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Efficacy of Colchicine and Trifluralin in Creating In Vitro Autotetraploid *Gaura lindheimeri* Engelm. and Gray

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***Abstract. Gaura lindheimeri* is a diploid herbaceous perennial species native to Texas and Louisiana and winter hardy only to USDA hardiness zone 5. A potential source of winter hardiness is *G. coccinea* Pursh., a polyploid widely distributed in North America; of particular interest are autotetraploid populations of *G. coccinea* from Minnesota. To facilitate interspecific hybridization, a tetraploid *G. lindheimeri* would be advantageous. Two *G. lindheimeri* genotypes, MN selections 443-1 and 01G-02, were treated with two different antimitotic agents at two concentrations, trifluralin—15 and 30 μM and colchicine—0.25 and 1.25 mM, along with appropriate controls, to determine the frequency of chromosome doubling. Two-node stem sections were treated for 12, 24, or 48 h and then rooted and grown to flowering. Pollen diameter was measured as an indicator of chromosome doubling in cell layer LII, and morphologic characteristics (days to flower, flower size, plant height, inflorescence height, and plant width) were recorded for all plants. Chromosome doubling was not observed in any plant treated with trifluralin. Based on pollen diameter, genotype 443–1 only had chromosome doubling in the colchicine 1.25 mM concentration when treated for 12 h. All durations of colchicine at 1.25 mM were successful for genotype 01G-02 as well as a small percent treated with colchicine at 0.25 mM treated for 48 h. Autotetraploid plants (2*n* = 4*x* = 28) had larger flowers in both genotypes, and autotetraploid derivatives of genotype 01G-02 flowered earlier and were taller than diploid plants. Conformation changes from three-lobed to four-lobed pollen grains were observed when pollen diameter approached that expected of 2*n* pollen. Visual screening of pollen for conformation changes can quickly determine if chromosome doubling in cell layer LII has occurred. With the autotetraploid *G. lindheimeri* derived from colchicine application, crosses can be performed with autotetraploid *G. coccinea* to introgress cold tolerance. Additional breeding can also be done at the tetraploid level to develop new autotetraploid cultivars of *G. lindheimeri*.**

The horticultural industry has used related species to integrate new characteristics into cultivated crops. For example, shrub chrysanthemums, which are much larger than standard cushion types, were created by using the species *Dendranthema weyrichii* (Maxim.) Tzvelev (2*n* = 6*x* = 54) as the female in a cross with the garden mum, *Dendranthema* ×*grandiflora* Tzvelev (2*n* = 6*x* = 54) (Anderson and Ascher, 2003). However, difficulties may occur if one species is diploid while the other is a higher ploidy level. In crosses in which ploidy differed, Buchholz and Blakeslee (1929) found that pollen tubes often failed to reach the egg, whereas Cooper and Brink (1945) noted that postpollination endosperm development was slowed and the embryo was unable to develop properly. To increase the likelihood that interspecific pollinations produce viable hybrids, one species must often be manipulated until its chromosome numbers are equal to those of the other parent. This has been accomplished in *Buddleia globosa* Hope for introgressing yellow flower color into the tetraploid cultivated species *B. davidii* Franch (Rose et al., 2000), *Rosa*-interspecific hybrids for integrating disease resistance into cultivated roses (Ma et al., 1997), and *Vaccinium elliottii* Chapm. for improving chilling response, disease resistance, early ripening, and drought tolerance in *V. corymbosum* L. (Dweikat and Lyrene, 1991).

Colchicine was first used in 1937 to treat *Datura* seeds for chromosome doubling (Blakeslee and Avery, 1937). Since then, colchicine has been widely used as a chromosome doubling agent in crops such as *Vaccinium* (Goldy and Lyrene, 1984; Perry and Lyrene, 1984), *Zantedeschia* (Cohen and Yao, 1996), *Buddleia* (Rose et al., 2000), *Rosa* (Ma et al., 1997), and *Humulus* (Roy et al., 2001) both in vivo and in vitro. Colchicine is classified as an antimitotic agent, inhibiting microtubule formation during mitosis (Nebel and Ruttle, 1938). More recently, antimitotic agents that are less toxic to humans such as oryzalin, trifluralin, amiprophos-methyl (APM), and pronamide have been used (Hansen et al., 1998; Wan et al., 1991). These chemicals were effective at much lower concentrations than the rates used for colchicine. APM was demonstrated as the best method for doubling both *Zea mays* L. anther-derived callus (Wan et al., 1991) and *Beta vulgaris* L. ovule culture (Hansen et al., 1998). Oryzalin has been extensively used in ornamental crops such as *Rosa* (Kermani et al., 2003), *Gerbera* (Tosca et al., 1995), *Lilium*, and *Nerine* (van Tuyl et al., 1992). In side-by-side comparisons, trifluralin displayed effects similar to those of oryzalin for most crops (Hansen et al., 1998; Hassawi and Liang, 1991; Wan et al., 1991).

Blakeslee and Avery (1937) reported that leaves, flowers, and stem diameter were greater on plants that were doubled compared with the original diploid plants. Satina et al. (1940) demonstrated that plants consist of three layers of tissue forming the epidermal layer (LI), the subepidermal layer (LII), and the central core (LIII) and that size increases are associated with an increase in cell volume in the doubled layer. Mixoploids tended to have crinkled leaves with one side growing at a faster rate than the other (Blakeslee and Avery, 1937). Rose et al. (2000) demonstrated larger leaf size in doubled *Buddleia* as well as a delay in flowering. Zlesak et al. (2005) reported no differences between *Rosa* diploids and mixoploids when only the LI was doubled; however, when LI and LII were doubled, plants were phenotypically similar to fully doubled plants. For breeding purposes, only LII needs to be doubled because the gametophytic tissue is formed in the subepidermal layer (Sussex, 1989).

The University of Minnesota breeding program is integrating cold tolerance into *Gaura lindheimeri*, a commercially popular herbaceous perennial that does not survive in USDA plant hardiness zones 3 and 4 (Anderson et al., 2001). Gaura is a member of Onagraceae, tribe Onagreae (Raven, 1964), which also contains *Oenothera*, a genus that has been extensively studied for cytologic abnormalities such as ring chromosomes and Renner complexes. Ring chromosomes have been reported in *Gaura* (Carr, 1980; Raven and Gregory, 1972b), although a Renner complex has not. Pollen size can be used to determine chromosome doubling in the LII (Bamberg and Hanneman, 1991). However, in *Oenothera*, where a Renner complex exists, pollen diameter may differ based on the chromosome set (Gambier and Mulcahy, 1996).

Gaura coccinea was selected as the best donor for cold tolerance because it has a wide geographic range from as far north as British Columbia in USDA plant hardiness zone 2 to as far south as Mexico in USDA plant hardiness zone 10) (Raven and Gregory, 1972a). The herbaceous perennial breeding program at the University of Minnesota includes several genotypes of *G. coccinea,* which were collected in western Minnesota. However, western Minnesota populations are tetraploid

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($2n = 4x = 28$), whereas *G. lindheimeri* is a diploid ($2n = 2x = 14$). Preliminary interspecific crosses were made but no viable seeds were produced (Pietsch, unpublished data). To increase the success of crosses between the two species, we decided to double the somatic chromosomes of *G. lindheimeri*.

Chromosome doubling has not previously been reported in *G. lindheimeri*. The objectives of this study were to determine effective concentrations for in vitro doubling of *Gaura lindheimeri* by using colchicine and trifluralin and to document the morphologic characteristics of doubled plants.

Table 1. Percent survival of excised nodes from two *Gaura lindheimeri* genotypes (443-1 and 01G-02) subjected to varying concentrations and duration of exposure to colchicine and trifluralin[z].

		Trifluralin (μM)			Colchicine (mM)		
Genotype	Duration (hrs)	0 (N[y])	15 (N)	30 (N)	0 (N)	0.25 (N)	1.25 (N)
443–1	12	90.9 (11)	81.8 (11)	90.9 (11)	91.7 (12)	66.7[y] (12)	91.7 (12)
	24	45.5 (11)	63.6 (11)	45.5 (11)	100.0 (12)	100.0 (12)	58.3 (12)
	48	77.8 (9)	30.0 (10)	36.4 (11)	40.0[x] (10)	45.5 (11)	25.0 (12)
01G-02	12	100.0 (6)	83.3 (6)	100.0 (6)	90.0 (10)	80.0 [y] (10)	90.9 (11)
	24	66.7 (6)	66.7 (6)	100.0 (6)	100.0 (10)	90.0 (10)	90.9 (11)
	48	85.7 (7)	14.3 (7)	57.1 (7)	70.0[y] (10)	80.0 (10)	55.6 (9)

[z]Logistic regression indicated significant differences for duration of treatment.
[y] Number of node pairs in each treatment.
[x]High amount of contamination in both genotypes. Contaminated vials were counted as surviving the treatment if alive but not subcultured.

Materials and Methods

Plant material. Two *G. lindheimeri* genotypes were selected from the breeding program for inclusion in this study, MN selection no. 443–1 (white-flowered) and MN selection no. 01G-02 (pink-flowered). Both plants are half-sibs from a cross between 'Siskyou Pink' (Siskyou Nursery, Medford, Ore.) and different white-flowered males from seed lot AE02129 (Applewood Seed Company, Arvada, Colo.) (Peters and Anderson, 2006). Plants were maintained in a glasshouse in St. Paul, Minn. (latitude 45°N) for the duration of the experiment at 25 °C day/20 °C night and 16-h photoperiod (0600–2200 HR) supplemented with 1000 W high-pressure sodium (HPS) lamps at 135 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Tissue culture. Stem cuttings (4 cm) were taken from stock plants, leaves and apical shoots removed, sterilized in a 10% bleach solution, and rinsed three times before being placed on autoclaved (20 min at 121 °C) MS (Murashige and Skoog, 1962) medium, pH 5.8, supplemented with 2.5% sucrose, 1 $mg \cdot L^{-1}$ BA (N^6-benzyladenine; Sigma Chemical Co., St. Louis, Mo.), and 0.8% Bacto Agar (Becton Dickinson and Co., Sparks, Md.). Shoots were subcultured on the same medium and multiplied until enough plant material was available for the experiment. Two chemicals were tested as chromosome doubling agents: colchicine (Sigma Chemical Co.) and trifluralin (Treflan, emulsifiable concentrate, 41.2% trifluralin; Dow AgroSciences, Indianapolis, Ind.). Two concentrations of each chemical (colchicine at 0.25 and 1.25 mM and trifluralin at 15 and 30 μM) along with appropriate controls were used based on the best results reported for other crops (Bouvier et al., 1994; Goldy and Lyrene, 1984; Ma et al., 1997; Perry and Lyrene, 1984; Rose et al., 2000; Roy et al., 2001). Three immersion times, 12, 24, and 48 h, were also tested to determine optimal duration.

As a result of the amount of time needed to prepare plant material for treatment, trifluralin treatments occurred 1 week before colchicine treatments. Two nodes per stem section (Table 1) were excised, placed in a flask containing 45 mL autoclaved (20 min at 121 °C) liquid MS medium, pH 5.8, supplemented with 2.5% sucrose and 1 $mg \cdot L^{-1}$ BA, and placed on an orbital shaker (Laboratory-line, Melrose Park, Ill.) at 1500 rpm under continuous lighting (fluorescent lamps, 19 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and 23 °C constant temperature for 24 h. The flasks were moved to a walk-in cooler (Volrath Company, Sheboygan, Wis.) at 4 °C and complete darkness for 48 h. Trifluralin was diluted to a concentration of 15 μM and 30 μM and mixed with 2% dimethyl sulfoxide (DMSO) as a carrier. Colchicine was dissolved in distilled, deionized water to a concentration of 0.25 mM and 1.25 mM. Controls lacking trifluralin or colchicine were also included in this study. An Acrodisc 13-mm syringe filter (Pall Corporation, Ann Arbor, Mich.) was used to filter sterilize each chemical solution by adding 5 mL of solution to each flask. Flasks were placed on an orbital shaker at 23 °C under continuous cool white fluorescent lights (19 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Node pairs were removed from solution after 12, 24, or 48 h in each treatment. As each node pair was removed, it was rinsed three times in sterile distilled, deionized water and placed into an 8-dram vial with autoclaved (20 min at 121 °C) MS medium, pH 5.8, supplemented with 2.5% sucrose, 1 $mg \cdot L^{-1}$ BA, and 0.8% Bacto agar. Vials were sealed with parafilm (Pechiney Plastic Packaging, Chicago, Ill.), labeled, and placed under continuous cool white fluorescent lights (34 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 22 °C. The number of surviving node pairs was recorded for each treatment. Contaminated vials were counted and discarded. Four weeks after treatment, new shoots were subcultured in the same medium. Shoots grew another 4 weeks and were then transferred to a stem elongation medium consisting of autoclaved (20 min at 121 °C) MS medium, pH 5.8, 2.0% sucrose, and 0.8% Bacto agar.

Greenhouse conditions. After 3 weeks, shoots were removed from vials, dipped in 8000 ppm Indole-3-butyric acid (IBA) in talc, transplanted into Sunshine plug mix #5 (Sun Gro Horticulture, Bellevue, Wash.), covered with a clear plastic dome, and placed under constant, cool white fluorescent lights (37 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 24 °C. Plantlets were hand-misted to keep them from drying out until roots formed. After 2 weeks, trays were uncovered and moved to a mist house (25 °C constant temperature, 16-h photoperiod supplemented with HPS lamps at 135 $\mu mol \cdot m^{-2} \cdot s^{-1}$, intermittent mist on 8 s every 8 min) for 1 week and then moved to a greenhouse with 25 °C day/20 °C night temperatures and 16-h photoperiod (0600–2200 HR) supplemented with HPS lamps at 135 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Plantlets were transplanted into 10-cm diameter pots and grown under the same conditions until they flowered. As plants came into flower, date of first flower, flower size for the first five flowers, and flower color were recorded. Flower color for the pink-flowered plants was defined by background color (pink or white) and veination color.

Ploidy analysis. Three flowers (replications) were collected from each individual plant in each treatment. Pollen was prepared for microscope examination by staining with 0.1% aniline blue (Anderson and Ascher, 1993) and adding a drop of glycerol for even distribution of the pollen (Zlesak and Thill, 2002). Digital images were collected for pollen from each flower by using a Zeiss light microscope (Goettingen, West Germany) with a digital camera (Diagnostic Instruments, Inc., Sterling Heights, Mich.). Ten pollen grains were measured by using Image Pro Plus software (v. 4.5.1.26; Media Cybernetics, Inc., Silver Spring, Md.) for each image. Theoretical pollen diameter for tetraploid plants was estimated based on the formula derived by Bamberg and Hanneman (1991). A conformation change from three-lobed to four-lobed pollen grains was noted for $2n$ pollen in diploid genotypes (Pietsch, unpublished data). Therefore, conformation changes were recorded when greater than 50%.

Field conditions. Cuttings were taken from each individual plant that survived to the greenhouse phase, dipped in 8000 ppm IBA in talc, placed in 72-cell plug trays filled with Sunshine Universal mix (Sun Gro Horticulture), and placed under intermittent mist (8 s every 8 min) for 2 weeks for rooting. As a result of space limitations, one cutting per plant was transplanted into the field in St. Paul, Minn. (latitude 45°N), week 25, 2004, for field evaluation. Flowering was recorded weekly and plant height, inflorescence height, and plant width were measured after 10 weeks.

Data analysis. Logistic regression with a forward likelihood ratio (SPSS for Windows v. 10; SPSS Inc., Chicago, Ill.) was used to determine significant differences between treatments for survival. Pollen size

was analyzed with analysis of variance (ANOVA). Independent sample *t* tests were used to test for differences between ploidy levels for days to flower, flower size, and field plant height, inflorescence height, and plant width.

Results

Survival. Forward log likelihood ratios were significant only when duration of treatments was used in the model regardless of chemical or genotype. The Wald test for significance varied at $P \leq 0.001$ for genotype 443-1 colchicine treatments and $P = 0.003$ for the corresponding trifluralin treatments, and at $P = 0.035$ for genotype 01G-02 colchicine treatments and $P = 0.010$ for the corresponding trifluralin treatments. Survival was higher in genotype 01G-02 for all treatments except trifluralin at 15 μM immersed for 48 h, colchicine control and at 1.25 mM immersed for 12 h, and colchicine at 0.25 mM immersed for 24 h (Table 1). Trends were similar for both genotypes; as the duration of the trifluralin at 15 μM and 30 μM and colchicine at 1.25 mM treatments increased, survival decreased (Table 1). However, genotype 01G-02 had no difference in survival between 12- and 24-h durations for the highest concentrations of trifluralin and colchicine. Colchicine at 0.25 mM and the control had the highest survival rate at 24 h, whereas the trifluralin control had the lowest survival rate at 24 h. In all treatments except the trifluralin control, the longest duration had the greatest mortality. Two of the treatments had large amounts of contamination, colchicine 0.25 mM at 12-h duration and the colchicine control at 48-h duration (Table 1).

Pollen analysis. When measuring pollen diameter, some viable pollen grains appeared slightly smaller than others. For consistency, only the larger pollen grains were measured. Trifluralin concentration did not significantly affect pollen diameter for either genotype ($P = 0.328$, 443-1; $P = 0.350$, 01G-02) for pollen diameter. Duration was significant for genotype 01G-02 ($P \leq 0.001$) with pollen diameter decreasing as duration increased. However, pollen diameter for all flowers measured was significantly smaller than the calculated expected size for doubled pollen (data not shown). Therefore, no chromosome doubling could be found in either genotype after using trifluralin.

Significant differences were found in colchicine concentration ($P = 0.050$), duration ($P = 0.001$), and in the interaction ($P \leq 0.001$) for genotype 443-1. Colchicine concentration and duration were highly significant for genotype 01G-02 ($P \leq 0.001$) with the 1.25 mM concentration having significantly larger pollen diameter than either the control or the 0.25 mM concentration.

Average pollen diameter from the original diploid plants was 89.8 μm for both genotypes (data not shown), so pollen diameter for tetraploid plants was calculated as 113.1 μm (Bamberg and Hanneman, 1991). For colchicine-treated plants, pollen diameter ranged from 74.9 to 131.3 μm for genotype 443-1 and 71.9 to 128.3 μm for genotype 01G-02 (Fig. 1). Normality tests for both genotypes demonstrated that kurtosis existed (443–1 = 5.73; 01G-02 = 7.22) (Fig. 1). However, when doubled pollen diameter data were removed, the kurtosis was much closer to normal (443-1 = –0.15; 01G-02 = 0.54) (data not shown). The trends were similar for skewness; when all pollen from treated plants was included, skewness was much greater than normal (443-1 = 1.53; 01G-02 = 2.10) (Fig. 1). When doubled pollen was removed, skewness was much closer to normal (443-1 = –0.32; 01G-02 = –0.15) (data not shown).

Average pollen diameter for genotype 443-1 was larger than genotype 01G-02 in all colchicine treatments except the 0.25 mM concentration immersed for 48 h (Table 2). However, the larger pollen diameter did not indicate tetraploids because the average was still well below the calculated diameter of 113.1 μm expected for doubled pollen. A total of four potential polyploids (autotetraploids) were identified from one treatment in 443-1 and 14 potential polyploids from four different treatments in 01G-02 (Table 2). Colchicine treatments were more effective in genotype 01G-02 with doubling occurring in all durations at the 1.25 mM concentration (Table 2). The total number of plants recovered from colchicine treatments was much greater in genotype 01G-02 than in genotype 443-1, with only 10 plants from the 24-hour duration and two plants from the 48-hour duration of 1.25 mM concentration recovered (Table 2). If chromosome doubling occurred, the plants did not survive.

Conformation changes were noted when pollen size approached that of tetraploids. Diploid *Gaura* pollen is shaped as a triad

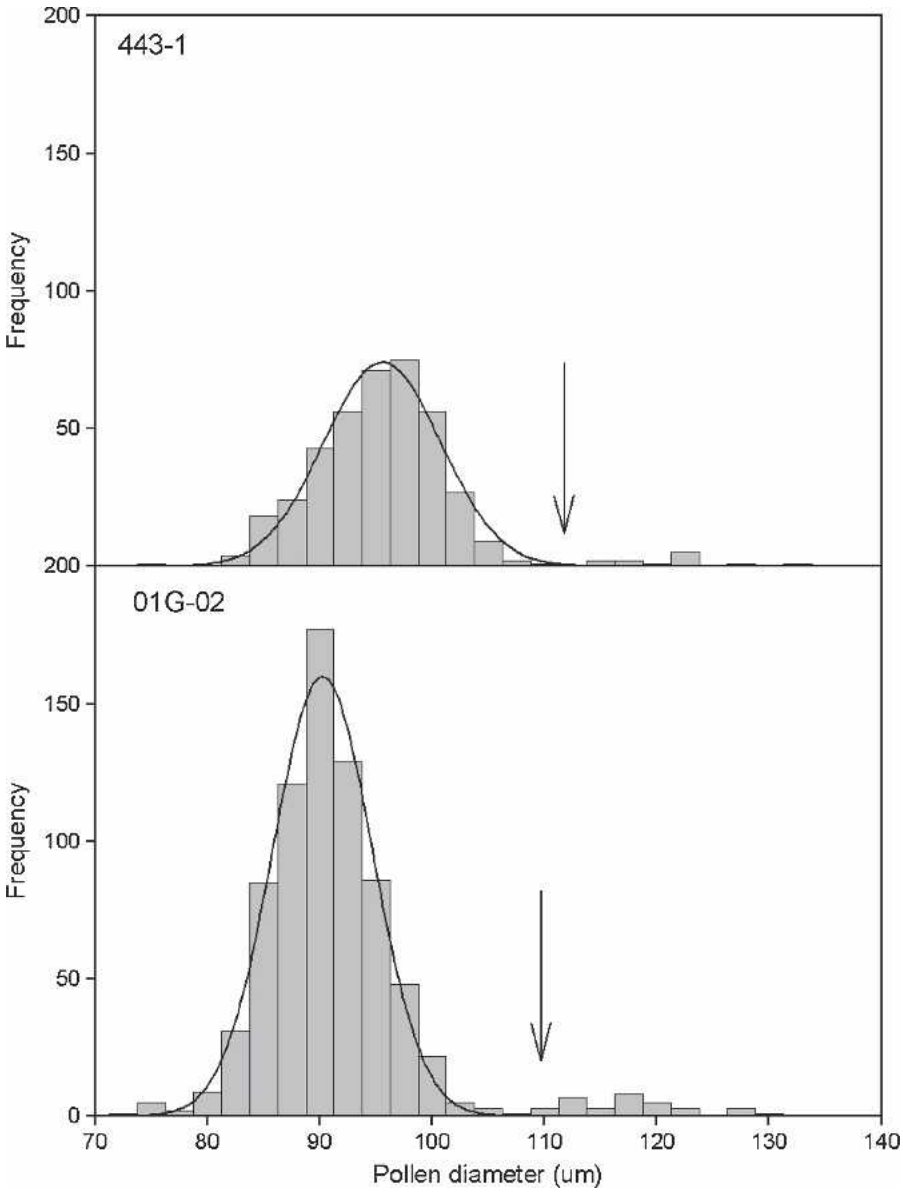

Fig. 1. Histogram with mean pollen diameter (n = 10 pollen grains/flower) of two *Gaura lindheimeri* genotypes (443-1, 01G-02) treated with colchicine at 0, 0.25, or 1.25 mM. Arrows indicate break between *n* and 2*n* pollen. Genotype 443-1 skewness = 1.53 ± 0.122, kurtosis = 5.73 ± 0.244. Genotype 01G-02 skewness = 2.10 ± 0.089, kurtosis = 7.22 ± 0.177.

Table 2. Mean pollen diameter (µM) ± SD based on n = 10 pollen grains/flower, 3 flowers/plant of two *Gaura lindheimeri* genotypes (443-1, 01G-02) treated with three colchicine concentrations and three time durations[z].

Colchicine concn	Duration (h)	Mean pollen diameter µM							
		443-1				01G-02			
		N[y]	*n*	N	2*n*	N	*n*	N	2*n*
0 mM	12	21	94.08 ± 3.78			21	90.57 ± 4.85		
0.25 mM	12	24	94.29 ± 4.24			0			
1.25 mM	12	12	92.48 ± 5.91	4	120.62 ± 5.08	8	90.74 ± 3.06	2	117.85 ± 8.96
0 mM	24	37	94.95 ± 5.27			51	88.61 ± 4.41		
0.25 mM	24	12	95.97 ± 4.62			53	88.57 ± 4.96		
1.25 mM	24	10	92.41 ± 6.51			40	87.82 ± 4.05	9	110.52 ± 12.26
0 mM	48	10	93.01 ± 5.46			28	89.89 ± 4.14		
0.25 mM	48	1	84.16 ± 1.40			25	89.07 ± 5.25	1	115.77 ± 6.68
1.25 mM	48	2	94.81 ± 4.82			12	90.60 ± 4.91	3	111.14 ± 5.59

[z]Diameter of 2*n* pollen was calculated as 113.1 µM (see text).
[y]Number of plants with *n* or 2*n* pollen.

(Fig. 2A), and when cell layer LII is doubled, a conformational shift to a tetrad or greater sided geometric shape is noted (Fig. 2B). Aborted pollen also exhibited a conformational shift in flowers with cell layer LII doubled. All plants listed as tetraploids in Table 2 had conformation changes in at least two-thirds of their flowers. Many flowers from the remaining diploids showed higher levels of 2*n* pollen, indicated by a conformation change, and aborted pollen grains (data not shown).

Morphology and field data. Autotetraploids from genotype 01G-02 had significantly larger flowers, flowered later, and were taller than were the diploids (Table 3). Diploid plants from both genotypes flowered an average of 8 to 10 d earlier and generally bloomed continuously both in the field and greenhouse (data not shown). Autotetraploids derived from genotype 443-1 had significantly larger flowers than did diploids with no significant differences for any other trait measured (Table 3). However, autotetraploids were generally taller and flowered later, similar to those derived from genotype 01G-02 (Table 3). Inflorescence height was slightly smaller for autotetraploids in genotype 443-1 while slightly greater for autotetraploids in genotype 01G-02. Plant width was the opposite with wider plants recorded for autotetraploid genotype 443-1 and narrower plants for autotetraploid genotype 01G-02.

Flower color for 01G-02 showed considerable variability, not just within the doubled plants, but in all plants that were treated in tissue culture. None of the plants was completely white, but petals ranged from a pale pink to a very dark pink, some with a white midvein. Flower color from the same clone in the field and greenhouse also varied. Tetraploids were not necessarily darker or lighter than the diploids.

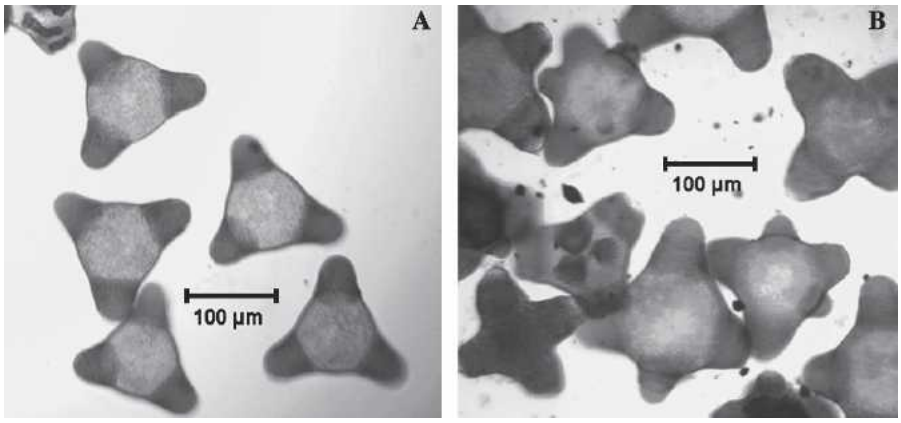

Fig. 2. Pollen from untreated *Gaura lindheimeri* genotype 01G-02 (**A**) and autotetraploid derivative treated with 1.25 mM colchicine for 24 h (**B**). A conformation change in the pollen grains as well as the larger size indicates successful chromosome doubling (**B**).

Discussion

Trifluralin was unsuccessful in creating autotetraploids from node sections, although preliminary experiments to double *G. lindheimeri* by applying a 0.086% solution to seedling cotyledons were successful (Pietsch and Zlesak, unpublished data). Trifluralin has not been as widely used as a doubling agent as have other mitotic inhibitors. When compared with oryzalin, pronamide, and amiprophos-methyl (APM), trifluralin induced chromosome doubling at comparable rates, but often had toxic effects at high concentrations (Hansen et al., 1998; Wan et al., 1991). The rates used in this experiment were higher than the optimum in vitro for *Beta vulgaris* (Hansen et al., 1998), *Zea mays* (Wan et al., 1991), and *Triticum aestivum* L. (Hansen and Andersen, 1998; Hassawi and Liang, 1991). *Gaura lindheimeri* may require higher concentrations to affect the meristem, or the method of application may have limited the amount of chemical in solution or absorbed. Another possibility is that although filter sterilization has been used successfully with colchicine (Anderson et al., 1991; Hassawi and Liang, 1991; Rose et al., 2000; Roy et al., 2001), oryzalin (Hassawi and Liang, 1991; Petersen et al., 2003), and trifluralin (Hassawi and Liang, 1991), when we used this procedure, the trifluralin solution that passed through the filters was observed to be milky to clear, whereas the original liquid was an orange color. This suggests that the pores in the filter used for sterilization may have been too small for the trifluralin molecule to pass through. Additional work needs to be done to verify this.

Chromosome doubling rates for genotype 01G-02 were ≈20% with colchicine at the 1.25 mM concentration at all durations and less than 5% being doubled with the 0.25 mM concentration when held for 48 h. Duration of treatment did not affect the percentage of plants doubled in genotype 01G-02 (Table 2). Fewer plants from genotype 443-1 were recovered for all treatments, which may account for the lack of doubling at longer durations with the colchicine 1.25 mM concentration. Doubled plants often display stunted or slowed growth (Blakeslee and

Table 3. Means ± SD for morphologic characteristics (number days to flower, flower size, height of plant, plant width, height of inflorescence) for diploid and autotetraploid *Gaura lindheimeri* plants pooled for chemical treatment.

Morphologic characteristic	443-1			01G-02		
	2*x*	4*x*	Sig difference	2*x*	4*x*	Sig difference
Number of days to flower	79.5 ± 9.5	89.7 ± 11.8	NS	74.1 ± 4.8	82.4 ± 8.5	***
Flower size (cm)	4.30 ± 0.26	4.85 ± 0.18	***	3.57 ± 0.28	3.74 ± 0.34	***
Plant height (cm)	10.33 ± 3.32	15.00 ± 7.39	NS	12.54 ± 3.78	16.00 ± 5.42	**
Plant width (cm)	20.24 ± 7.94	23.38 ± 9.51	NS	18.40 ± 6.19	16.35 ± 5.09	NS
Inflorescence height (cm)	36.65 ± 8.50	35.25 ± 13.12	NS	39.10 ± 10.14	40.54 ± 11.78	NS

NS,**, ***Nonsignificant, $P \leq 0.01$, $P \leq 0.001$, respectively.

Avery, 1937; Perry and Lyrene, 1984; Roy et al., 2001) and thus could have been lost when transferred to new tissue culture medium or soil. Many of the smallest plants died when transferred to soil. Goldy and Lyrene (1984) found genotypic differences in *Vaccinium* species for survival and chromosome doubling based on chemical concentration and duration.

Many of the autotetraploid gaura exhibited delayed flowering and larger flowers than did the diploids. Larger flowers have been observed in doubled *Tagetes* (Nebel and Ruttle, 1938), *Oenothera* (Renner, 1933), and *Vaccinium* (Dweikat and Lyrene, 1991). Thicker leaves and stems have also been associated with doubled plants (Blakeslee and Avery, 1937; Kermani et al., 2003; Nebel and Ruttle, 1938; Renner, 1933; Rose et al., 2000). Although, the LI cell layer was not examined, many of the plants showed thickened leaves and slower growth. Although only a few plants were doubled from 443-1, autotetraploid plants were not significantly later flowering or taller than the original plants in the greenhouse. However, plants in the field generally flowered much later and only flowered intermittently compared with the diploid plants.

Pollen grains of three different size classes were observed in tissue culture plants from this experiment with the smallest appearing aborted and slight differences between the two larger sizes. Bhaduri (1941) described two different-sized pollen grains in *G. lindheimeri* with smaller grains appearing empty. Gambier and Mulcahy (1996) noted three pollen size classes in *Oenothera villaricae* Dietrich and *O. picensis* Phil. ssp. *picensis* with the smallest appearing aborted. The two larger size classes corresponded to different Renner complexes found in the each species. In measuring the pollen from plants in this experiment, only the 10 largest pollen grains from each image were measured. These measurements were used to determine ploidy of each plant and were smaller than the calculated estimated size of tetraploid pollen. Tetraploids had a greater range in pollen size, probably as a result of incomplete meiosis or disruption of chromosome rings. Thus, a better measure of ploidy in chemically doubled *Gaura* might be the conformation change in the pollen.

Pollen conformation changes have been noted in some crops such as *Dendranthema* (Weddle, 1940), in which a change from oval to cubical or pyramidal was noted, and *Trifolium* (Anderson et al., 1991; Taylor et al., 1976), in which a change from oval or cylindrical to tetrahedral was noted in doubled plants. Polyploid *Oenothera* was reported to have four-lobed pollen compared with three-lobed pollen found in diploids (Renner, 1933). This same conformation change was observed with 2*n* pollen in *Gaura lindheimeri* and was more pronounced in pollen of chromosome-doubled plants (Fig. 2B). Measurements of pollen diameter confirmed that these pollen grains were doubled, according to Bamberg and Hanneman (1991). Screening flowers from rooted cuttings of diploid and tetraploid plants for conformation changes demonstrated that this method works well for classifying doubled plants. All of the tetraploid plants from 01G-02 showed the same conformation change, whereas there were some differences from 443-1.

Pollen viability or fertility has not been examined in autotetraploid *Gaura*. Renner (1933) found that autotetraploid *Oenothera* had high fertility and readily crossed with other autotetraploids. Initial crosses have been set up between genotype 443-1 and 01G-02 to determine fertility and crosscompatibility. Often, self incompatibility barriers can be overcome through chromosome doubling (Cohen and Yao, 1996; Perry and Lyrene, 1984), which can be tested by selfing or crossing among plants derived from the same genotype. Chromosome doubling was begun to determine if autotetraploid *G. lindheimeri* can be successfully crossed with the winter-hardy *G. coccinea* to introgress cold hardiness. These crosses will be initiated once fertility has been determined. Additional *G. lindheimeri* genotypes can be doubled for interspecific crosses as well as for breeding new varieties at the tetraploid level.

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