Inducing Polyploidy in *Rhododendron* Seedlings©

Jeff R. Jones, Thomas G. Ranney, and Thomas A. Eaker
Department of Horticultural Science, Mountain Horticultural Crops Research and Extension Center, North Carolina State University, Fletcher, North Carolina 28732–9244
Email: tom_ranney@ncsu.edu

Induction of polyploidy can be a valuable technique for breeding programs and developing improved nursery crops. *Rhododendron* seedlings, derived from controlled crosses, were treated with a single drop containing a 50-µM suspension of oryzalin in a warm agar mixture to temporarily inhibit mitosis and induce polyploidy. Multiple (1, 2, 3, or 4) applications, separated by 4 day intervals, were compared to determine the best efficacy. Ploidy analysis was completed using flow cytometry. This technique was successful in producing a range of polyploids including tetraploids, octoploids, and mixaploids (cytochimeras). The optimal treatment to induce tetraploidy was taxa dependent, but typically ranged from 2–4 applications.

INTRODUCTION

Polyploidy is the condition of having three or more complete sets of chromosomes and is relatively common in plants, including the genus *Rhododendron* (Jones et al., 2007). For horticulturalists and plant breeders, the implications of polyploidy are important and can influence crossability, fertility of hybrids, plant vigor, and gene expression (Ranney, 2006). The induction of artificial polyploids has many applications including fertility restoration, enhancing crossability, increasing levels of heterozygosity, and creating novel gene combinations (Ranney, 2006). In some cases, polyploids may also have enhanced ornamental characteristics (Kehr, 1996).

Oryzalin (3,5-dinitro-N4,N4-dipropylsufanilamide), a dinitroaniline herbicide, has been utilized as a desirable chromosome doubling agent due to its high specificity for tubulin binding sites in plants and low mammalian toxicity (Eeckhaut et al., 2001). Oryzalin only affects dividing cells; thus, prolonged contact with the apical meristem is crucial to inductive efficacy (Kehr, 1996).

The objectives of this project were to: (1) Develop a simple and effective, ex-vitro method for inducing polyploidy in *Rhododendron* seedlings by applying a suspension of oryzalin in an agar mixture directly to apical shoots; (2) Evaluate the effectiveness of repeated applications; and (3) Develop a population of new polyploidy rhododendron 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. Seeds were obtained from the following crosses:

Rhododendron 'Summer Lyric' (pollinated with either *R.* 'Millenium' or *R.* 'August Beauty') with the goal of tetraploid deciduous azaleas with fragrant flowers, a range of flower colors, and late-season flowering.

Rhododendron 'Cheyenne' × *Rhododendron* 'Capistrano' with a goal of an improved yellow-flowered tetraploid, elepidote, evergreen rhododendron with fragrant flowers.

Rhododendron 'Kimberly' × *Rhododendron* 'Nestucca' with a goal of a tetraploid, evergreen, elepidote, rhododendron 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 not treated (control) or received 1, 2, 3, or 4 applications of oryzalin separated by 4-day intervals. The preemergent herbicide Surflan® A.S. (40.4% oryzalin) was diluted with water to produce a 50-µM oryzalin suspension with 5.5-g·L^{-1} agar. Concentrations of oryzalin and agar were based on preliminary studies (data not presented). A single drop (2–4 µl) of the warm (~40 °C) oryzalin suspension was then pipetted between the cotyledons of each seedling to cover the emerging shoot. The pots were placed in a high humidity (approximately 100% relative humidity) growth chamber at 23 °C under constant light. The experimental design was completely randomized. Flow cytometry was utilized for ploidy determination using methods described in 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, North Carolina; SAS Institute, 1988).

RESULTS

Treatment of 'Summer Lyric' seedlings resulted in a range of ploidy levels including mixaploids (Table 1). The percentage of the different classes of polyploids followed various trends as a function of number of applications. The percentage of solid tetraploids (of primary interest) followed a quadratic trend in response to increasing number of applications with highest percentage, 41%, of solid tetraploids resulting after two successive applications of the oryzalin. A few higher level polyploids and mixaploids were also recovered, including octoploids, as the number of applications increased. Although a quadratic response was significant, the increasing number of applications did not increase death over that of the control. Two to three applications were ideal for inducing tetraploids.

Numerous polyploids resulted from the oryzalin treated *R.* 'Cheyenne' × *R.* 'Capistrano' hybrids (Table 2). Mixaploids increased with number of applications, while there was no significant trend for tetaploids. The percentage of dead plants increased linearly with increasing application number, suggesting sensitivity to oryzalin.

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

DISCUSSION

As suggested by Eiselein (1994), a certain percentage of meristematic cells are affected by a single application and a recovery period allows for the cell cycle to resume. Because the cell cycle is not synchronized among all the cells in the meristem,

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 50-µM 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 50-µM 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, $P \leq 0.01$.

later applications can induce polyploidy in different populations of cells. The higher level polyploids apparently resulted from mitotic inhibition of multiple cell cycles.

The shoot apical meristem is comprised of zones (Kerstetter and Hake, 1997). The peripheral zone gives rise to new cells leading to organ primordia and surrounds the central zone which contains the dividing stem cells for the shoot apex. Within the zones lay multiple histogenic layers. Since the central zone maintains the apex stem cell population, doubling cells in this region gives rise to polyploidy tissue distal to the point of treatment. The solid polyploids developed in this study appear to be the result of successful doubling of sufficient cells, in all histogenic layers, within the central zone to give rise to a homogeneous lineage of tetraploid cells. Mixaploids appear to be a conglomeration of cells of varying ploidy levels among or within the histogenic layers resulting from incomplete doubling. In some cases, e.g., 'Summer

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 50-µM 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[y]***; $R^2 = 0.67$
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[x]
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. [y]L = linear trend. [x]NS = trend not significant; *significant, $P \leq 0.10$; **significant, $P \leq 0.05$; ***significant, $P \leq 0.01$.

Lyric' and 'Kimberly' × 'Nestucca' seedlings, increasing the number of applications increased the number of solid tetraploids. Repeated applications over time most likely allows for multiple cells within the central zone to be affected during several asynchronous cell cycles.

The results of this study demonstrated that applying a suspension of oryzalin in an agar mixture to the shoots of *Rhododendron* seedlings can be an effective method for inducing polyploidy. Although single applications resulted in some polyploidy 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 at North Carolina State University.

LITERATURE CITED

Eeckhaut, T., G. Samyn, and **E. Van Bockstaele.** 2001. In vitro polyploidy induction in *Rhododendron simsii* hybrids. Med. Fac. Landbouww. 66:451–454.

Eiselein, J.E. 1994. A study of chromosome yields and growth responses in colchicine treated rhododendrons. J. Amer. Rhododendron Soc. 48(4):205–209.

Jones, J.R., T.G. Ranney, N.P. Lynch, and **S.L. Krebs.** 2007. Ploidy levels and genome sizes of diverse species, hybrids, and cultivars of *Rhododendron*. J. Amer. Rhododendron Soc. 81:220–227.

Kehr, A.E. 1996. Woody plant polyploidy. Amer. Nurseryman 183:38–47.

Kerstetter, R.A., and **S. Hake.** 1997. Shoot meristem formation in vegetative development. The Plant Cell. 9:1001–1010.

Ranney, T.G. 2006. Polyploidy: From evolution to new plant development. Comb. Proc. Intl. Plant Prop. Soc. 56:383–389.

SAS Institute Inc. 1988. SAS/STAT user's guide, release 6.03 edition. SAS Institute Inc., Cary, North Carolina.