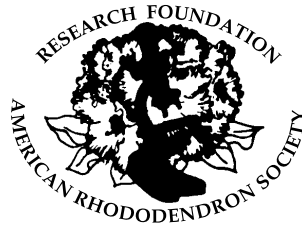


Fertility of Neopolyploid *Rhododendron* and Occurrence of Unreduced Gametes in Triploid Cultivars

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Introduction

Polyploidy, defined as an organism with three or more complete sets of chromosomes, is common in plants and widely recognized as an important mechanism of adaptation and speciation (Ramsey and Schemske, 2002; Soltis et al., 2003; Wendel, 2000). The prevalence of polyploidy in the genus *Rhododendron* has previously been documented (Jones et al., 2007). In some cases, polyploid plants, including *Rhododendron*, can have desirable features including enhanced vigor, thicker leaves, and larger flowers with thicker petals that persist longer (Kehr, 1996). Polyploidy can also have a profound influence on reproductive biology, including fertility (Allard, 1960; Ramsey and Schemske, 1998). As such, a greater understanding of reproductive behavior of polyploids is valuable to plant breeders.

The potential for utilizing polyploids in a breeding program is dependent upon fertility of specific taxa. The origin of polyploids can be a major factor in determining fertility. If a polyploid arises from somatic doubling in a meristem (spontaneous endoreduplication or chemical induction) or from the union of unreduced gametes from two **closely related** diploid parents, it will have four similar (homologous) versions of each chromosome and is often referred to as an autotetraploid (or polysomic tetraploid) (Ramsey and Schemske, 1998; Sanford, 1983). Autopolyploids may lack fertility due to the presence of multiple homologous chromosomes

that can result in multivalent pairing and unequal segregation in meiosis (Ranney, 2006; Riesberg, 2001; Stebbins, 1950). Polyploids that result from somatic doubling in a hybrid or from unreduced gametes from **different species** are referred to as allopolyploids (or sometimes amphidiploids or disomic polyploids). Allopolyploids are often fertile due to nonrandom, disomic pairing between two distinct sets (pairing among chromosomes derived from each parental species, but not between species) of chromosomes during meiosis (Ramsey and Schemske, 2002; Ranney, 2006). In many cases, however, polyploids fall somewhere between an autopolyploid and an allopolyploid; where there is partial chromosome homology resulting in a combination of disomic and polysomic pairing; these polyploids are referred to as segmental allopolyploids. Surveying a broad range of plant species, Ramsey and Schemske (2002) noted that fertility varied considerably within both neoauto- and neoallopolyploids (newly formed polyploids), emphasizing the need to determine fertility on a plant-by-plant basis.

Triploids are often found to be highly infertile, if not sterile (Allard, 1960; Ramsey and Schemske, 1998). Infertility of triploids results from the fact that three sets of chromosomes cannot be divided evenly during meiosis yielding unequal segregation of the chromosomes often resulting in aneuploid gametes or meiotic failure. A number of triploid ($2n = 3x = 39$) *Rhododendron* have been documented. De Schepper et al. (2001) confirmed

triploidy in the azaleas 'Euratom', 'Euratom Orange*', and 'Red Wing', and Jones et al. (2007) found 'Hallelujah', 'Taurus', 'White Ruffles*', and 'Crimson Majesty*' to be triploids. However, little is known about the fertility of triploid *Rhododendron*.

Although triploids are often infertile, meiotic restitution can circumvent this limitation resulting in functional, unreduced gametes (Dweikat and Lyrene, 1988; Ramsey and Schemske, 1998). Triploids that produce unreduced gametes can be utilized as bridges for the development of tetraploids by crossing them back with diploids (Bretagnolle and Thompson, 1995; Ramsey and Schemske, 1998). Moreover, hexaploids may also be produced by intercrossing among triploids that produce viable unreduced gametes (Ehlenfeldt and Vorsa, 1993). Frequencies of unreduced gamete production are highly variable, may differ considerably among individuals, and vary with environmental factors such as temperature (Bretagnolle and Thompson, 1995; Dweikat and Thompson, 1988; Ramsey and Schemske, 1998). Unreduced pollen has previously been documented within the genus in diploid clones including the elepidote *R. 'Hexe'* (De Schepper et al. 2001), evergreen azaleas (Eeckhaut et al., 2006), and deciduous azaleas with hybrids involving *R. x kosterianum* and *R. prinophyllum* displaying a range of unreduced pollen production from 0 – 21% (Widrechner et al., 1982). Circumstantial evidence also supports the role of unreduced gametes in the formation of various polyploid cultivars (Jones et al., 2007; Li, 1957; Widrechner et al., 1982; Willingham, 1976). Unreduced gametes may also be more prevalent in triploids than in diploids (Dweikat and Lyrene, 1988; Veilleux, 1985). Examination of the fertility of specific clones is therefore

necessary to determine the usefulness of incorporating triploids into a breeding program.

The pollen structure in *Rhododendron* and many Ericaceous species is typically a tetrad of grains that are tightly grouped at maturity (Copenhaver, 2005; Widrechner et al., 1982). However, the pollen of unreduced gamete producers is often a mix of sporads containing tetrads, dyads, and monads (Ortiz et al., 1992). The dyad grain structure is representative of $2n$ (the somatic chromosome complement) pollen that results from irregularities of the first or second meiotic division (Bretagnolle and Thompson, 1995; Widrechner et al., 1982). Unreduced pollen grains are also typically larger than reduced pollen grains (Bretagnolle and Thompson, 1995; Widrechner et al., 1982). Pollen staining, using acetocarmine, can be used to assess male fertility and reflects the potential for pollen to germinate and contribute to fertilization (Dafni and Firmage, 2000). Acetocarmine stains nuclei within the cells and has been utilized previously in studies of *Rhododendron* pollen (Contreras et al., 2007; Sakai et al., 2004).

The objectives of this project were to: 1) evaluate the effect of increased ploidy level on pollen fertility of selected *Rhododendron* and 2) evaluate pollen fertility of naturally occurring triploids found in the genus.

Materials and Methods

Comparing fertility between ploidy levels. Newly formed polyploids and their progenitor taxa were chosen to compare the fertilities between ploidy levels within the same genotype. The ploidy levels of all plants were determined by Jones et al. (2007) (Table 1). The tetraploid *R. 'Supernova'* and the octoploid *R. 'Fragrant Star'* were chemically induced at Briggs Nursery, Olympia, Wash., from *R. 'Nova Zembla'* and *R. 'Snowbird'*, respectively (Dan Meier, Olympia, Wash., per. comm.). *Rhododendron 'Vulcan'* (tetraploid form) is a $2x + 4x$ mixaploid (Jones et al., 2007) and the result of natural

Table 1. Pollen viability of polyploid *Rhododendron* and progenitor taxa.

Taxa	Genotype	Ploidy Level	Viability (%) ¹	Contrast ²
'Nova Zembla'	1	2x	33.4 ± 1.8	NS ³
'Super Nova'	1	4x	30.4 ± 2.5	
'Vulcan'	2	2x	48.0 ± 1.3	NS
'Vulcan' Tetraploid	2	4x	46.5 ± 6.4	
'Snowbird'	3	4x	63.8 ± 3.5	$P < 0.0001$
'Fragrant Star'	3	8x	16.4 ± 1.5	
'Fragrant Affinity'*	4	2x	1.5 ± 0.2	$P < 0.0001$
'Fragrant Affinity Tetra'*	4	4x	19.9 ± 2.3	
<i>fortunei</i>	5	2x	47.4 ± 4.0	$P < 0.0001$
<i>fortunei</i> Tetraploid	5	4x	7.7 ± 1.8	
PJM Group	6	2x	31.6 ± 1.4	$P < 0.0001$
'Northern Starburst'	6	4x	47.0 ± 1.3	
'The Honourable Jean Marie de Montague'	7	2x	28.7 ± 2.8	$P < 0.0001$
'Briggs Red Star'	7	4x	11.0 ± 1.1	
'Weston's Aglo'	8	2x	19.6 ± 0.9	$P < 0.0001$
'Bubblemum'	8	4x	58.8 ± 3.2	

¹Values represent means ± SEM for 10 replications.

²Contrast represents LSD_{0.05} mean separations between common (highlighted) genotypes of different ploidy levels.

³NS = Not significant.

somatic endoreduplication in a shoot of *R. 'Vulcan'* (Harold Greer, Eugene, Ore., per. comm.). *Rhododendron 'Briggs Red Star'* is also a $2x + 4x$ mixaploid (Jones et al., 2007) developed at Briggs Nursery through the chemical induction of *R. 'The Honourable Jean Marie de Montague'*. Both mixaploids appear to be tetraploid in the LII histogenic layers since they breed as tetraploids (Dr. Thomas G. Ranney, N.C. State University, NC, per. comm.) and gametogenesis occurs within the LII layer (Pratt, 1983; Ranney, 2006). *Rhododendron 'Fragrant Affinity Tetra'** was chemically induced from *R. 'Fragrant Affinity'** (Contreras et al., 2007). Dr. Max Byrkit chemically induced *R. fortunei* to produce the tetraploid form

(Kehr, 1996). *Rhododendron 'Northern Starburst'* and *R. 'Bubblemum'* are both chemically induced tetraploids of *R. PJM Group* and *R. 'Weston's Aglo'*, respectively, developed at Briggs Nursery (Dan Meier, Olympia, Wash., per. comm.).

Pollen viability was determined using staining procedures similar to those described by Contreras et al. (2007) and Olsen et al. (2006). All pollen was collected at anthesis from plants at the Mountain Horticultural Crops Research Station, dried at -21°C for 24 hrs, and stored at -25°C until testing. Pollen was placed on glass microscope slides, and the grains were stained with 1% acetocarmine (w/v) for 15 minutes. Pollen grains that stained a distinct red-pink color were

Table 2. Percent unreduced gametes in selected triploid *Rhododendron* taxa.

Taxa	Viable Unreduced Gametes (%) ¹
'Hallelujah'	2.87 ± 0.55 B
'Red Wing'	5.31 ± 0.81 A
'Taurus'	1.09 ± 0.13 C
'White Ruffles'	0.65 ± 0.19 C
Azaleodendron 94-28/2	0.45 ± 0.19 C
Azaleodendron 94-28/3	0.98 ± 0.27 C
Azaleodendron 94-28/7	0.60 ± 0.23 C
Azaleodendron 94-28/14	0.23 ± 0.01 C

¹Values represent means ± SEM. Means followed by a different letter are significantly different at $P < 0.05$.

scored as viable (Dafni and Firmage, 2000; Ramsey and Schemske, 2002). The tetrad nature of *Rhododendron* pollen required each individual grain in the tetrad to be analyzed. Each tetrad has the potential to contain four viable grains. The experimental design was a randomized complete block with ten replicates blocked over time. A minimum of 50 tetrads were randomly selected and analyzed per replicate. Pollen was observed at 300× using a light microscope (Nikon Eclipse 80i, Nikon, Melville, NY). Pollen viability percentages were calculated and the data were subjected to analysis of variance and pairwise means comparisons between ploidy levels for a given genotype (LSMEANS option, PROC GLM; SAS version 8.02, SAS Institute., Cary, N.C.; SAS Institute, 1988).

Triploid fertility. The cultivars *R.* 'Hallelujah', *R.* 'Red Wing', and *R.* 'Taurus' were confirmed as triploids by Jones et al. (2007). Recent analysis also determined that the azaleodendrons (crosses of an unnamed Ilam azalea by the evergreen *R.* 'Catalpa' provided by Dr. Steven Krebs at the Holden Arboretum, Kirtland, Ohio) 94-28/2, 94-28/3, 94-28/7, and 94-28/14 were also triploids (Krebs, S.L., unpub. material). Pollen was collected at anthesis, and the frequency of viable, unreduced gametes in the

triploid taxa was determined using pollen staining as described above and observed under a light microscope at 300×. The experimental design was a randomized complete block with 5 replicates blocked over time. At least 50 sporads (tetrads, dyads, or monads) were randomly selected and analyzed per replicate. Pollen was considered viable and unreduced if there was a well-stained monad or dyad and the pollen diameter was visibly larger (>120%) than normal (Shoemaker-Megalos and Ballington, 1988). The frequency of unreduced gametes was determined using the equation from Ortiz et al. (1992):

Unreduced pollen frequency = $[(2 \times \# \text{ of dyads}) + (\# \text{ of monads})] / (\# \text{ of total grains})$. Data were then subjected to analysis of variance and means compared using least significant differences (LSD) (PROC GLM; SAS version 8.02, SAS Institute., Cary, N.C.; SAS Institute, 1988).

Results and Discussion

Comparing fertility between ploidy levels. Pollen grains were readily apparent as being stained or unstained (Figure 1). Pollen viabilities for all taxa ranged from 1.5 – 63.8 % (Table 1). There was a significant effect of ploidy level ($P < 0.001$), genotype ($P < 0.0001$), and a ploidy-genotype interaction ($P < 0.0001$)

on fertility. The significant interaction indicated the effect of polyploidy on fertility depended on genotype.

In the genotypes, *R. fortunei*, 'The Honourable Jean Marie de Montague', and 'Snowbird', the increased ploidy level reduced fertility. The tetraploid form of *R. fortunei* is an induced autotetraploid and its reduced fertility most likely results from problems associated with polysomic chromosome pairing commonly found in autotetraploids. The diploid *R.* 'The Honourable Jean Marie de Montague' is a hybrid of unknown parentage believed to involve *R. griffithianum*. The reduced fertility of the tetraploid *R.* 'Briggs Red Star' suggests that it functions more like an autotetraploid or segmental allopolyploid and the increased ploidy level compromised gamete formation. *Rhododendron* 'Snowbird' is a naturally occurring tetraploid believed to be a hybrid between *R. atlanticum* and *R. canescens* (Galle, 1987), most likely the result of a normally reduced gamete from *R. atlanticum* combined with an unreduced gamete from *R. canescens*. The induced octoploid *R.* 'Fragrant Star' would thus be considered an autoallopolyploid, that contains four homologous chromosomes from each species, which may still result in polysomic chromosome pairing leading to reduced fertility.

In the genotypes, *R.* PJM Group, 'Weston's Aglo', and 'Fragrant Affinity*', the increased ploidy level significantly increased fertility. Both *R.* PJM Group and 'Weston's Aglo' are interspecific hybrids between *R. minus* × *R. dauricum*. The induced polyploids of these taxa, *R.* 'Northern Starburst' and 'Bubblegum', represent allopolyploids, and the increased fertility most likely results from restoration of chromosome homology and improved disomic pairing. The increased fertility of *R.* 'Fragrant Affinity Tetra*' over 'Fragrant Affinity*' represents a similar case as previously described by Contreras et al. (2007).

For the remaining genotypes, *R.* 'Nova Zembla' and *R.* 'Vulcan', increased ploidy

level had no apparent effect on fertility. Both of these genotypes are complex hybrids and the induced tetraploids most likely function as segmental allopolyploids with moderate to high fertility, regardless of ploidy level.

These results demonstrate that fertility of polyploid *Rhododendron* can be highly variable and that induced polyploidy may either enhance or compromise fertility. The effect of polyploidy on fertility most likely results from the level of homology among the chromosome sets and subsequent impacts on chromosome pairing during meiosis. Information on the fertility of specific cultivars will assist breeders in utilizing these plants in future breeding programs.

Triploid fertility. Viable dyad and monad grains (Figures 2 and 3) were observed in pollen samples from triploid taxa, ranging from 0.2 to 5.3% (Table 2), indicating the presence of unreduced pollen. The increased size of unreduced pollen diameter was also clearly evident (Figure 3). As in other triploid Ericaceae taxa (Ehlfeldt and Vorsa, 1993; Vorsa and Ballington, 1991), the small percentage of viable stained pollen was largely limited to monad or dyad grains. There was a significant effect ($P < 0.0001$) of taxa on the percentage of unreduced pollen. *Rhododendron* 'Red Wing' had the highest percentage of unreduced pollen at 5.3%, followed by *R.* 'Hallelujah' at 2.9%, while the remaining taxa were similar with less than 1.1% unreduced gametes. In studies of *Vaccinium* spp., also in the Ericaceae, significant differences in frequencies of unreduced pollen among taxa have also been reported (Ortiz et al., 1992; Shoemaker-Megalos and Ballington, 1988).

Dyad pollen grains are typically expected to be $2n$ and can result from a variety of meiotic irregularities including first or second division restitution (Bretagnolle and Thompson, 1995; Islam and Shepherd, 1980; Widrlechner, 1982). Monad pollen grains could result from different pathways including double

restitution ($4n$) or the combination of a restitution event and abnormal nuclear fusion of individual grains producing a $2n$ monad (Bretagnolle and Thompson, 1995; Veilleux, 1985).

The production of unreduced pollen by triploid taxa indicates the potential for utilizing certain taxa in breeding programs. The greater frequency of unreduced pollen found in *R.* 'Red Wing' and 'Hallelujah' may allow for successful hybridizations given adequate numbers of pollinations. Additional breeding studies with triploid parents would help elucidate the level of functional unreduced pollen and the presence of unreduced female gametes (Ehlfeldt and Vorsa, 1993).

Overall, the influence of polyploidy on fertility in *Rhododendron* is highly variable and appears to be influenced by the ploidy level, degree of homology among chromosomes, and in the case of triploids, the frequency of unreduced gamete formation. A greater understanding of fertility mechanisms in polyploid *Rhododendron* and information on fertility of specific clones will better allow breeders to incorporate and utilize polyploids in plant improvement programs.

* = name of plant is unregistered

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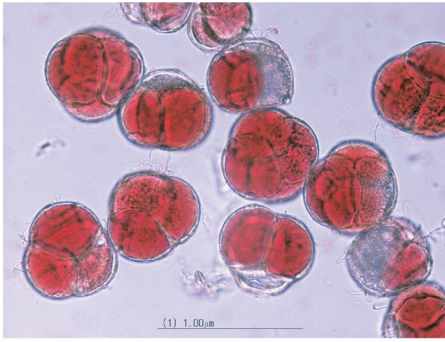


Figure 1. Photomicrograph of tetrad pollen grains of the tetraploid *Rhododendron* 'Northern Starburst'.



Figure 2. Photomicrograph of dyad pollen grain of the triploid *Rhododendron* 'Red Wing'.

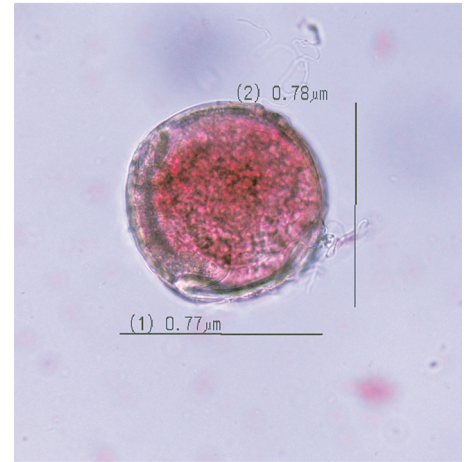


Figure 3. Photomicrograph of monad pollen grain of the triploid *Rhododendron* 'Hallelujah' illustrating size dimensions.

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