In Vitro Shoot Regeneration and Polyploid Induction of *Rhododendron* 'Fragrantissimum Improved'

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Abstract. Rhododendron L. 'Fragrantissimum Improved' is an attractive cultivar with showy, fragrant flowers but has limited potential for breeding because it is a sterile wide hybrid. Protocols for in vitro regeneration and polyploid induction were developed for this cultivar as a means to potentially restore fertility and enhance ornamental traits. Combinations of thidiazuron (TDZ) at 0, 5, 10, 15, or 20 μM and 1-naphthaleneacetic acid (NAA) at 0, 2.5, 5, or 10 μM were used to induce shoot regeneration from leaves. Shoot regeneration was optimized (68% of leaf segments produced shoots) using 8.8 μM TDZ and 10 μM NAA. To induce polyploidy, regenerative callus was treated with 7.5, 15, 30, 60, or 90 μM of the mitotic inhibitor oryzalin for 1, 3, 5, 7, or 14 d in various combinations. Oryzalin significantly affected survival and shoot regenerative capacity. A percentage of homogenous, tetraploid shoots was recovered from treatments of 30 μM oryzalin for 1 (13%) or 3 (13%) days and 7.5 μM oryzalin for 7 (20%) or 14 (7%) days.

The genus *Rhododendron* (Ericaceae) includes diverse species and phenotypes with a broad range of ornamental characteristics and environmental tolerances. These traits make the genus appealing for breeding novel cultivars for landscape use. *Rhododendron* 'Fragrantissimum Improved' is a unique wide hybrid between *R. edgeworthii* Hook. and *R. formosum* Wallich. var. *formosum* (American Rhododendron Society, 2009) that is an improvement on the leggy and sprawling cultivar *R.* Fragrantissimum, which has been in the nursery industry for over 100 years. 'Fragrantissimum Improved' exhibits a compact growth habit, attractive exfoliating bark, lush evergreen foliage, and clusters of large, white blushed pink, pleasantly fragrant flowers. These ornamental traits are highly desirable for breeding and development of an improved cold-hardy cultivar. Like many wide hybrids, however, *R.* 'Fragrantissimum Improved' is sterile (T.G. Ranney, personal experience).

Hybrid sterility or chromosomal sterility can result from structural differences in chromosomes between species, thus preventing proper alignment during metaphase I of meiosis. This can prevent formation of viable gametes as a result of the presence of univalents and laggard chromosomes (Contreras et al., 2007). Contreras et al. (2007) found laggard chromosomes and bivalent bridges during cell division in the sterile wide hybrid *R.* 'Fragrant Affinity' that also led to infertility. In many cases, fertility can be restored in wide hybrids by doubling the number of chromosomes to produce allotetraploids. Allotetraploids have homologous pairs of chromosomes that allow for disomic pairing and formation of balanced gametes during meiosis (Lu and Bridgen, 1997; Olsen et al., 2006; Ramsey and Schemske, 2002; Ranney, 2006).

Allopolyploids have been successfully induced in many genera, including *Buddleia* L. (butterfly bush), *Lilium* L. (lily), *Nerine* Herb. (cape flower), and *Syringa* L. (lilac) (Rose et al., 2000a, 2000b; van Tuyl et al., 1992). In addition to restored fertility, allotetraploids often possess improved ornamental characteristics such as thicker, darker-colored leaves, larger flowers, and improved pest or disease resistance, which makes them desirable to breeders (Comai, 2005; Ranney, 2006). Several studies have shown that allotetraploids can be developed successfully in rhododendrons (Jones et al., 2008; Pryor and Frazier, 1968; Sakai et al., 2004). Moreover, the development of allotetraploids of *R.* 'Fragrant Affinity' may restore fertility (Contreras et al., 2007).

In vitro regeneration protocols provide an excellent mechanism for the manipulation of ploidy level, mutation treatment, and transgenic applications. In vitro shoot regeneration protocols have been developed for several *Rhododendron* species and hybrids belonging to diverse subsections, including *R. canadense* (L.) Torr. (rhodora), *R. mucronulatum* Turcz. (Korean rhododendron), *R. schlippenbachii* Maxim. (royal azalea), *R. yedoense* var. *poukhanense* (Lev.) Nakai (Yodogawa azalea), *R.* 'Boule de Neige', and *R.* 'Gibraltar' (McCown and Lloyd, 1982); *R. ponticum* L. (pontic rhododendron) (Almeida et al., 2005); *R. simsii* Planch. 'Helmut Vogel' (Mertens, 1996); *R. catawbiense* Michx. 'English Roseum' (Sicuranza and Mitkowski, 2007); and *R.* P.J.M. Group (McCown and Lloyd, 1982; Preece and Imel, 1991). The majority of these species and hybrids belong to the subgenera *Hymenanthes*, *Pentanthera*, or *Tsutsusi* and represent various sections and subsections with only *R. mucronulatum* and the *R.* P.J.M. Group representing subgenus *Rhododendron* (subsections *Rhodorastra* and *Caroliniana*) (American Rhododendron Society, 2009). The parents of *Rhododendron* 'Fragrantissimum Improved' are members of subgenus *Rhododendron* subsection *Edgeworthii* (*R. edgeworthii*) and subsection *Maddenia* (*R. formosum* var. *formosum*). No research has been reported on tissue culture protocols for *Rhododendron* sp. in subsection *Edgeworthia* or subsection *Maddenia*.

In vitro shoot regeneration from leaves of *Rhododendron* sp. is most commonly stimulated by a combination of the cytokinins, 6-(γ,γ-dimethylallylamino) purine (2iP) or zeatin, and auxins, indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA). To maximize shoot regeneration from leaves, 2iP and zeatin are typically required in high concentrations often exceeding 50 μM (Iapichino and Chen, 1995; Iapichino et al., 1992; Mertens, 1996; Tomsone and Gertnere, 2003). In comparison, thidiazuron [6-(γ,γ-dimethylallylamino)] (TDZ) has been an effective cytokinin in some species and can be used in lower concentrations, often making it more efficient (Bates et al., 1992; Huetteman and Preece, 1993). Thidiazuron has been used successfully at lower concentrations in several *Rhododendron* sp. (Preece and Imel, 1991; Samyn et al., 2002). For *R.* P.J.M. Group, Preece and Imel (1991) found TDZ to be effective at low concentrations with 52 shoots per leaf explant obtained on media supplemented with 10 μM TDZ and 1 μM IBA compared with 21 shoots with 50 μM 2iP and 10 μM IBA.

Efficient in vitro regeneration systems provide an ideal platform for manipulation of ploidy levels. Early polyploidization experiments used colchicine for mitotic inhibition. However, oryzalin is often preferred to colchicine as a result of its reduced toxicity, higher affinity to plant tubulins, effectiveness

at lower concentrations, and higher survival of plantlets (Hansen and Anderson, 1996; Sree Ramulu et al., 1991; Väinölä, 2000; van Tuyl et al., 1992). Oryzalin has also been used successfully for in vitro ploidy manipulation in several genera, including *Buddleia*, *Hypericum* L. (hypericum), *Miscanthus* Anderss. (silver grass), and *Rosa* L. (rose) (Dunn and Lindstrom, 2007; Kermani et al., 2003; Meyer et al., 2009; Petersen et al., 2003; Rose et al., 2000b). However, there have been few studies investigating in vitro ploidy manipulation in *Rhododendron*. In an intersubgeneric hybrid of an evergreen and deciduous azalea, the highest tetraploid formation (85%) occurred when explants were treated with 300 µM oryzalin for 48 h (Sakai et al., 2004). In another study using one evergreen and two deciduous *Rhododendron* hybrids, the highest percentage of tetraploids (18%) was formed when explants were treated with 150 µM oryzalin for 24 h (Väinölä, 2000).

Development of an efficient in vitro regeneration system for *R.* 'Fragrantissimum Improved' would provide an avenue for ploidy manipulation for this cultivar and related species. Development of an allopolyploid of this cultivar could further enhance ornamental traits and restore fertility. Thus, the objectives of this research were to 1) develop an efficient methodology for in vitro shoot regeneration from leaves of *R.* 'Fragrantissimum Improved'; and 2) develop an oryzalin-mediated protocol for polyploid induction in 'Fragrantissimum Improved'.

Materials and Methods

Plant material. Recently expanded leaves of *Rhododendron* 'Fragrantissimum Improved' were collected from greenhouse-grown stock plants maintained at the Mountain Horticultural Crops Research Station, Mills River, NC. Leaves were rinsed under tap water for 4.5 h and then surface-sterilized for 25 min using a 20% (v/v) solution of commercial bleach (6.15% NaOCl) containing two drops of Tween 20 and agitated periodically followed by three rinses of 5 min each in sterile distilled water. Leaf explants were cut into 5-mm^2 segments and placed abaxial side down on shoot regeneration media.

Shoot regeneration. The base growth medium was Murashige and Skoog (MS) basal salts (Phytotechnology Laboratories, Shawnee Mission, KS) and vitamins (Murashige and Skoog, 1962) supplemented with sucrose at 30 g·L^{-1}, myoinositol at 0.1 g·L^{-1}, MES monohydrate at 0.1 g·L^{-1}, and agar at 8 g·L^{-1} (pH 5.75 to 5.80 before autoclaving). Media was supplemented with 0, 5, 10, 15, or 20 µM TDZ in combination with either 1-naphthaleneacetic acid (NAA) or IAA at 0, 2.5, 5, or 10 µM. Plant growth regulators were added to the media before autoclaving. Cultures were incubated at 23 ± 2 °C in complete darkness and the number of segments producing callus and number of segments producing shoots were recorded after 4 weeks. Each set of TDZ × auxin treatments was a separate experiment

with a completely randomized factorial design (five rates of TDZ × five rates of auxin = 25 total plant growth regulator treatment combinations) with replicates consisting of petri dishes receiving the same plant growth regulator combination. There were eight replications (petri dishes) per treatment combination, each with five subsamples (leaf segments). Data were analyzed using analysis of variance (ANOVA) and multiple regression procedures (PROC GLM; SAS Version 9.1; SAS Inst., Cary, NC).

Polyploid induction. Two experiments were conducted to determine an effective concentration and duration of oryzalin treatment on callus survival and polyploid induction in regenerated shoots. Shoot organogenic callus used in all experiments was maintained on basal media containing 5 µM TDZ and 10 µM NAA. In the first experiment, calli were submerged in a liquid MS medium supplemented with 0, 30, 60, or 90 µM oryzalin for 1, 3, or 5 d on a reciprocating shaker. A 1-mM stock solution of oryzalin was prepared in ethanol, filter-sterilized, and added to cooled autoclaved media. An equivalent amount of ethanol was added to all control treatments. In the second experiment, calli were submerged in 0, 7.5, or 15 µM oryzalin for 7 or 14 d using the same technique. After treatment with the mitotic inhibitor, calli were transferred and washed in liquid MS media for 24 h. Finally, the callus cultures were grown on solidified MS media supplemented with 5 µM TDZ and 10 µM NAA at 25 °C in complete darkness and survival was measured after 4 weeks. For shoot elongation, surviving calli were placed on Anderson's media (Anderson, 1984; Phytotechnology Laboratories) containing 10 µM 2iP and 1 µM IBA and supplemented with MS vitamins, sucrose at 30 g·L^{-1}, myoinositol at 0.1 g·L^{-1}, MES monohydrate at 0.1 g·L^{-1}, and agar at 8 g·L^{-1} (pH 5.50 before autoclaving). There were six replications (petri dishes), each with five callus pieces ($\approx$1 cm^2), for each treatment, arranged in a completely randomized design. ANOVA was used to determine treatment main effects and interactions and multiple regression techniques were used to determine responses to continuous variables (PROC GLM; SAS Version 9.1; SAS Inst.).

Determining tissue ploidy level. Holoploid, 2C DNA content (i.e., DNA content of the entire nonreplicated, chromosome complement), and associated ploidy level were determined through flow cytometry. Approximately 4 months after treatment, leaves ($\approx$0.5 cm^2) were collected from newly elongated shoots, finely chopped, and incubated in 0.4 mL nuclei extraction buffer (CyStain ultraviolet Precise P Nuclei Extraction Buffer®; Partec, Münster, Germany) for 1 to 2 min at 25 °C. The preparation was then filtered using Partec CellTrics® disposable filters to remove debris. Nuclei were stained with 1.5 mL 4′, 6-diamidino-2-phenylindole staining buffer and incubated again at 25 °C for 1 to 2 min. The preparation was analyzed for relative DNA content using a flow cytometer (Partec PA II; Partec). The genomic size of *R.* 'Fragrantissimum Improved' was de-

termined using an internal standard of *Pisum sativum* L. 'Ctirad' with a known genome size of 8.76 pg (Greihuber et al., 2005). Ploidy level was determined by DNA content of diploid *R.* 'Fragrantissimum Improved' from nontreated controls with the DNA content from each sample. Three shoots (subsamples) were chosen randomly from each replicate of the 0, 7.5, 15, or 30 µM oryzalin treatment and analyzed for ploidy level. All data were subjected to ANOVA and multiple regression analysis using PROC GLM and means were separated using Fisher's protected least significant difference (SAS Version 9.1; SAS Inst.).

Results and Discussion

Shoot regeneration. Leaf segments formed limited callus and shoots on media containing TDZ in combination with IAA. For the IAA treatments, only the combinations 5 µM TDZ and 10 µM IAA (6%), 5 µM TDZ and 2.5 µM IAA (3%), and 15 µM TDZ and 10 µM IAA (6%) produced shoots (data not presented). This is consistent with previous observations that IAA does not promote organogenesis in *R.* 'Little John' (D.H. Touchell, personal communication). In contrast, studies on *R. ponticum* L. subsp. *baeticum* (Boiss. & Reut.) Hand.-Mazz. showed IAA (11.4 µM) to be an effective auxin for shoot regeneration from both apical shoots and nodal segments (Almeida et al., 2005). As a result of limited shoot formation on media containing IAA, the remaining analyses focused on the effect of NAA and TDZ on callus formation and shoot regeneration.

Regression analysis showed TDZ and NAA concentrations and their interaction significantly affected callus and shoot formation ($P < 0.01$). For both the percentage of segments forming callus (Fig. 1) and shoots (Fig. 2), there was a significant quadratic response for TDZ and NAA concentrations as well as their interaction. The predicted optimal concentration for shoot production in *R.* 'Fragrantissimum Improved' was 8.8 µM TDZ in combination with 10 µM NAA with 68% of leaf segments producing shoots through a callus phase (Fig. 2). In comparison, other studies have reported that a broad range of cytokinin and auxin levels induce shoot formation, suggesting in vitro responses may be specific to subgroups or species. For example, floral explants of *Rhododendron* 'Irina' produced the maximum number of shoots per explant using a combination of 73.8 µM 2iP, 14.8 µM IBA, and 9.1 µM TDZ (Tomsone et al., 2004). In contrast, Preece and Imel (1991) found low concentrations of TDZ (0.1 µM) combined with 1.0 µM IBA to be optimal for shoot regeneration from leaves of *R.* P.J.M. Group.

Induction of polyploids. Rhododendron 'Fragrantissimum Improved' had a 2C DNA content of 1.42 ± 0.16 pg (mean ± SEM, n = 10), which is consistent with 2C DNA contents of diploid rhododendrons (Jones et al., 2007).

In the first experiment, the effect of higher concentrations for shorter durations was investigated. Percentage of surviving calli was
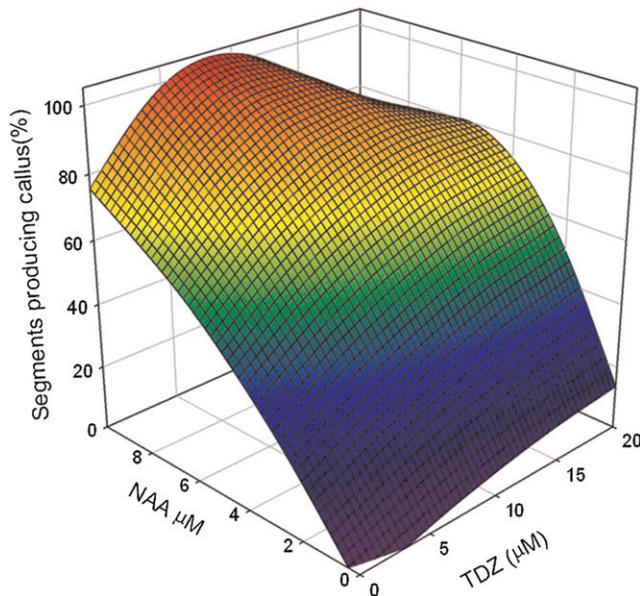
Fig. 1. Percent of leaf segments producing callus in response to 1-naphthaleneacetic acid (NAA) and thidiazuron (TDZ) concentrations. Percent of segments producing callus = −4.2 + 1.5 (TDZ), −0.03 (TDZ^2), +11.8 (NAA), −0.4 (NAA^2), +0.5 (TDZ) (NAA), −0.004 (TDZ^2) (NAA^2); $P < 0.0001$; $r^2 = 0.56$.
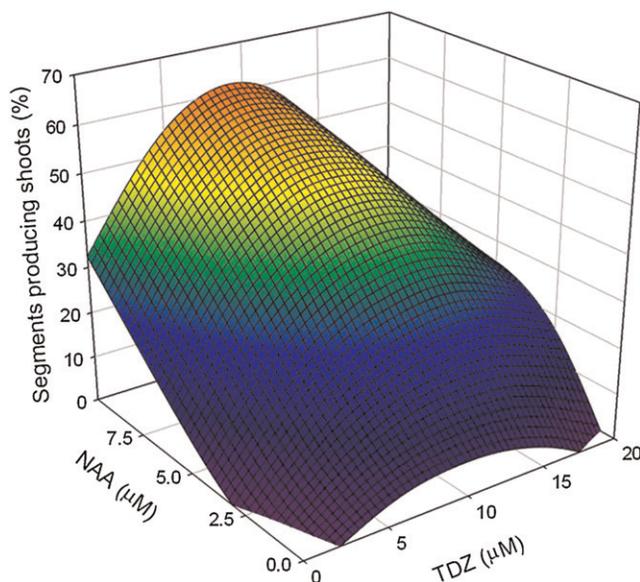
Fig. 2. Percentage leaf segments producing shoots in response to 1-naphthaleneacetic acid (NAA) and thidiazuron (TDZ) concentrations. Percent of leaf segments producing shoots = −1.34 + 1.71 (TDZ), −0.11 (TDZ^2), −1.99 (NAA), +0.47 (NAA^2), +0.76 (TDZ) (NAA), −0.004 (TDZ^2) (NAA^2); $P < 0.0001$; $r^2 = 0.62$.

affected by oryzalin concentration, duration, and their interaction ($P < 0.0001$) (Table 1). At concentrations of 0 or 30 µM, a duration of 5 d reduced callus survival compared with 1- and 3-d exposure. The lower callus survival for the 5-d control may be attributed to sensitivity to the ethanol added to the control treatments. At concentrations of 60 or 90 µM, however, survival was low (less than 35%) regardless of duration (Table 1). Callus that survived exposure to 60 µM or 90 µM oryzalin failed to produce shoots. The effect of oryzalin concentration on the number of

mixoploid and homogenous tetraploids was not significant. In addition, treatment duration did not influence ploidy level. As a result, there were no significant differences between treatments for the number of diploid, mixoploid, or tetraploid shoots recovered. For this experiment, two tetraploids (13.1%) was the maximum number attained with treatment of 30 µM oryzalin for 3 d (Table 1).

In a second experiment, the influence of lower concentrations of oryzalin combined with longer treatment durations was investi-

gated. An interaction between oryzalin concentration and treatment duration resulted in a significant decline in survival for the 15 µM oryzalin, 14-d duration treatment ($P < 0.05$) (Table 2). There was a significant effect of oryzalin concentration on the induction of polyploidy ($P < 0.05$) with both mixoploid and tetraploid shoots resulting from 7.5 µM oryzalin treatments. Three tetraploids (20%) were produced in the treatment 7.5 µM oryzalin for 7 d.

Rhododendron 'Fragrantissimum Improved' appears highly sensitive to oryzalin with only minimal survival obtained at the highest oryzalin concentration (90 µM) and polyploid induction occurring at concentrations as low as 7.5 µM (Tables 1 and 2). In comparison Sakai et al. (2004) found that explants from an interspecific hybrid between an evergreen (*R. kiusianum* Makino × *R. eriocarpum* Nakai) and a deciduous azalea (*R. japonicum* f. *flavum* Suringer) survived oryzalin concentrations of 30, 150, or 300 µM oryzalin for up to 72 h. In a study of an intersubgeneric *Rhododendron* hybrid, Sakai et al. (2004) determined the optimal treatment for tetraploid induction to be 300 µM oryzalin for 48 h yielding 85% tetraploids. Similarly, Väinölä (2000) found three *Rhododendron* hybrids could tolerate 150 µM for 48 h with the highest percentage of tetraploids (18%) occurring after 24 h.

Differences in sensitivity to oryzalin observed between *R.* 'Fragrantissimum' and other *Rhododendron* hybrids may be attributed to the growth and morphology of the different tissues used. Mitotic inhibitors such as oryzalin work by arresting cell division in actively growing tissues, and their effectiveness is dependent on the ability to penetrate those tissues. For example, Kermani et al. (2003) found that in *Rosa* 'Thérèse Bugnet', a higher proportion of tetraploids (66%) was obtained after treatment of thin nodal segments with 5 µM oryzalin for 1 d compared with shoots treated with 5 µM oryzalin for 14 d (40%). The higher success rate in the thin nodal segments was attributed to more efficient penetration of oryzalin into the dividing cells. For *Rhododendron* hybrids, high concentrations of oryzalin were required to penetrate nodal sections to reach meristematic tissues and induce polyploidy (Väinölä, 2000). In comparison, the shoot regeneration system developed here for *R.* 'Fragrantissimum' allowed developing meristems to be directly exposed to low concentrations of oryzalin to induce polyploidy.

In conclusion, effective in vitro shoot regeneration and polyploidy induction protocols were developed for *R.* 'Fragrantissimum Improved'. These procedures provide a foundation for in vitro regeneration and ploidy manipulation of other *Rhododendron* taxa, particularly in subgenus *Rhododendron* and subsections *Edgeworthia* and *Maddenia*. The polyploid shoots produced in this study will be rooted and the plantlets grown to maturity to determine fertility, evaluate ornamental traits, and potentially incorporate these traits into future breeding programs.

Table 1. Effects of short duration, high concentration oryzalin treatments on survival and polyploidy induction in callus cultures of *Rhododendron* 'Fragrantissimum Improved'.

Oryzalin concn (μM)	Duration (days)	Survival (%)	Ploidy level (%) 2x	Mix[y]	4x
0	1	100 a[z]	100 a	0 a	0 a
	3	87 a	100 a	0 a	0 a
	5	81 b	100 a	0 a	0 a
30	1	100 a	80 ab	7 a	13 b
	3	98 a	80 ab	7 a	13 b
	5	55 c	60 b	40 b	0 a
60	1	24 d	—	—	—
	3	35 d	—	—	—
	5	20 d	—	—	—
90	1	14 e	—	—	—
	3	0 f	—	—	—
	5	0 f	—	—	—

[z]Means separation within columns by Fisher's protected least significant difference at $P < 0.05$.
[y]Mixoploid (cytochimera) tissue.
— Shoots failed to regenerate from callus.

Table 2. Effects of long-duration, low-concentration oryzalin treatments on survival and polyploidy induction in callus cultures of *Rhododendron* 'Fragrantissimum Improved'.

Oryzalin concn. (μM)	Duration (days)	Survival (%)	Ploidy level (%) 2x	Mix[y]	4x
0	7	100 a[z]	100 a	0 a	0 a
	14	90 a	100 a	0 a	0 a
7.5	7	98 a	70 b	10 a	20 b
	14	83 a	87 ab	7 a	7 a
15	7	100 a	100 a	0 a	0 a
	14	40 b	100 a	0 a	0 a

[z]Means separation within columns by Fisher's protected least significant difference at $P < 0.05$.
[y]Mixoploid (cytochimera) tissue.

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