

# In Vitro Chromosome Doubling of *Prunus laurocerasus* ‘Otto Luyken’ and ‘Schipkaensis’

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**Abstract.** *Prunus laurocerasus* L. ( $2n = 22x = 176$ ), common cherrylaurel, is an evergreen shrub generally used as a hedge or screen. This species produces large drupes that are a nuisance when they drop on walkways or deposited by birds and also has escaped cultivation in parts of northwestern United States, which has raised concern about the invasive potential of common cherrylaurel. Therefore, a fruitless and sterile form of common cherrylaurel is desirable. As part of our efforts to develop sterile common cherrylaurel cultivars, we conducted two experiments to induce chromosome doubling using in vitro exposure of ‘Otto Luyken’ and ‘Schipkaensis’ cherrylaurel to oryzalin. For ‘Otto Luyken’ (Expt. 1), we tested the effects of treatment duration (1, 2, 14, or 28 days) and oryzalin concentration (0, 6.25, 12.5, 25, 50, 100, or 150  $\mu\text{M}$ ) applied in a liquid phase over explants. In Expt. 2, we treated ‘Schipkaensis’ cherrylaurel shoots with a single duration of 28 days and exposed explants to the same varying concentrations of oryzalin incorporated into the solidified medium. In Expt. 1, the 14-day treatment had reduced survival compared with 1- and 2-day treatments and there was still greater mortality in the 28-day treatment. Duration of the treatment affected mortality more than oryzalin concentration. Sixteen treatment combinations resulted in 44x plants. The percentage of 44x plants increased with concentration in the 1- and 2-day treatments up to 30% of treated shoots at 150  $\mu\text{M}$ . Overall, the longer duration treatments in Expt. 1 were less efficient for inducing 44x plants. Expt. 2 was less effective for inducing homogenous 44x plants. It is unclear if this is due to treatment or cultivar differences but the highest concentration was 8% in the 6.25  $\mu\text{M}$  treatment.

*Prunus laurocerasus* (common or English cherrylaurel) is an evergreen shrub or small tree in Rosaceae generally used as a hedge or screen. This species is a cytological anomaly due to its high ploidy ( $2n = 22x = 176$ ; Meurman, 1929). Common cherrylaurel grows to more than 6 × 9 m and has a coarse texture with large leaves that can be up to 20 cm or more in length and generally are oblong with serrated margins. The species produces prolific numbers of white flowers in axillary racemes up to 12 cm or longer. There are a number of cultivars of common cherrylaurel in the trade, but ‘Otto Luyken’ and ‘Schipkaensis’ are most common. ‘Otto Luyken’ is a compact form that generally grows to 1.2 m high and 1.8 m wide with narrower leaves than the species and the leaves for the most part lack serration. ‘Otto

Luyken’ maintains very dense growth and has prolific flower production even when shaded. ‘Schipkaensis’ is reportedly confused in the trade and often growers are producing what Dirr (2009) refers to as ‘West Coast Schipkaensis’. The original ‘Schipkaensis’ has leaves up to 11.4 cm long with entire margins or only a few teeth toward the apex. ‘West Coast Schipkaensis’, a name used by retailers to describe an alternate form of the original (M. Dirr, personal communication) is characterized by having more serrated leaves and a distinct upright habit.

Fruits of common cherrylaurel are large drupes of up to 1.25 cm and are produced in large numbers. In managed landscapes, they are a nuisance when they drop on walkways or are deposited by birds. Common cherrylaurel has escaped cultivation in parts of northwestern United States including western Oregon. It is regularly found in native forests and is ranked as a medium (M) to high (H) impact potential invasive species by the Emerald Chapter of the Native Plant Society of Oregon. Because of its characterization as an invasive species, a fruitless form of common cherrylaurel is desirable. Ploidy manipulation is a common technique used to reduce fertility by doubling chromosomes and then backcrossing to produce a plant with odd ploidy. Polyploidization often is performed in vitro due to the

relatively small meristem size and ability to treat many plants (or meristems). In vitro polyploidization has been successfully performed on a wide array of plant species in many families using various antimetabolic agents (Dhooghe et al., 2011). Although colchicine is the historical method of polyploidization and has proven effective for in vitro chromosome doubling, the dinitroaniline herbicide oryzalin has recently become more popular. Oryzalin has been used to double chromosomes of woody landscape plants in Rosaceae including *Chaenomeles japonica* (Thunb.) Lindl. ex Spach (Stanys et al., 2006), *Malus* Mill. (Bouvier et al., 1994), and *Rosa* L. (Kermani et al., 2003). Our goal was to induce whole genome duplication of ‘Otto Luyken’ and ‘Schipkaensis’ using in vitro application of oryzalin.

## Materials and Methods

*Plant material and medium.* In vitro cultures were initially established during April 2012, in glass culture tubes (30 × 200 mm) filled with 10 mL of multiplication medium consisting of MS salts (Murashige and Skoog, 1962; Phytotechnology Laboratories, Shawnee Mission, KS) supplemented with 0.5 mg·L<sup>-1</sup> benzylaminopurine (BAP), 0.1 mg·L<sup>-1</sup> gibberellic acid (GA<sub>3</sub>), and 0.01 mg·L<sup>-1</sup> indole butyric acid with pH adjusted to 5.7 and solidified with 7 g·L<sup>-1</sup> agar. This medium was used in establishment, multiplication, and during treatments for both ‘Otto Luyken’ and ‘Schipkaensis’. Shoots were



Fig. 1. *Prunus laurocerasus* ‘Otto Luyken’ explant during treatment in culture tubes (25 × 150 mm) filled with 10 mL of solidified MS medium into which the base of the explant was inserted before being covered with a liquid phase consisting of MS medium supplemented with varying concentrations of oryzalin. The color difference from the solid phase to liquid phase is due to the addition of oryzalin in the liquid phase.

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collected from container-grown plants received from Blue Heron Farms Nursery (Corvallis, OR) and maintained for four weeks in culture at 20 °C with 16-h daylength



Fig. 2. *Prunus laurocerasus* 'Schipkaensis' shoots in 100 × 15 mm sterile polystyrene dishes containing 25 mL of multiplication medium with varying concentrations of oryzalin added to the solidified medium. Pictured are shoots growing from explants following 50 μM oryzalin treatment.

provided using cool white fluorescent lamps (50 μmol·m<sup>-2</sup>·s<sup>-1</sup>). Plants labeled as 'Schipkaensis' are more upright than the description by Dirr (2009) and likely are what is referred to as 'West Coast Schipkaensis', which is a more upright form. However, for this report we will maintain the nomenclature of the plants used by the nursery and herein refer to it as 'Schipkaensis'. Each shoot was ≈1.5 cm long with two to three axillary and terminal meristems. Oryzalin solutions were prepared by dissolving oryzalin in 100% ethanol and dimethyl sulfoxide (DMSO) for a final concentration of 2.5% ethanol + 0.85% DMSO for each respective oryzalin concentration. Solutions were filter sterilized with a 0.45 μM polytetrafluoroethylene syringe filter before diluting to final concentrations in sterile liquid MS medium, pH 5.7 (Expt. 1) or being incorporated into solidified medium (Expt. 2).

*Experiment 1.* Individual shoots of 4-week-old cultures of *P. laurocerasus* 'Otto Luyken' were transferred to culture tubes (25 × 150 mm) filled with 10 mL of fresh multiplication medium. Shoots used were similar in length and meristem number to the original shoots described above for initi-

ation. After one week on multiplication medium, 10 mL of liquid MS basal medium supplemented with oryzalin treatments was added to 20 replications, of one shoot per tube, which represented an experimental unit (Fig. 1). Treatments included a control (MS medium + 2.5% ethanol and 0.85% DMSO), 6.25, 12.5, 25, 50, 100, and 150 μM oryzalin applied by submerging shoots for 1, 2, 14, and 28 d. After treatments were completed, the liquid oryzalin solution was removed by pouring off, and the shoots remained on the solid basal medium until 28 d from the start of the treatment. After 28 d, all shoots were transferred to fresh multiplication medium, including the 28-d treatment. Regression analyses for oryzalin concentration on ploidy levels were conducted for each treatment duration to identify significant linear and quadratic trends and to estimate the treatment combination(s) that resulted in the greatest percentage of chromosome doubling.

*Experiment 2.* Individual shoots of 4-week-old cultures of *P. laurocerasus* 'Schipkaensis' were transferred to 100 × 15 mm sterile polystyrene dishes containing 25 mL of multiplication medium with oryzalin incorporated

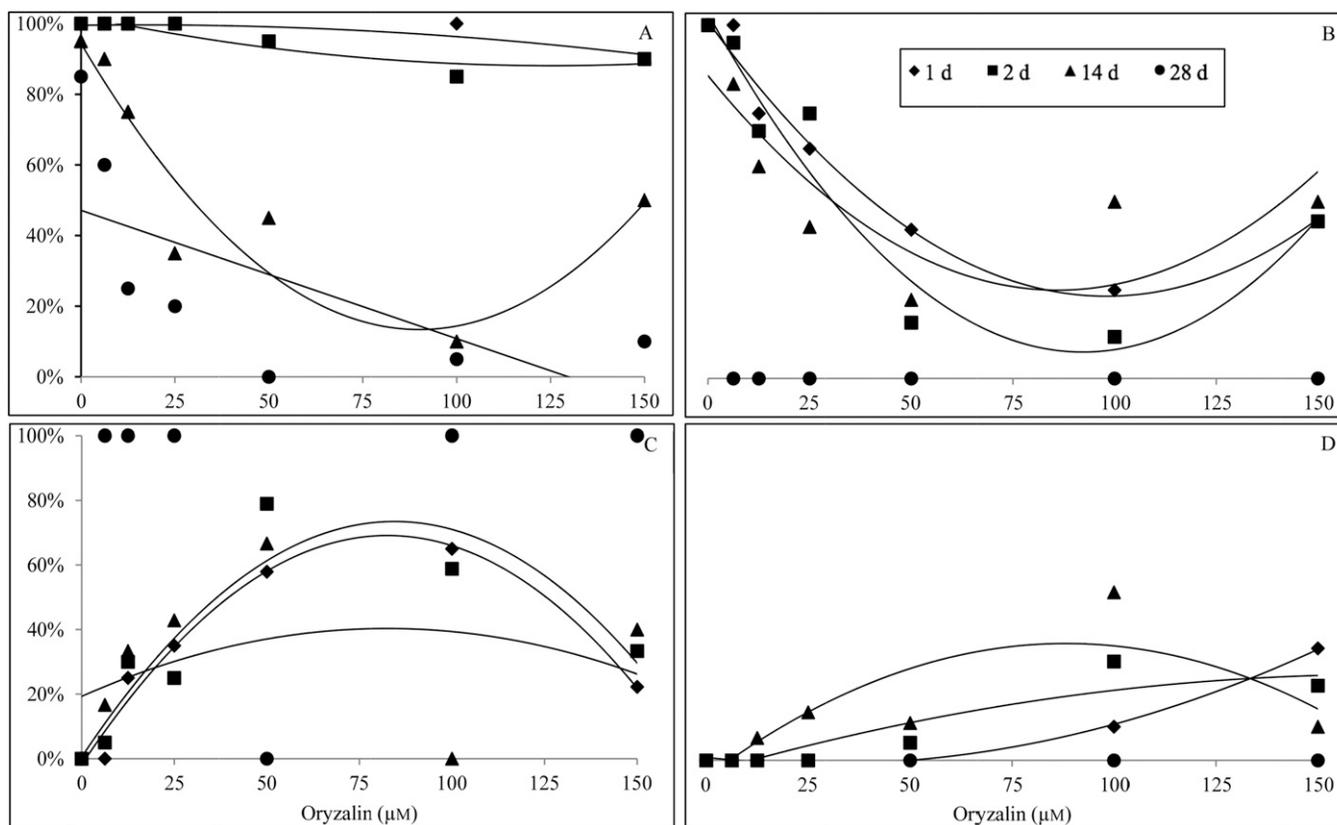


Fig. 3. Response of in vitro *Prunus laurocerasus* 'Otto Luyken' to exposure of 0, 6.25, 12.5, 25, 50, 100, or 150 μM of oryzalin for 1, 2, 14, or 28 d. These data are expressed as the percent of tubes that either survived or were found to be at each cytotype; most tubes had several meristems that grew following treatment and we further present effects on an individual meristem basis elsewhere. (A) survival percentage of shoots exposed to varying concentrations for 1 d ( $y = -5E-06x^2 + 0.0002x + 0.995$ ;  $r^2 = 0.6508$ ), 2 d ( $y = 9E-06x^2 - 0.0022x + 1.0214$ ;  $r^2 = 0.853$ ), 14 d ( $y = 1E-04x^2 - 0.018x + 0.944$ ;  $r^2 = 0.872$ ), or 28 d ( $y = 9E-05x^2 - 0.0166x + 0.652$ ;  $r^2 = 0.786$ ). (B) percentage of surviving shoots that were 22x following exposure to varying concentrations for 1 d ( $y = 8E-05x^2 - 0.0159x + 1.0098$ ;  $r^2 = 0.974$ ), 2 d ( $y = 0.0001x^2 - 0.0208x + 1.032$ ;  $r^2 = 0.930$ ), or 14 d ( $y = 8E-05x^2 - 0.0141x + 0.857$ ;  $r^2 = 0.681$ ); there were no 22x shoots observed at 28 d. (C) percentage of surviving shoots that were cytochimeras (22x + 44x) following exposure to varying concentrations for 1 d ( $y = -0.0001x^2 + 0.0172x - 0.0184$ ;  $r^2 = 0.968$ ), 2 d ( $y = -0.0001x^2 + 0.0173x + 0.0051$ ;  $r^2 = 0.841$ ), 14 d ( $y = -3E-05x^2 + 0.0051x + 0.193$ ;  $r^2 = 0.09295$ ), or 28 d (trend line not included, as it was not useful in describing response). (D) percentage of surviving shoots that were 44x following exposure to varying concentrations for 1 d ( $y = 2E-05x^2 - 0.0013x + 0.0086$ ;  $r^2 = 0.997$ ), 2 d ( $y = -1E-05x^2 + 0.0035x - 0.0372$ ;  $r^2 = 0.834$ ), or 14 d ( $y = -5E-05x^2 + 0.009x - 0.0495$ ;  $r^2 = 0.676$ ). No tubes were found to be composed solely of 44x tissue after 28-d exposure.

into the solidified medium (Fig. 2). We attempted to prepare single node cuttings but many had more than one meristem due to extremely short internodes. Treatments were 28 d of varying concentrations of oryzalin including control (MS medium + 2.5% ethanol and 0.85% DMSO), 6.25, 12.5, 25, 50, 100, and 150  $\mu\text{M}$  oryzalin. Each treatment was replicated four times with 16 individual shoots per polystyrene dish (experimental unit). Following treatments, shoots were transferred to multiplication medium for an additional 28 d. Data were analyzed by analysis of variance and mean separation was performed using Fisher's least significant difference ( $\alpha = 0.05$ )

**Ploidy analyses.** After transferring to multiplication medium elongating meristems were allowed to grow for 28 d and then leaves were collected from individual elongating meristems for flow cytometry analysis. Samples were not all prepared on the same day due to the number of experimental units. Analyses began 28 d after transfer and were completed in  $\approx 15$  d. Samples from controls and treated plants were collected directly from culture tubes or polystyrene dishes. About 1  $\text{cm}^2$  of sample tissue was collected using a scalpel and forceps, then finely chopped in an extraction buffer (CyStain<sup>®</sup> Ultraviolet Precise P Nuclei Extraction Buffer; Partec, Münster, Germany) with our standard (*Solanum lycopersicum* L. 'Stupicke';  $2\text{C} = 1.96$  pg) using a double-sided razor blade to extract nuclei. The nuclei suspension was passed through a 30  $\mu\text{M}$  filter (Partec), stained with 4',6-diamidino-2-phenylindole (DAPI; CyStain<sup>®</sup> Ultraviolet Precise P Staining Buffer; Partec), and nuclei were analyzed using a CyFlow<sup>®</sup> Ploidy Analyzer (Partec).

## Results

**Experiment 1.** Increasing concentration of oryzalin and duration of treatment generally reduced survival of 'Otto Luyken' shoots (Fig. 3A). Over 85% survival was obtained for 1- and 2-d durations for all oryzalin concentrations. Increased shoot death was observed in the 14-d treatment and reached a low of 10% at 100  $\mu\text{M}$  but interestingly had 50% survival at 150  $\mu\text{M}$ . The fewest shoots survived following 28-d treatment at all concentrations of oryzalin. In the 14- and 28-d control treatments, one and three shoots died, respectively, indicating that submerging shoots of 'Otto Luyken' cherry laurel for this duration did not impact survival and the increased mortality with other treatments was due to increased oryzalin concentration. We did not wash shoots following treatments, therefore it seems likely that our 1-, 2-, and 14-d treatments extended beyond these durations. We observed tinting of medium in some tubes and even liquefaction of medium in some 28-d treatments, which was due to the applied solution mixing with agar-solidified medium.

As oryzalin concentration increased, 1-, 2-, and 14-d treatments showed a similar

trend in the percentage of surviving 'Otto Luyken' cherry laurel shoots that remained 22x (Fig. 3B). The lowest percentage of surviving shoots that were 22x for 1- (25%) and 2-d (12%) treatments was at 100  $\mu\text{M}$ , while the 14-d treatment reached its lowest percentage (22%) of 22x shoots at 50  $\mu\text{M}$ . None of the shoots treated for 28 d were 22x. The percentage of shoots that were mixoploid was highest in the 28-d treatment, which had 100% mixoploids at all oryzalin concentrations beside 0  $\mu\text{M}$  (100% 22x) and 50  $\mu\text{M}$  (0% survival) (Fig. 3C). Trends for 1-, 2-, and 14-d treatments based on regression analysis were similar and the greatest number of mixoploids for these treatment durations were observed in the 50 or 100  $\mu\text{M}$  oryzalin treatments (Fig. 3C). No mixoploids were

recovered at 100  $\mu\text{M}$  in the 14-d treatment. The highest percentage of surviving shoots that were 44x was in the 14-d treatment at 100  $\mu\text{M}$  (50%) (Fig. 3D), though this treatment only had 50% survival. The percentage of 44x shoots resulting from the 1-d treatment increased with oryzalin concentration up to 150  $\mu\text{M}$  (33% of surviving shoots).

Analysis of individual meristems separated from surviving shoots provided a better indication of relative success and efficiency of inducing polyploids in 'Otto Luyken' cherry laurel (Fig. 4). The greatest number of subcultured shoots were recovered following the 2-d treatment with 50  $\mu\text{M}$  oryzalin, which produced 110 plants, of which 49 were 44x. Relatively high concentrations of oryzalin ( $\geq 50$   $\mu\text{M}$ ) for short durations (1 or 2 d) were

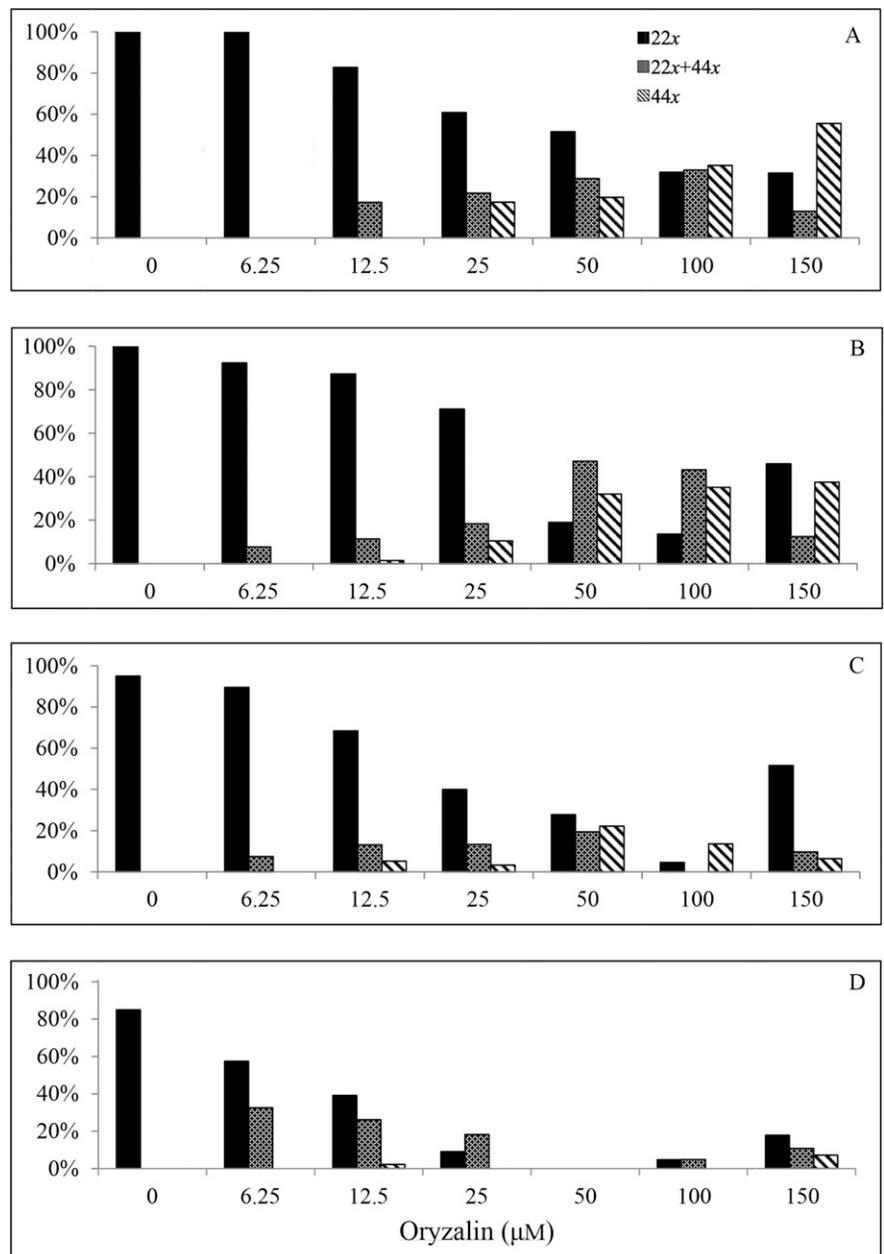


Fig. 4. Response of in vitro *Prunus laurocerasus* 'Otto Luyken' to exposure of 0, 6.25, 12.5, 25, 50, 100, or 150  $\mu\text{M}$  of oryzalin in a liquid phase applied to shoots for (A) 1 d, (B) 2 d, (C) 14 d, or (D) 28 d. Data are presented as the percent of individual surviving shoots, taken from culture tubes to which treatments were applied and analyzed separately, that were 22x, 22x + 44x, or 44x.

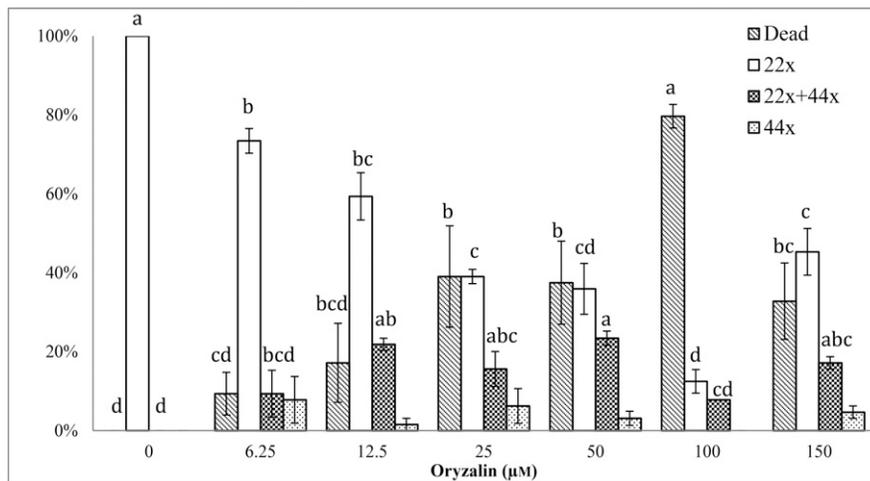


Fig. 5. Percent mortality and percentage of treated shoots representing each cytotype following in vitro exposure of *Prunus laurocerasus* 'Schipkaensis' to 0, 6.25, 12.5, 25, 50, 100, or 150  $\mu\text{M}$  of oryzalin incorporated into solidified MS medium for 28 d including. Different letters on each response (dead, 22x, 22x + 44x, 44x) indicates significant difference among oryzalin concentrations based on Fisher's least significant difference ( $\alpha = 0.05$ ).

more effective in producing greater shoot proliferation as well as inducing polyploids of 'Otto Luyken' compared with longer durations at all oryzalin concentrations.

**Experiment 2.** There was an increase in mortality with increasing oryzalin concentration for 'Schipkaensis' cherry laurel with the maximum mortality at 100  $\mu\text{M}$  (Fig. 5). As in the first experiment, it is interesting that there was greater shoot survival at 150  $\mu\text{M}$  (67%) oryzalin than at 100  $\mu\text{M}$  (20%). The percentage of 22x shoots declined to a minimum of 13% at 100  $\mu\text{M}$  but increased to 45% at 150  $\mu\text{M}$ . The percentage of mixoploids ranged from 8% to 24% without a clear relationship with concentration of oryzalin. There was not a high percentage of 44x plants induced with any treatment; the highest was at 6.25  $\mu\text{M}$  (8%) but there were no significant differences among oryzalin treatments.

## Discussion

We successfully induced higher level polyploidy in 'Otto Luyken' and 'Schipkaensis' cherry laurels using two methods of in vitro exposure of shoots to oryzalin. Our treatments spanned a range from low concentration and short duration to high concentration and long duration. A number of our treatments exceeded the  $LD_{50}$  of explants and resulted in low survival and even 100% mortality in the case of 50  $\mu\text{M}$  for 28 d in Expt. 1. Generally, other studies have used narrower ranges of oryzalin concentration, treatment duration, or both; and often had higher survival. Stanys et al. (2006) treated flowering quince [*Chaenomeles japonica* (Thunb.) Lindl. ex Spach] shoots by soaking explants for 2 d in varying concentrations of oryzalin solutions up to 144  $\mu\text{M}$ , which resulted in at least 84% survival and a maximum of 17% induced polyploids. In addition

to being more effective for inducing polyploidy in some treatments, oryzalin affected shoot regeneration less than colchicine in flowering quince (Stanys et al., 2006) and in our study we observed increased shoot regeneration in 'Otto Luyken' cherry laurel for the 1- and 2-d treatments with increasing oryzalin concentration up to 100 and 50  $\mu\text{M}$ , respectively. After treatment of callus from *Rhododendron* L. 'Fragrantissimum Improved' with 7.5  $\mu\text{M}$  oryzalin for 7 d, Hebert et al. (2010) observed 98% survival and 20% 4x; the highest observed in their study. It should be noted that many of our concentrations, as well as those of other published reports (reviewed by Dhooghe et al., 2011), exceed the solubility of oryzalin. We did not observe any precipitation of oryzalin in any of our treatments. Perhaps this was because we added oryzalin either to liquid MS medium (Expt. 1) or to solidified MS medium (Expt. 2). Whatever the reason, we demonstrated that increasing concentration above reported solubility generally reduces survival and results in chromosome doubling.

Our methods of exposing meristems varied between the studies, as did the relative size of material at the start of treatments. In Expt. 1, 'Otto Luyken' cherry laurel shoots were  $\approx 1.5$  cm long and had several to many meristems, whereas in Expt. 2 we attempted to prepare the 'Schipkaensis' cherry laurel shoots as single node cuttings, though a number of these had multiple meristems. However, the size of the cuttings did not appear to be a clear factor for survival. In both experiments, increased mortality of shoots was observed with increased oryzalin concentration. However, in Expt. 1, results indicated that submerging shoots for extended periods of time (14 or 28 d) did not appear to be detrimental to survival. Nevertheless, it is likely that our decision to simply pour off the oryzalin solution allowed continued exposure

of explants beyond the described treatment duration. Although we could not statistically compare the means between the experiments to determine if there were differences between cultivars, other studies have clearly demonstrated a genotype effect (Stanys et al., 2006). Based on our findings, the most efficient method for inducing polyploidy in *P. laurocerasus* is applying a relatively high concentration of oryzalin (100 or 150  $\mu\text{M}$ ) as a liquid that covers explants for 1 to 2 d. Using this method of application, we had up to 30% of treated shoots from which we subcultured 100% 44x plants. We will evaluate the higher order polyploids including mixoploids for fertility to assess if increasing ploidy to such a high degree has impact on fruit and seed production. To date, the only plants that have flowered are three mixoploids of 'Schipkaensis', which all have 22x L-II histogenic layer based on pollen measurements. Tetraploids of *Chitalpa tashkentensis* Elias and Wisura were delayed and more sporadic in flowering compared with diploids or mixoploids (Olsen et al., 2006; personal communication). Earlier and more consistent flowering of mixoploids has potential to benefit our breeding and evaluation efforts by expediting the process if we are able to identify individuals with 44x L-II histogenic layers.

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