HortScience 52(3):332–337. 2017. doi: 10.21273/HORTSCI11593-16

In Vivo Chromosome Doubling of *Prunus lusitanica* and Preliminary Morphological Observations

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Additional index words. Portuguese cherrylaurel, flow cytometry, colchicine, oryzalin, polyploid

***Abstract. Prunus lusitanica* (2*n* = 8*x*) and *Prunus laurocerasus* (2*n* = 22*x*) are evergreen woody shrubs commonly used in landscapes across the United States and Europe. To reduce the difference in ploidy between these species and with the expectation of successful hybridization, an experiment was performed to double the chromosome number of *P. lusitanica*. Colchicine was applied at 0%, 0.2%, 0.4%, and 0.8% (w/v), and 125 μM oryzalin as a viscous liquid to the apical meristem of open-pollinated *P. lusitanica* seedlings. Solutions were semisolidified using 0.55% agar (w/v). Cellular penetration was increased by adding 1% dimethyl sulfoxide (v/v) in all groups except oryzalin. As a result, three chromosome doubled (2*n* = 16*x*) plants, one 2*n* = 12*x* plant, and 14 cytochimeras (2*n* = 8*x* + 16*x*) were recovered. Application of 125 μM oryzalin had a meristem-survival rate of 17%, statistically lower than all other treatments. The oryzalin treatment also produced the highest number of altered ploidy seedlings. Oryzalin at 125 μM was the most effective chromosome doubling agent in this experiment. Phenotypic examination indicated that chromosome doubled (2*n* = 16*x*) plants displayed shorter stems, thicker leaves, and fewer but larger guard cells than the untreated controls.**

Portuguese cherrylaurel (*Prunus lusitanica*) and common cherrylaurel (*Prunus laurocerasus*) are popular landscape plants throughout the northern temperate zone. They are densely growing evergreen shrubs, commonly used in hedging. An important difference between the two species is that *P. lusitanica* is resistant to shot-hole disease, while *P. laurocerasus* is susceptible (Dirr, 2009; Williams-Woodward, 1998). Shot-hole disease refers collectively to a number of bacterial and fungal pathogens (e.g., *Pseudomonas syringae* pv. *syringae*, *Xanthomonas arboricola* pv. *pruni, Wisonomyces carpophilum*, *Microgloeum pruni*, and *Cercospora* sp.) which detract from ornamental appearance of leaves and may eventually kill diseased trees if cankers girdle stems (De Boer, 1980; Marchi et al., 2014; Pscheidt and Ocamb, 2014; Williams-Woodward, 1998). Symptoms typically present as numerous small holes in the leaves of affected plants through the loss of necrotic leaf tissue.

So far, there have been no reports of successful hybridization between *P. lusitanica* and *P. laurocerasus*. We believe this apparent sexual incompatibility is likely due, at least in part, to the difference in ploidy level. *Prunus laurocerasus* is a 22-ploid with a chromosome number of 2*n* = 22*x* = 176 (Meurman, 1929). *P. lusitanica* is an octoploid with a chromosome number of 2*n* = 8*x* = 64 (Darlington and Wylie, 1956).

We theorize that if we can double the ploidy level of *P. lusitanica* to 2*n* = 16*x*, an interspecific cross might be possible. Similar approaches have been successfully applied in *Rhododendron* (Kehr, 1996), *Rosa* (Debener et al., 2003), and *Vaccinium* (Lyrene, 2011) by doubling the chromosomes of one of the parents. Theoretically, the resulting hybrid would have a chromosome number of 2*n* = 19*x* = 152. This odd ploidy level, in tandem with being an interspecific hybrid, could produce a low-fertility plant.

In recent years, increasing attention has been given to the level of fertility in nursery and landscape plants (Niemiera and Von Holle, 2009). Legislation and regulation of weedy plants is becoming commonplace and some of these species are economically important for nursery growers. As such, reducing fertility has become a goal of breeders and may be regarded as a value-added trait since sterile or nearly sterile plants are less likely to escape from cultivation. A common goal of ornamental plant breeders is to create plants with odd ploidy levels (i.e., triploids). This typically reduces a plant's fertility and ability to develop seed. For example, this technique was used in the development of low-fertility *Hypericum androsaemum* without losing its ornamental appearances (Olsen et al., 2006).

Induction of polyploidy, or chromosome doubling, can be accomplished in several ways. Commonly, seedlings or shoots tips are treated with colchicine (in vitro or in vivo). Colchicine, a mitotic spindle inhibitor affecting chromosome separation during mitosis, has been used for chromosome doubling since the late 1930s (Blakeslee and Avery, 1937). The effectiveness of colchicine treatment in chromosome doubling has been seen in many woody species including *Acacia crassicarpa* (Lam et al., 2014), *Lagerstroemia indica* (Ye et al., 2010), *Platanus acerifolia* (Liu et al., 2007), *Pyrus pyrifolia* (Kadota and Niimi, 2002), and *Ziziphus jujuba* (Gu et al., 2005).

Oryzalin is another effective mitotic inhibitor for chromosome doubling in many woody plants including *Acacia crassicarpa* (Lam et al., 2014), *P. laurocerasus* (Contreras and Meneghelli, 2016), *Platycladus orientalis* (Contreras, 2012), *Rhododendron* (Jones et al., 2008), *Rosa* (Kermani et al., 2003), *Thuja occidentalis* (Contreras, 2012), and *Thuja plicata* (Contreras, 2012). Oryzalin is now commonly used in plants due to its binding affinity to plant tubulin rather than mammalian (Hugdahl and Morejohn, 1993), therefore reducing toxicity to humans. Oryzalin is also effective at much lower concentrations than colchicine. Colchicine is typically applied at a rate of 0.1% to 1%, while in one recent example, where treatment was applied to the meristem in vivo, Jones et al. (2008) doubled the chromosomes of *Rhododendron* using only 50 μM oryzalin.

A shot-hole disease resistant cherrylaurel hybrid with low fertility could have the potential to be widely adopted by the nursery industry. Establishing an effective chromosome doubling method in *P. lusitanica* is the first step in developing hybrids that realize these traits. The objectives of this study were to 1) develop methods for generating 2*n* = 16*x* *P. lusitanica* that can be used in future breeding projects and 2) assess morphological variation among different cytotypes.

Materials and Methods

Plant material. Open-pollinated fruit were collected from one *P. lusitanica* on the Oregon State University campus (lat. 44°34′04″N, long. 123°17′14″W) in Corvallis, OR, on 5 Sept. 2014. The exocarp and mesocarp were removed to expose the stony endocarp. Seeds were then cold-stratified the in moist perlite for 60 d at 5 °C. After cold-stratification, seeds were planted in soilless substrate (Metro-Mix 840PC; SunGro Horticulture, Agawam, MA) in 50.6 × 21.6 × 5.2 cm flats (T.O. Plastics, Clearwater, MN) and grown in a glasshouse with 24 °C day/9 °C night temperatures. Trays were hand-watered as needed and germination began to occur 19 d after sowing. Germinated seedlings were transferred each day to 36-cell trays (T.O. Plastics), and grown under soft fluorescent lights at 90 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at 22 to 25 °C with a 16-h photoperiod (0600 to 0000 HR) covered with clear humidity domes (T.O. Plastics).

Received for publication 17 Nov. 2016. Accepted for publication 6 Jan. 2017.

We would like to acknowledge Oregon Association of Nurseries for partial funding of this research. We also thank Shawn Mehlenbacher, Carolyn Scagel, John Lambrinos, and three anonymous reviewers for critical review to improve the manuscript.

This article is a portion of a thesis submitted by J. Schulze in partial fulfillment of the requirements for an MS.

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Table 1. Sources and quantities of altered-ploidy *Prunus lusitanica* seedlings.

Treatments	No. of treated seedlings	Survival rate (%)[z]	No. of cytochimera[y]	No. of $2n = 16x$	No. of $2n = 12x$
Control	84	98.8 a[x]	0	1	0
0.2% colchicine	84	98.8 a	2	1	1
0.4% colchicine	84	73.8 b	3	0	0
0.8% colchicine	84	60.7 b	4	0	0
125 µM oryzalin	84	16.7 c	5	1	0
Totals	420		14	3	1

[z]Survival rates refer to percentages of surviving apical meristem following treatment application.
[y]Cytochimera include both $2n = 8x$ and $2n = 16x$ cells ($2n = 8x + 16x$) in leaf tissue.
[x]Letters indicate significant ($P \leq 0.05$) differences in Kruskal–Wallis rank sum using Bonferroni-inequality adjusted least significant difference comparisons.

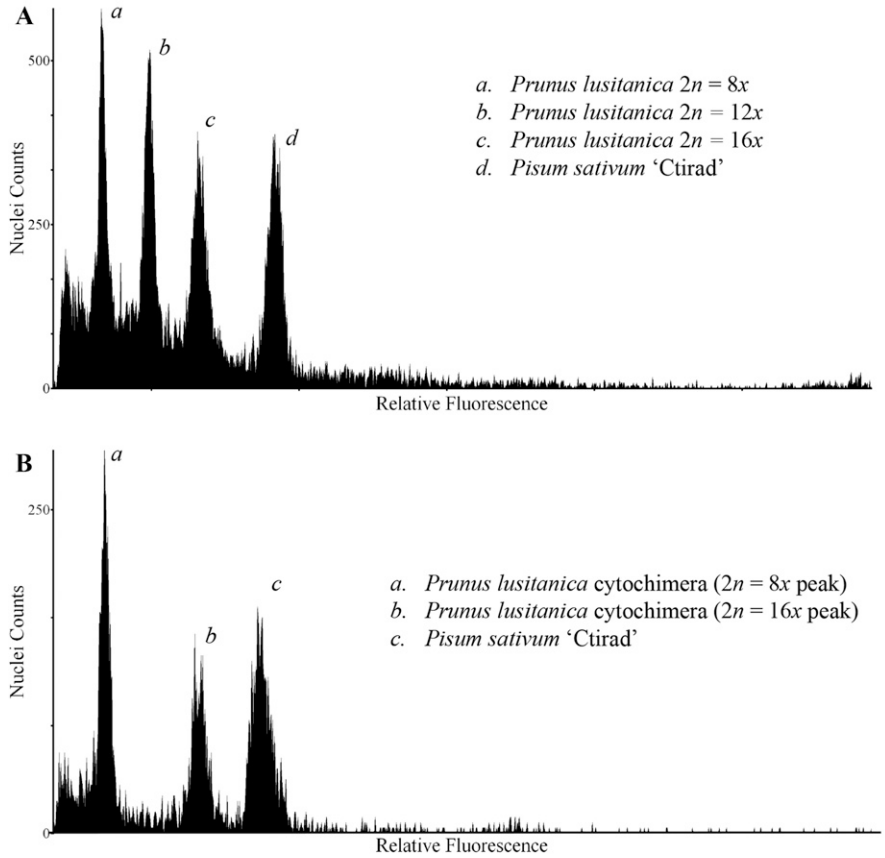

Fig. 1. Flow cytometric output of *Prunus lusitanica* and induced polyploids. (**A**) Over 6000 particles were run with $2n = 8x$, $2n = 12x$ and $2n = 16x$, plus the *Pisum sativum* 'Ctirad' internal standard. (**B**) Over 3000 particles were run of one cytochimera plus the *Pisum sativum* 'Ctirad' internal standard.

Inducing polyploidy. Seedlings were randomly placed into groups of 12 as they germinated, and each group was assigned a treatment. This occurred sequentially over time until each treatment was replicated seven times. A total of 420 seedlings (12 seedlings per replicate × 7 replicates × 5 treatments) were treated. Colchicine at a rate of 0% (control), 0.2%, 0.4%, 0.8% (Sigma-Aldrich, St. Louis, MO) (w/v), and 125 µM oryzalin (Surflan® AS; United Phosphorus, Trenton, NJ) were applied as mitotic spindle inhibitors. One percent dimethyl sulfoxide (Sigma-Aldrich) (v/v) was added to the colchicine and control treatments to improve cellular penetration, and 0.55% agar (Sigma-Aldrich) (w/v) was added to all the treatments as a congealing agent. All treatments were heated to 38 °C to dissolve the agar before application. Once the first true leaves opened, then 25 µL of treatment solution was applied to the exposed meristem. Treatments were maintained on the meristem for 10 d. Reapplication was conducted as droplets dried out, about every 3 to 4 d. After treatments, meristems were manually cleaned with running water to remove solution remains. The plants were then moved to a glasshouse equipped with high-wattage lamps with the ambient environmental conditions maintained as 12-h photoperiod and 24 °C day/19 °C night. Apical meristem survival rate was recorded one month after the completion of all treatments. In many instances, if the apical meristem was killed by the treatment, adventitious shoots would emerge just above the cotyledon, but such adventitious shoots were not counted.

Ploidy analysis. A flow cytometer (CyFlow Ploidy Analyzer; Partec, Münster, Germany) was used to screen all the surviving seedlings. *Pisum sativum* 'Ctirad' (2C = 8.76 pg) was used as an internal standard for genome size estimation of treated plants (Greilhuber et al., 2007). About 0.5 cm^2 of young leaf tissue from each sample and the internal standard was finely cochopped in 0.4 mL of extraction buffer (CyStain® Ultraviolet Precise P Nuclei Extraction Buffer; Partec) with a double-sided razor blade to extract nuclei. The nuclei suspension was passed through a 30 µm filter (Partec), and then stained with 1.6 mL of 4',6-diamidino-2-phenylindole (CyStain® ultraviolet Precise P Staining Buffer; Partec**)**. The stained nuclei solutions were incubated for ≈60 s before being fed into the flow cytometer. Genome size of *P. lusitanica* samples was calculated according to a formula: sample genome size = genome size of internal standard × (mean fluorescence value of sample ÷ mean fluorescence value of internal standard).

Pollen diameter measurements. Fresh pollen was collected from the mother tree and stored it over calcium sulfate desiccant (Drierite, Xenia, OH) at –20 °C until observation. At the time of observation, pollen was brushed onto a microscope slide in a drop of 2% acetocarmine solution. The stained pollen was then covered by a cover slip and observed under a compound light microscope (Zeiss Imager A1; Zeiss Microscopy, Oberkochen, Germany). Thirty photos from three slides at ×200 magnification were subjected to an image analysis software (AxioVision; Zeiss Microscopy), resulting that more than 1000 pollen grains were measured in diameter.

Stomatal measurements. After 1-year postgermination, the length of stomatal guard cells was measured using a compound light microscope (Zeiss Imager A1, Zeiss Microscopy). Two fully expanded leaves were randomly selected and only the abaxial leaf surfaces were used for microscopic observation. By using an image analysis software (AxioVision; Zeiss Microscopy), 300 stomata per plant were measured at ×200 magnification. The same imaging software with the same magnification setting was then used to count stomata per frame of view (449.2 × 336.5 µm), and determine stomatal density (stomata/mm^2). Six images per plant were analyzed and stomatal index was calculated per Li et al. (1996). For use in figures, the slide mounts of stomatal impressions were prepared by applying clear nail polish (nitrocellulose) to the abaxial surface of the leaf

and mounted on a microscope slide using clear tape.

Morphology measurements. When plants were 1.5 years old, plant height, plant width, leaf thickness, and leaf color were measured for all induced polyploids, and a random subset of the control group with the standard cytotype. Plant height and width were measured using a standard ruler to the nearest 0.5 cm. For plant width, the plant's widest spread was measured, followed by the plant's minimum spread, which was perpendicular to the widest spread. These two measurements were then averaged. Leaf thickness on three fully expanded leaves per plant was measured using digital calipers to the nearest 0.01 mm, making sure to avoid the leaf midvein.

Color measurements. Using three fully expanded leaves per plant, leaf color was measured using a colorimeter (BC-10, Konica Minolta, Ramsey, NJ) and three color coordinates; L*, a*, and b* were recorded. The L* coordinate represents the lightness level of the color, L* = 0 being pure black, and L* =100 being pure white. The a* and b* coordinates represents positive/negative correlation to the red/green component, and the yellow/blue component of color, respectively. These coordinates were used to calculate hue angle (H°) and Chroma (C*), and the resulting data sets were reported per McGuire (1992). By using the averaged color characteristic values for each cytotype, the values were then matched with corresponding color chips in the Royal Horticultural Society (RHS) Color Chart (Royal Horticultural Society, 2007). This was accomplished using a color difference equation which calculated differences in color to a single value (ΔE*). Color values from the RHS Color Chart were tested against that of the averaged L*, a*, and b* values from the leaves of the three cytotypes until ΔE* was minimized.

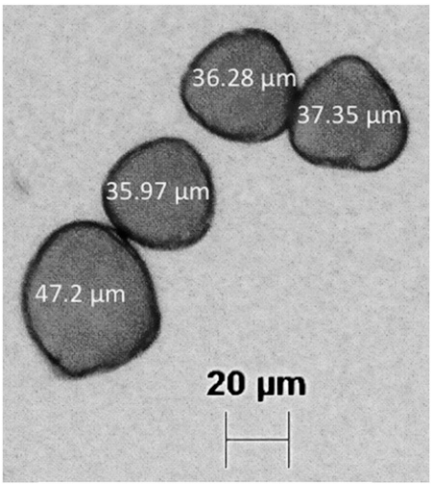

Fig. 2. Putative unreduced pollen grain (47.2 µm), which is a 29% increase over the mean of $n = 4x$ pollen (36.7 µm) of standard cytotype ($2n = 8x$) *Prunus lusitanica*, observed at ×200 magnification.

Data analysis. Data were analyzed using R version 3.3.1 (R Foundation for Statistical Computing, Vienna, Austria). Survival data were nonnormal with unequal variance and therefore analyzed using the nonparametric Kruskal–Wallis rank sum test. Treatments were then compared using least significant difference multiple comparisons test with the Bonferroni inequality adjustment. This method does not allow for the consideration of block effects, but variation among blocks was not significant ($P = 0.998$) and without trend. Treatment groups served as the experimental unit (n = 7) for this analysis. Stomata, morphology, and color were analyzed using two-sample *t* tests, using individual plants as sampling units (n = 3) and ploidy level as the independent variable. The single $2n = 12x$ plant was excluded from statistical analysis due to lack of replication, but data are reported.

Results and Discussion

Survival percentages of our treatments resulted in three levels of statistical significance (Table 1). The lowest survival rate was seen in 125 µM oryzalin at 17%, significantly lower than all other treatments and the control. *Prunus lusitanica* appeared to be less sensitive to colchicine than *Platanus acerifolia*, in which a single application of 0.5% colchicine solution to the apical meristem resulted in 0% survival rate (Liu et al., 2007).

Our treatments resulted in four cytotypes (Fig. 1), and altered ploidy was observed in every treatment group including the controls (Table 1). We estimated the 2C genome size of wild-type *P. lusitanica* to be 3.52 ± 0.02 pg (Mean ± SEM). This agrees with the previously reported genome size which was also determined by using flow cytometry

Fig. 3. Morphological variation within and between 1.5-year-old (**A**) $2n = 16x$ and (**B**) $2n = 8x$ *Prunus lusitanica*.

Table 2. Stomatal density and guard cell length in three cytotypes of 1-year-old *Prunus lusitanica.*

Ploidy	No. of plants	Guard cell length (µm)	Stomatal density (stomata/mm^2)	Stomatal index[z]
$2n = 16x$	3	26.2 ± 0.61 a[y]	112 ± 3.9 b	2,938
$2n = 12x$	1	23.6	142	3,362
$2n = 8x$	3	18.3 ± 0.33 b	194 ± 2.6 a	3,563

[z]Stomatal index = Guard cell length × stomatal density.
[y]Values are the mean ± SEM. Letters indicate significant differences ($P \leq 0.001$) using a two-sample *t* test. The $2n = 12x$ sample was excluded from statistical analysis due to lack of replication.

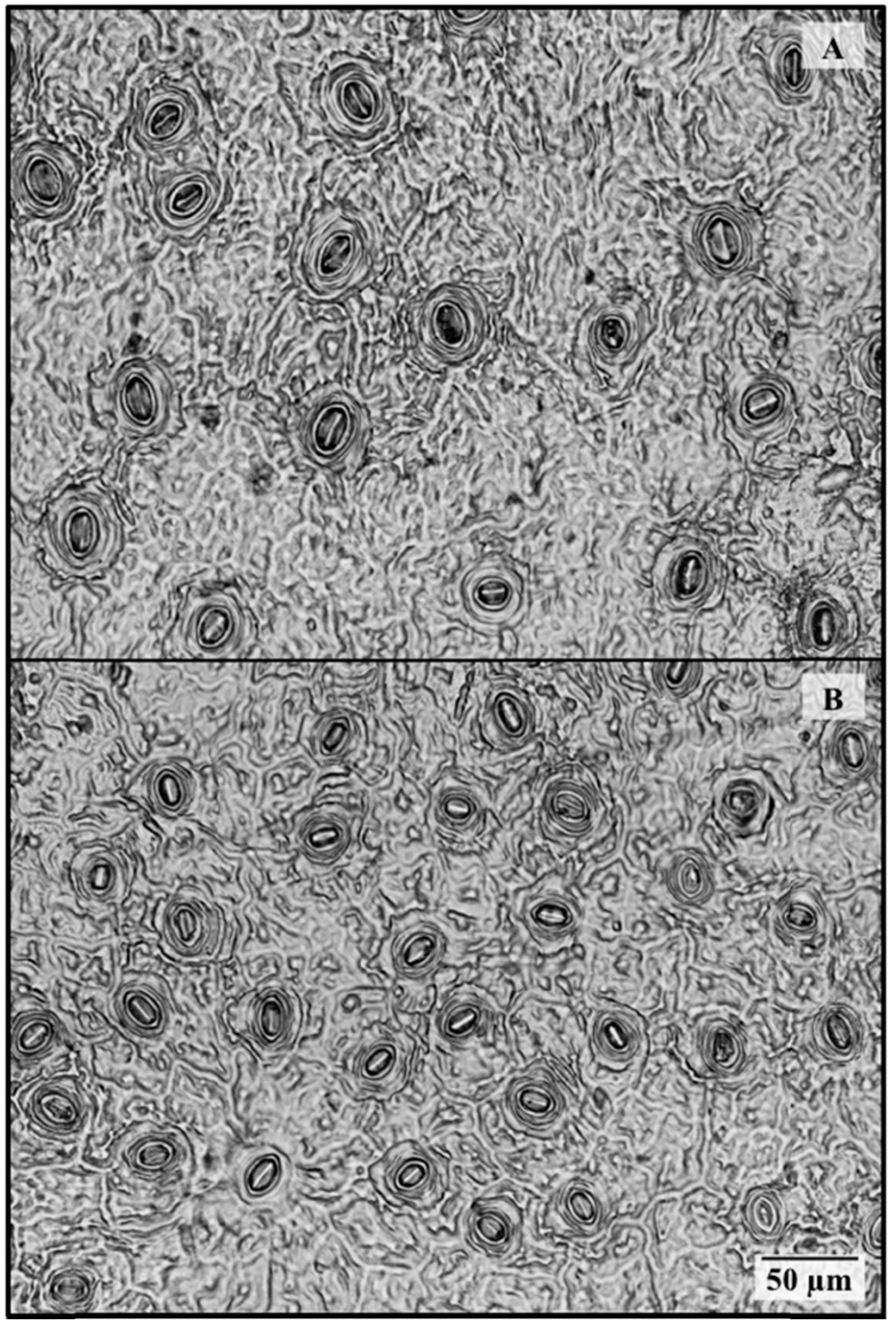

Fig. 4. Stomatal variation between (**A**) $2n = 16x$ and (**B**) $2n = 8x$ *Prunus lusitanica*, observed at ×200 magnification.

(García-Verdugo et al., 2013). We observed 14 cytochimeras ($2n = 8x + 16x$), one $2n = 12x$, and three $2n = 16x$ plants. Cytochimeras (mixoploids) commonly result from colchicine (Kadota and Niimi, 2002) and oryzalin (Contreras and Meneghelli, 2016) treatments of woody plants. The 125-µM oryzalin treatment produced the highest number of altered-ploidy seedlings (one $2n = 16x$ and five mixoploids) even with only 17% survival rate of treated meristems. The higher meristem survival percentages and lower altered-ploidy induction rates of other treatments indicated they were less efficient in obtaining altered-ploidy plants. The 125 µM oryzalin was more effective than any colchicine concentration tested here at altering ploidy in *P. lusitanica*.

We observed putative unreduced pollen (Fig. 2). The average diameter of a *P. lusitanica* pollen grain was 36.7 µm. When the volume of a sphere is doubled, the diameter increases by 26%. To account for variability in pollen shape, and inclusion of potential unreduced gametes in the calculation of average pollen size, we used 20% and 25% as cutoffs for identifying putative unreduced gametes. We found that 0.66% of pollen grains were 20% larger (>44.0 µm) than the average, and 0.47% were 25% larger (>45.8 µm). When flowering occurs in the $2n = 16x$ plants, measurements of pollen diameter may serve as a more precise cutoff for identification of putative unreduced pollen in the standard cytotype. As for now, our data support presence of unreduced gametes, which is the likely source of the $2n = 16x$ in the control group.

The control and 0.2% colchicine treatment produced seedlings with unexpected ploidies (control, $2n = 16x$; 0.2% colchicine, $2n = 12x$). We believe the $2n = 16x$ seedling from the control group may have been the result of bilateral sexual polyploidization, in which both parents contributed an unreduced gamete (Sleper and Poehlman, 2006). It is also possible that the $2n = 12x$ seedling seen in the 0.2% colchicine group was the result of unilateral sexual polyploidization, in which only one parent contributed an unreduced gamete (Sleper and Poehlman, 2006). To test the possible sexual origin of these plants, we performed flow cytometric analysis on the roots of all $2n = 12x$ and $2n = 16x$ plants as well as two $2n = 8x$ controls. Since the treatment was applied to the apical meristem, the roots of an induced polyploid should retain the ploidy of the standard ($2n = 8x$) cytotype. The roots of the $2n = 16x$ seedling from the control group and the $2n = 12x$ seedling had the same ploidy as the shoot apex. This indicates that these altered ploidy levels were not a result of the treatment, but occurred naturally. Occurrence of natural polyploids is not uncommon in *Prunus*, and the speculated source is often due to unreduced gametes (Tavaud et al., 2004). Furthermore, the two $2n = 16x$ plants falling into the 0.2% colchicine and 125-µM oryzalin treatments were therefore investigated and results showed that their root ploidy levels matched that of the standard cytotype ($2n = 8x$), thus confirming that the alteration in ploidy for these two $2n = 16x$ plants was a result of our treatments.

Morphological, stomatal, and color variability was not evident among the three apically $2n = 16x$ plants (data not reported). Therefore, $2n = 16x$ in the following sections will refer collectively to plants which possess an apical ploidy level of $2n = 16x$, regardless of root ploidy level.

Table 3. Morphological observations of three cytotypes of 1.5-year-old *Prunus lusitanica.*

Ploidy	No. of plants	Plant ht (cm)	Plant width (cm)	Leaf thickness (µm)
$2n = 16x$	3	39.8 ± 1.7 b[z]	40.2 ± 2.0 a	570 ± 4.0 a
$2n = 12x$	1	43.0	45.3	490
$2n = 8x$	3	50.3 ± 3.1 a	38.9 ± 3.4 a	470 ± 11 b

[z]Values are the mean ± SEM. Letters indicate significant differences ($P \leq 0.05$) using a two-sample *t* test. The $2n = 12x$ sample was excluded from statistical analysis due to lack of replication.

Table 4. Leaf color values in three cytotypes of 1.5-year-old *Prunus lusitanica* collected using a colorimeter (BC-10, Konica Minolta, Ramsey, NJ).

Ploidy	No. of plants	L*	a*	b*	H°[z]	C*[y]	RHS value[x]
$2n = 16x$	3	33.1 ± 1.2 a[w]	–7.5 ± 0.4 a	13.3 ± 1.5 a	120.0 ± 2.0 a	15.3 ± 1.5 a	N137A
$2n = 12x$	1	38.0	–10.5	22.1	115.3	24.5	146A
$2n = 8x$	3	37.0 ± 2.0 a	–9.9 ± 0.7 b	20.5 ± 3.1 a	116.3 ± 1.6 a	22.8 ± 3.1 a	N137D

[z]Hue angle = H° = arctangent (b*/a*).
[y]Chroma = $C^* = \sqrt{(a^{*2} + b^{*2})}$.
[x]Comparisons were made by minimizing ΔE* with corresponding color chart values, using the color difference equation; $\Delta E^*_{1,2} = \sqrt{[(L^*_2 - L^*_1)^2 + (a^*_2 - a^*_1)^2 + (b^*_2 - b^*_1)^2]}$.
[w]Values are the mean ± SEM. Letters indicate significant differences ($P \leq 0.05$) using a two-sample *t* test. The $12x$ sample was excluded from statistical analysis due to lack of replication.

We observed morphological variation among different cytotypes as the plants developed. The $2n = 16x$ cytotype appeared to have a more compact growth habit at one year (Fig. 3). When compared with the $2n = 8x$ cytotype, the $2n = 16x$ cytotype showed increased stomatal size by 43%, but decreased stomatal density by 42% (Table 2; Fig. 4). The $2n = 12x$ plants, although excluded from statistical analysis, fell in between the $2n = 8x$ and $2n = 16x$ cytotypes in all categories except for plant width. Previous work with *Betula papyrifera* reported that the enhanced drought tolerance in polyploids was possibly due in part to their lower stomatal index (Li et al., 1996). These $2n = 16x$ plants displaying reduced stomatal index will exhibit the same improved drought tolerance. After 1.5 years, $2n = 16x$ plants exhibited shorter stems and thicker leaves than the $2n = 8x$ plants (Table 3). In other Rosaceous plants, similar effects of chromosome doubling on leaf thickness and stomatal variation are seen in *Rosa* (Kermani et al., 2003) and *Pyrus* (Kadota and Niimi, 2002), respectively.

The $2n = 16x$ plants had a higher a* color component (Table 4), indicating that $2n = 16x$ plants had stronger red hue than the $2n = 8x$ plants. Perhaps more plants are required in the future to improve statistical power and detect apparent color differences. The RHS Color Chart, which has been considered as the standard reference for characterizing color in plants, was used to describe color of the three cytotypes. After the color difference equation was applied, the smallest ΔE* corresponded to three different shades of green. Results suggested a darker shade of green in the $2n = 16x$ plants. Other researchers have reported darker color in leaves of induced polyploids (Kermani et al., 2003; Liu et al., 2007), but none of these studies used this method to quantify and report this difference.

Application of 125 µM oryzalin to apical meristems of Portuguese cherrylaurel seedlings is an effective method to double their chromosomes. This adds to the body of evidence that oryzalin is an excellent alternative to the more hazardous colchicine for ploidy manipulation work. We speculate that this method would be applicable to different species of stone fruits, with variability in the effective concentrations of mitotic spindle inhibitors and application time. The $2n = 16x$ plants are now 2 years old and showing excellent ornamental qualities, with less leaf-folding, superior form, and less apical dominance for a more shrub-like appearance. Perhaps most importantly, the $2n = 16x$ plants may facilitate interspecific hybridization with *P. laurocerasus.*

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