International Journal of Plant Breeding ©2009 Global Science Books

Alteration of Capsule-Picking Time for Improving the Germination Rate through the Histological Observation of Zygote Development in Interspecific Crossing Kurume Azalea x *Rhododendron japonicum* f. *flavum*

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ABSTRACT

To improve the germination rate in interspecific crossing of Kurume azalea x *Rhododendron japonicum* f. *flavum*, Kurume azalea 'Yorozuyo' (seed parent) was crossed with *R. japonicum* f. *flavum* and Kurume azalea 'Wakakaede' (as a control). 1) Histological observation was carried out to clarify the zygote development process from 10 to 100 days after pollination (DAP). From 20 DAP on, the zygote length of Yorozuyo x *R. japonicum* f. *flavum* (YxJ) was less than that of 'Yorozuyo' x 'Wakakaede' (YxW). The zygotes of YxJ 60 DAP were the same length as those of YxW 30 DAP. At 100 DAP, the embryos of YxJ entered the late heart-shaped stage, and those of YxW reached the torpedo stage. These results suggest that zygote development of this interspecific cross appeared to be about one month later than that of the intraspecific crossing of Kurume azalea cultivars. 2) The capsules were collected from YxJ and YxW at 170 DAP and YxJ at 200 DAP, and the germination rate was compared among them. The germination rate increased in YxJ when the capsule-picking time was put off for 30 days. Also, the germination rates of YxJ capsules collected at 200 DAP had no statistically significant differences from those of YxW capsules collected at 170 DAP. These results imply that delaying the capsule-picking time effectively improves germination rates in this interspecific crossing.

Keywords: breeding, distant hybridization, embryo, *Pentanthera*, *Tsutsusi*
Abbreviations: DAP, days after pollination; **PPFD**, photosynthetic photon flux density

INTRODUCTION

Cultivated varieties of evergreen azalea originate from *Rhododendron* subgenus *Tsutsusi* section *Tsutsusi* species distributed in Japan and China. They are used for street planting, gardens and container use. Their flower colors are red, pink, purple and white but not yellow or blue. Kurume azalea, one of the evergreen azalea groups, is a brand name for Kirishima azalea (*R. obtusum*) and is named after the cultivars bred in Kurume, Fukuoka, in northern Kyushu (Akashi 1934). Although more than 700 cultivars of Kurume azalea can be found in horticultural books and seedling catalogs since the Edo era, the existing cultivars are estimated at about 360. They are characterized by small to medium-small flowers of bright color, and flowering is earlier than blanch growth and new spring leaves, so that the flowers cover the entire plant for the blooming time. The production of Kurume azalea has increased since the 1950s because of the great demand for public tree planting. At present, commercial azalea growers in Kurume desire to produce a yellow-flowered Kurume azalea cultivar.

To produce a yellow-flowered Kurume azalea cultivar, my laboratory conducted cross hybridization with yellow-flowered deciduous species *R. japonicum* f. *flavum* (subgenus *Pentanthera* section *Pentanthera*), used as a pollen parent. However, this cross was intersubgeneric hybridization between subgenus *Tsutsusi* and subgenus *Pentanthera*, so viable seedlings were rarely obtained. Thus my laboratory tried to develop a means of obtaining more viable seedlings. Some have reported that interspecific crossing was slower than intraspecific crossing for growing ovules or embryos (Chen and Adachi 1992; De Jeu and Garriga Caldere 1997; Li *et al*. 2000). In *Rhododendron*, foreign pollen tube growth appeared to be slower than the growth of self pollen tubes (Kaul *et al*. 1986). I suggest that the seeds obtained from the crossing of Kurume azalea x *R. japonicum* f. *flavum* may not have matured perfectly at the capsule-picking time selected in my laboratory and that causes other than germinating failure owing to zygotic isolation may have affected the germination rate. The objective of this study was to investigate the zygote development process in crossing of Kurume azalea x *R. japonicum* f. *flavum* and to verify the effectiveness of delaying the capsule-picking time for improving the germination rate.

MATERIALS AND METHODS

Kurume azalea 'Yorozuyo' was used as the seed parent since the crossability was high when *R. japonicum* f. *flavum* pollens were pollinated with this cultivar (Okamoto and Suto 2006). *R. japonicum* f. *flavum* accession no. 27026136 and Kurume azalea 'Wakakaede' as a control were used as the pollen parents. Anthers were collected from flower buds at the beginning of anthesis and preserved in a desiccator at room temperature in the dark until use. Flower buds were emasculated at the beginning of anthesis, and the stigma, covered with mucus, was hand-pollinated. The crossings were performed at the greenhouse of the Kurume Vegetable and Flower Research station, National Agricultural Research Center for Kyushu Okinawa Region, Japan, on 9 April 2006. This greenhouse reduced photosynthetic photon flux density (PPFD) by 23% (300-400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at noon).

To observe zygote development, three ovaries from each cross were collected from the seed parent at 10, 20, 30, 40, 60 and 100 days after pollination (DAP), fixed for 24 hr in FAA (1 formalin: 1

Received: 15 January, 2009. Accepted: 9 June, 2009.

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glacial acetic acid: 18 ethanol 70%, v/v/v; for the 10, 20, 30 and 40 DAP samples) and FPA_{50} (1 formalin: 1 propionic acid: 18 ethanol 50%, v/v/v; for the 60 and 100 DAP samples), then stored in 70% ethanol. Fixed samples were dehydrated by an ethanol-butanol series and embedded into paraffin blocks. Sections were cut to 10 µm thickness (10, 20, 30 and 40 DAP) or to 16-20 µm thickness (60 and 100 DAP) with a rotary microtome (Yamato Kohki, PR-50) and were stained with 1% fuchsin acid (Wako) and 0.2% fast green (Wako).

Five capsules were collected from the crossings of 'Yorozuyo' x *R. japonicum* f. *flavum* (YxJ) and 'Yorozuyo' x 'Wakakaede' (YxW, control) at 170 DAP (late September) and YxJ at 200 DAP (late October). After the capsules were dried artificially in a desiccator, more than 100 seeds from each crossing were sown on seedling beds that consisted of absorbent cotton in a plastic box (15.5 × 10.5 × 4.5 cm) and tested in triplicate on the 18th November 2006. The boxes were placed in a room facing south that reduced PPFD by 36.5% (350-500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at noon). The seeds were watered with tap water once a week. Germinating seeds were counted once every three weeks from December to March. The room temperature during the examination ranged from 3 to 18°C.

Numerical comparisons were carried out by analysis of variance. Dunnett's test (Dunnett 1955) was used to compare means of germination rate after arcsine transformation.

RESULTS

Table 1 lists the zygote lengths of YxJ and YxW. At 10 DAP, no statistically significant difference (P=0.05) was found between YxJ and YxW. From 20 DAP on, the zygotes of YxJ were significantly smaller than those of YxW. The zygotes of YxJ 40 DAP were the same length as those of YxW 20 DAP and the zygotes of YxJ 60 DAP were the same length as those of YxW 30 DAP.

An apical cell and a basal cell on the zygote could be discriminated at 60 DAP in YxJ (**Fig. 1A**) and at 30 DAP in YxW. Globular embryos were seen at 60 DAP in YxW (**Fig. 1B**). As of 100 DAP, the embryos of YxJ exhibited the late heart-shaped stage (**Fig. 1C**), and those of YxW reached the torpedo stage (**Fig. 1D**). These results suggest that the zygote growth of YxJ is about one month later than that of YxW.

Table 1 Zygote length in crossings of 'Yorozuyo' x *R. japonicum* f. *flavum* and 'Yorozuyo' x 'Wakakaede' 10, 20, 30, 40, 60, and 100 days after pollination.

Days after pollination	Yorozuyo x *R. japonicum* (µm)	Yorozuyo x Wakakaede (µm)	F value
10	14.4 ± 1.34	14.3 ± 1.59	0.004 n.s.[z]
20	21.8 ± 7.39	30.7 ± 7.78	11.368 **
30	27.1 ± 3.34	64.1 ± 13.46	88.429 ***
40	30.8 ± 4.60	112.8 ± 25.81	147.888 ***
60	67.1 ± 15.28	160.4 ± 23.72	84.267 ***
100	288.8 ± 80.16	520.2 ± 60.89	60.519 ***

[z] **, ***, and n.s. indicate significance at P = 0.01, 0.001, and non-significance, respectively (following analysis of variance).

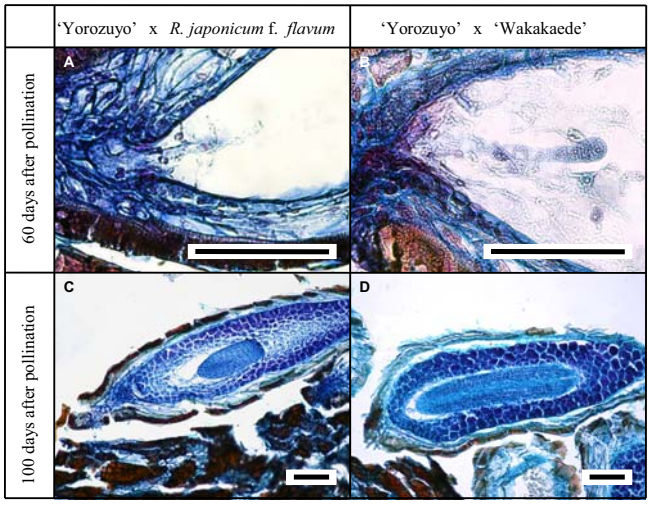

Fig. 1 Zygote development in crossings of 'Yorozuyo' x *R. japonicum* f. *flavum* and 'Yorozuyo' x 'Wakakaede'. (**A**) Apical cell and basal cell could be discriminated. (**B**) Globular embryo. (**C**) Late heart shaped-stage embryo. (**D**) Torpedo-stage embryo. Scale bar = 100 µm.

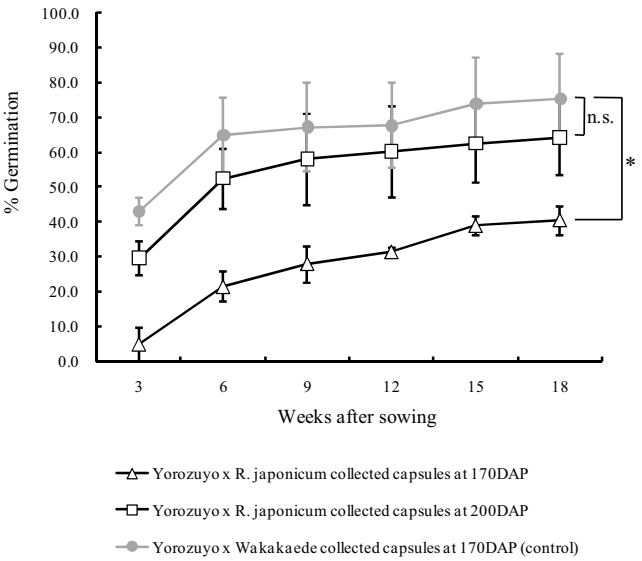

Fig. 2 Germination rate of seeds obtaining from crossing of 'Yorozuyo' x *R. japonicum* f. *flavum* as influenced by capsule-picking time. * and n.s. indicate significance at P = 0.05 and non-significance, respectively (by Dunnett's test for treatments vs. control).

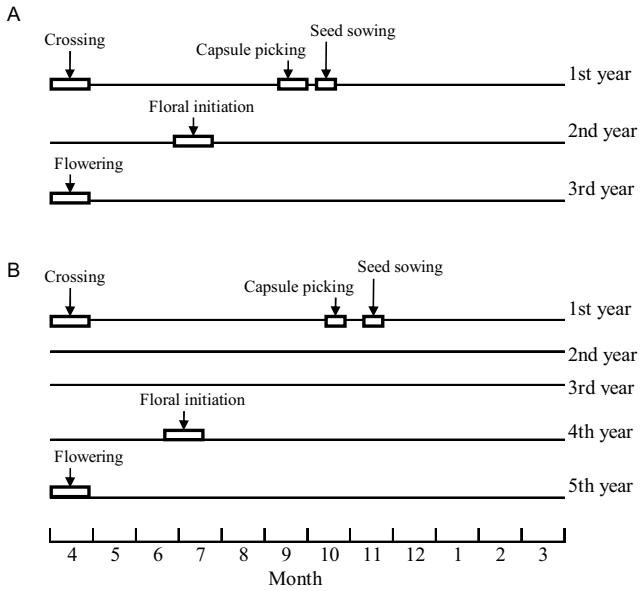

Fig. 3 Breeding program for azaleas (growth in a green house, more than 5°C). (**A**) Cross combination between evergreen azaleas conducted in my laboratory. (**B**) Cross combination between evergreen azalea and *R. japonicum* f. *flavum* proposed through the results of this study.

The germination rate of YxJ capsules collected at 170 DAP was significantly lower than that of YxW capsules collected at 170 DAP (**Fig. 2**). No statistically significant difference was found between YxJ capsules collected at 200 DAP and YxW capsules collected at 170 DAP (**Fig. 2**). When the capsules of YxJ were collected at 200 DAP, the germination rate at 18 weeks after sowing was 64.1%. This result indicates that it is possible to improve the YxJ germination rate by delaying capsule-picking time for 30 days.

DISCUSSION

Interspecific hybridization between evergreen azalea and *R. japonicum* f. *flavum* has been described since the Taisho era (1912 to 1926) in Japan (Miyazawa 1922), but no yellow-flowered evergreen azalea has ever been produced. When evergreen azalea was used as a pollen parent, pollen tube growth in the style was totally inhibited (Creech 1955; Ureshino *et al*. 2000). Furthermore, arrest of pollen tube growth, failure of pollen tube penetration into the ovules, lack of seed germination, chlorophyll defects in cotyledons, and death of young seedlings were detected when evergreen azalea was used as a seed parent (Noguchi 1932; Akabane *et al*. 1971; Okamoto and Suto 2004). These hybridization barriers overlapped in one cross combination, and each barrier reduced hybridization but did not arrest it perfectly (Okamoto and Suto 2004).

Okamoto and Suto (2006) found that the percentage of surviving seedlings was higher when some Kurume azalea cultivars which possessed dorsal leaf surface features similar to evergreen species *R. macrosepalum* and *R. ripense*, was used as the seed parent. If the germination rate could be improved in such cross combinations, it should be possible to increase the percentage of viable seedlings. The histological observation of the zygote development process revealed that the growth of YxJ zygotes progressed one month later than that of the control. Furthermore, when the capsule-picking time was delayed for one month, YxJ exhibited a relatively high germination rate, and this rate was not statistically significantly different from that of control crossing capsules collected at 170 DAP. I thus consider that delaying the capsule-picking time effectively improves the germination rate in Kurume azalea x *R. japonicum* f. *flavum*. In addition, it is evident that early collecting of capsules affects the germination in this crossing, although germination failure has been estimated by germination rate.

The reason the YxJ zygote growth was delayed is not clear from the results of this study. However, the pollen tube growth of both YxJ and YxW was 1.2 cm/day in the style (data not shown). At 10 DAP, no statistically significant difference was found between the zygote length of YxJ and that of YxW. I presume that the reason is not the inhibition of normal pollen tube growth by foreign pistil tissues (Kaul *et al*. 1986), but the suppression of zygote development by genetic differences after fertilization.

In azalea breeding, it is important to sow seeds as soon as possible and to grow seedlings quickly to shorten the first flowering age because azaleas have a long juvenile phase, and this phase continues until the azalea reaches a fixed tree size (pers. obs.). **Fig. 3A** illustrates the breeding program for evergreen azalea conducted in my laboratory. In cross combination between evergreen azaleas, azaleas were crossed in early to mid April, capsules were collected in mid to late September, and seeds were sown in mid October. Germination began two weeks after sowing, three or four true leaves grew until next spring, and flower buds were seen in autumn. Consequently, the seedlings reached first flowering two years after pollination. In interspecific crossings of Kurume azalea x *R. japonicum* f. *flavum*, however, first flowering began four years after pollination since the seedlings grew slowly. If capsules were picked too late, winter arrived before germination, and the growth of seedlings was delayed further. Therefore, I consider that collecting the capsules in late October, one month later compared with intraspecific crossings of evergreen azaleas is suitable for improving the germination rate and the growth of seedlings (**Fig. 3B**). As a result, seeds are sown in mid November. This is the optimal timing for the seeds to germinate normally, and subsequently the seedlings grew true leaves before winter.

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