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Reproductive Behavior of Diploid and Allotetraploid *Rhododendron* L. 'Fragrant Affinity'

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Abstract. **Wide hybridization can lead to recombination of diverse traits and creation of unique phenotypes, but the resultant hybrids are often sterile as is the case with the intersubgeneric hybrid *Rhododendron* L. 'Fragrant Affinity'. Sterility in wide hybrids can either be genic or chromosomal; the latter may be overcome by induction of polyploidy, which can restore chromosome homology and fertility. Cytologic studies of 'Fragrant Affinity' appear to support the presence of bridges between bivalents in metaphase I and laggard chromosomes in anaphase I. In the current study, an allotetraploid form of *R.* 'Fragrant Affinity' was developed using oryzalin (4-(dipropylamino)-3, 5-dinitro-benzenesulfonamide) as a mitotic inhibitor and chromosomal doubling agent. Genome sizes (2C) were determined using flow cytometry and found to be ≈1.6 and 3.2 pg for the diploid and allotetraploid, respectively. Pollen viability, determined by staining and germination tests, was 4% and 0%, respectively, for the diploid and 68% and 45%, respectively, for the allotetraploid. No seeds were produced when the diploid *R.* 'Fragrant Affinity' was used as a pistillate parent when pollinated with pollen from viable diploid and tetraploid parents. The allotetraploid produced viable seeds and seedlings when pollinated with pollen from either diploid or tetraploid parents, including self-pollination, demonstrating restored male and female fertility.**

Rhododendrons and azaleas (*Rhododendron* L.) are among the most widely grown ornamental plants in the world. There are over 1000 species recognized in eight subgenera (Chamberlain et al., 1996). Species within subgenera readily hybridize and have resulted in thousands of cultivars (Väinölä, 2000). Although intrasubgeneric hybridization is responsible for the majority of existing cultivars, species in different subgenera are sometimes capable of hybridizing.

Azaleodendrons are hybrids between deciduous azaleas (subgenus *Pentanthera* (G.Don) Pojarkova) and nonscaly leaved rhododendrons (subgenus *Hymenanthes* (Blume) K.Koch) and constitute some of the oldest hybrids within the genus. The first interspecific hybrid rhododendron reported was 'Azaleoides' resulting from a chance cross between *Rhododendron periclymenoides* (Michx.) Shinners and *R. ponticum* L. in London circa 1820 (Hillier Nurseries, 2002). Such hybrids have the potential to combine the fragrance of the deciduous azaleas with darker flower colors, larger flower size, and persistent foliage of evergreen rhododendrons. One such hybrid with breeding potential is *Rhododendron* 'Fragrant Affinity'. 'Fragrant Affinity' is an azaleodendron with semievergreen foliage, vigorous growth, good cold hardiness (–26 °C), and fragrant, lavender flowers (personal observations). This intersubgeneric hybrid, believed to be a cross between *R. ponticum* and *R. viscosum* (L.) Torrey (Contreras, 2006), possesses unique attributes that are desirable for breeding and development of superior, cold-hardy, fragrant azaleodendrons. Unfortunately, like many other wide hybrids, it appears to be sterile.

Hybrid sterility, referred to as chromosomal sterility or chromosomal hybrid sterility, is often the result of improper chromosome pairing during gametogenesis resulting from structural differences in parental chromosomes. This results in meiotic abnormalities such as univalents and lagging chromosomes; however, other mechanisms may also be involved in hybrid sterility (Lu and Bridgen, 1997). In a study using *Alstroemeria aurea* Graham × *A. caryophyllaea* Jacq., Lu and Bridgen (1997) determined that its sterility resulted from complex fertility/sterility-regulating mechanisms and was not simply the result of parental chromosome differences. In cases in which sterility is incited by improper chromosome pairing, doubling the chromosome complement (polyploidization) of sterile hybrids to produce allotetraploids provides a homolog for each chromosome to pair with during meiosis and can allow for the development of fertile gametes (Hadley and Openshaw, 1980; Lu and Bridgen, 1997; Stebbins, 1950; van Tuyl and De Jeu, 1997; Zadoo et al., 1975).

Natural polyploids exist in the genus *Rhododendron,* including triploids ($2n = 3x = 39$), tetraploids ($2n = 4x = 52$), hexaploids ($2n = 6x = 78$), octaploids ($2n = 8x = 104$), and dodecaploids ($2n = 12x = 156$) (Ammal, 1950; Ammal et al., 1950). Artificial polyploid rhododendrons have also been developed to increase ornamental characteristics such as flower size and texture, extend time of flowering, produce more compact plants, and facilitate crosses not possible at the diploid level (Eiselein, 1994; Kehr, 1996a, 1996b; Pryor and Frazier, 1968; Tolstead and Glencoe, 1991; Väinölä, 2000). Polyploid rhododendrons have been induced with various chemical doubling agents, including colchicine (Kehr, 1996a) and oryzalin (Väinölä, 2000).

The objective of this study was to evaluate fertility of diploid and allotetraploid forms of *R.* 'Fragrant Affinity' and to determine if induced polyploidy is an effective method for restoring fertility in hybrids of distantly related rhododendrons.

Materials and Methods

Plant material. A single plant of *Rhododendron* 'Fragrant Affinity' was received from Dr. August Kehr in 2000. Semihardwood cuttings were taken in late summer, treated with 5000 ppm K-IBA, set in 1 peat:1 perlite (by volume), and placed in a chamber with intermittent mist at a rate of 10 s every 10 min. After rooting, plants were grown in pine bark medium amended with 0.59 kg·m^{-3} dolomitic lime and 1.0 kg·m^{-3} micronutrient blend (Micromax; Scotts, Marysville, Ohio) under 50% shade. Plants were fertilized using 17N–7.4P–14.1K controlled-release fertilizer (Multicote; Vicksburg Chemical Co., Vicksburg, Mo.). Plants used in controlled crosses were container-grown with the same media and conditions. Plant material was maintained at the Mountain Horticultural Crops Research Station (MHCRS), Fletcher, N.C.

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Development of allotetraploids. Expanding leaves were removed from 20 actively growing shoots of diploid *Rhododendron* 'Fragrant Affinity' and shoot tips were submerged in 150 μM oryzalin (4-(dipropylamino)-3, 5-dinitro-benzenesulfonamide) (Surflan A.S.; Dow AgroScience LLC, Indianapolis, Ind.) solution for 24 h. Shoot growth temporarily stopped after treatment and ploidy levels were determined on individual shoots the next spring when new growth was present.

Determining ploidy level and genome size. Relative DNA content, approximate genome size, and ploidy level of control (untreated) and treated plants were determined using flow cytometry (De Schepper et al., 2001; Doležel et al., 1998; Galbraith, et al., 1983). Approximately 1 cm^2 of newly expanded leaf tissue was finely chopped with a razor blade in a Petri dish with 500 μL of nuclei extraction buffer (CyStain ultraviolet Precise P Nuclei Extraction Buffer; Partec, Münster, Germany). The solution was filtered using Partec CellTrics disposable filters with a pore size of 50 μm to remove leaf tissue. Nuclei were stained with 1.5 mL 4′, 6-diamidino-2-phenylindole (DAPI) staining buffer (CyStain ultraviolet Precise P Staining Buffer; Partec) and incubated for 1 to 2 min at ≈24 °C. The suspension was analyzed using a flow cytometer (Partec PA-I; Partec) to determine relative DNA fluorescence. Ploidy and genome size were determined by comparing mean relative fluorescence of each sample with the 2C peak of diploids and an internal standard of known genome size. *Pisum sativum* L. 'Ctirad' with a genome size of 9.09 pg (Bennett and Smith, 1976; Doležel et al., 1998) was used as an internal standard to calculate nuclear DNA content [(2C DNA content of sample = 9.09 pg × (mean fluorescence value of sample/mean fluorescence value of standard)].

Cytologic study. Flower buds were harvested from untreated control plants between 1000 and 1200 HR on warm, sunny days in the Fall. Buds were harvested and placed in ice water until individual flower buds were removed and fixed. Flower buds were fixed in Carnoy's solution (1 glacial acetic acid:3 chloroform:6 100% ethanol) for 24 to 30 h. After fixing, flower buds were transferred to 70% ethanol and stored at 4 °C. Flower buds were washed in sterile distilled water and anthers removed. Anthers were squashed in 1% acetocarmine stain on glass slides, debris removed, coverslip applied, and cells observed at metaphase I and anaphase I using a light microscope (Carl Zeiss photomicroscope; Carl Zeiss MicroImaging, Inc., Thornwood, N.Y.) under ×600 and ×1000 magnification.

Assessing pollen fertility. Pollen fertility was assessed using staining and germination tests (Sharma and Sharma, 1980). Pollen was collected from diploid and allotetraploid plants, dried at 25 °C for 24 h, and frozen at –25 °C. Staining was performed by adding 1% acetocarmine (w/v) solution and incubating pollen for 3 h under ambient conditions (Jahier, 1996). Tetrads that were well formed and had at least one pollen grain stained were scored as viable. The germination study was conducted using Brewbaker-Kwack media (Kearns and Inouye, 1993) with 5% sucrose (w/v). Pollen was added to the solution and incubated for 8 h under ambient conditions. Tetrads with pollen tubes equal to or greater than the width of a pollen grain were scored as germinated. Pollen was observed using a light microscope (Micromaster; Fischer Scientific, Pittsburgh, Pa.) under ×100 and ×400 magnification. The experimental design for the pollen viability study was a completely randomized design with two and three replicates (plants) for the diploids and allotetraploids, respectively, with five subsamples of at least 100 pollen tetrads scored per subsample. Data were subjected to analysis of variance using the PROC GLM procedure and means across treatments separated by LSD ($P \leq 0.01$) using SAS 9.1 software (SAS Institute, Cary, N.C.).

In addition to in vitro testing of male fertility, self- and cross-pollination tests were used to evaluate male and female fertility, self-compatibility, and interploid crossability. Diploid *R.* 'Fragrant Affinity' was self-pollinated and both diploid and allotetraploid *R.* 'Fragrant Affinity' plants were pollinated with pollen collected from a fertile diploid *R. catawbiense* and allotetraploid *R.* 'Fragrant Affinity'. A completely randomized design was used with between two and five replicates (plants) for each treatment, respectively, with a minimum of 50 flowers pollinated per plant with each pollen source. Average seed set per pollinated flower and average seed germination percentages were calculated for each treatment. Means across treatments were separated using LSD ($P \leq 0.05$).

Results and Discussion

Three allotetraploid shoots were identified on oryzalin-treated plants using flow cytometry. Approximate DNA content of diploid and allotetraploid *Rhododendron* 'Fragrant Affinity' was calculated as 1.6 and 3.2 pg, respectively (Fig. 1) confirming induced polyploidy. Väinölä (2000) reported 2C DNA content of a limited number of diploid *Rhododendron* taxa ranging from 1.1 to 1.5 pg, which is relatively close to the 2C value observed in the current study. Allotetraploid shoots also typically had larger flowers, leaves, and pollen compared with diploids.

Cytology of pollen mother cells (PMCs) showed that although there was proper bivalent pairing during metaphase I, there were bridges formed between bivalents and laggard chromosomes were present in anaphase I (Fig. 2A–C) in the limited number of cells observed. Further cytologic analysis was precluded primarily by timing of meiosis and difficulty in separating chromosomes in the genus *Rhododendron*. Apparent meiotic irregularities such as laggards further support the hypothesis that sterility in *R.* 'Fragrant Affinity' is chromosomal as opposed to genic, and fertility may be restored by doubling the chromosome complement.

Pollen staining and germination tests demonstrated improved pollen viability in the allotetraploids (Table 1). Diploids had well-formed tetrads but stained poorly and exhibited no germination, whereas the allotetraploids showed improved staining and germination rates.

Female fertility, self-compatibility, and interploid crossability were all improved in the allotetraploids (Table 2). Although no seeds were produced in crosses using diploid *R.* 'Fragrant Affinity' as a pistillate parent, seeds were obtained when allotetraploid 'Fragrant Affinity' was self-pollinated or used as a pistillate parent in crosses with *R. catawbiense*.

Doubling the chromosomes of sterile cultivars resulting from wide hybridization to create fertile allotetraploids has been used in a number of crops with varying success. Zadoo et al. (1975) reported an increase of pollen staining from 0% in diploids to a range of 91% to 98% in three induced allotetraploid *Bougainvillea* cultivars. These allotetraploids were reported to be male and female fertile when crossed with fertile diploids and other allotetraploids. Chen et al. (2003) produced a "fully fertile" interspecific hybrid (*Cucumis hystivus* Chen and Kirkbride, $2n = 4x = 38$) of *Cucumis hystrix* Chakr. ($2n = 2x = 24$) x *C. sativus* L. ($2n = 2x = 14$) by doubling an F_1 hybrid. Pollen grain germination increased from 0% to 2% in the diploid hybrid to 10% to 40% in the amphidiploid (allotetraploid). Chromosome doubling has also been used to develop fertile allotetraploid forms of the hybrids *Lilium henryi* × *L. candidum* (van Tuyl et al., 1992), *Passiflora incarnata* L. × *P. edulis* f. *flavicarpa* (Knight, 1991), and the kangaroo paw cultivar *Anigozanthos* 'Bush Ranger' (*Anigozanthos humilis* × *A. flavidus*) (Griesbach, 1990). In addition to these examples of interspecific hybridization within a genus, doubling was used to

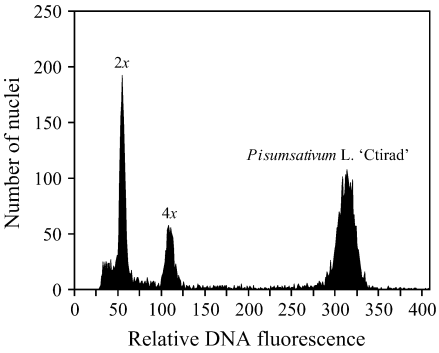

Fig. 1. Determination of ploidy level and DNA content of diploid (2*x*) and allotetraploid (4*x*) *R.* 'Fragrant Affinity' using flow cytometry with *Pisum sativum* 'Ctirad' used as an internal standard of known genome size (9.09 pg). Mean relative fluorescence was 55.42, 109.96, and 313.35 for the diploid, allotetraploid, and internal standard, respectively. Sample DNA contents were calculated using the formula: 9.09 pg * (mean fluorescence of sample/mean fluorescence of standard).

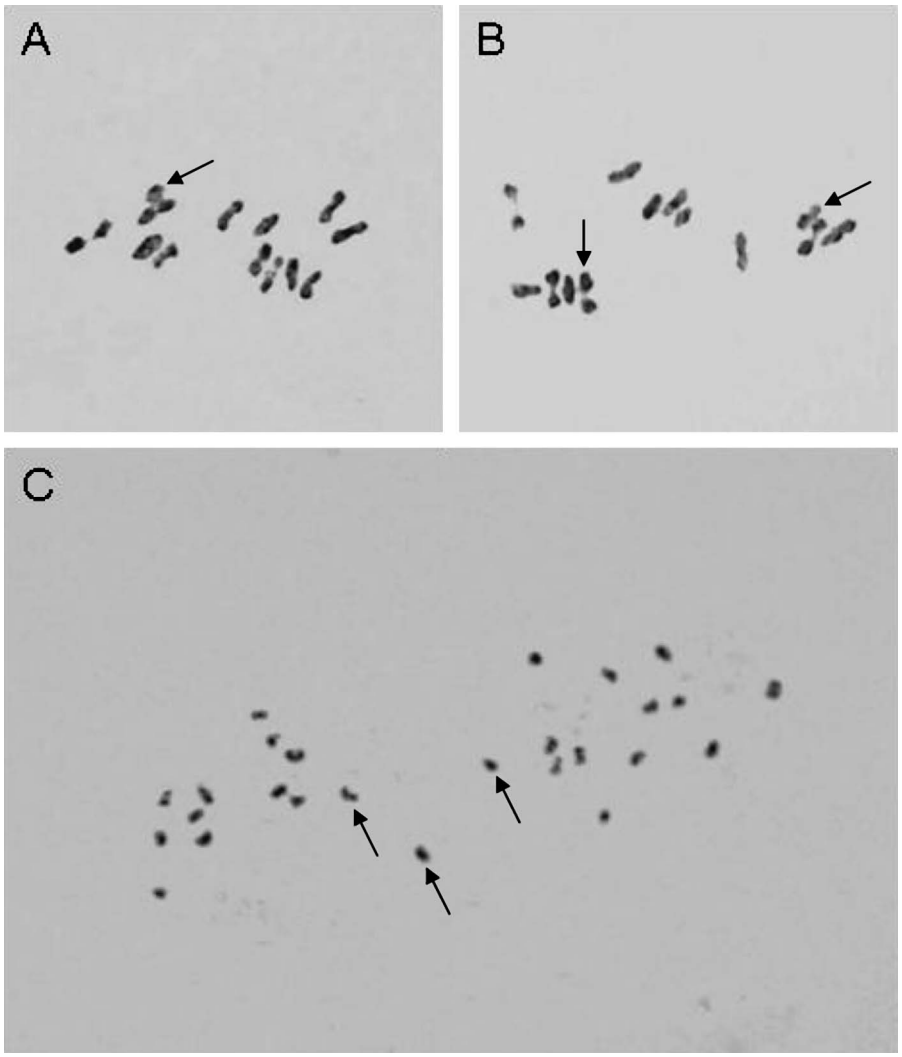

Fig. 2. Micrographs of meiotic cells from diploid ($2n = 2x = 26$) *Rhododendron* 'Fragrant Affinity'. Diploid cells in (**A** and **B**) metaphase I (MI) and (**C**) anaphase I (AI). Arrows indicate (**A** and **B**) bridges and (**C**) laggards.

Table 1. Pollen staining and germination of diploid ($2x$) and allotetraploid ($4x$) *Rhododendron* 'Fragrant Affinity'.

Ploidy	n^z	Staining (%)	Germination (%)
$2x$	2	3.7 ± 2.0[y] a[x]	0 a
$4x$	3	67.6 ± 19.9 b	44.9 ± 5.5 b

[z]n = number of replications (plants); each replication consisted of five subsamples of ≥100 pollen tetrads observed.
[y]Data are presented as mean ± one standard error of the mean.
[x]Means within columns followed by different letters are significantly different based on a LSD, $P \leq 0.01$.

restore fertility in ×*Chitalpa tashkentensis*, an intergeneric hybrid between *Catalpa bignoniodes* Walt. × *Chilopsis linearis* (Cav.) Sweet (Olsen et al., 2006). Olsen et al. (2006) reported an increase in pollen staining from <1.0% in the diploid to nearly 99% in the allotetraploid and an increase in pollen germination from <1.0% in the diploid to nearly 66% in the allotetraploid. Female fertility of this intergeneric hybrid was also confirmed through a crossing study.

The degree of fertility restoration reported for induced allopolyploids varies considerably. For example, Chen et al. (2003) had pollen germination as low as 10% in their allotetraploid, whereas Olsen et al. (2006) reported a mean pollen germination of 66%, and in the current study, nearly 45% pollen germination was observed. An explanation for this may be the degree of similarity between the genomes being combined. Genomes that are very similar behave more like autopolyploids, which often have marked reduction in fertility (Stebbins, 1950) resulting from improper pairing between similar (homologous) chromosomes from different genomes (Riesberg, 2001), whereas extreme allopolyploids (e.g., intergeneric) have a frequency of meiotic pairing of near one between homologous chromosomes and zero between homeologous chromosomes (Wu et al., 2001). *Rhododendron* 'Fragrant Affinity' appears to behave as an intermediate between these extremes. Stebbins (1950) termed these intermediates segmental allopolyploids, defined as "…a polyploid containing two pairs of genomes which possess in common a considerable number of homologous chromosomal segments or even whole chromosomes, but differ from each other in respect to a sufficiently large number of genes or chromosome segments, so that the different genomes produce sterility when present together at the diploid level." Cytologic analysis provides evidence that *R.* 'Fragrant Affinity' behaves as a segmental allopolyploid. The chromosomes of two cells observed during metaphase I exhibit 100% bivalent pairing in the cells observed, but laggard chromosomes were observed in an anaphase I cell, indicating that chromosomes of the two genomes are similar enough to pair but different enough that proper gametogenesis does not occur. There is a distinct advantage in using taxa that exhibit this intermediate manner. In allopolyploids formed from very similar genomes, the fertility is expected to be extremely low as a result of the formation of multivalents, making progress slow and difficult. On the other hand, in extreme allopolyploids formed from very disparate genomes, although fertility is high as a result of strict disomic pairing that results from 100% preferential pairing between homologous chromosomes (Wu et al., 2001), recombination between the two genomes is reduced or eliminated. Intermediate or segmental allopolyploids such as 'Fragrant Affinity' appear to allow for some crossing over between genomes while maintaining a level of fertility high enough to make it practical in a breeding program.

There are also examples of crops in which chromosome doubling is completely ineffective at restoring fertility as a result of a different mode of sterility. Lu and Bridgen (1997) developed allotetraploids of the hybrid *Alstroemeria aurea* × *A. caryophyllaea,* which were no more fertile than the diploids. In their study, they observed PMCs in the diploids and allotetraploids. They observed abnormal meiotic behavior such as no pairing, bridges, and laggard chromosomes in the

Table 2. Fertility and interploid crossability of diploid ($2x$) and allotetraploid ($4x$) *Rhododendron* 'Fragrant Affinity' ('FA').

Female	Male	No.[z]	No. of Flowers Pollinated	Average Seed Set[y]	Germination (%)
$2x$ *R.* 'FA'	$2x$ *R.* 'FA'	3	292	0.0 a[x]	—
$2x$ *R.* 'FA'	$4x$ *R.* 'FA'	3	403	0.0 a	—
$2x$ *R.* 'FA'	*R. catawbiense*	2	441	0.0 a	—
$4x$ *R.* 'FA'	*R. catawbiense*	4	436	0.1 a	25.0 a
$4x$ *R.* 'FA'	$4x$ *R.* 'FA'	5	409	2.0 b	12.3 a

[z]Number of replications (plants).
[y]Average number of seed set per pollinated flower.
[x]Means within columns followed by different letters are significantly different based on a LSD, $P \leq 0.05$.

diploids, whereas the induced allotetraploids exhibited nearly normal meiotic chromosome behavior. These findings indicate that in some cases, sterility is more complex than structural chromosomal differences. In light of successful restoration of fertility by chromosome doubling and the apparent meiotic irregularities observed in 'Fragrant Affinity', it can be concluded that the mode of sterility is chromosomal involving structural dissimilarity between parental chromosomes.

Azaleodendrons have not been used in breeding programs in the past as a result of hybrid sterility. However, the development of a fertile allotetraploid form provides new opportunities for integrating diverse traits among subgenera of *Rhododendron*. The induced allotetraploid *R.* 'Fragrant Affinity' is both male and female fertile and may serve as a valuable parent for developing new cold-hardy, evergreen rhododendrons with broad adaptability and desirable floral fragrance. Additionally, the development of triploids through interploid crosses could reduce fruit set and promote increased annual or remontant flowering. This approach may be valuable for restoring fertility in other wide hybrids within the genus, including other sterile azaleodendrons.

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