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Trifluralin-mediated polyploidization of *Rosa chinensis minima* (Sims) Voss seedlings

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Summary

Many diploid rose species and cultivars possess valuable traits that can be introgressed into modern tetraploid cultivars. Interspecific, interploidy crosses are possible, but triploid hybrids typically have limited fertility, hindering further breeding and selection. Tetraploidizing diploids before mating with tetraploids can alleviate fertility barriers. The efficiency of trifluralin was investigated for polyploidization of *Rosa chinensis minima* ($2n = 2x = 14$) seedlings. Treatments were trifluralin at 0.086% and 0.0086%, colchicine (0.5%), and distilled water and contained 2% dimethyl sulfoxide and a surfactant. Approximately 5 μl of the treatment solution was applied to the apical meristem of seedlings ($N = 337$, 82–85 per treatment) in the process of cotyledon expansion. Guard cell length, pollen diameter, and root tip squashes of rooted cuttings were used to detect polyploidy in meristematic layer (L)I, LII, and LIII, respectively. Trifluralin (0.086%) was the most effective treatment for polyploidization (LI 20.2%, LII 12.9%, LIII 12.9%), followed by trifluralin (0.0086%) (LI 10.6%, LII 7.1%, LIII 4.7%) and colchicine (LI 2.4%, LII 0%, LIII 0%). Polyploidization consistently occurred from LI inward. Polyploids as a group had reduced pollen stainability and a lower leaflet length to width ratio than diploids. In addition, two diploid seedlings were identified which produce 2*n* pollen. Considerations in selecting germplasm and generating somatically-induced polyploids from seedlings versus clones for use in breeding are discussed.

Introduction

Roses are one of the most widely grown and valued of all ornamental crops. For instance, for cut roses and potted florist roses in the United States alone, greater than 51 million stems and nine million pots are produced annually and have a combined wholesale value of over US$78 million (USDA, 2004). Rose culture can be challenging due to susceptibility to various pathogens and abiotic stresses (Horst, 1983). Fortunately, variability for resistance to biotic and abiotic stresses, as well as novel ornamental traits, are available within the genus *Rosa* for introgression into cultivars (Krüssmann, 1981; Wright, 1947).

Most cultivated garden, cut flower, and pot roses are tetraploid ($2n = 4x = 28$), while most species roses and some cultivars are diploid ($2n = 2x = 14$) (Cairns, 2000; Krüssmann, 1981). Hybrids are possible between diploid and tetraploid roses. However, triploid progeny often have reduced fertility, creating a bottleneck to additional breeding efforts (Rowley, 1960). In addition, some interspecific diploid hybrids have reduced fertility with respect to their diploid parents (Basye, 1990). One tool that can circumvent fertility bottlenecks is polyploidization of diploid or triploid germplasm.

Polyploidization of diploids before crossing with tetraploids allows for tetraploid progeny (Ma et al., 2000; von Malek & Debener, 1998). The use of colchicine on shoot meristems has been the primary method of somatic polyploidization in roses (Basye, 1990; Ma et al., 1997; Roberts et al., 1990; Seminiuk & Arisumi, 1968). Mechanisms of 2*n* gamete formation in roses for use in sexual polyploidization have only recently been reported, and the inheritance of these

mechanisms in roses is unknown (Crespel et al., 2002; El Mokadem et al., 2002a, b).

Colchicine has been widely used for polyploidization in plants since it was first reported in the 1930s to have this property (Blakeslee & Avery, 1937). Colchicine works by disrupting polymerization of microtubules which, in turn, disrupts spindle fiber development and mitosis (Bartels & Hilton, 1973; Eigsti & Dustin, 1957). Cells arrested at metaphase may recover and enter the next mitotic cycle with twice as many chromosomes. When using topically applied chromosome doubling agents, chimerism is common and isolating polyploid sectors and ploidy characteriztion of meristematic layers is necessary (Seminiuk & Arisumi, 1968; Tilney-Bassett, 1986).

Because colchicine is a carcinogen and is relatively expensive, anti-microtubule herbicides have been explored as an alternative for polyploidization, i.e. amiprophos-methyl, oryzalin, and trifluralin (Eeckhaut et al., 2004; Hansen & Anderson, 1998; Hansen et al., 1998; Hassawi & Liang, 1991; Ramulu et al., 1991; Wan et al., 1991). Some herbicides, such as trifluralin, have been shown to have greater affinity for binding to plant microtubules than animal microtubules (Bartels & Hilton, 1973). Compared to colchicine, the efficiency of such herbicides in polyploidization of plants has been variable but promising. Kermani et al. (2003) recently reported successfully using oryzalin to chromosome double diploid and triploid rose clones *in vitro*. Our study evaluates the effectiveness of trifluralin for chromosome doubling diploid *R. chinensis minima* seedlings. To the best of our knowledge, this is the first report evaluating the efficiency of trifluralin for polyploidization of a woody species.

Materials and methods

Germplasm and pre-treatment culture

Germinating seedlings used for polyploidization were obtained from two open-pollinated *R. chinensis minima* genotypes (95-1 and 95-2) selected from a commercial seed source (*Rosa* 'Polyantha Angel Rose Mixed', Thompson & Morgan Seedsmen, Inc., Jackson, NJ). *R. chinensis minima* was chosen because of its small stature and ability to flower quickly (<12 weeks) and repeatedly after germination. Achenes were removed from hypanthium tissue, soaked for 24 h in distilled water, and planted in an open seed flat using Fafard® Germination mix (Fafard®, Inc., Agawam, MA). Seeds were cold stratified in flats at 5 °C for one month and then moved to 16 °C. Upon emergence, seedlings were transplanted to 10 cm square pots containing Sunshine Mix #2 (Sun Gro® Horticulture, Bellevue, WA) with up to 16 seedlings per pot. Due to erratic germination, potting occurred over four weeks and eight potting dates. At each potting date, germinating seedlings from each female were evenly distributed among four pots in preparation for one of four treatments: altogether 337 seedlings were potted. After potting, seedlings were placed 20 cm below cool-white fluorescent lights (16 h photoperiod, $\sim$32 μmol m^{-2} s^{-1}). Treatments were applied one day following potting, after the hypocotyls straightened and cotyledons were in the process of expanding in response to light.

Treatments

Four treatments were used: Distilled water (82 seedlings), colchicine (0.5%) (85 seedlings), and trifluralin (0.0086% & 0.086%) (85 seedlings each). Trifluralin was obtained as Treflan* (emulsifiable concentrate, 43% trifluralin, Dow AgroSciences, Indianapolis, IN). Each treatment contained 2% dimethyl sulfoxide as a carrier and one drop ($\sim$20 μl) of Dawn® original dish detergent (Procter & Gamble, Cincinnati, OH) per 100 ml as a surfactant. The treatment solution was suspended ($\sim$5 μl) between the expanding cotyledons with a medicine dropper. After application, pots were sealed in a clear polyethylene bag to elevate humidity and prevent the treatment from drying. After 24 h the polyethylene bag was removed. Treated seedlings remained under fluorescent lights (16 h photoperiod; 16 °C; $\sim$32 μmol m^{-2} s^{-1}) for approximately two months, and surviving seedlings subsequently were transplanted to individual pots and moved to the greenhouse. Seedlings were given a four-part designator denoting the potting date (1–8), treatment (A = water; B = colchicine; C = trifluralin 0.0086%; D = trifluralin 0.086%), female (A = 95-1; B = 95-2), and seedling number with previous combination of factors, respectively.

Ploidy determination

Periclinal ploidy chimeras are possible using topically applied polyploidization agents, and characterization of polyploidization patterns in regard to meristematic layers requires a more sensitive method than macerated shoot tips and flow cytometry. Ploidy level was determined in LI using guard cell length. Due to difficulty obtaining epidermal peels in rose, epidermal imprints

were used. Two fully expanded terminal leaflets per seedling were pressed (abaxial side down) into a drop of fast drying glue (Kwik fix® Super glue plus™, Chemence, Inc., Alpharetta, GA) on a glass microscope slide. Leaf tissue was allowed to air dry before removal. A drop of acetocarmine and a cover slip were placed over the imprint adhered to the glass slide for greater contrast during examination. The length of five guard cell imprints (one measured per stomatal pair) were recorded per leaflet (10 measurements/seedling).

Ploidy level of LII was determined using pollen diameter. Microscopic preparation of pollen occurred using acetocarmine for staining and glycerin for uniform distribution according to Zlesak and Thill (2002). Pollen from $\geq$2 flowers was bulked and the diameters of 30 well-stained pollen grains were recorded per seedling. Pollen stainability was monitored to ascertain how polyploidization of this germplasm effected fertility. Seedlings were assigned to one of five pollen stainability categories: 1 = 1–20%, 2 = 21–40%, 3 = 41–60%, 4 = 61–80%, and 5 = 81–100% stainable pollen. The midpoint of the pollen stainability range for each seedling was used to analyze pollen stainability across grouping variables.

Terminal leaflet length to width ratio (L/W) was calculated to determine if it was an effective predictor of polyploidization. The L/W was calculated for five fully expanded terminal leaflets per seedling.

Semi-hardwood stem cuttings of seedlings that were polyploid in LI and LII as well as a sample of seedlings polyploid in LI and diploid in LII were rooted to assess ploidy in LIII. Adventitious roots typically arise from interfascicular parenchyma or in the vascular ray near the cambium (Esau, 1977), and are LIII-derived tissue. Root tip squashes were used for ploidy determination in LIII. Chromosomes of $n \geq 5$ metaphase cells were counted per seedling. Actively growing root tips were harvested and stored in glass vials of distilled water on ice for 24 h. Root tips were then fixed in Farmer's fixative (3:1 v/v, 95% ethanol: glacial acetic acid) and refrigerated. For samples that were not examined within two weeks, Farmer's fixative was replaced with 70% ethanol. Root tips were hydrolyzed in 5N HCl for 90 min at room temperature prior to squashing and acetocarmine was used for staining.

Statisitical analysis

Dependence between categorical variables was examined using Chi-squared (χ^2) analysis. Comparisons of quantitative data with two grouping variables were made using Student's unpaired *t*-tests, and more than two grouping variables were compared using ANOVA and Tukey's HSD. Comparison of proportions were made using two-tailed *z*-tests. Student's unpaired *t*-tests, ANOVA, and Tukey's HSD analysis were performed using SPSS software (SPSS Inc., 1997).

Results

Ploidy classification

A loosely bimodal distribution was observed with distinct breaks in the data for guard cell length and pollen diameter, allowing for separation of seedlings into diploid and polyploid groups for LI and LII (Figure 1). The separation between diploids and polyploids was less clear for LI than LII and was set in the break in guard cell length data between 21.9 and 23.1 μm (Table 1) because the first seedling to the right of the break (5DB4) was confirmed to be polyploid in LII (determined by pollen diameter) and LIII (determined by root tip analysis). Across all treatments 28 LI and 17 LII polyploids were recovered (Table 1). The observed pollen diameter ranges based on ploidy are in general agreement with those found by Erlanson (1931) and Seminiuk & Arisumi (1968).

Two genotypes, 5DA4 and 2AA2, did not produce anthers. Guard cell length and root tip squash analyses showed that 5DA4 was polyploid in LI and LIII. Therefore, 5DA4 was classified as polyploid in LII for statistical analysis. Because 2AA2 was diploid in LI and there were no confirmed seedlings that were diploid in LI and polyploid in LII, it was classified as diploid for LII. Three of the nine clones that were polyploid in LI and diploid in LII were available for ploidy assessment in LIII, and each was diploid.

Dependence was not found ($P > 0.05$) between female and polyploidization (LI, $\chi^2 = 2.59$, $P = 0.13$; LII, $\chi^2 = 0.76$, $P = 0.68$), female and survival ($\chi^2 = 1.85$, $P = 0.17$), potting date and polyploidization (LI, $\chi^2 = 9.24$, $P = 0.34$; LII, $\chi^2 = 5.56$, $P = 0.66$), and potting date and survival ($\chi^2 = 6.29$, $P = 0.60$), allowing data to be pooled over potting dates and females. Progeny from the two females differed significantly for pollen diameter, guard cell length, and leaflet L/W (Table 2), but ranges of progeny groups for pollen diameter and guard cell length overlapped and differences due to female were relatively small and did not impact ranges for ploidy classification.

Table 1. Guard cell length and pollen diameter ranges of *R. chinensis minima* parents and progeny used to determine polyploid conversion of seedlings in meristem layers I and II

	Layer I Guard cell length (μm)			Layer II Pollen diameter (μm)		
	Mean (S.D.)	Range	n[a]	Mean (S.D.)	Range	n[b]
Diploid female parents						
95-1	20.8 (1.6)			34.2 (1.7)		
95-2	19.1 (1.2)			34.7 (1.7)		
Seedling progeny						
Diploids	19.3 (1.2)	14.1–21.9	178	33.7 (1.1)	31.0–38.2	188
Diploids with 2n pollen						
4AB1	18.4 (0.9)			37.9 (4.4)		
4BA3	20.2 (1.5)			38.2 (3.1)		
Polyploids	28.8 (3.6)	23.1–39.3	28	41.3 (1.5)	39.7–45.2	16
Polyploid with unusual meiosis[c]						
5DB6	30.4 (2.2)			45.2 (4.3)		

[a]Number of genotypes assessed.
[b]Two seedlings, one diploid and one polyploid, were not included in Layer II data because the flowers did not contain anthers.
[c]Cytological examination revealed tetrads and unusual configurations involving five or more microspores.

Table 2. Pollen diameter, pollen stainability, guard cell length, and terminal leaflet length to width ratio (L/W) between diploid (LI & LII) seedling progenies of two open-pollinated *R. chinensis minima* female parents

	Female parent				
	95-1		95-2		Unpaired Student's $t-$test[b]
	Mean (S.D.)	n[a]	Mean (S.D.)	n	
Pollen diameter (μm)	33.9 (1.1)	65	33.6 (1.1)	112	*
Pollen stainability (%)	77.1 (21.0)	65	77.3 (15.4)	112	ns
Guard cell length (μm)	19.9 (0.9)	66	18.9 (1.3)	112	**
Terminal leaflet L/W	1.8 (0.1)	66	1.7 (0.1)	112	*

[a]One seedling of 95-1 did not produce anthers and is not included in the analysis of pollen-related traits.
[b]**, *, ns indicates significance at $P < 0.01$, $P \leq 0.05$, and not significant, respectively.

Treatment effects

Only trifluralin allowed for recovery of polyploid shoots in LII and LIII, while both trifluralin and colchicine stimulated polyploidy in LI (Table 3). Trifluralin (0.086%) had the highest mortality (65%), yet also the highest frequency of polyploidization of treated meristems (LI, 20.2%; LII, 12.9%; LIII, 12.9%). Trifluralin (0.086%) induced a significantly higher ratio of polyploids among surviving seedlings than trifluralin (0.0086%) for both LI ($z = 5.45$, $P < 0.001$) and LII ($z = 3.99$, $P < 0.001$). All polyploid individuals generated using trifluralin (0.086%) were polyploid in LII and LIII, while trifluralin (0.0086%) produced two periclinal chimeras polyploid for both LI and LII, but diploid in LIII. With either trifluralin treatment, $\sim$ two-thirds of the LI polyploids were polyploid in LII. Colchicine generated two LI polyploids, but no LII polyploids and had 15% mortality, which is significantly lower than the water control ($z = 2.99$, $P = 0.003$).

Aberrant genotypes

Two genotypes, 4AB1 and 4BA3, had mean pollen diameters which fell between the two ploidy ranges

Table 3. Survival and polyploid conversion frequency among survivors in meristem layers I, II, and III of diploid *R. chinensis minima* seedlings treated with water, colchicine, and trifluralin

Treatment	No. of seedlings treated	No. of survived	LI Polyploid	LII Polyploid	LIII[a] 4*x*	3*x*	2*x*
Water	82	53	0	0			
Colchicine (0.5%)	85	72	2	0			
Trifluralin (0.0086%)	85	51	9	6	3	1	2
Trifluralin (0.086%)	85	30	17	11	9	2[b]	0

[a]Semi-hardwood stem cuttings were rooted for all LII polyploids to assess ploidy of LIII using root tip squashes.
[b]One seedling was monosomic and had 20 chromosomes ($3x - 1$) instead of 21.

Table 4. Frequencies of dyads, triads and tetrads observed during microsporogenesis for diploid *R. chinensis minima* seedlings 4AB1 and 4BA3

Seedling	No. of dyads	No. of triads	No. of tetrads	Total observed
4AB1	2	6	53	61
4BA3	12	0	88	100

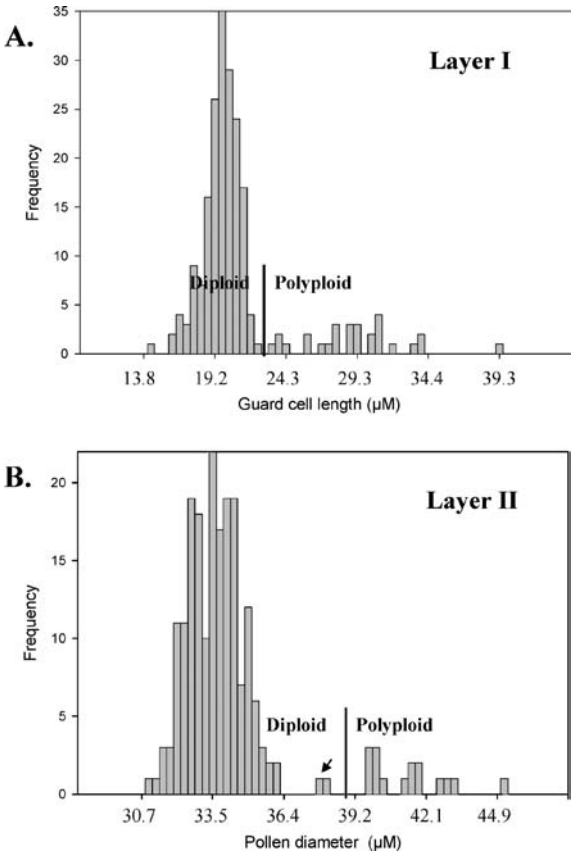

Figure 1. Distribution of (A.) guard cell length and (B.) pollen diameter in *R. chinensis minima* seedlings for ploidy classification of meristematic layers I and II. Arrow (B.) denotes two diploid genotypes that produce 2*n* pollen.

(Table 1 and Figure 1). They were diploid in LI based on guard cell length, and root tip squashes confirmed these clones are diploid in LIII. Approximately 30% of the pollen grains of 4AB1 and 4BA3 were the diameter expected from tetraploids, suggesting these clones may be producing relatively high levels of 2*n* pollen. Subsequent cytology confirmed 2*n* pollen production for both clones by the presence of dyads and triads (Table 4).

Seedling 5DB6 had exceptionally large pollen grains for a polyploid (Table 1). Guard cell length was not exceptionally long, compared to other polyploids, and root tip squashes confirmed it was tetraploid in LIII. Cytological examination did not reveal dyads or triads to suggest 2*n* pollen production through standard mechanisms like parallel spindles or premature cytokinesis. It did, however, reveal tetrads and unusual configurations involving five or more joined microspores (Figure 2).

One aneuploid seedling was identified, 6DB5. Root tip squashes revealed it had 20 chromosomes ($3x - 1$) in LIII. This seedling exhibited a reduced growth rate relative to other polyploids and had unusual, slightly twisted petioles.

Phenotypic changes due to polyploidization

Macroscopic evidence of sectoral ploidy chimerism was not observed in this study. Surviving seedlings either possessed typical traits of polyploidization around the whole circumference of the shoot (i.e. larger and darker green leaves and stems, thicker foliage and petals, and less branching) or did not. All LII polyploids showed typical macroscopic evidence of polyploidization, while LI polyploids/LII diploids generally did not differ in phenotype from LI–LII diploids. Layer II polyploids had a significantly lower leaflet L/W and a lower percent of stainable pollen than LII diploids (Table 5). Complete diploids (LI–LII) and polyploids (LI–LII–LIII) were significantly different for leaflet L/W, but were indistinguishable from ploidy chimeras (Table 6).

Table 5. Terminal leaflet length to width ratio (L/W) and pollen stainability between polyploid and diploid meristematic layers of *R. chinensis minima*

	Terminal leaflet L/W			% Pollen stainability		
	Mean (S.D.)[a]	*n*	Range	Mean (S.D.)	*n*[b]	Range
Diploid						
Layer I	1.8 (0.1)	178	1.1–2.2	77.2 (17.6)	177	10–90
Layer II	1.8 (0.1)	189	1.1–2.2	77.7 (17.3)	188	10–90
Polyploid						
Layer I	1.6 (0.2)	28	1.2–2.0	53.7 (31.9)	27	10–90
Layer II	1.5 (0.1)	17	1.2–1.9	32.5 (22.9)	16	10–70
Layer III	1.5 (0.2)	15	1.2–1.9	32.9 (23.3)	14	10–70

[a]All inter-ploidy, intra-layer comparisons for mean leaflet L/W and pollen stainability are significantly different ($P < 0.01$) using an unpaired Student's *t*-test.
[b]One diploid and one polyploid seedling were not included in pollen stainability data because flowers did not contain anthers.

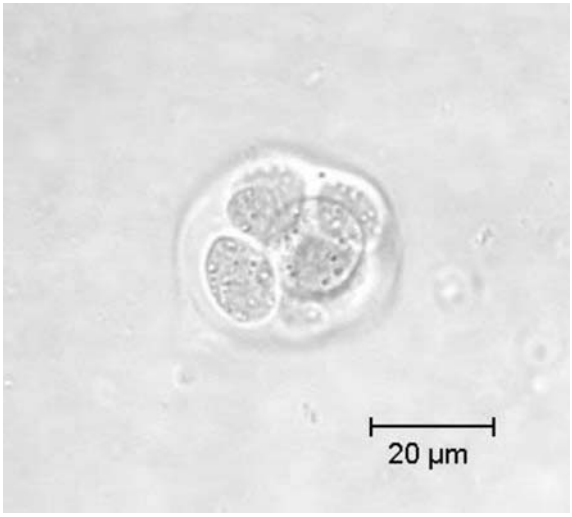

Figure 2. Pentad configuration from polyploid *R. chinensis minima* seedling 5DB6.

Ploidy chimeras differed in pollen stainability based on ploidy in LII (Table 6). Although significant differences were found between the diploid and polyploid groups for these traits, the ranges overlapped and were not reliable predictors of polyploidization.

Four seedlings that were polyploid in both LI and LII produced an adventitious diploid shoot below the cotyledons. Semi-hardwood stem cuttings were rooted from the diploid root suckers, and they were analyzed as all other plants in the study (Table 7). In LIII, two of the four LI and LII polyploid seedlings were tetraploid, one was triploid, and one was diploid. Pollen diameter, guard cell length, and leaflet L/W were significantly different ($P < 0.01$) between ploidy level for each genotype set using unpaired Student's *t* tests, and pollen stainability was consistently lower in the LII polyploid member of each set (Table 7 and Figure 3).

Discussion

The ability to induce polyploids as well as plants with unexpected numbers of chromosomes, common effects of colchicine, were also found in this investigation using trifluralin. Periclinal chimeras were generated in this study using both colchicine and trifluralin, as previously reported for *R. multiflora* using colchicine (Seminiuk & Arisumi, 1968). Effects other than polyploidization have also been reported using colchicine and include gene mutation and chromosomal loss, substitution, and rearrangements (Luckett, 1989; Sanders & Franzke, 1960; Simantel & Ross, 1963). In this study, not all LIII polyploids were tetraploid; there were two triploids and one aneuploid ($3x - 1$) (Table 3). One of the triploids (5DA2) generated a diploid root sucker, suggesting chromosome loss or incomplete polyploidization induced by trifluralin, rather than the seedling being inherently triploid through the union of an *n* and 2*n* gamete. More studies are necessary to assess the relative frequency of chromosomal abnormalities and to address the issue of induced gene mutation by trifluralin relative to colchicine.

Seedling growth stage can influence polyploidization success. Seedlings undergoing cotyledon expansion were used for this study, which has been described as the optimum seedling stage in roses for colchicine treatment (Basye, 1990). Unfortunately, Basye (1990) did not report mortality or conversion rates using colchicine on seedlings for comparison. Waiting beyond cotyledon expansion may make chemical penetration to the meristem more difficult because of additional leaf primordia. Using colchicine and axillary meristems of *R. multiflora ex vitro*, Semeniuk& Arisumi (1968) found frequent macroscopic evidence of sectoral polyploidization, and out of 125 treated axils isolated one shoot tetraploid in all layers and another

Table 6. Terminal leaflet length to width ratio (L/W) and pollen stainability among ploidy periclinal chimeras and complete diploid and polyploid *R. chinensis minima* seedlings

	Terminal leaflet L/W			% Pollen stainability		
Ploidy layer I/II/III	Mean (S.D.)[a]	*n*	Range	Mean (S.D.)	*n*[b]	Range
2*x*/2*x*/–	1.8 (0.1) a	178	1.1–2.2	77.2 (17.6) b	177	10–90
P[c]/P/P	1.5 (0.2) b	15	1.2–1.9	32.9 (23.3) a	14	10–70
P/2*x*/–	1.7 (0.2) ab	11	1.3–2.0	84.5 (9.3) b	11	70–90
P/P/2*x*	1.6 (0.2) ab	2	1.5–1.7	30.0 (28.3) a	2	10–50

[a]Values within columns followed by the same letter do not differ significantly using Tukey's HSD at $P = 0.05$.
[b]One diploid and one polyploid seedling were not included in pollen stainability data because flowers did not contain anthers.
[c]Polyploid.

Table 7. Pollen diameter, pollen stainability, guard cell length, and terminal leaflet length to width ratio (L/W) for four sets of diploid genotypes and their somatically-induced polyploids

Genotype (ploidyLIII)[a]	Pollen diameter (μm)	Pollen stainability[c]	Guard cell length (μm)	Terminal leaflet L/W
	Mean (S.D.)[b]		Mean (S.D.)	Mean (S.D.)
2CA1 (2*x*)[d]	34.6 (1.6)	5	20.9 (1.8)	2.1 (0.1)
2CA1 (2*x*)	43.0 (3.5)	1	27.1 (1.4)	1.5 (0.3)
5DA2 (2*x*)[d]	33.4 (1.9)	5	20.5 (3.8)	2.1 (0.1)
5DA2 (3*x*)	39.7 (4.6)	1	25.3 (2.3)	1.7 (0.1)
6CA2 (2*x*)[d]	33.8 (1.9)	5	20.8 (2.2)	1.7 (0.1)
6CA2 (4*x*)	42.8 (2.8)	1	33.8 (1.7)	1.5 (0.1)
6DA1 (2*x*)[d]	33.7 (2.0)	4	20.3 (2.9)	1.9 (0.1)
6DA1 (4*x*)	40.3 (3.7)	1	33.7 (4.0)	1.3 (0.1)

[a]Semi-hardwood stem cuttings were taken of polyploid shoots (Layers I and II) and shoots from diploid suckers for ploidy assessment of Layer III.
[b]All intra-genotype ploidy comparisons for pollen diameter, guard cell length, and leaflet L/W are significantly different ($P < 0.01$) using an unpaired Student's *t*-test.
[c]Pollen stainability: 1 = 1–20%, 4 = 61–80%, and 5 = 81–100% stainable pollen.
[d]Plant isolated from diploid adventitious shoot from below the cotyledons.

shoot tetraploid in LI, but diploid in LII and LIII. The use of very young seedlings, rather than axillary buds which have well-developed leaf primordia, may account for the lack of macroscopic symptoms of sectoral chimerism and the relatively high rate of ploidy conversion observed in the present study.

Polyploidization with topically applied trifluralin and colchicine occurred from LI inward as evidenced by the fact that there were no individuals that were polyploid in LII, but diploid in LI. Polyploidization from LI inward also has been found with *ex vitro* colchicine application to *R. multiflora* axillary meristems (Semeniuk & Arisumi, 1968). Since LII polyploids have reliably been polyploid in LI, one could first measure guard cell length of leaves grown from treated meristems and only bring LI polyploids into flower for assessment of LII. Recent papers on polyploidization of roses used *in vitro* axillary or apical shoot meristems with the polyploidization agent in the medium (Kermani et al., 2003; Ma et al., 1997). Primary site of absorbance of colchicine or oryzalin into the meristem and direction of polyploidization through characterization of each meristematic layer has not been reported in these studies. In an effort to identify LII polyploids, perhaps removing LII diploids by first screening for polyploidy in LI would be less effective using *in vitro* polyploidization methods.

Although the *in vitro* rose polyploidization experiments of Ma et al. (1997) using colchicine and Kermani et al. (2003) using oryzalin utilize different germplasm and treatment methods, the rate of recovered polyploids from treated meristems among their best treatments

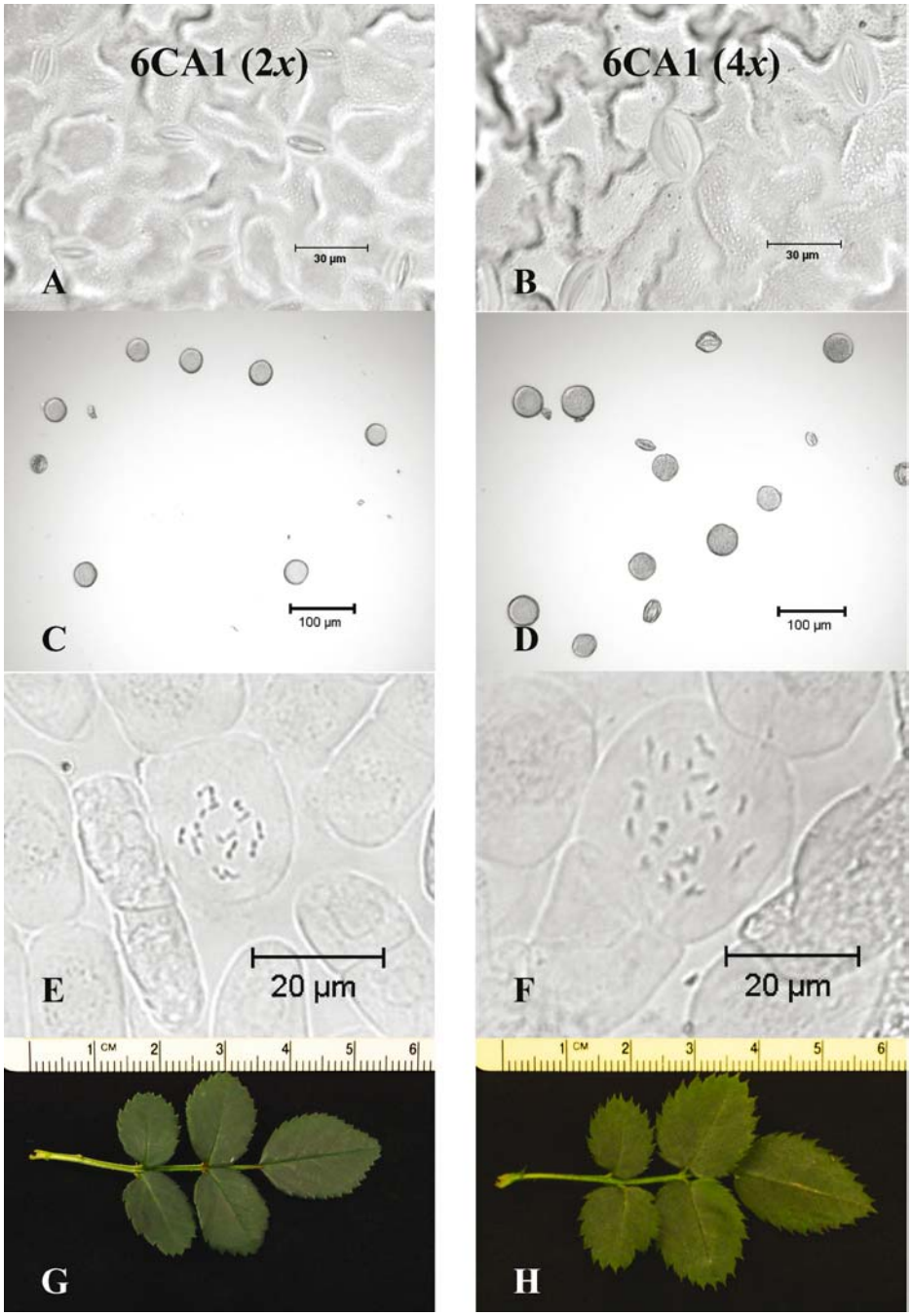

Figure 3. Stomatal imprints (A & B), pollen (C & D), mitotic root tip cells (E & F), and leaves (G & H) of diploid *R. chinensis minima* seedling 6CA1 (2*x*) and its trifluralin-induced tetraploid, 6CA1 (4*x*).

(≤20%) are generally comparable and may slightly favor oryzalin. Eeckhaut et al. (2004) found that oryzalin (10%B5M) and trifluralin (10%B5M) were comparable for polyploidization frequency and induced more polyploids than colchicine (100%B5M) in *Spathiphyllum wallisii*, however, treatment methods differed with colchicine supplied in liquid media for 16 hours and the herbicides supplied in solid media for six week. In

the current study both the trifluralin treatments induced more polyploids than colchicine (0.5%). Possible explanations include 0.5% may not be the optimum concentration of colchicine for polyploidization of rose seedlings, although it is what has been used by others (Basye, 1990), or that trifluralin is a more efficient polyploidization agent under the conditions imposed by this study. In addition, it is unclear why there was significantly greater seedling mortality within the water treatment than the colchicine treatment.

Although leaflet L/W was not a reliable predictor of polyploidization among segregating seedlings in this study due to overlapping diploid and polyploid ranges, leaflet L/W has been a helpful tool in the identification of induced polyploids from originating diploid and triploid clones (Kermani et al., 2003; Ma et al., 1997). In this study leaflet L/W proved useful for ploidy classification among polyploid genotypes and diploid plants derived from adventitious shoots arising from below treated tissue. The two members of each of the four polyploid/diploid seedling sets were significantly different for this ratio (Table 7).

The effects of polyploidization on phenotypic traits for clones is difficult to predict. Kermani et al. (2003) found significantly more or less petals and significantly shorter or longer internodes within induced polyploids relative to the original clone among half the original clone/induced-polyploid comparisons. If polyploidization effects are deleterious, especially in the case of fertility, using the newly generated polyploids in breeding may be challenging. In the current study, for instance, polyploidization of LII significantly reduced pollen stainability, a common occurrence when generating autopolyploids (Eigsti & Dustin, 1957). This study generated 17 LII polyploids with only two having $\geq$61% stainable pollen. If autopolyploids were generated using multiple axils of a few superior *R. chinensis minima* clones, there would only be those few genotype $\times$ polyploidization interactions to assess and from which to find individuals possessing relatively high fertility.

Genetic variability among seedling populations used for polyploidization, relative to using multiple axillary meristems of typically few diploid clones, has a number of advantages. Genetic variability among polyploid individuals allows for an increased probability of finding genotypes that facilitate gene transfer through higher fertility and combining ability with other parents, or even induced polyploids that may be useful as cultivars. One of the strongest arguments for using diploid clones proven to be superior parents for polyploidization is the expectation that they will continue to be superior parents and produce superior progeny with predictable phenotypic distributions after polyploidization. This expectation may be tenuous when considering the effect polyploidization can have on fertility, Kermani et al. (2003) finding unpredictable phenotypic changes among clones in response to polyploidization, and the complexity of polyploid genetics. On the other hand, each germinated rose seedling is a new genotype with an unknown breeding value and phenotype. Testing the hypothesis that superior diploid clones tend to produce superior tetraploid parents and is more efficient for gene transfer and cultivar development relative to the same amount of resources invested in polyploidization of genetically comparable seedling populations is worthy of investigation.

Widespread identification of 2*n* gamete production among rose genotypes would allow for new possibilities to study and maximize heterosis and epistasis in breeding programs. Higher quality progeny with greater vigor has been obtained using meiotic versus somatic polyploidization in potatoes, another primarily outbreeding species that benefits from heterozygosity (Tai & DeJong, 1997). This study unexpectedly identified two diploids (4AB1 and 4BA3) which consistently produced 2*n* pollen; $\sim$30% according to pollen diameter and $<$10% according to frequencies of dyads, triads, and tetrads (Table 4). Obtaining variable frequencies of 2*n* pollen across samples of 2*n*-pollen producing genotypes is common (Zlesak & Thill, 2002).

When using somatic polyploidization as a tool to aid a breeder in crop improvement, there are a few general considerations that need to be addressed to maximize success. They include choices regarding which germplasm to invest in, if germinating seedlings or meristems of selected clones will be used, if polyploidization will occur *in vitro* or *ex vitro*, and which polyploidization agent will be used. Optimally, germplasm is available which possesses multiple traits of interest for introgression along with as few undesirable traits as possible to minimize drag. In addition, it would be valuable to obtain polyploids with relatively high fertility to more easily generate progeny, an outcome which tends to be more common when generating allopolyploids than autopolyploids (Basye, 1990; Eigsti & Dustin, 1957). What route a breeder chooses is made weighing breeding objectives with available resources of germplasm, labor, supplies, and time. We propose that breeders consider the advantages of generating polyploids by using trifluralin on germinating seedlings due to the widespread availability of

trifluralin (Tomlin, 1997), relatively high rates of polyploids that can be recovered, and because tissue culture facilities are not necessary.

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