In Vitro Regeneration of *Rhododendron* 'Fragrantissimum Improved'

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Significance to Industry: *Rhododendron* 'Fragrantissimum Improved' is a wide hybrid that exhibits attractive exfoliating bark, lush foliage, and clusters of large, white and pink, pleasantly fragrant flowers. This combination of traits makes 'Fragrantissimum Improved' appealing to breeders for the development of novel cultivars for use in the landscape. Like many wide hybrids, however, 'Fragrantissimum Improved' is sterile. *In vitro* chromosome doubling can be used to develop new allopolyploids with restored fertility, providing new opportunities to use these plants in breeding programs (7, 9). This approach first requires the development of an *in vitro* regeneration system. In this study we developed an effective *in vitro* regeneration protocol from leaves of *R*. 'Fragrantissimum Improved' that was optimized using 8.8 µM thidiazuron (TDZ) and 10 µM α-naphthaleneacetic acid (NAA).

Nature of Work: The development of *in vitro* regeneration protocols requires the optimization of plant growth regulators (PGR). For *Rhododendron* species, thidiazuron has been effective in stimulating shoot regeneration and several studies have successfully utilized it for *in vitro* production of shoots of difficult to propagate species (1, 4, 5, 6). For example, shoot regeneration of *R.* 'P.J.M.' was 250 times higher on media containing TDZ and IBA (indolebutyric acid) than on media containing 2iP (2-isopentyl adenine) and IBA (6). In contrast, shoot production of *R. catawbiense* 'English Roseum' was optimized using 4 µM IAA (3-acetic acid) combined with 15 µM 2iP (8). Therefore, the objective of this study was to develop an *in vitro* regeneration system by determining the optimal PGR concentrations for *in vitro* callus formation and shoot regeneration from leaves of *R*. 'Fragrantissimum Improved'.

Recently expanded leaves of *R.* 'Fragrantissimum Improved' were collected from glasshouse-grown stock plants maintained at the Mountain Horticultural Crops Research Station in Mills River, NC. The leaves were rinsed under tap water for 4.5 hrs, then washed in 20% commercial bleach (5.25% NaClO) containing a few drops of Tween 20 for 25 minutes. Before being cultured on regeneration media, plants were rinsed three times in sterile, distilled water for 5 minutes per rinse.

The effect of hormone concentration on callus production and shoot regeneration was tested using TDZ (0, 5, 10, 15 and 20 µM) in combination with either NAA or IAA (0, 2.5, 5 and 10 µM). The basal medium consisted of MS salts and vitamins supplemented with 3% sucrose and 0.8% agar (pH 5.75-5.80 prior to autoclaving). Cultures were

maintained in the dark at 23°C (73.4°F). Experiments were conducted as two separate completely randomized factorial designs, using either IAA or NAA as the auxin source. The 20 possible PGR treatment combinations between each auxin and cytokinin were replicated with 8 petri dishes, each containing 5 leaf segments (subsamples). After 8 weeks, data were collected for the number of leaf segments producing callus tissue and shoots. Responses to PGRs were analyzed using multiple regression analyses (SAS version 9.1; SAS Institute, Cary, NC).

Results and Discussion: *In vitro* callus formation and shoot regeneration protocols were successfully developed from leaf segments of *R.* 'Fragrantissimum Improved' (Table 1). Leaf segments formed callus on media containing TDZ in combination with either NAA or IAA (Table 1; IAA data not shown). Shoot formation readily occurred on leaf segments exposed to NAA, but shoot production was limited on media containing IAA. Of the IAA treatments, only the combinations 10 µM TDZ and 2.5 µM IAA (0.08% segments forming shoots), 5 µM TDZ and 2.5 µM IAA (0.03% segments forming shoots), and 5 µM TDZ and 10 µM IAA (0.06% segments forming shoots) produced shoots (data not shown). This is consistent with previous observations that IAA did not promote organogenesis in *R.* 'Little John' (Dr. Darren Touchell, personal observations). Therefore, our analysis focused on the effect of NAA and TDZ on callus formation and shoot regeneration.

Regression analysis indicated that the interaction between the concentrations of TDZ and NAA significantly affected shoot formation ($P<0.01$). From the regression model, a surface response was generated to highlight the interaction between TDZ and NAA and its affect on shoot regeneration (Figure 1). The optimal range of PGR concentrations to maximize shoot production in *R*. 'Fragrantissimum Improved' was 8.8 µM TDZ in combination with 10 µM NAA (Figure 1). This is similar to previous research, which found concentrations of 0.1-10 µM TDZ was optimal for shoot regeneration of *R*. 'P.J.M.' (6). Following the experiment, shoots were transferred to Anderson's media supplemented with 10 µM 2iP and 4 µM IBA for elongation. The *in vitro* regeneration protocol for *R*. 'Fragrantissimum Improved' developed in this study will be used in future experiments in attempts to develop new allopolyploids with restored fertility.

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Table 1. Percentage of leaf segments (out of five subsamples) of *R*. 'Fragrantissimum Improved' producing callus or shoots when cultured on media with different concentrations of TDZ and NAA.

		Regeneration response	
TDZ (µM)	NAA (µM)	Segments with callus(%)[1]	Segments with hoots (%)
0	0	0 ± 0	0 ± 0
5	0	0 ± 0	0 ± 0
10	0	0 ± 0	0 ± 0
15	0	0 ± 0	0 ± 0
20	0	0 ± 0	0 ± 0
0	2.5	20 ± 12	0 ± 0
5	2.5	70 ± 30	2 ± 8
10	2.5	0 ± 0	0 ± 0
15	2.5	88 ± 16	38 ± 14
20	2.5	96 ± 8	20 ± 16
0	5	6 ± 10	0 ± 0
5	5	100 ± 0	72 ± 10
10	5	46 ± 32	10 ± 16
15	5	78 ± 26	32 ± 18
20	5	46 ± 34	14 ± 18
0	10	88 ± 18	0 ± 0
5	10	100 ± 0	84 ± 16
10	10	100 ± 12	70 ± 16
15	10	94 ± 10	48 ± 30
20	10	66 ± 36	18 ± 16

[1]Means (n=8) ± SEM.

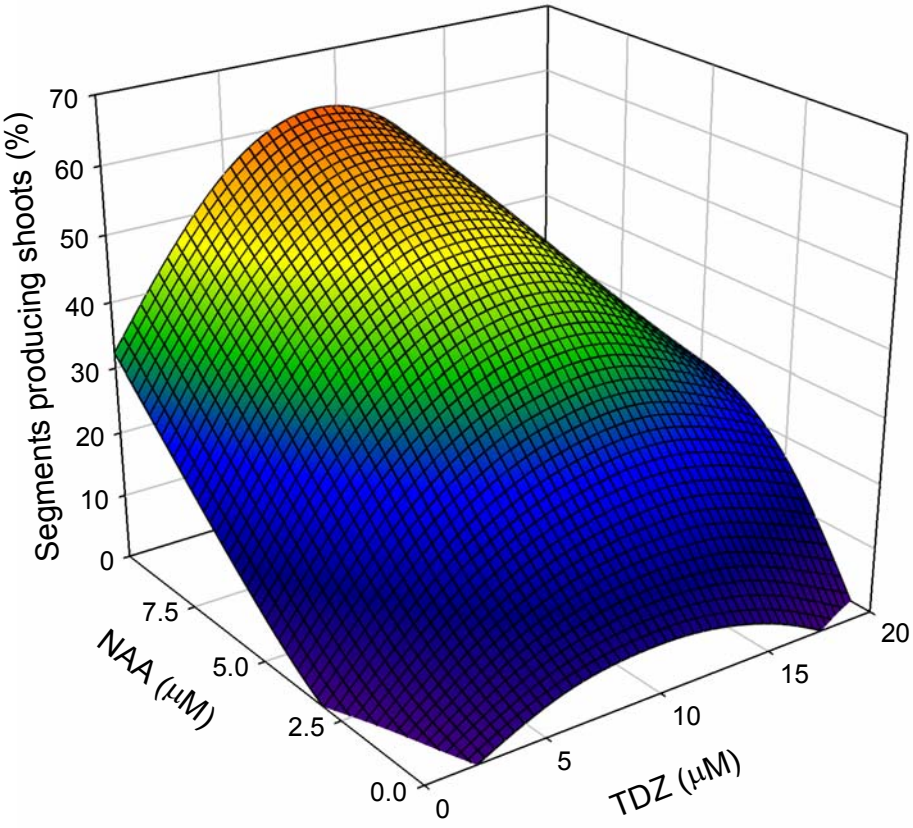

Figure 1. Percent of leaf segments per dish (out of 5) producing shoots in response to α-naphthaleneacetic acid (NAA) and thidiazuron (TDZ). Percent of leaf segments producing shoots= -1.34 +(1.708*TDZ) -(0.107*TDZ^2) -(1.988*NAA) +(0.47*NAA^2) +(0.76*TDZ*NAA) -(0.0039*TDZ^2*NAA^2); P<0.0001; r^2 = 0.62.