A Novel Method for Inducing Polyploidy in *Rhododendron* Seedlings

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Introduction

Polyploidy (having three or more complete sets of chromosomes) is relatively common in plants. By some estimates as many as 70% of all angiosperms are natural polyploids (Masterson, 1994). The importance and prevalence of polyploidy is especially evident in the genus *Rhododendron*. Polyploidy occurs naturally in many species and hybrids of *Rhododendron*, particularly in the *Rhododendron* and *Pentanthera* subgenera (Ammal et al., 1950; Jones et al., 2007).

Polyploidy is considered to be a major pathway for plant evolution and can contribute to reproductive isolation and abrupt speciation (Ramsey and Schemske, 1998; Soltis et al., 2003; Wendel, 2000). The effects of polyploidy on plant traits are also important to horticulturists and plant breeders. Ploidy levels can influence crossability, fertility of hybrids, plant vigor, and gene expression (Ranney, 2006). The induction of artificial polyploids has been utilized in the development of allopolyploids to restore fertility in sterile hybrids, enhance crossability and fertility of progeny, create seedless triploids, produce novel gene combinations, and increase the expression and diversity of secondary metabolites (Chen and Ni, 2006; Contreras et al., 2007; Olsen et al., 2006; Soltis and Soltis, 1993; Wendel, 2000). In some cases, polyploids may also have additional desirable ornamental characteristics including thicker leaves and larger flowers with thicker petals that persist longer (Barlup, 2002; Hosoda et al., 1953; Kehr, 1996a; Leach, 1961).

Polyploidy can arise naturally through multiple pathways including spontaneous chromosome doubling in somatic meristem cells and sexual fertilization with unreduced gametes (Jones et al., 2007; Ramsey and Schemske, 1998; Widrlechner et al., 1982). Polyploidy can also be induced through the use of various chemical doubling agents (mitotic inhibitors) (Contreras et al., 2007; Sanford, 1983; van Tuyl, 1992). Colchicine (N-(5,6,7,9-tetrahydro-1,2,3,10-tetra-methoxy-9-oxobenzo(a)heptalen-7-yl)acetamide) was first discovered as an effective mitotic inhibitor in 1937 and has been extensively utilized for inducing polyploidy in a wide range of species (Eigsti and Dustin, 1955; Hancock, 1997). The dinitroaniline herbicide oryzalin (3,5-dinitro-N4,N4-dipropylsufanilamide) has also been effectively utilized as a doubling agent and is considerably less toxic than colchicine (van Tuyl, 1992). Both agents have a similar mode of action: inhibiting microtubule polymerization and arresting mitosis at metaphase thus preventing the replicated chromosomes from separating into daughter cells. When mitosis resumes, a lineage of polyploid cells with double the normal chromosome number can be established. Studies comparing the effectiveness of colchicine versus oryzalin as induction agents have produced mixed results. Oryzalin is more effective at lower dosages than colchicine due to a higher specificity for tubulin binding sites in plant material (Eeckhaut et al., 2001; Eiselein, 1994; Morejohn et al., 1987; van Tuyl, 1992). Undesirable side effects of colchicine, including sterility, abnormal growth, and deformed tissue can be avoided when using oryzalin (Bouvier, 1994; van Tuyl, 1992). Eeckhaut et al. (2001) found that *in-vitro* treatment of seedlings of rhododendron with 0.05% and 0.25% colchicine had no effect on ploidy while treatment with 0.01% and 0.05% oryzalin yielded some polyploids and numerous cytochimeras. Väinölä (2000) compared the efficacy of colchicine (0.025% or 0.05%) and oryzalin (0.001% or 0.005%) for 24 or 48 hour durations on chromosome doubling in rhododendron seedlings. Plant survival was higher with colchicine, but oryzalin was more efficient in the induction of polyploidy (18% of the surviving plants at 0.005% with the 24 hr exposure). The use of mitotic inhibitors often produces polyploids that are cytochimeras (mixaploids) whereby different cells or histogenic layers vary in ploidy level (Pratt, 1983; Pryor and Frazier, 1968; Väinölä, 2000). The ploidy of the LII histogenic layer is crucial to breeding as this layer gives rise to reproductive tissue (Pratt, 1983; Ranney, 2006).

Polyploidy has been induced in many woody ornamental plant genera such as ×*Chitalpa* (Olsen et al., 2006), *Citrus* (Lee, 1988), *Rosa* (Semeniuk and Arisumi, 1968), *Prunus* (Dermen, 1953), and *Pyrus* (Kadota and Niimi, 2002). Attempts to

induce polyploidy in rhododendrons, through an assortment of methods both *ex-vitro* and *in-vitro,* have been met with varying degrees of success (Contreras et al., 2007; Eeckhaut et al., 2001; Eiselein, 1994; Kehr, 1996b; Paden, 1990; Pryor and Frazier, 1968; Sakai et al., 2004; Väinölä, 2000). Mitotic inhibitors only affect actively dividing cells; therefore, prolonged contact with the apical meristem is crucial for inducing polyploidy, yet over-exposure results in death (Kehr, 1996a). Pryor and Frazier (1968) successfully applied colchicine to actively growing shoots to obtain tetraploid azaleas. Kehr (1996b) developed a protocol for misting seedlings with colchicine after the cotyledons developed but before the first true leaves were evident; however, efficacy of the treatment was never determined. Contreras et al. (2007) developed an allotetraploid form of *Rhododendron* 'Fragrant Affinity' by submerging actively growing shoots tips in 150µM oryzalin for 24 hours. Eiselein (1994) compared single applications of 1% colchicine for 0, 24, 48, 72, and 96 hours with repeated applications of a 24-hour exposure, interrupted by 2-5 day recovery periods, totaling 96 hours of treatment. Percentage of tetraploids (determined on root tips—no data presented on shoots) with the single treatments averaged approximately 20% with no effect of duration, while the repeated applications resulted in a 79% conversion rate. The higher conversion rate for the repeated applications may have resulted from impacting a greater number of cells in metaphase, over time, while allowing for periodic recovery periods (Eiselein, 1994).

Confirmation of ploidy levels in treated seedlings is essential to determine efficacy of these techniques. Although determination of ploidy level in *Rhododendron* by counting chromosomes is possible, the chromosomes of *Rhododendron* are small and particularly difficult to view and discern using standard cytological techniques (Eiselein, 1994; Tolstead and Glencoe, 1991). Flow cytometry provides a fast and efficient method for determining relative genome size and associated ploidy level of *Rhododendron* (De Schepper et al., 2001; Eeckhaut et al., 2004; Jones et al., 2007). An additional advantage of flow cytometry for evaluating efficacy of mitotic inhibitors is the ability to sample thousands of cells and also determine the presence of cytochimeras (De Schepper et al., 2001; Jones et al., 2007).

The objectives of this project were to 1) develop a simple and effective, *ex-vitro* method for inducing polyploidy in *Rhododendron* seedlings, 2) evaluate the effectiveness of using repeated treatments of an oryzalin suspension in a warm agar solution applied directly to apical shoots of *Rhododendron* seedlings to induce polyploidy, and 3) develop a population of new polyploid rhododendrons and azaleas for use in future breeding projects.

Materials and Methods

Controlled pollinations were completed to produce new hybrids with desirable ornamental characteristics for use in this study. All parents were confirmed diploids (Jones et al, 2007). Seeds were obtained from the following crosses:

1) *R.* 'Summer Lyric' (*R. prunifolium* × *R. arborescens*) [pollinated with either *R.* 'Millennium' (*R.* 'Weston's Sparkler'* x *R.* 'Weston's Parade'*) or *R.* 'August Beauty'*(*R. prunifolium* × *R. arborescens*)] with the goal of producing tetraploid deciduous azaleas (subgenus *Pentanthera*) with fragrant flowers, a range of flower colors, and late-season flowering.

2) *R.* 'Cheyenne' (*R.* Jalisco Group × *R.* Loderi Group) × *R.* 'Capistrano' (*R.* 'Hindustan' × {{[*R. catawbiense* × (*R. fortunei* ssp. *discolor* × *R.* Fabia Group)] × (*R.* 'Russell Harmon' × *R.* 'Goldsworth Orange')} × *R.* 'Golden Gala'}) with the goal of producing a tetraploid elepidote rhododendron (subgenus *Hymenanthes*) with fragrant, yellow flowers.

3) *R.* 'Kimberly' (*R. williamsianum* × *R. fortunei* ssp. *fortunei*) × *R.* 'Nestucca' (*R. fortunei* ssp. *fortunei* × *R. degronianum* ssp. *yakushimanum*) with the goal of producing a tetraploid elepidote rhododendron (subgenus *Hymenanthes*) with a compact habit, good cold hardiness, and fragrant flowers.

Seedlings from each cross were germinated in five separate pots with approximately 100 seeds per pot. When seedlings were at the cotyledon stage, all of the plants (subsamples) in an individual pot were either treated with 1, 2, 3, or 4 applications of oryzalin separated by 4-day intervals or left untreated (control). The preemergent herbicide Surflan® A.S. (40.4% oryzalin) was diluted to produce a suspension containing 50µM oryzalin with 5.5g/L agar at 50 °C. Concentrations of oryzalin and agar were based on preliminary studies (data not presented). A single drop (2-4 µL) of warm (~40 °C) oryzalin suspension was then pipetted on top of the cotyledons of each seedling to cover the emerging shoot. Pots were placed in a high humidity (approximately 100% relative humidity) growth chamber at 23ºC under constant light to preserve the integrity of the agar droplet. Subsequent applications were made after the 4-day interval. After treatment, plants were grown under standard greenhouse conditions prior to analysis. The experimental design was completely randomized. Flow cytometry was utilized to determine ploidy levels approximately 3 months after treatment using methods described by Jones et al. (2007). Data on percent death and ploidy level were subjected to regression analysis (PROC REG; SAS version 8.02, SAS Institute., Cary, N.C.; SAS Institute, 1988).

Results and Discussion

The semi-solid agar appeared to be an effective medium for applying oryzalin to the apical shoots of rhododendron seedlings (Figure 1, p. 135). The agar drop typically rested on the cotyledons and solidified around the elongating shoots, thus allowing for sufficient contact between the oryzalin and the meristem. Drops persisted for 2 to 3 days before deteriorating. There were no visual phytotoxic symptoms over the treatment periods.

Treatment of 'Summer Lyric' seed-

lings resulted in a broad range of ploidy levels including mixaploids (Table 1). Induction percentages for the different classes of polyploids varied as a function of number of applications. The percentage of homogeneous tetraploids (of primary interest) followed a quadratic trend in response to increasing number of applications with the highest percentage, 41%, resulting after two successive applications of oryzalin. A few higher level polyploids, including octoploids, and mixaploids were also recovered as the number of applications increased. Although a quadratic response was significant for seedling death, the increasing number of applications did not increase death over that of the control. Overall, it appeared that 2 to 3 applications were ideal for inducing tetraploids.

A few polyploids resulted from the oryzalin treated *R.* 'Cheyenne' × 'Capistrano' hybrids (Table 2). Mixaploids increased with number of applications, while there was no significant trend for tetraploids. The percentage of dead plants increased linearly with increasing application number, suggesting a sensitivity to oryzalin. Oryzalin treatments did result in some polyploids, but it remains unclear if there was any benefit from multiple applications to induce tetraploid plants.

Among the *R.* 'Kimberly' × 'Nestucca' seedlings, oryzalin treatment resulted in a range of polyploids including tetraploids, several octoploids, and three classes of mixaploids (Table 3). Diploids decreased linearly with each additional application, and conversely, the percentage of 2x+4x mixaploids and solid tetraploids increased linearly. The octoploid, higher level mixaploids, and death percentages were random in their distribution with no significant trend. For induction of tetraploids, 2-4 applications were optimal.

The shoot apical meristem (Figure 2, p. 135) is comprised of zones. The central zone includes a group of cells at the distal end of the meristem. These cells function as intitial cells that give rise to other cells, other regions of the meristem, and ultimately the shoot (Francis, 1997; Kerstetter and

Table 1. Ploidy levels and death of seedlings from *Rhododendron* 'Summer Lyric' following treatment of apical shoots with 0, 1, 2, 3, or 4 applications of 50µM oryzalin in agar separated by 4 day intervals.

	Number of Applications					
Ploidy	**0**	**1**	**2**	**3**	**4**	**Trend**
2x	89[z]	59	24	31	19	Q[y]***;R^2=0.95
2x + 4x	0	21	26	26	31	Q**; R^2=0.92
4x	0	12	41	33	24	Q***; R^2=0.86
4x+8x	0	0	4	0	8	L[x]**; R^2=0.49
8x	0	0	1	2	0	NS[w]
2x+8x	0	0	1	0	5	L**; R^2=0.53
2x+4x+8x	0	0	0	0	3	Q*; R^2=0.85
Dead	11	8	2	9	10	Q*; R^2=0.66

[z]Data in percent.
[y]Q=quadratic trend.
[x]L=linear trend.
[w]NS=trend not significant.
*significant, $P \leq 0.10$
**significant, $P \leq 0.05$
***significant, $P \leq 0.01$

Table 2. Ploidy levels and death of *Rhododendron* 'Cheyenne' × *R.* 'Capistrano' seedlings following treatment of apical shoots with 0, 1, 2, 3, or 4 applications of 50µM oryzalin in agar separated by 4 day intervals.

	Number of Applications					
Ploidy	**0**	**1**	**2**	**3**	**4**	**Trend**
2x	69[z]	59	24	31	19	Q[y]***; R^2=0.88
2x+4x	0	8	7	6	2	Q**; R^2=0.88
4x	0	2	7	8	4	NS[w]
Dead	31	68	65	68	80	L[x]***; R^2=0.70

[z]Data in percent.
[y]Q=quadratic trend.
[x]L=linear trend.
[w]NS=trend not significant.
*significant, $P \leq 0.10$
**significant, $P \leq 0.05$
***significant, $P \leq 0.01$

Table 3. Ploidy levels and death of *Rhododendron* 'Kimberly' × R. 'Nestucca' seedlings following treatment of apical shoots with 0, 1, 2, 3, or 4 applications of 50µM oryzalin in agar separated by 4 day intervals.

	Number of Applications					
Ploidy	**0**	**1**	**2**	**3**	**4**	**Trend**
2x	67[z]	30	43	33	20	L[x]***; R^2=0.67
2x+4x	0	6	9	11	11	L**; R^2=0.85
4x	0	4	12	11	12	L***;R^2=0.81
4x+8x	0	0	0	1	1	NS[w]
8x	0	0	0	4	0	NS
2x+8x	0	0	0	0	1	NS
Dead	33	60	36	40	55	NS

[z]Data in percent.
[x]L=linear trend.
[w]NS=trend not significant.
*significant, $P \leq 0.10$
**significant, $P \leq 0.05$
***significant, $P \leq 0.01$

Hake, 1997; Tax and Durbank, 2006). Within the central zone lay multiple histogenic layers: L1, L2, and L3, that are distinct and give rise to separate cell lines and tissues (Hudson and Goodrich, 1997). Development of homogeneous polyploids requires the successful doubling of the initial cells, in all histogenic layers, within the central zone of the apical meristem. In constrast, mixaploids appeared to be a conglomeration of cells of varying ploidy levels, among or within the histogenic layers, resulting from incomplete doubling of initial cells within the meristem. As suggested by Eiselein (1994), only a certain percentage of meristematic cells may be affected by any single application of a mitotic inhibitor. Pryor and Frazier (1968) also observed mixaploids following a single application of colchicine on evergreen azaleas. Poor penetration or asynchronous cell cycling within the meristem could result in only partial doubling of the meristem. A gradient of cell size, relative growth rates and cell cycling times can exist within a meristimatic zone (Francis, 1997). Because the cell cycle is not synchronized among all the cells in the meristem, multiple applications may induce polyploidy in different populations of cells. In some cases, e.g., 'Summer Lyric' and 'Kimberly' × 'Nestucca' seedlings, increasing the number of applications (from 2 to 3 or 1 to 2, respectively) increased the number of homogeneous tetraploids. Thus, repeated applications over time most likely allowed for doubling of different initial cells during several asynchronous cell cycles.

Stability of the mixaploids developed here and the specific nature of their chimeral arrangement is uncertain. If all the cells in an individual histogenic layer are uniformly one ploidy level, e.g., a periclinal chimera, the confirmation may be more stable. Limited sampling two months after initial testing revealed that many of the higher level mixaploids eventually reverted to their lower ploidy level (data not presented). Väinölä (2000) reported similar results in which one third of the induced mixaploids shifted to diploidy. If the meristem is composed of a mosaic of cells with different ploidy levels mixed within histogenic layers, some cell types may multiply faster (those cells of lower ploidy) and effectively overrun the other cell type (those of higher ploidy) in a phenomenon known as diplontic selection (Broertjes and Keen, 1980; Pratt, 1983). Cell types of higher DNA content typically take longer to cycle through mitosis (Singh, 1993) and selection will then favor reversion to the faster proliferating, lower ploidy level, cytotype. The higher level polyploids (e.g., octoploids) likely resulted from mitotic inhibition of multiple cell cycles whereby diploid meristematic cells became tetraploid, and those tetraploid cells were doubled again to become octoploid. Such occurrences have been previously noted in polyploid induction of apple (Tilney-Bassett, 1986).

The results of this study demonstrate that the method of applying a suspension of oryzalin in warm, semi-solid agar to the shoots of *Rhododendron* seedlings is an effective method for inducing polyploidy. Although single applications resulted in some polyploid plants, multiple applications increased efficacy for some of the taxa studied. Polyploid plants developed in this study will be further evaluated for desirable traits and incorporated into an ongoing rhododendron breeding program.

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Figure 1. Oryzalin-agar treatment of hybrid *Rhododendron* seedling.

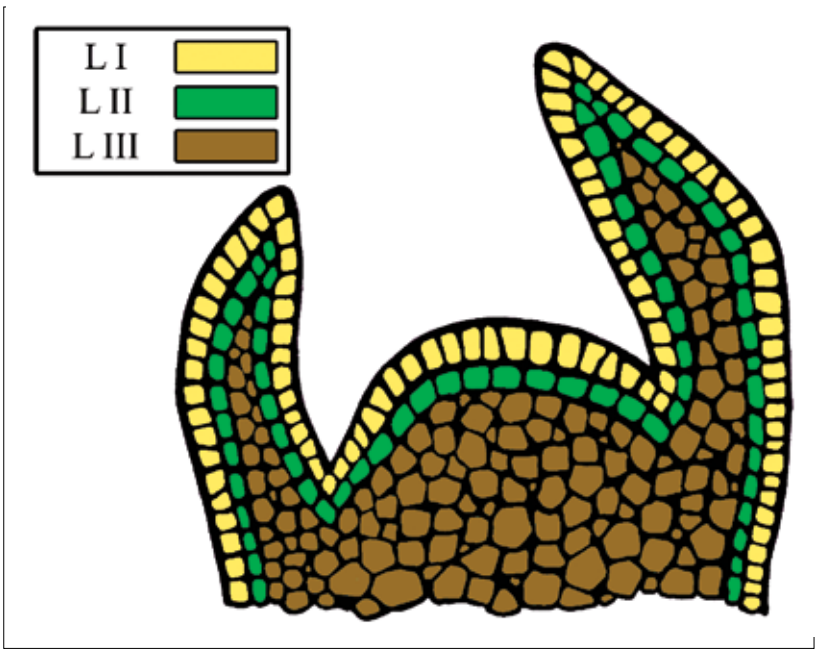

Figure 2. Diagram of the shoot apical meristem highlighting the three histogenic layers.

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