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Heat Stress Tolerance in *Rhododendron simsii* Planch.

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The wild type of *Rhododendron simsii* is mainly distributed in eastern Asia. In Vietnam, this species grows along streamsides or riversides in northern and central parts at above 800–1400 m altitude. In contrast, Japanese *R. simsii* species is distributed in sunny and grassy slopes on the islands of Kyushu and Ryukyu Archipelago. Plants grow in different environmental conditions may perform different morphological or physiological characteristics. In this study, the heat stress tolerance of Vietnamese and Japanese *R. simsii* was evaluated by electrolyte leakage technique to measure the cell membrane thermostability (CMT) using fresh leaf discs. In addition, *R. eriocarpum*, which is considered as one of the most heat resistance evergreen azaleas species in Japan, and the F1 hybrids with Vietnamese *R. simsii* were also used as materials. In the results, the relationship between the relative injury (RI) value in the leaf discs and the water bath temperature treatments was sigmoidal in all samples. A single temperature treatment of 50ºC for 30min resulted in 38.9% and 31.7% RI value in Vietnamese and Japanese *R. simsii*, respectively, but only 14.5% in *R. eriocarpum.* Interestingly, the hybrids showed the moderate value from their parents (24.8%). In greenhouse condition, there was no significant difference in shoot dry weight between samples collected in June (27/20ºC) and that in August (34/26ºC) of *R. eriocarpum* while two *R. simsii* accessions and F_1 hybrids showed significant differences. It suggests that *R. eriocarpum* seemed to be a heat resistant species. Regressing the shoot dry weight ratio versus their RI values at 50ºC of all species was linear relationship. The negative correlation existed between two values indicated that the electrolyte leakage technique is appropriate for evaluating the heat stress tolerance among evergreen azalea species. Heat stress tolerance of *R. eriocarpum* (seed parents) seems to be inherited to its F_1 offspring.

Key words: cell membrane thermostability (CMT), heat stress tolerance, hybrids, *R. eriocarpum*, *R. simsii*

INTRODUCTION

Rhododendron simsii is one of the most important wild azaleas, which is known as the mother species of the Belgian 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). In Vietnam, *R. simsii* distributes along streamside or riversides in the mountainous areas of northern and central part of Vietnam above 800–1400 m altitude and it has narrow lanceolate shape leaves (Ho, 1991) (Fig. 1A). In contrast, Japanese *R. simsii* grows in sunny and grassy slopes on the islands of Ryukyu Archipelago and it has relatively small and broadly obovate shape leaves (Yamazaki, 1996) (Fig. 1B). Thus, it seems that these two accessions may have not only different morphological characteristics but also physiological characteristics.

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 plant 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*

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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.

Thus, the authors concerned about heat stress resistance of two accessions of *R. simsii* distributed in Japan and Vietnam. It is 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* (Fig. 1C), which is distributed abundantly in Tokara Islands of Japan and considered as a high heat stress resistant species among Japanese evergreen azaleas (Sakata *et al*., 2006), and the hybrids between *R. eriocarpum* and *R. simsii* was also evaluated.

In this study, the heat tolerance of two accessions of *R. simsii* species, *R. eriocarpum* and their interspecific hybrids were evaluated using electrolyte leakage method to measure CMT. 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.

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 1). Cutting branches were planted by pumice soil and placed under the mist house condition 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.

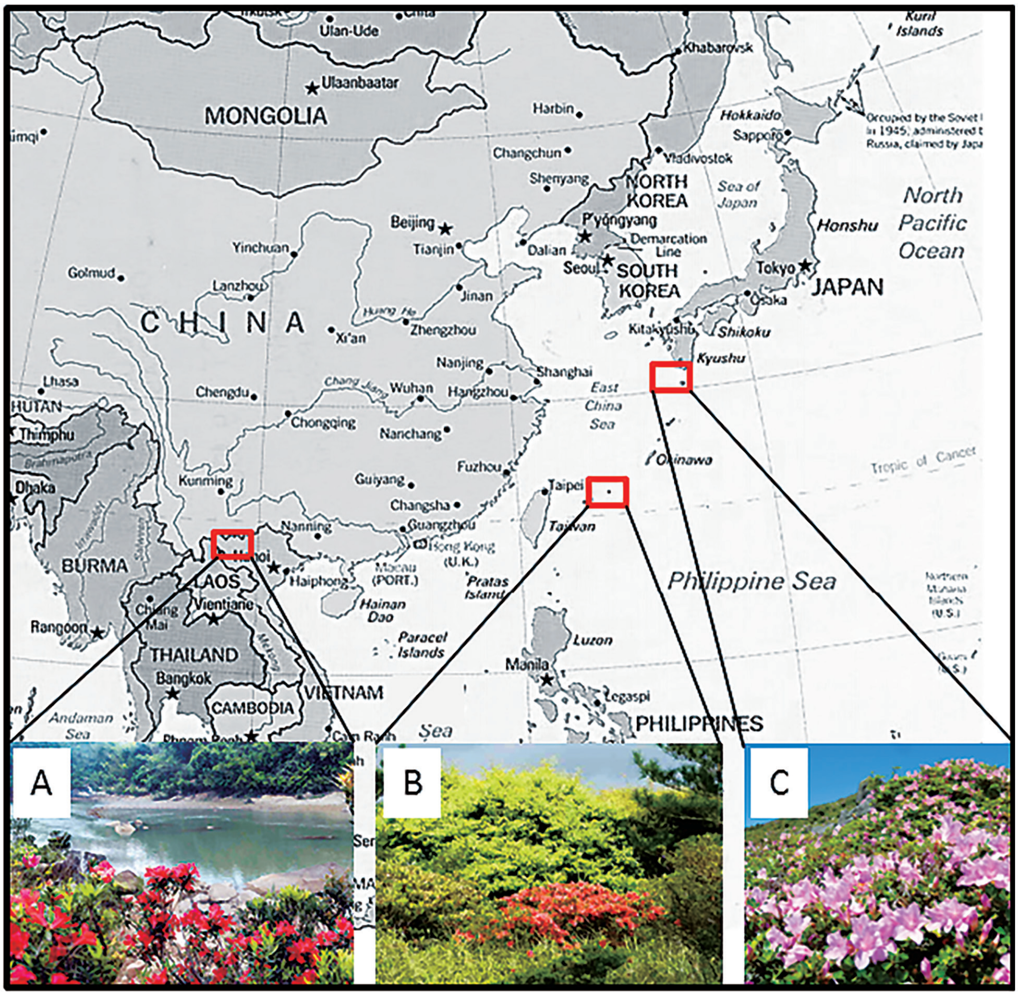

Fig. 1. Collection sites of the plant materials used in this study. (A) *R. simsii* in Than Uyen, Lai Chau, Vietnam, (B) *R. simsii* in Iriomotejima Islands, Japan and (C) *R. eriocarpum* in Tokara Islands, Japan.

Table 1. Plant materials used in this study

Samples	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)[z]	SIM (JP)	Showanomori Park, Umimachi, Fukuoka, Japan	Iriomotejima Island, Okinawa, 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 cross

[z] VN: Vietnam, JP: Japan

Leaf cell membrane thermostability (CMT) measurement.

From 23[th] June to 14[th] July, 2016, fully expanded leaves (leaves three to six from 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 at 25, 30, 35, 40, 45, 50, 55, 60, 65 and 70ºC for 30 min in the water bath.

A sample for assay consisted of a paired set (25ºC as control and another temperature treatment) of 6 mm diameter leaf discs samples cut from group of leaves of each species by 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 treatment, all tubes were added 15 mL of distilled water and shaken well by a shaker for 24 hours at room temperature (25ºC). Solution conductivity was measured by electrical conductivity meter (model ES–71; Horiba, Ltd, Kyoto, Japan). Then, test tubes were autoclaved for 15 min at 121ºC, cooled to 25ºC, 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 (25ºC) 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.

Temperature treatment in greenhouse condition

Three rooted cuttings of each material were placed in the greenhouse during June to August 2016 to evalu-

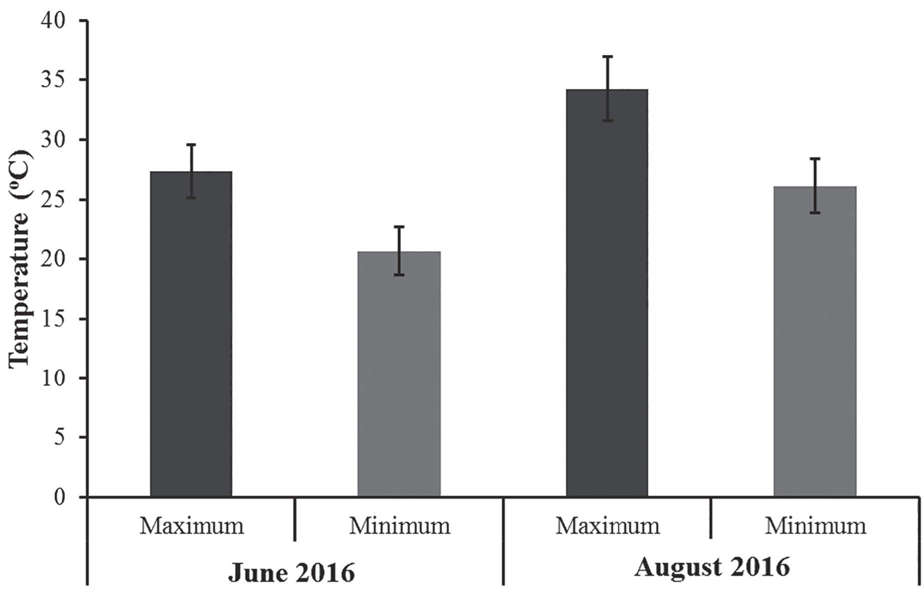

Fig. 2. Monthly mean maximum temperature and monthly mean minimum temperature in June and August, 2016 under greenhouse condition at Hakozaki campus, Kyushu University. Vertical bars indicate standard error.

ate the shoot dry weight under different temperature. The day and night temperature in June and August were 27/20°C and 34/26°C, respectively (Fig. 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°C for 48 hours (Jamal *et al*., 2014). Dry weight of all samples was also measured. Shoot dry weight ratio was calculated by shoot dry weight of samples collected in August divide to those in June.

Statistical analysis

Statistical analysis of the results was performed with IBM SPSS Statistics 21 software. The least significant difference (LSD) test and Tukey test ($P \leq 0.05$) 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 (Fig. 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°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°C, the relative injury percentage increased sharply up to above 75%. It means above 50°C, leaf tissue suffered severe heat stress resulting high percentage of leakage.

Relative injury values were ranked from low to high as ERI 18 × SIM (VN) < ERI 18 < SIM (JP) < SIM (VN) at 25°C (Table 2). However, at 50°C and calibrated 50°C, this rank was changed as ERI 18 < ERI 18 × SIM (VN)

Table 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 F_1 hybrids

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

[z] VN: Vietnam; JP: Japan.
[y] Calculated as (initial conductivity/final conductivity) × 100.
[x] Calibrated RI= $\{1-[1-(T_1/T_2)]/[1-(C_1/C_2)]\} \times 100$.
[w] Means with different letters within columns represent statistical difference by Tukey's test ($P < 0.05$).

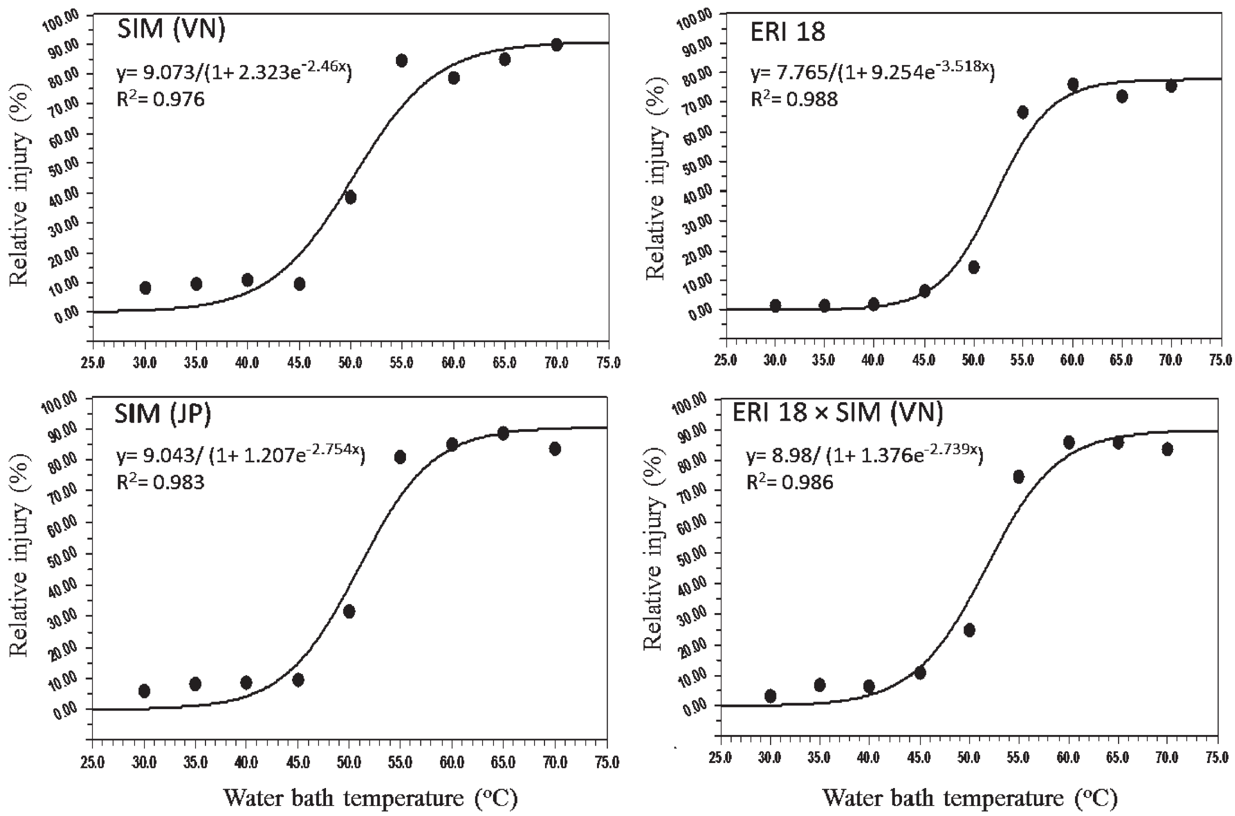

Fig. 3. Effect of water bath temperature on relative injury on leaf disks of *R.simsii*, *R. eriocarpum* and their F_1 hybrids. Response curves are established by Sigmoidal model in CurveExpert program version 1.4 based on the data of each species.

< SIM (JP) < SIM (VN). Among water bath treatments, 50°C resulted in the best differentiation between the heat tolerance of all samples. It suggests that a single heat treatment at 50°C can be used to evaluate the heat tolerance of evergreen azalea species without testing with different temperature treatment. A treatment temperature at 50°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 condition, there was no significant difference in shoot dry weight between samples collected in June (27/20°C) and that in August (34/26°C) of *R. eriocarpum*, while two *R. simsii* accessions and F_1 hybrids showed significant differences (Table 3). At high temperature of 34/26°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°C) (data not shown).

In present study, regressing the shoot dry weight ratio of all samples versus their RI value at 50°C was a linear relationship and negative correlation (Fig. 4). 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 as a primary limiting factor in the distribution and adaptability of not only wild species but also cultivated plants. Heat stress is often defined as the rise in temperature beyond a threshold level for a period of time sufficient to 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). Thus, it is a worldwide serious threat to crop production and ornamental production as well (Hall, 2001).

Like other organisms, plants have evolved different 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 induced 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°C suggested that the electrolyte leakage method is appropriate and effective as a rapid *in vitro* technique to evaluate plant heat stress tolerance using leaf discs instead of whole plant.

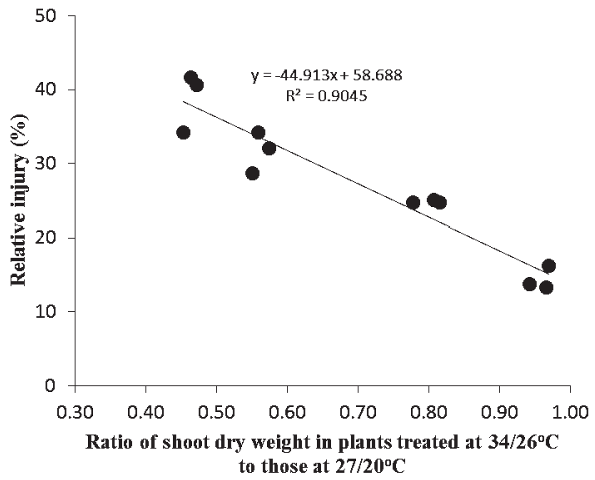

Fig. 4. The relationship between relative injury at 50°C and the ratio of shoot dry weight in *R. simsii*, *R. eriocarpum* and their F_1 hybrids grown at 34/26°C (August, 2016) to those at 27/20°C (June, 2016).

Table 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 to those in June 2016
	June 2016 (27/20°C)	August 2016 (34/26°C)	
SIM (VN)[z]	157.3 a[y]	72.8 b	0.46 D
SIM (JP)[z]	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] VN: Vietnam, JP: Japan

[y] Means with different letters within rows (small letters) and columns (capital letters) represent statistical difference by Tukey's test ($P < 0.05$).

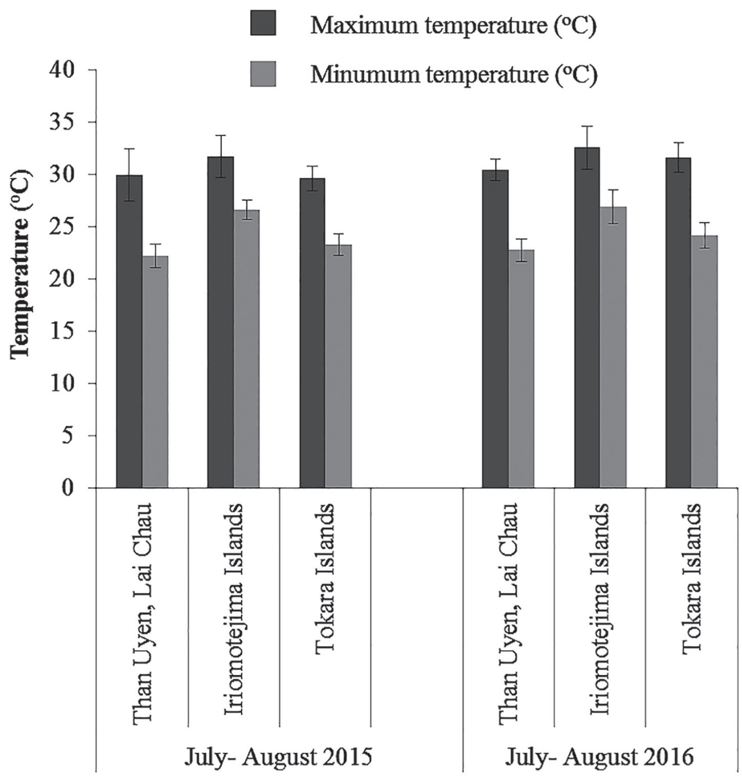

Fig. 5. Monthly mean maximum temperature and monthly mean minimum temperature during July to August 2015 and 2016 in Than Uyen, Lai Chau, Vietnam (source: Hydrometeorological center of Lai Chau province); Iriomotejima Islands and Tokara Islands (source: Japan meteorological agency). Vertical bars indicate standard error.

In natural growing habitat, SIM (VN) grows near riverside or streamside, indicating that these plants seem to be adapted under high moisture condition. Moreover, the temperature in Than Uyen district, where is above 800– 1400 m altitude, is not so hot with 30°C and 22°C of average highest temperature and lowest temperature, respectively (Fig. 5). Based on this growing condition, plants from Vietnamese accessions may have low heat stress tolerance. This is confirmed by 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). It is possible to agree with this result because this accessions grow in sunny and glassy slopes of Iriomotejima Islands in southern Japan at 32°C and 27°C of average highest temperature and lowest temperature during July to August, respectively (Fig. 5). This condition requires plants to be both drought and heat tolerant because it is rarely rainy during these two months, and plants have to withstand long term drought due to high temperature. In case of *R. eriocarpum* species, it performed the best resistance to heat stress condition as previous report (Sakata and Hashimoto, 2006). *Rhododendron eriocarpum* grows wildly at the sea level in the area composed 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 relating to heat 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 parents.

In the further study, an experiment about heat 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.

AUTHOR CONTRIBUTION

1. Dao Thi Thanh HUYEN, designed and conducted the experiments about heat stress tolerance of *R. simsii* and other related evergreen azalea species. Based on the results of this study, this author has contributed more knowledge about heat stress tolerance of *R. simsii* species and figured out that the electrolyte leakage method is appropriate and effective as a rapid in vitro technique to evaluate plant heat stress tolerance using leaf discs instead of whole plant.
2. Yuki MIZUNOE, contributed meaningful suggestion

and followed up the progress of this study steps by steps.

3. Dao Thanh VAN, cooperated with other authors to collect plant materials in Vietnam for this study. This author also gave valuable suggestion during writing the manuscript.
4. Ikuo MIYAJIMA, advised as a main supervisor in every corner of this study, and revised the manuscript very carefully up to final version.

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