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Autotetraploid Induction of *Cercis canadensis* by Oryzalin and Colchicine Agar Drop Technique

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Abstract. **Eastern redbud, *Cercis canadensis* L., is a deciduous flowering tree species with great ornamental value. In 2019, redbuds had an estimated economic value of $28.4 million in the United States, with *C. canadensis* comprising the majority of this market. Ploidy manipulation is a powerful tool in ornamental plant breeding for developing cultivars with novel traits; however, no studies of polyploidy induction in *C. canadensis* have been reported to date. To develop a genome doubling protocol, two mitotic inhibitors—oryzalin and colchicine—were applied to *C. canadensis* seedlings using the agar drop method, with varying concentrations and treatment durations. The ploidy levels of surviving plants were evaluated using flow cytometry. In the first experiment, oryzalin treatments yielded a tetraploid conversion rate three-times higher than that of colchicine treatments, despite colchicine being applied at concentrations 100-times greater. In the second experiment, increasing concentrations of oryzalin (150, 300, and 450 µM) did not significantly affect polyploid induction, and no statistically significant differences were observed in either survival or tetraploid conversion ratios. In the third experiment, extending treatment duration led to increased frequencies of tetraploid and mixoploid seedlings, although the increase in tetraploid conversion ratio was not statistically significant. Among all treatments, the 150 µM oryzalin treatment applied for 9 days yielded the optimal results: a 55.15% survival rate and 15.38% of surviving seedlings confirmed as tetraploid. This study presents the first established protocol for polyploidy induction in *C. canadensis* and offers a valuable tool for breeding novel cultivars of eastern redbuds and redbud species.**

Cercis L. is an early diverging genus in the family Fabaceae consisting of eight accepted species of small trees and shrubs distributed throughout the northern hemisphere (Govaerts 2023). *Cercis canadensis* ($2n = 2x = 14$) is grown worldwide as an ornamental crop for its precocious spring flowers, colorful heart-shaped foliage, various architecture, and wide adaptability. In the United States, the crop has a total economic value of $28.4 million (US Department of Agriculture, National Agricultural Statistics Service 2019) according to the 2019 horticultural specialty crops census. The majority of these sales are attributed to cultivars of *C. canadensis* L. and *C. chinensis* Bunge. As of 2018, 36 cultivars of *C. canadensis* were registered (Kidwell-Slak and Pooler 2018), and many notable cultivars have been released since then. *Cercis* cultivars have experienced rapid popularization in the US and European markets in the last few decades because of advances in valuable traits such as foliage colors, weeping forms, double flowers, and dwarf forms. Its popularity can also be attributed to its wide adaptability, with an example being its ability to handle conditions and soils (Griffin et al. 2004). Two subspecies of *C. canadensis*, *C. canadensis* var. *texensis* and *C. canadensis* var. *mexicana*, have been incorporated into hybrids to improve heat and drought adaptability and novel leaf surface structures. The increase in awareness of the importance of and demand for native ornamental plants has also contributed to the success of *C. canadensis* as a major nursery crop in recent years. Continued development of advanced *C. canadensis* cultivars building on previous successes in breeding efforts for this group will be essential.

The occurrence of polyploidy in plants is a fascinating driver of plant speciation (Alix et al. 2017) and has broad application in plant improvement (Ranney 2006). Polyploidy can bring about physiological and morphological changes in plants (Doyle 2012), which usually result in novel visible traits or changes in environmental adaptable ranges (Perera-Castro et al. 2023; Saleh et al. 2008). A commonly noted effect of increased ploidy is the enlargement of cells and organs, which is associated with traits preferred in many ornamental crops (Lutz 1907; Tupper and Bartlett 1916). Because polyploid plants have duplicated alleles, both gene expression and regulatory mechanisms can change, often leading to new phenotypic variations (Adams and Wendel 2005; Chen 2007). It has been frequently reported that ploidy changes influence key ornamental traits in nursery crops, including leaf thickness, flower size, petal structure, flower color and intensity, bloom timing, and plant compactness (Chen and Contreras 2022; Fetouh et al. 2016; Manzoor et al. 2019; Olsen 2007). Another important effect of polyploidy is the emergence of new chromosomal pairing patterns, which often affect fertility (Ranney 2006). In some cases, these changes restore chromosome pairing and fertility, enabling successful hybridization. Furthermore, polyploidy, with additional sets of chromosomes, allows for greater allelic variation compared with diploids. For instance, the cultivated sweetpotato (*Ipomoea batatas*, a hexaploid; $2n = 6x = 90$) can contain loci with more than two allele types and complex dosage combinations (Zhao et al. 2024). Such genetic flexibility increases variation and enhances breeders' ability to generate diverse phenotypes. The applications of polyploidy manipulation have great value for the cultivar improvements of the essential ornamental crop, *C. canadensis*.

Limited known ploidy-level diversity among *Cercis* cultivars exists. Ploidy-level diversity is commonly found in many asexually propagated ornamental crops, such as hibiscus (Chen and Contreras 2022; Lattier et al. 2019), roses (Harmon et al. 2023), lilacs (Lattier and Contreras 2017), vacciniums (Redpath et al. 2022), and camellias (Hembree et al. 2019). However, no ploidy-level diversity was reported among cultivars of *Cercis canadensis*, a valuable crop in the horticultural industry (Roberts and Werner 2016). A genome size and ploidy survey covering 30 *Cercis* taxa with 18 *C. canadensis* cultivars found that all tested taxa had diploid genome sizes ranging from 0.70 to 0.81 pg (Roberts and Werner 2016). No research of ploidy manipulation for this important nursery crop has been reported. The absence of polyploidy in the *Cercis* taxa is unusual given the widespread occurrence of polyploidy in family Fabaceae and the fact that all other genera in the tribe Cercidoideae exhibit polyploidy (Doyle 2012). Because the ploidy changes, such as sterility, flower size, leaf characters, and environmental adaptabilities (Touchell et al. 2020), frequently contribute to the ornamental value, the development of tetraploid *C. canadensis* could lead to breeding breakthroughs. One straightforward approach for manipulating ploidy in the group would be the first step of developing seedless triploid cultivars, because seed pods are viewed as unattractive by some, and stopping the tree from seeding into landscapes.

Over the years, various mitotic inhibitor application methods have been used in plant breeding to artificially induce polyploidy. Protocols can be developed in tissue culture,

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in vitro environments (Jones et al. 2008; Nadler et al. 2012; Touchell et al. 2020), or ex vitro environments (Crawford et al. 2021; Suppa et al. 2024). In vitro approaches are thought to result in fewer cytochimeras and greater efficiency because of improved chemical uptake and nutrient support to plant tissues. Ex vitro methods, however, offer the advantage of not requiring tissue culture protocols and typically involve lower labor demands. To prevent excessive damage to whole plant tissues—especially nonmeristematic regions—ex vitro approaches must be designed so that mitotic inhibitors target only the meristem for a controlled duration. Typically, ex vitro techniques apply mitotic inhibitors to the meristem of young seedlings—often located between the cotyledons of newly germinated seeds (Lehrer et al. 2008; Madon et al. 2005; Suppa et al. 2024)—or to internode tissues (Crawford et al. 2021; Teng and Leonhardt 2009). The agar drop technique, which is a commonly used method, delivers mitotic inhibitor-infused agar directly to the seedling meristem (Contreras and Hoskins 2020). The technique is especially effective when a receptive surface exists between the cotyledons to hold the agar drop.

Knowledge of ploidy manipulation methods for *Cercis* is limited. No genome doubling protocol is currently available for *C. canadensis* to support cultivar development; however, two studies have reported genome doubling in other *Cercis* species. In *C. siliquastrum* genome doubling research, seeds were treated with aqueous colchicine, and putative tetraploids were identified based on morphological traits without further cytogenetic confirmation (Suliman and Asander 2019). Because of the lack of a standard genome size or ploidy measurement method, this method is difficult to repeat in related species. In *C. glabra* genome doubling research, aqueous treatment of shoot cultures grown in vitro was used, and the approach relies on a mature tissue culture system for the plant material.

Efficient methods of polyploid manipulation for *C. canadensis* have great potential in the development of cultivars with novel traits. To develop a genome doubling protocol, mitotic inhibitor types, chemical concentration, and time duration of the treatment on diploid seedlings were tested in this study. The ploidies of treated plants were analyzed by flow cytometry and chromosome counting. The results recommend the optimal protocol for agar drop on *C. canadensis* seedlings for developing tetraploids.

Materials and Methods

Plant material. Mature seeds of seven *C. candensis* families were used. The accession number and family information of seeds included in this research are listed in Table 1. Because *Cercis canadensis* is a self-incompatible species, the F_2 populations were generated by bulk pollination among F_1 siblings (Roberts and Werner 2016). A modified seed scarification treatment was used (Chen and Werner 2021). Harvested seeds were stored at 4 °C. Stored seeds were scarified by sulfuric acid treatment. The dry seeds were submerged in concentrated (18.4 M) sulfuric acid for 30 min, gently stirred briefly every 2 min for the first 10 min, and then gently stirred briefly every 5 min. Acid-scarified seeds were rinsed with water and mixed with wet perlite for 6 to 8 weeks for stratification. Seed sowing methods and mitotic inhibitor agar drop treatments were addressed in each experiment (Fig. 1).

Expt. 1: mitotic inhibitors test. The agar drop method was used on seedlings of two redbud families. Seeds were sown in 8-× 5-inch nursery pots filled with Sun Gro Metro Professional Growing Mix (Sun Gro Horticulture, Anderson, SC, USA). A tent with increased humidity and 70% shadecloth was erected in a greenhouse (Raleigh, NC, USA). The seeds of H2021-001 and *C. canadensis* 'Floating Clouds' OP families were sown. These seedlings were assigned to agar drop treatments of water control, 10 mM colchicine, 30 mM colchicine, 100 μM oryzalin, and 300 μM oryzalin, each with 0.3% agar. A volume of approximately 50 to 100 μL agar drop was applied to each seedling, depending on the maximum capacity of the space between the cotyledons. A total of 562 seedlings were treated (Table 2). All drops were made using distilled water and 0.3% agar that was heated to melt it; mitotic inhibitors were added after the agar buffer had heated and before solidifying. The oryzalin was sourced from the Surflan preemergent herbicide Weed Impede® (40.4% active ingredient oryzalin, <30% glycerin), with other ingredients not disclosed. The colchicine was sourced from Product C226 (PhytoTech Laboratories, Lenexa, KS, USA). The seedlings were adjusted so that all plants received the first drop at a similar meristematic stage when no true leaves had begun to emerge. Plants were monitored so that no new seeds could germinate and grow after treatment. Drops were applied to plants in all treatments and maintained for the following 72 h (∼3 d); this required six sessions of drops in the humidity setup in which this was performed. At the end of the treatments, plants were gently and thoroughly washed to remove the residue of the mitotic inhibitors and agar. Plants were given 30 d (∼4.5 weeks) before survivability data were taken. At 30 d after the treatment, plants were classified as surviving if the treated meristem was growing and true leaves had emerged. Plants that had died or ceased growing for 30 d were classified as dead. Surviving plants were given an additional 2 months to recover before the ploidy analysis by flow cytometry.

Expt. 2: oryzalin concentration tests. The oryzalin drop method was selected for treating seedlings of H2020-01, H2020-07, and H2020-09 redbud families. The stratified seeds were sown and grown in an incubator at 25 °C with a 16-h photoperiod to maintain a consistent climate for this experiment. The seeds of each family were divided into six 8- × 5-inch nursery pots. To maintain humidity, plastic drainage saucers were placed on top of the bulb pans (Fig. 1). The treated seed number, survivorship, and ploidy of each seedling were recorded. The drop treatments consisted of water control, 150 μM oryzalin, 300 μM oryzalin, and 450 μM oryzalin. All drops were made using distilled water and 0.3% agar. Only seedlings at the early meristematic stage, when cotyledons were just open and no true leaves had emerged, were used to receive the drop treatment. Seedlings that germinated too early (where true leaves had already appeared) were removed. Any seedlings that germinated after treatment with agar drops had begun were removed. A total of 245 seedlings were treated (Table 3). Drops were applied daily on each seedling across all treatments and maintained for the following 72 h. At the end of the treatment, seedlings were thoroughly washed to remove the residue of the mitotic inhibitors and agar. The number of surviving plants was recorded 30 d after the treatment finished, and the ploidy of each surviving plant was analyzed 2 months after the treatment finished.

Expt. 3: treatment duration tests. The 150 μM oryzalin agar drop method was selected for treating seedlings of H2024-136 and *C. canadensis* 'Covey' OP seed families. The treatment conditions were similar to those of Expt. 2. Seedlings were treated with 150 μM oryzalin for 3, 6, and 9 d, and nontreated seedlings were used as a 0-d control group. A total of 460 seedlings were used in this experiment.

Flow cytometry. For each surviving seedling, two 0.28-cm^2 discs of true leaf tissue obtained using a hole puncher were chopped using a razor with 200 μL of nuclei extraction buffer (Cystain ultraviolet Precise P Nuclei Extraction Buffer; Sysmex, Görlitz, Germany). The chopped sample was filtered using a 50-μm gauge filter (Celltrics; Sysmex America Inc., Lincolnshire, IL, USA) and collected in a 3.5-mL plastic tube (Sarstedt Ag & Co., Nümbrecht, Germany); then, 1000 μL of stain buffer (Cystain ultraviolet Precise P Staining Buffer) was added to the tube. Then, the nuclei were analyzed using a flow cytometer (Quantum P Ploidy Analyzer; QuantaCyte, Lincolnshire, IL, USA). The ploidy of each seedling was analyzed twice using new tissue to further confirm the ploidy observed. Diploid *C. canadensis* and the internal reference standard *Pisum sativum* 'Ctirad' were used to set peak placement. Seedlings were run independently, and putative ploidy was determined by the placement of the peaks.

Chromosome counts. Chromosome counts were conducted to confirm the relationship between genome size and ploidy. The protocol is modified from a rose chromosome counting method (Harmon et al. 2023). A flowcytometry estimated diploid H2020-001 seedling and a flowcytometry estimated tetraploid H2020-009 seedling were used to represent each estimated ploidy. Five to 10 young shoot tips with a size of 0.5 cm from each seedling were collected in Spring 2025. Tissue was directly placed in microcentrifuge vials with 2 mM 8-hydroxyquinoline and 70 $mg \cdot L^{-1}$ cycloheximide buffer at room temperature for 3 h and then transferred to a refrigerator overnight before being transferred to Farmer's fixative

Table 1. Plant materials and pedigree information of *Cercis canadensis* seedlings used in each experiment and the experiment treatment summary.

Seed family used	Expt.[i]	Parent A	Parent B	Affinity
H2021-001	Expt. 1: mitotic inhibitors	NC 2014-10[i]	NC2016-8	F2
Floating Clouds OP	Expt. 1: mitotic inhibitors	Floating Clouds	Open-pollinated	OP
H2020-001	Expt. 2: oryzalin conc.	NC2016-2	NC2014-5	F2
H2020-007	Expt. 2: oryzalin conc.	Ruby Falls	Florida wild collection	F2
H2020-009	Expt. 2: oryzalin conc.	Oklahoma	NC 2014-10[ii]	F2
H2024-136	Expt. 3: duration	Oklahoma	Ace of Hearts	F2
Covey OP	Expt. 3: duration	Covey	Open-pollinated	OP

[i]Expt. 1: control and two colchicine concentrations and two oryzalin concentration agar drop treatments; Expt. 2: three oryzalin concentration agar drop treatments; Expt. 3: control and three durations of 150 μM oryzalin agar drop treatment.
[ii] An F2 plant of 'Texas White' × Hearts of Gold.

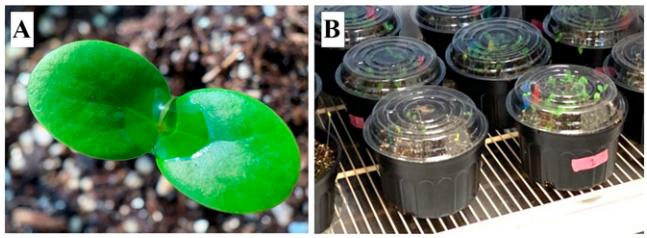

Fig. 1. Agar drop method mitotic inhibitor treatment on *Cercis canadensis* seedlings. (**A**) One drop of semi-solid agar (0.3%) containing mitotic inhibitor types and concentration was applied to the seedling apical meristem. (**B**) Seedlings in a growth chamber incubator environment for the agar drop treatment. Treated seedlings were grown in a growth chamber with the pot covered by drainage dishes to maintain humidity.

solution containing 95% ethanol and glacial acetic acid (3:1 v/v) and maintained at 4 °C until further processing. Fixed tissues were rinsed with deionized water for 1 min three times before the enzyme treatment. In the enzyme treatment, terminal meristem tissue (≤0.2 cm) of one shoot was collected and placed on a microscope slide with 50 μL of a cell wall digestive enzyme, 6% pectinase (Sigma Chemical; St. Louis, MO, USA), and 6% cellulase (Onozuka R-10; Yakult Honsha, Tokyo, Japan) in 75 mM KCl buffer (pH 4.0). The slides were placed in an enclosed container with moistened paper towels and incubated at 37 °C for 5 h after enzyme treatment. The treated meristem tissue was broken up using a small spatula to spread the macerated tissue evenly on the slides. An additional drop of the modified fixative buffer containing 95% methanol and glacial acetic acid (3:1 v/v) was added on each corner of the slide before using an alcohol lamp to ignite the modified fixative buffer and macerated tissue mix on the slide. Other steps, including after-treatment, DAPI stain, and microscopy work, were identical to the rose protocol (Harmon et al. 2023).

Statistics and ratio comparisons. To evaluate the effect of treatments, survival ratios (surviving seedling number/treated seedling

Table 2. Expt. 1 with mitotic inhibitor agar drop treatments in *Cercis canadensis* seedling genome doubling test results.

Seedling family	Treatment	Treated (*n*)	Surviving (*n*)	Survival ratio[iii]	Mixoploid (*n*)	Tetraploid (*n*)	4× Conversion ratio[i, iii]	Affected ratio[ii,iii]
H2021-001	Control	42	41	97.62%^{A}	0	0	0.00%^{A}	0.00%^{A}
H2021-001	10 mM Colchicine	46	43	93.48%^{A}	2	2	4.65%^{AB}	9.30%^{AB}
H2021-001	30 mM Colchicine	52	40	76.92%^{B}	3	1	2.50%^{AB}	10.00%^{AB}
H2021-001	100 μM Oryzalin	58	22	37.93%^{C}	4	2	9.09%^{BC}	27.27%^{AB}
H2021-001	300 μM Oryzalin	46	22	47.83%^{C}	5	3	13.64%^{BC}	36.36%^{BC}
Floating Clouds OP	Control	54	51	97.73%^{A}	0	0	0.00%^{A}	0.00%^{A}
Floating Clouds OP	10 mM Colchicine	80	75	94.56%^{A}	0	2	2.67%^{AB}	2.67%^{AB}
Floating Clouds OP	30 mM Colchicine	67	55	82.26%^{B}	5	4	7.27%^{BC}	16.36%^{BC}
Floating Clouds OP	100 μM Oryzalin	65	22	33.76%^{C}	5	2	9.09%^{BC}	31.82%^{BC}
Floating Clouds OP	300 μM Oryzalin	52	18	33.47%^{C}	1	3	16.67%^{C}	22.22%^{C}
Total	Control	96	92	95.83%^{a}	0	0	0%^{a}	0%^{a}
Total	10 mM Colchicine	126	118	93.65%^{a}	2	0	3.39%^{a}	5.08%^{a}
Total	30 mM Colchicine	119	95	79.83%^{b}	8	4	5.26%^{ab}	13.68%^{b}
Total	100 μM Oryzalin	123	44	35.77%^{c}	9	5	9.06%^{ab}	29.55%^{c}
Total	300 μM Oryzalin	98	40	40.82%^{c}	6	6	15.00%^{c}	30.00%^{c}

[i]The ratio of seedlings converted to the tetraploid condition out of the surviving seedlings.
[ii] The ratio of seedlings converted to either the tetraploid or mixoploid condition out of the surviving seedlings, indicating a doubling effect.
[iii] Tukey's honestly significant difference for the multiple ratio comparisons analysis after the binomial analysis of variance (ANOVA) ($P \leq 0.05$). Different characters indicate the statistical differences in ratios across treatments. Uppercase and lowercase letters indicate a separate independent binomial ANOVA analysis of multiple ratio comparisons.

Table 3. Expt. 2 with oryzalin concentration agar drop treatments in *Cercis canadensis* seedling genome doubling test results.

Seedling family	Oryzalin (μM)	Treated (*n*)	Surviving (*n*)	Survival ratio[iii]	Mixoploid (*n*)	Tetraploid (*n*)	4× Conversion ratio[i,iii]	Affected ratio[ii,iii]
2020-001	150	30	21	70.00%	4	3	14.29%	33.33%
2020-001	300	26	13	49.40%	4	2	15.38%	46.15%
2020-001	450	33	20	60.11%	1	2	10.00%	15.00%
2020-007	150	35	20	57.35%	4	4	20.00%	40.00%
2020-007	300	30	14	45.70%	4	2	14.29%	42.86%
2020-007	450	50	25	47.86%	1	1	4.00%	8.00%
2020-009	150	9	6	65.00%	1	1	16.67%	33.33%
2020-009	300	15	10	66.67%	2	1	10.00%	30.00%
2020-009	450	17	11	68.94%	4	0	0.00%	36.36%
Total	150	74	47	64.12%	9	8	17.02%	36.17%
Total	300	71	37	53.92%	10	5	13.51%	40.54%
Total	450	100	56	58.97%	6	3	5.36%	16.07%

[i]The ratio of seedlings converted to the tetraploid condition out of the surviving seedlings.
[ii] The ratio of seedlings converted to either the tetraploid or mixoploid condition out of the surviving seedlings, indicating a doubling effect.
[iii] Binomial analysis of variance showed that there is no statistically significant treatment effect of oryzalin on the ratios of survival ratio, tetraploid conversion ratio, and affected ratio ($P > 0.05$); therefore, Tukey's honestly significant difference for the multiple ratio comparisons analysis was not applied.

number), tetraploid conversion ratios (tested tetraploid seedling number/surviving seedling number), and effected ratios (sum of tested tetraploid and mixoploid seedling number/surviving seedling number) were compared using an analysis of variance with "treatments" as a fixed effect in each experiment, and the data were modeled using a binomial distribution with a logit function to account for the proportional nature of the response. While treatment effects showed significance, Tukey's honestly significant difference-style group was used in pairwise comparisons. Treatments were assigned group letters (e.g., a, b, c); treatments sharing the same letter were not significantly different ($P < 0.05$) (Tables 2–4).

Results

Expt. 1: mitotic inhibitors test. The survival ratios, tetraploid conversion ratios, and the effect ratios of the mitotic inhibitors test are listed in Table 2. The average survival ratios of the control, 10 mM colchicine, 30 mM colchicine, 100 μM oryzalin, and 300 μM oryzalin were 95.83%, 93.65%, 79.83%, 35.77%, and 40.82%, respectively, and the average tetraploid conversion ratios were 3.39%, 5.26%, 9.06%, and 15.00%, respectively. Overall, oryzalin treatment showed lower survival ratios and a higher tetraploid conversion ratio than those of colchicine. The 300 μM oryzalin treatment resulted in six tested tetraploid plants from 40 surviving plants. The survival ratio of 100 μM oryzalin treatment showed a lower survival ratio than that of 300 μM oryzalin treatment (not statistically different). In comparison, treatment with 300 μM oryzalin showed a significantly higher tetraploid conversion ratio. A similar result was also found in the effect ratios among the treatments, while the two concentrations of oryzalin showed no statistical difference. The results revealed that oryzalin showed a better genome doubling effect than colchicine, while the concentration of colchicine was approximately 100-times higher than that of oryzalin. The results of this study determined that the following studies would be performed with oryzalin.

Expt. 2: oryzalin concentration tests. The results of the three oryzalin concentration agar drop tests are listed in Table 3. The average survival ratios of the 150 μM, 300 μM, and 450 μM oryzalin treatments were 64.12%, 53.92%, and 58.97%, respectively. The tetraploid conversion ratios of the 150 μM, 300 μM, and 450 μM oryzalin treatments were 17.02%, 13.51%, and 5.36%, respectively. The conversion tetraploid ratios decreased as oryzalin concentration increased, but they were not statistically different. The result indicated that increasing the oryzalin concentration might not improve the tetraploid conversion ratio. The results of this study determined that the following studies would not be performed with increasing oryzalin concentration.

Expt. 3: treatment duration tests. The results of the three durations of the 150 μM oryzalin agar drop treatment test are listed in Table 4. The average survival ratios of the control, 3-d, 6-d, and 9-d treatments were 89.83%, 76.12%, 60.87%, and 55.15%, respectively. The average tetraploid conversion ratios of the control, 3-d, 6-d, and 9-d treatments were 0%, 12.75%, 12.24%, and 15.38%, respectively. The average affected ratios of the control, 3-d, 6-d, and 9-d treatments were 0%, 33.33%, 39.80%, and 49.45%, respectively. Although the survival ratios showed a statistically significant reduction with an elongated treatment duration, the tetraploid conversion did not show a statistically significant difference. However, the 9-d treatment showed the highest effect ratio, with 49.45% of surviving plants becoming mixoploid or tetraploid after 9 d of treatment.

Flow cytometry and chromosome count. The chromosome count result matched the flow cytometry result (Fig. 2). A flow cytometry-tested diploid H2020-001 seedling showed 14 chromosomes in metaphase ($2n = 2x = 14$). In addition, a flow cytometry-tested diploid H2020-009 seedling showed 28 chromosomes in metaphase ($2n = 4x = 28$). The cytology results confirmed that the ploidy estimation was performed with flow cytometry.

Discussion

Polyploid induction in C. canadensis. In Expt. 1, the study investigated the effects of oryzalin and colchicine treatments on the survivorship and genome doubling rate of *C. canadensis* seedlings. The results (Table 2) showed that oryzalin had a more substantial effect on genome doubling and reduced survivorship compared with colchicine. In this *C. canadensis* seedling mitotic inhibitor test, the two most commonly used chemicals, colchicine and oryzalin, were compared. In contrast, 100-times concentrated colchicine (10 mM and 30 mM) was used for comparisons with oryzalin (100 μM and 300 μM). As a result, the two oryzalin treatments significantly reduced survival ratios and increased genome doubling event ratios. The 300 μM oryzalin showed the most optimal results and had the highest tetraploid conversion ratio compared with all other treatments. Oryzalin with better polyploid induction is also found in other plants; for example, the mitotic inhibitor treatments on pregerminated *Berberis thunbergii* seeds (Ebrahimzadeh et al. 2018; Lehrer et al. 2008) on common cherry laurel (*Prunus laurocerasus*) and apical meristems (Schulze and Contreras 2017) as well as on haploid cucumber (*Cucumis sativus* L.) embryo (Ebrahimzadeh et al. 2018) and seedling meristem of *Hibiscus acetosella* 'Panama Red' (Contreras et al. 2009).

However, the sensitivities to colchicine and oryzalin for genome doubling can be species-specific. In a study that induced autotetraploids of three wild peanut species (*Arachis* spp), seedlings showed more colchicine sensitivity, and tetraploids from oryzalin treatments frequently reverted to diploids. In contrast, tetraploids received from colchicine showed much more stability (Suppa et al. 2024). Research of hemp (*Cannabis sativa*) also showed a higher affected ratio and a higher tetraploid conversion ratio in colchicine treatments (McLeod et al. 2023). In most cases, the survival ratio is significantly negatively correlated to the polyploid induction ratio;

Table 4. Expt. 3 with treatment duration tests of the 150 μM Oryzalin agar drop treatment for *Cercis canadensis* seedling genome doubling test results.

Seedling family	Period (d)	Treated (*n*)	Surviving (*n*)	Survival ratio[iii]	Mixoploid (*n*)	Tetraploid (*n*)	4× Conversion ratio[i,iii]	Affected ratio[i,ii]
H2024-136	0	89	83	93.26%	0	0	0.00%	0.00%
H2024-136	3	95	70	73.68%	17	10	14.29%	38.57%
H2024-136	6	101	59	58.42%	16	9	15.25%	42.37%
H2024-136	9	33	49	54.44%	14	9	18.37%	46.94%
Covey OP	0	29	23	79.31%	0	0	0.00%	0.00%
Covey OP	3	39	32	82.05%	4	3	9.38%	21.88%
Covey OP	6	60	39	65.00%	11	3	7.69%	35.90%
Covey OP	9	75	42	56.00%	17	5	11.90%	52.38%
Total	0	118	106	89.83%^{A}	0	0	0.00%^{A}	0.00%^{A}
Total	3	134	102	76.12%^{A}	21	13	12.75%^{B}	33.33%^{B}
Total	6	161	98	60.87%^{B}	27	12	12.24%^{B}	39.80%^{B}
Total	9	165	91	55.15%^{C}	31	14	15.38%^{B}	49.45%^{C}

[i] The ratio of seedlings converted to the tetraploid condition out of the surviving seedlings.

[ii] The ratio of seedlings converted to either the tetraploid or the mixoploid condition out of the surviving seedlings, indicating a doubling effect.

[iii] A binomial analysis of variance showed no significant treatment effect of each family and duration combination on the ratios ($P > 0.05$), but duration (combined data of all family) showed a significant effect on the conversion ratio and an effect on the ratio ($P < 0.05$). Different characters indicated the statistical differences in ratios across the treated period.

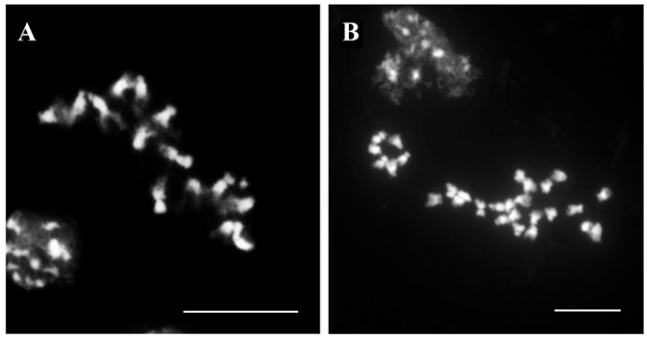

Fig. 2. Visualization of chromosomes of flow cytometry-tested diploid and tetraploid *Cercis canadensis* seedlings. (**A**) A metaphase cell of a diploid H2020-001 seedling ($2n = 2x = 14$). (**B**) A metaphase cell of a tetraploid H2020-009 seedling ($2n = 4x = 28$). Bar = 10 μm.

therefore, using the survival ratio to screen mitotic inhibitor sensitivity can be an efficient method before the concentration and duration treatment test (Suppa et al. 2024). The results of Expt. 1 concluded that *C. canadensis* seedlings have a better tetraploid conversion ratio in oryzalin treatments than in colchicine treatments.

Building off Expt. 1, Expt. 2 investigated the effects of oryzalin concentrations on survivorship and genome tetraploid conversion ratios. The results showed that an increased oryzalin concentration in the agar drop treatment did not increase the tetraploid induction ratio (Table 3). Usually, increasing mitotic inhibitor concentrations result in increased lethal and polyploid conversion ratios, for example, *Berberis thunbergii* (Lehrer et al. 2008) and *Populus* (Zeng et al. 2019). An explanation for the consistency in responses between the concentrations tested could be found in the saturation point of oryzalin. The 150 μM treatment is approximately 51 ppm of oryzalin, which is well past this saturation point. When considering the agar drop, it is more of a supersaturated suspension than a supersaturated solution in which the oryzalin precipitates out, leaving only a saturated solution at 7.2 μM (2.5 ppm) of oryzalin (US Environmental Protection Agency 2011). This would mean that treatments beyond the saturation point are effectively similar in the concentration that interacts with the plant cells. This explains why 150 μM, 300 μM, and 450 μM responded with similar survivorship and tetraploid conversion ratios. These findings have important implications for the development of a protocol for generating polyploid *C. canadensis* genotypes. The observed differences in the conversion rate and event rate among the different oryzalin concentrations can guide the selection of optimal treatment conditions for inducing genome doubling in *C. canadensis* seedlings. The results of Expt. 1 and Expt. 2 suggest that a rate ranging from 150 to 300 μM will be similarly effective at generating polyploid *C. canadensis*, and the 450 μM treatment resulted in lower polyploid rates.

The duration of the elongated oryzalin treatment showed positive effects on reducing the surviving ratio and increasing polyploid inductions; however, some potential improvements remain possible. Expt. 3 showed generally increased tetraploid and mixoploid conversion ratios when the treatment duration increased. Although not statistically significant, the longest duration treatment (9 d) showed the highest tetraploid conversion ratios in the two tested seedling families. A total of 15.38% of surviving seedlings tested as tetraploids. In addition, the affected ratio [(tetraploid + mixoploid number)/surviving seedling number] was 49.45%, which was the highest affected ratio among all treatments in the three experiments. An increased treatment duration might result in an improved polyploid induction ratio, but it could also further decrease survivability in seedlings. Considering the total survival ratios of the control and 9-d treatment were 89.83% and 55.15%, the 9-d treatment only reduced the survival ratio 38%; however, most mutagenesis research or polyploid induction research targets treatment that reduces the survival ratio 50% to receive the optimal result. A lower survival rate could lead to a higher conversion rate with fewer treated seedlings to test further, thus optimizing the protocol.

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