glycosides in different solvent systems.

Flavonol glycosides	R_f (× 100) in		
	BAW [z]	H_2O	15% AcOH
Pigment 1	69	06	27
Pigment 2	84	12	40
Quercetin 3-glucoside (isoquercetin)	68	07	29
Quercetin 3-galactoside (hyperoside)	64	09	31
Quercetin 3-rutinoside (rutin)	61	35	53
Quercetin 3-rhamnoside (quercitrin)	84	13	40

[z] *1*-butanol: AcOH: DW (4:1:2; v/v/v)

Table II-5. Retention times of P1, P2 and four authentic standard quercetin glycosides in different solvent system of HPLC analysis.

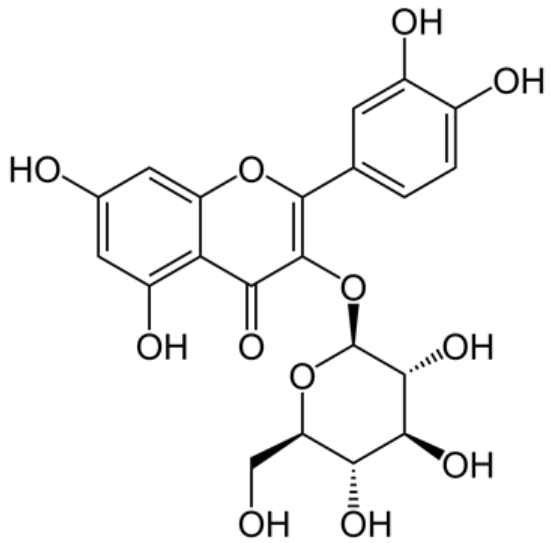
Flavonol glycosides	Solvent system [z]		
	I	II	III
Pigment 1	23.2	13.5	7.2
Pigment 2	27.8	18.0	11.1
Quercetin 3-glucoside (isoquercetin)	23.9	13.9	7.7
Quercetin 3-galactoside (hyperoside)	21.7	15.1	7.4
Quercetin 3-rutinoside (rutin)	23.3	12.6	6.4
Quercetin 3-rhamnoside (quercitrin)	27.7	17.8	11.4

[z] I: solvent A (1.5% H_3PO_4) and solvent B (1.5% H_3PO_4: 20% AcOH: 25% CH_3CN); B conc.: 20% - 85% at 40 min.
II: solvent A (4 % H_3PO_4) and solvent B (Acetonitrile); B conc.: 15% - 30% at 30 min.
III: solvent A (0.1 M AcOH) and solvent B (Acetonitrile); B conc.: 20% (constant flow).

Table II-6. Molecular weight of flavonol glycosides of P1 and P2.

Flavonol glycosides	Molecular weight (g/mol)
Pigment 1 ($M + H^+$)	465.09
Pigment 2 ($M + H^+$)	449.10
Quercetin 3-glucoside (isoquercetin)	464.37
Quercetin 3-rutinoside (rutin)	610.52
Quercetin 3-rhamnoside (quercitrin)	448.38

P1

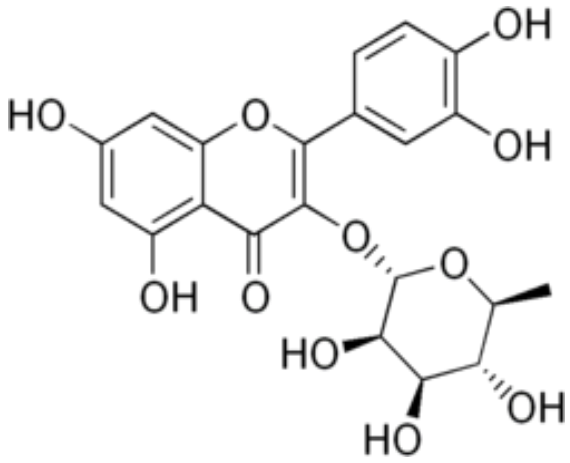


Quercetin 3-glucoside

(Isoquercetin)

$C_{12}H_{20}O_{12}$

P2



Quercetin 3-rhamnoside

(Quercitrin)

$C_{12}H_{20}O_{11}$

Figure II-12. Chemical structures of P1 and P2 in upper petals of *R. simsii* flowers.

report about flavonol composition in wild *R. simsii* distributed in Vietnam.

The flavonol composition of several wild red flowered species such as *R. simsii* dítributed in Iriomotejima Island of Japan, *R. indicum* (Yakushima Island, Japan), *R. oldhamii* (Taiwan) and *R. scabrum* (Okinawa Island, Japan) were also surveyed by HPLC analysis. The results showed that upper petals of these species also contained two major flavonols (Qu 3Gl and Qu 3 Rh), which were identified in *R. simsii* flowers from Vietnam in this study (Figure II-13).

From these results, quercetin 3-glucoside (isoquercetin) and quercetin 3-rhamnoside (quercitrin) present as the two major flavonols in the reddish-purple blotch areas of the flowers of *R. simsii* and several red-flowered evergreen azalea species. These two flavonols play an important role on co-pigmentation phenomenon. Reddish-purple color of blotch areas is due to quercetin 3-glucoside and quercetin 3-rhamnoside co-pigmented with cyanidin 3-galactoside and cyanidin 3-arabinoside. This detailed understanding about pigmentation of flowers will contribute necessary knowledge for flower color breeding in the further study using Vietnamese wild *R. simsii* as the important genetic resources.

Summary

Whole petals of *R. simsii* contained two major anthocyanins while two major flavonols were detected only in upper petals, especially in reddish-purple blotch areas. Two major flavonols were identified as quercetin 3-glucoside and quercetin 3-rhamnoside. These flavonols seem to be popular pigments in the reddish-purple blotches of red-flowered evergreen azalea species.

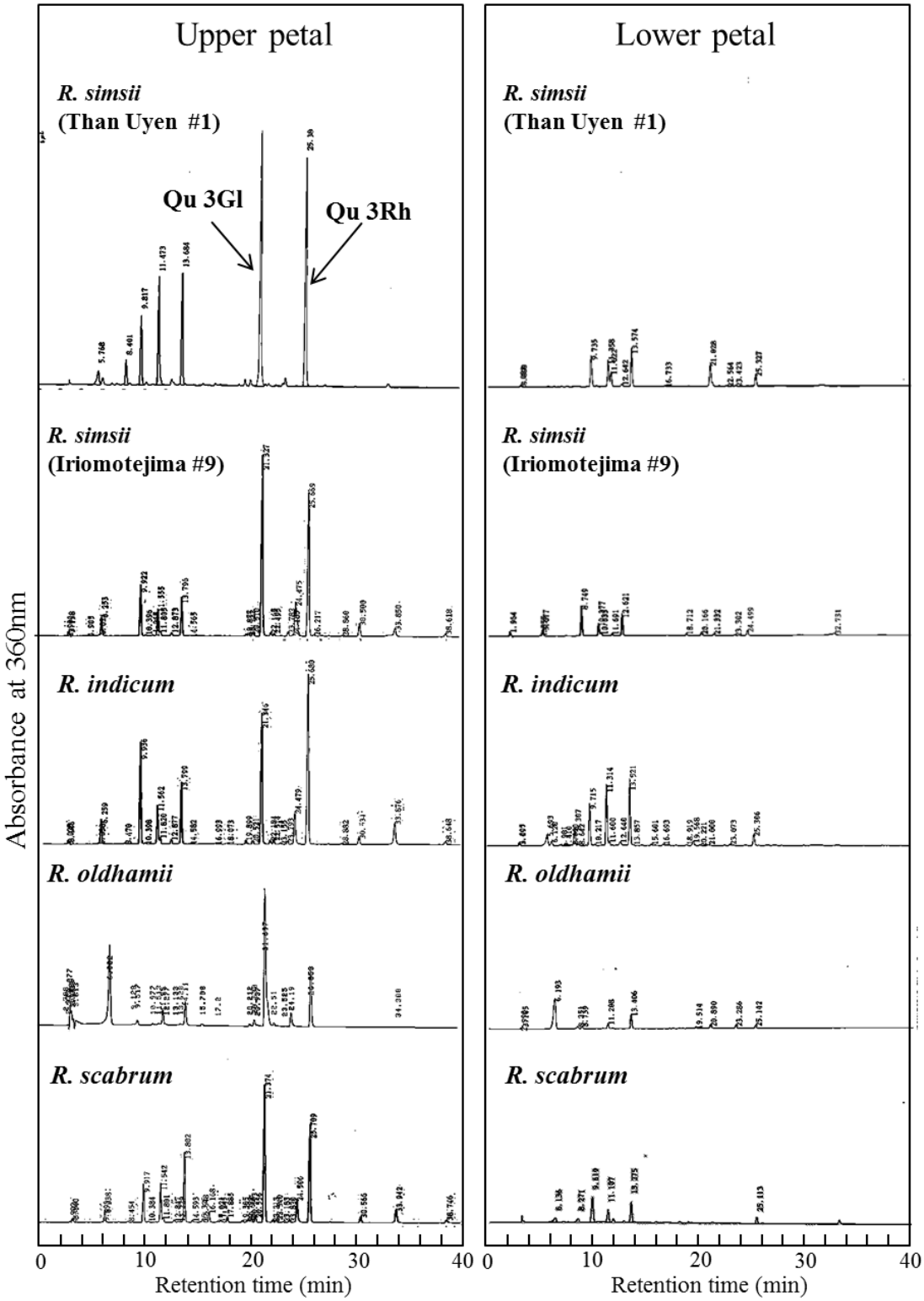


Figure II-13. HPLC analysis of extracts from upper and lower petals of *R. simsii* and several red-flowered evergreen azalea species. Qu 3Gl: quercetin 3-glucoside; Qu 3Rh: quercetin 3-rhamnoside.

Chapter III

INTERSPECIFIC CROSS COMPATIBILITY BETWEEN VIETNAMESE *RHODODENDRON SIMSII* AND SEVERAL EVERGREEN AZALEA SPECIES

Introduction

Evergreen azaleas are important ornamental plants in many regions in the world such as USA, European countries and Japan. They are usually planted for decorating gardens, streets, parks or public infrastructures. For hundred years, azaleas have been hybridized and over 10,000 different cultivars or cultivated varieties have been registered or named (Azalea Society of America, 2007). They provide a wide variety of plant habits, size, colors and blooming times to meet almost every landscaping need or personal preference (Kunishige, 2002).

Among evergreen azaleas, interspecific hybridization is known to be one of the most effective means of expanding morphological variations among cultivars (Michishita et al., 2003). Mizuta et al. (2014) reported that interspecific hybridization between wild species with purple series flowers and wild species with red series flowers resulted in variations of pigment composition and anthocyanin synthesis-related gene expression. Several studies reported about the cross compatibility among evergreen azaleas distributed in Japan (Akabane et al., 1971; Kunishige, 1983; Yamaguchi et al., 1985; Michishita et al., 2003). The cross compatibility among Japanese evergreen azaleas is generally high; thus resulting in natural hybridization occurrence in southern Kyushu (Tagane, 2008). Hang (2011) has confirmed the cross compatibility and hybridity between *R. simsii* distributed in

the mountainous areas of central part of Vietnam and several evergreen azalea species in Japan. Hence, the information of cross compatibility in different accessions of *R. simsii* is necessary for establishing breeding program in Vietnam.

In this chapter, the interspecific hybridization between wild *R. simsii*, collected from northern Vietnam and several evergreen azalea species originated in Japan and Taiwan has been conducted.

Materials and Methods

Plant materials

Three accessions of *R. simsii* distributed in Than Uyen district, Lai Chau province, Vietnam and several evergreen azalea species (*R. eriocarpum*, *R. kaempferi* var. *macrogemma*, *R. kiusianum*, *R. oldhamii* and *R. tosaense*) cultivated in a greenhouse of Kyushu University were used in this study. Their collected site and origin are described in Table III-1 and Figure III-1.

Pollen fertility

Flower buds of three accessions of wild *R. simsii* (pollen parents) were collected from Than Uyen, Lai Chau, Vietnam in March 2013, 2014, 2015 and 2016. Anthers were separated from flower buds and kept in the transparent capsules at -20^{o}C until use. Pollen grains were sown in the petri dish which contained 0.5% agar and 5% sucrose media. All dishes were placed into an incubator for 5 hours at 25^{o}C. After 5 hours, observation of pollen germination was carried out under a digital microscope (Leica DM-2500; Leica Microsystems GmbH, Wetzlar, Germany). The pollen grains were defined as germinated

Table III-1. Plant materials used in this study and their source.

Species	Code	Source
Pollen parent		
R. simsii #1	SIM 1	Than Uyen district, Lai Chau province, Vietnam
R. simsii #2	SIM 2	
R. simsii #3	SIM 3	
Seed parent		
R. eriocarpum #18	ERI 18	Tokara Islands, Japan
R. kaempferi var. *macrogemma* #1	KAM 1	Izuoshima Island, Japan
R. kiusianum #2	KIU 2	Kirishima Mountains, Japan
R. oldhamii #1	OLD 1	Alishan Mountain, Taiwan
R. tosaense #1	TOS 1	Kitakata, Miyazaki, Japan

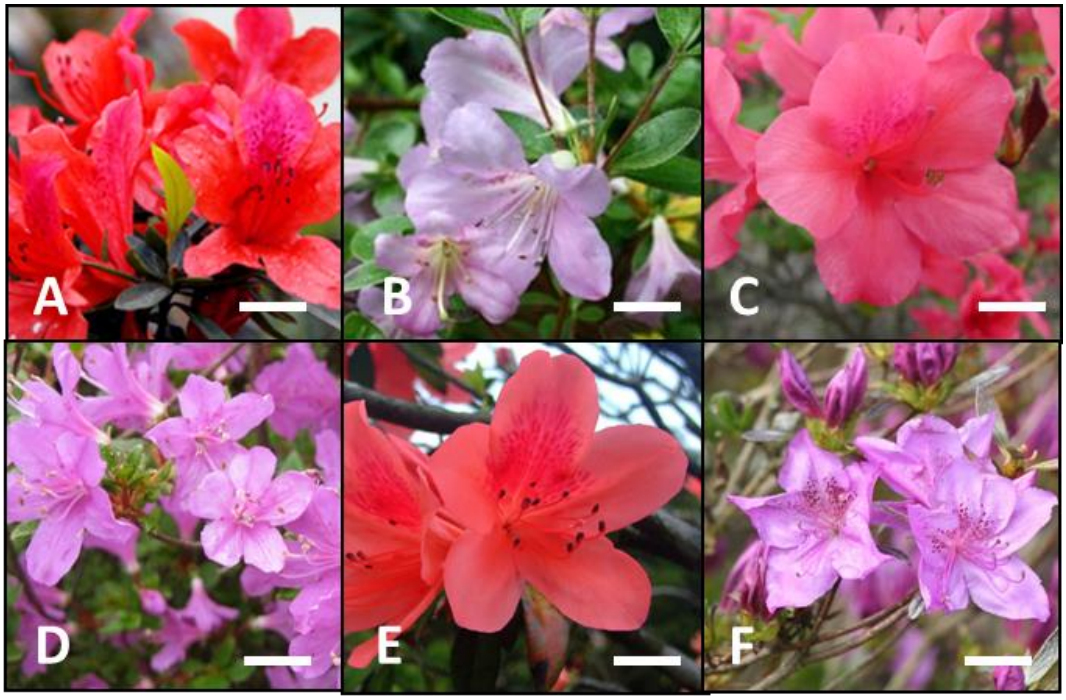


Figure III-1. Flowers of *R. simsii* (A), *R. eriocarpum* (B) *R. kaempferi* var. *macrogemma* (C), *R. kiusianum* (D), *R. oldhamii* (E) and *R. tosaense* (F). Bar = 3 cm.

when the pollen tube length reached the pollen diameter (Brown, 1958). Germination rate was also calculated by the following formula:

$$\text{Germination rate (\%)} = \frac{\text{Number of germinated pollen grains}}{\text{Total number of pollen grains observed}} \times 100\%$$

Germination test was replicated three times in each accession.

Interspecific crossing

Interspecific crosses between Vietnamese *R. simsii* and Japanese and Taiwanese evergreen azaleas were carried out from October to November in 2013, from April to June 2014 and from April to June 2015. Crossing method was followed as the method of Sakai *et al.* (2006). Before anthesis, selected flowers of seed parent plants were emasculated and covered by paper bags to avoid contamination of other pollen source. At anthesis, flowers were pollinated with pollen of *R. simsii* by hand and recovered by paper bags to prevent contamination by undesirable pollen.

Capsule set, number of seeds per capsule

Capsule setting was observed about 90 days after pollination. Set capsules were harvested 5-6 months after pollination. Harvested capsules were allowed to dry at room temperature (25^{o}C) until they naturally opened. Capsules setting rate and number of seeds per capsule in each cross were recorded**.**

Seed germination of F_1 progenies

One hundred seeds per cross were sown on sphagnum moss in April 2014 and 2015.

They were incubated in a greenhouse. Three replications were carried out. Number of germinated seeds was counted after one month of culture. Germination rate was calculated by (number of germinated seeds/ number of sown seeds) × 100%. Two months after seed germination, green and vigorous seedlings were transplanted on the pumice soil tray and transplanted on 7 cm diameter pots containing pumice soil six months after germination.

Molecular analysis of hybridity of F_1 offspring

In the present study, RAPD analysis was used to confirm the hybridity of F_1 offspring obtained from the crosses between several evergreen azalea species and Vietnamese *R. simsii*. Detailed materials used in this analysis were shown in Table III-2. Total genomic DNA was extracted from approximately 80 mg of fresh leaves by a modified CTAB method (Kobayashi et al., 1998). Random amplified polymorphic DNA (RAPD) analysis was conducted with CMN-A02 (5'-GCCAGCTGTACG-3') and CMN-B27 (5'-CGCAGCCGAGAT-3') common primers (BEX, Co., Ltd., Tokyo, Japan) to confirm hybridity of the seedlings followed the method of Okamoto and Ureshino (2015). Amplifications were performed in 25 μL of reaction solution containing 20 ng of genomic DNA, 0.5 μM primers, 0.1 mM dNTPs, 1× the original reaction buffer including 2 mM $MgCl_2$, and 0.5 units EX *Taq* DNA polymerase (Takara Bio Inc., Otsu, Japan). DNA was amplified with a DNA thermal cycler dice gradient (TP600; Takara Bio Inc., Otsu, Japan) as follows: 1 cycle of 30 sec at 90°C; 45 cycles of 30 sec at 94°C, 2 min at 37°C, and 3 min at 72°C. The amplified products were electrophoresed in 1.5% agarose gels (agarose I™ Amresco Inc., Solon, Ohio, USA) with 1× TAE. The gel was stained with a Midori Green Advance DNA stain solution (Nippon Genetics, Tokyo, Japan) and observed under LED

Table III-2. Plant materials used for molecular analysis of hybridity of F_1 hybrids and their parents.

Pollen parent, seed parent and F_1 hybrid	Code	Number of samples examined
Pollen parent		
R. simsii #1	SIM 1	1
R. simsii #2	SIM 2	1
R. simsii #3	SIM 3	1
Seed parent		
R. eriocarpum #18	ERI 18	1
R. kaempferi var. *macrogemma* #1	KAM 1	1
R. kiusianum #2	KIU 2	1
R. tosaense #1	TOS 1	1
F_1 hybrid		
R. eriocarpum #18 × *R. simsii* #1	ERI 18× SIM 1	5
R. eriocarpum #18 × *R. simsii* #2	ERI 18 × SIM 2	5
R. kaempferi var. *macrogemma* #1 × *R. simsii* #1	KAM 1 × SIM 1	5
R. kaempferi var. *macrogemma* #1 × *R. simsii* #2	KAM 1 × SIM 2	5
R. kaempferi var. *macrogemma* #1 × *R. simsii* #3	KAM 1 × SIM 3	1
R. kiusianum #2 × *R. simsii* #1	KIU 2 × SIM 1	5
R. tosaense #1× *R. simsii* #2	TOS 1 × SIM 2	4

100 illumination (AMZ System Science, Osaka, Japan). Each polymorphic primer was tested at least three times to ensure reproducibility of polymorphism and the banding patterns.

Leaf shape index

Leaves of *R. simsii*, several evergreen azalea species and their hybrids were measured for length and width by using a digital caliper (model 19971, Shinwa rules Co., LTD. Japan). Then leaf shape index was calculated by dividing leaf length by leaf width. Statistical analysis was performed using Tukey test.

Results and Discussion

Pollen quality is very important for growers and breeders. Pollen grains were viable if they had germinated in the medium (Sulugoglu, 2014). The pollen tube germination of three accessions of *R. simsii* was observed under microscope (Figure III-2). The results showed that the viability of pollen of three *R. simsii* accessions in 2013, 2014 and 2015 was high with the range from 61.8 to 70.1% (Table III-3). Statistical analysis using Tukey test showed that no significant difference was found among pollen germination rate of three accessions at the $P < 0.05$. Based on this result, *R. simsii* collected from Vietnam can be considered as good pollen donors when we breed it with several Japanese and Taiwanese evergreen azalea materials.

Capsule set was observed in all of the crosses (Table III-4). In total of all crosses from 2013 to 2015, 162 of 190 pollinated flowers (85.3%) set capsules. Especially, in 2014, 100% capsule setting rates were obtained in the crosses between KAM 1 and SIM 1, 2, 3.

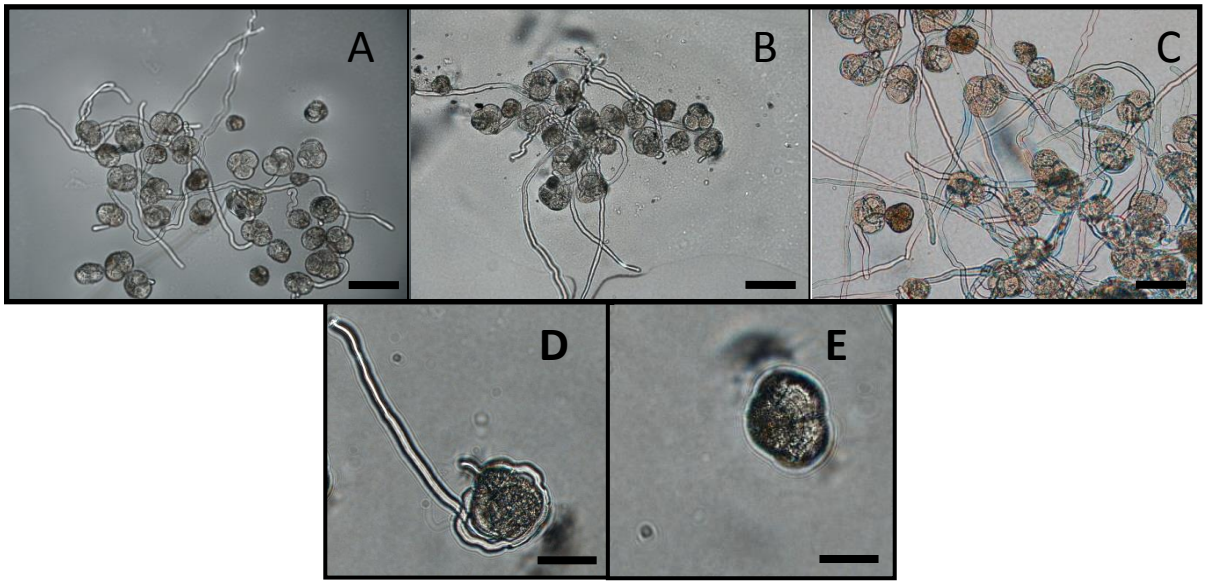


Figure III-2. Pollen germination in SIM 1 (A), SIM 2 (B) and SIM 3 (C) on 0.5% agar + 5% sucrose media. Pollen tube growth in germinated pollen (D) and ungerminated pollen (E). Bar= 100 μm in (A), (B) and (C); bar = 40 μm in (D) and (E).

Table III-3. Germination rate of pollens of three *R. simsii* accessions collected in Vietnam in 2013, 2014 and 2015.

Code [z]	2013			2014			2015		
	Total number of pollen grains observed	Number of pollen grains germinated	Germination rate [y] (%)	Total number of pollen grains observed	Number of pollen grains germinated	Germination rate (%)	Total number of pollen grains observed	Number of pollen grains germinated	Germination rate (%)
SIM 1	313	185	59.1 a [x]	374	189	50.5 a	NE	NE	NE
SIM 2	403	266	66.0 a	255	171	67.1 a	630	465	73.8 a
SIM 3	338	289	79.6 a	462	313	67.7 a	492	327	66.5 a
Mean	351.3	240	68.2	363.7	224.3	61.8	561.0	396.0	70.1

[z] See Table III-1.

[y] (number of germinated pollen/ total number of observed pollen) x 100.

[x] Difference between means were compared by Tukey's test (P=0.05) for data of 2013 and 2014, and by T-test (P=0.05) for data of 2015. Means within a column with the same letter are not significantly different.

NE: not examined

Table III-4. Capsule setting rate in interspecific crosses between Vietnamese *R. simsii* and Japanese and Taiwanese evergreen azalea species.

Year	Seed parent	pollen parent [z]	No. of obtained capsules/ No. of pollinated flowers	Capsule setting rate[y] (%)
2013	ERI 18	SIM 1	5/6	83.3
		SIM 2	5/8	62.5
		SIM 3	NE	NE
	OLD 1	SIM 1	10/13	76.9
		SIM 2	12/17	70.6
		SIM 3	NE	NE
2014	KAM 1	SIM 1	7/7	100.0
		SIM 2	7/7	100.0
		SIM 3	2/2	100.0
	KIU 2	SIM 1	9/9	100.0
		SIM 2	7/7	100.0
		SIM 3	11/12	91.7
	TOS 1	SIM 1	6/6	100.0
		SIM 2	16/18	88.9
		SIM 3	10/10	100.0
2015	ERI 18	SIM 1	6/7	85.7
		SIM 2	4/5	80.0
		SIM 3	3/4	75.0
	KAM 1	SIM 1	4/4	100
		SIM 2	4/5	80.0
		SIM 3	4/5	80.0
	KIU 2	SIM 1	3/3	100.0
		SIM 2	3/4	75.0
		SIM 3	2/3	66.7
	OLD 1	SIM 1	6/8	75.0
		SIM 2	3/4	75.0
		SIM 3	4/6	66.7
	TOS 1	SIM 1	5/5	100.0
		SIM 2	4/5	80.0
		SIM 3	NE	NE
Total			162/190	85.3

[z] Pollen parents were collected at Than Uyen district, Lai Chau, Vietnam in March 2013 and 2014.

[y] (No. of obtained capsules/ No. of pollinated flowers) x 100%.

NE: not examined.

In 2015, the capsule setting rates were slightly reduced in these same crosses but still at the high rates. The lowest capsule setting rate was observed in 2013 for the crosses of ERI 18 × SIM 2 (62.5%), in 2015 for OLD 1× SIM 3 (66.7%) and in 2015 for KIU 2 × SIM 3 (66.7%). Tamura (1963), Kunishige (1983), Yamaguchi et al. (1985) and Michishita et al. (2003) also reported that in interspecific hybridization between evergreen azaleas capsule setting rate is generally high and in agreement with the result of this study.

Number of seeds per capsule was different in cross combinations (Figure III-3, 4). In 2013 and 2014, number of seeds per capsule was less than 300 in three crosses: OLD 1 × SIM 2, KIU 2 × SIM 2 and KIU 2 × SIM 3. Above 500 seeds in a capsule were obtained in two crosses: OLD 1 × SIM 1 and KAM 1 × SIM 3 (Figure III-3). The result of seed number per capsule in 2015 crosses showed almost the same as that of 2013 and 2014 (Figure III-4). In 2015, number of seeds per capsule was less than 300 in five crosses: KIU 2 × SIM 1, KIU 2 × SIM 2, KIU 2 × SIM 3, TOS 1 × SIM 1 and TOS 1 × SIM 2. Above 500 seeds in a capsule were obtained in four crosses: OLD 1 × SIM 1, KAM 1 × SIM 1, KAM 1 × SIM 2 and KAM 1 × SIM 3. These results indicated that *R. kiusianum* always produces capsules with small number of seeds compared to other species used in this study. According to William et al. (1990), *R. kiusianum* has small flowers with short styles and small ovaries caused small number of seeds per capsule. In contrast, *R. kaempferi* var. *macrogemma* and *R. oldhamii* having relatively big flowers with medium-sized styles and big ovaries can produce numerous number of seeds per capsule. The present data agrees with the experiment of Michishita et al. (2003). In their study, the cross between *R. kaempferi* var. *macrogemma* and *R. simsii* originated in Japan also produced approximately 770 seeds per capsule. Michishita et al. (2003) also pointed out that the male/female style length ratio

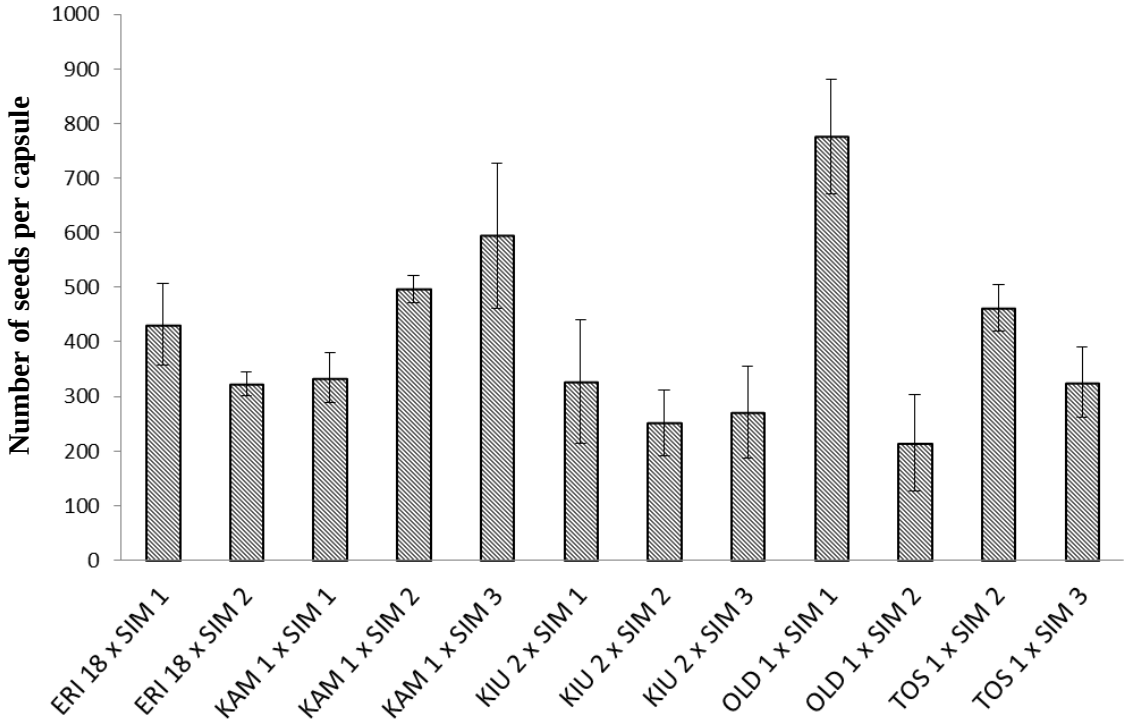


Figure III-3. Number of seeds per capsule in F_1 hybrids obtained in 2013 and 2014 crosses. Bars indicate the standard errors.

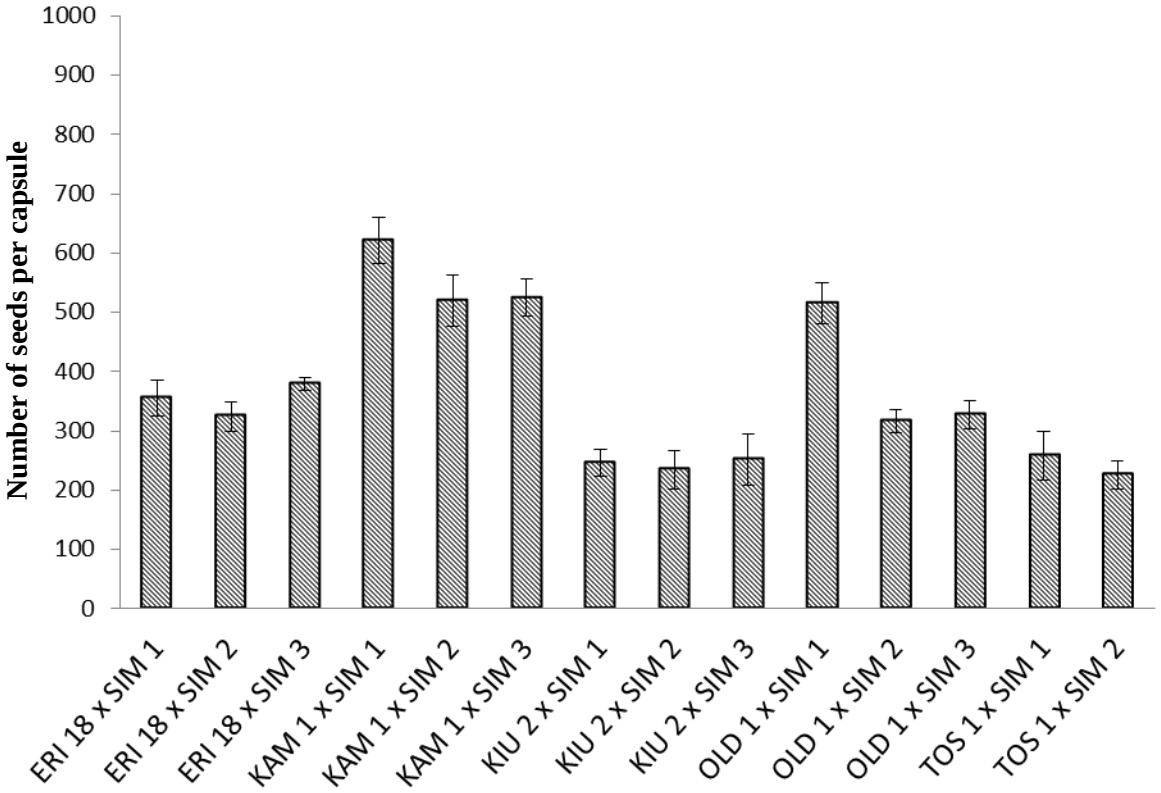


Figure III-4. Number of seeds per capsule in F_1 hybrids obtained in 2015 crosses. Bars indicate standard errors.

(SLR) is a key factor affecting capsule set and number of seed per capsule in every cross between evergreen azalea species. If the range of SLR was 0.7-2.3, about 500 seeds per capsule can be obtained in a cross. By observation, the SLR of KAM and SIM used in this study has almost the same ratio with previous study data (Michishita et al., 2003). Thus, SLR can be considered as a good indicator to evaluate the capsule set and number of seed per capsule in the cross between evergreen azaleas.

Seeds germinated in all crosses and the germination rate varied in different cross combinations (Figure III-5, 6). In 2013 and 2014, more than 50% of seed germination rates were observed in four crosses: OLD 1 × SIM 1, KIU 2 × SIM 1, KAM 1 × SIM 2 and ERI 18 × SIM 1 (Figure III-5). On the other hand, low germination rates of seed (< 30%) were obtained in the crosses of OLD 1 × SIM 2, KIU 2 × SIM 3, KAM 1 × SIM 1, KAM 1 × SIM 3 and TOS 1 × SIM 3. In 2015, almost crosses show higher germination rates compared to those of 2013 and 2014. In details, more than 50% of seed germination rates were observed in almost crosses except for crosses of OLD 1 × SIM 3, TOS 1 × SIM 1 and ERI 18 × SIM 3 (Figure III-6). Seedlings were vigorous and developed green leaves in all crosses. No albino or pale green colored seedlings was observed among the seedlings (Figure III-7). High cross compatibility among evergreen azaleas was reported by Noguchi (1932), Akabane et al. (1971) and Yamaguchi et al. (1985). In comparison to the cross compatibility of *R. simsii* distributed in central Vietnam, which was discussed by Hang (2011), northern Vietnamese accessions also performed relatively similar result. It indicates that all known accessions of Vietnamese *R. simsii* have high cross compatibility with Japanese and Taiwanese evergreen azaleas.

Molecular markers generated from randomly amplified polymorphic DNA (RAPD)

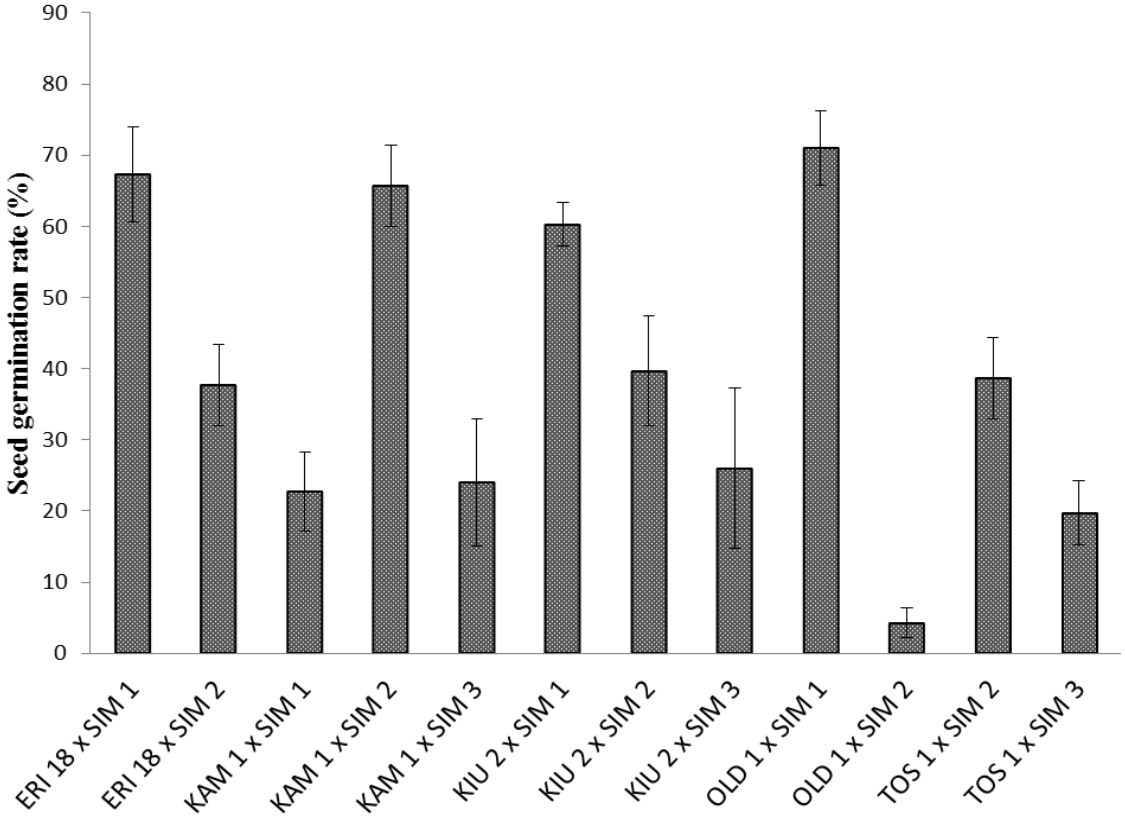


Figure III-5. Seed germination rate of F_1 hybrids obtained from crosses in 2013 and 2014. Bars indicate standard errors.

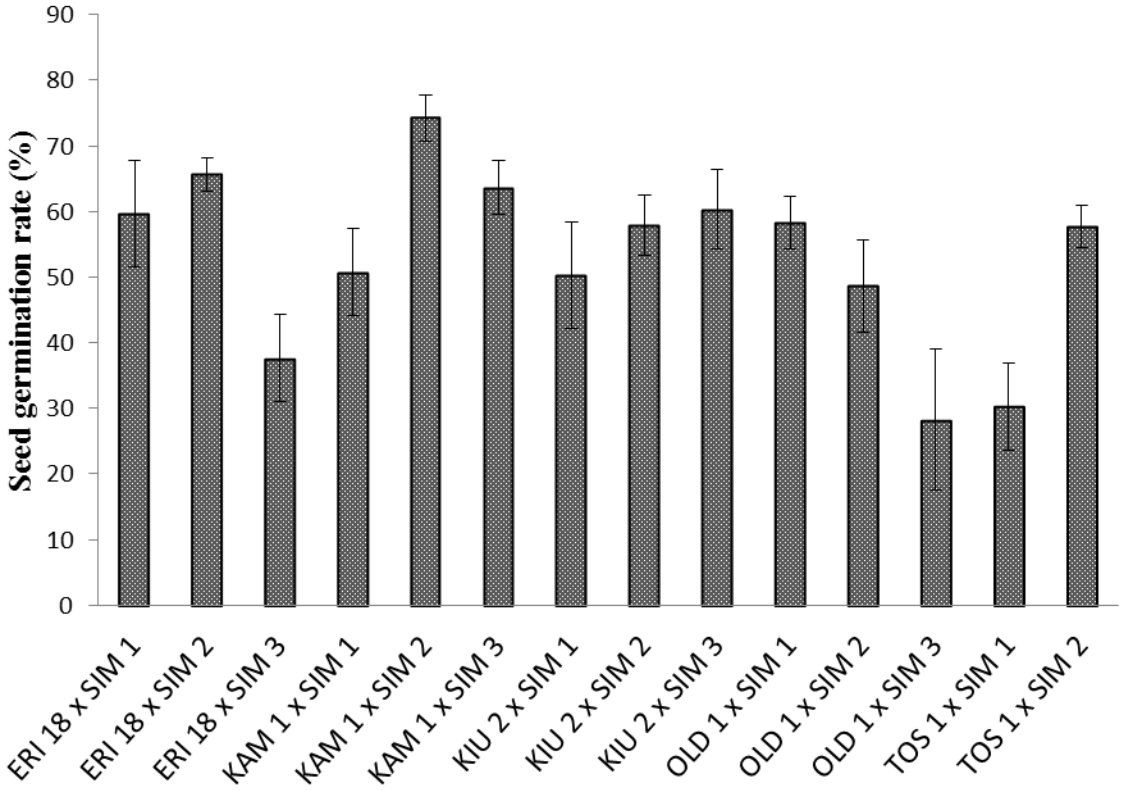


Figure III-6. Seed germination rate of F_1 hybrids obtained from crosses in 2015. Bars indicate standard errors.

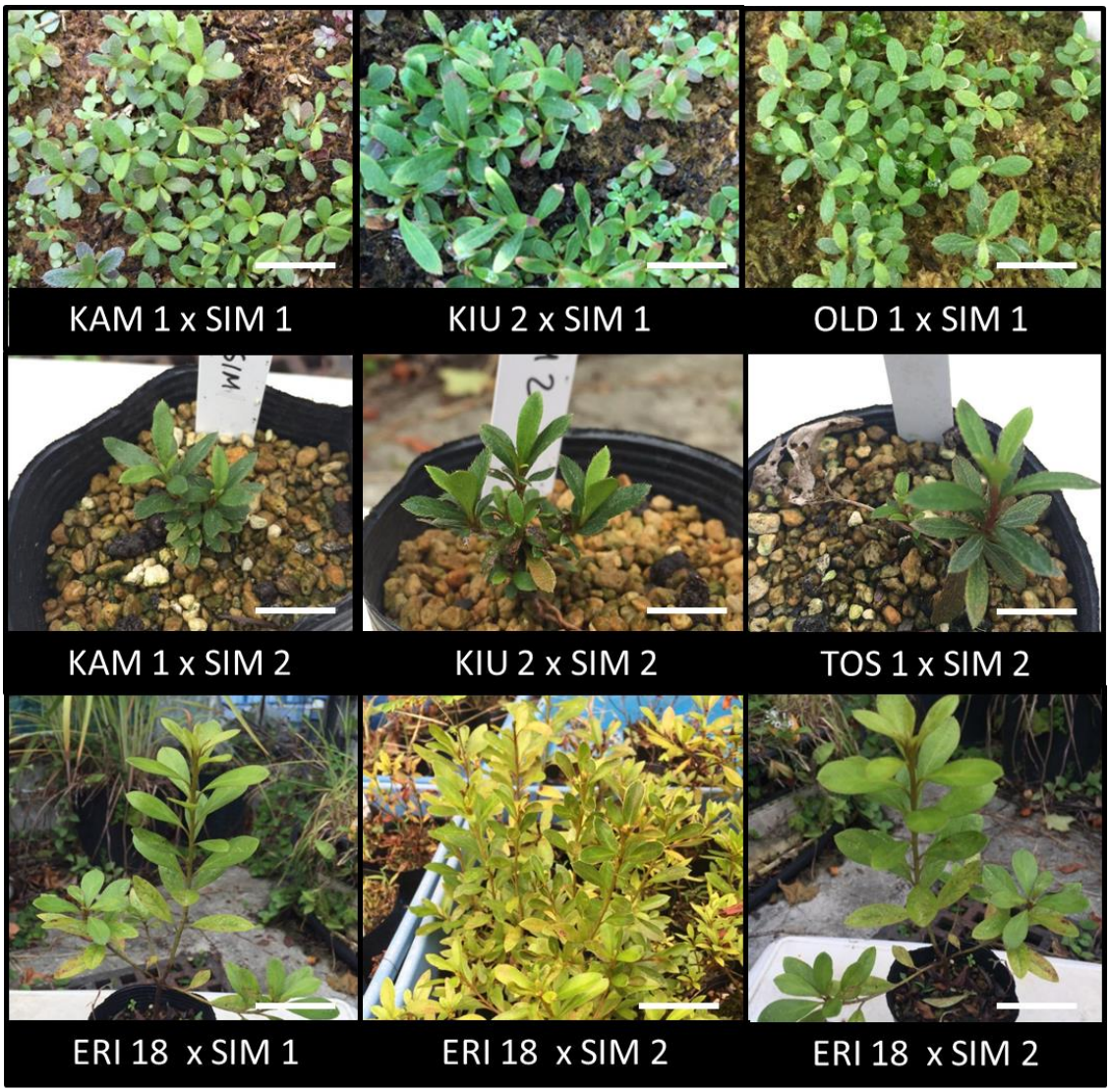


Figure III-7. Green vigorous seedlings obtained from interspecific hybridization between Vietnamese *R. simsii* and several evergreen azalea species originated in Japan and Taiwan. Top: six-month-old seedlings; middle: One-year-old seedlings; bottom: two-year-old seedlings. Bar = 10 cm.

(Williams et al., 1990) have been used extensively to study genetic diversity and to confirm hybrids and their parental species (Amy et al., 2008; Anuj et al., 2007; Lee et al., 2006). To confirm the hybridity of the F_1 seedlings, RAPD analysis on the putative hybrids and their parents was conducted using CMN-A02 and CMN-B27 primers. Almost seedlings were confirmed to be hybrids by CMN-A02 primer because they yielded specific bands derived from both parents. They were ERI 18 × SIM 1, ERI 18 × SIM 2, KIU 2 × SIM 1, KIU 2 × SIM 2, KIU 2 × SIM 3 and TOS 1 × SIM 2 (Figure III-8, 10, 11 (upper)). For the cross combination of KAM 1 and SIM 1, 2, 3, only the specific band from seed parents appeared in the hybrids, and that of pollen parents was not detected (Figure III-9 (upper)). On the other hand, using CMN-B27 primer, specific band from both parents were observed only in seedlings obtained from the crosses of ERI 18 × SIM 1, ERI 18 × SIM 2 and KIU 2 × SIM 2 (Figure III-8, 11 (lower)). For remained cross combinations, F_1 hybrids showed specific bands from seed parents or pollen parents only (Figure III-9, 10, 11 (lower)). However, when the results analyzed by both primers were combined together, it is obvious to understand that F_1 seedlings still yielded specific bands from both parents, for examples, in cases of KAM 1 × SIM 2, KAM 1 × SIM 3, KIU 2 × SIM 1 and KIU 2 × SIM 3 (Figure III-9, 10, 11). From these results, almost F_1 seedlings were confirmed for their hybridity using CMN-A02 and CMN-B27 primers, except seedlings from the cross of KAM 1 × SIM 1. Okamoto and Ureshino (2015) reported that the hybridity of F_1 hybrids of the crosses between *R. uwaense* and five evergreen azalea species were confirmed successfully by RAPD analysis using the same two primers. Thus, RAPD analysis seems to be an effective mean to reveal the hybridity of the offsprings obtained from interspecific hybridization between evergreen azalea species.

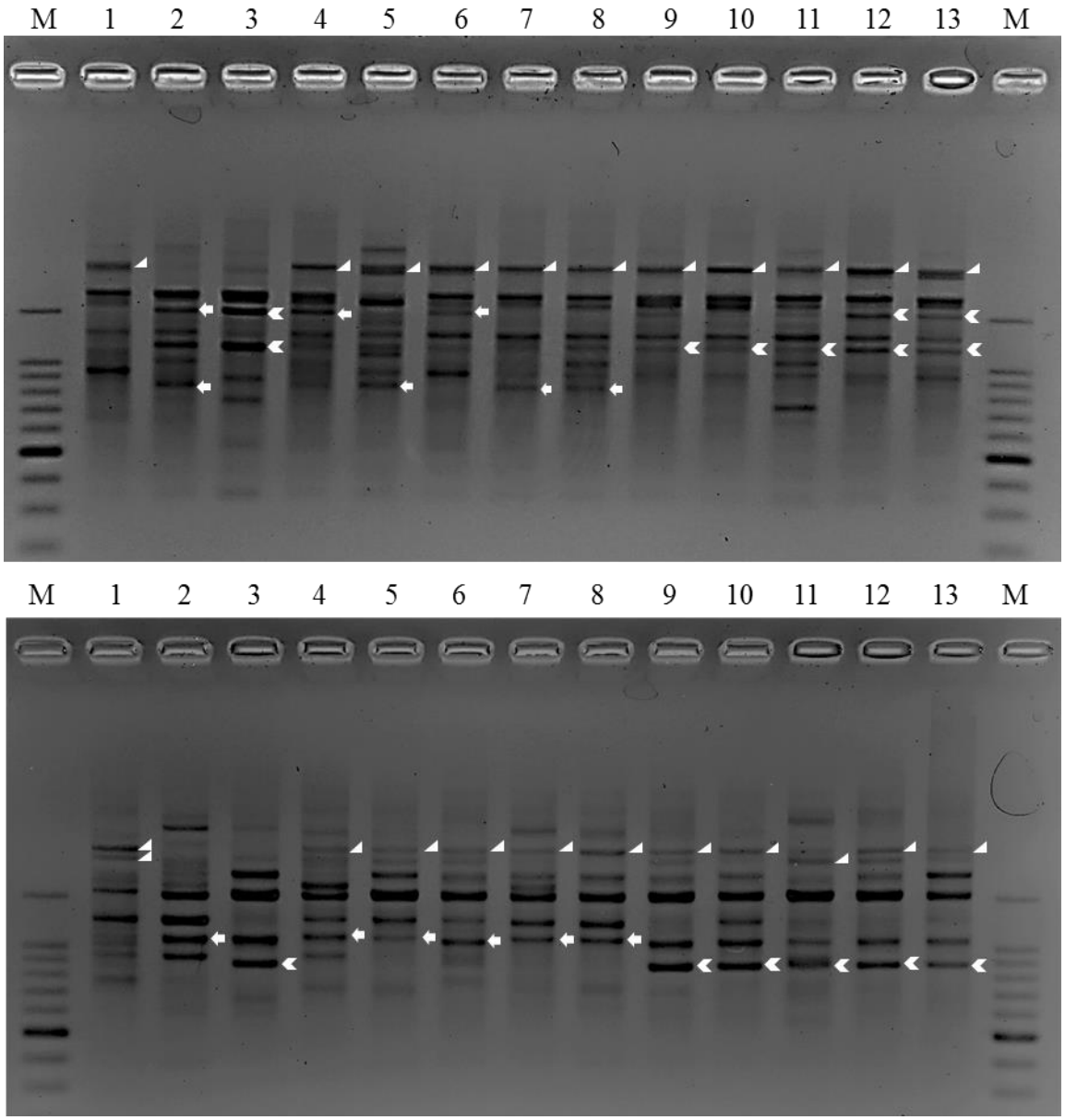


Figure III-8. Amplified polymorphisms of putative hybrid seedlings obtained from the crosses of *R. eripcarpum* #18 with two accessions of *R. simsii* using primer CMN-A02 (upper) and CMN-B27 (lower). Triangulars indicate the specific bands for ERI 18. Arrows indicate the specific band for SIM 1. Chevrons indicate the specific bands for SIM 2. Lane M, 100 bp ladder DNA size markers; Lane 1, ERI 18; Lane 2, SIM 1; Lane 3, SIM 2; Lane 4 to 8, seedlings from ERI 18 × SIM 1; Lane 9 to 13, seedlings from ERI 18 × SIM 2.

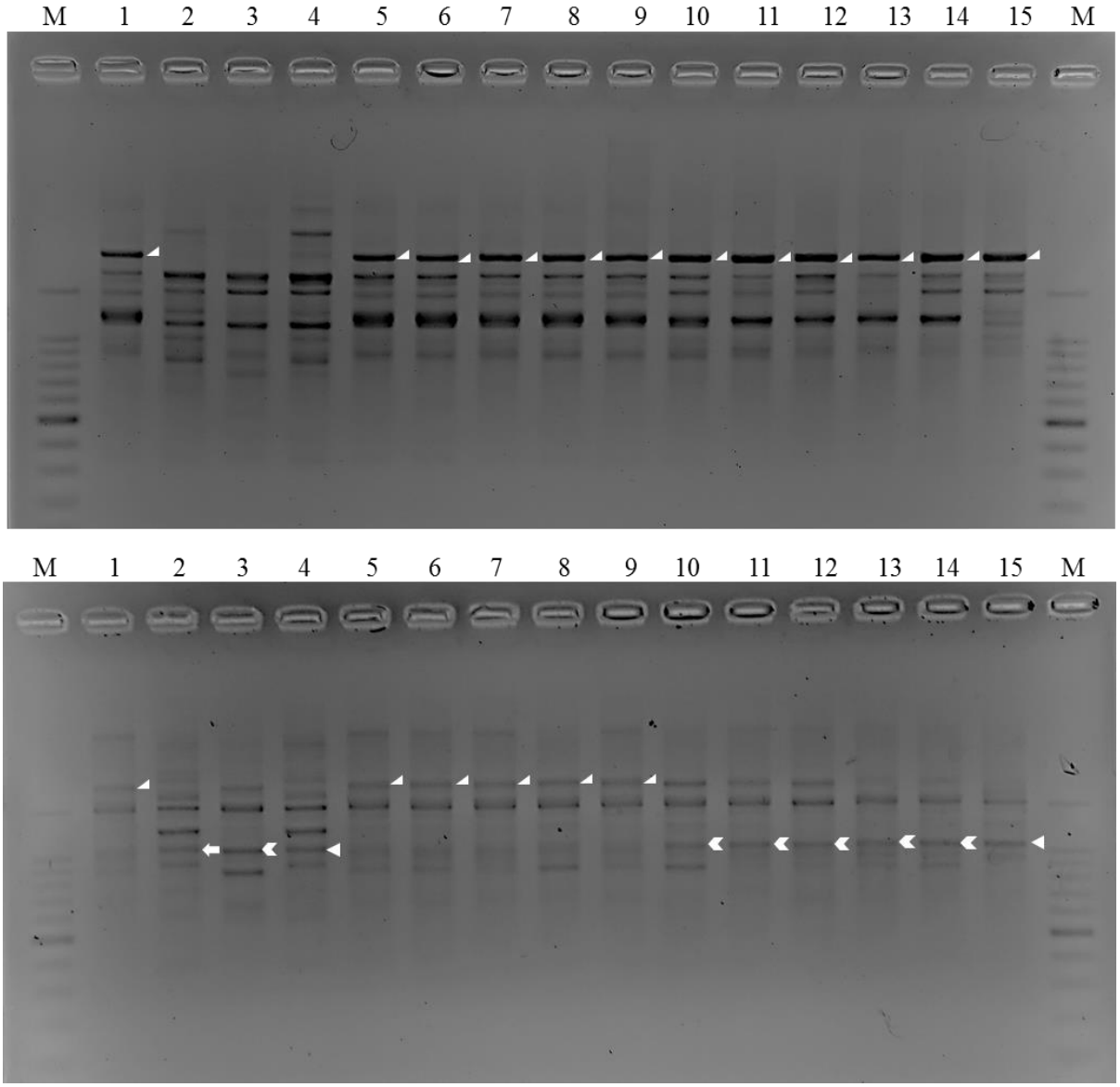


Figure III-9. Amplified polymorphisms of putative hybrid seedlings obtained from the crosses of *R. kaempferi* var. *macrogemma* #1 with three accessions of *R. simsii* using primer CMN-A02 (upper) and CMN-B27 (lower). Triangulars indicate the specific bands for KAM 1. An arrow indicates a specific bands for SIM 1. Chevrons indicate a specific band for SIM 2. Isosceles triangulars indicate the specific bands for SIM 3. Lane M, 100 bp ladder DNA size markers; Lane 1, KAM 1; Lane 2, SIM 1; Lane 3, SIM 2; Lane 4, SIM 3; Lane 5 to 9, seedlings from KAM 1 × SIM 1; Lane 10 to 14, seedlings from KAM 1 × SIM 2; Lane 15, a seedling from KAM 1 × SIM 3.

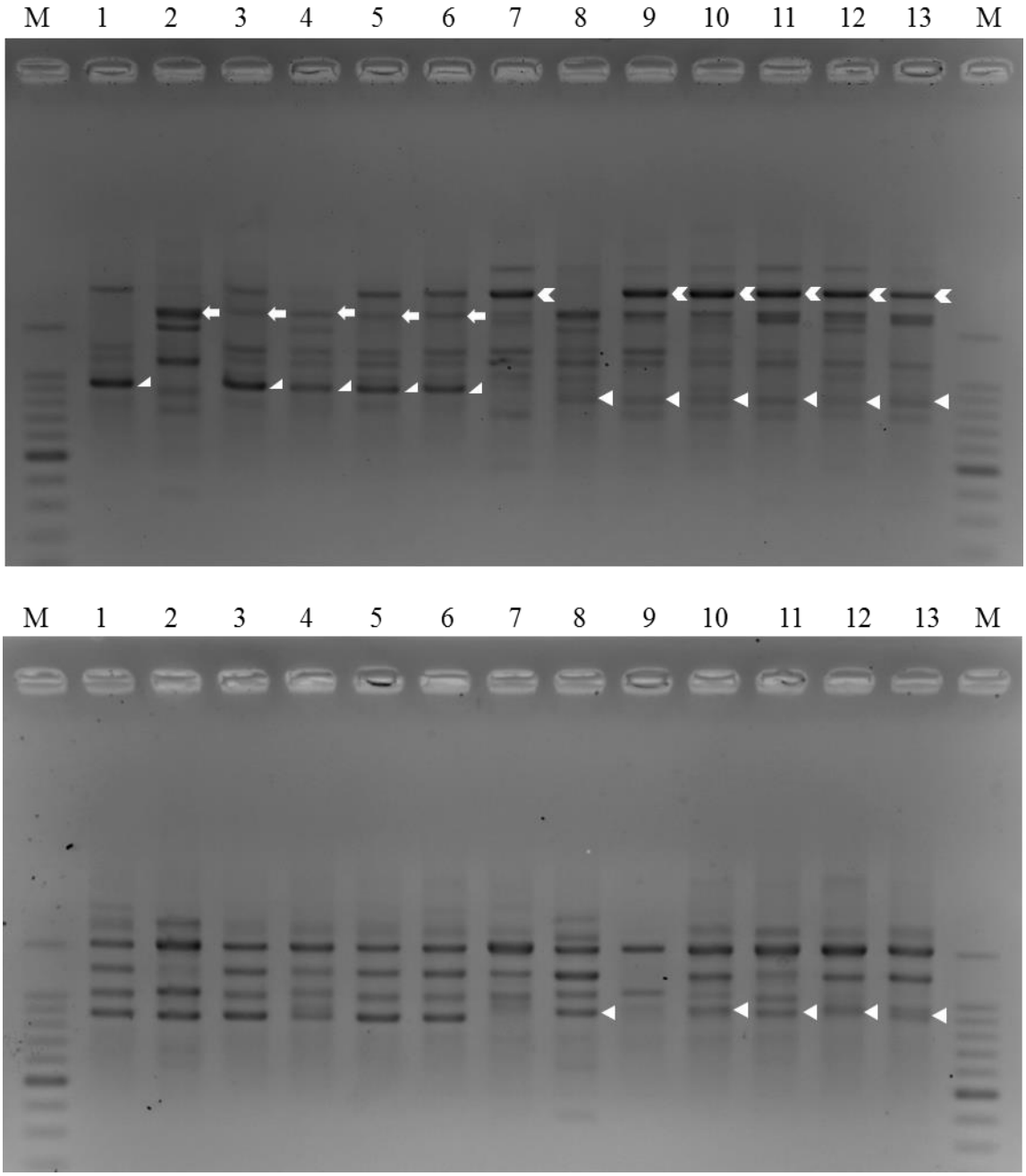


Figure III-10. Amplified polymorphisms of putative hybrid seedlings obtained from the crosses of *R. tosaense* #1 × *R. simsii* 2 and *R. kiusianum* #2 × *R. simsii* 1 using primer CMN-A02 (upper) and CMN-B27 (lower). Triangulars indicate the specific band for TOS1. Arrows indicate a specific band for SIM 2. Chevrons indicate a specific band for KIU 2. Isosceles triangulars indicate the specific bands for SIM 1. Lane M, 100 bp ladder DNA size markers; Lane 1, TOS 1; Lane 2, SIM 2; Lane 3 to 6, seedlings from TOS 1 × SIM 2; Lane 7, KIU 2; Lane 8, SIM 1; Lane 9 to 13, seedlings from KIU 2 × SIM 1.

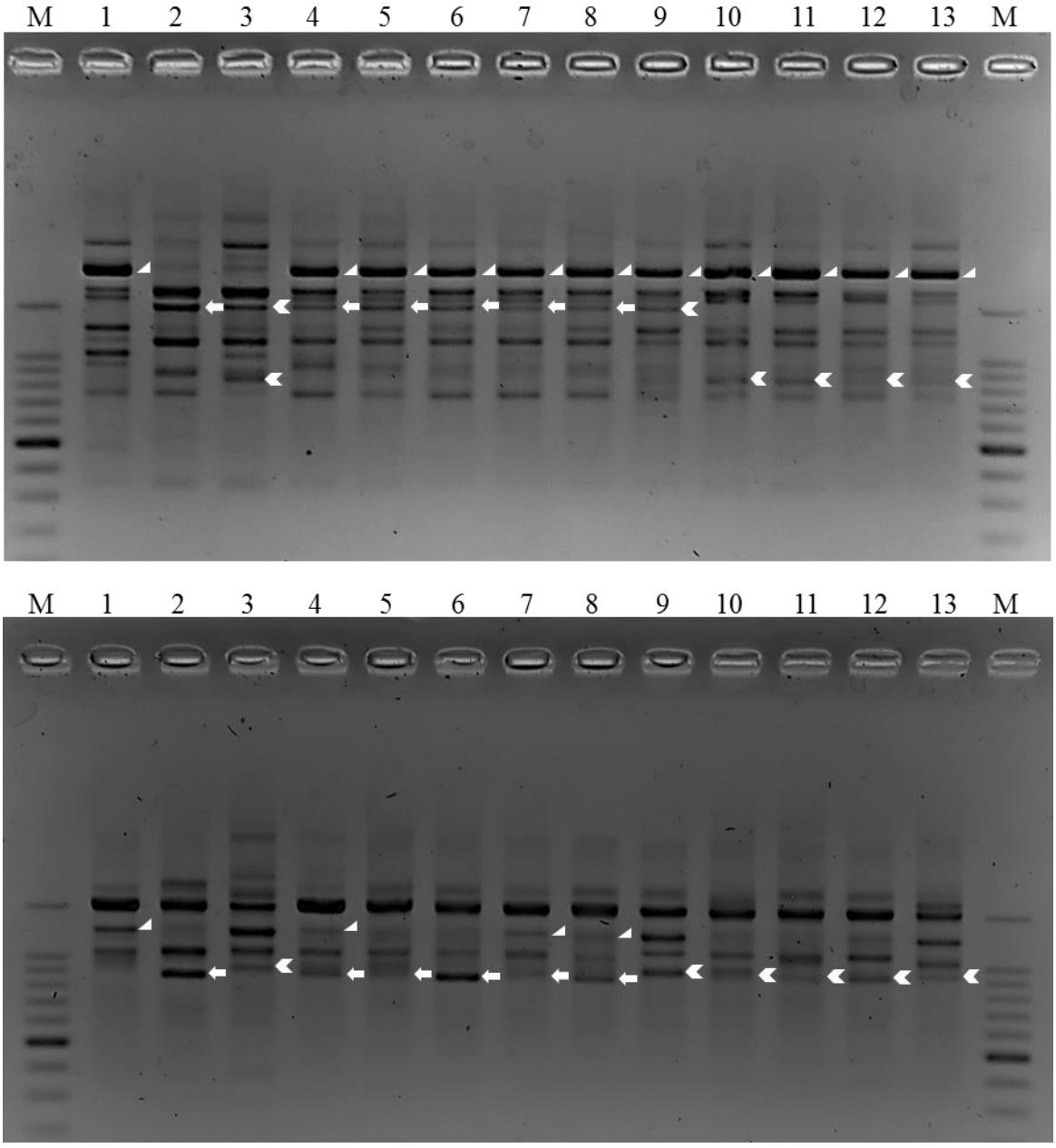


Figure III-11. Amplified polymorphisms of putative hybrid seedlings obtained from the crosses between *R. kiusianum* #2 and *R. simsii* #2, 3 using primer CMN-A02 (upper) and CMN-B27 (lower). Triangulars indicate the specific bands for KIU 2. Arrows indicate the specific bands for SIM 2. Chevrons indicate the specific bands for SIM 3. Lane M, 100 bp ladder DNA size markers; Lane 1, KIU 2; Lane 2, SIM 2; Lane 3, SIM 3; Lane 4 to 8, seedlings from KIU 2 × SIM 2; Lane 9 to 13, seedlings from KIU 2 × SIM 3.

In addition, leaf morphology of seed parents, pollen parents and F_1 offsprings was observed and compared. The leaves of three accessions of *R. simsii* were narrow lanceolate shape with approximately 40 mm length and 10 mm width (Figure III-12A). Among seed parents, *R. eriocarpum*, *R. kaempferi* var. *macrogemma* and *R. kiusianum* have leaves of broadly obovate shape (Figure III-12B, C, D) while *R. tosaense* performed lanceolate shape, which is quite similar with *R. simsii* leaves (Figure III-12E). Based on the leaf shape index, three accessions of *R. simsii* from Vietnam showed the highest ratio of leaf length and leaf width (Table III-5). The leaf shape index of offspring from all crosses almost showed a uniform intermediate ratio (2.82- 3.64) compared to that of their seed parents (1.84-2.20) and pollen parents (3.84- 4.41) except progenies from the cross of TOS 1 × SIM 2 (Figure III-12(F-N) and Table III-5). Because *R. simsii* and *R. tosaense* possess narrow lanceolate leaves, the offspring inheritably performed same leaf morphology with their parents (Figure III-12L). Statistical analysis using Tukey test showed that significant differences were found between the leaf shape index of pollen parents, seed parents and their offspring (Figure III-13). As mentioned above, progeny obtained from the cross of TOS 1 × SIM 2 showed same leaf morphology as their parents, so there was no significant difference in leaf shape index among them.

In some reports, the morphology of the hybrids have been described to be intermediate between the parents (Gleba and Hoffmann, 1980; Schenck and Röbbelen, 1982; Sundburg et al., 1887; Terada et al., 1987), or to be a combination of parental characteristics (Toriyama et al., 1987). Jaynes (1976) also reported the results in his experiment that the seedlings obtained from the crosses between evergreen and deciduous azaleas performed intermediate leaf size and shape compared to their parent plants.

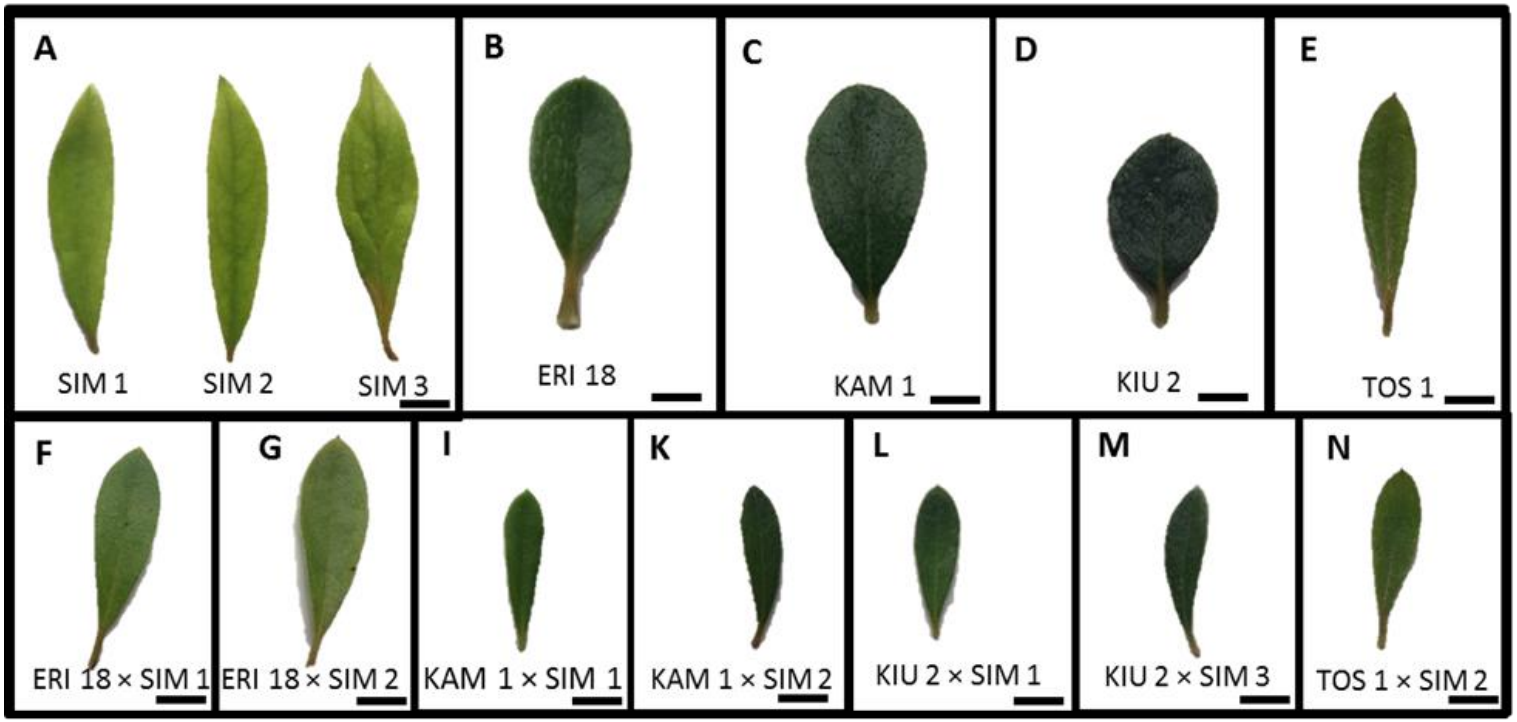


Figure III-12. Leaf shapes of pollen parents (A), seed parents (B-E) and their offsprings (F-N). Bar = 3 cm.

Table III-5. Comparison of leaf morphological characteristics between *R. simsii* in Vietnam, several evergreen azalea species and their hybrids.

Species and F_1 hybrid	Leaf size (mm)		Leaf shape index [z]
	Leaf length [y]	Leaf width	
SIM 1	41.50 ± 0.80	10.27 ± 0.82	4.04 ± 0.41
SIM 2	41.72 ± 3.14	9.45 ± 1.03	4.41 ± 0.37
SIM 3	42.70 ± 3.04	11.12 ± 0.91	3.84 ± 0.34
ERI 18	18.89 ± 1.05	9.81 ± 0.59	1.93 ± 0.16
KAM 1	28.43 ± 1.27	15.42 ± 1.44	1.84 ± 0.10
KIU 2	24.44 ± 2.18	11.10 ± 0.86	2.20 ± 0.07
TOS 1	28.13 ± 0.43	7.10 ± 0.16	3.96 ± 0.15
ERI 18 × SIM 1	28.71 ± 1.05	10.16 ± 1.14	2.82 ± 0.42
ERI 18 × SIM 2	33.69 ± 3.82	11.68 ± 1.72	2.89 ± 0.72
KAM 1 × SIM 1	21.06 ± 3.82	6.60 ± 0.72	3.19 ± 0.57
KAM 1 × SIM 2	15.39 ± 1.61	4.88 ± 0.26	3.15 ± 0.47
KAM 1 × SIM 3	15.72 ± 0.00	4.85 ± 0.00	3.24 ± 0.00
KIU 2 × SIM 1	14.89 ± 1.22	4.83 ± 0.30	3.08 ± 0.23
KIU 2 × SIM 2	17.92 ± 2.45	4.92 ± 0.98	3.64 ± 0.28
KIU 2 × SIM 3	16.27 ± 0.95	5.07 ± 0.84	3.21 ± 0.38
TOS 1 × SIM 2	16.48 ± 0.26	4.14 ± 0.10	3.98 ± 0.05

[z] Leaf shape index = Leaf length/ leaf width.

[y] Mean ± S.E.

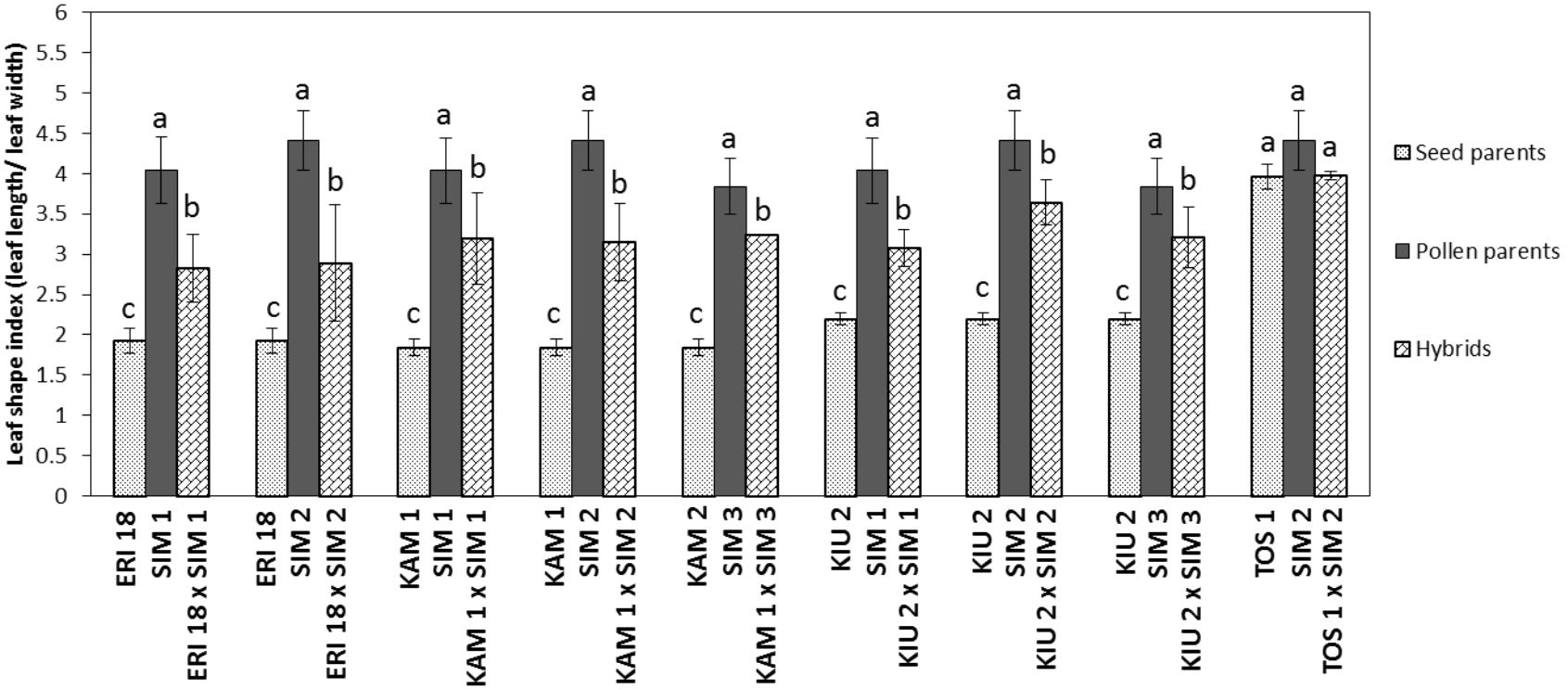


Figure III-13. Leaf shape index of *R. simsii* from Vietnam, several evergreen azalea species and their hybrids. Leaf shape index = leaf length/ leaf width. Statistical analysis was performed using Tukey test. Means followed by the same letter are not significant different at the value of $P < 0.05$. Vertical bars indicate standard error.

In my experiment, the offsprings from interspecific hybridization are seemed to obtain various traits from both parents, since they showed the same inherited pattern of intermediate morphological characters as reported in previous studies. Furthermore, RAPD analysis proved that these seedlings were hybrids with specific polymorphic bands from both parents.

Summary

The cross compatibility between Vietnamese *R. simsii* and several evergreen azalea species originated in Japan and Taiwan was elucidated by relatively high set capsules and seed numbers from every crosses. Germination rates of the seeds showed low to high percentages or varied considerably in different cross combinations. No albino or pale green seedlings were obtained from all crosses. The RAPD analysis using two specific primers indicated that almost seedlings obtained from these crosses were hybrids. In addition, leaves of F_1 plants evaluated by leaf shape index showed intermediate shape compared to those of their parents.

CHAPTER IV

HEAT STRESS TOLERANCE IN *RHODODENDRON SIMSII*

Introduction

Stress caused by high temperature has been much less studied in crop plants such as wheat (Stone and Nicolás, 1994), corn (Thompson, 1986), cotton (Rehman et al., 2004) and rice (Morita et al., 2004) or in woody species such as *Rhododendron* (Ranney, 1995), redbud (*Cercis canadensis* L.) (Griffin et al., 2004) and sunflower (*Helianthus annuus*L.) (Senthil- Kumar et al., 2003). The most popular and rapid method to evaluate heat stress tolerance effectively in plants is electrolyte leakage for measuring cell membrane thermostability (CMT) from leaf discs over a range of temperatures (Wu and Wallner, 1993). Electrolyte leakage is a hallmark of stress response in intact plant cells. This phenomenon is widely used as a test for the stress-induced injury of plant tissues and a measure of plant stress tolerance (Levitt, 1972; Blum and Ebercon, 1981; Bajji et al., 2002; Lee and Zhu, 2010). The electrolyte leakage is ubiquitous among different species, tissues and cell types, and can be triggered by all major stress factors, including pathogen attack (Ebel and Mithofer, 1998; Maffei et al., 2007), salinity (Shabala et al., 2006; Demidchik et al., 2010), drought (Shcherbakova and Kacperska, 1983), heat (Liu and Huang, 2000), waterlogging (Shabala, 2011), and others. Several studies have shown the effectiveness of CMT testing in detecting genetic variability for heat tolerance among several agronomic crops, fruits, vegetables and floricultural plants (Chen et al., 1982; Ingram and Buchanan, 1984; Lester, 1985; Martineau et al., 1979; Saadalla et al., 1990; Sullivan and Ross, 1979;

Yeh and Lin, 2003). However, in evergreen azaleas, especially in *R. simsii*, information of heat tolerance using CMT is still lacking.

On the breeding point of view, I am interested in heat stress resistance of two accessions of *R. simsii* distributed in Japan and Vietnam. In comparison of growing condition perspective, Vietnamese *R. simsii* distributes along streamside or riversides in the mountainous areas of northern and central part of Vietnam above 800- 1400 m altitude (Ho, 1991) (Figure IV-1C). In contrast, Japanese *R. simsii* grows vigorously in sunny and grassy slopes on the islands of Ryukyu Archipelago (Yamazaki, 1996) (Figure IV-1B). The author hypothesized that growing in two different environmental conditions leads different morphological and physiological characteristics between two accessions. Even though the morphological variation of leaves in these two accessions has been investigated and compared (Hang et al., 2010), information of heat stress tolerance seems to be limited. It is necessary to conduct the study to evaluate this important characteristic in different accessions of *R. simsii*. In addition, the heat tolerance of *R. eriocarpum* (Figure IV-1A), which is considered as a high heat stress resistant species among Japanese evergreen azaleas (Sakata and Hashimoto, 2006), and the hybrids between *R. eriocarpum* and *R. simsii* was also evaluated.

In this chapter, the heat stress tolerance of two accessions of *R. simsii* species, *R. eriocarpum* and their interspecific hybrids were evaluated using electrolyte leakage method to measure cell membrane thermostability (CMT). In addition, shoot dry weight of these plant materials were also measured under different temperature of a greenhouse condition.

This information will be useful for pre-selection of breeding program of evergreen azaleas to select good plant parents, and obtain hybrids with desirable traits.

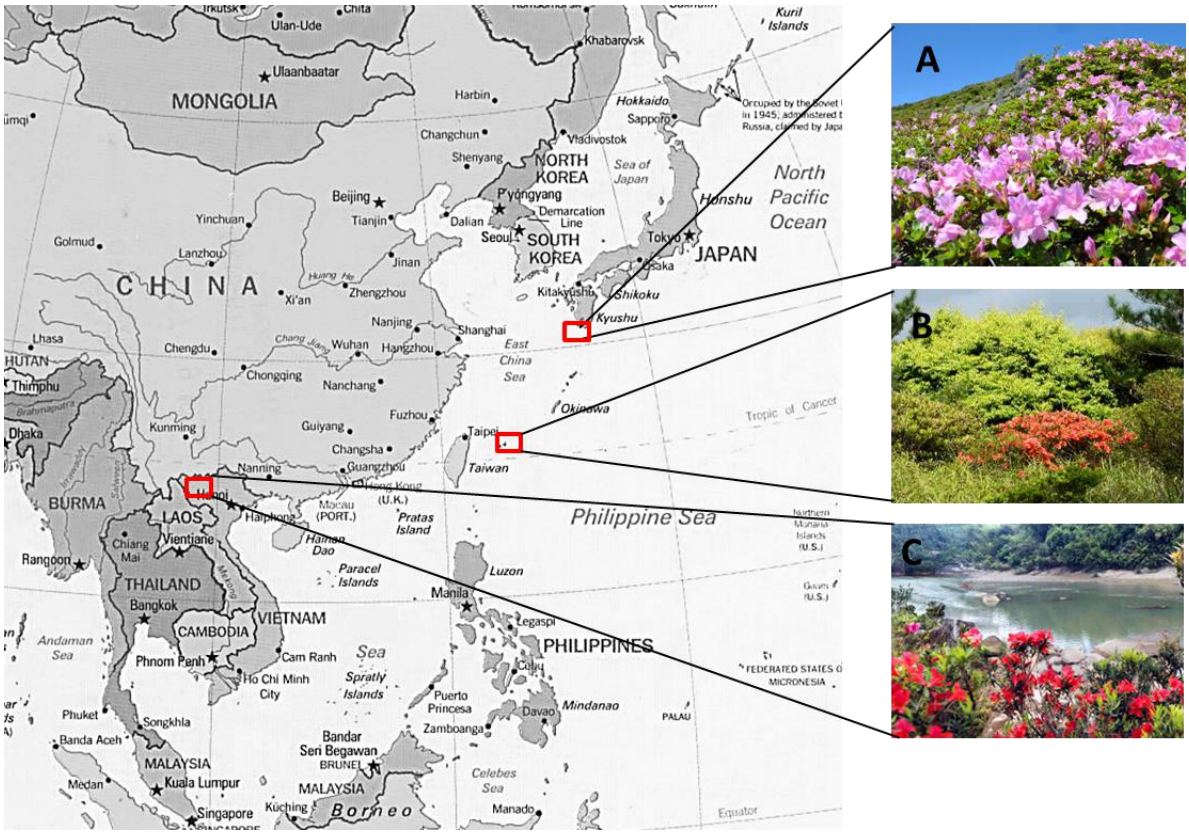


Figure IV-1. Collection sites of the plant materials used in this study. (A) *R. eriocarpum* in Tokara Islands; (B) *R. simsii* in Iriomotejima Island and (C) *R. simsii* in Than Uyen, Lai Chau, Vietnam.

Materials and Methods

Plant materials

Cutting branches of two accessions of *R. simsii* and an accession of *R. eriocarpum* were collected in March and April 2015 (Table IV-1). Cutting branches were planted by pumice soil and placed under the mist house conditions for two months. After rooting, they were transplanted into 8 cm diameter plastic pots. The seeds from interspecific cross between *R. eriocarpum* #18 and Vietnamese *R. simsii* were sown in March 2014, and then vigorous seedlings were transplanted into pots. All plant materials were placed under a netted-house in Kyushu University, Hakozaki campus with natural light source and temperature.

Leaf cell membrane thermostability (CMT) measurement.

From 23th June to 14th July, 2016, fully expanded leaves (leaves three to six from shoot apex) were collected from stock plants. These leaves were harvested for CMT following procedure described by Yeh and Lin (2003). Cell membrane thermostability was measured from 25^{o}C to 70^{o}C with 5^{o}C intervals for 30 min each in the water bath.

A sample for assay consisted of a paired set (25^{o}C as control and another temperature as treatment) of 6 mm diameter leaf discs samples cut from group of leaves of each species by a hole puncher. Before conducting, the leaves were rinsed thoroughly with distilled water for three times. Leaf discs were then placed into 50 mL test tubes containing 1 mL distilled water to prevent secondary water stress. Tubes were placed in a heated water bath for 30 min at each treatment temperature. After the treatment, all tubes were added 15 mL of distilled water and shaken well by a shaker for 24 hours at room temperature (25^{o}C).

Table IV-1. Plant materials used in this study.

Species, accession No. and hybrid	Code	Collected site	Origin
R. simsii (VN) [z]	SIM (VN)	Than Uyen district, Lai Chau province, Vietnam	Than Uyen district, Lai Chau province, Vietnam
R. simsii (JP)	SIM (JP)	Showanomori Park, Umimachi, Fukuoka, Japan	Iriomotejima Island, Japan
R. eriocarpum #18	ERI 18	Kyushu University greenhouse	Tokara Islands, Kagoshima, Japan
R. eriocarpum #18 × *R. simsii* (VN)	ERI 18 × SIM (VN)	Kyushu University greenhouse	Interspecific crossing

[z] Origin: VN;Vietnam, JP: Japan.

Solution conductivity was measured by electrical conductivity meter (model ES-71; Horiba, Ltd, Kyoto, Japan). Then, test tubes were autoclaved for 15 min at 121^{o}C, cooled to 25^{o}C, and incubated for an additional 24 hours before taking final conductivity measurement. In final measurement, the injury was expressed as a percentage of total leakage.

The degree of relative injury (RI) induced by the temperature treatment was calculated as follows (Yeh and Lin, 2003):

$$RI\ (\%) = \{1 - [1 - (T_1/T_2)]/\ [1 - (C_1/C_2)]\} \times 100$$

Where, T and C refer to conductivity values for treatment and control (25^{o}C) vials, respectively, and subscripts 1 and 2 refer to initial and final conductivity readings, respectively.

The relationship between RI value and water bath temperature treatments was performed by response curves, which were determined with logistic analysis using sigmoidal model in CurveExpert 1.4 program based on the data of each sample.

Shoot dry weight measurement under different temperature of a greenhouse conditions

Three rooted cuttings of each material were placed in a greenhouse during June to August 2016 to evaluate the shoot dry weight under different temperature. The day and night temperature in June and August were 27/20^{o}C and 34/26^{o}C, respectively (Figure IV-2). Three shoots per plant were harvested at the end of June and August, 2016 for measurement of shoot dry weight. Fresh shoots were immediately measured for fresh weight by a digital scale, and dried by the oven at 70^{o}C for 48 hours (Jamal et al., 2014). Dry weight of all samples was also measured. Shoot dry weight ratio was calculated by dividing shoot dry weight of samples collected in August by that in June.

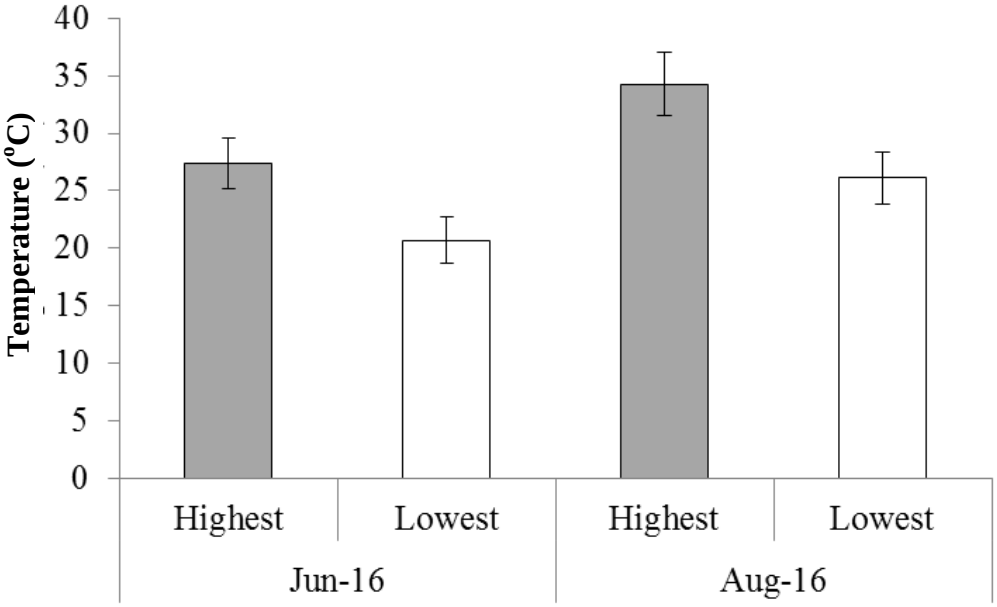


Figure IV-2. Average of the highest and lowest temperatures each in June and August, 2016 in greenhouse conditions. Vertical bars indicate standard error.

Statistical analysis

Statisctical analysis of the results was performed with IBM SPSS Statistics 21 software. The data was subjected to one way analysis of variance (ANOVA). The least significant difference (LSD) test and Tukey test (P = 0.05) were done to compare the means and determine whether there were any significant differences between treatments.

Results and Discussion

The relationship between the relative injury (RI) value in the leaf discs and the water bath temperature treatments was sigmoidal in all samples (Figure IV-3). Similar response curves have been reported for a number of plants and crops (Chen et al., 1982; Ismail and Hall, 1999; Lester, 1985; Inaba and Crandall, 1988). In 50^{o}C water bath treatment, SIM (VN) and SIM (JP) showed the injury level at 38.9 and 31.7 %, respectively. In contrast, ERI 18 performed low level of RI as 14.5%. Interestingly, the hybrid of ERI 18 × SIM (VN) showed the moderate value (24.8%) of RI under heat stress condition. The low relative injury value of ERI 18 indicated that this species can tolerate well under heat stress condition. From 55^{o}C, the relative injury percentage increased sharply up to above 75%. It means above 50^{o}C, leaf tissue suffered severe heat stress resulting high percentage of leakage.

Relative injury values were ranked from low to high: ERI 18 × SIM (VN) < ERI 18 < SIM (JP) < SIM (VN) at 25^{o}C (Table IV-2). However, at 50^{o}C and calibrated 50^{o}C, this rank was changed: ERI 18 < ERI 18 × SIM (VN) < SIM (JP) < SIM (VN). Among water bath treatments, 50^{o}C showed in the maximum difference in the heat tolerance among all samples. This suggests that a single heat treatment at 50^{o}C can be used to evaluate the

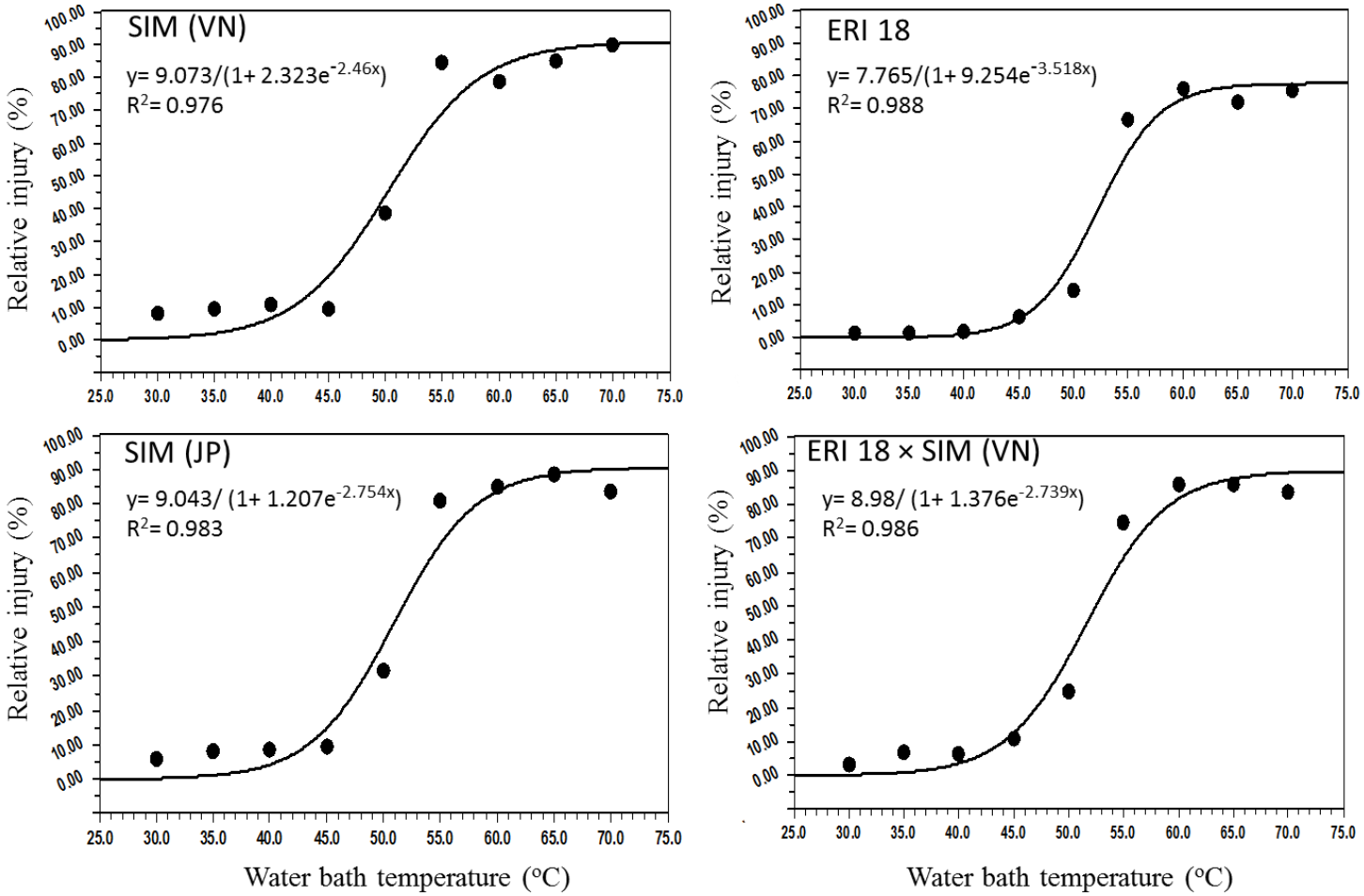


Figure IV-3. Effect of water bath temperature on relative injury of leaf disk of *R. simsii*, *R. eriocarpum* and their F_1 hybrids. Response curves are established by Sigmoidal model in CurveExpert program version 1.4 based on the data of each species.

heat stress tolerance of evergreen azalea species without testing with different temperature treatments. A treatment temperature at 50^{o}C has been also successfully used to screen for heat tolerance in pepper (Anderson et al., 1990), wheat (Saadalla et al., 1990) or in some woody plants such as holly (*Ilex aquifolium* L.) (Ruter, 1993). This treatment temperature may be considered as standard temperature index and useful to evaluate the heat tolerance of number of cultivars or species before hybridizations or for commercial production of potted evergreen azaleas in hot regions.

In greenhouse conditions, there was no significant difference in shoot dry weight between samples collected in June (27/20^{o}C) and those in August (34/26^{o}C) of *R. eriocarpum*, while two *R. simsii* accessions and F_1 hybrids showed significant differences (Table IV-3). At high temperature of 34/26^{o}C in August, shoot dry weight of *R. eriocarpum* #18 was not reduced much, indicating that this species can withstand in hot condition better than others species in this study. SIM (VN) performed lowest ratio of shoot dry weight, and this result agrees with the result of RI value. High temperature of August induced sunburns on leaf tips of SIM (VN), and they were easy to be withered and yellowish compared to plants grown in June (27/20^{o}C) (data not shown).

In present study, regressing the shoot dry weight ratio of all samples versus their RI value at 50^{o}C was a linear relationship and negative correlation (Figure IV-5). It means that when the ratio of shoot dry weight decreases, relative injury percentage will increase resulting low heat stress tolerance.

Heat stress is considered to be a primary limiting factor affecting the distribution and adaptability not only of wild species but also of cultivated plants. Heat stress is often defined as the rise in temperature beyond a threshold level for a period of time sufficient to

Table IV-2. Relative injury (RI) as determined by the cell membrane thermostability test at 25 and 50°C for 30 min of *R. simsii*, *R. eriocarpum* and their hybrids.

Sample	Relative injury (%)		
	25°C [z]	50°C [z]	Calibrated [y]
SIM (VN)	50.3 a [x]	69.6 a	38.9 a
SIM (JP)	30.5 b	52.6 b	31.7 b
ERI 18	12.5 c	25.2 d	14.5 d
ERI 18 x SIM (VN)	10.0 c	34.0 c	24.8 c

[z] Calculated as (initial conductivity/final conductivity) × 100.

[y] Calibrated RI= $\{1-[1-(T_1/T_2)]/[1-(C_1/C_2)]\} \times 100$.

[x] Means with different letters within column represent statistical difference by One-way ANOVA ($P<0.05$).

VN: Vietnam; JP: Japan.

Table IV-3. Effect of temperature on shoot dry weight of *R.simsii*, *R. eriocarpum* and their F_1 hybrids.

Species	Shoot dry weight (mg)		Shoot dry weight ratio measured in August and June 2016
	June 2016 (27/20°C)	August 2016 (34/26°C)	
SIM (VN)	157.3 a [z]	72.8 b	0.46 D
SIM (JP)	224.2 a	125.6 b	0.56 C
ERI 18	317.1 a	304.1 a	0.96 A
ERI 18 × SIM (VN)	197.9 a	158.3 b	0.80 B

[z] Means with different letters within rows (small letter) and columns (capital letter) represent statistical difference by One-way ANOVA (P<0.05).

cause irreversible damage to plant growth and development. In general, a transient elevation in temperature, usually 10–15°C above ambient, is considered heat shock or heat stress (Wahid et al. 2007). Therefore, it is a worldwide serious threat to crop production and ornamental production as well (Hall, 2001).

Like other organisms, plants have evolved various mechanisms to withstand heat stress. Heat tolerance is generally defined as the ability of the plant to grow and produce higher yield, better growth performance or greater plant survival under high temperatures than standard species or cultivars (Hall, 1990a).These include a wide variety of long-term evolutionary adaptations affecting their morphology and ecophysiology, as well as shorter term acclimation mechanisms such as transpiration cooling, changes in leaf orientation, or alterations in membrane lipid composition (Larkindale et al., 2005a).

Many studies indicate that heat condition inducing loss of membrane stability is a major reason for decreased growth performance of various plant species (Blum and Ebercon, 1981; Bajji et al., 2002 and Iba, 2002). In grapes (*Vitis vinifera*), heat stress severely damaged the mesophyll cells and increased permeability of membrane causing solute leakage in plant tissues (Zhang et al., 2005). The correlation between shoot dry weight and relative injury at 50^{o}C suggested that the electrolyte leakage method using leaf discs instead of whole plant is an appropriate, effective and rapid *in vitro* technique to evaluate plant heat stress tolerance.

In natural growing habitat, SIM (VN) grows near riverside or streamside, indicating that these plants seem to be adapted under high moisture condition. Moreover, Than Uyen district, where is above 800- 1400 m altitude, average highest temperature of 30^{o}C and lowest temperature of 22^{o}C, is not so hot area as in the other areas (Figure IV-5). Based on

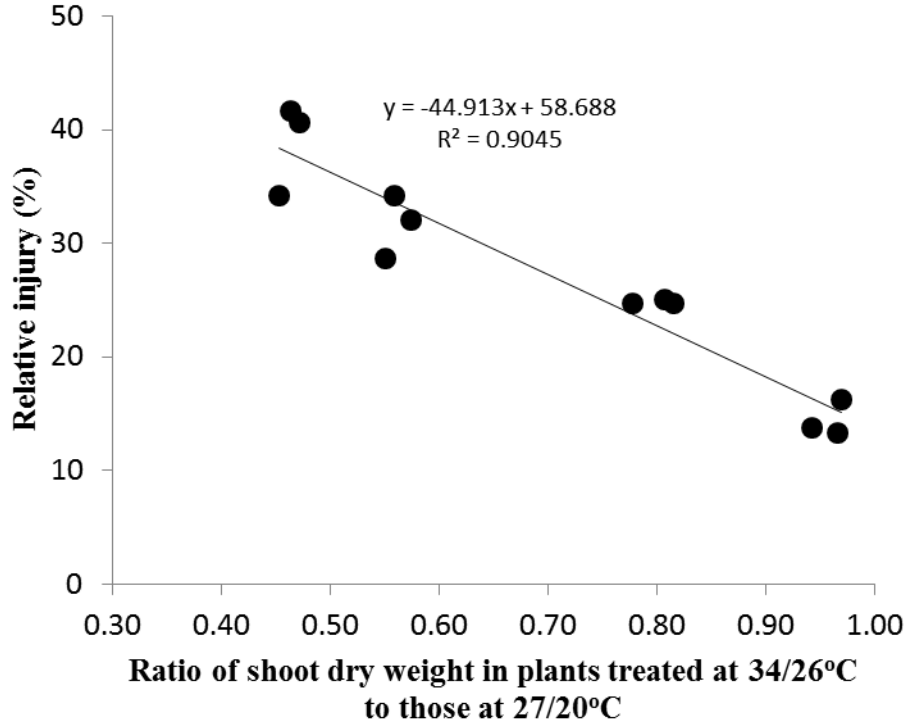


Figure IV-4. The relationship between relative injury at 50°C and the ratio of shoot dry weight in *R. simsii*, *R. eriocarpum* and their F_1 hybrids grown at 34/26°C (August, 2016) to shoot dry weight in those grown at 27/20°C (June, 2016).

this growing temperature condition, plants from Vietnamese accessions may have low heat stress tolerance. This is confirmed by the above results that SIM (VN) performs lowest heat stress tolerance among treated species. In comparison, SIM (JP) showed higher resistance to heat condition than SIM (VN). This result agrees with the fact that SIM (JP) accessions grow in sunny and glassy slopes of Iriomotejima Island in southern Japan at 32^{o}C and 27^{o}C of average highest and lowest temperature during July to August (Figure IV-5). This condition requires plants to be both drought and heat tolerant because it is rarely rainy during the two months, and plants have to withstand long term drought due to high temperature. In case of *R. eriocarpum* species, it performed the best tolerance to heat stress condition as has been shown in the previous report (Sakata and Hashimoto, 2006). *Rhododendron eriocarpum* grows wildly at the sea level in the area composed of volcano mountains of Tokara Islands in Kagoshima prefecture, and it also has high resistance to sulfur dioxide (Sakata and Hashimoto, 2006). From all results of this study, heat stress tolerance of *R. eriocarpum* (seed parents) seems to be inherited to its F_1 offspring due to their moderate performance in CMT and shoot dry weight ratio. According to Sakata and Hashimoto (2006), a gene related to heat stress tolerance can be transferred into a heat-intolerant azalea plant by crossings. Thus, F_1 hybrids seemed to be received this good trait from its seed parent.

In further study, an experiment about heat stress tolerance related gene expression in these species and their offspring should be concerned to clarify heat stress tolerant inheritance molecularly. This information is expected to be useful for breeders to select good materials for production of evergreen azaleas. Furthermore, interspecific hybridization is a best way to introduce heat stress tolerant trait into the hybrids.

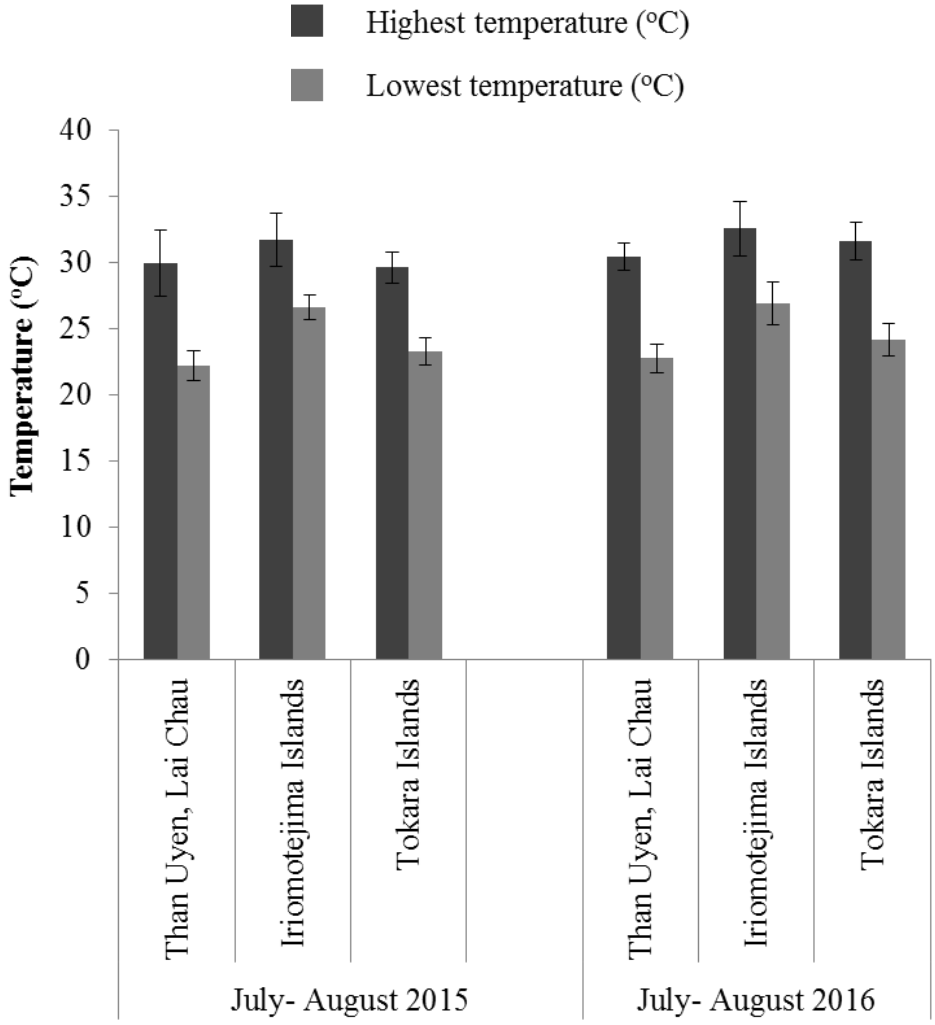


Figure IV-5. Average highest and lowest temperature during July to August 2015 and 2016 in Than Uyen, Lai Chau, Vietnam (source: Hydrometeorological center of Lai Chau province); Iriomotejima Islands and Tokara Islands (source: Japan meteorological agency). Vertical bars indicate standard error.

Summary

In this chapter, heat stress tolerance of *R, simsii*, *R. eriocarpum* and their F_1 hybrids were evaluated using electrolyte leakage method for measuring cell membrane thermostability (CMT). The results showed that a sigmodal curve existed between the relative injury (RI) values occurring in the leaf tissue discs of all samples and water bath treatment temperature. A single heat treatment at 50^oC can be used to screen the heat tolerance of large number of species without testing with different temperature treatment. Regressing the shoot dry weight ratio of all samples versus their RI value at 50^oC was a linear relationship and negative correlation. It suggested that the electrolyte leakage method using leaf discs instead of whole plant is an appropriate, effective and rapid *in vitro* technique to evaluate plant heat stress tolerance.

Chapter V

GENERAL DISCUSSION

For the purpose of understanding physiological characteristics and confirming the interspecific cross compatibility of *R. simsii*, which is mainly distributed in eastern Asia, this study was carried out based on three main experiments. Hereafter, I would like to have a summary and general discussion about all issues of this study.

Evergreen azaleas are one of the most important flowering pot plants in Western Europe, USA and Japan. Nowadays, they are widely utilized and planted for landscaping in temperate region of the world because of their colorful and graceful flowers with desirable traits. Among evergreen azaleas, *R. simsii* is considered to be an ornamental valuable species, and it was used for breeding many famous cultivars and varieties of Belgium pot evergreen azaleas (Eeckhaut et al., 2013). Even though the importance of this species was recognized, the detailed information of flower color, high-temperature stress tolerance and breeding of wild *R. simsii* in subtropical and tropical regions is still limited. In chapter I, information related to history of *R. simsii* species and unknown knowledges of several physiological and breeding of this species were reviewed. In chapter II, pigmentation in the reddish-purple blotches of Vietnamese wild *R. simsii* was firstly identified, and the chemical factors affecting the co-pigmentation phenomenon in blotch areas were also elucidated. In chapter III, interspecific hybridization between *R. simsii* from Vietnamese population and several evergreen azaleas originated in Japan and Taiwan was carried out, and in Chapter IV, evaluation of heat stress tolerance in wild *R. simsii*, *R. eriocarpum* and their hybrids was performed.

Flavonol composition and co-pigmentation of anthocyanin-flavonol were elucidated clearly in Chapter II. Microscopic observation indicated that reddish-purple cells were mainly distributed in the adaxial subepidermis of blotch areas in three upper petals. This observation agrees with a schematic illustration described by Benjamin (1992). In case of *Rhododendron* flowers, weak colored cells commonly tent to be distributed in epidermis, and subepidermis always contains strong colored cells (Figure V-1). This structure seems to be common and universal for blotch structure among genus *Rhododendron*.

In general, pigmentation of red colored flowers of evergreen azaleas was investigated and identified by number of researchers or horticulturists. However, research of blotch structure or pigment composition is really limited even though blotches are available in almost flowers of evergreen azaleas species. A single part of a flower has its own function. The blotches expressed distinctive color to attract more pollinators for spreading the pollens to other plants because color of the blotches was always prominent and wider color variation than petal background color, and the pigment composition seemed to be more complex as well (Martins et al., 2013). This fact has been scientifically confirmed by the differences in L^* a^* b^* color coordinate between blotch areas in upper petals and lower petals, and by chromatographic analyses shown in Section II-2 of Chapter II. The evidences of pollinators for distinguishing blotches flowers from non-blotches flowers and for visiting frequencies for each of red and purple flowers have been also reviewed. This result suggested that blotch areas not only contribute to flower morphological variation for human, but also play an important role for attraction of pollinators to maintain the lineage of the species.

In the plant breeding, the knowledge of cross compatibility between species is

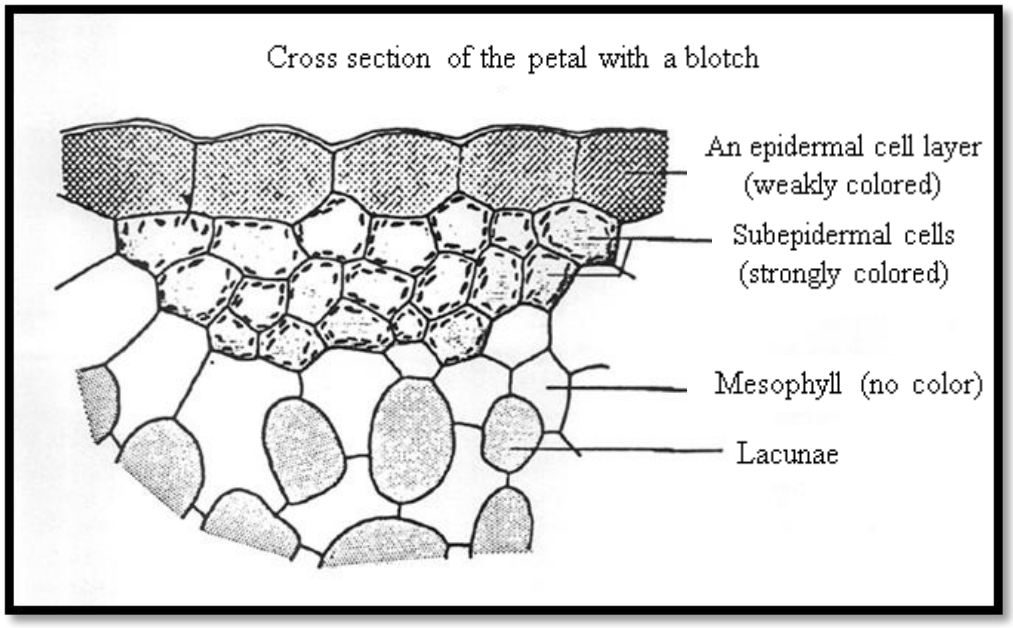


Figure V-1. Schematic illustration of pigment distribution and its contribution to petal color in the blotches of *Rhododendron* flowers (Benjamin, 1992).

important and needed for their effective breeding, particularly in the simultaneous selection of several desirable traits (Michishita et al., 2003). In Chapter III, interspecific hybridization with other perspective evergreen azalea species was carried out to improve flower color of *R. simsii*. The progenies confirmed to be hybrids have mostly intermediate characters of the parents with respect to leaf size index. The occurrence of intermediate phenotypes of the progeny is a typical feature in interspecific hybridization, and has been described for *Rhododendron* (Jaynes, 1976) and several plant genera such as *Dianthus* (Nimura et al., 2003), *Chrysanthemum* (Cheng et al. 2001), *Streptocarpus* (Afkhami-Sarvestani et al. 2012) and *Kalanchoë* (Kuligowska et al., 2015a). The present study has revealed the cross compatibility between Vietnamese *R. simsii* and Japanese and Taiwanese evergreen azaleas. In future work, flower color of F_1 progenies should be confirmed. In previous study, Miyajima et al. (1995) reported that color variation occurred in flowers of natural hybrids between reddish-purple blotch red flowered *R. kaempferi* and non-blotch reddish-purple flowered *R. kiusianum*. These hybrids showed wide variation in size of flowers with prominent blotches on the petals. Hence, it is also expected that the progenies obtained from this study will perform the variation in the phenotype, and will be promising materials for future breeding.

In addition to pigmentation and cross compatibility of *R. simsii*, this study has also focused on the heat stress tolerance of this species and, especially, the obtained F_1 hybrids. In Chapter IV, heat stress tolerance of *R. simsii*, *R. eriocarpum* and their F_1 hybrids has been evaluated using the electrolyte leakage method for measuring cell membrane thermostability (CMT). The results have indicated that heat stress tolerance of all species is able to estimate with relative injury (RI) percentage of leaf discs. Each species has shown

different RI percentages under heat stress of water bath temperature. This means high temperature has induced cell membrane damages in the leaf tissues. The function of cell membrane under the high temperature stress is very important for plant physiological activities such as photosynthesis and respiration (Blum, 1988). It has been considered that heat stress condition accelerates the kinetic energy and movement of molecules across membranes, and thereby loses chemical bonds within molecules of the biological membranes. This makes the lipid bilayer of biological membranes easy to damage by either denaturation of proteins or an increase in unsaturated fatty acids (Savchenko et al., 2002). This suggests that cell membrane is very sensitive to heat stress. If high temperature affects and alters the structures of membrane proteins, the higher the temperature is, the more quickly the relative permeability of membranes increases causing high electrolyte leakage. In this study, the experiment has been focused on the relative injury of cell membrane. However, saturated fatty acid content in membranes should be concerned too in further analysis. Because it has been determined that an increase in saturated fatty acids in leaves elevated melting temperature of cell membranes and thus reducing heat tolerance in the plant (Somerville and Browse, 1991). Even though these results just reflected one of major aspects of heat stress tolerant determination as CMT, electrolyte leakage using a standard temperature index at 50^{o}C can quickly and simply evaluated heat stress tolerance for large number of selected plants. It would be useful for choose good candidates of heat stress tolerant *Rhododendron* and the other plant species for ornamental production in tropical regions.

In conclusion, the above-mentioned findings will contribute more or less to knowledge about Vietnamese *R. simsii* in physiological and breeding aspects. Although it is

necessary to wait for several years to know the results of further growth and flower expression of the interspecific seedlings obtained in Chapter III, the information of pigmentation, cross compatibility and heat stress tolerance would help to establish and improve an effective breeding program in Vietnam using *R. simsii* plants as the important genetic resources.

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