

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

Sakai, Kaori

Laboratory of Horticultural Science, Division of Agricultural Botany, Department of Plant Resources, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University

Miyajima, Ikuo

Crop Production Section, Institute of Tropical Agriculture, Kyushu University

Ureshino, Kenji

Laboratory of Olericultural and Floricultural Science, Faculty of Agriculture, Iwate University

Ozaki, Yukio

Laboratory of Agricultural Ecology, Division of Agricultural Ecology, Department of Plant Resources, Faculty of Agriculture, Kyushu University

他

<https://doi.org/10.5109/4589>

---

出版情報：九州大学大学院農学研究院紀要. 49 (2), pp.293-299, 2004-10-01. Faculty of Agriculture, Kyushu University

バージョン：

権利関係：

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

Kaori SAKAI<sup>1\*</sup>, Ikuo MIYAJIMA<sup>2</sup>, Kenji URESHINO<sup>3</sup>,  
Yukio OZAKI<sup>4</sup> and Hiroshi OKUBO

Laboratory of Horticultural Science, Division of Agricultural Botany,  
Department of Plant Resources, Faculty of Agriculture,  
Kyushu University, Fukuoka 812–8581, Japan  
(Received June 30, 2004 and accepted July 13, 2004)

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

---

<sup>1</sup> Laboratory of Horticultural Science, Division of Agricultural Botany, Department of Plant Resources, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University

<sup>2</sup> Crop Production Section, Institute of Tropical Agriculture, Kyushu University

<sup>3</sup> Laboratory of Olericultural and Floricultural Science, Faculty of Agriculture, Iwate University, Morioka 020–8550, Japan

<sup>4</sup> Laboratory of Agricultural Ecology, Division of Agricultural Ecology, Department of Plant Resources, Faculty of Agriculture, Kyushu University, Fukuoka 811–2307, Japan

\* Corresponding author (k\_sakai@agr.kyushu-u.ac.jp)

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-carmin 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 ( <i>R. kiusianum</i> X <i>R. eriocarpum</i> ) (No. 1, 2) <sup>2</sup>	E1, 2	White-flowered interspecific hybrid, subgen. Tsutsusi X subgen. Tsutsusi
Deciduous azalea <i>R. japonicum</i> f. <i>flavum</i> (No. 1, 2)	D1, 2	Yellow-flowered species, subgen. Pentanthera
Hybrid ( <i>R. kiusianum</i> X <i>R. eriocarpum</i> ) (No. 2) X <i>R. japonicum</i> f. <i>flavum</i> (No. 2)) (No. 1, 2)	(E2 X D2) 1, 2	Pale yellow-flowered intersubgeneric hybrid

<sup>2</sup> ( ) = 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 mg l<sup>-1</sup> of gib-

berellic acid (pH5.0). After seed germination, green and vigorous seedlings were transplanted on the Anderson's rhododendron medium with  $10 \text{ mg l}^{-1}$  of  $\text{N}^6$ -(2-isopentenyl) adenine (2ip) to induce multiple shoots. The multiplied 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  $10 \text{ mg l}^{-1}$  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  $25^\circ\text{C}$  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  $25^\circ\text{C}$ . The shoots obtained from the explants were transferred to the sphagnum in plastic pots, and incubated in the light at  $25^\circ\text{C}$ . 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  $50 \mu\text{m}$  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 * (%)
Evergreen azalea	
E2	$79.5 \pm 4.5$
Deciduous azalea	
D2	$87.4 \pm 2.8$
Hybrid	
(E2 X D2) 1	$0.6 \pm 0.2$
(E2 X D2) 2	$0.1 \pm 0.1$

\* Mean  $\pm$  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, representing 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 <sup>a</sup>
Hybrid x evergreen azalea	
(E2 X D2) 1 X E1	0/5 <sup>y</sup>
(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

<sup>a</sup> Capsule sets were observed 3 months after crossing

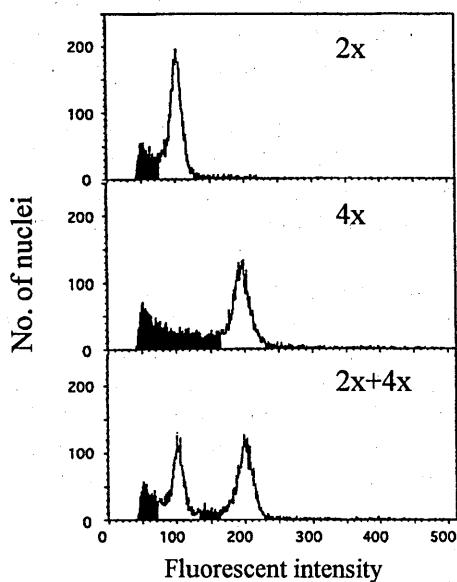
<sup>y</sup> No. of capsules / No. of pollinated flowers

**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 <sup>a</sup> (%)	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

<sup>a</sup> 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.

## ACKNOWLEDGEMENT

The authors wish to thank Mr. Masaru Akabane for his donation of the white-flowered evergreen azalea. We also thank to Associate Professor Dr. Koichiro Gyokusen in the Laboratory of Silviculture, Faculty of Agriculture, Kyushu University for cooperation in flow cytometric analysis.

## REFERENCES

- Akabane, M. 1993 On the cross compatibility F<sub>1</sub>-plants (*Rhododendron kiusianum* Mak. X *R. indicum* Sweet) X *R. japonicum* Suringar. *J. Japan. Soc. Hort. Sci.*, **62** (Suppl. 1): 344-345 (in Japanese)
- Akabane, M., A. Yamanaka, D. Takashima, T. Nakatsue and Y. Nakamura 1971 On the fertility of interspecific crossing and the growth of F<sub>1</sub> seedlings in rhododendron species. *Bull. Tohigi Pref. Agri. Expt. Stn.*, **15**: 95-102 (in Japanese)
- Ali, M., H. Okubo and K. Fujieda 1992 Production and characterization of *Solanum* amphidiploids and their resistance to bacterial wilt. *Scientia Hortic.*, **49**: 181-196
- Anderson, W. C. 1984 A revised tissue culture medium for shoot multiplication of rhododendron. *J. Amer. Soc. Hort. Sci.*, **109**: 343-347

- Asano, Y. 1982 Overcoming interspecific hybrid sterility in *Lilium*. *J. Japan. Soc. Hort. Sci.*, **51**: 75–81
- Baird, W. V., A. S. Estager and J. K. Wells 1994 Estimating nuclear DNA content in peach and related diploid species using laser flow cytometry and DNA hybridization. *J. Amer. Soc. Hort. Sci.*, **119**: 1312–1316
- Dermen, H. 1947 Periclinal cytochimera and histogenesis in cranberry. *Amr. J. Bot.*, **34**: 32–43
- Dermen, H. and R. N. Stewart 1973 Ontogenic study of floral organs of peach (*Prunus persica*) utilizing cytochimeral plants. *Amr. J. Bot.*, **60**: 283–291
- De Schepper, S., L. Leus, M. Mertens, E. Van Bockstaele and M. De Loose 2001 Flow cytometric analysis of ploidy in *Rhododendron* (subgenus *Tsutsusi*). *HortScience*, **36**: 125–127
- Heursel, J. 1981 Diversity of flower colors in *Rhododendron simsii* Planch. and prospects for breeding. *Euphytica*, **30**: 9–14
- Miyajima, I., K. Ureshino, N. Kobayashi and M. Akabane 2000 Flower color and pigments of intersubgeneric hybrid between white-flowered evergreen and yellow-flowered deciduous azaleas. *J. Japan. Soc. Hort. Sci.*, **69**: 280–282
- O'Brien, I. E. W., D. R. Smith, R. C. Gardner and B. G. Murray 1996 Flow cytometric determination of genome size in *Pinus*. *Plant Sci.*, **115**: 91–99
- Ozaki, Y., K. Narikiyo, M. Hiramatsu, K. Ureshino and H. Okubo 1998 Application of flow cytometry for rapid determination of ploidy levels in asparagus (*Asparagus officinalis* L.). *J. Fac. Agr., Kyushu Univ.*, **43**: 83–88
- Pinheiro, A. A., M. T. Pozzobon, C. B. do Valle, M. I. O. Penteado and V. T. C. Carneiro 2000 Duplication of the chromosome number of diploid *Brachiaria brizantha* plants using colchicine. *Plant Cell Rep.*, **19**: 274–278
- Pryor, R. L. and L. C. Frazier 1968 Colchicine-induced tetraploid azaleas. *HortScience*, **3**: 283–286
- Ramulu, K. S., M. Devreux, G. Ancora and U. Laneri 1976 Chimerism in *Lycopersicon peruvianum* plants regenerated from *in vitro* cultures of anthers and stem internodes. *Z. Pflanzenzüchtg.*, **76**: 299–319
- Sakai, K., Y. Ozaki and H. Okubo 2003 Intra- and inter-ploid cross compatibility among diploid triploid and tetraploid Satsuki azaleas. *J. Japan. Soc. Hort. Sci.*, **72** (Suppl. 2): 205 (in Japanese)
- Tosca, A., R. Pandlfi, S. Citterio, A. Fasoli and S. Sgorbati 1995 Determination by flow cytometry of the chromosome doubling capacity of colchicine and oryzalin in gynogenetic haploids of *Gerbera*. *Plant Cell Rep.*, **14**: 455–458
- Ureshino, K. and I. Miyajima 1998 The relationship between appearance of albino seedlings and their ploidy level in intersectional crossings among azalea species (tentatively translated into English by K. Sakai. from the original Japanese title). *J. Japan. Soc. Hort. Sci.*, **67** (Suppl. 2): 389 (in Japanese)
- Ureshino, K., I. Miyajima and M. Akabane 1998 Effectiveness of three-way crossing for the breeding of yellow-flowered evergreen azalea. *Euphytica*, **104**: 113–118
- Vainola, A. 2000 Polyploidization and early screening of *Rhododendron* hybrids. *Euphytica*, **112**: 239–244
- Van Tuyl, J. M., B. Meijer and M. P. Dien 1992 The use of oryzalin as an alternative for colchicine in *in-vitro* chromosome doubling of *Lilium* and *Nerine*. *Acta Hort.*, **325**: 625–630
- Yamaguchi, S. 1986 *In-vitro* culture of remote hybrid seedlings aiming to breed new yellow flowered evergreen azalea. *Plant Cell Incompatibility Newslett.*, **18**: 50–51
- Yamaguchi, S., M. Kunishige and T. Tamura 1985 Interspecific compatibility in Japanese rhododendrons. *Bull. Veg. and Orn. Crop Res. Stn. Japan, Ser. B*, **8**: 87–97 (in Japanese with English summary)