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# Oryzalin–induced Allotetraploids of an Intersubgeneric Hybrid between Evergreen and Deciduous Azaleas

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Pollen viability of intersubgeneric hybrids between evergreen and deciduous azaleas was much lower than that of their parents. No capsule set in the crossings with the hybrids when used either as seed or pollen parents. *In vitro* chromosome doubling with oryzalin was attempted for restoring the fertility of the hybrid. Survival rates of the explants were more than 50% in oryzalin treatments, though high concentration and long term treatments brought the survival rates and number of shoots per explant low, under which number of shoots per explant also decreased. Twenty tetraploid and 28 mixoploid (2x+4x) plants were obtained by the treatments from 123 individuals. The most suitable conditions for obtaining tetraploids appeared to be 0.01% oryzalin treatment for 48 hours.

## INTRODUCTION

Evergreen azaleas have been grown as garden trees and potted plants for hundreds of years in Japan because of their beautiful flower colors and evergreen leaves. There are many evergreen azalea species growing wild in Kyushu Island, Japan, from which several hundred varieties such as Kurume Azaleas and Hirado Azaleas have been bred. Their flower colors have been, however, restricted to white, pink, red, reddish–purple and purple, and yellow–flowered cultivars have not yet been obtained.

Numbers of breeders have tried to produce yellow–flowered evergreen azaleas using intersubgeneric crossings between evergreen species and yellow–flowered deciduous species such as *Rhododendron japonicum* f. *flavum* Suringer or *R. molle* G. Don (Akabane *et al.*, 1971; Heursel, 1981; Yamaguchi *et al.*, 1985). Most of these crossings, however, failed to obtain progenies because of low frequency of capsule set and viable seeds. Almost of the progenies from the crossings, if obtained, were albino or pale–green plants, which withered away within one year (Heursel, 1981; Yamaguchi, 1986). Akabane (1993) obtained intersubgeneric hybrids from the crosses between white–flowered

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evergreen azalea and yellow-flowered *R. japonicum* f. *flavum*. Flowers of the hybrids were pale yellow at full bloom stage, and the carotenoid pigment, the main agent of the deep yellow flower color of *R. japonicum* f. *flavum*, was detected in the petals of the hybrids (Miyajima *et al.*, 2000). Thus, these hybrids may be favorable breeding materials for yellow-flowered evergreen azalea cultivars.

The sterility of distant hybrids is common phenomenon, and it may be considered as the results of the genetic unbalance. In such sterility caused from chromosomal structural differences, doubling of chromosomes, particularly in somatic tissue, results in normal meiosis of complete homologous chromosomes.

Flow cytometry has been established for rapid and efficient estimation of ploidy levels in some crops (Van Tuyl *et al.*, 1992; Tosca *et al.*, 1995; Ozaki *et al.*, 1998). Ploidy analysis by flow cytometry has been also accepted in *Rhododendron* (Vainola, 2000; De Schepper *et al.*, 2001; Ureshino and Miyajima, 1998, Sakai *et al.*, 2003).

The objective of this study is to assess the gametic fertility of intersubgeneric hybrids and to establish the methods for obtaining allotetraploid azaleas through *in vitro* oryzalin treatment.

## MATERIALS AND METHODS

### Pollen fertility of intersubgeneric hybrids

Pollen fertility of evergreen azalea (E), *R. japonicum* f. *flavum* (D) and their intersubgeneric hybrids (E X D) was examined (Table 1). Pollen grains were collected at their full bloom stage, and observed with an optical microscope after staining with aceto-carmine for 10 minutes. The pollen grains were classified to perfect pollen with densely stained cytoplasm or to empty pollen that was not stained. More than 400 pollen grains in three replications were observed in each accession.

**Table 1.** Evergreen and deciduous azaleas and their hybrids used as cross parents in this study.

| Parents | Code | Description |
|---|---|---|
| Evergreen azalea<br>(*R. kiusianum* X *R. eriocarpum*) (No. 1, 2)[z] | E1, 2 | White-flowered interspecific hybrid, subgen. Tsutsusi X subgen. Tsutsusi |
| Deciduous azalea<br>*R. japonicum* f. *flavum* (No. 1, 2) | D1, 2 | Yellow-flowered species, subgen. Pentanthera |
| Hybrid<br>((*R. kiusianum* X *R. eriocarpum*) (No. 2)<br>X *R. japonicum* f. *flavum* (No. 2)) (No. 1, 2) | (E2 X D2) 1, 2 | Pale yellow-flowered intersubgeneric hybrid |

[z] ( )=individual number

### Induction of multiple shoots

The artificial crossings between evergreen azalea (E) and *R. japonicum* f. *flavum* (D) were carried out on May 1997. The obtained seeds were sown in October 1997 on Anderson's rhododendron medium (Anderson, 1984) supplemented with 50 $mgl^{-1}$ of gib-

berellic acid (pH5.0). After seed germination, green and vigorous seedlings were transplanted on the Anderson's rhododendron medium with 10 $mgl^{-1}$ of $N^6$–(2–isopentenyl) adenine (2ip) to induce multiple shoots. The multiplicated shoots from one seedling were cut into segments and supplied for *in vitro* oryzalin treatments after confirming their hybridity (Ureshino *et al.*, 1998).

**In vitro oryzalin treatments**

The autoclaved nutrient solution (Anderson's rhododendron medium with 10$mgl^{-1}$ 2ip and 3% sucrose) with pH 5.0 was supplemented with filter–sterilized 0, 0.001, 0.005 or 0.01% oryzalin dissolved in 1% DMSO (dimethyl sulfoxide) after being dispensed into 200 ml plastic vessels. Three segments obtained from the multiple shoots were incubated in the light at 25 °C for 24, 48 or 72 hours in the vessels. After the treatment, the segments were washed with distilled water three times. Then the shoots of the segments were cut into 8–10 mm length, and cultured for five months on the multiplication medium in the light at 25 °C. The shoots obtained from the explants were transferred to the sphagnum in plastic pots, and incubated in the light at 25 °C. The rooted plants were placed to a greenhouse.

**Ploidy levels in the regenerated plants**

The assessment of ploidy levels was done by flow cytometry. Young leaves of the generated plants were chopped with a sharp razor blade in nuclei extraction buffer (High resolution DNA kit, Partec), and the suspension containing released nuclei was passed through a 50 $\mu$m filter. Then, the nuclei in filtrate were stained with four times volume of staining solution (High resolution DNA kit, Partec) containing 4'–6–diamidino–2–phenylindole (DAPI). After shaking the solution gently, samples were analyzed with a flow cytometer (PA Ploidy Analyzer, Partec). Relative DNA content was estimated according to the prominent peak in each measurement.

## RESULTS

Pollen fertility of evergreen azalea (E) and *R. japonicum* f. *flavum* (D) was high (79.5–87.4%), whereas that of their hybrids (E X D) was quite low (0.1–0.6%) (Table 2). No capsule set when the hybrids were used either as seed or pollen parents in the crossings with evergreen azalea and *R. japonicum* f. *flavum* (Table 3).

**Table 2.** Pollen fertility of evergreen and deciduous azaleas and their hybrids.

| Parents | Pollen fertility [z] (%) |
|---|---|
| Evergreen azalea | |
| E2 | 79.5±4.5 |
| Deciduous azalea | |
| D2 | 87.4±2.8 |
| Hybrid | |
| (E2 X D2) 1 | 0.6±0.2 |
| (E2 X D2) 2 | 0.1±0.1 |

[z] Mean±SE

The survival rates of the explants were more than 50% in each treatment with oryzalin though high concentration and long term treatments brought low survival rates and decreased the number of shoots per explant (Table 4).

Diploid and tetraploid plants were easily identified by the flow cytometric analysis as reported by Sakai *et al.* (2003), i.e., fluorescent intensities at prominent peaks in diploids and tetraploids were about 100 and 200, respectively (Fig. 1). The treated plants, representeing two peaks of fluorescent intensities 100 and 200, were judged to be mixoploids

**Table 3.** Frequency of capsule set in the crosses with intersubgeneric azalea hybrids.

| Cross | Capsule set[z] |
|---|---|
| Hybrid x evergreen azalea | |
| (E2 X D2) 1 X E1 | 0/5[y] |
| (E2 X D2) 2 X E1 | 0/3 |
| Evergreen azalea X hybrid | |
| E1 X (E2 X D2) 1 | 0/4 |
| E1 X (E2 X D2) 2 | 0/2 |
| E2 X (E2 X D2) 1 | 0/2 |
| E2 X (E2 X D2) 2 | 0/4 |
| Deciduous azalea X hybrid | |
| D1 X (E2 X D2) 1 | 0/3 |
| D1 X (E2 X D2) 2 | 0/5 |
| Hybrid X hybrid | |
| (E2 X D2) 1 X (E2 X D2) 2 | 0/3 |

[z] Capsule sets were observed 3 months after crossing
[y] No. of capsules / No. of pollinated flowers

**Table 4.** Effects of oryzalin treatments on the survival rates and shoot formation in the intersubgeneric azalea hybrid.

| Concentration (%) | Duration (hours) | No. of explants treated | Survival rate[z] (%) | No. of shoots / explant |
|---|---|---|---|---|
| 0 | 24 | 17 | 100 | 9.5 |
| | 48 | 21 | 95.2 | 20.0 |
| | 72 | 21 | 100 | 18.6 |
| 0.001 | 24 | 20 | 90.0 | 39.4 |
| | 48 | 21 | 95.7 | 15.7 |
| | 72 | 18 | 94.0 | 28.5 |
| 0.005 | 24 | 21 | 71.4 | 31.0 |
| | 48 | 21 | 76.2 | 35.8 |
| | 72 | 21 | 80.1 | 13.3 |
| 0.01 | 24 | 21 | 100 | 33.2 |
| | 48 | 21 | 52.4 | 11.3 |
| | 72 | 21 | 62.0 | 11.6 |

[z] Data were collected after 5 months of culture

(2x+4x). In total 123 plants investigated, controls with DMSO maintained the diploid status, and 20 tetraploids and 28 mixoploid plants were obtained (Table 5). The most suitable condition for inducing tetraploid plants was the treatment with 0.01% oryzalin for 48 hours.

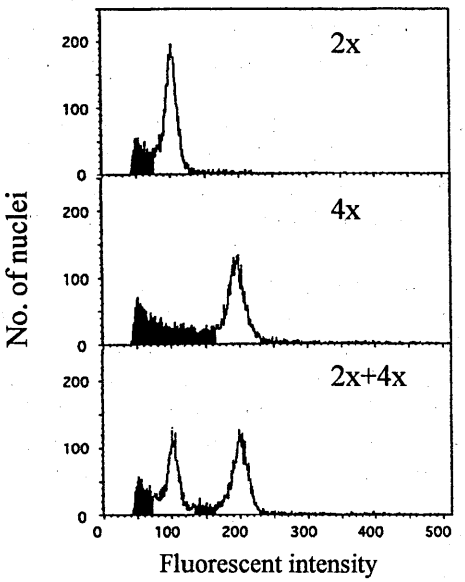


**Fig. 1.** Flow cytometric histograms of diploid, tetraploid and mixoploid (2x+4x) obtained from the *in vitro* oryzalin treatments for the intersubgeneric azalea hybrid.

**Table 5.** Effects of oryzalin treatments on the rate of tetraploid induction in the intersubgeneric azalea hybrid.

| Concentration (%) | Duration (hours) | No. of individuals examined | Ploidy levels (%) | | |
|---|---|---|---|---|---|
| | | | 2x | 2x+4x | 4x |
| 0 | 24 | 5 | 5 (100 ) | 0 ( 0 ) | 0 ( 0 ) |
| | 48 | 1 | 1 (100 ) | 0 ( 0 ) | 0 ( 0 ) |
| | 72 | 9 | 9 (100 ) | 0 ( 0 ) | 0 ( 0 ) |
| 0.001 | 24 | 12 | 10 ( 83.3) | 2 (16.7) | 0 ( 0 ) |
| | 48 | 13 | 6 ( 46.2) | 3 (23.1) | 4 (30.8) |
| | 72 | 11 | 6 ( 54.5) | 4 (36.4) | 1 ( 9.1) |
| 0.005 | 24 | 12 | 1 ( 8.3) | 11 (91.7) | 0 ( 0 ) |
| | 48 | 11 | 11 (100 ) | 0 ( 0 ) | 0 ( 0 ) |
| | 72 | 9 | 8 ( 88.9) | 1 (11.1) | 0 ( 0 ) |
| 0.01 | 24 | 12 | 12 (100 ) | 0 ( 0 ) | 0 ( 0 ) |
| | 48 | 13 | 2 ( 15.4) | 0 ( 0 ) | 11 (84.6) |
| | 72 | 15 | 4 ( 26.7) | 7 (46.7) | 4 (26.7) |
| Total | | 123 | 75 ( 61.0) | 28 (22.8) | 20 (16.3) |

## DISCUSSION

The hybrid (E X D) of evergreen azalea (E) X *R. japonicum* f. *flavum* (D) used in this study may not be used in further breeding. The sterility in hybrids derived from distant hybridization has been considered to be a result of the genetic unbalance as has been found in this study and also reported in many plants such as *Lilium* (Asano, 1982) and *Solanum* (Ali *et al.*, 1992). In such cases, chromosome doubling is effective for restoring fertility of the hybrids.

Pryor and Frazier (1968) obtained the colchicine–induced tetraploid azaleas with great potential of improving flower size and petal texture. Although colchicine has been successfully used to induce polyploidy in many horticultural crops, oryzalin is considered superior to colchicine because of its lower phytotoxicity and the absence of long term effects (Tosca *et al.*, 1995).

There are many reports that flow cytometry is a useful method for ploidy level determination and this technique is as a practical and rapid tool for confirming the ploidy levels of plants (Baird *et al.*, 1994; O'Brien *et al.*, 1996; Ozaki *et al.*, 1998; Pinheiro *et al.*, 2000). It was also recognized that the effectiveness of flow cytometry for the ploidy determination of rhododendron hybrids (Vainola, 2000; Sakai *et al.*, 2003). In our study, the *in vitro* oryzalin treatments of multiple shoots in an intersubgeneric azalea hybrid were proved to be effective for inducing polyploids, and the tetraploid plants, which are predicted to be fertile, was obtained.

Stomata, pollen and roots originate from histogenic layers LI, LII and LIII, respectively (Dermen, 1947; Dermen and Stewart, 1973; Ramulu *et al.*, 1976). The plants, determined to be tetraploid by flow cytometry, had larger guard cells than diploid plants (data not shown). The *in vitro* oryzalin treatments in this study induced 28 mixoploid plants. Chromosome doubling in LII layer would be confirmed. If the LII of the plants are tetraploid, the regaining of fertility of these chimeric plants is hopeful like tetraploid plants.

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