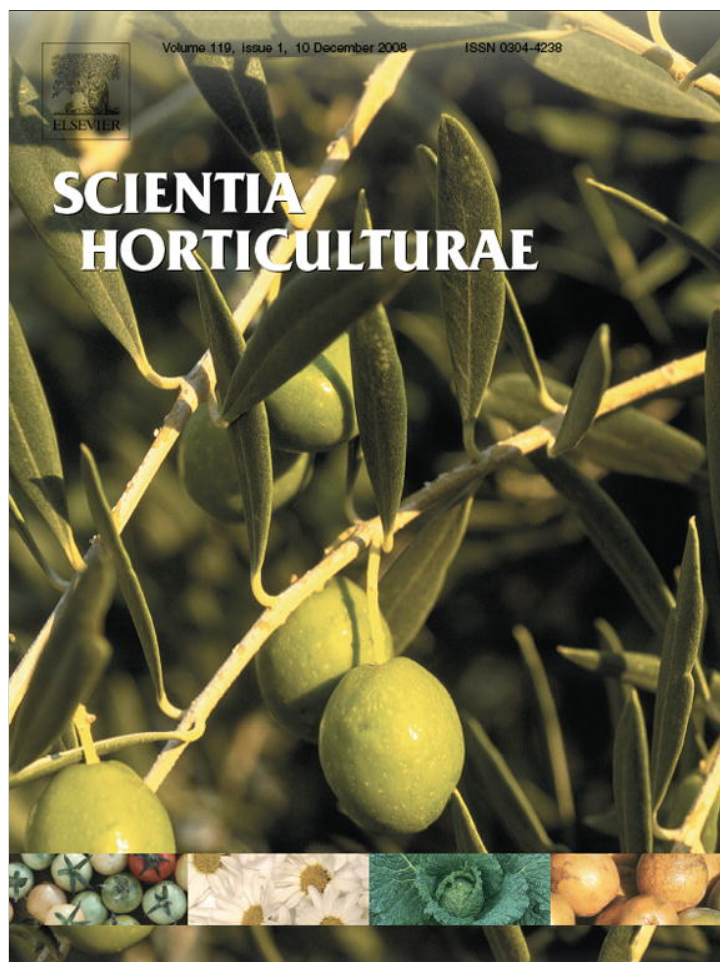


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Induction of tetraploidy in meristematically active seeds of Japanese barberry (*Berberis thunbergii* var. *atropurpurea*) through exposure to colchicine and oryzalin

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ABSTRACT

Japanese barberry (*Berberis thunbergii* DC) is an invasive shrub, widely naturalized across the United States, whose numerous cultivars remain an important horticultural commodity. Maintaining this crop for the future necessitates the development of sterile clones. Exposure to the mitotic inhibitors colchicine and oryzalin is a traditional method for inducing tetraploidy in breeding lines as a precursor to creating sterile genotypes. Treatments utilized pre-germinated *B. t.* var. *atropurpurea* seeds with emerged radicles. Seeds were immersed in aqueous solutions of colchicine (.02%, .05%, .1% and .2%) and oryzalin (.002%, .005%, .01% and .02%) dissolved in 1% DMSO for 6, 12 and 24 h durations. Seedling ploidy level was determined via flow cytometry following 6 and 52 weeks of growth in the greenhouse. Both anti-mitotic chemicals proved effective at inducing tetraploidy and produced comparable efficiency rates. The survival rate of treated seeds decreased in response to both increased mitotic inhibitor concentration and longer exposure duration. While exposure to oryzalin produced greater seed mortality than colchicine, most seedlings that survived had altered ploidy levels. The most efficient oryzalin concentration was 0.002% with a rating of 28%, while the most efficient colchicine concentrations were in the range from 0.05% to 0.2%. Duration of exposure to mitotic inhibitor was not a significant factor over the range from 6 to 24 h. Reversion of tetraploid plants to the diploid state occurred at a low frequency following a dormancy period. Some tetraploid seedlings derived from exposure to both chemicals displayed foliar abnormalities including irregular leaf margins and mottled lamina. The primary advantage of colchicine was low seedling toxicity, while oryzalin was notable for its ability to induce tetraploidy at low concentrations.

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1. Introduction

The invasive plant issue is a primary concern for representatives of the nursery industry (Harrington et al., 2003) since many species of horticultural importance are included on lists of invasive species compiled by state and regional Exotic Pest Plant Councils, conservation groups and native plant societies (Bell et al., 2003). One key plant of interest is Japanese barberry (*Berberis thunbergii* DC.), a shrub introduced in Boston as a landscape ornamental in 1875 that has escaped from cultivation (Silander and Klepeis, 1999) and is currently considered naturalized across the central and eastern United States (USDA, 2008). Japanese barberry remains an important landscape plant (Steffey, 1985) represented

in cultivation by more than 40 cultivars with novel traits such as colored foliage and unique growth habit (Dirr, 1998). These ornamental genotypes constitute important nursery commodities across the United States (Steffey, 1985) worth \$5 million annually in Connecticut alone (Heffernan, 2005). Recent work has demonstrated that some popular ornamental barberry forms display high levels of reproductive potential (Lehrer et al., 2006a), though gene flow from cultivated *B. thunbergii* to feral *B. thunbergii* is yet unproven.

The results of a recent survey of nursery professionals in Connecticut suggest that the development of sterile genotypes which lack invasive potential and possess desirable ornamental attributes is as a preferred method for addressing the invasive plant issue (Gagliardi and Brand, 2007). Gardening consumers also show interest in sterile plants as a means of reducing reliance on invasive species (Kelley et al., 2006). An expedient and cost effective model to induce sterility in horticultural plants involves first deriving polyploid genotypes (Ranney, 2003). These tetraploid plants are then crossed with diploid

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genotypes to produce triploid progeny which are often sterile due to unequal segregation of chromosome pairs during meiosis (Ranney, 2003; Recupero et al., 2005). This strategy has been utilized successfully to produce seedless varieties of various pomological crops for the world market including citrus, banana, plantain, watermelon and grapes (Predieri, 2001; Recupero et al., 2005). Unlike biotechnology approaches, mutation breeding with mitotic inhibitors does not alter DNA coding within the genome (van Harten, 1998). Plants generated through this form of traditional breeding, therefore, face little public or regulatory scrutiny.

Changes in chromosome number may be induced via exposure of plant tissue to various chemical preparations and forms of electromagnetic radiation (van Harten, 1998). Utilization of mitotic inhibitors such as colchicine and oryzalin is commonplace because applying these substances does not require special equipment and is relatively safe (van Harten, 1998). Colchicine is an alkaloid derived from the bulbous plant *Colchicum autumnale* L. (autumn crocus) (Ranney, 2003; Eigsti and Dustin, 1955) whereas oryzalin is the active ingredient in the herbicide Surflan® (Dow AgroSciences, Indianapolis, IN) (van Harten, 1998). Both chemicals induce tetraploidy by disrupting the metaphase stage of cellular mitosis through inhibition of the spindle fibers that segregate replicated chromosomes into daughter cells (Eigsti and Dustin, 1955; van Harten, 1998; Recupero et al., 2005). Successful use of these agents targets areas of high meristematic activity to yield polyploid cells containing multiple chromosome sets (van Harten, 1998).

Soaking pre-germinated seeds with newly emerged radicles in solutions containing anti-mitotic agents is a practical strategy because large samples may be treated simultaneously (Ranney, 2003) to compensate for low rates of ploidy conversion (van Harten, 1998). Notable ornamental plant breeders including Fiala (1988), Leach (1961) and McEwen (1990) have employed this technique to generate tetraploid forms of *Syringa* spp. L. (lilac), *Rhododendron* spp. L. (rhododendron) and *Iris* spp. L. (iris), respectively, through informal experimentation. Academic workers have refined the methodology by evaluating parameters of anti-mitotic chemical concentration and application duration to generate tetraploid forms of many agronomic and horticultural species, including *Catharanthus roseus* L. (common periwinkle) (Kobza and Qing, 2000), *Portulaca grandiflora* Hook. (moss rose) (Mishiba and Mii, 2000), *Trifolium alexandrinum* L. (Egyptian clover) (Khushk et al., 1987) and *Zea mays* L. (maize) (Gayen et al., 1994). This report details *in vivo* treatment of meristematically active seeds of *B. thunbergii* var. *atropurpurea* with colchicine and oryzalin.

2. Methods

2.1. Plant material

Seeds were collected in 2003–2005 from a large planting of *B. thunbergii* var. *atropurpurea* (purple-leaved Japanese barberry) located at Smith College in Northampton, Mass. Previous work had revealed that purple-leaved Japanese barberry plants located adjacent to peers with similar phenotype produce a high percentage of progeny with purple leaves (Lehrer et al., 2006b), a desirable ornamental quality for our breeding work. After fruit collection the seeds were cleaned by maceration and stratified in moist sterilized sand for 4–6 weeks in a cooler held at 4 °C (following Dirr and Heuser, 2006). Pre-germinated seeds were prepared for treatment once radicle emergence of 5–7 mm was evident indicating high levels of meristematic activity.

2.2. Treatment protocol

The experimental design was a three way factorial with two mitotic inhibitors, five concentrations and three time durations. There were three replications per treatment. After being washed to remove residual sand and other debris, 42 seeds per mitotic inhibitor per concentration were placed in 15 ml conical Falcon™ tubes (Becton, Dickinson and Co., Franklin Lakes, NJ) holding 5 ml of modified liquid woody plant media (WPM) (30 g l⁻¹ sucrose, complete micro- and macro-nutrients, pH 5.8) (Lloyd and McCown, 1981) with dissolved aqueous colchicine (.02%, .05%, .1% and .2%) or oryzalin (.002%, .005%, .01%, .02%) dissolved in 1% dimethyl sulfoxide (DMSO). Control groups contained only WPM and WPM plus 1% DMSO. For the duration of treatment all tubes were placed on a rotary shaker in a growth chamber maintained at 25 ± 3 °C with a 16-h photoperiod and a photosynthetic photon flux density of 20 μmol m⁻² s⁻¹. At 6, 12 and 24 h after exposure, 14 seeds were removed per treatment per replication. Removed seeds were rinsed for 10 min using three 10 ml changes of deionized water. Control treatments were maintained and treated for 24 h. Seeds were planted in a randomized complete block design in heavy plastic flats (Kadon Corp., Dayton, OH) filled with Metro Mix 360 Growing Medium (Scotts Co., Marysville, OH).

The flats were placed in a greenhouse with set points of 21 °C day and 17 °C night and natural lighting. Emergent seedlings were irrigated and weeded as needed until they had produced three to five true leaves approximately 6 weeks following anti-mitotic chemical exposure at which time they were evaluated for ploidy through flow cytometric analysis. Seedlings which produced a tetraploid profile from this initial test were maintained and transferred to individual 1040-ml black plastic square pots (Belden Plastics, Roseville, Minn.) using Metro Mix 510 Growing Medium (Scotts, Co., Marysville, OH). These seedlings were grown through the season, maintained in a dormant state for 12 weeks and then brought back into growth to be retested for ploidy confirmation 52 weeks post-exposure. The experiment was conducted twice.

2.3. Ploidy analysis via flow cytometry

Fresh leaves were harvested and nuclei suspensions were prepared by chopping approximately 50 mg of young leaf tissue with a fresh razor blade in 55 mm plastic Petri dishes containing extraction buffer prepared according to Arumuganathan and Earle (1991). The procedure was modified in accordance with Meng and Finn (1999) by adding 2 g of PVP-10 per 50 ml of extraction buffer and fluorescently staining released nuclei with propidium iodide after filtering, rather than during the chopping process. Relative fluorescence of total DNA (FL2) for each stained nucleus was determined with a Becton-Dickinson FACS Calibur Dual Laser Flow Cytometer (Becton, Dickinson and Co., Franklin Lakes, NJ) located at the Flow Cytometry and Confocal Imaging Facility at the University of Connecticut in Storrs, CT. The cytometer was equipped with an argon ion laser emitting radiation at 488 nm. For each sample 10,000–20,000 particles were measured. Fluorescent emission data was collected and displayed by BD CellQuest™ software (Becton, Dickinson and Co., Franklin Lakes, NJ) in histograms of nuclei number according to fluorescence intensity, which was proportional to DNA content. The peaks of test samples were compared to peaks derived from control *B. thunbergii* containing 2N DNA to determine relative ploidy levels as either diploid, tetraploid, or mixoploid (having both diploid and tetraploid peaks) (Fig. 1). Tetraploid seedlings were maintained and retested 46 weeks later, as previously described. Those accessions that twice tested tetraploid were retained for

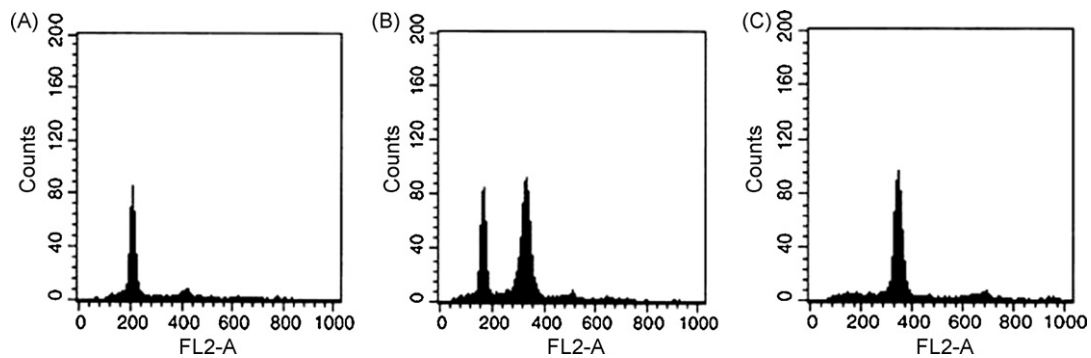


Fig. 1. Flow cytometric histograms representing Japanese barberry (*Berberis thunbergii* var. *atropurpurea*) seedlings with (A) a diploid ($2n$) profile, (B) a mixoploid ($2n + 4n$) profile, and (C) a tetraploid ($4n$) profile. Histograms reflect measurement of 10,000–20,000 particles.

observation of growth/morphology abnormalities and to assess future breeding potential.

2.4. Data analysis

To assess the chromosome doubling capacity of colchicine and oryzalin it was informative to compute the survival rate of treated seeds and the rate of tetraploidy induction for different treatments. A sprouted seed was considered to have survived if it persisted to be evaluated by flow cytometry 6 weeks following chemical exposure. Tetraploidy induction efficiency was calculated as follows (after Bouvier et al (1994): efficiency = % seed survival \times % tetraploidy induction. Efficiency values could range from 0 to 100, where 100 would indicate that all treated seeds survived and later tested tetraploid by flow cytometric analysis. The data from both replications in time were combined and subjected to analysis of variance using Statistical Analysis Systems software version 9.1 for Windows (SAS Institute, 2003) and the mixed (PROC MIXED) procedure. Fisher's least significant difference test was used as the mean separation test.

3. Results and discussion

Both colchicine and oryzalin proved effective at inducing tetraploidy in meristematically active seeds of *B. thunbergii* var. *atropurpurea*, though great variation was observed in post-exposure survival rates, rates of tetraploidy induction and overall efficiency (Table 1). Each anti-mitotic chemical also produced small numbers of chimeric mixoploid seedlings containing comparable quantities of diploid and tetraploid nuclei (Fig. 1, Table 1).

Seed survival rates for oryzalin exposure were depressed overall compared to colchicine (Table 1). Only 30% of oryzalin-treated seeds survived, while 58% of colchicine-treated seeds survived. In part, the reduced survival of oryzalin-treated seeds may be attributed to 1% DMSO needed as a solvent (Table 1). The control colchicine treatment, which required only liquid WPM, had 82% seed survival, while the oryzalin control containing 1% DMSO had only 58% seed survival. While it is difficult to judge the relative toxicity of the two anti-mitotic chemicals due to different working concentrations and solvents, it appears that oryzalin displayed somewhat greater overall toxicity relative to colchicine. This effect may be attributed to oryzalin's higher affinity for plant tubulin, a protein that comprises the microtubules of the mitotic spindle apparatus (Hart and Sabnis, 1976; Okamura, 1980; van Harten, 1998). Oryzalin is also believed to interfere with calcium ions involved in microtubule assembly (Weisenberg, 1972). For these reasons, lower con-

centrations of oryzalin relative to colchicine are typically employed for ploidy alteration in plants.

Seed survival was also depressed by increasing concentrations of both mitotic inhibitors and by increasing exposure time to the chemicals (Table 1). For colchicine at the lowest concentration of 0.02%, seed survival was 76%. The survival rate dropped to 26% at the highest concentration of 0.2%. For oryzalin, seed survival went from 40% at the lowest concentration to 14% at the highest concentration. With both mitotic inhibitors, seed survival at 24 h of exposure was approximately two thirds that of 6 h exposure. These results show that the toxicity of these anti-mitotic chemicals is proportional to concentration and exposure duration.

Across all concentrations and exposure durations, 38% of surviving colchicine-treated seed were tetraploid, while 61% of surviving oryzalin-treated seed were tetraploid (Table 1). Both mitotic inhibitors produced 7–8% mixoploid seedlings. For oryzalin-treated seeds a greater percentage of polyploid-creating events (90%) yielded tetraploid seedlings than mixoploid seedlings compared to colchicine exposure (83%) (Table 1). This finding supports previous investigations (van Harten, 1998) and suggests that polyploid creation tends to be more complete and stable for oryzalin in comparison to colchicine.

Colchicine treatment at concentrations of 0.05–0.2% produced 40–76% tetraploid seedlings and was superior to 0.02% colchicine (Table 1). Although not statistically significant, it did appear that increasing colchicine concentration within the effective range converted more seedlings to tetraploidy. These findings parallel the work of Gayen et al. (2003) and Khushk et al. (1987) who exposed seeds to a range of colchicine concentrations from 0.02% to 0.1% and found that 0.06% and 0.04%, respectively, were the optimal treatments. For oryzalin, any concentration from 0.002% to 0.02% produced 69–90% tetraploid seedlings (Table 1). Duration of exposure to colchicine or oryzalin had no statistical effect on the percentage of tetraploid seedlings produced, although longer exposure to colchicine appeared to produce a greater percentage of tetraploid seedlings than shorter exposures (Table 1). The solvent DMSO employed with oryzalin treatments did not alter ploidy since no tetraploid plants arose from the oryzalin control.

Tetraploid induction efficiency percentage is a good measure of which treatments will most effectively produce tetraploid plants because it considers both seedling survival and rate of conversion to tetraploidy. Overall, colchicine and oryzalin treatments yielded similar tetraploid induction efficiencies of around 20% (Table 1). The best colchicine efficiencies were obtained at concentrations from 0.05% to 0.2% and ranged from 20% to 27%. The most efficient oryzalin concentration was 0.002% which produced a 28% efficiency rating. Oryzalin at 0.005%, with an efficiency of 22%

Table 1
Survival, ploidy and tetraploid induction efficiency of colchicine and oryzalin at various concentrations (%) and durations (h) using seeds of *Berberis thunbergii* var. *atropurpurea*

Treatment	Seed survival (%) ^a	Seedling ploidy (%)			Tetraploid induction efficiency (%) ^b
		Diploid (2n)	Mixoploid (2n + 4n)	Tetraploid (4n)	
Mitotic inhibitor (MI)					
Colchicine	58 a ^c	54 a	8 a	38 b	22 a
Oryzalin	30 b	32 b	7 a	61 a	18 a
MI × conc.					
Colchicine					
0	82 a	100 a	0 a	0 b	0 b
0.02	76 a	81 b	8 a	11 b	8 b
0.05	63 b	49 c	11 a	40 a	25 a
0.1	42 c	24 d	11 a	65 a	27 a
0.2	26 d	16 d	8 a	76 a	20 a
Oryzalin					
0	58 a	100 a	0 a	0 b	0 d
0.002	40 b	25 bc	5 a	70 a	28 a
0.005	29 c	17 c	6 a	77 a	22 b
0.01	12 d	7 c	3 a	90 a	11 c
0.02	14 d	12 c	19 a	69 a	10 c
MI × duration					
Colchicine					
6	71 a	64 a	10 a	26 a	18 a
12	59 b	58 a	7 a	35 a	21 a
24	45 c	40 b	6 a	54 a	24 a
Oryzalin					
6	36 a	36 a	3 a	61 a	22 a
12	32 a	34 a	12 a	54 a	17 a
24	24 b	27 b	5 a	68 a	16 a

^a Seedlings which survived to be tested 6 weeks following exposure to mitotic inhibitor.

^b Calculated as % seed survival × % tetraploid seedlings.

^c Mean separation within columns within treatments according to Fisher's LSD, $p \leq 0.05$.

Table 2
Tetraploid stability and percent abnormal leaf morphology for seedlings of *Berberis thunbergii* var. *atropurpurea* exposed to colchicine and oryzalin

Mitotic inhibitor	Number of tetraploids at 6 weeks	Number of tetraploids at 52 weeks	Reversion to diploid (%)	Abnormal leaf morphology (%)
Colchicine	166	159	4	15
Oryzalin	135	132	2	6

was the next most efficient concentration. Oryzalin at 0.01%, which converted 90% of surviving seedlings to tetraploidy, achieved only 11% efficiency because seedling survival was limited to 12%. Exposure time did not have an effect on tetraploid efficiency for either mitotic inhibitor (Table 1).

Observation of tetraploid seedlings 52 weeks post-exposure to oryzalin and following a dormancy period revealed the presence of both ploidy reversion and foliar abnormalities at low frequencies (Table 2, Fig. 2). Only 2% of seedlings that yielded tetraploid flow cytometry profiles 6 weeks post-treatment exhibited mixoploid or

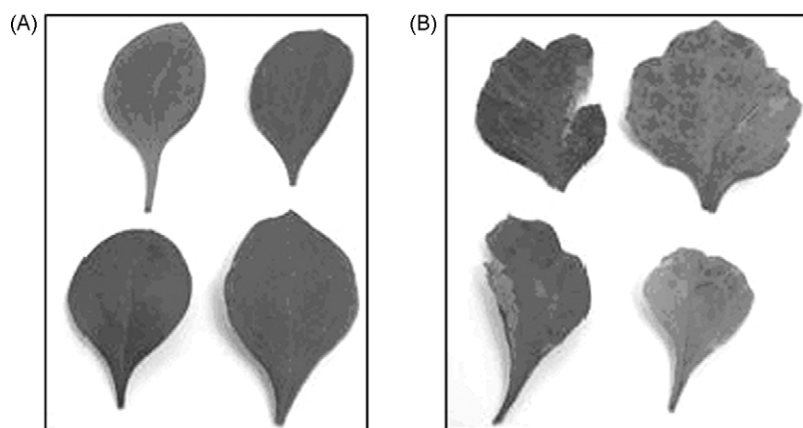


Fig. 2. Representative leaves from Japanese barberry (*Berberis thunbergii* var. *atropurpurea*) depicting: (A) normal leaf morphology typical of diploid (2n) plants and most tetraploid (4n) plants, and (B) abnormal leaf morphology sometimes found on tetraploid (4n) plants.

diploid results when tested 46 weeks later. All plants that reverted had been treated with the lowest oryzalin concentration of 0.002%. The absence of ploidy reversion at higher oryzalin concentrations suggests that these treatments may be preferable since they yield more complete ploidy conversion. Plants with foliar abnormalities exhibited thicker leaves that were oddly shaped and often possessed irregular sectors of white tissue (Fig. 2). Similar phenotypic alterations in plants exposed to anti-mitotic chemicals have been reported by other investigators (van Harten, 1998). It was also observed that initial seedling growth of oryzalin tetraploids was often stunted with plants producing roots that were abnormally thickened and/or few in number (data not shown).

Tetraploid plants produced by colchicine treatment exhibited slightly higher rates of ploidy reversion and foliar abnormalities than oryzalin-treated plants. Reverted plants were present throughout the range of colchicine concentrations examined and totaled 4% of tetraploid plants produced (Table 2). The slightly higher reversion rate for colchicine-treated tetraploids may reflect this mitotic inhibitor's reduced relative affinity for plant spindle fibers compared to oryzalin. Nonetheless, only 7 of 166 tetraploid plants produced through colchicine exposure had changed ploidy when retested (Table 2). Fifteen percent of colchicine-induced tetraploid individuals exhibited leaf abnormalities (Table 2) which were similar in appearance to affected oryzalin tetraploids (Fig. 2). Unlike oryzalin-treated seedlings, colchicine-treated seedlings did not exhibit stunted and/or thickened roots.

4. Conclusion

Observations of initial seedling development revealed that growth stunting among colchicine tetraploids was reduced relative to oryzalin-treated plants. This observation reflects the reduced phytotoxicity of colchicine, which proved to be the primary advantage of using this anti-mitotic chemical for induction of barberry tetraploids. While both colchicine and oryzalin can effectively induce tetraploidy, a program that uses colchicine may avoid delays in completing plant evaluation resulting from oryzalin-induced seedling stunting. Despite the drawback of seedling stunting associated with oryzalin, it still provides high rates of tetraploidy induction at low working concentrations and a slightly lower rate of reversion to diploidy. Safety concerns should also be taken into consideration when choosing a strategy for ploidy conversion since it has long been established that colchicine poses a much greater human health risk than oryzalin.

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